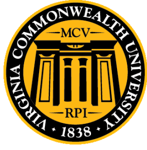

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This is to certify that the dissertation prepared by Todd L. Kelson entitled "Sjögren-Larsson Syndrome: Genetic Studies and Biochemical Characterization of Human Fatty Aldehyde Dehydrogenase" has been approved by his committee as satisfactory completion of the dissertation requirements for the degree of Doctor of Philosophy.

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Sjögren-Larsson Syndrome:

Genetic Studies and Biochemical Characterization of Human Fatty Aldehyde Dehydrogenase

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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LIST OF ABBREVIATIONS

ADH: Alcohol dehydrogenase
ALDH: Aldehyde dehydrogenase
AMP: 2-Amino-2-methyl-1-propanol
5'AMP: Adenosine 5'-monophosphate
AMPSO: 3-[(1,1-Dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid
BSA: Bovine serum albumin
CVS: Chorionic villus sampling
DEAE: Diethylaminoethyl
DOPAL: 3,4-Dihydroxyphenylacetaldehyde
EDTA: Ethylenediaminetetraacetic acid
FADH: Fatty alcohol dehydrogenase
FALDH: Fatty aldehyde dehydrogenase
FAO: Fatty alcohol: NAD^+ oxidoreductase
GABA: gamma-Aminobutyraldehyde
HCl: Hydrochloric acid
HPLC: High pressure liquid chromatography
H_2SO_4: Sulfuric acid
kDa: Kilodaltons
LTB_4: Leukotriene B_4
MEM: Modified Eagle's medium
MES: 2-[N-Morpholino]ethanesulfonic acid
MOPS: 3-[N-Morpholino]propanesulfonic acid
NaCl: Sodium chloride
PAF: Platelet aggregating factor
PAGE: Polyacrylamide gel electrophoresis
p-CMB: para-Chloromercuribenzoate
PMSF: Phenylmethylsulfonyl fluoride
SDH: Succinate dehydrogenase
SDS: Sodium dodecyl sulfate
SLS: Sjögren-Larsson syndrome
TCA: Trichloroacetic acid
TCDD: 2,3,7,8-Tetrachlorodibenzo-p-dioxin
TES: N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid
$TiOSO_4$-H_2SO_4: Titanium oxysulfate-sulfuric acid complex
TLC: Thin-layer chromatography
XLRI: X-linked Recessive Ichthyosis

Sjögren-Larsson syndrome: Genetic studies and biochemical characterization of human fatty aldehyde dehydrogenase

ABSTRACT

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Todd L. Kelson

Virginia Commonwealth University

Advisor: William B. Rizzo, M.D.

Sjögren-Larsson syndrome (SLS) is an autosomal recessive disorder due to deficiency of the fatty aldehyde dehydrogenase (FALDH) component of fatty alcohol:NAD^{+} oxidoreductase (FAO). We investigated the enzymatic defect in SLS in order to elucidate the role of FALDH in fatty aldehyde and fatty alcohol metabolism.

Genetic studies were performed to investigate carrier detection for SLS. Cultured skin fibroblasts from normal controls, SLS obligate heterozygotes, and SLS homozygotes were assayed for FAO and FALDH activities using 18-carbon substrates. In SLS homozygotes, mean FAO and FALDH activities were 8% of normal, and there was no overlap between the homozygote and heterozygote ranges. We demonstrated that mean FAO activity in SLS obligate heterozygotes was 78 ± 16% of normal (mean ± SD); whereas mean FALDH activity was 60 ± 15% of normal. Using both FAO

and FALDH assays in concert, none of the SLS obligate heterozygotes (n=11) had both FAO and FALDH activities which overlapped the normal range (n=12). Therefore, we conclude that measurement of FAO and FALDH activities in cultured skin fibroblasts using 18-carbon substrates is useful for SLS carrier detection.

Prenatal diagnosis was undertaken to monitor FAO and FALDH activities in four pregnancies at-risk for SLS. Enzymatic results in one case using cultured amniocytes obtained during the second trimester predicted an affected SLS fetus, which was confirmed at termination of the pregnancy. Another at-risk fetus was predicted to be affected with SLS using cultured chorionic villi cells obtained during the first trimester, and fetal skin fibroblasts obtained after termination of the pregnancy confirmed a profound deficiency of FAO and FALDH activities. Two other fetuses were correctly predicted to be unaffected. These results demonstrate that SLS can be diagnosed prenatally during either the first or second trimester of pregnancy using enzymatic methods.

In order to better understand the role of FALDH in fatty alcohol oxidation, we determined the subcellular localization of FALDH in human liver, a human cultured hepatocyte cell line (HepG2), and human cultured skin fibroblasts. Differential centrifugation and density gradient centrifugation in Nycodenz were utilized to

separate subcellular organelles. Organelle-specific enzyme markers confirmed the subcellular separations that were attained. FALDH activity was primarily localized to the microsomes in human liver, a cultured HepG2 cells, and human cultured skin fibroblasts.

FALDH was solubilized from human liver microsomes and purified 167-fold by column chromatography. Purified FALDH had a subunit molecular weight estimated by SDS-PAGE to be 54,000 daltons. Gel filtration and nondenaturing polyacrylamide gel electrophoresis of purified FALDH indicated that the enzyme formed large, polymeric protein aggregates with a molecular weight greater than 500,000 daltons. FALDH was NAD^+-dependent, had optimal catalytic activity at pH 9.8, and was thermolabile at 47°C. K_m and V_{max} values were determined for saturated and unsaturated aliphatic aldehydes ranging from 2 to 24 carbons in length, as well as dihydrophytal, a 20-carbon branched chain aldehyde. FALDH was active towards long-chain fatty aldehydes greater than 6 carbons in length. FALDH was sensitive to inhibition by disulfiram, iodoacetamide, iodoacetate, α,p-dibromoacetophenone, and p-chloromercuribenzoate, but it was unaffected by magnesium ions. These investigations represent the first purification and characterization of human microsomal FALDH.

CHAPTER 1

Introduction

SJÖGREN-LARSSON SYNDROME

HISTORICAL BACKGROUND

In 1957, two groups of investigators independently described a genetic syndrome that was subsequently called Sjögren-Larsson syndrome (SLS) (Sjögren and Larsson, 1957; Soderhjelm and Enell, 1957). Sjögren and Larsson reported 28 patients from northern Sweden with congenital ichthyosiform erythrodermia, low-grade oligophrenia (mental retardation), and spastic pyramidal symptoms (spasticity). Soderhjelm and Enell described three other children from northern Sweden who had similar symptoms to those described by Sjögren and Larsson. Prior to this time, a patient with ichthyosis and spastic diplegia was described by Pisani and Cacchione in Italy in 1935 (Pisani and Cacchione, 1935), although the clinical features of that patient were not typical of SLS. In 1940, Bredmose in Denmark probably reported the first patients with typical SLS (Bredmose, 1940). To date, over 200 cases of SLS from throughout the

world among many ethnic and racial groups have been described (Jagell *et al.*, 1981).

CLINICAL FEATURES

SLS is characterized by a triad of cardinal symptoms: congenital ichthyosis, mental retardation, and spasticity. Congenital ichthyosis was present in all of the 28 cases studied by Sjögren and Larsson (Sjögren and Larsson, 1957). The characteristic pattern of scaling is usually apparent at birth, but in some cases ichthyosis did not develop until the patient was several months old. Affected individuals demonstrate varying degrees of ichthyosis from mild to moderate in severity. The distribution of the skin lesions are generalized, with predilection for the neck, elbows, knees, and lower extremities. These lesions do not usually occur on the central face, hands, and feet (Chaves-Carballo, 1987; Williams, 1990; Williams and Elias, 1986).

Intelligence was studied in a cohort of 35 Swedish patients (Jagell and Heijbel, 1982). All were moderately retarded with an IQ lower than 70. The majority had IQs below 50 and were thus classified as severely mentally retarded. There was no history indicating a progression of the mental retardation in these patients.

Spastic paralysis (spastic di- or tetraplegia) is usually manifested by three years of age (Chaves-Carballo, 1987; Theile, 1974). The patients' gait is severely

disturbed with pronounced contractures in the hip and knee joints, thus giving these patients the appearance that they are walking on their toes. About 75% of patients with SLS are unable to walk without assistance. SLS is not a neurodegenerative disorder because neurologic function of the upper and lower extremities and mental retardation do not worsen with increasing age (Jagell and Heijbel, 1982).

In addition to the three cardinal symptoms there may be other clinical signs and symptoms. These include glistening white dots on the retina, speech defects, seizures, kyphosis of the thoracic spine, and short stature (Chaves-Carballo, 1987). Retinal glistening white dots and other changes in retinal pigmentation have been reported in about half of the patients (Theile, 1974). Speech defects consisted of stammering and single, often incomprehensible, syllables (Chaves-Carballo, 1987). It is not known if the speech abnormalities are due to mental retardation or to a separate neurological abnormality. Epileptic seizures or convulsions (not due to febrile illness) characterized by abnormal electroencephalograms were noted in some patients (Chaves-Carballo, 1987). Kyphosis and scoliosis were seen in a small number of patients, with muscular weakness not being a contributing factor (Theile, 1974). Most of the patients with SLS are shorter than average for their population, in part due to leg contractures (Chaves-Carballo, 1987). Height may be difficult to measure

accurately because of the hip and knee joint contractures.

PATHOLOGY OF SLS

The dermatopathology of SLS is well characterized (Goldsmith *et al*., 1971; Matsuoka *et al*., 1982; Jagell *et al*., 1987; Ito *et al*., 1991; Williams, 1990). Affected infants show varying degrees of erythroderma, fine scaling along with larger, plate-like scales; or follicular hyperkeratosis without scales; however, a true collodion membrane rarely occurs. There is acanthosis and papillomatosis. The epidermis is hyperproliferative and the stratum granulosum is thickened. Goldsmith *et al*. (1971) observed a small degree of vasodilatation and perivascular infiltration of round cells and fibroblasts. Studies of the skin by electron microscopy revealed within keratinocytes prominent Golgi apparatus and increased numbers of mitochondria (Matsuoka *et al*., 1982). In both affected and unaffected skin, laminated membranous inclusions were present in cornified cells of the epidermis (Ito *et al*., 1991).

Pathologic examination of the central nervous system in clinically typical SLS patients has been reported in two autopsies (Baar and Galindo, 1965; Sylvester, 1969) and has been summarized in two other Swedish SLS patients (Wester *et al*., 1991). Baar and Galindo (1965) found asymmetrical atrophy of the caudate and demyelination in the frontal

lobes. In contrast, Sylvester (1969) found no apparent loss of neurons in the caudate or hypothalamus. Considerable loss of myelin was noted in the centrum semiovale. Astrocytes were increased in number. Both medullary pyramids showed poor myelin content. Wester *et al.* (1991) reported one patient with mild loss of cerebellar cortical neurons, whereas another patient, who developed Parkinsonian signs, showed widespread loss of myelin and the presence of macrophages containing myelin breakdown products. Mental retardation and spasticity in SLS may be a result of demyelination as evidenced by neuropathological examinations.

GENETICS AND PREVALENCE

SLS is inherited as an autosomal recessive trait with 100% penetrance (Sjögren and Larsson, 1957). The prevalence of SLS in all of Sweden (based on 52 patients born between 1901-1977) is 1 in 167,000. The prevalence is 1 in 37,000 in Norbotten county, a neighboring county where this disorder was first described; 1 in 10,000 in Vasterbotten county where all of the cases originated; and 1 in 6,000 in a subregion of Vasterbotten and Norbotten counties. Jagell *et al.* (1981) reported that 50 of 58 patients in their study were either born in the region of Vasterbotten and Norbotten counties or had ancestors born there. The SLS gene frequencies estimated from the

prevalence rates are 0.002 in the whole of Sweden, 0.005 in the county of Norbotten, 0.010 in the county of Vasterbotten, and 0.013 in the SLS region of Vasterbotten and Norbotten (Jagell *et al*., 1981). The heterozygote frequencies calculated from these figures are 1 in 200 in Sweden, 1 in 100 in Norbotten county, 1 in 50 in Vasterbotten county, and 1 in 39 in the SLS region (Jagell *et al*., 1981).

The high frequency of SLS in northern Sweden may be due to a founder effect. The major mutation of the SLS gene may have been introduced into this population from a settler moving into the area or may have arisen as a new mutation from an inhabitant of this region (Iselius and Jagell, 1989). Census records dating to A.D. 1413 indicate that there were about 600 persons living in this region. Because of the geographic isolation of northern Sweden, consanguineous marriages and, hence, inbreeding may have been more common and therefore contributed to the high incidence of this recessive disorder.

Cytogenetic studies, including chromosome G-banding, were normal on primary cell cultures from a skin biopsy of an affected male (Book *et al*., 1963). No chromosomal abnormalities were found in blood cultures from one other male proband (Hooft *et al*., 1967).

TREATMENT

Treatment of SLS is mainly supportive. Physical therapy and orthopedic care may prolong mobility, but most patients still require leg braces or wheelchair assistance (Chaves-Carballo, 1987).

Seven SLS patients treated with etretinate, a synthetic retinoid, showed improvement of their ichthyosis after one month (Jagell and Lidén, 1983). The use of this drug, however, is limited to adults because it has potent, growth-inhibiting side effects in children.

Dietary therapy has also been investigated (Hooft *et al.*, 1967; Chaves-Carballo *et al.*, 1981; Maaswinkel-Mooij *et al.*, 1993). Hooft *et al.* (1967) described an 8-year-old SLS patient with exudative enteropathy who was given a diet containing only 8-carbon to 10-carbon fatty acids as fat. The exudative enteropathy disappeared within the first few weeks on the diet. A marked improvement in psychic and motoric condition were noted. Furthermore, the ichthyosiform rash completely disappeared. This improvement persisted after more than a year on the diet.

Chaves-Carballo *et al.* (1981) reported an 8-year-old SLS patient who was placed on a diet consisting of 20% of total daily caloric intake as the 8-carbon and 10-carbon saturated fatty acids, which was increased to 40% of total daily caloric intake after 5 months on the diet. During the 12-month period of treatment, skin scaliness and

dryness markedly improved; but there was no improvement in neurologic dysfunction.

Maaswinkel-Mooij *et al*. (1993) recently described the clinical results of dietary treatment in five SLS patients ranging in age from 5 months to 8 years. The patients were treated for about one year with a diet that was restricted in fat (15-20% of total caloric intake) and supplemented with medium-chain triglyceride oil to a total fat caloric intake of 45%. Clinical examinations showed no improvement of the skin lesions or the neurological symptoms in any of the five patients after one year on this diet. Moreover, in two patients (aged 5 and 8 months at the start of dietary therapy), mental retardation and spasticity developed progressively in the second year of life while on this diet.

These results indicate that a low fat diet supplemented with medium-chain fatty acids showed inconsistent and negative results in several SLS patients. The dietary therapies reported to date were attempted before the primary defect in SLS was elucidated. Now that the biochemical defect is known, an appropriate diet selectively deficient in fatty alcohol should be devised.

METABOLIC STUDIES IN SLS

Several disorders of ichthyosis, such as Refsum disease, X-linked recessive ichthyosis, Chanarin syndrome,

infantile Gaucher disease, and autosomal recessive rhizomelic chondrodysplasia punctata, are caused by defects in lipid metabolism (Williams and Elias, 1986). The ichthyosis of patients with SLS has some features in common with that seen in patients with essential fatty acid deficiency. To determine if there was an abnormality in essential fatty acid metabolism or absorption, Hernell *et al.* (1982) studied the fatty acid patterns of plasma phospholipids, cholesteryl esters, triglycerides, and free fatty acids in 11 SLS patients. Their findings indicated that all 11 SLS patients had decreased products of delta-6 desaturation. Avigan *et al.* (1985) subsequently reported that the relative concentrations of 18:3 (n6), 20:3 (n6), and 20:4 (n6) fatty acids, and the activity of delta-6 desaturase were normal in cultured skin fibroblasts from SLS patients. No errors in amino acid, organic acid, or carbohydrate metabolism were found (Holmgren *et al.*, 1981).

Because of the distinctive association of cardinal symptoms (ichthyosis, mental retardation, and spasticity), Rizzo *et al.* (1989) suspected a defect in fatty alcohol metabolism in SLS. Plasma fatty alcohol concentrations were measured in eight SLS patients (Rizzo *et al.*, 1989). The concentration of tetradecanol (14-carbon) was normal in all but one patient; whereas the mean concentration of plasma hexadecanol (16-carbon) was increased two-fold above normal and plasma octadecanol (18-carbon) was increased

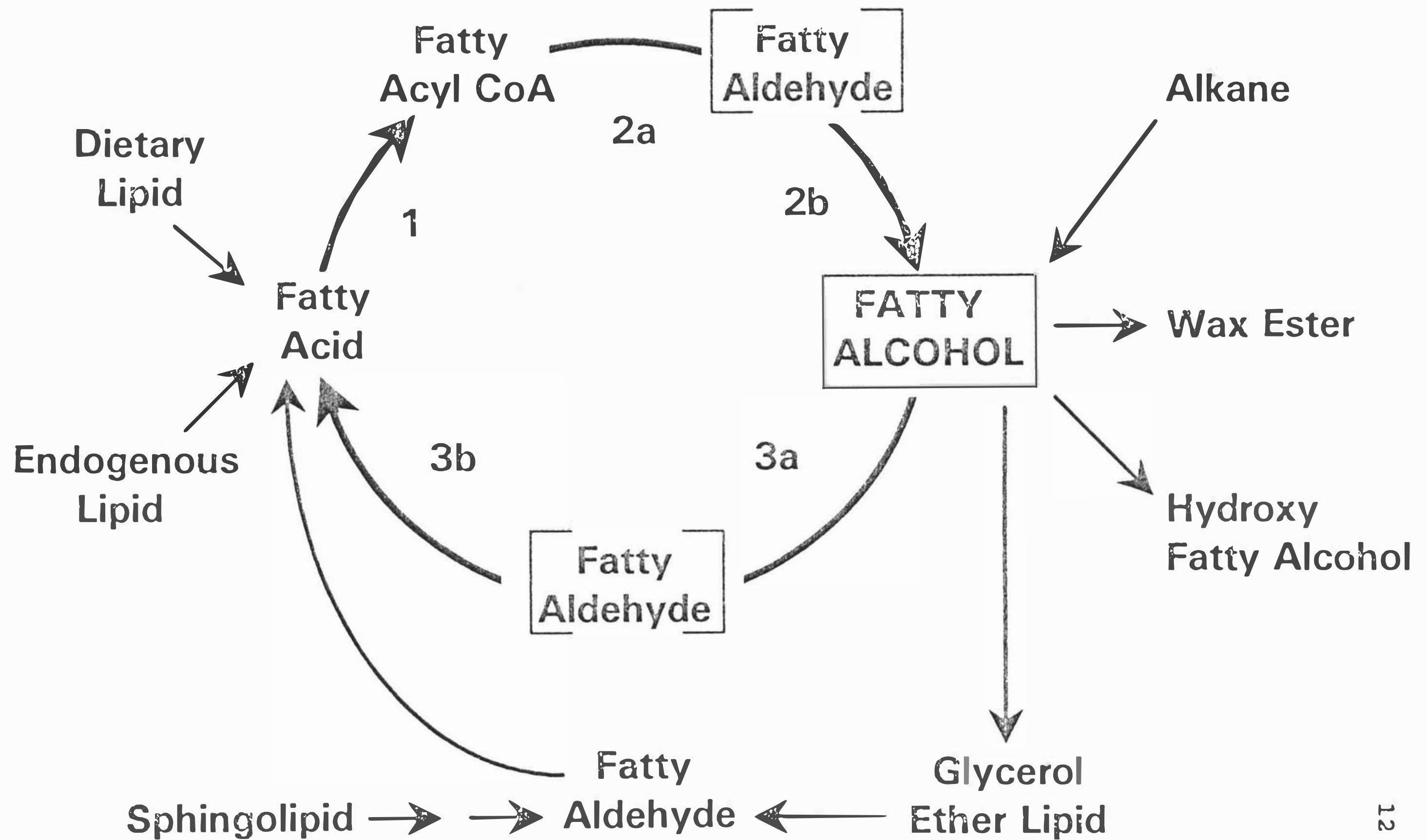
almost three-fold above normal. In order to understand the role of fatty alcohols and their accumulation in SLS, fatty alcohol synthesis and degradation were investigated.

FATTY ALCOHOL METABOLISM

Fatty alcohols (carbon chain length ≥ 12) have been detected in bacteria (Singer and Finnerty, 1985; Naccarato *et al.*, 1972; Day *et al.*, 1970), insects (Tulloch, 1970), plants (Moreau and Huang, 1979; Kolattukudy, 1970), and mammals (Takahashi, T. and Schmid, 1970). Wax esters are abundant in germinating seeds in some plant species. These wax esters are catabolized to provide carbon skeletons and energy for growth (Moreau and Huang, 1979). In marine organisms, free and esterified fatty alcohols are very abundant and play an important role in energy storage (Nevenzel, 1970). Long-chain fatty alcohols and aldehydes are components of sex pheromones in insects and play a role in species attraction (Blatter *et al.*, 1990).

Fatty alcohols are synthesized from either fatty acid or alkane precursors. The main pathway for generating fatty alcohol is through the enzymatic reduction of fatty acids (Riendeau and Meighen, 1985). Based on studies in human cultured skin fibroblasts, a fatty alcohol cycle has been proposed to describe fatty alcohol metabolism (Rizzo *et al.*, 1987) (Figure 1).

Figure 1. The fatty alcohol cycle in mammals. The enzymes shown are as follows: 1) acyl-CoA synthetase; 2a and 2b) acyl-CoA reductase; 3a) fatty alcohol dehydrogenase component of FAO; and 3b) fatty aldehyde dehydrogenase component of FAO.


Fatty
Acyl CoA
Fatty
Aldehyde
Alkane
2a
Dietary
Lipid
2b
1
Fatty
Acid
FATTY
ALCOHOL
Wax Ester
Endogenous
Lipid
3b
3a
Hydroxy
Fatty Alcohol
Fatty
Aldehyde
Fatty
Aldehyde
Sphingolipid
Glycerol
Ether Lipid

In the fatty alcohol cycle, fatty alcohol synthesis begins with the activation of fatty acid precursors obtained either from the diet or endogenous sources, such as triglyceride stores. Fatty acid is activated to fatty acyl-CoA via the enzyme, acyl-CoA synthetase (see Figure 1, enzyme 1). This reaction requires ATP and CoA.

Fatty acyl-CoA, the product of acyl-CoA synthetase, is the substrate for acyl-CoA reductase (see Figure 1, enzyme 2a and 2b), resulting in the formation of fatty alcohol. Acyl-CoA reductase is NADH-dependent in cytosol, and NADPH-dependent in membrane fractions. Fatty aldehyde is an intermediate in the reduction of fatty acyl-CoA to fatty alcohol; however, fatty aldehyde is not released as a free intermediate because aldehyde-trapping agents (such as semicarbazide) and excess unlabeled aldehyde have little effect on the complete reduction of labeled fatty acyl-CoA to fatty alcohol (Bishop and Hajra, 1981). The enzyme is particularly active with 16- and 18-carbon acyl-CoA substrates, and is not inhibited by fatty alcohol.

Fatty alcohol can be utilized in the synthesis of hydroxy fatty alcohol, wax esters (prominent in skin), and glyceryl ether lipids (abundant in nerve tissue). The metabolism of hydroxy fatty alcohol (Miura *et al.*, 1987) and wax esters (Kolattukudy and Rogers, 1986; Wykle *et al.*, 1979) is not well characterized.

Glyceryl ether lipids (including plasmalogens) are

synthesized from fatty alcohol by the enzyme alkyl-dihydroxyacetone phosphate synthase. Glyceryl ether lipids are catabolized to release fatty aldehyde, which is subsequently oxidized to fatty acid via an NAD^+-dependent enzyme system. This reaction occurs via a free fatty aldehyde intermediate. Therefore, fatty alcohol can be indirectly oxidized to fatty acid via glyceryl ether lipid synthesis and degradation.

Fatty alcohol is directly oxidized to fatty acid by fatty alcohol:NAD^+ oxidoreductase (FAO, see Figure 1, enzyme 3a and 3b). FAO activity has been demonstrated in human cultured skin fibroblasts (Rizzo *et al.*, 1987, 1988), human leukocytes (Rizzo *et al.*, 1989), and in rat liver (Lee, 1979). FAO activity is present in most tissues of rat, and is highest in liver (Lee, 1979). FAO is membrane-bound (Rizzo and Craft, 1991) and shows an absolute requirement for NAD^+ (Lee, 1979). The results in rat liver suggest that fatty aldehyde is an intermediate in the oxidation of fatty alcohol to fatty acid. Fatty aldehyde produced by FAO in rat liver is not released as a free intermediate because semicarbazide, an aldehyde trapping agent, included in the *in vitro* reaction buffer did not inhibit oxidation of fatty alcohol to fatty acid (Lee, 1979). Aldehyde trapping could also not be demonstrated in human fibroblast homogenates (Rizzo, personal

communication).

The demonstration of a fatty aldehyde intermediate in the rat liver *in vitro* assay suggests that FAO may consist of an enzyme complex with one enzyme functioning as a fatty alcohol dehydrogenase (FADH) and the other enzyme functioning as a fatty aldehyde dehydrogenase (FALDH). Ichihara *et al.* (1986a) solubilized and chromatographically separated FADH from FALDH in rabbit intestinal mucosal cells. In reconstitution experiments, they found that both enzymatic components are necessary for the complete oxidation of fatty alcohol to fatty acid.

SUBCELLULAR LOCALIZATION OF FATTY ALCOHOL-METABOLIZING ENZYMES

The enzymes involved in fatty alcohol metabolism have been localized using either differential centrifugation (which separates organelles based on size) or density gradient centrifugation. Separation by density is more precise and less contamination between subcellular organelles is demonstrated.

Fatty acids derived from the diet or triglyceride stores are activated in the fatty alcohol cycle. Fatty acid ß-oxidation occurs in the mitochondria (Bremer and Osmundsen, 1984) and peroxisomes (Lazarow and de Duve, 1976).

Fatty acids are activated to acyl-CoA by acyl-CoA

synthetase. Acyl-CoA synthetase activity has been demonstrated in rat liver mitochondria (Tanaka *et al.*, 1981), rat liver peroxisomes (Singh, H. and Poulos, 1988), and rat liver microsomes (Singh, H. and Poulos, 1988), as well as in rat brain microsomes (Singh, I. *et al.*, 1985; Reddy *et al.*, 1984).

The enzyme catalyzing the reduction of fatty acyl-CoA to fatty alcohol, acyl-CoA reductase, is localized to the soluble fraction of bacteria (Naccarato *et al.*, 1972; Day *et al.*, 1970), broccoli leaves (Kolattukudy, 1971), and bovine cardiac muscle (Johnson and Gilbertson, 1972); the microsomes of fish (Griffith *et al.*, 1981), birds (Kolattukudy and Rogers, 1978), rat brain (Bishop and Hajra, 1981, 1978), bovine meibomian glands (Kolattukudy and Rogers, 1986), and mouse preputial gland tumors (Wykle *et al.*, 1979); and the peroxisomes of guinea pig intestinal mucosal cells (Burdett *et al.*, 1991).

Fatty alcohols can be used as substrates for the synthesis of glyceryl ether lipids. This step is catalyzed by the enzyme alkyl-dihydroxyacetone phosphate synthase. This enzyme was initially described in microsomal preparations of rat brain (Natarajan and Schmid, 1978), rat liver (Lee *et al.*, 1980), and two rat tumors: sarcoma and hepatoma (Lee *et al.*, 1980). However, more recent studies using density gradient centrifugation indicate that alkyl-dihydroxyacetone phosphate synthase activity sediments with

peroxisomal-enriched fractions of rat and guinea pig liver (Hajra and Bishop, 1982; Singh, H. *et al.*, 1993).

Fatty alcohol oxidation, catalyzed by FAO, has been demonstrated in rat liver microsomes (Lee, 1979).

ENZYMATIC DEFECT IN SLS

Fatty alcohol accumulation has been found in plasma from SLS patients (Rizzo *et al.*, 1989). SLS patients also show symptoms involving both the skin and the nervous system, sites where wax esters and glycerol ether lipids are abundant, respectively. The fatty alcohol accumulation in SLS patients is not due to a block in glycerol ether lipid synthesis nor is it due to an increase in fatty alcohol synthesis (Rizzo *et al.*, 1987). Rather, intact cultured skin fibroblasts from SLS patients show impaired oxidation of fatty alcohol to fatty acid (Rizzo *et al.*, 1988).

The enzyme catalyzing the oxidation of fatty alcohol to fatty acid, FAO, is deficient in SLS patients. Using hexadecanol, a 16-carbon saturated fatty alcohol, as substrate, mean FAO activity in cultured skin fibroblasts from 8 SLS patients was 18% of normal and enzyme activity in peripheral blood leukocytes from three patients was 22% of normal (Rizzo *et al.*, 1989). Using a histochemical staining technique, deficient fatty alcohol oxidation was confirmed in epidermis and jejunal mucosa from seven SLS

patients (Judge *et al.*, 1990; Lake *et al.*, 1991). Fibroblasts and leukocytes from SLS heterozygotes showed mean FAO activities that were intermediate between those activities seen in SLS homozygotes and normal controls (Rizzo *et al.*, 1989).

