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REVIEW

Serum bile acids as a sensitive biological marker for evaluating hepatic effects of organic solvents

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Serum bile acids (SBAs) are suggested as a potentially sensitive and specific indicator of liver function which, accordingly, could provide an early indication of hepatobiliary dysfunction. This offers advantages over more traditional parameters of liver integrity/function. Recent studies have shown that occupational exposure to low levels of halogenated aliphatic or non-halogenated aromatic solvents is associated with significant increases in SBA levels. As this has often been evident in the absence of any effect on conventional parameters of hepatobiliary integrity/function, elevated SBA levels may well be regarded as a sensitive biological marker of exposure/effect of these compounds. In addition, it may be considered that they provide an early indicator of solvent-induced changes in hepatobiliary function. Extensive studies with experimental animals have also provided supporting evidence for these observations in solvent-exposed individuals. Investigations of the mechanisms at cellular and subcellular levels by which these increases occur have suggested that these effects are likely to be the result of selective, dose-related and reversible inhibition of bile acid uptake at the sinusoidal domain of the hepatocyte plasma membrane. Increased concentrations of SBA under low levels of exposure to different solvents have been demonstrated to be a short-lived and reversible effect which is not accompanied by any other evidence of liver damage. Therefore, it could be assumed that it is unlikely that there would be pathological sequelae to these effects, although the longer term ramifications of such effects have not been thoroughly investigated. Nevertheless, the available evidence indicates that investigation of SBA in solvent-exposed workers could provide useful indications of exposure and effect.

Keywords: serum bile acids, biological marker, hepatotoxicity, organic solvents.

Abbreviations: AIB, α-aminoisobutyric acid; ALT, alanine aminotransferase; ALP, alkaline phosphatase; ANIT, α-naphthylisothiocyanate; AST, aspartate aminotransferase; CA, cholic acid; CDC, chenodeoxycholic acid; DC, deoxycholic acid; FC 113, 1,1,2-trichloro-1,2,2-trifluoroethane; GC, glychocholic acid; GCDC, glychochenodeoxycholic acid; GDC, glychodeoxycholic acid; GGT, γ-glutamyltransferase; GLC, glycholithocholic acid; GLC–MS, gas–liquid chromatography–mass spectrometry; HPLC, high performance liquid chromatography; ISBA, individual serum bile acids; LDH, lactate dehydrogenase; OMG, 3-*O*-methyl-D-glucose; SBA, serum bile acids; SDH, sorbitol dehydrogenase; TC, taurocholic acid; TCDC, taurochenodeoxycholic acid; TCE, 1,1,1-trichloroethane; TDC, taurodeoxycholic acid; TET, tetrachloroethylene; TRI, trichloroethylene; TUDC, tauroursodeoxycholic acid; UDC, ursodeoxycholic acid.

Introduction

A large variety and number of chemicals are used as organic solvents which have great commercial and industrial importance. Extensive use of organic solvents

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in different industries and many commercial products, as well as the release of these volatile substances into the environment from different sources, have led to a broad potential for occupational and public exposure to these compounds (Swedish Work Environment Fund Report 1980). It has been estimated that in 12 member countries of the European Community, about one million workers are potentially exposed to ten commonly used chlorinated aliphatic hydrocarbon solvents (Commission of the European Communities 1986). The potential for occupational and public exposure to other organic solvents such as the non-chlorinated aromatic hydrocarbons, toluene and xylene is even greater. In the USA, it has been estimated that 1.1 and 1.3 million workers in 1981–1983 were potentially exposed to xylene and toluene, respectively (IARC 1989). Owing to their wide application, the toxicity and health effects of organic solvents have been the subject of extensive studies and scientific reviews in both humans and experimental animals. The similarities in physical and chemical characteristics of most organic solvents, such as high volatility, fat solubility and low molecular weight, confer on them certain common properties and effects upon contact with living organisms (Bergman 1979). While there are some variations in symptoms and effects with different chemical structures, the results of exposure to high levels of solvents are very similar, and include disturbances of the central nervous system manifested by disorientation, euphoria, giddiness and confusion progressing to unconsciousness, paralysis, convulsion and death. Additionally, a number of studies have suggested that frequently used solvents at sub-anaesthetic doses may cause behavioural toxicity (Snyder and Andrews 1996).

Prolonged dermal exposure to solvents in liquid form has been associated with a sensation of burning, reversible erythema, irritation and dryness of skin and contact dermatitis due to their defatting properties (WHO 1981, Low *et al.* 1989).

Distinct from skin effects and general acute CNS depressant actions of solvents, are their specific organ toxicities. These effects may result from repeated exposure to tolerable levels of solvents rather than from acute exposure to very high levels (Snyder and Andrews 1996). As the liver concentrates highly lipophilic compounds, it usually is a target organ for toxic effects of such substances and vulnerable to injury by these agents (Farrell 1994).

Detection and evaluation of solvent-induced hepatotoxicity in humans

Although the hepatotoxic potential of some halogenated aliphatic hydrocarbon solvents has been well documented (Commission of the European Communities 1986, Rouisse and Chakrabarti 1986, Farrell 1994, Lundberg *et al.* 1994), for some other solvents this issue is the subject of some debate and remains unresolved (Franco *et al.* 1986, Guzelian *et al.* 1988). These uncertainties may have been further complicated by the substitution of many of the solvents used in the early days of the chemical industry, which were hepato-renal toxicants, by newer and less toxic compounds (Mitchell 1987).

Limitations of plasma or serum enzymes in detecting solvent-induced hepatotoxicity

Due to the ease of measurement and experience with plasma or serum enzymes in various states of hepatobiliary disease (Harrison 1990), these measurements have

been widely used in screening for hepatotoxicity associated with exposure to organic solvents (Kurppa and Husman 1982, Waldron *et al.* 1982, Lundberg and Hakansson 1985). The frequency of abnormal results using conventional serum enzyme tests for screening occupational liver injury, however, is believed to be low (Edling and Tagesson 1984). These tests have been criticized for non-specificity and poor positive predictive value in identifying true occupational liver injury (Harrison 1990). Moreover, they are thought to be of limited use for screening workers exposed to solvents for which overt hepatotoxicity is not a major outcome (Mitchell 1987).

The lack of specificity and sensitivity of enzyme tests prevents detection of subtle liver changes at an early stage in chemical hepatotoxicity (Dossing and Skinhoj 1985, Whitby 1986). Serum enzymes primarily reflect acute disruption of cell membrane integrity (liver cell 'leaking') rather than uptake, metabolism, storage and excretion functions of the liver cells (Zimmerman and Seef 1970). Therefore, they cannot accurately reflect more subtle accumulative damage until late in the injury process (Tamburro and Greenberg 1981). The failure of serum enzyme tests to identify the early phases or progression of liver injury has been shown in numerous studies (Tamburro 1979, Tamburro and Greenberg 1981, Liss *et al.* 1985, Bai *et al.* 1992b, Azer *et al.* 1993).

Interpretation of common enzyme measurements for individuals with potential occupational exposure to hepatotoxicants, is made more difficult for several reasons (Hodgson *et al.* 1990), which include:

(1) The true predictive value of serum enzyme tests for liver disease and the prevalence of liver diseases in most populations is unknown.

(2) Prediction of long-term prognosis from transient or minor elevations in serum enzyme tests is questionable.

(3) The mathematical foundations for surveillance (repeated measures, longitudinal analysis) are poorly developed.

(4) Changes in the activities of serum enzymes may be due to non-specific liver diseases, such as acute viral illnesses, and cardiac and skeletal muscle injury.

These limitations are further highlighted by the studies among workers exposed to mixtures of commonly used aromatic and aliphatic hydrocarbon solvents that have failed to detect any abnormality in serum enzyme tests (Hane *et al.* 1977, Tahti *et al.* 1981, Kurppa and Husman 1982, Waldron *et al.* 1982, Dossing 1983, Dossing *et al.* 1983).

These studies, however, did not employ a test to assess hepatic function. Additionally, they do not rule out the possibility that long term exposure to low levels of organic solvents may eventually lead to chronic liver damage. Some circumstantial evidence (Dossing and Skinhoj 1985) indirectly suggests:

(1) An increased mortality rate due to liver cirrhosis among workers with long-term daily exposure to different organic solvents.

(2) Liver cirrhosis associated with long-term occupational exposure to several chlorinated hydrocarbon solvents including carbon tetrachloride (Pointdexter and Greene 1934), tetrachloroethane (Willcox 1915), trichloroethylene and 1,1,1-trichloroethane (Thiele *et al.* 1982).

The uncertainties regarding the evaluation of liver function and concern among exposed workers has led to a demand for a simple, reliable, practical and more sensitive non-invasive test of liver function that can accurately identify and quantitate exposure and detect subtle chronic changes which could be used for

early diagnosis and prevention of occupational liver disease (Tamburro 1979, Gopinath *et al*. 1980, Anonymous 1982a,b, Edling and Tagesson 1984, Tamburro and Liss 1986, Franco *et al*. 1989).

Bile acids

Bile acids have been suggested as useful indicators of hepatobiliary dysfunction, as almost all aspects of hepatobiliary function are involved in bile acid metabolism (Palmer 1982). Their sensitivity and specificity in a variety of clinical hepatobiliary conditions as well as liver dysfunction associated with occupational exposure to chemicals have been reported or suggested in several studies (Kaplowitz *et al*. 1973, Barnes *et al*. 1975, Stiehl *et al*. 1977, Skrede *et al*. 1978, Davidson *et al*. 1979, Gopinath *et al*. 1980, Tobiasson and Boeryd 1980, Douglas *et al*. 1981, Tamburro and Greenberg 1981, Edling and Tagesson 1984, Vihko *et al*. 1984, Liss *et al*. 1985, Franco *et al*. 1986, Mitchell 1987, Bruijne and Rothuizen 1988, Evans 1988, Wang and Stacey 1990, Franco *et al*. 1991, Bai *et al*. 1992a, 1992b, Driscoll *et al*. 1992, Azer *et al*. 1993, Hamdan and Stacey 1993, Neghab and Stacey 1996a, 1997a, Neghab *et al*. 1997).

Determination of bile acids

The potential use of bile acids as a test of liver function for subjects occupationally exposed to industrial chemicals and as a sensitive and specific indicator of liver disease (Wang *et al*. 1990) have led to major advances in the measurement of these molecules in biological samples over the last three decades. Although immunological and enzymatic methods provide rather simple and sensitive methods for determination of total or selected sub-groups of bile acids, neither is able to provide both quantitative and qualitative estimation for each of the individual bile acids in biological fluids (Azer *et al*. 1997b).

The development of sensitive methods such as high performance liquid chromatography (HPLC) and gas–liquid chromatography–mass spectrometry (GLC–MS) have offered reliable techniques for identification and determination of individual bile acids and their conjugates in biological fluids. For instance, using an HPLC assay, Wang *et al*. (1990) have determined 12 individual bile acids in serum (table 1) with high recovery and reproducibility rates (the reader is referred to an excellent review on biochemical assays of serum bile acids which has been published by Azer *et al*. (1997b)).

Determination of individual bile acids in serum (ISBA) has indicated that some of these molecules, in particular cholic acid (CA) and taurocholic acid (TC) levels are more sensitive to solvent exposure (Wang and Stacey 1990, Bai *et al*. 1992a,b, Driscoll *et al*. 1992, Hamdan and Stacey 1993, Neghab and Stacey 1997a, Neghab *et al*. 1997). Given this observation, some enzymatic and radioimmunoassay methods used for determination of total or only one or two particular bile acids (Hotz *et al*. 1990, Chen *et al*. 1991, Lundberg *et al*. 1994) may fail to detect some of the significantly elevated ISBA.

Interestingly, raised serum bile acid (SBA) levels following exposure to chemicals have been shown to be reversible. For instance, preliminary information from individuals occupationally exposed to organic solvents has shown that elevated SBA levels in exposed subjects returned to normal 2 weeks after cessation

Table 1. Recovery and reproducibility for the determination of free and conjugated bile acids (CA: cholic acid, UDC: ursodeoxycholic acid, CDC: chenodeoxycholic acid, DC: deoxycholic acid, GC: glychocholic acid, GCDC: glychochenodeoxycholic acid, GDC: glychodeoxycholic acid, GLC: glycholithocholic acid, TC: taurocholic acid, TUDC: tauroursodeoxycholic acid, TCDC: taurochenodeoxycholic acid, TDC: taurodeoxycholic acid) in human serum ($n = 6$).

Bile acids	Mean (%) ± SD	Coefficient of variation (%)	Reference
Free bile acids			
CA	83 ± 9.2	11.1	Wang *et al*. (1990)
UDC	96 ± 4.9	5.1	
CDC	92 ± 7.8	8.5	
DC	86 ± 8.1	9.4	
Glycine conjugated			
GC	85 ± 6.1	7.2	
GCDC	87 ± 6.0	6.9	
GDC	87 ± 10.5	12.1	
GLC	88 ± 9.0	10.2	
Taurine conjugated			
TC	81 ± 5.8	7.2	
TUDC	75 ± 5.7	7.6	
TCDC	72 ± 6.6	9.2	
TDC	83 ± 10.4	12.5	

of exposure (Neghab *et al*. 1997). Similarly, Sotaniemi *et al*. (1982) found that 3–6 weeks after cessation of exposure to solvents, bile acid levels and other biochemical abnormalities returned to normal. Similar conclusions have been reached by other studies (Lundberg *et al*. 1994) in which normal concentrations of SBA were found in a group of workers previously exposed to high atmospheric concentrations of solvents, with negligible or no current exposure.

Serum bile acids in fasting and postprandial states

The use of SBA concentrations as a measure of hepatic function is based on the same assumption as that for the use of serum creatinine as an index of renal functional reserve (Azer *et al*. 1997b). The efficiency of hepatic clearance from portal blood maintains SBA concentrations at low levels. During fasting, no significant change in the levels of SBA [CA and chenodeoxycholic acid (CDC)] has been noted in healthy volunteers (Ponz de Leon *et al*. 1978). After meals, however, SBA have been reported to increase slightly in healthy individuals and markedly in patients with hepatic disease (Vlahcevic *et al*. 1971). At the physiological level, both hepatocellular uptake and hepatocanalicular secretion of bile acids are the major determinants of peripheral bile acid concentration (Azer *et al*. 1997b). The effects induced by gall-bladder contraction and intestinal movement can be minimized by adjusting the time of blood sampling (i.e. fasting state or 2-h postprandial). Additionally, as the postprandial rise in SBA levels is largely governed by non-hepatic factors, in the assessment of liver function, fasting concentrations of SBA are considered more reliable (Franco *et al*. 1986).

Serum bile acids (SBAs) for evaluating hepatic effects of chemicals with particular reference to organic solvents

In 1984, two groups (Edling and Tagesson 1984, Vihko *et al*. 1984) demonstrated that SBA levels could provide an early indication of liver dysfunction following occupational exposure to organic solvents. Edling and Tagesson (1984) measured serum bile acid (CA and CDC) concentrations and determined the activities of serum enzymes in a group of workers occupationally exposed to styrene. Of the laboratory tests used, serum bile acids were the most frequently elevated parameters (table 2). Similar findings were reported by Vihko *et al*. (1984) in groups of subjects exposed to one of the following chemicals, styrene, multiple solvents (cresol, toluene, xylene), polyvinyl chloride, and vinyl chloride monomer. These observations were later supported by the findings of two additional studies in which occupational exposure to low levels of a mixture of organic solvents (Franco *et al*. 1986, 1989) was shown to produce a significant increase in serum conjugated chenodeoxycholic acid and total SBA levels in the absence of any alterations in other hepatobiliary function measurements (table 3).

Further studies (Wang and Stacey 1990), using an HPLC assay, also demonstrated that i.p. injection or inhalation exposure to trichloroethylene (TRI) in rats was associated with increased concentrations of serum bile acids, in

Table 2. Frequency of abnormal liver laboratory test results (LDH:lactate dehydrogenase, ALP: alkaline phosphatase, GGT: γ-glutamyltransferase, AST: aspartate aminotransferase, ALT: alanine aminotransferase, CA: fasting serum cholic acid, CDC: fasting serum chenodeoxycholic acid) in 23 men exposed to styrene (exposure level, 41-202 mg^{m3}).

	Abnormal test results		
Laboratory test	No.	%	Reference
LDH	0	0	Edling and Tagesson (1984)
ALP	0	0	
GGT	1	4	
AST	1	4	
ALT	3	13	
CA	9	39	
CDC	9	39	

Table 3. Mean levels of fasting total serum bile acids (SBA) and direct bilirubin concentrations, and serum activities of ALT (alanine aminotransferase), AST (aspartate aminotransferase) and GGT (γ-glutamyltransferase) in 30 men exposed to a mixture of organic solvents (mainly, toluene, xylene, acetone, *n*-butyl acetate, *n*-butanol and ethyl acetate) and in a reference group ($n = 20$).