FAO is believed to consist of 2 enzyme moieties: FADH which oxidizes fatty alcohol to fatty aldehyde, and FALDH which oxidizes fatty aldehyde to fatty acid. To determine which enzymatic component was responsible for FAO deficiency in SLS, activities of FADH and FALDH were assayed separately in seven unrelated SLS patients (Rizzo and Craft, 1991). Using 16-carbon substrates, the mean FADH activity in cultured skin fibroblasts from SLS patients was 92% of the mean activity measured in normal controls, whereas mean FALDH activity in the SLS cells was reduced to 18% of the mean activity seen in normal controls. FALDH deficiency of the FAO enzyme complex has been demonstrated in all SLS patients studied to date (Rizzo and Craft, 1991). FAO deficiency has also been demonstrated in a mutant Chinese hamster ovary cell line (James *et al.*, 1990). This mutant strain was also deficient in FALDH activity.

Fatty alcohol metabolism has been investigated in cultured skin fibroblasts from SLS patients (Rizzo and Craft, 1991). When intact fibroblasts from SLS patients were incubated with radioactive-labeled long-chain fatty

alcohol, there was a deficiency in the production of radioactive-labeled fatty acid, but no accumulation of radioactive-labeled long-chain fatty aldehyde was seen. Intact fibroblasts from SLS patients were incubated in the presence of radioactive-labeled fatty aldehyde and its oxidation to radioactive-labeled fatty acid was measured. Oxidation of free fatty aldehyde was not impaired in SLS fibroblasts. These results suggest that fatty aldehyde oxidation occurs by more than one mechanism, since FALDH deficiency in SLS fibroblasts did not lead to an accumulation of fatty aldehyde derived from fatty alcohol.

FALDH in cultured skin fibroblasts can utilize a broad spectrum of aldehyde substrates. When saturated aldehydes and alcohols of different carbon chain lengths were used as substrate for FALDH, Rizzo and Craft (1991) found that mean FALDH activity in SLS fibroblasts was reduced to 62% of normal using 3-carbon substrates, 30% of normal using 6-carbon substrates, 25% of normal using 8-carbon substrates, 27% of normal using 12-carbon substrates, and 8% of normal using 18-carbon substrates. A similar trend was seen for FAO activity. These results suggest 2 possible situations: 1) there are multiple aldehyde dehydrogenases (ALDHs) in human fibroblasts with overlapping substrate specificities, only one of which is deficient in SLS, or 2) a single FALDH is responsible for oxidizing short-, medium-, and long-chain aliphatic aldehydes and the mutant FALDH in SLS is

more impaired in oxidizing long-chain substrates than medium- and short-chain substrates.

ALDEHYDE-METABOLIZING ENZYMES

Aldehydes are reactive molecules that can be generated from a large number of endogenous and exogenous sources. Many organisms have evolved enzyme systems that will metabolize aldehydes to less reactive forms. In mammals, aldehydes can be metabolized by three different enzyme systems: aldehyde oxidase, aldo-keto reductase, and aldehyde dehydrogenases (ALDHs). These three systems have broad and sometimes overlapping substrate requirements (Lindahl, 1992).

ALDEHYDE OXIDASE

Aldehyde oxidase, a specific isozyme of the oxidase family, is a molybdenum-containing hydroxylase that uses oxygen as the electron acceptor, generating the oxidized products, H_2O_2 and O_2 (Beedham, 1987). It is a large, cytosolic flavoprotein with a molecular mass of approximately 300,000 daltons consisting of two apparently identical subunits. The physiological function of aldehyde oxidase *in vivo* is believed to be the oxidation of purines, pyrimidines, and other nitrogen-containing heterocyclic

compounds.

ALDO-KETO REDUCTASE

Aldo-keto reductase consists of a family of enzymes that reduce a variety of aldehydes and ketones to their corresponding alcohols (Flynn, 1982). They are small, monomeric (30,000-40,000 daltons), NADPH-dependent enzymes localized to the cytosol. There are three families of reductase known, with multiple isozymes within each family. The first family, aldehyde reductase, catalyzes the reduction of aldehydes, especially the aldehyde form of uronic acid, semialdehydes, and ketones. The second family, aldose reductase, has overlapping substrate specificity with aldehyde reductase, but preferentially reduces aldohexoses. Structurally, the aldehyde and aldose reductase families are closely related. The third family, carbonyl reductase, catalyzes the reduction of quinones, prostaglandins, and ketosteroids. This third family is structurally more distinct from the other two families.

ALDEHYDE DEHYDROGENASE

ALDHs are a family of $NADP^+$- and NAD^+-dependent enzymes that oxidize a wide variety of aromatic and aliphatic aldehydes to their corresponding carboxylic acids by an irreversible reaction (Lindahl, 1992; Yoshida *et al.*,

1991). Several isozymes have been purified and characterized from different species including bacteria, plants, moth, mouse, rat, rabbit, sheep, pig, horse, and human (for review see Yoshida *et al.*, 1991; Lindahl, 1992; Pietruszko, 1983; Weiner, 1980). The enzymes are distributed in most tissues within these organisms and have been found in virtually every subcellular compartment.

HUMAN ALDHs

In humans, ALDH families exist which differ from one another in subcellular localization, kinetic properties (low or high K_m for acetaldehyde or propionaldehyde), isoelectrophoretic mobility, tissue distribution, disulfiram sensitivity, and molecular structure (see Tables 1, 2, and 3). Recently, a nomenclature was established which classifies ALDHs by their kinetic and physical properties.

Sensitivity to disulfiram and other compounds has been used to differentiate ALDH isozymes and to elucidate some of the amino acid residues implicated in enzyme catalysis. Specifically, disulfiram, iodoacetamide, iodoacetate, N-ethylmaleimide, and p-chloromercuribenzoate are all sulfhydryl reagents which specifically react with cysteine residues. Bromoacetophenone reacts with a specific glutamic acid residue thereby inhibiting enzyme activity of

Table 1. Biochemical properties of human ALDH isozymes.

Class	Sub-cell[a] Location	Major Tissues	pI[b]	subunit MW (# subunits)	Ref.
1	cyto	liver	5.1	54 (4)	c
3	cyto	stomach, lung	5.9-6.4	54 (2)	d
gamma	cyto	liver	5.3, 5.45	54 (4)	e
	cyto	cornea	6.8	54 (1)	f
	cyto	saliva	6.5-7.0	48 (?)	g
2	mito	liver	4.9	54 (4)	h
4	mito	liver, kidney	6.8, 6.9	70.6 (2)	i
2a	mito	brain	4.9	54 (4)	j
2b	mito	brain	5.0	54 (4)	j
	mito	testis, liver		54 (?)	k
	micro	leukocytes			l

[a] cyto - cytosol,
mito - mitochondria,
micro - microsomes
[b] isoelectric point
[c] Greenfield and Pietruszko, 1977; Ikawa *et al.*,
[d] Yin *et al.*, 1989
[e] Kurys *et al.*, 1989
[f] Gondhowiardjo *et al.*, 1991; King and Holmes, 1993
[g] Harada *et al.*, 1989
[h] Greenfield and Pietruszko, 1977; Ikawa *et al.*, 1983
[i] Forte-McRobbie and Pietruszko, 1986
[j] Ryzlak and Pietruszko, 1987, 1989
[k] Hsu and Chang, 1991
[l] Sutyak *et al.*, 1989

Table 2. Kinetic properties of human ALDH isozymes.

		K_m (pH)		
Class	Major Tissues	acet-aldehyde	optimal substrate	Ref.
1	liver	22 μM (7.5)		a
3	stomach, lung	83 mM (8.5)	11 μM[b] (8.5)	c
gamma	liver	50 μM (7.4)	14 μM[d] (7.4)	e
	cornea	67 mM (7.4)	1 mM[f] (8.5)	g
	saliva	106 μM (8.0)		h
2	liver	4 μM (7.5)		i
4	liver, kidney	2.3 mM (8.5)	100 μM[j] (7.0)	k
2a	brain	1 μM (9.0)	0.5 μM[l] (9.0)	m
2b	brain	1 μM (9.0)	1.0 μM[l] (9.0)	m
	testis, liver			n
	leukocytes		3 μM[o] (6.5)	p

continued on next page

Table 2 (continued from previous page).

[a] Greenfield and Pietruszko, 1977; Ikawa *et al.*, 1983
[b] heptanal
[c] Yin *et al.*, 1989
[d] gamma-aminobutyraldehyde
[e] Kurys *et al.*, 1989
[f] benzaldehyde
[g] Gondhowiardjo *et al.*, 1991; King and Holmes, 1993
[h] Harada *et al.*, 1989
[i] Greenfield and Pietruszko, 1977; Ikawa *et al.*, 1983
[j] glutamic-gamma-semialdehyde
[k] Forte-McRobbie and Pietruszko, 1986
[l] 3,4-dihydroxyphenyl acetaldehyde
[m] Ryzlak and Pietruszko, 1987, 1989
[n] Hsu and Chang, 1991
[o] 20-carbon aldehyde leukotriene B_4
[p] Sutyak *et al.*, 1989

Table 3. Disulfiram sensitivity and thermostability of human ALDH isozymes.

Class	Major Tissues	Disulfiram Sensitivity % Activity Remaining	Disulfiram Sensitivity Conc.	Thermo-stability[a]
1	liver	5-10%	20 μM	+
3	stomach, lung			-
gamma	liver	insensitive		-
	cornea	insensitive		-
	saliva			
2	liver	50%	20 μM	-
4	liver, kidney			-
2a	brain	0%	3.3 μM	
2b	brain	insensitive		-
	testis, liver			
	leukocytes			

[a] + indicates greater than 70% activity remaining after incubation at 55°C for 5 minutes

some ALDH isozymes.

In this dissertation, subcellular localization is discussed at length. In all discussions, we refer to microsomes as consisting of the rough endoplasmic reticulum, the smooth endoplasmic reticulum, and the Golgi apparatus.

Class 1 ALDH consists of a cytosolic, low K_m (acetaldehyde as substrate) NAD^+-dependent form (Yoshida *et al.*, 1991). It is constitutively expressed and ubiquitously distributed (except in erythrocytes). It is inhibited by disulfiram (90-95% inhibition at 20 μM; Hempel *et al.*, 1982), iodoacetamide (90% inhibition at 33 μM; Hempel and Pietruszko, 1981), and bromoacetophenone (93% inhibition at 10 μM; MacKerrell *et al.*, 1986). It is thermostable at 55°C. Retinaldehyde is oxidized exclusively by this isozyme and not by members of class 2 or 3 (Yoshida *et al.*, 1992), and the K_m of class 1 isozymes for retinal is 600 times less than for acetaldehyde, suggesting that retinal is the major physiological substrate.

The class 2 ALDH is a mitochondrial, low K_m NAD^+-dependent enzyme that is also constitutively expressed and distributed ubiquitously (except in erythrocytes) (Yoshida *et al.*, 1991; Pietruszko, 1983). This isozyme is not completely inhibited by disulfiram (50% inhibition at high

concentrations; Hempel *et al.*, 1981), bromoacetophenone (53% inhibition at 10 µM; MacKerrell *et al.*, 1986), or iodoacetamide (80% inhibition at 31 µM; Hempel and Pietruszko, 1981). Deficiency of the class 2 isozyme has been implicated in alcohol sensitivity in Oriental and Native American populations (Goedde *et al.*, 1983). The symptoms of alcohol sensitivity include facial flushing, palpitation, dizziness, and increased skin temperature (Goedde *et al.*, 1983; Agarwal and Goedde, 1987). Higher blood acetaldehyde concentrations have been demonstrated in individuals experiencing these symptoms. The frequency of ALDH class 2 isozyme deficiency in these populations ranges from 25-44% in Orientals, 41-43% in South American Indians, 2-5% in North American Indians, and 0% in Europeans and Middle Easterners, as determined by enzyme assays. This ALDH variant appears to be responsible for the low incidence of alcoholism in those populations with a high frequency of enzyme deficiency (Yoshida *et al.*, 1991).

Class 3 ALDH represents a cytosolic-inducible isozyme with a high K_m for acetaldehyde (Yoshida *et al.*, 1991). This enzyme has been identified following exposure in rats to certain xenobiotics, including drugs and carcinogens, the two most significant compounds being phenobarbital and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). This isozyme can utilize both NAD^+ and $NADP^+$ as coenzyme; however, NAD^+

is required in lower concentrations. The optimal substrates are benzaldehyde and heptanal. It is resistant to inhibition by disulfiram.

The class 4 isozyme is a mitochondrial, inducible high K_m enzyme which is also induced by xenobiotics (Forte-McRobbie and Pietruszko, 1986). It can utilize NAD^+ or $NADP^+$ as coenzyme as does the class 3 isozyme above, and it requires NAD^+ in smaller quantities. It is irreversibly inhibited by iodoacetamide, and actively uses benzaldehyde and heptanal as substrates, but it is most active with glutamic-gamma-semialdehyde. As a result, it has been classified as glutamic-gamma-semialdehyde dehydrogenase.

Other ALDH isozymes have been characterized which differ from those classes previously described. An ALDH has recently been purified from liver cytosol that has a low K_m for gamma-aminobutyraldehyde (GABA) as substrate at physiological pH (Kurys *et al.*, 1989). At pH 9.5, this same isozyme also oxidizes propionaldehyde, albeit to a lesser extent than GABA. It uses NAD^+ as cofactor and is resistant to disulfiram inhibition. It reacts with anti-class 1 and anti-class 2 antibodies thereby having some structural similarities to these ALDHs.

Human corneal extracts contain one prominent ALDH (Gondhowiardjo *et al.*, 1991). This soluble isozyme is $NADP^+$-dependent, utilizing benzaldehyde and hexanal as

substrates. This ALDH is abundant in the corneal epithelium and is thought to be important in detoxifying lipid peroxides generated by UV light.

An ALDH isozyme has been purified and characterized from human saliva (Harada *et al.*, 1989). It also is present in liver and kidney, but to a lesser extent. The limited information reported, based on isoelectric point, subunit molecular weight, and K_m values, suggests that it is a novel isozyme.

Two mitochondrial ALDH isozymes have been purified from the brain (Ryzlak and Pietruszko, 1987, 1989). Their kinetic and isoelectric focusing properties are almost identical to the class 2 isozyme, but, one of these isozymes is completely inhibited by disulfiram at a concentration of 20 μM, whereas the other is not. Both isozymes utilize acetaldehyde, but the substrate K_m is lowest with the dopamine derivative 3,4-dihydroxyphenylacetaldehyde (DOPAL) as substrate. One isozyme has a slightly lower K_m for this substrate than the other. These isozymes are mainly found in the cerebellum and corpus striatum of the brain.

A new ALDH isozyme cDNA was recently cloned by screening a human testis cDNA library using a class 2 ALDH DNA probe (Hsu and Chang, 1991). When expressed in Chinese hamster ovary cells, this ALDH was capable of oxidizing

propionaldehyde, but not benzaldehyde or gamma-aminobutyraldehyde.

An ALDH has been described in the microsomes of polymorphonuclear leukocytes that catalyzes the conversion of the 20-carbon aldehyde of leukotriene B_4 to its carboxylic acid (Sutyak *et al.*, 1989). This isozyme has a specificity for NAD^+ over $NADP^+$ as coenzyme, and acts at the terminal step of inactivation for the LTB_4 metabolic pathway.

MOLECULAR STRUCTURE OF HUMAN ALDHs

Class 1 and 2 mature ALDHs are homotetrameric proteins with subunits composed of 500 amino acid residues (Yoshida *et al.*, 1991). The genes encoding the human class 1 and 2 liver ALDHs have been cloned and sequenced (Hsu *et al.*, 1988; Hsu *et al.*, 1989). Both contain many introns. Although the amino acid sequences are highly homologous (68%), no homology is found in the first 21 amino acid residues. An intron exists between the bases coding for the 20th and 21st amino acids. Thus it appears that a common ancestor could have existed and that a gene rearrangement occurred at the 5' end of the gene so that one exon was replaced by another that coded for the first 21 amino acid residues, as well as a mitochondrial-targeting signal peptide. The mitochondrial class 2

enzyme is synthesized with a 17 amino acid residue signal peptide that is cleaved upon insertion in the mitochondria.

Mammalian class 1 and class 2 isozymes share a large degree of amino acid sequence homology between and within species (see Table 4). In all cases there is more homology between amino acid sequences of the class 2 isozyme isolated from different species (93-96%) than between class 1 and class 2 isozymes isolated from the same species (63-66%).

The hydropathic property differences between class 1 and 2 isozymes are greatest in amino acid positions 249-255, 335-340, and 390-394 (Yoshida *et al.*, 1991, Hempel *et al.*, 1993). It is thought that possible subunit interactions occur at these residues since heterotetramers have not been found between class 1 and 2 subunits. Cys-302 of mammalian class 1 and 2 ALDHs has been implicated to have a catalytically essential role because iodoacetamide (Hempel *et al.*, 1982; Hempel and Pietruszko, 1981; Hempel *et al.*, 1993) and disulfiram (Santisteban *et al.*, 1985) modify enzyme activity. Of the many cysteine residues found in ALDHs this is the only one strictly conserved both within and among species (Hempel *et al.*, 1993). The horse liver class 2 mitochondrial isozyme contains 2 cysteine residues (cys-49 and cys-162) that are reactive towards N-ethylmaleimide and, therefore, may also play a role in catalysis (Tu and Weiner, 1988). Affinity labeling with

Table 4. Comparison of amino acid sequences of mammalian class 1 and class 2 ALDHs.

	human	horse	bovine	rat
human	68%[b]	94%[a]	93%[a]	96%[a]
horse		66%[b]	94%[a]	95%[a]
bovine			---	94%[a]
rat				---

[a] Figures listed are the percentages of identity in the class 2 ALDH from different species. Reproduced from Yoshida *et al.*, 1991

[b] Figures listed are the percentages of identity between class 1 and class 2 ALDHs from the same species.

bromoacetophenone suggested that glu-268 was an important residue for catalysis (Abriola *et al*., 1987; MacKerrell *et al*., 1986). The characteristic glycine distribution at positions 223, 225, 229, 245, and 250 has been implicated in the NAD-binding domain (Hempel *et al*., 1984). Sequence alignments across species provide some useful information to define consensus sequences and identify putative residues important in maintaining structural stability and providing enzyme catalysis; however, X-ray crystallography and tertiary structural models would be optimal in understanding the ALDH structure-function relationships.

MAMMALIAN MICROSOMAL ALDHs

Microsomal ALDHs have been purified and characterized from rat liver (Nakayasu *et al*., 1978; Mitchell and Petersen, 1989; Lindahl and Evces, 1984) and rabbit intestine (Ichihara *et al*., 1986a, 1986b), but never from human tissues. Nakayasu *et al*. (1978) first reported the purification of a rat liver membrane-bound microsomal ALDH. This enzyme has a molecular weight of 51,000 daltons, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Sucrose density gradient centrifugation revealed large, polymeric aggregates with apparent molecular weights above 360,000 daltons. This enzyme showed little activity towards acetaldehyde as substrate, but required straight-chain aliphatic aldehydes,

at least up to 12-carbons in length.

Mitchell and Petersen (1989), using the same purification scheme as Nakayasu *et al*. (1978), isolated the same isozyme. The K_m of the purified enzyme decreased from 800 μM to 3 μM as the substrate chain length increased from three carbons to nine carbons. They did not characterize the enzyme for substrates longer than nine carbons.

Lindahl and Evces (1984) demonstrated two rat liver microsomal isozymes which differed from each other by the inability of one (isozyme I) to bind DEAE-cellulose at 1 mM phosphate, pH 7.3. Isozyme I had an isoelectric point (pI) of pH 6.4, and isozyme II has a pI of pH 5.6. Both isozymes were similar in molecular weight, K_m for NAD^+, K_m for substrates (octanal having the highest maximum velocity), inhibition to p-chloromercuribenzoate, and thermolability (less than 10% activity after incubation for 5 minutes at 55°C). They were not inhibited by disulfiram or chloral hydrate.

The cDNA for the rat liver microsomal ALDH was recently cloned and sequenced (Miyauchi *et al*., 1991). The deduced amino acid sequence, 484 amino acid residues, revealed a hydrophobic segment at the carboxy terminus which may be important in signaling insertion into the microsomal membrane. The deduced amino acid sequence of the cloned DNA shares 64% homology with the rat liver class

3 inducible, cytosolic ALDH amino acid sequence, but only 23% homology to the human liver class 1 and 2 ALDHs (Hempel *et al.*, 1993).

A rabbit intestinal microsomal ALDH has also been purified and characterized (Ichihara *et al.*, 1986a, 1986b). This isozyme did not elute from a 5'AMP-Sepharose affinity column with NAD^+, where NAD^+ is used to elute the rat liver microsomal isozyme. It did elute with 200 mM phosphate buffer. The relative enzyme activity was determined for aldehyde substrate chain lengths ranging from 1- to 12-carbons. The enzyme had its highest relative activity with 10- and 12-carbon substrates. The subunit molecular weight was estimated to be 60,000 daltons by SDS-PAGE, and the apparent native molecular weight was estimated to be 360,000 daltons as determined by gel filtration. Disulfiram, $MgCl_2$, and $CuSO_4$ did not affect enzyme activity ($MgCl_2$ and $CuSO_4$ inhibit the rabbit intestinal class 1 cytosolic ALDH).

PHYSIOLOGICAL ROLE OF ALDHs

The physiological roles of ALDHs have not yet been clearly established. The class 1 enzyme has a low K_m for acetaldehyde and is thought have a role in the oxidation of acetaldehyde, a product of ethanol oxidation during alcohol ingestion. Recently it has been suggested that

retinaldehyde is the major physiological substrate of this isozyme, implying that its primary biological role is the generation of retinoic acid, resulting in modulation of cell differentiation (Siegenthaler *et al.*, 1990; Dockham *et al.*, 1992; Yoshida *et al.*, 1992). The gamma class ALDH catalyzes gamma-aminobutyraldehyde, a metabolite of putrescine, to gamma-aminobutyric acid, a well-studied neurotransmitter (Kurys *et al.*, 1989). Putrescine is metabolized to spermidine during proliferation in chick embryo brain, but it is preferentially converted to gamma-aminobutyric acid during differentiation (Kurys *et al.*, 1989). Therefore, it is possible that this isozyme has a regulatory effect on cell metabolism. Microsomal ALDHs are particularly active against medium- and long-chain substrates and are especially suited to lipid peroxidation because these membrane-bound enzymes are compartmentalized in a membrane-lipid bilayer microenvironment, where lipid-soluble aldehydic products of lipid peroxidation are produced (Antonenkov *et al.*, 1987; Mitchell and Petersen, 1989).

The primary objective of this dissertation project was to investigate the role of FALDH in fatty alcohol and fatty aldehyde metabolism to gain a better understanding of the biochemical defect in SLS. The major aim of my project was to purify FALDH from human tissues and characterize it with respect to its biochemical properties. Because the rat

liver microsomal ALDH had previously been purified by other investigators (Nakayasu *et al.*, 1978), I modified their purification scheme to isolate a fatty aldehyde dehydrogenase from human liver. I also wanted to determine the subcellular localization of FALDH in several different human tissues in order to better define the role of FALDH in fatty alcohol oxidation as it relates to the overall regulation of cellular fatty alcohol levels. During the course of my studies, I analyzed FAO and FALDH activities using cultured skin fibroblasts obtained from normal controls, SLS patients and their obligate heterozygous parents in order to improve carrier detection for SLS. Finally, I also assayed FALDH in cultured chorionic villi cells and cultured amniocytes to diagnose SLS prenatally in pregnancies at-risk for this disease.

CHAPTER 2

Carrier Detection for Sjögren-Larsson Syndrome

SUMMARY

Sjögren-Larsson syndrome (SLS) is an autosomal recessive disorder associated with reduced activity of the fatty alcohol:NAD$^+$ oxidoreductase complex (FAO). Recent studies indicate that SLS patients are specifically deficient in the fatty aldehyde dehydrogenase (FALDH) component of FAO. To investigate the possibility of carrier detection for SLS, FAO and FALDH activities were measured in cultured skin fibroblasts from normal controls, obligate SLS heterozygotes, and SLS homozygotes using the 18-carbon substrates, octadecanol and octadecanal. FALDH activity (nmol/min/mg protein) in normal controls was 8.5 ± 1.2 (mean ± SD; range 7.0-11; n=12) and in SLS heterozygotes was 5.1 ± 1.3 (range 3.3-7.0; n=11), or 60 ± 15% of mean normal activity. Three of 11 SLS heterozygotes had FAO activities that were within the normal range. One of 11 SLS heterozygotes had FALDH activity within the lower range of normal; this heterozygote had FAO activity below

normal. None of the 11 SLS heterozygotes had FAO or FALDH activities within the range of SLS homozygotes. These results indicate that measurement of both FAO and FALDH activities in cultured skin fibroblasts using 18-carbon substrates is useful for SLS carrier detection.

INTRODUCTION

Sjögren-Larsson syndrome (SLS) is an autosomal recessive disorder characterized by congenital ichthyosis, mental retardation, and spastic di- or tetraplegia (Sjögren and Larsson, 1957; Chaves-Carballo, 1987). SLS patients have been reported to have macular degeneration, glistening white dots in the optic fundus, speech defects, seizures, kyphosis of the thoracic spine, brain atrophy, and short stature (Chaves-Carballo, 1987; Jagell *et al.*, 1981). Generalized hyperkeratosis is present at birth and is usually the first sign of SLS. Commonly, the diagnosis of SLS is made in patients who present with congenital ichthyosis and show signs of spasticity and mental retardation before 3 years of age (Jagell and Heijbel, 1982).

Patients with SLS are deficient in fatty alcohol:NAD^+ oxidoreductase (FAO), an enzyme which catalyzes the oxidation of fatty alcohol to fatty acid (Rizzo *et al.*

1988, 1989; Judge *et al.* 1990). It has been postulated that FAO is an enzyme complex: one component functioning as a fatty alcohol dehydrogenase (FADH) necessary for the oxidation of fatty alcohol to fatty aldehyde, and another enzyme closely associated with FADH which functions as a fatty aldehyde dehydrogenase (FALDH) catalyzing the oxidation of fatty aldehyde to fatty acid (Ichihara *et al.*, 1986). Recent investigations have indicated that the primary biochemical defect in SLS patients is a deficiency of the FALDH component of FAO (Rizzo and Craft, 1991). In SLS fibroblasts, the extent of FALDH and FAO deficiency is distinctly more profound with 18-carbon substrates than with shorter chain substrates.

The demonstration of partial FAO deficiency in some obligate SLS heterozygotes raises the possibility of using this test for SLS carrier detection. However, heterozygote detection for SLS is unreliable when FAO activity is measured in cultured fibroblasts using the 16-carbon substrate, hexadecanol (Rizzo *et al.*, 1989). The present study was undertaken in an effort to improve carrier detection by measuring total FAO and FALDH activities using 18-carbon substrates. We investigated both crude homogenates and subcellular fractions in cultured skin fibroblasts from normal controls, obligate SLS heterozygotes, and affected homozygous patients.

MATERIALS AND METHODS

CHEMICALS

[1-^{14}C] Stearate (58 mCi/mmol) was obtained from ICN Radiochemicals, Irvine, CA. [1-^{14}C] Octadecanol was synthesized from [1-^{14}C] stearate and purified by thin-layer chromatography as described (Rizzo *et al.*, 1987). Thin-layer chromatography plates composed of silica gel G were obtained from Whatman Inc., Clifton, NJ. Organic solvents, either reagent-grade or high-performance liquid chromatography-grade, were from J.T. Baker Inc., Phillipsburg, NJ. n-Octadecanal was prepared from octadecanol according to the Ferrell and Yao method (Ferrell and Yao, 1972). Unless otherwise specified, all other biochemicals were purchased from Sigma Chemical Co., St. Louis, MO.

CELL LINES

Cultured skin fibroblasts were grown from skin punch biopsies taken from normal controls, parents of affected patients who are obligate heterozygotes for this autosomal recessive disorder, and affected homozygous patients. Cells were routinely grown at 37°C in an atmosphere of 5% CO_2, 95% air in Dulbecco's MEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and

streptomycin (100 μg/ml). All experiments were performed on cells at passage 2 through 12.

ENZYME ASSAYS

Human skin fibroblasts were grown to near confluence on 100 mm tissue culture dishes, and harvested by trypsinization. The cells were pelleted by centrifugation at 1500g for 5 minutes and washed twice with phosphate-buffered saline. The final cellular pellet was resuspended in 25 mM tris-HCl (pH 8.0), 0.25 M sucrose and homogenized with 10 strokes using a motor-driven, glass-teflon homogenizer. These crude homogenates, in some cases, were further purified as follows. Homogenates were centrifuged at 1000g for 10 minutes to pellet unbroken cells and nuclei. The supernatant was removed and centrifuged at 128,000g for 30 minutes. The pellet was resuspended in the above homogenization buffer prior to assay.

Total FAO activity, which catalyzes the complete conversion of radioactive fatty alcohol to the fatty acid, was assayed as described (Rizzo *et al.*, 1990), except that 12 μM [1-^{14}C] octadecanol was used as substrate. The amount of [1-^{14}C] octadecanal accumulating in the FAO assay was determined after extracting and purifying the reaction products by thin-layer chromatography (Rizzo and Craft, 1991). Protein was determined by the method of Lowry

(Lowry *et al.*, 1951).

Fatty aldehyde dehydrogenase activity was assayed fluorometrically by measuring the fatty aldehyde-dependent production of NADH (Rizzo and Craft, 1991).

STATISTICAL ANALYSIS OF DATA

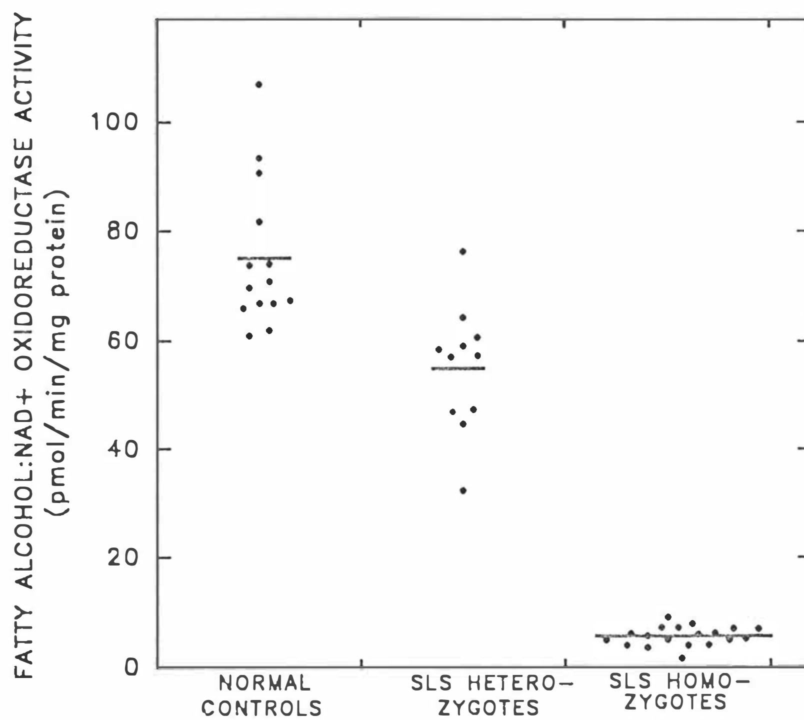
Results were expressed as mean (pmol/min/mg protein) ± standard deviation and differences between means were evaluated by Student's *t* test. The correlations between enzyme activity, donor age, and age of fibroblasts in cell culture were analyzed by linear regression.

RESULTS

No significant correlation was observed in normal controls between enzyme activities and either the age of the donor at the time the skin punch biopsy was taken or the number of passages (2-12) of the fibroblasts in tissue culture.

Figure 2 illustrates the FAO activities measured in crude fibroblast homogenates from normal controls, obligate SLS heterozygotes, and SLS homozygotes. The mean FAO specific activity in affected SLS patients was reduced to 8% of normal when an 18-carbon substrate (octadecanol) was used. Mean FAO activity in crude homogenates from SLS

Figure 2. FAO activity in crude fibroblast homogenates from normal control subjects, SLS obligate heterozygotes, and SLS homozygotes. Each cell line was assayed between 1 and 4 times on separate occasions and the average value was expressed. Horizontal lines correspond to the mean FAO activities (pmol/min/mg protein) which were 75 in normal controls, 55 in obligate SLS heterozygotes, and 6 in SLS homozygotes.


FATTY ALCOHOL:NAD+ OXIDOREDUCTASE ACTIVITY
(pmol/min/mg protein)
100
80
60
40
20
0
NORMAL CONTROLS
SLS HETERO-ZYGOTES
SLS HOMO-ZYGOTES

heterozygotes was 73 ± 16% of mean normal activity. Although the mean FAO activities in normals and SLS heterozygotes were statistically different ($p<0.01$), 3 of 11 heterozygotes had FAO activities which overlapped into the normal range.

FAO in rat liver (Lee, 1979) and human skin fibroblasts (Rizzo, unpublished observations) is a membrane-bound enzyme. Some of the residual FALDH activity measured in crude fibroblast homogenates from SLS homozygotes is due to the presence of cytosolic enzyme (Rizzo and Craft, 1991). To eliminate as much of this residual activity as possible, we measured FAO activity in 128,000g pellets from cultured fibroblasts (Table 5). In this particulate fraction, the mean residual FAO activity in SLS homozygotes was further reduced to 1.5% of normal, and the heterozygotes had a mean activity of 58 ± 16% of normal. Nevertheless, 3 of 8 heterozygotes had activities which overlapped the low normal range.

FAO sequentially oxidizes fatty alcohol to fatty aldehyde and fatty acid. Since SLS patients are selectively deficient in the FALDH component of FAO, assays for FAO may show both accumulation of fatty aldehyde and deficient production of fatty acid in SLS heterozygotes and homozygotes as compared to normal controls. We therefore determined whether the ratio of octadecanoic acid to octadecanal produced in the FAO assay would better

Table 5. Fatty alcohol:NAD^+ oxidoreductase (FAO) activity and radioactive fatty acid/fatty aldehyde ratios in membrane fractions from human cultured skin fibroblasts.

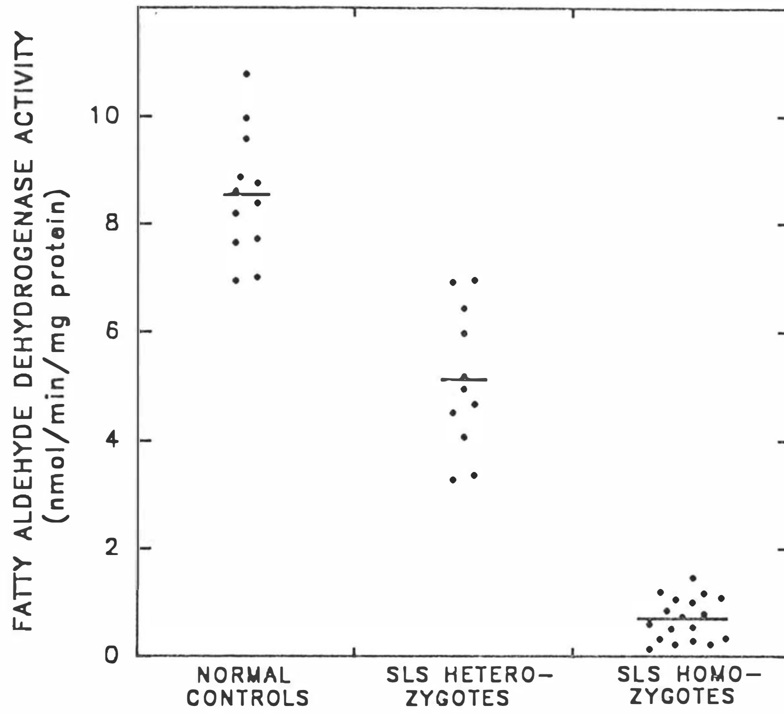
Cell Line	N	FAO Activity[a]	Ratio of $[^{14}C]$-Octadecanoic Acid to $[^{14}C]$-Octadecanal
Normal Controls	9	67 ± 26 (37-111)	4.19 ± 1.59 (2.26-7.40)
SLS Heterozygotes	8	39 ± 11 (24-56)	1.62 ± 0.74 (0.80-2.67)
SLS Homozygotes	5	0.71 ± 1.59 (0-3.56)	0.014 ± 0.031 (0-0.074)

[a] data are expressed as pmol/min/mg protein, mean ± standard deviation. Numbers in parentheses represent the range of values.

discriminate SLS heterozygotes from normal controls. To eliminate as much FAO residual activity as possible, fibroblast 128,000g pellets were used as enzyme source. These data are expressed in Table 5 as the ratio of radioactive octadecanoic acid to octadecanal produced when cell fractions were incubated with [1-^{14}C] octadecanol. Overall, the fatty acid to fatty aldehyde ratios better distinguished SLS heterozygotes from normal controls than did the measurement of FAO activity alone. The mean fatty acid to fatty aldehyde ratio in membrane fractions from SLS heterozygotes was 39% of normal, but 2 of 8 heterozygotes had ratios that fell within the lower range of normal. This overlap of SLS heterozygote ratios into the low normal range was also observed when crude homogenates were tested (data not shown).

Since the primary defect in SLS is deficiency of the FALDH component of FAO, FALDH activity was measured in crude homogenates from normal controls, obligate SLS heterozygotes, and SLS homozygotes (Figure 3). Mean FALDH activity in SLS heterozygotes was 60 ± 15% of normal and mean activity in SLS homozygotes was 8 ± 5% of normal. The difference between normals and heterozygotes was statistically significant ($p<0.001$). FALDH activity from one heterozygote was within the lower range of normal (Figure 3). This subject, however, had an FAO activity

Figure 3. FALDH activity in crude fibroblast homogenates from normal control subjects, SLS obligate heterozygotes, and SLS homozygotes. Each cell line was assayed between 1 and 4 times on separate occasions and the average value was expressed. Horizontal lines correspond to the mean FALDH activities (nmol/min/mg protein) which were 8.54 in normal controls, 5.12 in obligate SLS heterozygotes, and 0.69 in SLS homozygotes.


FATTY ALDEHYDE DEHYDROGENASE ACTIVITY
(nmol/min/mg protein)
10
8
6
4
2
0
NORMAL CONTROLS
SLS HETERO-ZYGOTES
SLS HOMO-ZYGOTES

that was below the normal range.

DISCUSSION

Although FAO and FALDH deficiency may be diagnosed in SLS homozygotes using 16-carbon substrates (Rizzo *et al.*, 1988, 1989; Judge *et al.*, 1990; Rizzo and Craft, 1991), overlap between FAO activities measured in fibroblasts from normal controls and SLS heterozygotes has precluded reliable carrier detection. There are several possible reasons for this overlap. First, other dehydrogenases that act on fatty aldehydes up to 12-carbons in length (Nakayasu *et al.*, 1978; Ichihara *et al.*, 1986) may act on 16-carbon substrates and could contribute to the residual FAO activity measured in SLS heterozygotes and homozygotes (Rizzo *et al.*, 1989). Utilizing an 18-carbon substrate in enzyme assays led to a decrease in the residual activity measured in SLS homozygotes, and allowed better discrimination between normal controls and SLS heterozygotes. Second, FADH appears to be the rate-limiting enzyme for the FAO complex (Rizzo and Craft, 1991). As a result, the variation in FAO activity among heterozygotes may largely reflect variation in FADH activity. This may explain why FAO activity alone discriminates SLS heterozygotes from normal controls less well than does FALDH activity. Third, mutant alleles may

vary in the amount of residual enzyme activity present in both SLS homozygotes and heterozygotes. Finally, the variation in enzyme activity may be due to biologic variation in the human population or to nongenetic factors, such as cell culture conditions.

In summary, we found that carrier detection for SLS can best be made when both FAO and FALDH activities are determined in fibroblast crude homogenates using 18-carbon substrates. Carrier detection may be useful for siblings of SLS homozygotes and their parents because these siblings are at an increased risk for being heterozygous for SLS.

CHAPTER 3

Prenatal diagnosis for Sjögren-Larsson syndrome using enzymatic methods

SUMMARY

Sjögren-Larsson syndrome (SLS) is an autosomal recessive disorder characterized by the presence of congenital ichthyosis, mental retardation and spasticity. The primary biochemical defect in SLS has recently been identified to be a deficiency of fatty aldehyde dehydrogenase (FALDH), which is a component of fatty alcohol:NAD^+ oxidoreductase (FAO). We monitored four pregnancies at-risk for SLS by measuring FAO and FALDH in cultured amniocytes or cultured chorionic villi cells. Enzymatic results in one case using amniocytes obtained during the second trimester predicted an affected SLS fetus, which was confirmed at termination of the pregnancy. Another at-risk fetus was predicted to be affected with SLS using cultured chorionic villi cells obtained in the first trimester, and fetal skin fibroblasts obtained after termination of the pregnancy confirmed a profound

deficiency of FAO and FALDH. Two other pregnancies were correctly predicted to be unaffected. These results demonstrate that SLS can be diagnosed prenatally either during the first or second trimester of pregnancy using enzymatic methods.

INTRODUCTION

Sjögren-Larsson syndrome (SLS) is a neurocutaneous disorder characterized by the presence of ichthyosis, mental retardation and spastic di- or tetraplegia (Sjögren and Larsson, 1957). The ichthyosis is usually present at birth, whereas the neurological symptoms develop later during infancy. Mental retardation is often moderate to profound, and the spasticity typically impairs or prevents ambulation. SLS is inherited in an autosomal recessive fashion.

Recent studies indicate that SLS is an inborn error of lipid metabolism. Affected patients have impaired fatty alcohol oxidation due to deficient activity of fatty aldehyde dehydrogenase (FALDH), a component of fatty alcohol:NAD^+ oxidoreductase (FAO) (Rizzo and Craft, 1991; Rizzo, 1993). Measurement of FAO and FALDH activities in cultured skin fibroblasts provides a reliable means to diagnose SLS postnatally.

Prenatal diagnosis of SLS has been accomplished by detecting histological evidence of ichthyosis in a fetal skin biopsy obtained by fetoscopy at 23 weeks gestation (Kousseff *et al.*, 1982). These investigators removed 4 pieces of skin from the back of the fetus and under light microscopy demonstrated hyperkeratosis and papillomatosis. The pregnancy was carried to term and an affected female was subsequently born. Fetoscopy is complicated by a relatively high (4-5%) risk of fetal loss (Elias, 1987). Furthermore, the timing of the skin biopsy may be critical for proper diagnosis. Fetal skin keratinization normally occurs at the end of the second trimester (Holbrook, 1979), and, in one case, multiple fetal skin biopsies obtained at 19 weeks gestation failed to detect an affected SLS fetus (Tabsh *et al.*, 1993). Moreover, fetal skin biopsy may not be a reliable method for diagnosis even when performed later during pregnancy because the ichthyosis is not invariably present at birth (Ito *et al.*, 1991).

We now report our initial experience with prenatal diagnosis of SLS using enzymatic methods. Our results show that prenatal diagnosis can be accomplished in the first and second trimester by measuring FAO and FALDH activities in cultured chorionic villi cells and amniocytes.

MATERIALS AND METHODS

CELL LINES

Amniocyte cultures were grown from amniotic fluid cells obtained at 16 to 19.5 weeks gestation. Cells were grown in minimal essential medium containing 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) under an atmosphere of 95% air, 5% CO_2. Cultured chorionic villi cells were grown from chorionic villi biopsies obtained at 9 to 11 weeks gestation. Control amniocytes and chorionic villi cells were obtained from women undergoing amniocentesis or chorionic villus sampling (CVS) for advanced maternal age. Fetal skin fibroblast cultures were established and grown using standard methods as described (Rizzo *et al.*, 1988).

ENZYME ASSAYS

FALDH activity was measured fluorometrically using n-octadecanal as substrate as described in the Materials and Methods section of Chapter 5 (Rizzo and Craft, 1991). FAO was assayed by Dr. W. Rizzo using [^{14}C]-octadecanol as substrate as described (see Materials and Methods, chapter 2, Rizzo *et al.*, 1989). Acyl-CoA reductase was assayed using palmityl-CoA as substrate according to the method of Rizzo *et al.* (1993). The protein concentration of cell

homogenates was assayed according to Lowry *et al.* (1951) .

RESULTS

We have previously shown that FALDH activity in cultured skin fibroblasts from 18 SLS patients varied from 2% to 17% of mean normal activity using octadecanal as substrate (see Chapter 2; Kelson *et al.*, 1992); FAO activity using octadecanol as substrate showed a similar deficiency. The range of FAO and FALDH activity in SLS heterozygotes does not overlap with the range of FAO and FALDH activity in SLS homozygotes (see Chapter 2). Since FAO and FALDH activities were readily measured in cultured amniocytes and chorionic villi cells obtained from control pregnancies, we attempted prenatal diagnosis by measuring the activities of these enzymes in cultured cells from pregnancies at-risk for SLS.

SECOND TRIMESTER DIAGNOSIS USING CULTURED AMNIOCYTES

Family 1: The clinical details of this case have been previously reported (Tabsh *et al.*, 1993). Briefly, a Lebanese mother presented to the University of California, Los Angeles Medical Center with a history of having two sons with SLS; both were confirmed to have FAO deficiency in cultured skin fibroblasts. Using fetoscopy at 19 weeks gestation, fetal skin biopsies were taken from 6 different

sites and amniocyte cultures were established. The fetal skin histology was normal. As shown in Table 6, however, amniocytes were profoundly deficient in FAO (9% of mean normal activity using octadecanol as substrate); FALDH was 5% of mean normal activity. Activity of a control enzyme, acyl-CoA reductase, was slightly below the normal range (Table 6). An affected fetus was predicted. The woman returned at 23.5 weeks gestation, at which time repeat fetal skin biopsies were taken and a fetal skin fibroblast culture was established. The skin biopsies at this time showed hyperkeratosis. The pregnancy was terminated and the fetus was observed to have ichthyosis. Fetal skin fibroblast cultures showed 2% of mean normal FAO activity.

Family 2: This family presented to the Hôpital Necker in Paris when the mother was 6 weeks pregnant. There was a history of an affected 19 month old boy, who was born with ichthyosis, and subsequently developed mental retardation and spastic diplegia. FAO activity in his cultured skin fibroblasts was 5% of mean normal activity, confirming the diagnosis of SLS. The mother underwent amniocentesis at 16 weeks gestation. As shown in Table 6, the FAO activity in cultured amniocytes was normal, and an unaffected fetus was predicted. A normal infant was subsequently born, and the FAO activity in cultured skin fibroblasts from this child was consistent with a heterozygote (56% of normal).

Table 6. Enzyme activities measured in cultured amniocytes and cultured chorionic villi cells from pregnancies at-risk for SLS

	Specific Activity[a]		
Cells	FAO	FALDH	Acyl-CoA Reductase
AMNIOCYTES			
Family 1 At-risk fetus	4	67	4.9
Family 2 At-risk fetus	45	NT[b]	NT[b]
Normal Controls			
Mean	47	1227	9.8
SD	13	419	2.4
Range	26-78	591-1561	6.6-12.9
N	16	5	6
CHORIONIC VILLI CELLS			
Family 3 At-risk fetus	4	84	7.6
Family 4 At-risk fetus	74	NT[b]	5.0
Normal controls			
Mean	88	1031	6.4
SD	25	219	1.7
Range	41-119	730-1358	4.6-8.7
N	19	8	8

[a] data are expressed as pmol/min/mg protein
[b] not tested

FIRST TRIMESTER DIAGNOSIS USING CULTURED CHORIONIC VILLI CELLS

Family 3: These consanguineous Italian parents presented to the Instituto Giannina Gaslini with a son who had congenital ichthyosis, mental retardation, spastic tetraplegia, kyphosis and glistening white dots on the retina. FAO activity in cultured skin fibroblasts from the propositus was reduced to 9% of mean normal activity. His mother was 10 weeks pregnant. CVS was performed at 10.5 weeks gestation. Cultured chorionic villi cells were profoundly deficient in FAO and FALDH activities, whereas the control enzyme (acyl-CoA reductase) was normal (Table 6). The pregnancy was terminated at 18.5 weeks. The fetus did not show ichthyosis on physical examination. Cultured skin fibroblasts obtained from the fetus, however, confirmed a profound deficiency of FAO (2% of mean normal activity).

Family 4: This family presented to Christchurch Hospital in New Zealand with a history of having a 6 year old daughter with congenital ichthyosis, spastic diplegia, and normal intelligence. Her fibroblast FAO activity was 7% of mean normal activity. The mother was pregnant and underwent CVS at 10.5 weeks gestation. The FAO activity in cultured chorionic villi cells was normal (Table 6), and a healthy infant was subsequently born.

DISCUSSION

Our initial experience indicates that the enzymatic measurement of FAO and FALDH in cultured fetal-derived cells was capable of detecting 2 affected SLS fetuses. This method permitted the diagnosis of SLS in the first trimester using chorionic villi cells and in the second trimester using amniocytes. The enzymatic approach should provide a considerably safer and earlier method for prenatal diagnosis than can be achieved using fetal skin biopsy. The use of fetal cells obtained by CVS has the advantage of permitting the diagnosis of SLS in the first trimester, and it also affords the possibility of follow-up amniocentesis if initial results are ambiguous. The time from CVS to enzyme assay was several weeks, which consisted entirely of time required for cell culture. Elimination of the period for chorionic villi cell culture was not possible, because enzymatic assay of uncultured control villi gave highly variable results. The direct assay of uncultured chorionic villi, therefore, cannot be recommended.

The reliability of the enzymatic approach for prenatal diagnosis of SLS is still not established and will require more experience. Since enzyme activities in cultured skin fibroblasts from heterozygotes for SLS do not overlap with those measured in SLS homozygotes (see Chapter 2), false

positive results based on misdiagnosis of a heterozygous fetus should be minimized. Confirmation of the correct diagnosis, however, will rest on histological and enzymatic examination of aborted fetal tissues. In family 1, the diagnosis of an affected SLS fetus was histologically and clinically evident when the pregnancy was terminated. Cultured skin fibroblasts were used to enzymatically confirm the diagnosis. In case 3, however, the fetus was aborted at a gestational age that preceded the onset of skin disease; the age of onset of histological changes in the nervous system in SLS is unknown. In such cases of prenatal diagnosis using cells derived by CVS or amniocentesis, the diagnosis of an affected fetus prior to the end of the second trimester can only be confirmed by performing enzymatic studies on fetal tissues or cultured cells.

In all attempts at prenatal diagnosis of SLS by enzymatic methods, it is important to establish that the pregnancy is truly at-risk for FAO deficiency. This enzyme deficiency appears to be specific for SLS and is not seen in other forms of ichthyosis or neurological disease (Rizzo, 1993). Clinically atypical SLS patients with FAO deficiency exist, and some "SLS-like disorders" have clinical features that resemble SLS, including the presence of ichthyosis, mental retardation and spasticity, but are associated with normal FAO activity (Koone *et al.*, 1990;

Scalais *et al.*, 1992). For those SLS-like disorders that are not deficient in FAO activity, the histological examination of a fetal skin biopsy remains a potential method for attempting prenatal diagnosis (Arnold and Anton-Lamprecht, 1987).

CHAPTER 4

Subcellular localization of human fatty aldehyde dehydrogenase in liver, a cultured hepatocyte cell line (HepG2), and cultured skin fibroblasts

SUMMARY

Sjögren-Larsson syndrome (SLS) is an inborn error of fatty alcohol oxidation due to deficiency of fatty aldehyde dehydrogenase (FALDH), a component of the fatty alcohol:NAD^+ oxidoreductase (FAO) enzyme complex. To better understand fatty alcohol oxidation, we determined the subcellular localization of FALDH in human liver, a cultured human hepatocyte cell line (HepG2), and in human cultured skin fibroblasts. Subcellular organelles were separated by differential centrifugation and by density gradient centrifugation in Nycodenz. FALDH was assayed using a saturated 18-carbon aliphatic aldehyde substrate and subcellular separations were monitored using organelle specific marker enzymes. In human liver, cultured hepatocytes, and cultured skin fibroblasts, FALDH activity co-sedimented largely with the microsomal marker. These

results were confirmed with studies performed on rat liver. There was no FALDH activity in the peroxisomes and very little activity in cytosol.

INTRODUCTION

Sjögren-Larsson syndrome (SLS) is an autosomal recessive disorder characterized by congenital ichthyosis, mental retardation, and spastic di- or tetraplegia (Lindahl, 1992). SLS results from an impairment in fatty alcohol oxidation due to the deficiency of fatty alcohol:NAD^+ oxidoreductase (FAO) (Rizzo *et al.*, 1988). FAO catalyzes the oxidation of fatty alcohol to fatty acid via a fatty aldehyde intermediate and consists of two components: a fatty alcohol dehydrogenase and a fatty aldehyde dehydrogenase (FALDH). Rizzo and Craft (1991) have previously demonstrated that patients with Sjögren-Larsson syndrome are selectively deficient in the FALDH component of FAO.

FAO is involved in a metabolic pathway referred to as the fatty alcohol cycle (Rizzo *et al.*, 1987). In this cycle, fatty acid is reduced to fatty alcohol via an acyl CoA intermediate. Fatty alcohol is a substrate for the biosynthesis of 2 major lipid classes: wax esters and glycerol ethers. Wax esters are prominent in the skin, and

glycerol ether lipids (especially plasmalogen) are abundant in myelin. FAO completes the fatty alcohol cycle by oxidizing fatty alcohol to fatty acid.

The subcellular location of the enzymes involved in the fatty alcohol cycle have not been unambiguously established. In order to better understand the role of FALDH in the regulation of fatty alcohol levels in humans, we determined the subcellular localization of FALDH in liver, a cultured hepatocyte cell line (HepG2), and cultured skin fibroblasts. Differential centrifugation and density gradient separation of subcellular organelles in Nycodenz, an iodinated density gradient media, were utilized to separate peroxisomes, mitochondria, and microsomes.

MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Potassium phosphate (di- and mono-basic) was obtained from Mallinckrodt (Paris, KY). Nycodenz (now sold as Accudenz) was purchased from Accurate Chemical Co. (Westbury, NY). Nycodenz was dissolved in homogenization buffer to a final concentration of 50% (w/v) (see below). Cell culture materials were the same as those described in Materials and Methods, chapter 2. Protein concentrations were determined

using the method of Lowry (Lowry *et al.*, 1951) or the Bio-Rad microtiter plate protein assay (Bradford) according to the manufacturer's instructions. $TiOSO_4$-H_2SO_4 was prepared by dissolving 3 g $TiOSO_4$-H_2SO_4-H_2O (Mallinckrodt, Paris, KY) in 0.3 liters of 1 N H_2SO_4 (Mallinckrodt, Paris, KY).

Enzyme activity data were expressed as relative activity of the enzyme assayed. For the Nycodenz gradients, enzyme relative activities were calculated by dividing the total enzyme activity in each fraction by the mean activity of each fraction as if no separation had occurred (mean activity divided by the number of fractions collected). In the differential centrifugation experiments, the relative specific activity was determined by dividing the percent total activity for each fraction by the percent total protein for that same fraction.

PREPARATION OF CELL HOMOGENATES

Human liver was received from 2 different transplant donors and was homogenized separately in 3 volumes of homogenization buffer (0.25 M sucrose, 1 mM tris-HCl, pH 7.4, 1 mM EDTA) with 10 strokes in a Dounce homogenizer (A and B pestles, Corning, NY). The homogenate was centrifuged twice at 500g for 10 minutes. The post-500g supernatants were further fractionated by density centrifugation on a 12-ml, bottom-loaded linear Nycodenz

gradient (20-40%) with a 1 ml 50% Nycodenz buffer at the bottom. The gradient tube was centrifuged using a Beckman vertical tube rotor (VTi 65.1) at 94,000g for 25 minutes at 4°C with low acceleration and deacceleration. The gradient fractions were collected in 0.5 ml aliquots by puncturing the bottom of the centrifuge tube.

Cultured hepatocytes (HepG2) were prepared as follows: Cells from four confluent flasks (150 cm^2) were trypsinized and sedimented at 500g for 5 minutes. After centrifugation, the pellets were washed in PBS three times and then homogenized in 1 ml homogenization buffer (0.25 M sucrose, 1 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% ethanol, 0.05 μg/ml leupeptin, 0.7 μg/ml pepstatin A). Cells were lysed using a Dounce homogenizer (B pestle) until about 90% of the cells were broken as determined by phase-contrast microscopy. The homogenate was centrifuged twice at 500g for 10 minutes. The post-500g supernatant was layered onto a 12-ml, bottom-loaded Nycodenz gradient (20-40%) with a 1 ml 50% Nycodenz buffer. The gradient tube was centrifuged using a Beckman vertical tube rotor (VTi 65.1) at 94,000g for 25 minutes at 4°C with low acceleration and deacceleration. The gradient fractions were collected in 0.5 ml aliquots by puncturing the bottom of the tube.

Cultured skin fibroblasts were prepared as follows: Cells from 10 or more confluent flasks (150 cm^2) were

trypsinized and sedimented at 500g for 5 minutes. The cells were incubated at 37°C for one hour in medium supplemented with 5% fetal calf serum. The cell pellet was washed in PBS three times and then homogenized in 1 ml of homogenization buffer (0.25 M sucrose, 1 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% ethanol, 0.05 μg/ml leupeptin, 0.7 μg/ml pepstatin A) using a Dounce homogenizer (B pestle) until about 90% of the cells were broken as determined by phase-contrast microscopy. The homogenate was centrifuged at 500g for 5 minutes and the post-500g supernatant was again centrifuged.

For gradient density centrifugation of cultured skin fibroblasts, the final post-nuclear supernatant was loaded onto a 40-ml, bottom-loaded Nycodenz gradient (0-30%) with a 4 ml 35% Nycodenz buffer at the bottom of the tube. Gradients were centrifuged in a Beckman vertical tube rotor (Vti 65.1) at 94,000g for 25 minutes at 4°C with low acceleration and deacceleration. The gradient fractions were collected in 0.5 ml aliquots by puncturing the bottom of the tube.