Experimental group	SBA[a] (μmol/L)	Bilirubin (μmol/L)	ALT (μKat/L)	AST (μKat/L)	GGT (μKat/L)	Reference
Exposed[b] group	8 ± 6*	1.9 ± 1.5	0.41 ± 0.23	0.31 ± 0.13	0.40 ± 0.24	Franco *et al.* (1986)
Controls	2.8 ± 1.4	1.9 ± 0.5	0.33 ± 0.21	0.28 ± 0.15	0.45 ± 0.33	

[a] Values are mean ± standard deviation.

[b] Exposure level in mg/m^3 (toluene:3–311, xylene:8-–658, acetone:5–1448, *n*-butyl acetate 18–1683, *n*-butanol 8–96 and ethyl-acetate:24–739).

* Significantly different from the respective control ($p<0.01$).

particular TC and CA. The effects were dose-dependent and were present at doses below those that resulted in increases of serum enzymes (table 4).

Elevated SBA levels in response to solvent exposure in rats were also demonstrated for a wide range of other chlorinated solvents including carbon tetrachloride, chloroform, hexachlorobutadiene and tetrachloroethylene (Bai *et al.* 1992a,b). These solvents were administered via intraperitoneal injection to rats on each of three consecutive days and 4 h after the last treatment blood samples were taken. Analysis of the samples revealed that SBA levels were increased at doses that did not cause any changes of enzyme tests or tissue injury when examined by electron microscopy (tables 5 and 6).

1,1,2-Trichloro-1,2,2-trifluoroethane (FC 113)

A more recent study, although small in sample size, demonstrated that occupational exposure to FC 113 (1,1,2-trichloro-1,2,2-trifluoroethane), a fully halogenated hydrocarbon, at an atmospheric concentration of about 7% of its current TLV, resulted in a two-fold increase in total SBA (as measured by HPLC assay), and up to a five-fold increase in some individual serum bile acids (Neghab *et al.* 1997). Elevated SBA levels, however, were not accompanied by any demonstrable change of serum enzymes or bilirubin (table 7).

1,1,1-Trichloroethane (TCE) and TRI

SBA levels in workers exposed to 1,1,1-trichloroethane (TCE) were reported to be significantly higher than unexposed workers ($p<0.05$). This was evident in the absence of any alteration in serum enzyme tests. Mean levels of total SBA of exposed and unexposed workers were 3.91 ± 0.45 μM ($n = 5$) and 2.63 ± 0.52 μM ($n = 6$), respectively (Hamdan and Stacey 1995). Similar findings were reported for TRI-exposed individuals (Driscoll *et al.* 1992, table 8). Additionally, Neghab *et al.* (1997) showed that in a group of workers occupationally exposed to low levels of

Table 4. Mean levels of significantly elevated serum bile acids (GDC: glychodeoxycholic acid, CA: cholic acid, CDC: chenodeoxycholic acid, DC: deoxycholic acid, TC: taurocholic acid, TUDC: tauroursodeoxycholic acid, TDC: taurodeoxycholic acid) in rats exposed to TRI.

	TRI dose (mmol kg^{-1} body wt, i.p.) [a]				Exposure conditions		
Bile acids	0	0.01	1	5	0 ppm	200 ppm[b]	Reference
GDC	0.3 ± 0.2[c]	0.1 ± 0.1	1.1 ± 0.4*	3.8 ± 0.7*	0.04 ± 0.0	0.04 ± 0.0	Wang and
CA	3.2 ± 0.8	5.8 ± 1.5*	8.9 ± 1.1*	8.4 ± 1.8*	2.3 ± 0.8	23.9 ± 16.9*	Stacey (1990)
CDC	0.8 ± 0.4	0.6 ± 0.3	1.2 ± 0.2*	1.4 ± 0.3*	1.9 ± 0.2	2.1 ± 0.4	
DC	0.6 ± 0.1	1.5 ± 0.3	2.3 ± 0.4	2.3 ± 0.5*	1.5 ± 0.3	2.1 ± 0.5	
TC	0.3 ± 0.1	1.6 ± 0.5*	3.2 ± 0.6*	1.8 ± 0.2*	2.7 ± 0.6	12.8 ± 6.7*	
TUDC	0.1 ± 0.0	0.04 ± 0.0	0.2 ± 0.1*	0.3 ± 0.1*	0.3 ± 0.1	1.1 ± 0.5	
TDC	0.2 ± 0.0	0.2 ± 0.1	0.4 ± 0.1	0.6 ± 0.1*	0.8 ± 0.2	1.9 ± 0.6	
Total	7.3 ± 1.4	10.1 ± 1.9	19.5 ± 1.1*	18.1 ± 4.7*	11.4 ± 2.0	49.1 ± 24.3*	

[a] None of the above doses resulted in any significant increase in serum activities of alanine aminotransferase (ALT), sorbitol dehydrogenase (SDH) and alkaline phosphatase (ALP).

[b] Continuous exposure to TRI over a period of 28 days.

[c] Values are mean ± SE ($n = 4$, 5, or 6), μmol/L.

* Significantly different from the respective control ($p<0.05$).

Table 5. Mean levels of significantly elevated serum bile acids(CA: cholic acid, DC: deoxycholic acid, GC: glychocholic acid, GCDC: glychochenodeoxycholic acid, GDC: glychodeoxycholic acid, GLC: glycholithocholic acid, TC: taurocholic acid, TCDC: taurochenodeoxycholic acid) in rats treated with carbon tetrachloride or chloroform.

	CCl_4 dose (mmol kg^{-1}, i.p.)[a]			$CHCl_3$ dose (mmol kg^{-1}, i.p.)[a]			
Bile acids	0	1	5	0	1	7.5	Reference
CA	1.81 ± 1.34[b]	19.58 ± 7.69*	15.14 ± 7.09*	1.41 ± 0.71	8.81 ± 2.28*	27.10 ± 9.79*	Bai *et al.* (1992b)
DC	0.04 ± 0.0	0.21 ± 0.11	1.30 ± 0.77*	0.23 ± 0.09	0.69 ± 0.31	0.56 ± 0.33	
GC	0.12 ± 0.07	3.67 ± 1.97	3.04 ± 1.41	0.04 ± 0.0	0.86 ± 0.16*	2.88 ± 2.11*	
GCDC	0.04 ± 0.0	0.04 ± 0.0	0.04 ± 0.0	0.11 ± 0.08	0.11 ± 0.05	17.57 ± 8.36*	
GDC	0.12 ± 0.07	3.89 ± 0.39*	6.35 ± 1.38*	0.23 ± 0.19	0.38 ± 0.15	8.44 ± 8.13*	
GLC	0.04 ± 0.0	0.04 ± 0.0	0.32 ± 0.25	0.04 ± 0.0	0.04 ± 0.03	2.54 ± 1.37*	
TC	1.09 ± 0.15	2.81 ± 0.77*	2.55 ± 0.29*	0.61 ± 0.35	2.20 ± 0.57*	5.69 ± 0.77*	
TCDC	0.04 ± 0.0	1.18 ± 0.44*	0.78 ± 0.46*	0.93 ± 0.54	1.08 ± 0.26	0.39 ± 0.24	
Total	3.28 ± 1.66	34.36 ± 10.60*	32.90 ± 9.21*	5.64 ± 1.64	17.25 ± 3.63*	67.47 ± 25.66*	

[a] Only the highest dose of CCl_4 and $CHCl_3$ resulted in a significant rise in serum activities of alanine aminotransferase and aspartate aminotransferase as well as histological alterations in the liver of treated rats.

[b] Values are mean ± SE (n=4), μmol/L.

* Significantly different from the respective control (p<0.05).

Table 6. Mean levels of significantly elevated serum bile acids (CA: cholic acid, CDC: chenodeoxycholic acid, GC: glychocholic acid, GDC: glychodeoxycholic acid, GLC: glycholithocholic acid, TC: taurocholic acid) in rats treated with hexachloro-1,3-butadiene (HCBD) and 1,1,2,2-tetrachloroethylene (TET).

	HCBD dose (mmol kg^{-1}, i.p.)[a]			TET dose (mmol kg^{-1}, i.p.)[b]			
Bile acids	0	0.2	0.4	0	1	10	Reference
CA	1.41 ± 0.71[c]	3.73 ± 1.54*	7.56 ± 2.27*	2.51 ± 1.46	9.94 ± 3.56*	3.72 ± 1.36*	Bai *et al.* (1992a)
CDC	0.65 ± 0.25	0.49 ± 0.23	0.62 ± 0.26	0.33 ± 0.10	1.14 ± 0.40*	1.37 ± 0.48*	
GC	0.04 ± 0.0	0.59 ± 0.34	2.60 ± 2.19	0.46 ± 0.05	1.31 ± 0.78*	2.46 ± 0.57*	
GDC	0.23 ± 0.19	0.12 ± 0.08	0.19 ± 0.11	0.10 ± 0.01	0.20 ± 0.02*	0.34 ± 0.09*	
GLC	0.04 ± 0.0	0.04 ± 0.0	0.04 ± 0.0	0.18 ± 0.03	0.28 ± 0.04	0.41 ± 0.04*	
TC	0.62 ± 0.34	0.96 ± 0.37	3.25 ± 1.22*	0.84 ± 0.18	1.21 ± 0.49	0.66 ± 0.28	
Total	4.29 ± 0.97	7.19 ± 1.69	14.63 ± 3.73*	5.61 ± 1.77	15.54 ± 4.43*	11.15 ± 2.94*	

[a] HCBD at 0.2 mmol kg^{-1} produced a significant increase in serum activity of alkaline phosphatase (ALP) and at 0.4 mmol kg^{-1} resulted in a significant increase in serum activities of both ALP and aspartate aminotransferase (AST).