For differential centrifugation of cultured skin fibroblasts, the post-nuclear supernatant volume was increased to 5 ml with homogenization buffer and ultracentrifuged in a SW50.1 swinging bucket rotor (Beckman) at 10,000g for 30 minutes. The supernatant was carefully removed and recentrifuged under the same

conditions. The 10,000g pellets (labeled as mitochondrial-enriched) from both preparations were combined and resuspended in 0.5 ml homogenization buffer. The post-10,000g centrifugation supernatant was centrifuged at 100,000g for 30 minutes. The supernatant was carefully removed and recentrifuged under the same conditions. The 100,000g pellets (labeled as microsomal- and peroxisomal-enriched) were combined and resuspended in 0.5 ml homogenization buffer. The post-100,000g centrifugation supernatant was designated cytosolic.

ENZYME ASSAYS

Catalase, a marker for peroxisomes and cytosol, was assayed by combining 100 μl of each fraction with a reaction mixture containing 10 μg/ml BSA, 20 mM imidazole buffer, pH 7.0, 0.1% Triton X-100, and 0.06% hydrogen peroxide in a final volume of 1 ml (Storrie and Madden, 1990; with some modifications mentioned above). The reaction was run at room temperature for 15 minutes and stopped by the addition of 0.5 ml of saturated titanium oxysulfate-sulfuric acid complex hydrate ($TiOSO_4$-H_2SO_4-H_2O) followed by 2 ml of water. In this assay, $TiOSO_4$ reacts with H_2O_2 forming a yellow complex. The loss of yellow color over time indicates the hydrolysis of H_2O_2 by catalase. The reaction was quantitated by measuring

absorbance at 405 nm on a spectrophotometer (Bausch and Lomb Spectronic 1001, Milton Roy Co., Rochester, NY).

Succinate dehydrogenase, an inner mitochondrial membrane enzyme marker, was assayed by combining 10 μl of each fraction with a reaction buffer containing 15 mM sodium succinate, 25 mM sodium phosphate, pH 7.7, and 0.5 mg/ml p-iodonitrotetrazolium violet in a final volume of 0.5 ml (Davis and Bloom, 1973). The reaction was run at 37°C for 30 minutes and stopped by the addition of 1.5 ml ethyl acetate/ethanol/100% trichloroacetic acid (5:5:1). The reaction was quantitated by measuring absorbance at 490 nm on a spectrophotometer.

Monoamine oxidase, an outer mitochondrial membrane enzyme marker, was assayed fluorimetrically by mixing 10 μl of each fraction with 1 ml of 10 mM potassium phosphate, pH 7.2 (Morinan and Garratt, 1985). The reaction was initiated by the addition of 30 μl of a 1 mg/ml kynuramine stock solution and was incubated for 30 minutes at 37°C. The reaction was stopped with the addition of 1 ml of 2 N HCl. The tubes were briefly centrifuged (9,000g for 5 minutes) to pellet any precipitated proteins, and the supernatant was carefully transferred and mixed with 2 ml of 1 N NaOH. The fluorescence was measured on a Perkin-Elmer LS 50 luminescence spectrometer (Perkin-Elmer Ltd., Beaconsfield, England) at an excitation wavelength of 315 nm (slit length = 10 nm) and an emission wavelength of 380

nm (slit length = 10 nm). A standard curve was run using 4-hydroxyquinoline (2-100 nmol).

NADPH cytochrome c reductase, a microsomal enzyme marker, was assayed by combining 10 μl of each fraction with a reaction buffer containing 40 mM sodium phosphate, pH 7.4, 32 mM niacinamide, 0.25 mM sodium cyanide, and 124 μg oxidized cytochrome c in a final volume of 0.2 ml (Beaufay *et al.*, 1974). The reaction was initiated by adding NADPH to a final concentration of 22.5 μM and monitored at 550 nm on a spectrophotometer (MAXline microplate reader, Molecular Devices Corp., Menlo Park, CA). The rate of the reaction was followed for 10 minutes with the final results expressed as the reduction of cytochrome c per unit of time.

FALDH activity was measured fluorometrically by assaying 10 μl of each fraction and following the fatty aldehyde-dependent production of NADH using n-octadecanal as substrate (see Materials and Methods, chapter 5; Rizzo and Craft, 1991).

RESULTS

By using density gradient centrifugation of cell and tissue homogenates in Nycodenz, we hoped to adequately separate subcellular organelles. Enzyme markers were used to confirm the efficiency of organelle separation: catalase

(particulate) for peroxisomes and free catalase for cytosol; succinate dehydrogenase for the inner mitochondrial membrane; monoamine oxidase for the outer mitochondrial membrane; and NADPH cytochrome c reductase for microsomes. Intact peroxisomes sediment at the higher density of the gradient and cytosol at the lower density (high to low, left to right). Between 90 and 110% of the marker enzymes and FALDH were recovered in each of the gradients assayed and in the fractions from differential centrifugation.

The subcellular distribution of FALDH and marker enzymes is shown in figure 4 for human liver. Mitochondrial (peak in fraction 13) and microsomal (peak in fraction 18) markers are well separated, and FALDH co-sediments primarily with the microsomal marker. Some FALDH activity (13% of total FALDH activity) in the peak mitochondrial fractions (13 and 14) can not be completely accounted for by microsomal contamination which contributes 6% of total microsomal marker enzyme activity in these two fractions. The microsomal and cytosolic markers overlap. There is no FALDH activity in peroxisomes.

The subcellular distribution of mitochondrial and microsomal marker enzymes as well as FALDH is shown in figure 5 for a human cultured hepatocyte cell line (HepG2). The mitochondrial enzyme marker showed the greatest activity in fractions 16 and 17, whereas the microsomal

Figure 4. Distribution of FALDH in human liver fractions separated on 20-40% Nycodenz gradients (fractionation of the post-nuclear supernatant and all enzyme assays are described in the text). The relative activity was plotted against fraction number. Homogenates from 2 different liver samples were separated on gradients with both gradients showing similar results; therefore, only the results from one gradient are shown. The enzyme markers used are as follows: catalase as a peroxisomal and cytosolic marker, succinate dehydrogenase as an inner mitochondrial marker, and NADPH cytochrome c reductase as a microsomal marker.

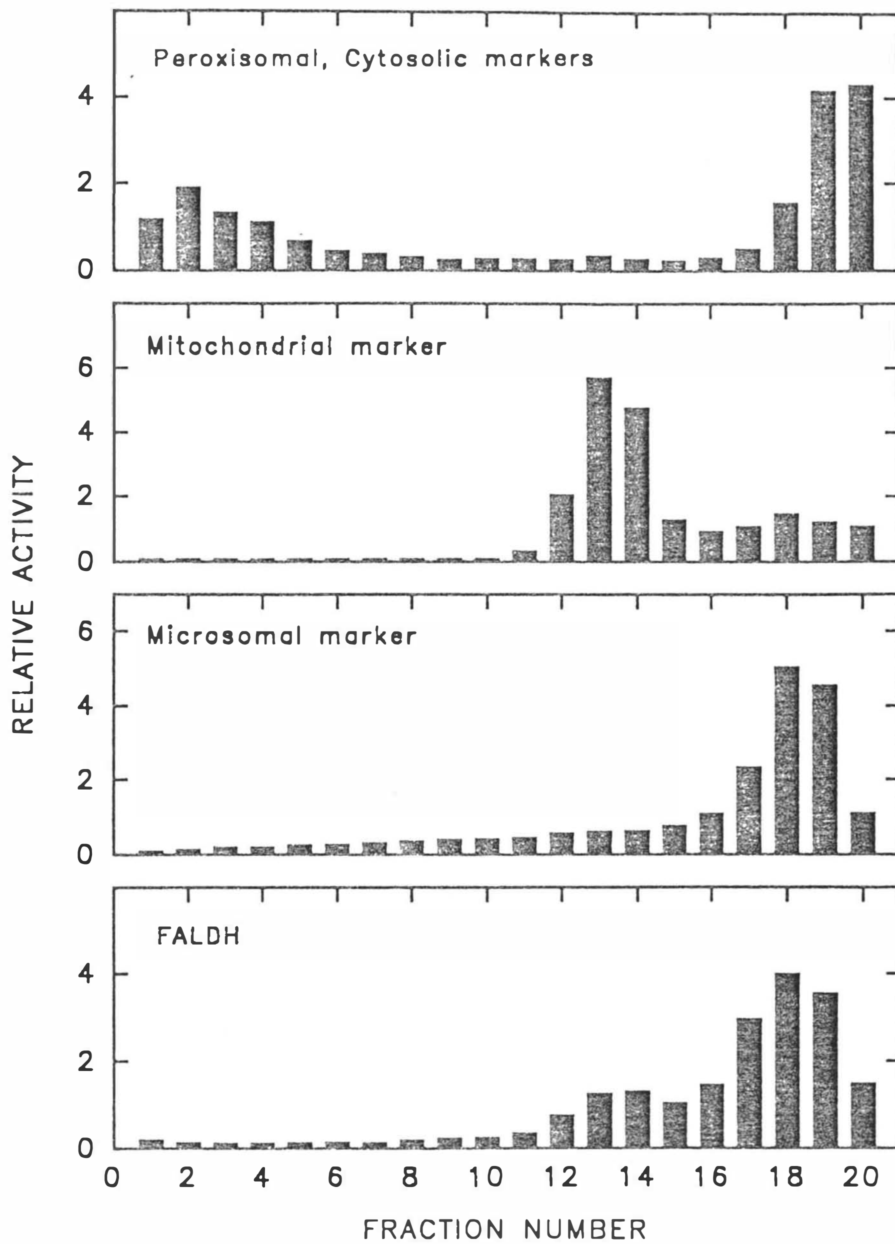
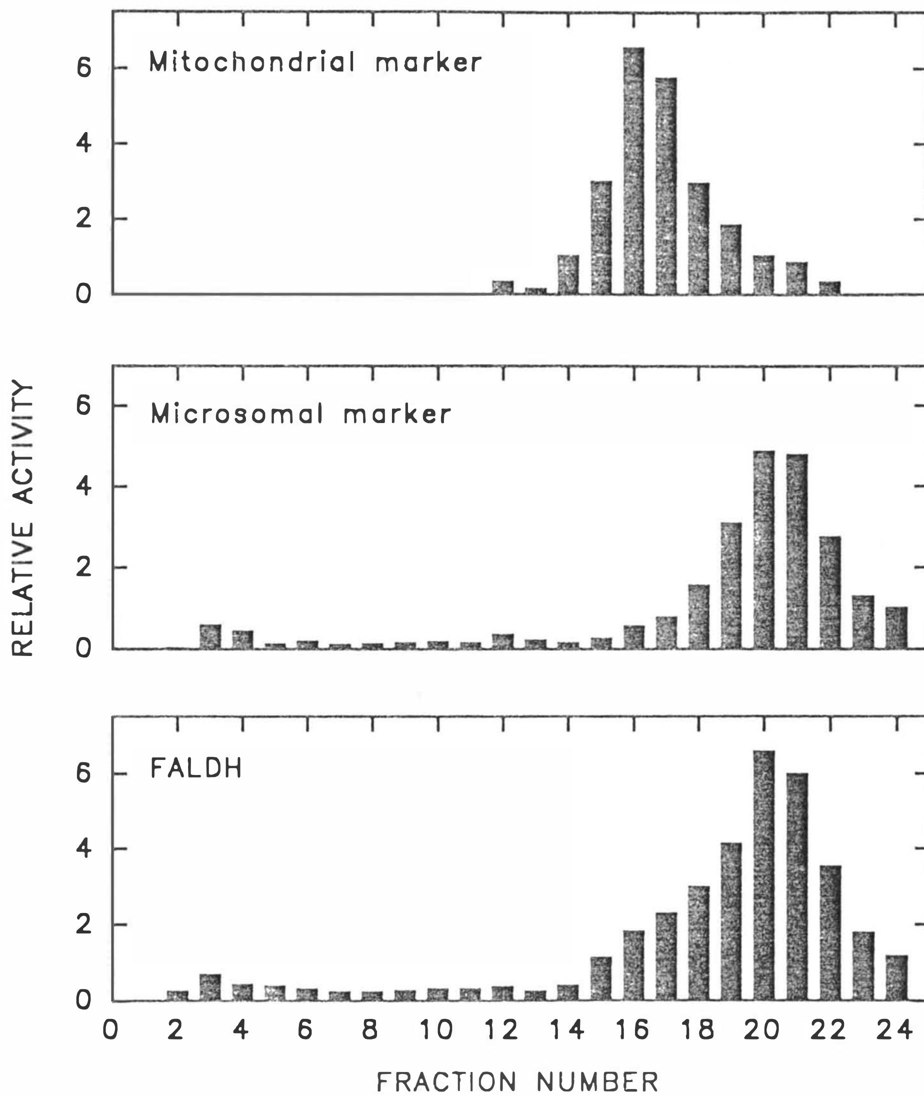

HUMAN LIVER
Peroxisomal, Cytosolic markers
Mitochondrial marker
Microsomal marker
FALDH
RELATIVE ACTIVITY
FRACTION NUMBER
0
2
4
6
0
2
4
6
8
10
12
14
16
18
20

Figure 5. Distribution of FALDH in human cultured hepatocyte fractions separated on 20-40% Nycodenz gradients (fractionation of the post-nuclear supernatant and all enzyme assays are described in the text). The relative activity was plotted against fraction number. The enzyme markers used are as follows: succinate dehydrogenase as an inner mitochondrial membrane marker and NADPH cytochrome c reductase as a microsomal marker.


HUMAN CULTURED HEPATOCYTES (HEPG2)
Mitochondrial marker
Microsomal marker
FALDH
RELATIVE ACTIVITY
FRACTION NUMBER
0
2
4
6
8
10
12
14
16
18
20
22
24

enzyme marker peaked in fractions 20 and 21. FALDH activity was primarily found in the microsomal fractions. Some FALDH activity, however, in fractions 16 and 17 (11% of total activity) could not be totally accounted for by microsomal contamination in these fractions (6% of total NADPH cytochrome c reductase activity in fractions 16 and 17).

Figure 6 illustrates the organelle-enriched fractions of cultured skin fibroblasts separated by differential centrifugation. FALDH activity co-sedimented predominantly with the microsomal enzyme marker. All of the FALDH activity in the mitochondria-enriched fraction (41%) could be accounted for by microsomal contamination (45% of total microsomal enzyme marker). Half of the FALDH activity in the cytosol (32%) could be accounted for by microsomal contamination (16%).

Cultured skin fibroblasts were harvested, homogenized, and loaded onto Nycodenz density gradients in an attempt to get an adequate separation of subcellular organelles. Using 21 gradients, we were unable to separate organelles adequately. The results from one such gradient are shown in figure 7. The mitochondrial enzyme marker activity peaked in fractions 5 through 7. 6% of the total FALDH activity was found in fractions 5-7. The microsomal contamination in these fractions consisted of 2% of the total microsomal enzyme marker activity. In contrast to

Figure 6. Distribution of FALDH in human cultured skin fibroblast fractions separated by differential centrifugation. The relative enzyme activities were plotted against the enriched fraction type, being enriched for either mitochondria, peroxisomes and microsomes, or cytosol. The enzyme markers used are as follows: succinate dehydrogenase as an inner mitochondrial membrane marker, monoamine oxidase as an outer mitochondrial membrane marker, catalase as a peroxisomal marker, and NADPH cytochrome c reductase as a microsomal marker.

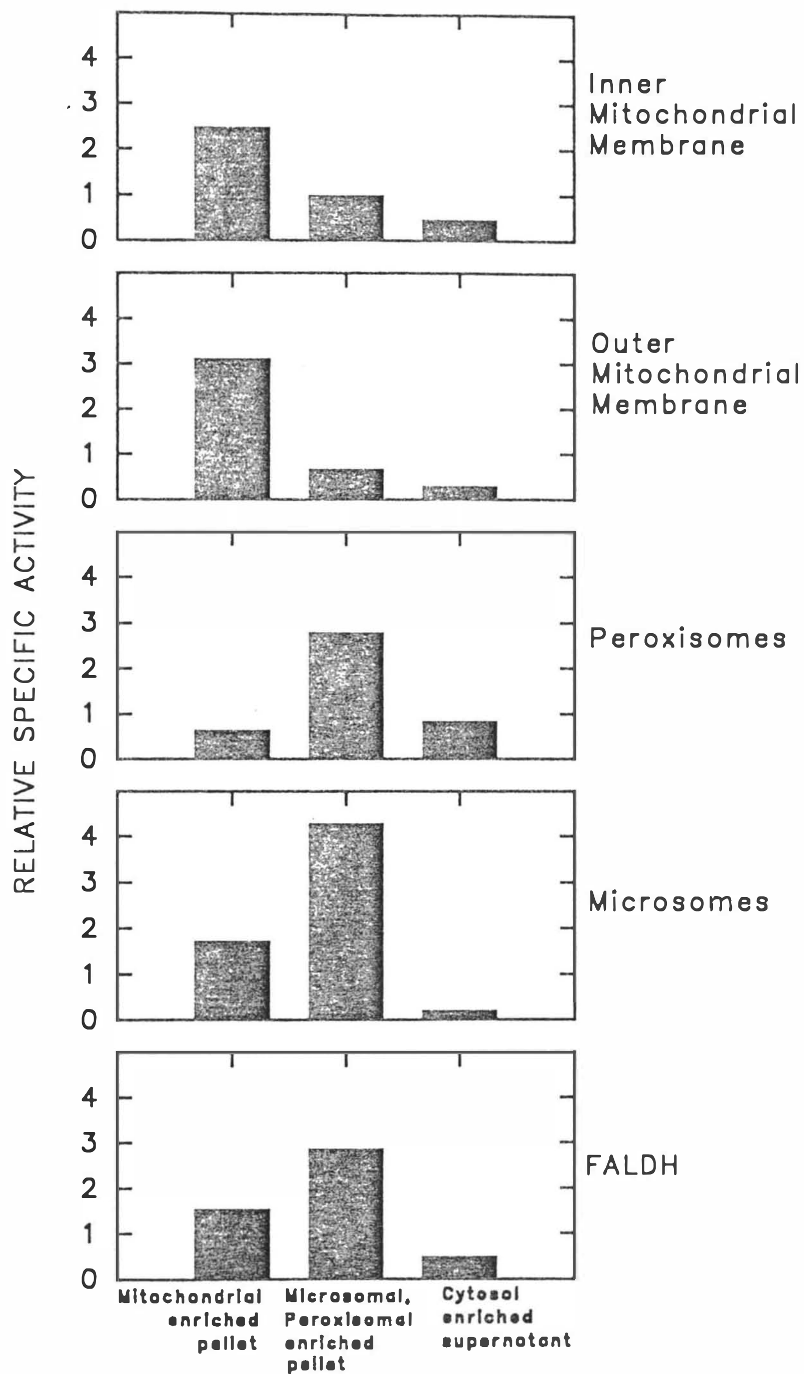
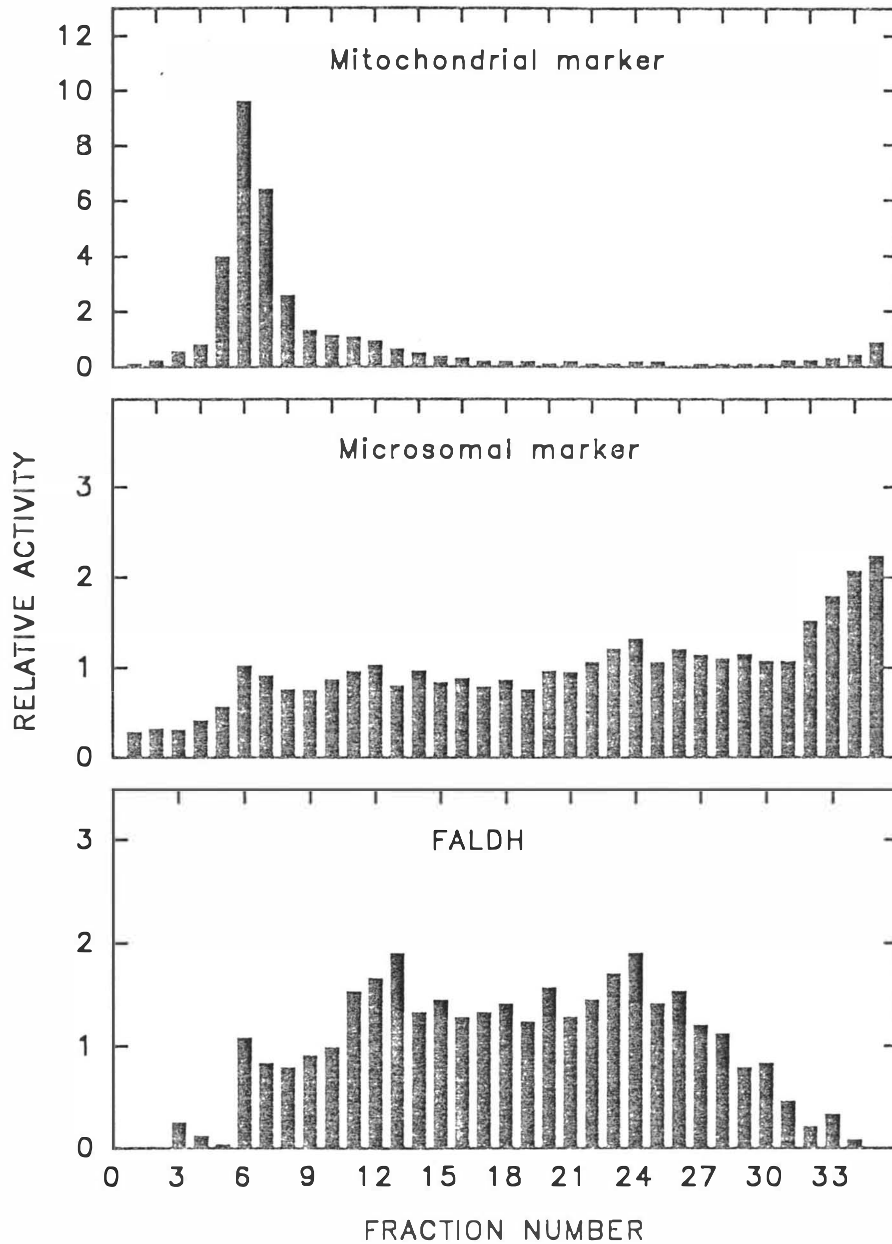

HUMAN CULTURED SKIN FIBROBLASTS
RELATIVE SPECIFIC ACTIVITY
4
3
2
1
0
Inner Mitochondrial Membrane
Outer Mitochondrial Membrane
Peroxisomes
Microsomes
FALDH
Mitochondrial enriched pellet
Microsomal, Peroxisomal enriched pellet
Cytosol enriched supernatant

Figure 7. Distribution of FALDH in human cultured skin fibroblast homogenates separated on a 10-30% Nycodenz gradient. The relative enzyme activity was plotted against fraction number. Two experiments involving different normal cell lines were performed and the results from one experiment are shown. The enzyme markers used are as follows: succinate dehydrogenase as the inner mitochondrial membrane marker and NADPH cytochrome c reductase as the microsomal marker.


HUMAN CULTURED SKIN FIBROBLASTS
Mitochondrial marker
Microsomal marker
FALDH
RELATIVE ACTIVITY
FRACTION NUMBER
0
2
4
6
8
10
12
1
3
9
15
18
21
24
27
30
33

the mitochondrial enzyme marker, the microsomal enzyme marker did not have a clear peak in any region of the gradient. FALDH appeared to follow the same pattern as that of the microsomal marker without any clear peak.

DISCUSSION

Enzymes involved in fatty alcohol metabolism have been described in several subcellular compartments (Tanaka *et al.*, 1981; Bishop and Hajra, 1981; 1982; Burdett *et al.*, 1991; Lee *et al.*, 1980; Singh *et al.*, 1993). Fatty alcohol metabolism in general occurs via membrane-bound enzymes. Fatty alcohol synthesis requires the reduction of fatty acid to fatty alcohol via acyl-CoA. Both of the enzymes (acyl-CoA synthetase and acyl-CoA reductase) involved in fatty alcohol synthesis are particulate. Acyl-CoA synthetase has been described in the microsomes, mitochondria and peroxisomes of the rat (Tanaka *et al.*, 1981). The three acyl-CoA synthetase enzymes in these compartments are indistinguishable based on several physicochemical, catalytic, and immunological properties (Tanaka *et al.*, 1979). Using differential centrifugation methods, acyl-CoA reductase has been described as a microsomal enzyme in rat brain (Bishop and Hajra, 1981), bovine meibomian gland (Kolattukudy and Rogers, 1986), and mouse preputial gland (Wykle *et al.*, 1979), but a

peroxisomal enzyme location was reported in guinea pig intestine when density gradient organelle separations were performed (Burdett *et al.*, 1991). Subcellular localization based on density gradient centrifugation appears to be more precise in separating organelles than differential centrifugation which separates organelles according to size.

Using differential centrifugation methods, the synthesis of glyceryl ether lipids from fatty alcohol has been reported to occur in the microsomes of rat liver (Lee *et al.*, 1980), rat brain (Natarajan and Schmid, 1978), rat sarcoma (Lee *et al.*, 1980), rat hepatoma (Lee *et al.*, 1980), and rabbit intestinal mucosa cells (Soodsma *et al.*, 1972). However, more refined techniques of subcellular fractionation using density gradient separations have clearly shown that the enzyme responsible for ether lipid synthesis (alkyl dihydroxyacetone phosphate synthase) is only present in the peroxisomes of rat liver (Hajra and Bishop, 1982; Singh *et al.*, 1993). Thus, differential centrifugation, which separates organelles according to size, appears to be an inadequate method for precise determination of the subcellular localization of enzymes, because it does not permit the unequivocal separation of organelles.

Studies of FAO and FALDH in rat liver, rabbit intestine, and human cultured skin fibroblasts have

consistently demonstrated that these are particulate enzymes (Lee, 1979; Ichihara *et al.*, 1986a; Mitchell and Petersen, 1989; Rizzo and Craft, 1991). Our results in human liver, HepG2 cells, and cultured skin fibroblasts demonstrate that the majority of FALDH activity parallels the microsomal enzyme marker. These results on human liver and cultured hepatocytes are supported by Nycodenz gradients in rat liver (data not shown). Although we did not separate the cytosolic and microsomal fractions in the fibroblast gradient, we know from prior results that very little FALDH activity in fibroblasts is soluble (Rizzo and Craft, 1991). It is significant that FALDH was not present in the peroxisomes of liver or HepG2 cells.

In all gradients analyzed above, there was a small amount of FALDH activity in the mitochondrial peak fractions that could not be fully accounted for by microsomal contamination. The enzyme marker used to determine the mitochondrial peak fractions (SDH) was specific for the inner mitochondrial membrane. If, during the homogenization process, we had disrupted some of the mitochondria such that the outer membranes were removed and had the same density as microsomes, and if FALDH were localized to the outer membrane of mitochondria, then one might see 2 peaks of activity -- one co-sedimenting with microsomes (broken mitochondrial outer membranes) and the other with mitochondria (intact outer membranes). In the

fibroblast differential centrifugation experiment, we included an outer mitochondrial membrane marker, monoamine oxidase, and demonstrated that this enzyme marker paralleled activity of the inner membrane enzyme marker and did not selectively appear in the microsomal-enriched fraction. Therefore, the localization of FALDH to the outer mitochondrial membrane could not account for FALDH activity seen in the microsomal and peroxisomal-enriched fraction of cultured skin fibroblasts.

There are at least 2 reasons to explain FALDH activity in the mitochondrial fraction being greater than can be explained due solely to microsomal contamination. First, FALDH may be predominantly localized to the microsomes with a subpopulation of enzyme localized to the mitochondria. There is no precedent for one ALDH isozyme localized to 2 different subcellular compartments in humans or other organisms. Theoretically, alternative splicing of one gene product could account for the same isozyme being found in different subcellular compartments. Mitochondrial-targeted proteins contain a leader signal peptide at the N terminus (Hurt and Schatz, 1987), whereas a protein localized to the microsomes has been shown to contain a region of hydrophobic amino acid residues at the carboxy terminus that may be important for insertion and anchoring into the endoplasmic reticulum (Miyauchi *et al.*, 1991). Takagi *et al.* (1985) have demonstrated that rat liver microsomal ALDH

mRNA is translated on free polysomes and the mature enzyme becomes membrane-bound only in the rough and smooth endoplasmic reticulum. Their *in vitro* expression system demonstrated that the authentic and mature enzyme have the same subunit molecular weight as determined by SDS-PAGE. These results from rat liver microsomes suggest that ALDH is localized to the microsomes.

Second, other ALDH isozymes may exist in mitochondria that utilize octadecanal as substrate. ALDHs have been localized to virtually every subcellular compartment with some degree of overlap between substrate specificities (Lindahl, 1992; Yoshida *et al.*, 1991). The FALDH activity seen in the mitochondrial peak is more likely due to other mitochondrial ALDHs with overlapping substrate specificities.