[b] TET at 10 mmol kg^{-1} significantly increased the activity of serum AST.

[c] Values are mean ± SE ($n = 4$), μmol/L.

* Significantly different from the respective control ($p<0.05$).

Table 7. Fasting concentrations (μmol/L) of significantly elevated serum bile acids (GC: glychocholic acid, GCDC: glychochenodeoxycholic acid, TC: taurocholic acid, TUDC: tauroursodeoxycholic acid, TCDC: taurochenodeoxycholic acid) in control and FC 113-exposed individuals.

	Experimental groups				
		FC 113-exposed[a, b]			
Bile acids	Control	Pre-exposure	Post-exposure	*p*-value	Reference
GC	0.46 ± 0.23[c]	0.46 ± 0.16	2.44 ± 0.43***	0.0003	Neghab *et al.* (1997)
GCDC	1.98 ± 0.34	2.76 ± 0.31	3.91 ± 0.47*	0.007	
TC	0.33 ± 0.06	0.18 ± 0.05	0.78 ± 0.20***	0.009	
TUDC	0.15 ± 0.04	0.12 ± 0.04	0.27 ± 0.03**	0.05	
TCDC	0.59 ± 0.13	0.85 ± 0.19	1.53 ± 0.18***	0.001	
Total	7.10 ± 1.00	7.06 ± 0.74	13.35 ± 1.48***	0.002	

[a] Exposure level 68.2 ± 12.6 ppm (mean ± SE).
[b] Other liver function tests did not differ between exposed workers and controls.
[c] Values are mean ± SE (n = 4,6, or 11).
* Significantly different from control only, ** pre-exposure only, *** both control and pre-exposure (p<0.05).

Table 8. Fasting concentrations (μmol/L) of significantly elevated plasma bile acids (GC: glychocholic acid, GCDC: glychochenodeoxycholic acid, CDC: chenodeoxycholic acid, TC: taurocholic acid, TCDC: taurochenodeoxycholic acid) in TRI-exposed individuals.

Bile acids	Unexposed (n = 6)	Exposed (n = 16)[a,b]	Reference
GC	0.14 ± 0.18	0.57 ± 0.28****	Driscoll *et al.* (1992)
GCDC	0.52 ± 0.39	1.23 ± 0.97**	
CDC	0.28 ± 0.19	0.61 ± 0.48**	
TC	0.00 ± 0.00	0.19 ± 0.22***	
TCDC	0.00 ± 0.00	0.25 ± 0.32**	
Total	2.43 ± 1.29	5.03 ± 2.45***	

[a] Regular exposure of less than 5 ppm for most workers, with peak exposure for two workers over 250 ppm on occasions.
[b] Other liver function tests did not differ between exposed workers and controls.
** p<0.01; *** p<0.001; **** p<0.0001.

TRI (8-h TWA exposure 8.9 ± 3.1 ppm, mean ± SE), the extent to which bile acids were elevated was directly related to the atmospheric concentration of this solvent and there was a very good correlation (r = 0.94) between the degree of increase in serum concentration of total bile acids and level of exposure to TRI. These data for the first time delineated the relationships between exposure (atmospheric concentration of the solvents) and SBA concentration and allowed the issue of the dose–response relationship to be addressed on a quantitative basis.

Toluene

While in several studies a causal association between exposure to halogenated solvents and a rise in SBA levels has been strongly indicated, until recently it was not clear whether this association is exclusive to these particular chemicals or an effect which might be associated with solvents more generally. *In vivo* studies with the

non-halogenated aromatic hydrocarbon solvent, toluene (Neghab and Stacey 1997a), showed that raised SBA levels are not exclusively associated with exposure to halogenated solvents. The study also demonstrated the advantages provided by measurement of total and individual SBA in evaluating the hepatic effects of this solvent. Injection of toluene to rats (i.p.) at a dose that did not change any other parameters of liver integrity, caused a two-fold increase in total SBA and up to a three-fold increase in some individual bile acids (table 9). With increasing dose, a further rise in SBA levels was noted (table 10). This rise, however, was accompanied by significant increases in serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

The effects of halogenated aliphatic and non-halogenated aromatic solvents on hepatobiliary transport of bile acids have been shown to be largely, if not entirely, similar. Therefore, it could be assumed that these effects are general, reversible and non-specific physical effects which may be related to the lipid solubility of these chemicals and their consequent ability to dissolve in the hepatocyte plasma membranes (the site of bile acid uptake).

Table 9. Concentrations of significantly elevated serum bile acids (CA: cholic acid, CDC: chenodeoxycholic acid, DC: deoxycholic acid, TC: taurocholic acid, TCDC: taurochenodeoxycholic acid, TDC: taurodeoxycholic acid) in control and toluene-treated rats.

	Experimental groups			
Bile acids	Control[a]	Toluene-treated[b]	*p*-value	Reference
CA	5.24 ± 0.69[c]	8.34 ± 0.73	0.0039	Neghab and Stacey (1997a)
CDC	0.72 ± 0.22	1.62 ± 0.16	0.0021	
DC	0.97 ± 0.08	1.40 ± 0.21	0.0373	
TC	1.36 ± 0.21	3.62 ± 0.50	0.0005	
TCDC	0.17 ± 0.05	0.51 ± 0.06	0.0006	
TDC	0.29 ± 0.05	0.55 ± 0.08	0.0069	
Total	9.72 ± 0.81	17.24 ± 1.15	<0.0001	

[a] Corn oil treated rats (2 ml kg^{-1} body weight, i.p., for three consecutive days).
[b] Dose (2.3 mmol kg^{-1} body weight, i.p., for three consecutive days).
[c] Values are mean ± SE (n = 8) µmol/L.

Table 10. Mean levels of serum total bile acids, total bilirubin and other liver function tests (ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, 5′-ND: 5′-nucleotidase) in control and toluene treated rats.

Parameter	Control[a]	Treated[b]	Reference
Total bile acids (µmol l^{-1})	10.0 ± 0.9[c]	23 ± 7[*]	Neghab and Stacey 1997a
Total bilirubin (µmol l^{-1})	1.3 ± 0.2	1.6 ± 0.4	
ALT (U l^{-1})	40 ± 4	55 ± 4[*]	
AST (U l^{-1})	52 ± 2	120 ± 33[*]	
ALP (U l^{-1})	135 ± 8	168 ± 20	
5′-ND (U l^{-1})	12 ± 0.4	13 ± 0.4	

[a] Corn oil treated rats (2 ml kg^{-1} body weight, i.p., for three consecutive days).
[b] Dose (9.2 mmol kg^{-1} body weight, i.p., for three consecutive days).
[c] Values are mean ± SE (n = 7 or 8).
[*] Significantly different from the respective control (Student's t-test, p<0.05).

α-Naphthylisothiocyanate (ANIT)

Studies with the non-solvent, cholestatic agent, α-naphthylisothiocyanate (ANIT) showed that direct addition of ANIT to isolated rat hepatocyte suspensions had no effect on *in vitro* transport of bile acids by these cells (Neghab and Stacey 1996a). Hepatocytes isolated from the liver of ANIT-pretreated rats also preserved their uptake and excretory functions. This was evidenced by the fact that the Michaelis constant (K_m) and maximum velocity (V_{max}) for uptake of TC by control and treated cells were similar (K_m values for control and treated cells were 17.7 and 16.9 μM, respectively, whereas mean values for V_{max} for control and treated cells were 2.53 and 2.49 nmol min^{-1} mg^{-1} protein, respectively). Similarly, the initial rate of efflux of TC from hepatocytes isolated from the liver of treated rats was not different from that of controls (0.12 ± 0.01 vs 0.11 ± 0.01 nmol min^{-1} mg^{-1} protein, respectively). *In vivo* treatment of rats with ANIT (50 μmol kg^{-1} i.p. for three consecutive days), however, resulted in a marked increase (five-fold) in total SBA levels in the absence of any remarkable effect on serum enzyme tests (table 11). These observations have provided further support for the proposition that the effects of solvent on SBA are likely to be related to lipid solubility of these compounds.

In vitro and *in vivo* mechanistic studies

The possible mechanisms by which chlorinated solvents may cause an increase in SBA were first investigated by Kukongviriyapan *et al.* (1990). Utilizing isolated rat hepatocytes as an experimental tool, the authors demonstrated that the chlorinated solvents, 1,1,1-trichloroethane (TCE) and tetrachloroethylene (TET), at non-cytotoxic doses inhibited energy-dependent uptake of TC, ouabain and α-aminoisobutyric acid (AIB) which are transported by different systems. The effect was shown to be dose-dependent and reversible and was evident in the absence of any abnormal ultrastructural alteration in solvent-exposed hepatocytes. These solvents, however, had no effect on uptake of cadmium and 3-*O*-methyl-D-glucose (OMG) which are predominantly taken up by non-energy dependent processes (Craik and Elliott 1979, Stacey and Klaassen 1980).

Bai and Stacey (1993a) examined the *in vitro* uptake and efflux of CA and TC by isolated rat hepatocytes in the presence of non-cytotoxic doses of carbon tetrachloride and chloroform. Both *in vitro* uptake and accumulation of bile acids in

Table 11. Mean levels of total serum bile acids, total bilirubin, ALP (alkaline phosphatase), ALT (alanine aminotransferase), AST (aspartate aminotransferase) and LDH (lactate aminotransferase) in control and ANIT-treated rats.

Experimental groups	Total bile acids (μmol/L)	Total bilirubin (μmol/L)	ALP (U/L)	ALT (U/L)	AST (U/L)	LDH (U/L)	Reference
Control[a]	14.7 ± 2.1[b]	1.5 ± 0.1	130 ± 14	40 ± 3	42 ± 2	79 ± 8	Neghab and Stacey (1996a)
ANIT-treated[c]	74.1 ± 9.1[*]	2.1 ± 0.1[*]	173 ± 6[*]	44 ± 6	37 ± 5	95 ± 18	

[a] Corn oil-treated rats (1 ml kg^{-1}, i.p. for three consecutive days).
[b] Values are mean ± SE (n = 4, 6 or 7).
[c] Dose (50 μmol kg^{-1}, i.p. for three consecutive days).
[*] Significantly different from the respective control (Student's t-test, $p<0.05$).

the hepatocytes were shown to be inhibited in the presence of carbon tetrachloride and chloroform. The processes of bile acid transport were also studied in hepatocytes isolated from the liver of rats pretreated with non-hepatotoxic doses of TRI (Bai and Stacey 1993b). These hepatocytes had significantly reduced capacities for uptake and accumulation of bile acids. The effect was dose-dependent and reversible, but there was no effect on efflux of bile acids. Similar findings (table 12) have been reported for the fully halogenated hydrocarbon, FC 113 (Neghab and Stacey 1996b) and the non-halogenated aromatic solvents, toluene (Neghab and Stacey 1997a) and xylene (Neghab and Stacey 1997b). Therefore, the mechanism of solvent-induced elevation of SBA levels has been suggested to be the result of reduced hepatocellular uptake rather than hepatocanalicular secretion (efflux) of bile acids.

Although it is difficult to be sure if efflux from isolated hepatocytes represents the process of bile secretion or a reverse movement across the sinusoidal domain of hepatocytes, or both (Stacey and Kotecka 1988), a few lines of evidence suggest that efflux is occurring in a manner similar to biliary excretion in the intact organ. Firstly, cellular polarity has been demonstrated in freshly isolated hepatocytes by examining cytoskeletal structures (Nickola and Frimmer 1986). Additionally, canalicular marker enzymes have been shown to retain a restricted distribution in the isolated cells (Vonk *et al.* 1978). Moreover, dibromosulphthalein efflux was shown to be similar, on a pharmacokinetic basis, to excretion from the intact organ (Vonk *et al.* 1978, Blom *et al.* 1981). Similar conclusions have been reached by other studies (Schwarz *et al.* 1979, Gewirtz *et al.* 1981, Tarao *et al*, 1982). Finally, different inhibitors have been shown to selectively inhibit either uptake and/or efflux. For instance chlorpromazine inhibits only efflux (Stacey 1988), ethinylestradiol inhibits uptake (Stacey 1986) and cyclosporin A both (Stacey and Kotecka 1988).

Table 12. Initial rates (nmol min^{-1} mg^{-1} protein) of uptake (10 μM) and efflux (25 μM) of taurocholic acid (TC) and cholic acid (CA) by isolated rat hepatocytes in the presence of non-cytotoxic doses of toluene, xylene and 1,1,2-trichloro-1,2,2-trifluoroethane (FC 113).

Solvent	TC uptake	CA uptake	TC efflux	CA efflux	Reference
Toluene (μl/flask)[a]					
0	0.83 ± 0.14[b]	0.31 ± 0.03	0.084 ± 0.02	0.057 ± 0.01	Neghab and
2.5	0.72 ± 0.15	0.16 ± 0.03*	0.090 ± 0.01	0.054 ± 0.01	Stacey (1997a)
10	0.82 ± 0.20	0.08 ± 0.01*	0.073 ± 0.01	0.064 ± 0.01	
Recovery rate (%)[c]	–	88	–	–	
Xylene (μl/flask)[a]					
0	1 ± 0.04	0.31 ± 0.03	0.13 ± 0.01	0.09 ± 0.01	Neghab and
2.5	0.52 ± 0.04*	0.20 ± 0.03*	0.10 ± 0.02	0.08 ± 0.002	Stacey (1997b)
Recovery rate (%)[c]	100	94	-	-	
FC 113 (μl/flask)[a]					
0	0.87 ± 0.05	0.30 ± 0.03	0.08 ± 0.01	0.09 ± 0.03	Neghab and
20	0.57 ± 0.06*	0.22 ± 0.01*	0.07 ± 0.01	0.10 ± 0.02	Stacey (1996b)
Recovery rate (%)[d]	95	85	–	–	

[a] 2.8×10^6 cells/flask in uptake and 1×10^6 cells/flask in efflux experiments.
[b] Values are mean ± SE ($n = 3,4$ or 5).
[c] Recovery of uptake 30 min after reincubation of the exposed cells in clean vessels in open air.
[d] Self recovery 30 min after commencement of the uptake experiments.
* Significantly different from the corresponding control value ($p<0.05$).

Despite the fact that these studies have provided substantial evidence in favour of a distinct relationship between efflux from isolated cells and bile secretion by the intact organ, controversy still exists due to a number of reasons:

1. Because efflux studies in isolated hepatocytes may be distorted by confounding variables such as intracellular metabolism, binding to cytosolic proteins, loss of polarity, etc. (Inoue *et al.* 1982, Duffy *et al.* 1983, Moseley *et al.* 1990).

2. These experimental approaches do not clearly separate transmembrane transport mechanisms from intracellular events (Inoue *et al.* 1982, Duffy *et al.* 1983).

3. Efflux in these experimental models represents both canalicular excretion and sinusoidal back diffusion (Willson *et al.* 1989).

To study the definitive inhibitory effect of solvents on transport of bile acids (in the absence of confounding variables), Neghab *et al.* (1996) have investigated the effects of different doses of FC 113 and TRI on uptake of TC into basolateral and canalicular rat liver plasma membrane vesicles. The authors selected the doses based on the following criteria:

1. A significant inhibition of bile acid uptake by isolated rat hepatocytes in the presence of such doses had been previously demonstrated (Bai and Stacey 1993b, Neghab and Stacey 1996b).

2. They elicit no toxicity or membrane-damaging effects, as indicated by the absence of any effect on integrity of hepatocytes in biochemical and morphological assays (Bai and Stacey 1993b, Neghab and Stacey 1996b).

3. They are physiologically relevant and similar to those found in the plasma of solvent-treated rats (Hamdan and Stacey 1993) with significantly elevated SBA.

Interestingly, both solvents induced significant inhibitory effects on transport of TC into hepatocyte plasma membrane vesicles. The features of this action were in full agreement with those observed in intact cells; that is:

1. Only uptake into basolateral liver plasma membrane vesicles (representing uptake by intact hepatocytes) was inhibited, while no effect on uptake of TC into canalicular plasma membrane vesicles (representing efflux from intact cells) was apparent (table 13).

2. The inhibitory effect was rapid in onset.

3. The effect was clearly dose-related.

Table 13. Effects of non-toxic doses of 1,1,2-trichloro-1,2,2-trifluoroethane (FC 113) and trichloroethylene (TRI) on the initial rate of uptake (V_o) of taurocholate (5 μM) into basolateral (blLPM) and canalicular (cLPM) rat liver plasma membrane vesicles.