It is well-known that biochemical pathways may involve enzymes located in more than one subcellular organelle. Fatty acid ß-oxidation occurs in the mitochondrial and peroxisomal matrices of mammals (Bremer and Osmundsen, 1984; Lazarow and de Duve, 1976). Long-chain fatty acids are completely oxidized to acetyl-CoA in the mitochondria, whereas long-chain and very long-chain fatty acids are only oxidized to medium-chain fatty acids (8-carbon chain length) and acetyl-CoA in peroxisomes. These medium-chain products of peroxisomal ß-oxidation are then transferred to mitochondria for subsequent oxidation. Thus, interactions

between subcellular compartments occur in fatty acid ß-oxidation. Similarly, activation of trihydroxy cholestanoic acid to its acyl-CoA ester occurs at the microsomal membrane and it is then transported, possibly via a carrier protein, to the peroxisome to be ß-oxidized (Prydz *et al.*, 1988).

Enzymes that participate in fatty alcohol metabolism have been localized to different subcellular organelles. This is logical because synthesis and degradation of fatty alcohols in one subcellular compartment would seem counterproductive. Activation of fatty acid to its acyl-CoA occurs in the mitochondria, peroxisomes, and microsomes. It is probable that reduction of the acyl-CoA to fatty alcohol is peroxisomal (Burdett *et al.*, 1991) where the fatty alcohol could be used for glyceryl ether lipid synthesis. Oxidation of fatty alcohol to fatty acid is microsomal. This would require that fatty alcohol be transported between subcellular organelles in those cells in which it is both synthesized and degraded. Fatty alcohol transport may be mediated by carrier proteins, but there is no evidence as yet for such proteins.

Tissue specific regulation of enzymes involved in the fatty alcohol cycle might also occur. This would permit fatty alcohol synthesis to occur in tissues where fatty alcohols are used as building blocks (for example, the brain where glyceryl ether lipids are abundant). It is

possible that fatty alcohols are transported via the blood to other tissues where enzymes responsible for fatty alcohol degradation are expressed.

Although the synthesis and degradation of fatty alcohol by enzymes in different organelles may seem complex, fatty alcohol metabolism can be regulated by organelle specific factors. The enzymes in the fatty alcohol metabolic pathway may be regulated together, possibly by key control enzymes or carrier proteins which shuttle the fatty alcohols between the microsomes and peroxisomes. Future investigations into the regulation of fatty alcohol levels in the cell may elucidate the factors crucial in controlling the enzymes involved in the fatty alcohol cycle.

CHAPTER 5

Purification and biochemical characterization of microsomal fatty aldehyde dehydrogenase from human liver

SUMMARY

Fatty aldehyde dehydrogenase (FALDH) was solubilized from human liver microsomes and purified by chromatography on columns consisting of omega-Aminohexyl-Agarose resin and 5'AMP-Sepharose 4B resin. The enzyme has an apparent subunit size of 54,000 kDa as determined by SDS-PAGE. FALDH requires NAD^+ as coenzyme, has optimal activity at pH 9.8, and is thermolabile at 47°C. The enzyme has high activity towards saturated and unsaturated aliphatic aldehydes ranging from 6 to 24 carbons in length, as well as dihydrophytal, a 20-carbon branched chain aldehyde. In contrast, acetaldehyde, propionaldehyde, crotonaldehyde, glutaraldehyde, benzaldehyde, and retinaldehyde are poor substrates. FALDH is inhibited by disulfiram, iodoacetamide, iodoacetate, α,p-dibromoacetophenone, and p-chloromercuribenzoate, but is unaffected by magnesium. Comparison of the kinetic and physical properties of this

microsomal FALDH to other human ALDH isozymes (classes 1, 2, 3, 4, and others) suggest that FALDH represents a different class of ALDH isozyme. These studies constitute the first purification and characterization of a human fatty aldehyde dehydrogenase and suggest a role for this enzyme in fatty aldehyde metabolism.

INTRODUCTION

Aldehyde dehydrogenases (ALDHs) comprise a family of enzymes that exhibit substrate specificities toward a wide variety of aliphatic and aromatic aldehydes (Lindahl, 1992; Yoshida *et al*., 1991). Most ALDHs are constitutively expressed, but two ALDHs are induced in rat liver by treatment with phenobarbital or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD); one of these is a tumor-associated rat liver ALDH that is also expressed during carcinogenesis (Yin *et al*., 1989; Forte-McRobbie and Pietruszko, 1986).

Mammalian ALDHs have been classified according to their subcellular localization, and biophysical and kinetic properties. ALDHs have been identified in mitochondrial, microsomal, and cytosolic compartments of the cell. Cytosolic and mitochondrial isozymes have been extensively studied (Yoshida *et al*., 1991; Pietruszko, 1983), whereas little is known about microsomal ALDH. A human microsomal ALDH that catalyzes the conversion of 20-CHO-leukotriene B_4

(LTB_4) to 20-COOH-LTB_4 has been reported in polymorphonuclear leukocytes (Sutyak *et al.*, 1989). Microsomal ALDHs have also been reported in rat liver and rabbit intestine (Ichihara *et al.*, 1986a, 1986b; Mitchell and Petersen, 1989; Nakayasu *et al.*, 1978; Lindahl and Evces, 1984), and are capable of oxidizing medium- and long-chain fatty aldehydes to the corresponding fatty acids. Although little is known about their physiological role, microsomal ALDHs may be important in the oxidation of aldehydes formed during fatty alcohol metabolism and in the detoxification of aldehydic products of lipid peroxidation.

Fatty alcohol is oxidized to fatty acid by action of fatty alcohol:NAD^+ oxidoreductase (FAO). This enzyme complex consists of separate activities that sequentially oxidize fatty alcohol to fatty aldehyde catalyzed by fatty alcohol dehydrogenase (FADH), and finally to fatty acid catalyzed by fatty aldehyde dehydrogenase (FALDH). Genetic deficiency of FALDH has been demonstrated in Sjögren-Larsson syndrome (SLS), an autosomal recessive disorder characterized by the presence of congenital ichthyosis, mental retardation, and spastic di- or tetraplegia (Rizzo and Craft, 1991). Deficiency of FALDH activity causes deficiency of FAO activity, and the accumulation of fatty alcohol in patients affected with SLS (Rizzo et *al.*, 1988; 1989).

Subcellular localization studies in human liver and cultured skin fibroblasts have indicated that FALDH is localized to the microsomal compartment (see Chapter 4), and prior studies using differential centrifugation have shown that cultured skin fibroblasts from SLS patients are most profoundly deficient in the 120,000g pellet (Rizzo and Craft, 1991). These findings suggest that SLS is due to a deficiency of microsomal FALDH.

To provide insight into the biochemical abnormalities that may be associated with SLS and understand fatty alcohol and fatty aldehyde metabolism, the purification and characterization of human liver microsomal FALDH was undertaken. Comparison of the kinetic, structural, and biophysical properties of FALDH with other human ALDHs suggests that this FALDH is a novel isozyme.

MATERIALS AND METHODS

MATERIALS

Thin-layer chromatography plates composed of silica gel G were obtained from Whatman Inc., Clifton, NJ. Organic solvents, either reagent-grade or high-performance liquid chromatography-grade, were from J.T. Baker Inc., Phillipsburg, NJ. Potassium phosphate (monobasic and dibasic) was obtained from Mallinckrodt (Paris, KY). α,p-dibromoacetophenone was purchased from Aldrich Chemical

Co., Inc. (Milwaukee, WI). α,p-dibromoacetophenone, p-chloromercuribenzoate (p-CMB), and disulfiram were resuspended in 100% dimethyl sulfoxide. omega-Aminohexyl-Agarose and 5'AMP-Sepharose 4B were purchased from Sigma Chemical Co. All other chemicals were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise noted.

A p-hydroxyacetophenone affinity ligand column was constructed according to the method described by Ghenbot and Weiner (1992). p-Hydroxyacetophenone was coupled to Sepharose 4B through an epoxy linkage.

The nomenclature used in naming the aldehydes is derived from "Definitive Rules for the Nomenclature of Organic Chemistry". Acetaldehyde, octanal, dodecanal, tetradecanal, and crotonaldehyde were purchased from Aldrich Chemical Co. They were diluted in 100% ethanol to the final desired concentration (80 mM). Glutaraldehyde (grade 1, 70% aqueous), propionaldehyde, hexanal, benzaldehyde, decanal, and retinaldehyde were purchased from Sigma Chemical Co. and were diluted in 100% ethanol to the final desired concentration (35.2 mM for retinaldehyde and 80 mM for all other aldehydes). Technical grade phytol (3,7,11,15-Tetramethyl-2-hexadecene-1-ol) was obtained from Analabs, The Foxboro Co., North Haven, CT.

Adult male Wistar rats (200-250 g) were killed by decapitation without any anesthesia and the organs were

used immediately. Human livers that, for one reason or another, could not be transplanted into patients were used for enzyme purification. Livers were stored at -70°C.

ENZYME ASSAYS

FALDH was assayed fluorometrically by measuring the fatty aldehyde-dependent production of NADH. The reaction was monitored using a filter fluorometer (Model 111: Turner Designs, Sunnyvale, CA); or by measuring fluorescence with a microtiter plate reader (LS50: Perkin Elmer Ltd., Beaconsfield, England). The excitation wavelength was 365 nm and the emission wavelength was 460 nm. In the Turner fluorometer, primary filter 7-60 was used, and secondary filters 2A and B440 were used (Baxter Diagnostics Inc., McGaw Park, IL).

Reaction tubes (6 X 50 mm disposable glass test tubes, Baxter) contained 50 mM glycine-NaOH buffer, pH 9.8, 10 mM pyrazole, 0.5 mg/ml fatty acid-free BSA, 1.5 mM NAD^+, 0.1% Triton X-100, and less than 6 μg of homogenate protein in a final volume of 0.2 ml (for the microtiter plate reader) or 0.4 ml (for the Turner fluorometer). Reaction tubes were preheated for 2 minutes at 37°C, and the assay was initiated by the addition of 3-5 μl of aldehyde substrate dissolved in 100% ethanol. In control incubations, the reactions were initiated by the addition of ethanol lacking

aldehyde. The reaction was stopped by the addition of p-chloromercuribenzoate (solubilized in 100% dimethyl sulfoxide to a final concentration of 1.25 mM) and the fluorescence was measured. The aldehyde-dependent activity was calculated by subtracting the change in fluorescence measured in the absence of aldehyde (control reaction) from that measured in the presence of aldehyde.

FALDH activity using acetaldehyde, propionaldehyde, benzaldehyde, glutaraldehyde, and crotonaldehyde could not be assayed under the same reaction conditions as long-chain aldehydes, because these substrates caused an increase in fluorescence even in the absence of added enzyme. Instead, activity was measured using 25 mM Tris-HCl, pH 8.8, in place of glycine-NaOH buffer without any Triton X-100 in the reaction mixture.

FAO activity was assayed by measuring the oxidation of radioactive octadecanol to radioactive stearate (Rizzo *et al.*, 1988; 1989). Activity was measured in a total volume of 0.2 ml containing 50 mM glycine-NaOH buffer, pH 9.5, 2.5 mM NAD^+, 0.5 mg/ml fatty acid-free bovine serum albumin, 12 μM [1-^{14}C]-octadecanol (220,000 cpm added in 3 μl ethanol), and 10-60 μg of cell homogenate protein; control incubations lacked homogenate. After 30 minutes at 37°C, reactions were stopped by the addition of 2 ml hexane, 2 ml water, and 2 ml 0.3 M NaOH in 95% ethanol. Reaction tubes

were agitated for 1 minute in a vortex mixer and centrifuged for 5 minutes at 2700g. The upper phase was discarded and the lower phase was extracted again with 2 ml hexane. One ml of 2N HCl was added to the remaining lower phase, and [^{14}C]-stearate was extracted twice with 2 ml hexane. The combined extracts were dried, and labeled stearate was isolated by thin-layer chromatography (TLC). To each lane of a silica gel G plate, 15 μg of carrier palmitate and hexadecanol were applied. The dried lipids were dissolved in chloroform/methanol (1/1) and spotted on individual lanes on the plate. Fatty acid was separated from fatty alcohol by chromatography in hexane/diethyl ether/water (60/40/1) solvent. The fatty acid spot was visualized under UV light after staining the plate with rhodamine 6G (1 mg/ml methanol). The areas of silica gel containing palmitate were scraped into scintillation vials and measured for radioactivity. The background radioactivity from control incubations was subtracted from that in incubation reactions containing homogenate protein to determine actual enzyme activity.

Protein concentrations were measured according to Lowry *et al.* (1951), absorbance at 245 nm (column chromatography) using a spectrophotometer (Bausch and Lomb Spectronic 1001, Milton Roy Co., Rochester, NY), or by the Bio-Rad DC protein assay when detergent was present in the

protein mixture. Succinate dehydrogenase and NADPH cytochrome c reductase were assayed by the method described previously in this text (see Chapter 4, Materials and Methods).

ALDEHYDE SYNTHESES

Long-chain aldehydes (15 to 24 carbons) were synthesized by oxidation of the primary alcohol to the fatty aldehyde. The saturated aldehydes were synthesized according to the method of Ferrell and Yao (1972). Briefly, 5 mg fatty alcohol was reacted with 4 mg 1-chlorobenzotriazole in 40 μl dichloromethane and 15 μl pyridine overnight in a 60°C water bath. The fatty aldehyde product was separated from unreacted fatty alcohol by TLC using two different solvent systems. The first system consisted of hexane/isobutanol/methanol (100/3/3). The fatty aldehyde was localized by staining the plate with rhodamine 6G and examining under UV light. The fatty aldehyde and fatty alcohol migrate to the middle of the plate, with the fatty aldehyde being the band with an R_f of 0.5. The fatty aldehyde spot was carefully scraped and eluted from the silica gel by extraction with 5 ml hexane/benzene (3/2). The hexane/benzene extract was dried under a stream of nitrogen, and the fatty aldehyde was purified on a second TLC plate using a solvent system

consisting of hexane/chloroform/methanol (73/25/2). In this system, the fatty aldehyde migrates near the top of the plate with an R_f of 0.8. The purified fatty aldehyde band was visualized, scraped, and eluted from silica gel as described above. The final fatty aldehyde concentrations were determined by gas liquid chromatography with flame ionization detection using response factors for fatty alcohol standards of similar carbon chain-length as that of the aldehydes. The response factors were determined by measuring the linear relationship of the system response as a function of the amount of standard injected.

Unsaturated alcohols (cis-9-hexadecene-1-ol (16:1), cis-9-octadecene-1-ol (18:1), and cis,cis-9,12-octadecadiene-1-ol (18:2)) were oxidized to aldehydes via their mesylate intermediates (Baumann and Mangold, 1964; Mahadevan, 1965). Briefly, mesylate synthesis was accomplished by dissolving 20 mg of the fatty alcohol in 100 μl pyridine, the tube was chilled on ice for 10 minutes. Then, 10 μl methanesulfonyl chloride was added. The mixture was again incubated on ice for 1 hour, and then removed from the ice bath and left at room temperature for 5 hours with shaking. Deionized water and diethyl ether (1 ml of each) were added. After vortexing for 1 minute, the ether phase (upper layer) was removed and extracted with 1 ml of the following solutions (in this order): deionized

water; 2 N H_2SO_4; deionized water; 1% potassium carbonate solution; and finally deionized water. The ether layer was dried under a stream of nitrogen. The mesylate product, thus formed, was then dissolved in 100 μl dimethyl sulfoxide to which 10 mg solid sodium bicarbonate was added. The mixture was closed in a nitrogen atmosphere and immersed in a 170°C oil bath. The reaction was complete within 5 minutes and the tube was cooled to room temperature, after which 1 ml ice water was poured into the tube. The mixture was extracted with 1 ml diethyl ether, and washed with deionized water. The organic ether layer was dried under nitrogen and the fatty aldehyde was dissolved in 100% ethanol. The fatty aldehyde was quantitated by gas liquid chromatography and dissolved in ethanol to a concentration of 80 mM. The final yield was between 9% and 15%.

Phytol (3,7,11,15-Tetramethyl-2-hexadecene-1-ol) was used as starting material to synthesize dihydrophytal (3,7,11,15-Tetramethyl-hexadecanal). The technical grade phytol was first purified by TLC in a solvent system consisting of hexane/chloroform/methanol (73/25/2). The TLC plate was sprayed with rhodamine 6G and visualized under UV light. The phytol spot with an R_f of 0.3 was scraped and eluted from silica gel using hexane/benzene (3/2). Dihydrophytol was synthesized from phytol by

hydrogenation in the presence of Raney nickel as catalyst (Jellum *et al.*, 1966). Approximately 100 mg of TLC-purified technical grade phytol was dissolved in 8 ml ethanol and 100 μl of a slurry of Raney nickel was added. The reaction proceeded under an atmosphere of hydrogen on a shaking platform at room temperature overnight. The ethanol containing dihydrophytol was carefully removed so as to avoid the Raney nickel and then the ethanol was dried under nitrogen. Dihydrophytol was purified by TLC and developed in hexane/chloroform/methanol (73/25/2). Dihydrophytol migrated near the middle of the plate with an R_f of 0.4, and was localized by spraying the plate with rhodamine 6G and examining under UV light. The dihydrophytol spot was scraped and eluted with hexane/benzene (3/2). A 20% yield of dihydrophytol was obtained. Dihydrophytol was oxidized to dihydrophytal using the method of Ferrell and Yao as described above (Ferrell and Yao, 1972). The yield of dihydrophytal from dihydrophytol was 50%, therefore, the overall yield of dihydrophytal from phytol was 10%.

ENZYME PURIFICATION

Human microsomal FALDH was purified by modification of the procedure described by Nakayasu *et al.* (1978). All steps were performed at 4°C. Potassium phosphate buffer

used in this purification was pH 7.5 and contained 1 mM EDTA, 10 mM ß-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (PMSF), except where noted.

Three volumes of homogenization buffer (0.25 M sucrose, 1 mM Tris-base, pH 7.4, 1 mM EDTA) were added to each gram of wet human liver. The tissue was homogenized with a Tissumizer (Tekmar, Cincinnati, OH) having a coarse probe for several minutes until the large chunks of tissue were dispersed, and then was further homogenized using a motor-driven teflon-glass homogenizer attached to a rheostat (set at 70% power), for 10 strokes. The homogenate was centrifuged at 500g for 10 minutes. The supernatant was collected and centrifuged again for 10 minutes at 500g. The supernatant was then poured through 4 layers of gauze and centrifuged at 19,000g for 30 minutes (L5-50 Ultracentrifuge, Beckman Instruments, Inc., Palo Alto, CA; SW27 swinging bucket rotor). The supernatant was decanted and then centrifuged at 100,000g for 30 minutes. The pellet from this centrifugation step was enriched for microsomes. The cytosolic supernatant was carefully removed with a transfer pipet. The microsomal pellet was re-suspended in homogenization buffer and centrifuged a second time under the same conditions. The purified microsomal pellet was re-suspended at a final concentration of 5-8 mg protein/ml in solubilization buffer consisting of 100 mM potassium phosphate, pH 7.5, 1 mM EDTA, 10 mM ß-

mercaptoethanol, 1 mM PMSF, 20% glycerol, and 0.5% sodium cholate. The microsomes were solubilized by gently stirring at 4°C for one hour and then were centrifuged at 100,000g for one hour. The supernatant (soluble microsomes) was carefully removed with a transfer pipet.

The solubilized microsomes were slowly loaded (25-30 ml/hour) onto an omega-aminohexyl-agarose column (2.5 X 8 cm) previously equilibrated with solubilization buffer. The column was washed extensively (10 bed volumes) with solubilization buffer lacking glycerol. FALDH was then eluted with 100 mM potassium phosphate, pH 7.5, 1 mM EDTA, 10 mM ß-mercaptoethanol, 1 mM PMSF, 0.3% sodium cholate, and 0.2% Triton X-100. After eluting for approximately 1 bed volume, the salt concentration was increased to 500 mM potassium phosphate, with the concentration of all other ingredients remaining the same, in order to elute FALDH activity. The fractions collected after elution began that contained the most FALDH activity were pooled and designated the hydrophobic column eluate. The eluate was dialyzed against 10 volumes of 100 mM potassium phosphate, pH 7.5, 1 mM EDTA, 10 mM ß-mercaptoethanol, and 1 mM PMSF for 24 hours with at least two changes of buffer.

The hydrophobic column eluate was loaded onto a 5'-AMP-Sepharose 4B affinity column (1.5 X 4 cm) previously equilibrated with the 100 mM potassium phosphate dialysis buffer. The affinity column was loaded at a flow rate of

0.5 ml/minute, washed with 25 mM potassium phosphate buffer, pH 7.5, for 5-10 bed volumes, and eluted with 25 mM potassium phosphate buffer containing 0.2 mM NAD^+. Column fractions were assayed for FALDH activity and the eluate fractions containing the majority of activity were pooled and designated as purified FALDH.

Regeneration of columns The omega-aminohexyl-agarose hydrophobic column was regenerated by washing with 3-5 bed volumes of the following solutions in this order: 0.05 M NaOH; 0.1 M sodium acetate, pH 4.5; deionized water; and 2 M NaCl. The 5'AMP-Sepharose-4B affinity column was regenerated by washing with 3-5 bed volumes of the following solutions in the order given: 0.05 M Tris-HCl, pH 8.5, 0.25 M NaCl; 0.05 M sodium acetate, pH 4.5, 0.25 M NaCl; deionized water; and 2 M NaCl. If the columns were not to be reused within a month, they were stored in 2 M NaCl with thimerosal (10 mg/100 ml).

KINETIC AND THERMOSTABILITY STUDIES

Enzyme kinetic studies were performed using approximately 12.5 ng of purified FALDH and assaying for 30 minutes with varying concentrations of substrate (range: 60 nmol-5 mmol). Lineweaver-Burk plots were generated and analyzed using the software program Enzfitter (Biosoft, Cambridge, United Kingdom) to determine K_m and V_{max}.

Inhibition and activation studies were performed by assaying purified microsomal FALDH (12.5 ng) using octadecanal as substrate with various inhibitors or activators at the final concentrations indicated.

Thermostability of FALDH was studied by incubating purified enzyme in 200 mM glycine buffer, pH 9.8 in a water bath at the various temperatures for various times. The enzyme was removed and immediately chilled on ice. After 5 minutes on ice, FALDH activity was assayed at 37°C for 30 minutes using n-octadecanal as substrate.

MOLECULAR WEIGHT DETERMINATION

Sodium dodecyl sulfate discontinuous polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels (Bio-Rad Laboratories, Richmond, CA) was performed according to Laemmli (1970). Native (nondenaturing) gel electrophoresis was performed in 6% linear and 4-24% gradient polyacrylamide gels (Bio-Rad Laboratories). Gels were stained either with Coomassie Brilliant Blue R-250 or silver nitrate (Bio-Rad) according to manufacturer's instructions.

SDS-PAGE gels were scanned and integrated by densitometry on an LKB 2222-020 UltroScan XL Laser Densitometer (Pharmacia, Uppsala, Sweden).

Gel filtration was performed by low pressure chromatography using Bio-Rad Bio-Gel A-0.5m (1.5 X 100 cm)

and Bio-Rad Bio-Gel A-1.5m (1.5 X 50 cm and 1.5 X 100 cm) with a Rainin Rabbit peristaltic pump (flow rate 25 ml/hour); and by high-pressure liquid chromatography (HP 1090, Hewlett Packard, Avondale, PA) using a Rainin Hydropore-5-sec column (10 mm X 25 cm) with 83-S13-C5 as a guard column. The column buffers contained 200 mM potassium phosphate buffer, pH 7.5, and either 0.2% Triton X-100, 1.0% Triton X-100 or 0.5% sodium cholate.

RESULTS

ENZYME DISTRIBUTION IN RAT TISSUE

FAO and FALDH were assayed in crude homogenates from various rat tissues in order to determine the tissue enzyme distribution (Table 7). Each enzyme was assayed in duplicate and the average value was expressed. FAO and FALDH activities were highest in the liver. FALDH specific activity was at least 20-fold higher than FAO in every rat tissue, but there was not always a direct correlation between FAO and FALDH activities. Oxidation of fatty alcohol to fatty aldehyde by FADH is the rate-limiting reaction in the FAO complex (Rizzo and Craft, 1991), and FAO activity may therefore reflect FADH activity more than FALDH activity. This may explain why FAO and FALDH activities do not correlate in every tissue. There may also be other non-microsomal ALDHs with overlapping

Table 7. Tissue distribution of fatty aldehyde dehydrogenase (FALDH) and fatty alcohol:NAD^+ oxidoreductase (FAO) specific activity in the rat using 18-carbon substrates[a].

Rat Tissue	FALDH	FAO
	nmol/min/mg	pmol/min/mg
Liver	40.9	1800
Muscle	18.8	5
Heart	13.2	10
Intestine	12.0	600
Kidney	7.4	90
Stomach	6.2	20
Lung	6.1	140
Testis	4.1	130
Brain	1.7	5
Skin	1.3	20

[a] The results from one rat are given.

substrate specificities in the crude homogenates of those rat tissues studied. These non-microsomal ALDHs may contribute to FALDH activity but not FAO activity.

HUMAN LIVER FALDH

The optimal assay conditions for measuring FALDH activity were initially established using crude liver soluble microsomal protein as enzyme source and octadecanal as substrate. Figure 8 shows that FALDH activity is linear with respect to protein concentration up to at least 6 μg per assay. Figure 9 demonstrates the time course of the enzyme assay using a protein concentration of less than 5 μg. FALDH activity increases linearly with time up to 150 minutes. All subsequent FALDH assays were, therefore, performed with protein concentrations and incubation times within the linear range.

Differential centrifugation of liver homogenates demonstrated that 60% of FALDH activity using octadecanal as substrate was located in the 19,000g pellet. This 19,000g pellet was sonicated to release organelle matrix proteins into the supernatant. After re-centrifugation at 19,000g, most of the FALDH activity (95%) was contained in the pellet. FALDH activity in the 19,000g pellet could be solubilized with 1% Triton X-100. These results suggest that FALDH is a membrane-bound protein in the 19,000g pellet.

Figure 8. FALDH activity with respect to protein concentration. Human liver microsomes solubilized with 0.5% sodium cholate were assayed for 60 minutes. The results are expressed as nanomoles of NADH formed per minute.

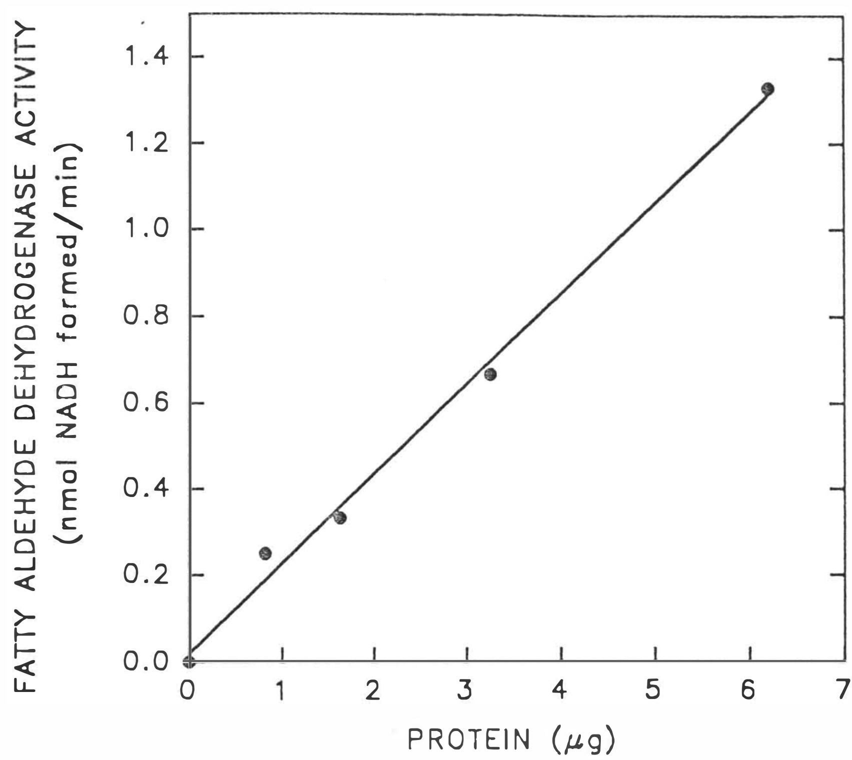
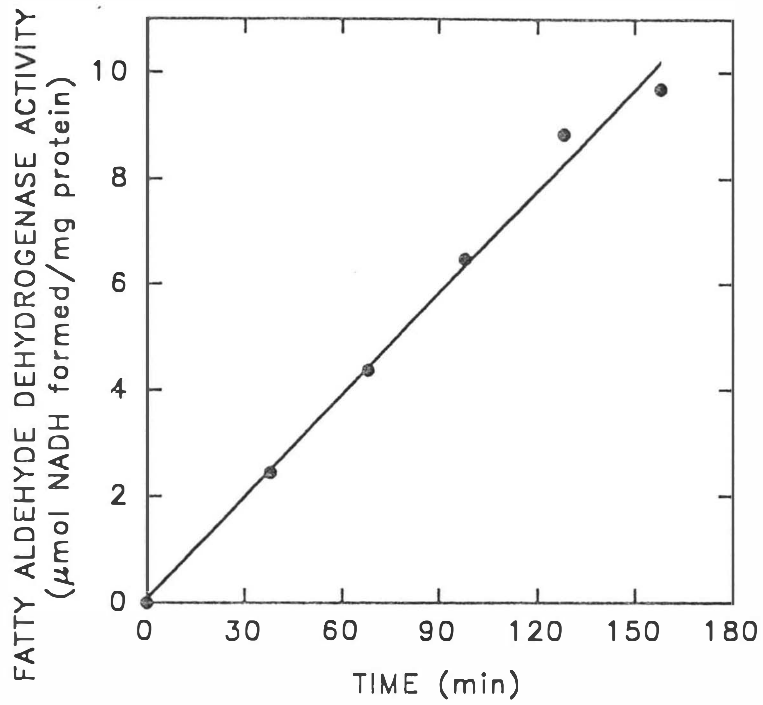

FATTY ALDEHYDE DEHYDROGENASE ACTIVITY
(nmol NADH formed/min)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
7
PROTEIN (μg)

Figure 9. FALDH activity with respect to time of incubation. Human liver microsomes (1.63 μg) solubilized with 0.5% sodium cholate were incubated for varying lengths of time. The results are expressed as micromoles of NADH formed per milligram soluble protein.