Treatment	Solvent dose	V_o (pmole/15s/mg protein) blLPM	V_o (pmole/15s/mg protein) cLPM	Reference
Control	0 μl/tube (0 μM)	60.7 ± 16.1[a]	35.8 ± 10.3	Neghab *et al.* (1996)
FC 113	1 μl/tube (30 μM)	64.4 ± 14.8	35.5 ± 3.2	
FC 113	2.5 μl/tube (72 μM)	23.4 ± 5.8*	35.1 ± 8.0	
Control	0 μl/tube (0 μM)	21.4 ± 2.4	28.6 ± 1.2	
TRI	0.3 μl/tube (1.5 mM)	29.2 ± 10.4	30.7 ± 2.0	
TRI	0.9 μl/tube (3 mM)	6.7 ± 3.2*	32.4 ± 5.0	

[a] Values are mean ± SE ($n = 3$ or 4).

* Significantly different from the corresponding control value (Student's t-test, $p<0.05$).

4. Upon extending the incubation time to 1 h, the effect of FC 113 was reversible. This is consistent with the effect of FC 113 in intact cells (table 12) and is presumably because of the very high volatility and rapid elimination from biological systems (Neghab and Stacey 1996b). However, the effect of TRI, was still present 1 h after incubation. This is also in line with the studies in isolated hepatocytes showing that TRI-exposed hepatocytes required additional treatment (washing and incubation in fresh medium) to restore their uptake function (Bai and Stacey 1993b).

These findings, which are entirely consistent with observations in intact cells, are physiologically relevant and conclusive and provide corroborating evidence to further substantiate the contention that, under low levels of exposure, the mechanism of solvent-induced elevation of SBA is the result of selective, dose-dependent and reversible inhibition of bile acid uptake at the sinusoidal domain of hepatocyte plasma membrane by parent compounds.

The exact reason(s) as to why solvents do not interfere with hepatocellular efflux of bile acids is not known. However, it is possible that the efflux receptor is not sensitive enough to solvents under these conditions (Bai 1993). There is some evidence to support this hypothesis. Structurally, the canalicular liver plasma membrane domain is significantly more rigid than its counterpart, sinusoidal domain (Mills *et al*. 1987), and is less perturbable by chemical agents, probably due to the higher cholesterol and sphingomyelin content (Meier *et al*. 1984). This may explain why this domain is more resistant to change in its fluid state after exposure to solvents (Mills *et al*. 1987) which might be related to a preserved capacity of transport activity. If these hypotheses are true, then upon increasing the dose of solvents, some effects on efflux of bile acids from solvent-exposed hepatocytes would be expected. Application of high doses of solvents is subject to some limitations (for instance due to induction of cytotoxicity which will obviously compromise the results, or reservations about the physiological relevance of such high doses). However, in experiments with FC 113 (Neghab and Stacey 1996b) where a relatively high dose (due to low degree of toxicity) of this solvent (20 µl 1×10^6 cells) was applied to the cell suspensions a significant inhibition of efflux of TC from pre-loaded hepatocytes at some early time points was demonstrated (table 14).

The precise mechanisms by which solvents exert their inhibitory effects on hepatocellular uptake of bile acids are yet to be determined. Inhibition of bile acid uptake by hepatocytes exposed to TCE and TET was shown to be associated with dose-dependent depletion of cellular ATP and reduced activities of the membrane-

Table 14. *In vitro* effects of 1,1,2-trichloro-1,2,2-trifluoroethane (FC 113) on efflux (nmol mg^{-1} protein) of taurocholate (25 µM) from isolated rat hepatocytes.

	Incubation time (min)					
Treatment	1	2	3	4	5	Reference
Control	0.61 ± 0.01[a]	0.46 ± 0.02	0.38 ± 0.03	0.32 ± 0.03	0.28 ± 0.04	Neghab and Stacey (1996b)
FC 113 exposed cells (20 µl per 10^6 cells)	0.64 ± 0.03	0.53 ± 0.03*	0.46 ± 0.01*	0.40 ± 0.02*	0.36 ± 0.03	

[a] Values are mean ± SE (n=4).
* Significantly different from the corresponding control value (Duncan's test, p<0.05).

bound enzymes Na^+-K^+-ATPase and Mg^{2+}-ATPase (Kukongviriyapan *et al.* 1990). The sodium pump is known to be the ultimate driving force for most of the secondary active transport systems (Ronquist 1975) and it is sensitive to inhibition by organic solvents (Pritchard 1979). Uptake of bile acids across the basolateral domain of hepatocytes is mainly a sodium dependent carrier-mediated process, driven by the inwardly directed Na^+ gradient generated by Na^+-K^+-ATPase (Duffy *et al.* 1983). Similarly, it has been shown that uptake of CA was markedly reduced when isolated hepatocytes were equilibrated with nitrogen/carbon dioxide (N_2/CO_2) instead of oxygen/carbon dioxide (O_2/CO_2). This effect was reversible upon reoxygenation. Moreover, depletion of intracellular ATP via replacement of glucose by fructose or by metabolic inhibitors induced similar effects on CA uptake. These effects are believed to be due to impairment of the Na^+ gradient, which is maintained by Na^+-K^+-ATPase (Frimmer and Ziegler 1988). Therefore, a decrease in ATP level and/or depressed activity of cell membrane ATPase may be causally linked with the inhibitory effects of solvent on bile acid uptake (Kukongviriyapan *et al.* 1990).

The exact mechanisms by which solvents may deplete cellular ATP and inhibit the activity of membrane-bound enzymes are not known. However, it has been shown that chlorinated solvents, at non-cytotoxic doses, significantly inhibit hepatocellular oxygen consumption (Berger *et al.* 1986) which can ultimately cause depletion of cellular ATP (Kukongviriyapan *et al.* 1990). Enzyme-inhibiting potencies of organic solvents have also been shown to be a general, reversible, non-specific physical effect based on their lipid solubility and there appears to be no specific chemical interaction between these molecules and membrane components (Dorling and Le page 1972, Sharom and Mellors 1980, Korpela and Tahti 1987). Since cell membranes are a 'primary target' for lipophilic compounds, it is expected that solvents initially affect these structures. Changes in membrane components have dramatic effects on allosteric properties of membrane-bound enzymes (Dorling and Le Page 1972, Barnett and Palazotto 1974, Schneider 1975, La Rocca and Carlson 1979, Sharom and Mellors 1980) including Na^+-K^+-ATPase as an allosteric enzyme in which Na and K act as allosteric effectors (Barnett and Palazotto 1974). This may provide a plausible explanation for altered activity of Na^+-K^+-ATPase at the cell membrane following exposure to solvents.

Thalhammer *et al.* (1993) have shown that a wide range of solvents reversibly inhibit hepatobiliary transport, in isolated perfused rat liver and *in vivo.* These effects were independent of the chemical structure of the solvent, and were largely related to the lipophilicity of the compound, indicating that a physical, non-specific interaction of solvents with membrane lipids may be responsible for the inhibitory effect. Interestingly, these effects were associated with an increase in membrane fluidity and a significant alteration in the activity of membrane-bound enzymes, Na^+-K^+-ATPase and Mg^{2+}-ATPase. Changes in membrane fluidity are known to modulate the function of a variety of membrane-bound properties, including transporter proteins and membrane-bound enzymes (Dorling and Le Page 1972, Barnett and Palazotto 1974, Schneider 1975, La Rocca and Carlson 1979, Sharom and Mellors 1980, Smith and Ploch 1991). With an increase in membrane fluidity, the active site of a functional membrane protein can be buried in the lipid microenvironment as a result of vertical displacement. These conformational changes may result in inactivation of transporter protein and reduction in transport activity (Smith and Ploch 1991).

Taken together, it may be concluded that interference with bile acid transport by solvents is the result of both changes in membrane fluidity as well as disturbance of the energy system at the cell membrane, by modulating the activity of membrane-bound enzymes which ultimately provide the driving force(s) for hepatobiliary transport of bile acids.

Discussion

Utilizing isolated rat hepatocytes and hepatocellular membrane vesicles as experimental tools, it has been demonstrated that reversible inhibition of hepatocellular uptake and accumulation of bile acids, rather than hepatocanalicular secretion of these molecules, is likely to be the cause of raised SBA levels following *in vivo* administration of the organic solvents to experimental animals or after occupational exposure to these compounds.