FATTY ALDEHYDE DEHYDROGENASE ACTIVITY
(μmol NADH formed/mg protein)
0
2
4
6
8
10
0
30
60
90
120
150
180
TIME (min)

In order to determine the extent of subcellular organelle contamination in these pellets, organelle-specific enzyme marker assays were performed on the 19,000g pellet, the 100,000g pellet, and the 100,000g supernatant (Table 8). Succinate dehydrogenase (SDH), an inner mitochondrial membrane enzyme, and NADPH cytochrome c reductase, a microsomal enzyme were utilized as organelle specific markers. In the case of SDH, 50% of total enzyme activity was located in the 19,000 g pellet and 25% was found in the 100,000 g pellet. For NADPH cytochrome c reductase, 55% of the total activity was found in the 19,000 g pellet and 45% of the total activity was found in the 100,000 g pellet. FALDH activity in these fractions closely mirrored that of the microsomal marker. Previous results using Nycodenz gradients (see Chapter 4) indicated that FALDH is mainly a microsomal enzyme. FALDH was purified separately from both the 19,000g and 100,000g pellets, and the enzyme in either pellet was found to be identical with respect to subunit molecular weight (54 kDa) and substrate specificity (as defined by the ratio of specific activity between 3-carbon and 18-carbon aldehyde substrates which was 0.23 in both pellets). Taken together, these results strongly suggest that the FALDH activity in the 19,000g pellet was due to microsomal contamination.

Our goal was to purify a microsomal ALDH that was

Table 8. Differential centrifugation of human liver. Each fraction was assayed for the enzyme indicated and results are expressed as the % total activity.

Enzyme	19,000g pellet	100,000g pellet	100,000g supernatant
SDH[a]	50%	25%	25%
NADPH cytochrome c reductase[b]	45%	55%	0
FALDH[c]	53%	47%	0[d]

[a] mitochondrial inner membrane enzyme marker
[b] microsomal enzyme marker
[c] FALDH was assayed using octadecanal as substrate
[d] The aldehyde-independent activity was equal to the aldehyde-dependent activity, so the overall activity is zero.

active against long-chain substrates, such as octadecanal, because previous studies indicated that the enzyme that is deficient in SLS is active against longer-chain substrates (Rizzo and Craft, 1991). A key to this enzyme purification was the ability to detect FALDH activity in column eluates. Our experience with the FALDH assay suggests that detergent is required in the reaction mixture in order for FALDH to optimally oxidize long-chain aldehydes. Table 9 shows the effects of 0.1% Triton X-100 in the reaction buffer when either soluble microsomal homogenates or purified FALDH were assayed. FALDH utilizes medium-chain aldehydes (dodecanal) equally well in the presence or absence of detergent. However, the enzyme assay requires the presence of detergent to measure activity against the more hydrophobic long-chain fatty aldehydes (octadecanal, dihydrophytal), especially when purified FALDH is used. Addition of detergent may be necessary to solubilize the long-chain fatty aldehyde substrates so as to make them available to the enzyme for oxidation. If there was no Triton X-100 in the reaction buffer, then FALDH activity could not be easily detected when long-chain aldehyde substrates were used. Therefore, all fractions through-out the purification process were assayed in the presence of 0.1% Triton X-100 using octadecanal as substrate.

Table 9. FALDH activity using medium- and long-chain substrates in the presence or absence of detergent in the reaction buffer.

	Soluble Microsomes[a]		Pure Enzyme	
Substrate	+ 0.1% Triton X-100	no detergent	+ 0.1% Triton X-100	no detergent
n-dodecanal	540	470	41,100	36,500
n-octadecanal	590	100	46,900	3,700
dihydrophytal	100	24	8,000	1,600

[a] microsomes were solubilized with 0.5% sodium cholate, data expressed as nmol/min/mg protein

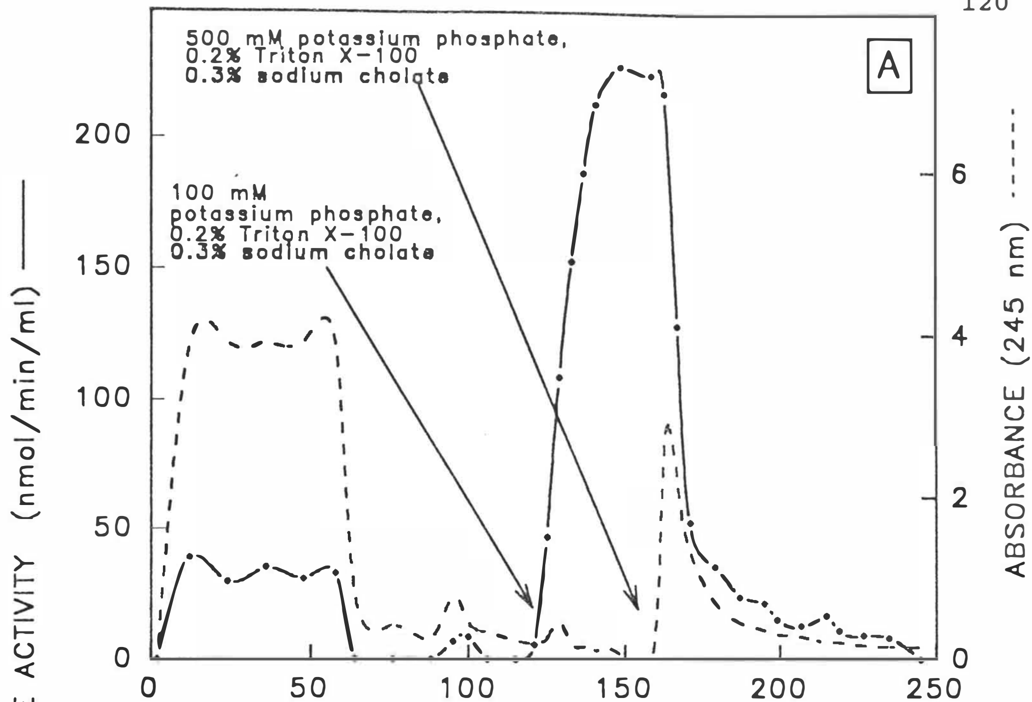
PURIFICATION OF HUMAN LIVER MICROSOMAL FALDH

FALDH activity was readily solubilized using two different classes of detergents: Triton X-100 (non-ionic) and sodium cholate (anionic). Triton X-100, at a final concentration of 0.2%, solubilized 57 ± 27% (n=4) of total FALDH activity from the 19,000g pellets, whereas sodium cholate, at a final concentration of 0.5%, solubilized 80 ± 27% (n=8) of total FALDH activity from both the 19,000g and the 100,000g pellets. Sodium cholate was used routinely to solubilize microsomal FALDH.

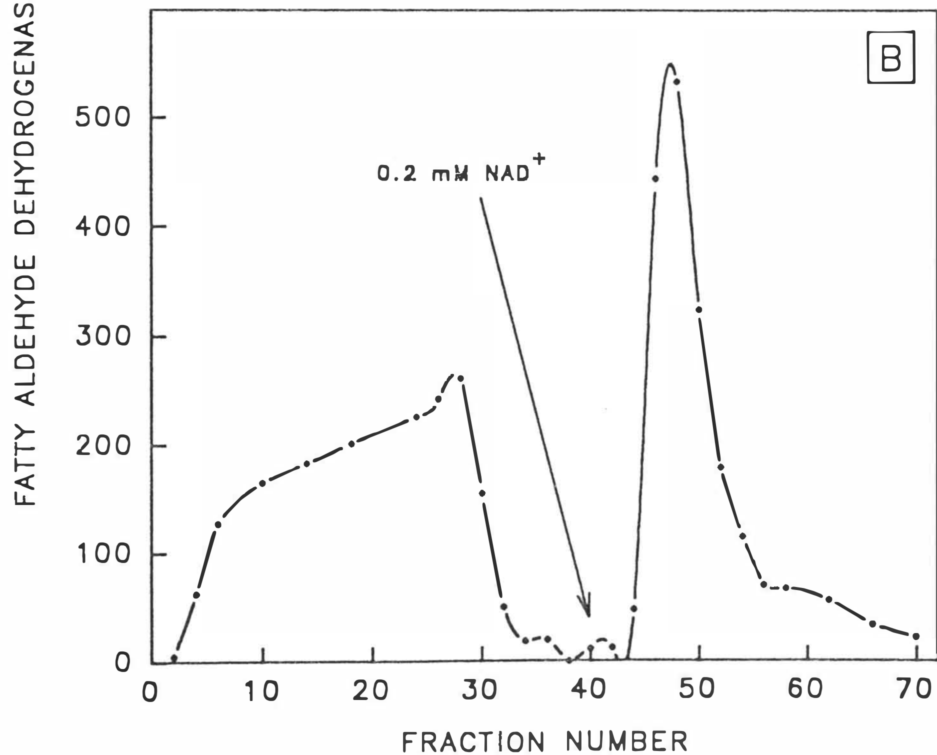
Purification of the hydrophobic, NAD^{+}-dependent microsomal FALDH required the use of two chromatography columns, an omega-aminohexyl-agarose (hydrophobic) column followed by a 5'AMP-Sepharose-4B affinity column. Figure 10 shows the binding and elution of FALDH from the hydrophobic and 5'AMP affinity columns. FALDH binds to the hydrophobic column and begins to elute when the detergent concentration is changed so that the potassium phosphate buffer contains 0.3% sodium cholate and 0.2% Triton X-100 (FALDH also elutes in the presence of 0.1% Triton X-100 and 0.4% sodium cholate). FALDH elutes as a very broad peak, so in order to decrease the elution volume, the elution buffer was changed to 500 mM potassium phosphate with no change in detergent concentrations after the first peak of protein eluted from the column (approximately one bed

Figure 10. Chromatography on omega-aminohexyl-agarose (A) and 5'AMP-sepharose 4B (B). Human liver solubilized microsomes (soluble in 0.5% sodium cholate, see description in the Materials and Methods section) were loaded onto the omega-aminohexyl-agarose column (A), washed free of unbound protein, and eluted first with addition of 0.2% Triton X-100 (beginning at fraction 110), and finally with the same elution buffer above but with 500 mM potassium phosphate buffer, pH 7.5 (beginning at fraction 150). Fractions were collected (8 ml) and assayed for FALDH activity (——) using octadecanal as substrate and protein absorbance (----) at 245 nm.

The fractions containing the most FALDH activity were pooled, dialyzed against 100 mM potassium phosphate buffer, pH 7.5, and loaded onto a 5'AMP-Sepharose affinity column (B). The column was extensively washed, and FALDH activity was eluted with 25 mM potassium phosphate buffer, pH 7.5 and 0.2 mM NAD^+ beginning at fraction 42. Fractions were collected (2 ml) and FALDH was assayed (——) using octadecanal as substrate. Proteins could not be detected in the eluate fractions due to the low concentration of proteins present in each fraction.



500 mM potassium phosphate, 0.2% Triton X-100 0.3% sodium cholate
100 mM potassium phosphate, 0.2% Triton X-100 0.3% sodium cholate
A
ABSORBANCE (245 nm)
0
50
100
150
200
250
2
4
6



B
0.2 mM NAD+
FATTY ALDEHYDE DEHYDROGENASE ACTIVITY (nmol/min/ml)
FRACTION NUMBER
0
10
20
30
40
50
60
70
100
200
300
400
500

volume). Some FALDH activity flowed through the hydrophobic column. When this flow-through activity was reapplied to a regenerated column, FALDH bound to the resin and elute with Triton x-100 in the buffer. This suggests that protein overloading occurred with the hydrophobic column.

FALDH bound to the affinity resin 5'AMP-Sepharose 4B and was eluted by NAD^+; however, a large amount of FALDH activity flowed through the column. This flow-through may have been due in part to overloading the column. When flow-through fractions were pooled and reloaded onto the column, FALDH activity bound to the resin and was eluted with NAD^+. This recycling process was continued in one instance up to 5 times, and FALDH activity was eluted with NAD^+ each time. All eluates contained similar specific activities of FALDH and a similar subunit size on SDS-PAGE. The molar binding capacities for this affinity column were determined to be sufficient for the amount of FALDH activity loaded onto the column assuming that every binding site in the affinity resin was available for protein interaction. Resin packing in these columns may have resulted in some 5'AMP molecules being too close together in the column to allow stoichiometric binding of FALDH molecules. Therefore, we conclude that the 5'AMP column overflow may have been due to insufficient binding capacity

of the column resin.

Attempts to purify FALDH with affinity resins other than 5'AMP-Sepharose 4B in order to get more efficient binding were not successful. FALDH did not bind to any of the following ß-NAD^+-Agarose affinity resins: attached through C-8 (6 or 9 carbon spacer arm), attached through ribose hydroxyls (6 or 11 carbon spacer arm), or attached through N-6 (8 or 11 carbon spacer arm). In addition, FALDH did not bind to any of the following colored dye resins: red 120, yellow 3, yellow 86, green 19, green 5, brown 10, blue 4, blue 72, or blue 3GA. FALDH was loaded onto these columns in 10 mM or 100 mM potassium phosphate buffers, pH 7.5, pH 9.0, or pH 9.5 with or without detergents, sodium cholate or Triton X-100.

When 5'AMP-Sepharose 4B purified FALDH was dialyzed against 10 mM potassium phosphate, the enzyme could be bound to hydroxyapatite, washed with 100 mM potassium phosphate, and eluted with 300 mM potassium phosphate. Extensive washing of the protein bound to the column was undertaken in an attempt to remove residual detergent and NAD^+ associated with FALDH. This hydroxyapatite column also concentrated the enzyme, but did not appear to improve the purity of FALDH.

Other chromatographic columns were used in an attempt to purify FALDH. Purified or partially purified FALDH did

not bind to DEAE-cellulose in 10 mM potassium phosphate buffer, pH 7.5, in the presence of buffer and 0.2% Triton X-100. However, detergent-free purified FALDH in 10 mM potassium phosphate buffer bound to DEAE-cellulose, and was eluted with 500 mM potassium phosphate, pH 7.5 and 0.1% Triton X-100 in the elution buffer. This step tended to eliminate some minor protein contaminants.

FALDH did not bind to a specific affinity column containing the ligand p-hydroxyacetophenone coupled to Sepharose 4B via an epoxy linkage. This affinity column was used previously to purify the rat mitochondrial class 2 enzyme (Ghenbot and Weiner, 1992). p-Hydroxyacetophenone inhibits rat liver class 2 ALDH activity and has a slight inhibitory effect on human FALDH as well (see Table 13).

Purified FALDH was loaded onto a concanavalin-A column. Concanavalin-A is a lectin which preferentially binds α-linked mannose sugars. The detergent solubilized FALDH did not bind to concanavalin-A. These results suggest that mature FALDH does not contain α-linked mannoses.

Table 10 summarizes the results of a typical purification of FALDH from human liver microsomes. The procedure used in these experiments resulted in a 167-fold purification (range: 167-322-fold for all five purifications).

Table 10. Purification of fatty aldehyde dehydrogenase from human liver microsomes.

Step	Protein (mg)	Total Activity (μmol/min)	Specific Activity (μmol/min/mg)	Yield (%)	Purification (-fold)
crude	7,780	1,350	0.2	100	1
microsomes	740	340	0.5	25	3
0.5% sodium cholate soluble microsomes	380	210	0.6	16	3
hydrophobic column eluate	50	150	3.0	11	15
5'AMP column eluate	0.15	5	33.3	0.4	167

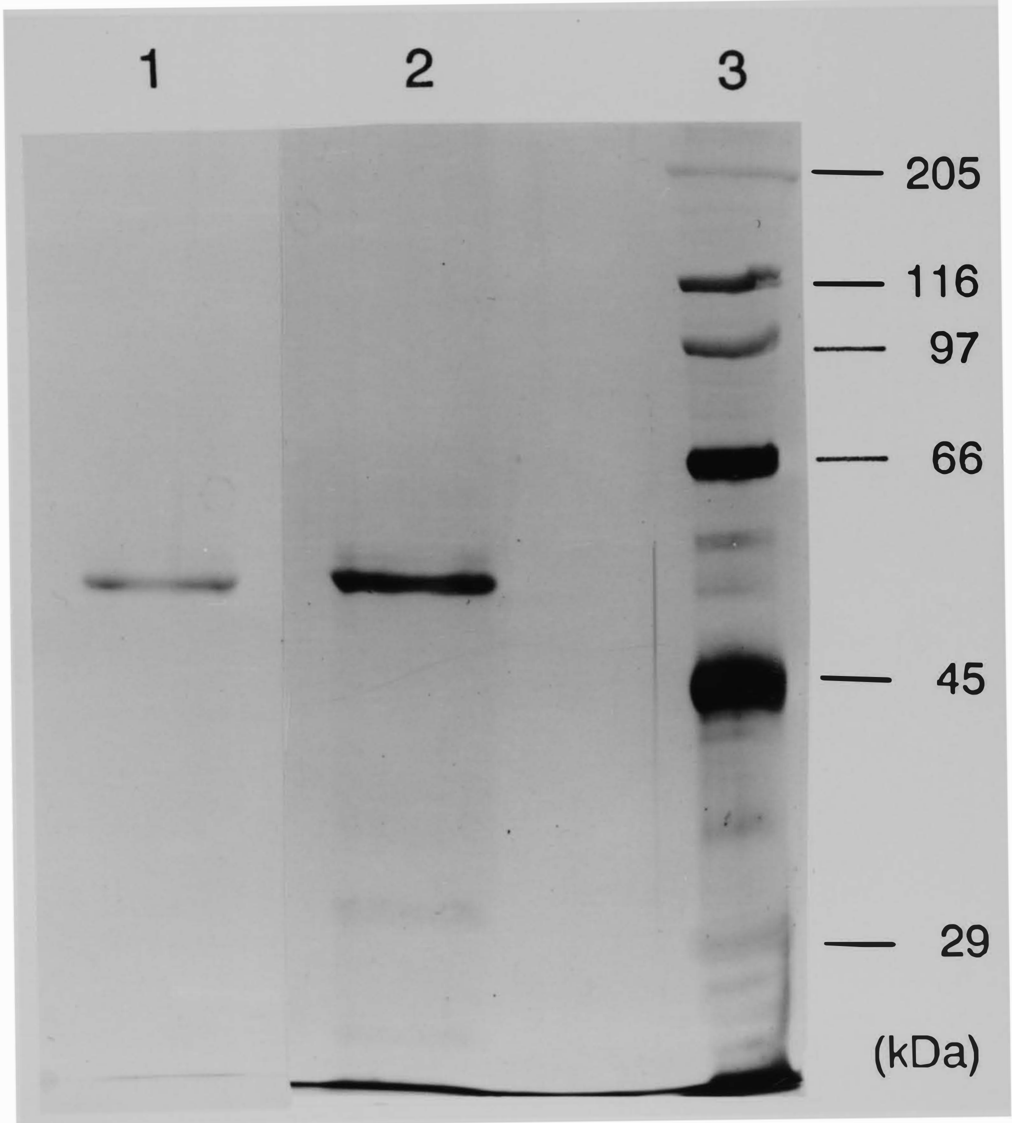
MOLECULAR WEIGHT

As shown in Figure 11, SDS-PAGE of FALDH purified from human liver microsomes exhibited one major polypeptide band when the gel was stained with Coomassie brilliant blue. Silver staining revealed the presence of several minor contaminating polypeptides smaller than 30 kDa in size. These minor bands constituted less than 20% of the total protein based on densitometry of the silver stained SDS gel. Subunit molecular weight of the purified enzyme was estimated to be 54,000 daltons (Figure 12).

Gel filtration was investigated using three different resins, Bio-gel A-0.5m, Bio-gel A-1.5m, and Hydropore-5-sec, in the presence of 200 mM potassium phosphate and either 0.2% Triton X-100, 1.0% Triton X-100, or 0.5% sodium cholate. In all cases, a large proportion of the enzyme activity eluted in larger molecular weight fractions near the void volume. The results from the Hydropore-5-sec chromatography column (HPLC) are shown in Figure 13.

Several attempts at electrophoresing purified FALDH on nondenaturing PAGE (using either a concentration of 6% polyacrylamide or a concentration gradient from 4-24% polyacrylamide) were undertaken to determine the native molecular weight of FALDH purified from human liver microsomes. In all cases, after staining the gel with either Coomassie or silver, a smear was apparent at the interface of the stacking and separating gels and in the

Figure 11. Electrophoresis of purified FALDH on SDS-polyacrylamide gels for subunit molecular weight determination. The gel (10%) was run according to Laemmli (1970). FALDH (approximately 1 μg) purified from human liver microsomes was precipitated with 100% trichloroacetic acid, resuspended in 0.5 M Tris-HCl, pH 6.8, and loaded onto lanes 1 and 2. Lane 1 was stained with coomassie brilliant blue. Lane 2 was silver stained. Lane 3 was also silver stained and contains a mixture of protein standards, with bands starting from the top of the gel corresponding to myosin (205 kDa); ß-galactosidase (116 kDa); phosphorylase B (97.4 kDa); bovine serum albumin (66 kDa); egg albumin (45 kDa); and carbonic anhydrase (29 kDa).



1
2
3
205
116
97
66
45
29
(kDa)

Figure 12. The subunit molecular weight versus mobility plot. The protein standards are identified as, from top to bottom, ß-galactosidase (116 kDa); phosphorylase B (97.4 kDa); bovine serum albumin (66 kDa); egg albumin (45 kDa); and carbonic anhydrase (29 kDa). FALDH molecular weight (○) was determined to be 54 kDa.

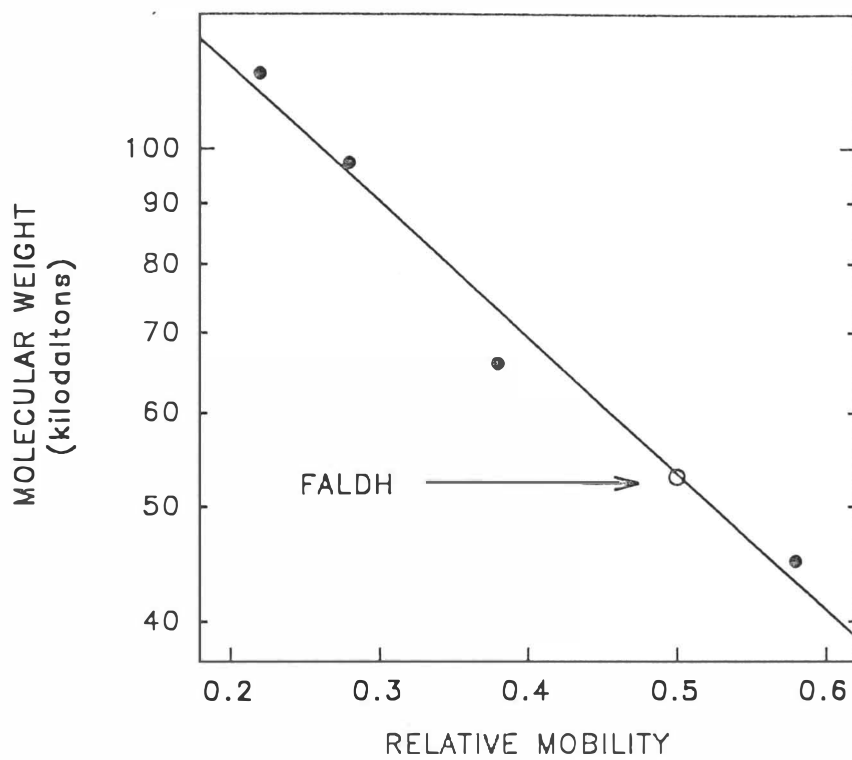
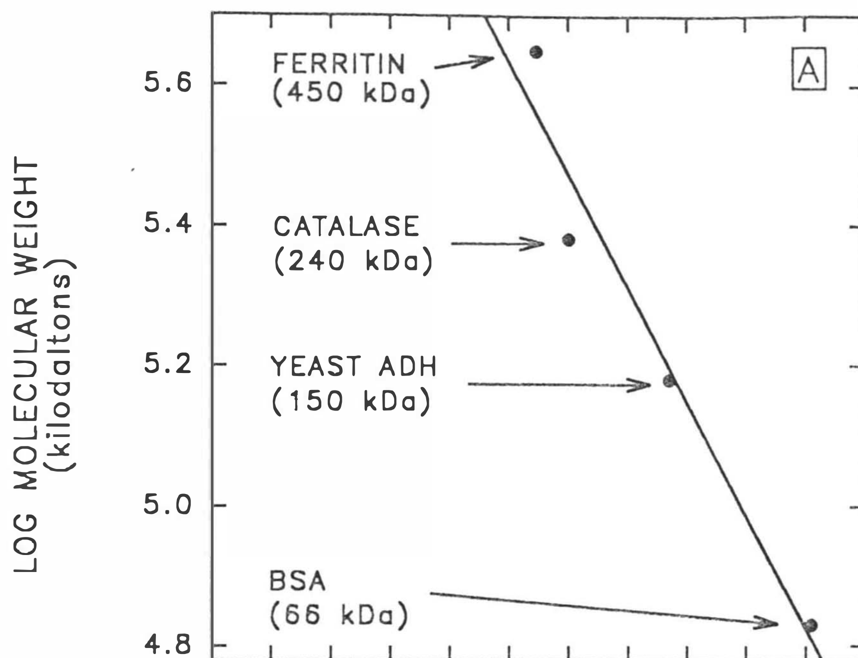
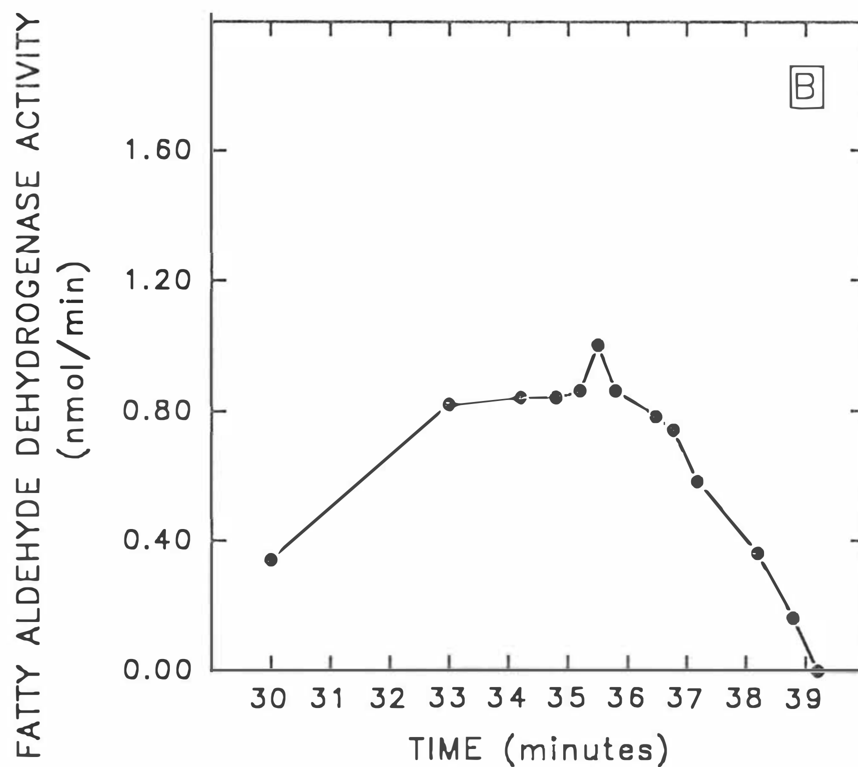

MOLECULAR WEIGHT
(kilodaltons)
100
90
80
70
60
50
40
FALDH
0.2
0.3
0.4
0.5
0.6
RELATIVE MOBILITY

Figure 13. Gel filtration of purified human liver microsomal FALDH. FALDH was purified and concentrated in a buffer containing 25 mM potassium phosphate, 1 mM EDTA, and 0.2% Triton X-100. Approximately 50 μg of purified protein was loaded onto an HPLC sizing column. The upper figure (A) demonstrates the standards run separately on this column. The peak retention time for each standard was determined from the protein absorbance at 245 nm. The standards used were ferritin (450 kDa), catalase (240 kDa), yeast alcohol dehydrogenase (150 kDa), and bovine serum albumin (66 kDa). The lower figure (B) shows the elution pattern of FALDH. 0.5-1.0 ml fractions were collected and an aliquot from each fraction was assayed for FALDH activity.


FERRITIN
(450 kDa)
CATALASE
(240 kDa)
YEAST ADH
(150 kDa)
BSA
(66 kDa)
A
LOG MOLECULAR WEIGHT
(kilodaltons)
5.6
5.4
5.2
5.0
4.8


B
FATTY ALDEHYDE DEHYDROGENASE ACTIVITY
(nmol/min)
1.60
1.20
0.80
0.40
0.00
30
31
32
33
34
35
36
37
38
39
TIME (minutes)

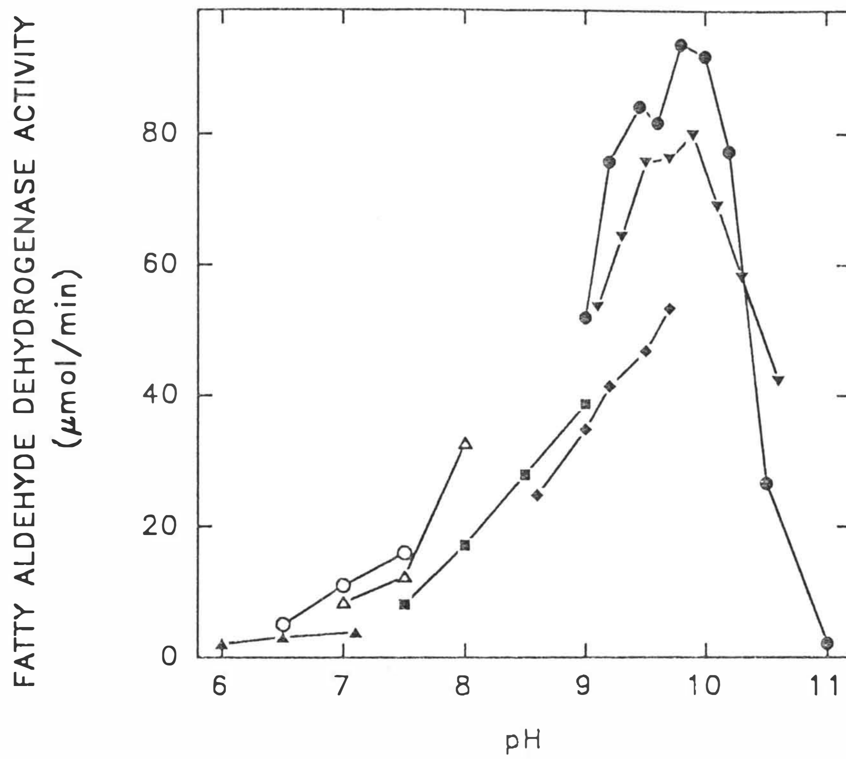
high molecular weight region of the gel (>500 kDa). The results from one nondenaturing gel (6% polyacrylamide) demonstrated 2 protein bands and a smear in the high molecular weight region of the gel. One of the protein bands noted on the stained gel had a native molecular weight of approximately 250 kDa and the other one had a molecular weight of approximately 500 kDa. In this one instance, however, the purified protein had been precipitated with 10% trichloroacetic acid and resuspended in electrophoresis buffer (0.5 M Tris-HCl, pH 6.8) prior to gel electrophoresis.

Altogether, these findings indicate that the subunit molecular weight of FALDH is 54 kDa. FALDH forms large, polymeric aggregates which have an apparent molecular weight much larger than 500 kDa. The native molecular weight, therefore, could not be determined.

pH OPTIMUM

The effect of pH on the enzyme activity (using octadecanal as substrate) was studied by utilizing several biological buffers with pK_a values ranging from pH 6.1 to pH 9.7. As shown in Figure 14, microsomal FALDH had an optimal enzyme activity near pH 9.8 in glycine buffer. All subsequent FALDH assays were performed in the presence of glycine buffer, pH 9.8.

Figure 14. FALDH activity as a function of pH. Human liver microsomal FALDH (10 ng) was assayed in the presence of 50 mM of each of the following biological buffers: MES (▲); MOPS (○); TES (△); Tris-HCl (■); AMPSO (◆); AMP (▼); and Glycine (●).


FATTY ALDEHYDE DEHYDROGENASE ACTIVITY
(μmol/min)
80
60
40
20
0
6
7
8
9
10
11
pH

STABILITY OF FALDH ACTIVITY

Purified FALDH, stored in 25 mM potassium phosphate, pH 7.5, 5% glycerol, and 0.2 mM NAD^+, retained 67% of its original activity when stored at 4°C for 1 month, whereas more than 80% of FALDH activity was retained after storage at -20°C for 3 months.

FALDH is a thermolabile enzyme. After 5 minutes at 47°C, microsomal FALDH retained only 13% of its original activity (Figure 15). At 52°C, all FALDH activity was lost within the first 5 minutes.

SUBSTRATE SPECIFICITY

Microsomal FALDH requires NAD^+ as cofactor in aldehyde oxidation. The comparative kinetic parameters of FALDH with NAD^+ or $NADP^+$ and a single reference substrate (octadecanal with 0.1% Triton X-100 in the assay buffer), are presented in Table 11. These data demonstrate that FALDH has greater specificity for NAD^+ as cofactor than $NADP^+$. All subsequent enzyme assays were performed with saturating concentrations of NAD^+ as the cofactor to determine the kinetic parameters of microsomal FALDH.

Various aldehydes with different chain lengths were tested for substrate specificity. The range of substrate concentrations used to determine the kinetic parameters varied from 1/8 of the K_m to eight times the K_m for each

Figure 15. Thermal stability of human liver microsomal FALDH. FALDH was incubated at the temperatures indicated for the times shown, chilled on ice for 5 minutes, and assayed as outlined in the Materials and Methods section.

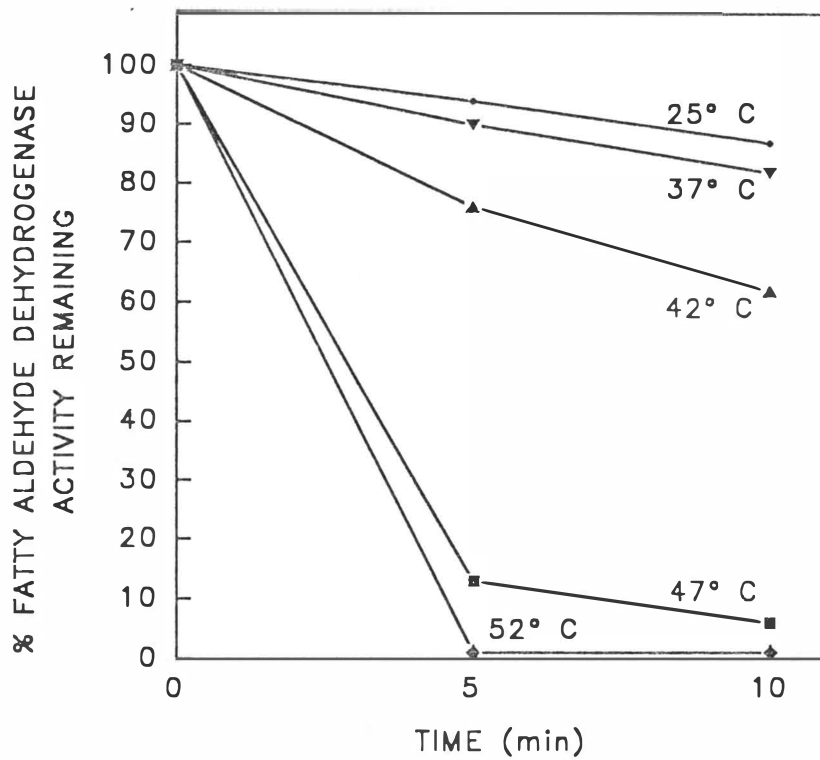

% FATTY ALDEHYDE DEHYDROGENASE ACTIVITY REMAINING
100
90
80
70
60
50
40
30
20
10
0
0
5
10
TIME (min)
25° C
37° C
42° C
47° C
52° C

Table 11. Apparent coenzyme kinetic properties of human liver microsomal FALDH.

Coenzyme	K_m (μM)	V_{max} (μmol/min)	V_{max}/K_m
NAD^+	280	390	1.40
$NADP^+$	8,700	120	0.02

substrate. Each substrate was assayed on at least two different occasions and the mean apparent K_m, V_{max}, and V_{max}/K_m are shown in Table 12. FALDH showed no activity towards retinaldehyde as substrate (final concentration ranging from 60 nM to 970 μM).

FALDH oxidized medium- and long-chain aldehydes (ranging from 6 to 24 carbons in length) with greater specificity than the short chain aldehydes (2 or 3 carbons), as measured by the specificity constant V_{max}/K_m. It is noteworthy that the unsaturated 18-carbon aldehydes had a lower apparent K_m than the saturated aldehydes of the same carbon chain length. Dihydrophytal, a 20-carbon branched chain aldehyde, appeared to be a good substrate for FALDH. Purified microsomal FALDH, when assayed in Tris-HCl buffer, pH 8.8, had a very high apparent K_m for acetaldehyde, propionaldehyde, crotonaldehyde, glutaraldehyde, and benzaldehyde.

Purified FALDH was unable to oxidize [^{14}C]-octadecanol to fatty acid. This suggests that the purified FALDH requires fatty alcohol dehydrogenase for oxidation of fatty alcohol to fatty acid.

EFFECT OF ACTIVATORS AND INHIBITORS

The effects of known ALDH activators and inhibitors were studied on microsomal FALDH (Table 13). FALDH

Table 12. Substrate kinetic properties of human liver microsomal FALDH.

Apparent Aldehyde	Apparent K_m (μM)	V_{max} (μmol/min)	V_{max}/K_m
Saturated			
2	2,500	2	0.01
3	1,300	12	0.01
6	50	29	0.6
8	32	63	2.0
10	23	73	3.2
12	19	45	2.4
14	23	42	1.8
15	28	43	1.5
16	32	40	1.3
18	21	46	2.2
20	38	55	1.4
22	36	25	0.7
24	18	12	0.7
Unsaturated			
16:1[a]	12	40	3.3
18:1[b]	11	39	3.5
18:2[c]	6	35	5.8
Other			
Dihydrophytal	6	8	1.3
Benzaldehyde	510	20	0.04
Crotonaldehyde	800	6	0.01
Glutaraldehyde	1,700	2	0.01

[a] cis-9-hexadecenal
[b] cis-9-octadecenal
[c] cis,cis-9,12-octadecadienal

Table 13. Influence of various effectors on human liver microsomal FALDH activity.

Effector	Concentration	% Uninhibited FALDH Activity[a]
none		100
cyanamide	1 mM	90
	4 mM	88
chloral hydrate	1 mM	105
	25 mM	71
α,p-dibromoacetophenone	10 μM	20
	31 μM	0
p-hydroxyacetophenone	10 μM	102
	31 μM	84
	250 μM	54
N-ethylmaleimide	0.5 mM	89
	1.0 mM	75
	2.0 mM	57
iodoacetate	0.5 mM	100
	1.0 mM	86
	5.0 mM	78
	10.0 mM	8
iodoacetamide	0.5 mM	73
	1.0 mM	15
	5.0 mM	4
	10.0 mM	4
disulfiram	12 μM	25
	25 μM	16
	50 μM	12
p-chloromercuribenzoate	10 μM	0

[a] % Uninhibited activity is the activity determined in the presence of effector relative to activity measured under identical conditions in the absence of effector. Octadecanal was utilized as substrate in all assays.

(continued on next page)

Table 13 (continued from previous page). Influence of various effectors on human liver microsomal FALDH activity.

Effector	Concentration	% Uninhibited FALDH Activity[a]
potassium cyanide	1 mM	67
sodium azide	1 mM	90
	50 mM	89
zinc chloride	0.1 mM	84
	1.0 mM	53
magnesium chloride	0.1 mM	135
	1.0 mM	127
cupric sulfate	0.1 mM	108
	1.0 mM	92

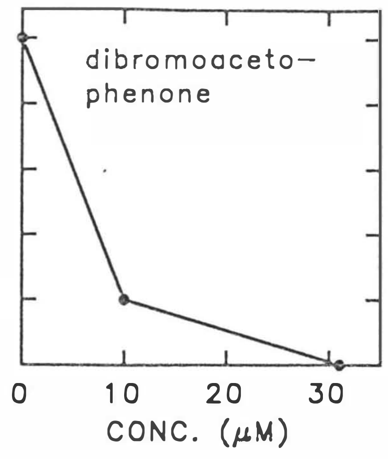
[a] % Uninhibited activity is the activity determined in the presence of effector relative to activity measured under identical conditions in the absence of effector. Octadecanal was utilized as substrate in all assays.

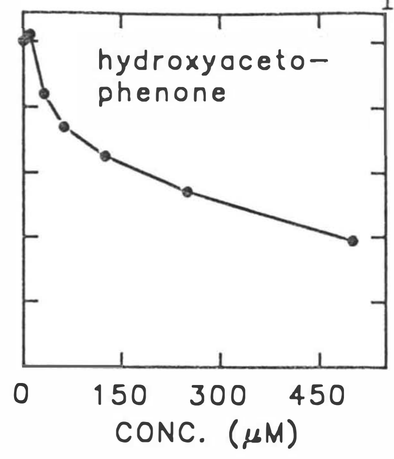
activity was significantly inhibited by disulfiram at concentrations less than 25 μM (Figure 16). p-Chloromercuribenzoate (p-CMB) was also a potent inhibitor of FALDH activity (Table 13). Cyanamide and chloral hydrate, *in vivo* inhibitors of rat liver class 1 and 2 ALDHs, did not significantly affect the activity of FALDH *in vitro*. Potassium cyanide and sodium azide at final concentrations of 1 mM did not inhibit FALDH activity. N-ethylmaleimide did not inhibit FALDH activity at concentrations less than 0.5 mM, but inhibited FALDH activity by 43% at 2 mM. The heavy metals, Zn^{++}, Mg^{++}, and Cu^{++}, did not inhibit FALDH activity at 0.1 mM in a pH 9.8 reaction buffer, rather, Mg^{++} stimulated FALDH activity slightly.

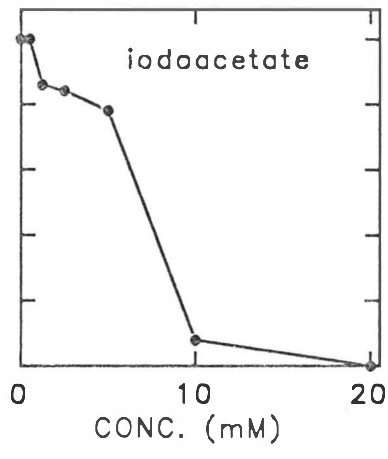
Bromoacetophenone and iodoacetamide have been implicated in binding ALDHs at glutamic acid and cysteine residues, respectively. These amino acid residues may be critical for enzyme catalysis, thereby inhibiting enzyme activity when bound by these compounds (Hempel *et al.*, 1982; Hempel and Pietruszko, 1981; Abriola *et al.*, 1987; MacKerrell *et al.*, 1986). p-Hydroxyacetophenone, α,p-dibromoacetophenone, iodoacetamide, and iodoacetate had an inhibitory effect on FALDH activity (Figure 16).

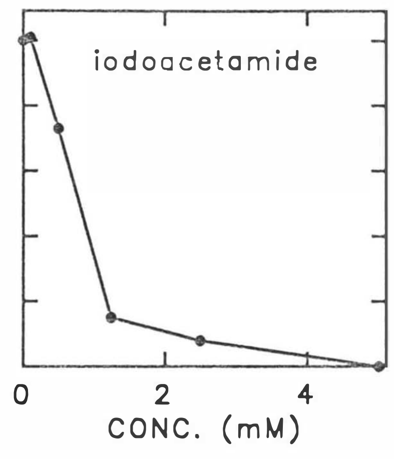
Figure 16. Inhibition of FALDH by α,p-dibromoacetophenone, p-hydroxyacetophenone, iodoacetate, iodoacetamide, and disulfiram. Human liver microsomal FALDH (12.5 ng) was assayed for 30 minutes in the presence of the compounds at the final concentrations indicated. % FALDH activity remaining is the activity determined in the presence of inhibitor relative to activity measured under identical conditions in the absence of inhibitor. Octadecanal was utilized as substrate in all assays.

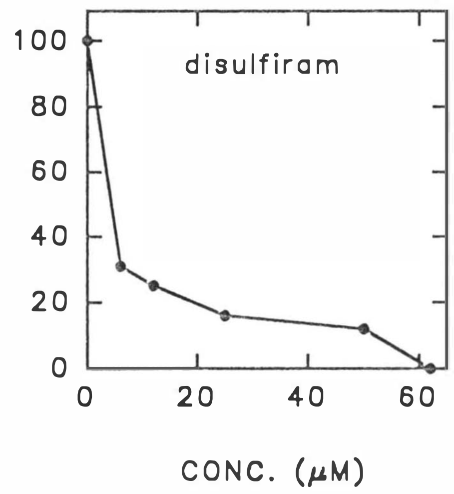
% FATTY ALDEHYDE DEHYDROGENASE ACTIVITY REMAINING


dibromoaceto-
phenone
0
10
20
30
CONC. (μM)


100
80
60
40
20
0
hydroxyaceto-
phenone
0
150
300
450
CONC. (μM)


iodoacetate
0
10
20
CONC. (mM)


100
80
60
40
20
0
iodoacetamide
0
2
4
CONC. (mM)


100
80
60
40
20
0
disulfiram
0
20
40
60
CONC. (μM)

DISCUSSION

Numerous studies have provided evidence for multiple ALDH isozymes in mammals (Greenfield and Pietruszko, 1977; Ikawa *et al.*, 1983; Yin *et al.*, 1989; Forte-McRobbie and Pietruszko, 1986; Ryzlak and Pietruszko, 1987, 1989; Guan *et al.*, 1988; Feldman and Weiner, 1972). These isozymes have been localized to various subcellular organelles within each tissue. Microsomal ALDHs have been described in rat liver (Mitchell and Petersen, 1989; Nakayasu *et al.*, 1978; Lindahl and Evces, 1984), rabbit intestine (Ichihara *et al.*, 1986a, 1986b), and human polymorphonuclear leukocytes (Sutyak *et al.*, 1989). Human microsomal ALDH has not previously been purified.

Nakayasu *et al.* (1978) and Mitchell and Petersen (1989) purified rat liver ALDH by first subjecting the microsomes to ammonium sulfate precipitation. The precipitate formed between 30 and 70% ammonium sulfate saturation was solubilized in 0.5% sodium cholate. The soluble microsomal proteins were chromatographically separated on omega-aminohexyl-Sepharose 4B. Lindahl and Evces (1984) solubilized rat liver microsomes with 0.5% Triton X-100 and 0.3% sodium desoxycholate. The soluble microsomes were dialyzed in phosphate buffer, pH 7.3 containing 0.2% Triton X-100. They chromatographically separated the soluble microsomes on a DEAE-cellulose

column. They purified two ALDHs that were active against octanal as substrate. They found that one isozyme bound to the DEAE-cellulose resin, whereas the other isozyme did not bind. These isozymes differed in isoelectric point (pH 6.4 versus pH 5.6) and cofactor specificity (one isozyme having a lower K_m for $NADP^+$ as coenzyme). Ichihara *et al.* (1986b) purified rabbit intestinal ALDH by first solubilizing microsomes with 0.4% sodium cholate. The soluble microsomes were separated on an aminohexyl-Sepharose column. All microsomal ALDH purifications reported above used a 5'AMP or NAD^+ affinity resin for the final purification step.

Our purification of human microsomal FALDH involves, first, the separation of FALDH from ALDHs localized to other subcellular organelles. This step resulted in a large loss of FALDH activity, but we separated FALDH from other ALDHs in cytosol and mitochondria. Subsequent chromatography of FALDH on a hydrophobic and 5'AMP affinity column resulted in a purified protein.

The purified human FALDH appeared as one major polypeptide band at 54 kDa on SDS-PAGE with a few minor protein bands less than 30 kDa in molecular weight. These polypeptides may either be unrelated to FALDH or might represent proteolytic products of FALDH.

To characterize the substrate specificity of FALDH, we

studied a wide variety of aldehydes: aliphatic (saturated and unsaturated), branched, and aromatic. Other microsomal ALDHs have not been investigated with respect to aldehyde substrates longer than 12 carbons. FALDH was inactive against retinaldehyde, and showed little activity towards the shorter chain substrates, acetaldehyde, propionaldehyde, crotonaldehyde, glutaraldehyde, and benzaldehyde. Because of their increased fluorescence, short-chain aldehydes were assayed in Tris-HCl buffer, pH 8.8. This pH is not optimal for FALDH activity. When FALDH was assayed in Tris-HCl buffer, pH 8.8 using octadecanal as substrate, we found that the apparent K_m did not differ from that measured at pH 9.8; however, the apparent V_{max} decreased from 41 μmol/min at pH 9.8 to 13 μmol/min at pH 8.8. Therefore, we are not able to accurately compare the apparent V_{max} of short-chain and longer chain aldehydes because they were assayed under different conditions.

Human microsomal FALDH had a low apparent K_m and a relatively high apparent V_{max} for 6-24-carbon aldehydes. The decreased apparent K_m for long-chain fatty aldehydes (>16 carbons) as compared with medium-chain aldehydes (12-16 carbons) could be attributed to an increase in hydrophobicity of the long-chain aldehydes, thereby enhancing the binding of the substrate with the hydrophobic

regions of FALDH, or to limitations in substrate solubility with increasing carbon chain length.

FALDH utilizes dihydrophytal as substrate and may, therefore, be involved in phytol metabolism. FALDH has a relatively low apparent K_m for dihydrophytal, a 20-carbon branched chain aldehyde, as compared to hexadecanal or eicosanal, the saturated 16- and 20-carbon aliphatic aldehydes, respectively. Phytol, a 20-carbon monounsaturated, branched chain fatty alcohol, has been shown to be oxidized to phytanic acid in rat liver (Muralidharan and Muralidharan, 1986), bovine rumen (Patton and Benson, 1966), and human tissues (Steinberg *et al.*, 1965). The phytol-phytanate conversion may involve a dihydrophytal intermediate being formed (Muralidharan and Muralidharan, 1985). The phytol-phytanate conversion activity was reported to be present in the mitochondrial-enriched and microsomal-enriched fractions obtained by differential centrifugation of rat liver (Muralidharan and Muralidharan, 1986). Our results have demonstrated that human cultured skin fibroblast mitochondria and microsomes are not separated well by differential centrifugation. We found that FALDH is localized to rat liver microsomes (see chapter 4) suggesting that the activity found in the mitochondrial-enriched fraction of rat liver separated by differential centrifugation by Muralidharan and

Muralidharan (1986) may have been due to microsomal contamination. The involvement of microsomal FALDH in dihydrophytal oxidation implicates a role for FALDH in phytol oxidation in humans.

Some ALDH isozymes are readily inhibited by disulfiram, whereas others are not (see Table 3). Disulfiram is believed to be a general reagent for the modification of protein sulfhydryl groups (Vallari and Pietruszko, 1982; Sanny and Weiner, 1987; Kitson, 1983; Yourick and Faiman, 1991). One report has shown that although it inhibits horse liver cytosolic ALDH activity, disulfiram is not an active site-directed reagent (Vallari and Pietruszko, 1982). Our results showed that human microsomal FALDH was inhibited 88% by 50 μM disulfiram.

Iodoacetamide, iodoacetate, p-CMB, and N-ethylmaleimide also interact with protein sulfhydryl groups (Tu and Weiner, 1988; Hempel and Pietruszko, 1981; Hempel *et al.*, 1982). Human FALDH was inhibited by p-CMB (10 μM) and iodoacetamide (1 mM), but not significantly by N-ethylmaleimide (2 mM) or iodoacetic acid (5 mM). Studies of ALDHs using those sulfhydryl reagents in other organisms suggest that there are critical cysteine residues in or near the active site of ALDH. To date, important cysteine residues have been located at the conserved amino acid positions 49, 162, and 302 (Hempel *et al.*, 1993). Sulfhydryl reacting reagents that inhibit FALDH activity

may bind to cysteine residues important for substrate interactions, cofactor interactions, or enzyme structural stability.

Acetophenone compounds are known to inhibit class 1 and class 2 human ALDHs (MacKerrell *et al.*, 1986; Abriola *et al.*, 1987; Woenckhaus *et al.*, 1987). Labeling studies have indicated that a specific amino acid residue, glutamic acid-268 of human liver class 1 and 2 ALDHs, is specifically labelled with radioactive bromoacetophenone (MacKerrell *et al.*, 1986). These authors suggest that glu-268 is involved with ALDH catalytic activity. We found that FALDH was completely inhibited by α,p-dibromoacetophenone (10 μM) and partially inhibited by p-hydroxyacetophenone (250 μM). Human FALDH may therefore contain glutamate residues that, when bound by bromoacetophenone, interfere with FALDH catalytic function or substrate/cofactor binding.

Cyanamide (200 μM final concentration) inactivates rat liver class 1 and 2 ALDHs *in vivo* but not *in vitro* (Prunonosa *et al.*, 1991; Deitrich *et al.*, 1976). It is believed that ALDH inhibition is not due to the presence of cyanamide, but rather to the presence of its metabolites. Our *in vitro* studies of human FALDH demonstrated that cyanamide did not inhibit enzyme activity at concentrations up to 4 mM.

Metal ions have different effects on ALDH isozymes

(Venteicher *et al.*, 1977; Takahashi, K. and Weiner, 1980). Magnesium ions have a stimulatory effect on rat mitochondrial ALDH purified from testis as well as the class 2 horse liver ALDH (Bedino *et al.*, 1992; Takahashi, K. and Weiner, 1980). In the presence of less than 40 μM Mg^{++}, the tetrameric enzyme dissociates into dimers that subsequently have greater overall enzyme activity. Magnesium ions have no effect on microsomal ALDH from rabbit intestine (Ichihara *et al.*, 1986b) and have an inhibitory effect on horse liver class 1 ALDH (Venteicher *et al.*, 1977). Human FALDH was slightly stimulated in the presence of 1 mM magnesium chloride.

Table 14 summarizes the different microsomal ALDH isozymes purified and characterized, as well as the isozyme described in human leukocyte microsomes. All of the enzymes are active against aliphatic substrates, but the range of substrates tested varied among reports. Human liver FALDH differs significantly from human leukocyte ALDH in its pH profile and coenzyme specificity. Rabbit intestinal ALDH has a subunit molecular size and pH optimum different from human liver FALDH. ALDHs isolated from rat liver differ from each other in their cofactor and substrate specificities, and these isozymes are different from human liver FALDH in cofactor specificity and its pH optimum. A significant finding is that human liver

Table 14. Comparison of mammalian microsomal ALDHs.

Microsomal Source	Molecular Weight: Subunit (kDa)	Molecular Weight: Native Protein (kDa)	K_m (μM): NAD^+	K_m (μM): $NADP^+$	Aldehyde Range (# carbons)	pH Optimum	Sensitivity to Disulfiram[a]
RAT LIVER							
Nakayasu, Mitchell	51	>360	70	20	5-12	?	?
Lindahl1	54	250	100	440	3-8	8.8	-
Lindahl2	54	250	100	270	3-8	8.8	-
RABBIT INTESTINE							
Ichihara	60	370	?	?	4-12	9.0	-
HUMAN LEUKOCYTES[b]							
Sutyak	?	?	25	70	20	6.5	?
HUMAN LIVER							
Kelson	54	?	280	8,700	6-24	9.8	+

[a] (+) signifies greater than 80% inhibition of enzyme activity at a final concentration of 50 μM disulfiram

[b] assays were performed in crude microsomes, not with a purified enzyme

microsomal FALDH is inhibited by disulfiram and the other microsomal ALDHs are not (no results are available for the rat liver microsomal isozyme purified by Nakayasu and Mitchell). Disulfiram sensitivity and its pH optimum suggest that the human liver microsomal FALDH is distinct from the human leukocyte microsomal ALDH described by Sutyak *et al.* (1989).

Microsomes are involved in the metabolism of long-chain fatty alcohols and fatty aldehydes (Lee, 1979; Bishop and Hajra, 1981; Singh, H. and Poulos, 1988). ALDHs isolated from microsomes are believed to have physiological roles in peroxidation of microsomal membranes (Mitchell and Petersen, 1989; Nakayasu *et al.*, 1978; Antonenkov *et al.*, 1987). We have demonstrated that FALDH isolated from human liver microsomes oxidized saturated, unsaturated, and branched chain aliphatic aldehydes. Further investigation is required to better define its physiological role in fatty alcohol and fatty aldehyde metabolism.

CHAPTER 6

Conclusions and future directions

SLS is an inborn error of metabolism characterized by the presence of congenital ichthyosis, mental retardation, and spasticity. SLS patients are deficient in the FALDH component of FAO (Rizzo and Craft, 1991). FAO plays a major role in the fatty alcohol cycle (Rizzo *et al*., 1987) by catalyzing the oxidation of fatty alcohol to fatty acid. Consequently, SLS patients accumulate fatty alcohol, particularly octadecanal and hexadecanal, in plasma (Rizzo *et al*., 1989). One hypothesis is that the symptoms seen in SLS may result from lipid accumulation in skin and nerve tissue.