As discussed elsewhere in this review, a number of studies have been reported during recent years showing that bile acids are elevated in workers exposed to solvents, often when conventional parameters of hepatobiliary function are unaltered (Edling and Tagesson 1984, Liss *et al*. 1985, Franco *et al*. 1986, 1989, Driscoll *et al*. 1992, Hamdan and Stacey 1995, Liu *et al*. 1996, Neghab *et al*. 1997). This has led to the suggestion that subclinical liver disease may be experienced by such workers. The longer term ramifications of such effects have not been confidently determined. Studies with experimental animals have provided data consistent with the observations that exposed workers had increases in serum bile acids on exposure to solvent but without evidence of liver injury as measured by conventional parameters. In the absence of any evidence of liver damage under these conditions it could be assumed that it is unlikely that there would be pathological sequelae to these effects.

These observations are important when deciding on the meaning and consequences of the increases in serum bile acids in exposed workers. At face value, with serum bile acids being regarded as sensitive indicators of liver injury, one might assume that there is liver dysfunction and that this would eventually result in damage to the organ. From the mechanistic data, however, there is the indication that this interference with bile acid levels is readily reversible (table 12), short-lived and without long-term adverse effects (Kukongviriyapan *et al*. 1990, Bai and Stacey 1993a,b, Neghab and Stacey 1996b,1997a,b). Therefore, the observations that solvent-exposed individuals had elevated SBA levels, in the absence of any alterations in serum enzyme tests, provide further support in favour of the notion that SBA levels could potentially be considered as a sensitive biological marker of exposure/effect of solvents and a sensitive tool for early detection of subtle changes in hepatobiliary function of asymptomatic exposed individuals.

Interestingly, while a causal association between occupational exposure to commonly used organic solvents and a significant rise in SBA levels has been found in some studies, varying results have also been reported (Hotz *et al*. 1990, Chen *et al*. 1991, Lundberg *et al*. 1994). These apparent discrepancies may have arisen for different reasons. For instance, the inhibition of bile acid transport by hepatocytes after exposure to solvents, which might account for the raised SBA levels in solvent-exposed workers, has been demonstrated to be a short-lived (table 12) and reversible effect (Kukongviriyapan *et al*. 1990, Bai and Stacey 1993a,b, Neghab and Stacey 1996b, 1997a,b). Therefore, it may be that the negative studies in workers

exposed to solvents have failed to detect a significant rise in SBA concentrations, not because the effect had not occurred, but simply because being reversible, the effect was no longer present (Franco 1991). This assumption is further supported by the results found with ethanol. Utilizing isolated rat hepatocytes (Yipu *et al.* 1987) and rat liver plasma membrane vesicles (Mills *et al.* 1987), it has been shown that this compound interferes with the hepatocellular transport of bile acids. Furthermore, alcohol intake is known to increase SBA levels (Milstein *et al.* 1976, Ahlegren *et al.* 1988). This effect, however, was reported to be reversible within a week after cessation of heavy alcohol intake (Ahlegren *et al.* 1988). Similar observations among solvent-exposed workers have been reported by other studies (Sotaniemi *et al.* 1982, Lundberg *et al.* 1994).

The effects of different solvents on SBA and their hepatic transport have been shown to be largely, if not entirely, similar. Typical chlorinated solvents, carbon tetrachloride and chloroform, at low doses (1 mmol kg^{-1} i.p.) have been shown to induce a significant rise in SBA levels (table 5). Elevated SBA levels, however, were not accompanied by histological alterations or by increased serum activities of liver enzymes. Upon increasing the dose (about five-fold) histological alterations in the liver of the treated rats as well as significant increases in the activities of serum enzymes were noted. These findings suggest that the sensitivity of total SBA as a screening tool is greater than the classical serum enzyme tests. Similar observations were made with the non-halogenated aromatic solvent, toluene (tables 9 and 10). Administration of this solvent at a low dose to rats (2.3 mmol kg^{-1} i.p.) resulted in a significant rise in the concentrations of total and individual SBA. Upon increasing the dose of toluene to a level (9.2 mmol kg^{-1} i.p. or four-fold higher) which has been shown to cause histopathological alterations in the liver (Ungvary *et al.* 1976), significant increases in the activity of serum enzymes (ALT and AST) were also noted (Neghab and Stacey 1997a). These results, on a quantitative basis, also support the previous findings in which total SBA levels were shown to be about five-fold more sensitive than the classical serum enzyme tests (Bai *et al.* 1992b).

Determination of individual SBA and subtotalling into groups based on their chemistry (i.e. taurine-conjugated, glycine-conjugated and unconjugated bile acids) has been shown to provide even greater sensitivity than simply using total SBA levels. In particular, some individual bile acids, notably CA and TC, have been suggested to be two of the bile acids (tables 4, 5 and 6) where changes in levels are the most sensitive response to exposure (Wang and Stacey 1990, Bai *et al.* 1992a,b, Hamdan and Stacey 1993). Serum cholate levels are also thought to be more sensitive and discriminant than other routine liver function tests for the detection of various hepatobiliary diseases as well as being a valid index of the severity of liver disease (Azer *et al.* 1997a). Although the molecular mechanism for the high sensitivity and specificity of individual bile acids in general, and TC and CA in particular awaits elucidation, the fact that CA and TC were also two of the most significantly elevated bile acids in the serum of toluene-treated rats (table 9) as well as in response to the cholestatic agent, α-naphthylisothiocyanate (Wang and Stacey 1990, Thompson *et al.* 1993) and the potent immunosuppressive agent, cyclosporin A (Azer and Stacey 1994), indicates that these bile acids could be considered as potentially valuable and sensitive tools in studying and evaluating the hepatobiliary effects of chemicals (Wang and Stacey 1990).

The great similarities between the effects of different solvents on hepatobiliary transport of bile acids indirectly indicate that these effects, similar to enzyme-

inhibiting potencies, are likely to be general, reversible and non-specific physical effects related to lipid solubility of these chemicals. Furthermore, these findings support the notion that the ability of solvents to interfere with hepatocellular transport of bile acids, with corresponding rise in serum concentrations, may be a general effect of these compounds which could be attributed to lipophilic parent compounds with solvent properties.

The following argument may provide further support for these assumptions. A wide range of chlorinated aliphatic hydrocarbon solvents (carbon tetrachloride, TRI, trichloroethane, tetrachloroethylene, etc.) as well as non-chlorinated aromatic hydrocarbon solvents (toluene, benzene, xylene, etc.) at non-cytotoxic or membrane-damaging doses have been shown to alter the fluidity of the hepatocyte plasma membrane and inhibit the activities of membrane-bound enzymes, in particular the basolateral enzyme marker, Na^+-K^+-ATPase (Dorling and Lepage 1972, Kukongviriyapan *et al*. 1990, Tahti and Naskali 1992, Thalhammer *et al*. 1993, Vaalavirta and Tahti 1995). These effects have been shown to be dose-related and reversible and mainly dependent upon the lipophilicity of the solvent regardless of its chemical structure. These changes have been implicated as the underlying mechanisms which should explain the ability of solvents to interfere with hepatocellular uptake of bile acids (Kukongviriyapan *et al*. 1990). In the light of this information, one might speculate that there should be a correlation between the degree of lipophilicity of a solvent and its ability to inhibit hepatocellular uptake of bile acids. In fact, this was found, at least qualitatively, to be the case as:

(1) Xylene, an aromatic hydrocarbon, possesses greater lipid solubility properties than its monomethylated homologue toluene (oil/water partition coefficient for different isomers of xylene at 37 °C varies between 1658 and 2353, whereas the corresponding value for toluene is only 659, Sato and Nakajima 1979b). This solvent, at a dose of 2.5 µl/2.8 × 10^6 cells, significantly inhibited both the initial rates of uptake (table 12) and hepatocellular accumulation of CA and TC (Neghab and Stacey 1997b), whereas accumulation of these bile acids by hepatocytes (Neghab and Stacey 1997a) and the initial rate of uptake of TC (table 12) in the presence of a similar dose of toluene were unaltered. Upon increasing the dose, however, toluene was able to significantly inhibit hepatocellular accumulation of both CA and TC. Similarly, FC 113, a highly volatile solvent with relatively poor lipid solubility (octanol/water partition coefficient of 46, Hazardous Substances Data Bank 1994) was able to produce similar effects on bile acid transport only at a dose (20µl/2.8 × 10^6 cells) eight-fold higher (table 12) than that of toluene (Neghab and Stacey 1996b).

(2) Similar conclusions could also be reached when the data obtained by other studies are analysed. The effects of non-cytotoxic doses of TCE and TET on hepatocellular uptake of TC and other actively-transported substrates and on the activities of membrane-bound enzymes Na^+-K^+-ATPase and Mg^{2+}-ATPase were investigated (Kukongviriyapan *et al*. 1990). TET, which is a more lipid soluble solvent (oil/water partition coefficients for TET and TCE at 37°C are 4458 and 383, respectively, Sato and Nakajima 1979a) was a more potent inhibitor of enzyme activity. Similarly, initial rates of uptake of TC, ouabain and AIB in the presence of TET (2 µl/2.8 × 10^6 cells) were inhibited to a greater extent, whereas a higher dose (up to 2.5-fold) of TCE was required to produce similar results. Comparison of chloroform and carbon tetrachloride (oil/water partition coefficients at 37 °C, 115 and 1444, respectively, Sato and Nakajima, 1979a) also revealed that the minimum

dose, sufficient to induce a significant decrease in the initial rate of uptake of bile acids, is at least two times higher for chloroform than for carbon tetrachloride (Bai and Stacey 1993a). These studies provided evidence to show that one of the physical properties of the solvents (fat solubility) is likely to be a contributing factor to the inhibitory effects of these chemicals on hepatocellular transport of bile acids. Furthermore, these data, although *in vitro*, for the first time provided plausible biochemical and cellular bases to explain elevated SBA levels associated with exposure to organic solvents in the absence of any other pathological effects.

Based on the experiments with TCE and TET, one might also tentatively conclude that:

(1) Solvents do not selectively interfere with transport systems at the cell membrane as interference with at least three different systems by TCE and TET has been noted (see '*In vitro* and *in vivo* mechanistic studies').

(2) Energy systems at the cell membrane may be involved in the inhibitory effect of solvents on the transport of bile acids. Moreover, a decrease in ATP levels and/or depressed activity of cell membrane ATPase may be causally linked with this inhibition.

A few other lines of circumstantial evidence have also provided further support for the proposition that interference with hepatocellular transport and accumulation of substrates by solvents may be an effect that is associated with intrinsic properties of these compounds based on their solvent properties. These include:

(1) The findings of *in vitro* and *in vivo* studies with the non-solvent, cholestatic agent ANIT. Direct *in vitro* addition of sub-cytotoxic doses of ANIT (up to 100 μM) had no effect (Neghab and Stacey 1996a) on either uptake or efflux of TC (one of the most significantly elevated bile acids in the serum of ANIT-treated rats (Wang and Stacey 1990, Thompson *et al.* 1993)). An uninterrupted enterohepatic pathway in intact animals has been shown to be essential for ANIT to express its cholestatic properties and toxic effects (Plaa and Priestly 1977, Traiger *et al.* 1984). Therefore, the authors reasoned that it is possible that the effects of ANIT on bile acid transport, if any, may not necessarily occur directly under *in vitro* exposure. Accordingly, the transport of bile acids by hepatocytes isolated from ANIT-pretreated rats was also studied. Although *in vivo* administration of ANIT to rats (50 μmol kg^{-1} i.p.) resulted in a marked (five-fold) increase in total SBA levels (table 11), hepatocytes isolated from the liver of these rats preserved both their uptake and excretory capacities. This was evidenced by the fact that both uptake and efflux of TC by these cells were similar to those of control hepatocytes. These results not only provide indirect evidence to further support a role for fat solubility of solvents as a contributing factor to the effects of these compounds on hepatocellular transport of bile acids, but also are consistent with the postulated mechanism of action of ANIT which involves an increased permeability of hepatic tight junctions as a causal factor in elevated SBA levels associated with this hepatotoxicant (Krell *et al.* 1982, Kan and Coleman 1986). Moreover, they show that SBA may not only be considered as a sensitive biological marker of exposure/effect of halogenated and non-halogenated organic solvents, but also extend their sensitivity in response to another hepatotoxicant with a different mechanism of action.

(2) The studies with TRI (Hamdan and Stacey 1993) showing that after *in vivo* administration of this solvent to experimental animals there is a close correlation and temporal relationship between the peak plasma concentrations of the solvent

and the peak SBA levels. However, no such association exists between SBA levels and more hydrophilic and polar metabolites of the solvent (peak plasma concentrations of TRI, but not its metabolites, and bile acids occurred simultaneously, at 4 h after dosing). These findings suggest that increased SBA levels following exposure to solvents are likely to be a property that could be attributed to the lipophilic parent compounds with solvent properties rather than their more hydrophilic metabolite(s).

(3) The findings that the non-metabolizable solvent, FC 113, both induced a significant rise in SBA levels (table 7) of exposed individuals (Neghab *et al.* 1997) and inhibited hepatocellular uptake (table 12) of bile acids (Neghab and Stacey 1996b).

(4) The observations that both TRI and FC 113, without undergoing metabolism, inhibited TC uptake into basolateral rat liver plasma membrane vesicles (table 13), as a preparation devoid of metabolic activity (Neghab *et al.* 1996).

Collectively, these results suggest that increased SBA levels, following *in vivo* administration of solvents to experimental animals or after occupational exposure to these compounds, are likely to be a consequence of reduced hepatocellular uptake of bile acids rather than an interference with bile acid efflux.

The nature of the inhibitory action of solvents on hepatocellular uptake of bile acids, on a kinetic basis, has been investigated in a few studies. Regardless of the chemical structure, FC 113 (Neghab and Stacey 1996b), toluene (Neghab and Stacey 1997a), and TRI (Bai and Stacey 1993b) were shown to non-competitively inhibit uptake of TC and CA (table 15). This suggests that solvents do not bind to membrane receptors for the uptake of bile acids (Kukongviriyapan and Stacey 1988). Rather, it reflects a reduced number of translocation carrier sites with a similar apparent affinity which is consistent with conformational changes in transporter proteins at the cell membrane, reduction in binding sites and inactivation and reduction of transport activities as a result of solvent exposure (Smith and Ploch 1991). The non-competitive inhibition is also in line with the view that organic solvents, as lipophilic compounds, are generally taken up by

Table 15. Effects of toluene, 1,1,2-trichloro-1,2,2-trifluoroethane (FC 113) and trichloroethylene (TRI) on kinetic parameters of taurocholic acid (TC) and/or cholic acid (CA) uptake by isolated rat hepatocytes.

Bile acid	Treatment	V_{max} (nmol min^{-1} mg^{-1} protein)	K_m (μM)	Reference
CA	Control	1.57 ± 0.25[a]	31.1 ± 2.9	Neghab and Stacey
	Toluene (2.5 μl/2.8 × 10^6 cells)	0.97 ± 0.07*	33.2 ± 4.0	(1997a)
TC	Control	2.26 ± 0.35	25.5 ± 3.5	Neghab and Stacey
	FC 113 (20 μl/2.8 × 10^6 cells)	1.31 ± 0.12*	21.7 ± 2.9	(1996b)
CA	Control	1.98 ± 0.06	90.98 ± 3.98	Bai and Stacey
	TRI[b]	1.30 ± 0.07*	87.27 ± 3.58	(1993b)
TC	Control	0.81 ± 0.08	27.25 ± 5.23	Bai and Stacey
	TRI[b]	0.61 ± 0.03*	27.78 ± 2.99	(1993b)

[a] Values are mean ± SE (n = 4, 5, or 6).

[b] Parameters of uptake by hepatocytes isolated from the liver of rats 4 h after dosing with 1 mmol kg^{-1} TRI.

* Significantly different from the corresponding control value (p<0.05).

hepatocytes by simple diffusion (Astrand 1975) and there is no competition between these molecules and bile acids for binding to membrane components.

Conclusions

(1) SBA levels provide a sensitive tool for detection of early, minor and subtle subclinical changes in liver function associated with occupational exposure to low levels of halogenated and non-halogenated solvents. Therefore, SBA are clearly superior to traditional liver function tests for screening liver effects in asymptomatic workers with current occupational exposure to organic solvents.

(2) Studies with membrane vesicles have provided corroborating evidence to support the notion that, under low levels of exposure, the mechanism of solvent-induced elevation of SBA is the result of selective, dose-related and reversible inhibition of bile acid uptake at the sinusoidal domain of hepatocytes by parent compounds. Additionally, these studies lend some support to the hypothesis that the effect of a solvent on hepatobiliary transport of bile acids is a property that can be attributed to the lipophilic parent compound rather than a more hydrophilic metabolite(s).

(3) The great similarities between the effects of different solvents on hepatocellular transport of bile acids suggest that these may be general effects of these compounds. Studies with the non-solvent, cholestatic agent ANIT provided further support in favour of a role for physical properties of solvents (fat solubility) as a contributing factor to the inhibitory effects of these chemicals on hepatocellular transport of bile acids and the subsequent rise in serum concentrations of these molecules among exposed individuals. Additionally, these studies directly demonstrated that SBA levels could also provide a valuable and sensitive indicator of hepatobiliary dysfunction associated with exposure to other hepatotoxicants with different mechanisms of action.

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