The mechanism by which fatty alcohol accumulation might cause the symptoms in SLS is not established. The major clinical symptoms in SLS involve the skin (congenital ichthyosis) and nervous system (mental retardation and spasticity). The neuropathology in SLS has not been well defined (Baar and Galindo, 1965; Sylvester, 1969; Wester *et al*., 1991) and little is known about the neurological effects of fatty alcohols. However, the dermatopathology

has been documented in SLS (Goldsmith *et al*., 1971; Matsuoka *et al*., 1982; Jagell *et al*., 1987; Ito *et al*., 1991), and it is well known that the lipid composition of the epidermis is important in the pathophysiology of ichthyosis (Williams, 1991). Advances in knowledge of the biochemical abnormalities underlying other lipid disorders that cause ichthyosis may provide insights into understanding the skin pathology in SLS. Therefore, I will briefly review what is already known about these other ichthyotic disorders.

The epidermis of the skin consists of several different cell layers. At the outer most layer of the epidermis resides the stratum corneum, a compartment that serves as a barrier to water loss - the most important function of the skin. The stratum corneum is portrayed as a two-compartment system composed of bricks (corneocytes) and mortar (intercellular material) (Williams and Elias, 1986). The corneocytes are largely dead proteinaceous cells. Protein abnormalities have been described for some ichthyotic disorders (Williams and Elias, 1986; Williams, 1990, 1991). The intercellular material consists of lipid lamellae which is largely composed of neutral lipids and sphingolipids. Lipid abnormalities have been described in several inherited disorders of ichthyosis, including Refsum disease (phytanic acid storage disease), X-linked recessive ichthyosis (steroid sulfatase deficiency), Chanarin

syndrome (neutral lipid storage disease), infantile Gaucher disease (glucocerebrosidase deficiency), and autosomal recessive rhizomelic chondrodysplasia punctata (multiple peroxisomal enzyme defects).

In order to better comprehend the relationship between FAO deficiency in SLS and the pathology of the skin and nervous system, it may help to understand how abnormal lipid metabolism in two of the ichthyotic disorders, Refsum disease and X-linked recessive ichthyosis, results in the pathogenesis of these diseases.

Refsum disease is an inborn error of phytanic acid metabolism (Steinberg, 1989). The clinical manifestations of this disorder include peripheral neuropathy, cerebellar ataxia, and ichthyosis. Phytanic acid accumulates in blood and tissues of Refsum disease patients due to a deficiency in fatty acid α-oxidation. Phytanic acid is a branched chain 20-carbon fatty acid derived from phytol.

Three hypotheses have been advanced to account for the pathogenesis of the skin disorder in Refsum disease (Williams, 1991). First, phytanate accumulation may incorporate into membrane lipids in place of the normal straight-chain fatty acids. Abnormal accumulation of phytanate may upset the membrane structure and function. Second, phytanate accumulation may substitute in the membrane lipids and result in a relative deficiency of essential polyunsaturated fatty acids normally present in

the epidermal layer of skin. Third, Refsum disease is caused by a defect in α-hydroxylation of phytanate, and perhaps deficiency of α-hydroxylation may be important for metabolism of other fatty acids that make up the stratum corneum bilayer.

X-linked recessive ichthyosis (XLRI) is an inborn error of steroid sulfate metabolism with accumulation of cholesterol sulfate reported in serum, skin, and red blood cell membranes (Shapiro, 1989). Deficiency of the enzyme, steroid sulfatase, results in ichthyosis and mild corneal opacities. Cholesterol sulfate is normally a minor constituent in many mammalian tissue membranes. It may play a critical role in maintenance of the intercellular lipid bilayers in the stratum corneum. Cholesterol sulfate hydrolysis in the stratum corneum of the skin is necessary for normal desquamation (Williams, 1991). Cholesterol sulfate is localized within the intercellular membrane region of the stratum corneum. In normal epidermis, steroid sulfatase hydrolyzes cholesterol sulfate, allowing for destabilization of the lipid lamellae between the outer layers of the stratum corneum. This destabilization, in turn, results in desquamation of the stratum corneum. However, in XLRI, absence of steroid sulfatase activity leads to accumulation of cholesterol sulfate in the stratum corneum and failure of normal desquamation (Williams and Elias, 1986). Abnormal desquamation leads to loss of

epidermal integrity, which results in dry skin because the epidermal water barrier is no longer maintained. Indeed, topical application of cholesterol sulfate to the skin of normal mice produces ichthyosis (Williams and Elias, 1986).

From the two examples described above, one may begin to understand the importance of the lipid composition of the stratum corneum for the pathology of the skin. The membrane lipid constitution is vital to the function and stability of the membrane. Acute perturbations in the membrane structure may result in the loss of normal water barrier function, causing the skin to dry out and become ichthyotic. Studies in XLRI suggest that an overabundance of cholesterol sulfate destabilizes stratum corneum membrane structure. In Refsum disease patients, phytanate accumulation may result in a deficiency of other lipids normally present in the cell membrane that are crucial to proper membrane structure and function. Together, these results suggest that molecular substitution of one lipid for another may alter the structural and functional properties of the epidermis.

The pathological changes in skin and nerve tissue in SLS may also be caused by accumulation of free fatty alcohol or related lipids coinciding with normal membrane constituents. Fatty alcohols are constituents of wax esters and ether lipids (including plasmalogens). Wax esters are synthesized predominantly by sebaceous glands in

the skin, however, these glands do not seem to be the locus for the cutaneous phenotype because the ichthyosis in SLS is present at birth, an age when sebaceous glands are normally quiescent (Williams, 1991). Wax ester products have not been measured in SLS patients. Ether lipids are prominent in myelin and may function as both membrane components and as cellular regulators. Erythrocyte glycerol ether linkages derived from hexadecanol were measured in 4 SLS patients and found to be either normal or slightly elevated (Rizzo, 1993; Rizzo *et al.*, 1989). The block in fatty alcohol oxidation in SLS does not appear to lead to accumulation of fatty alcohols in erythrocyte glycerol ether lipids. Whether ether lipids accumulate in myelin of skin is not known.

The mental retardation and spasticity in SLS may result from neuronal membrane instability. Although we have not found fatty alcohol accumulation in erythrocyte glyceryl ether lipids, there may be fatty alcohol accumulation in nerve tissue membrane lipids. An abnormal accumulation of these lipids may possibly lead to membrane instability and, hence, neurological dysfunction.

Fatty alcohol accumulation has been demonstrated in plasma from SLS patients (Rizzo *et al.*, 1989). FALDH deficiency might be expected to lead to fatty aldehyde accumulation, but fatty aldehydes have not been found to be elevated (Rizzo, 1993). Because FADH activity, a component

of FAO, is normal in SLS patients (Rizzo and Craft, 1991), one might expect to see normal levels of fatty alcohol in SLS, whereas fatty aldehyde levels may be increased. Fatty aldehyde accumulation could not be demonstrated in intact fibroblasts from SLS patients grown *in vivo* in the presence of excess radioactive fatty alcohol (Rizzo and Craft, 1991). However, under FAO assay conditions, SLS fibroblasts did accumulate fatty aldehyde derived from fatty alcohol substrates. These results suggest that in intact fibroblasts from SLS patients, fatty aldehyde derived from fatty alcohol oxidation may either 1) be reduced back to fatty alcohol by reversal of FADH in the presence of NADH, 2) react with other molecules in the cell and become incorporated into other molecules, or 3) may not accumulate because FADH is inhibited under conditions where FALDH is not active. Fatty aldehyde is a potentially reactive molecule and may form stable adducts with other molecules including lysine residues of proteins, or be further converted to other metabolites (Austin and Fraenkel-Conrat, 1992; Tuma *et al*., 1987). Currently, we are unable to distinguish between any of the 3 possibilities presented above.

Results from this dissertation research help to understand one of the roles of FALDH in human metabolism. FALDH oxidizes long-chain aliphatic aldehydes, including saturated, unsaturated (16:1, 18:1, and 18:2), and branched

chain molecules. These may arise from fatty alcohol oxidation, wax ester metabolism, and catabolism of sphingolipids and glycerol ether lipids. In addition, microsomal FALDH may play a role in lipid peroxidation (Mitchell and Petersen, 1989; Nakayasu *et al.*, 1978).

FALDH may be involved in the oxidation of other classes of fatty alcohols not studied in this research project, including dolichols (80-115 carbons), long-chain omega-hydroxy carboxylic acids (carboxylic acids that contain an omega-terminal alcohol which can be oxidized to give a dicarboxylic acid), leukotriene B_4 (the 20-carbon alcohol), and phytol (the precursor of dihydrophytol, containing a double bond at the 2,3 carbon position).

FALDH deficiency may theoretically result in the loss of specific fatty acid products of this enzymatic reaction. If FALDH oxidizes a specific class of aldehyde to fatty acid and this fatty acid class is synthesized by FALDH oxidation alone, then a deficiency of FALDH may result in a deficiency of one class of fatty acids.

The FALDH that I purified from human liver microsomes has not been positively identified as that same enzyme which is deficient in SLS patients. To date, FALDH activity has not been demonstrated to be deficient in the liver of an SLS patient. However, purified microsomal FALDH has characteristics consistent with the enzyme that

is deficient in SLS. FALDH activity is localized to the microsomes in human liver, rat liver, human HepG2 cells, and probably cultured human fibroblasts. Both the liver FALDH and the fibroblast enzyme deficient in SLS prefer long-chain and branched chain fatty aldehydes as substrates. However, there remain yet other ways to definitively show that the purified human liver microsomal FALDH is the same FALDH deficient in SLS fibroblasts. First, an antibody raised against human liver microsomal FALDH could be used as a probe to investigate the presence of cross-reacting material in normal and SLS fibroblasts. Proteins separated by either SDS or isoelectric focusing polyacrylamide gel electrophoresis could be probed using this FALDH-specific polyclonal antibody. If fibroblast proteins from SLS patients did not demonstrate cross-reactive material or if cross-reactive material were shown to have a subunit size or isoelectric point different from normal control fibroblast FALDH, this would constitute strong proof that the purified liver microsomal FALDH was the same enzyme affected in SLS.

Molecular genetic studies will soon be underway to clone the SLS gene. The cDNA of the SLS gene may be cloned using a traditional approach. The amino acid sequence of purified FALDH can be determined. Comparison of the FALDH sequence with that reported for other ALDHs may indicate regions of the protein that are not conserved in other

isozymes. These sequences unique to FALDH can be utilized to construct DNA primers. A cDNA library can be produced from human liver cells that have been induced for the synthesis of microsomal proteins. The primer could then be used to probe this library in search of plaques harboring the SLS cDNA. Sequencing the cloned DNA would then give a putative amino acid sequence for the encoded translation product of FALDH. Comparison of this sequence to the known amino acid sequence would help to verify that the SLS gene has been cloned (and not another ALDH). By sequencing the SLS cDNA in a number of different normal controls and comparing these sequences to DNA in SLS patients, it may be possible to demonstrate mutations in SLS patients and distinguish these from polymorphisms present in the normal population. Site-directed mutagenesis and *in vitro* expression studies of the SLS cDNA may demonstrate that these mutations in SLS patients do indeed cause loss of FALDH activity.

The SLS cDNA can be further utilized in *in vitro* expression studies and in transgenic animals to further elucidate the role of FALDH in fatty alcohol oxidation as well as in the pathogenesis of the skin and the nervous system in SLS.

REFERENCES

REFERENCES

Abriola, D.P., Fields, R., Stein, S., MacKerrell, J., AD and Pietruszko, R.: Active site of human liver aldehyde dehydrogenase. Biochemistry 26 (1987) 5679-5684.

Agarwal, D.P. and Goedde, H.W.: Human aldehyde dehydrogenase isozymes and alcohol sensitivity. In: Rattazi, M.C., Scandalios, J.G. and Whitt, G.S. (eds.), Isozymes: Current topics in biological and medical research, Vol. 16. Alan R. Liss, Inc., New York, 1987, pp. 21-48.

Antonenkov, V.D., Pirozhkov, S.V. and Panchenko, L.F.: On the role of microsomal aldehyde dehydrogenase in metabolism of aldehydic products of lipid peroxidation. FEBS Letters 224 (1987) 357-360.

Austin, J.E. and Fraenkel-Conrat, H.: Tryptophan analogues form adducts by cooperative reaction with aldehydes and alcohols or with aldehydes alone: Possible role in ethanol toxicity. Proc. Natl. Acad. Sci. USA 89 (1992) 8439-8442.

Avigan, J., Campbell, B.D., Yost, D.A., Hernell, O., Holmgren, G. and Jagell, S.: Sjögren-Larsson syndrome: Delta 5- and delta 6- fatty acid desaturases in skin fibroblasts. Neurology 35 (1985) 401-403.

Baar, H.S. and Galindo, J.: Pathology of the Sjögren-Larsson syndrome. J. Maine Med. Assoc. 56 (1965) 223-226.

Baumann, W.J. and Mangold, H.K.: Reactions of aliphatic methanesulfonates. I. Syntheses of long-chain glyceryl-(1) ethers. J. Org. Chem. 29 (1964) 3055-3057.

Beaufay, H., Amar-Costesec, A., Feytmans, E., Thines-Sempoux, D., Wibo, M., Robbi, M. and Berthet, J.: Analytical study of microsomes and isolated subcellular membranes from rat liver. J. Cell Biol. 61 (1974) 188-200.

Bedino, S., Testore, G. and Obert, F.: Kinetic behaviour and properties of aldehyde dehydrogenase from rat testis mitochondria. Effect of Mg^{++} ions. Int. J. Biochem. 24 (1992) 1175-1182.

Beedham, C.: Molybdenum hydroxylases: Biological distribution and substrate-inhibitor specificity. Progress in Medicinal Chemistry 24 (1987) 85-127.

Bishop, J.E. and Hajra, A.K.: Specificity of reduction of fatty acids to long chain alcohols by rat brain microsomes. J. Neurochem. 30 (1978) 643-647.

Bishop, J.E. and Hajra, A.K.: Mechanism and specificity of formation of long chain alcohols by developing rat brain. J. Biol. Chem. 256 (1981) 9542-9550.

Blatter, E.E., Tasayco, M.L., Prestwich, G.D. and Pietruszko, R.: Chemical modification of aldehyde dehydrogenase by a vinyl ketone analogue of an insect pheromone. Biochem. J. 272 (1990) 351-358.

Book, J.A., D'Avignon, M., Gustavson, K.H. and Soderhjelm, L.: The karyotype in T. Sjögren's Syndrome. Acta Psychiat. Scand. 39 (1963) 114-118.

Bredmose, G.V.: Et tilfaelde af mongoloid idioti og ichthyosis med neurohistologiske forandringer. Nord. Med. 5 (1940) 440-442.

Bremer, J. and Osmundsen, H.: Fatty acid oxidation and its regulation. In: Numa, S. (ed.), Fatty acid metabolism and its regulation. Elsevier, Amsterdam, 1984, pp. 113-154.

Burdett, K., Larkins, L.K., Das, A.K. and Hajra, A.K.: Peroxisomal localization of acyl-coenzyme A reductase (long chain alcohol forming) in guinea pig intestine mucosal cells. J. Biol. Chem. 266 (1991) 12201-12206.

Chaves-Carballo, E.: Sjögren-Larsson syndrome. In: Gomez, M.R. (ed.), Neurocutaneous Diseases. Butterworths, Boston, 1987, pp. 219-224.

Chaves-Carballo, E., Frank, L.M. and Bason, W.M.: Treatment of Sjögren-Larsson syndrome with medium-chain triglycerides. Ann. Neurol. 10 (1981) 294.

Davis, G.A. and Bloom, F.E.: Subcellular particles separated through a histochemical reaction. Anal. Biochem. 51 (1973) 429-435.

Day, J.I.E., Goldfine, H. and Hagen, P.-O.: Enzymic reduction of long-chain acyl-CoA to fatty aldehyde and alcohol by extracts of Clostridium butyricum. Biochim. Biophys. Acta 218 (1970) 179-182.

Deitrich, R.A., Troxell, P.A., Worth, W.S. and Erwin, V.G.: Inhibition of aldehyde dehydrogenase in brain and liver by cyanamide. Biochem. Pharmacol. 25 (1976) 2733-2737.

Dockham, P.A., Lee, M.-O. and Sladek, N.E.: Identification of human liver aldehyde dehydrogenases that catalyze the oxidation of aldophosphamide and retinaldehyde. Biochem. Pharmacol. 43 (1992) 2453-2469.

Feldman, R.I. and Weiner, H.: Horse liver aldehyde dehydrogenase. I. Purification and characterization. J. Biol. Chem. 247 (1972) 260-266.

Ferrell, W.J. and Yao, K.-C.: Reductive and oxidative synthesis of saturated and unsaturated fatty aldehydes. J. Lipid Res. 13 (1972) 23-26.

Flynn, T.G.: Aldehyde reductases: monomeric NADPH-dependent oxidoreductases with multifunctional potential. Biochem. Pharmacol. 31 (1982) 2705-2712.

Forte-McRobbie, C.M. and Pietruszko, R.: Purification and characterization of human liver "high K_m" aldehyde dehydrogenase and its identification as glutamic gamma-semialdehyde dehydrogenase. J. Biol. Chem. 261 (1986) 2154-2163.

Ghenbot, G. and Weiner, H.: Purification of liver aldehyde dehydrogenase by p-hydroxyacetophenone-Sepharose affinity matrix and the coelution of chloramphenicol acetyl transferase from the same matrix with recombinantly expressed aldehyde dehydrogenase. Protein Expression and Purification 3 (1992) 470-478.

Goedde, H.W., Agarwal, D.P. and Harada, S.: Aldehyde dehydrogenase deficiency and alcohol sensitivity. In: Rattazi, M.C., Scandalios, J.G. and Whitt, G.S. (eds.), Isozymes: Current topics in biological and medical research, Vol. 8. Alan R. Liss, Inc., New York, 1983, pp. 175-193.

Goldsmith, L.A., Baden, H.P. and Canty, T.G.: Sjögren-Larsson syndrome. Diversity of cutaneous manifestations. Acta Dermatovener. (Stockholm) 51 (1971) 374-378.

Gondhowiardjo, T.D., van Haeringen, N.J., Hoekzema, R., Pels, L. and Kijlstra, A.: Detection of aldehyde dehydrogenase activity in human corneal extracts. Curr. Eye Res. 10 (1991) 1001-1007.

Greenfield, N.J. and Pietruszko, R.: Two aldehyde dehydrogenases from human liver: Isolation via affinity chromatography and characteristics of the isozymes. Biochim. Biophys. Acta 483 (1977) 35-45.

Griffith, T.W., Sand, D.M. and Schlenk, H.: Reduction of fatty acids to alcohols in Roe of Gourami (Trichogaster cosby). Biochim. Biophys. Acta 665 (1981) 34-39.

Guan, K.L., Pak, Y.K., Tu, G.C., Cao, Q.N. and Weiner, H.: Purification and characterization of beef and pig liver aldehyde dehydrogenases. Alcoholism: Clin. Exp. Res. 12 (1988) 713-718.

Hajra, A.K. and Bishop, J.E.: Glycerolipid biosynthesis in peroxisomes via the acyl dihydroxyacetone phosphate pathway. Ann. N.Y. Acad. Sci. 386 (1982) 170-182.

Harada, S., Muramatsu, T., Agarwal, D.P. and Goedde, H.W.: Polymorphism of aldehyde dehydrogenase in human saliva. In: Weiner, H. and Flynn, T.G. (eds.), Enzymology and molecular biology of carbonyl metabolism 2, Vol. 290. Alan R. Liss, Inc., New York, 1989, pp. 133-139.

Hempel, J. and Pietruszko, R.: Selective chemical modification of human liver aldehyde dehydrogenases E1 and E2 by iodoacetamide. J. Biol. Chem. 256 (1981) 10889-10896.

Hempel, J., Nicholas, H. and Lindahl, R.: Aldehyde dehydrogenases: Widespread structural and functional diversity within a shared framework. Prot. Sci. 2 (1993) 1890-1900.

Hempel, J., Vallari, R. and Pietruszko, R.: On the interaction of human liver aldehyde dehydrogenase E1 isoenzyme with disulfiram and iodoacetamide. In: Thurman, R. (ed.), Alcohol and aldehyde metabolizing systems, Vol. 4. Plenum Press, New York, 1980, pp. 41-50.

Hempel, J., Pietruszko, R., Fietzek, P. and Jornvall, H.: Identification of a segment containing a reactive cysteine residue in human liver cytoplasmic aldehyde dehydrogenase (isoenzyme E1). Biochemistry 21 (1982) 6834-6837.

Hempel, J., Bahr-Lindström, H. and Jörnvall, H.: Aldehyde dehydrogenase from human liver. Primary structure of the cytoplasmic isoenzyme. Eur. J. Biochem. 141 (1984) 21-35.

Hernell, O., Holmgren, G., Jagell, S., Johnson, S.B. and Holman, R.T.: Suspected faulty essential fatty acid metabolism in Sjögren-Larsson syndrome. Pediatr. Res. 16 (1982) 45-49.

Holmgren, G., Jagell, S., Seeman, H. and Steen, G.: Urinary amino acids and organic acids in the Sjögren-Larsson syndrome. Clin. Genet. 20 (1981) 64-66.

Hooft, C., Kriekemans, J., van Acker, K., Devos, E., Traen, S. and Verdonk, G.: Sjögren-Larsson syndrome with exudative enteropathy. Influence of medium-chain triglycerides on the symptomatology. Helv. paediat. Acta 22 (1967) 447-458.

Hsu, L.C. and Chang, W.-C.: Cloning and characterization of a new functional human aldehyde dehydrogenase gene. J. Biol. Chem. 266 (1991) 12257-12265.

Hsu, L.C., Chang, W.C. and Yoshida, A.: Genomic structure of the human cytosolic aldehyde dehydrogenase gene. Genomics 5 (1989) 857-865.

Hsu, L.C., Bendel, R.E. and Yoshida, A.: Genomic structure of the human mitochondrial aldehyde dehydrogenase gene. Genomics 2 (1988) 57-65.

Hurt, E.C. and Schatz, G.: A cytosolic protein contains a cryptic mitochondrial targeting signal. Nature 325 (1987) 499-503.

Ichihara, K., Kusunose, E., Noda, Y. and Kusunose, M.: Some properties of the fatty alcohol oxidation system and reconstitution of microsomal oxidation activity in intestinal mucosa. Biochim. Biophys. Acta 878 (1986a) 412-418.

Ichihara, K., Noda, Y., Tanaka, C. and Kusunose, M.: Purification of aldehyde dehydrogenase reconstitutively active in fatty alcohol oxidation from rabbit intestinal microsomes. Biochim. Biophys. Acta 878 (1986b) 419-425.

Ikawa, M., Impraim, C.C., Wang, G. and Yoshida, A.: Isolation and characterization of aldehyde dehydrogenase isozymes from usual and atypical human livers. J. Biol. Chem. 258 (1983) 6282-6287.

Iselius, L. and Jagell, S.: Sjögren-Larsson syndrome in Sweden: distribution of the gene. Clin. Genet. 35 (1989) 272-275.

Ito, M., Oguro, K. and Sato, Y.: Ultrastructural study of the skin in Sjögren-Larsson syndrome. Arch. Dermatol. Res. 283 (1991) 141-148.

Jagell, S. and Heijbel, J.: Sjogren-Larsson syndrome: physical and neurological features. Helv. paediat. Acta 37 (1982) 519-530.

Jagell, S. and Lidén, S.: Treatment of the ichthyosis of the Sjögren-Larsson syndrome with etretinate (Tigason). Acta Dermatovener. (Stockholm) 63 (1983) 89-92.

Jagell, S., Gustavson, K.-H. and Holmgren, G.: Sjögren-Larsson syndrome in Sweden: a clinical, genetic and epidemiological study. Clin. Genet. 19 (1981) 233-256.

Jagell, S., Holmgren, G. and Hofer, P.Å.: Congenital ichthyosis with alopecia, eclabion, ectropion and mental retardation-a new genetic syndrome. Clin. Genet. 31 (1987) 102-108.

James, P.F., Rizzo, W.B., Lee, J. and Zoeller, R.A.: Isolation and characterization of a chinese hamster ovary cell line deficient in fatty alcohol:NAD^+ oxidoreductase activity. Proc. Natl. Acad. Sci. USA 87 (1990) 6102-6106.

Jellum, E., Eldjarn, L. and Try, K.: Conversion of phytol into dihydrophytol and phytanic acid. Acta Chem. Scand. 20 (1966) 2535-2538.

Johnson, R.C. and Gilbertson, J.R.: Isolation, characterization, and partial purification of a fatty acyl coenzyme A reductase from bovine cardiac muscle. J. Biol. Chem. 247 (1972) 6991-6998.

Judge, M.R., Lake, B.D., Smith, V.V., Besley, G.T.N. and Harper, J.I.: Depletion of alcohol (hexanol) dehydrogenase activity in the epidermis and jejunal mucosa in Sjögren-Larsson syndrome. J. Invest. Dermatol. 95 (1990) 632-634.

Keenan, R.W. and Maxam, A.: The in vitro degradation of dihydrosphingosine. Biochim. Biophys. Acta 176 (1969) 348-356.

Kelson, T.L., Craft, D.A. and Rizzo, W.B.: Carrier detection for Sjögren-Larsson syndrome. J. Inherit. Metab. Dis. 15 (1992) 105-111.

King, G. and Holmes, R.S.: Human corneal aldehyde dehydrogenase: Purification, kinetic characterization and phenotypic variation. Biochem. Mol. Biol. Int. 31 (1993) 49-63.

Kitson, T.M.: Mechanism of inactivation of sheep liver cytoplasmic aldehyde dehydrogenase by disulfiram. Biochem. J. 213 (1983) 551-554.

Kolattukudy, P.E.: Plant waxes. Lipids 5 (1970) 259-263.

Kolattukudy, P.E.: Enzymatic synthesis of fatty alcohols in Brassica oleracea. Arch. Biochem. Biophys. 142 (1971) 701-709.

Kolattukudy, P.E. and Rogers, L.: Biosynthesis of fatty alcohols, alkane-1,2-diols and wax esters in particulate preparations from the uropygial glands of White-Crowned Sparrows (Zonotrichia leucophrys). Arch. Biochem. Biophys. 191 (1978) 244-258.

Kolattukudy, P.E. and Rogers, L.: Acyl-CoA reductase and acyl-CoA: Fatty alcohol acyl transferase in the microsomal preparation from the bovine meibomian gland. J. Lipid Res. 27 (1986) 404-411.

Kurys, G., Ambroziak, W. and Pietruszko, R.: Human aldehyde dehydrogenase: Purification and characterization of a third isozyme with low K_m for gamma-aminobutyraldehyde. J. Biol. Chem. 264 (1989) 4715-4721.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (1970) 680-685.

Lake, B.D., Smith, V.V., Judge, M.R., Harper, J.I. and Besley, G.T.N.: Hexanol dehydrogenase activity shown by enzyme histochemistry on skin biopsies allows differentiation of Sjögren-Larsson syndrome from other ichthyoses. J. Inherit. Metab. Dis. 14 (1991) 338-340.

Lazarow, P. and de Duve, C.: A fatty acyl-CoA oxidizing system in rat liver peroxisomes: Enhancement by clofibrate, a hypolipidemic drug. Proc. Natl. Acad. Sci. USA 73 (1976) 2043-2046.

Lee, T.-C.: Characterization of fatty alcohol: NAD^+ oxidoreductase from rat liver. J. Biol. Chem. 254 (1979) 2892-2896.

Lee, T.-C., Fitzgerald, V., Stephens, N. and Snyder, F.: Activities of enzymes involved in the metabolism of ether-linked lipids in normal and neoplastic tissues of rat. Biochim. Biophys. Acta 619 (1980) 420-423.

Lindahl, R.: Aldehyde dehydrogenases and their role in carcinogenesis. Crit. Rev. Biochem. Molec. Biol. 27 (1992) 283-335.

Lindahl, R. and Evces, S.: Rat liver aldehyde dehydrogenase. I. Isolation and characterization of four high K_m normal liver isozymes. J. Biol. Chem. 259 (1984) 11986-11990.

Lowry, O.H., Rosebrough, J.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193 (1951) 265-275.

Maaswinkel-Mooij, P.D., Brouwer, O.F. and Rizzo, W.B.: Unsuccessful dietary therapy in five children with Sjögren-Larsson syndrome. manuscript submitted (1993).

MacKerrell, J., AD, MacWright, R.S. and Pietruszko, R.: Bromoacetophenone as an affinity reagent for human liver aldehyde dehydrogenase. Biochemistry 25 (1986) 5182-5189.

Mahadevan, V.: Conversion of oleyl and elaidyl tosylates to aldehydes. The Journal of the American Oil Chemists' Society 41 (1965) 520.

Matsuoka, L.Y., Kousseff, B.G. and Hashimoto, K.: Studies of the skin in Sjögren-Larsson syndrome by electron microscopy. Am. J. Dermatopathol. 4 (1982) 295-301.

Mitchell, D.Y. and Petersen, D.R.: Oxidation of aldehydic products of lipid peroxidation by rat liver microsomal aldehyde dehydrogenase. Arch. Biochem. Biophys. 269 (1989) 11-17.

Miura, Y., Hisaki, H., Siems, W. and Oda, S.: Hydroxylation of fatty acids and alcohols by hepatic microsomal cytochrome P-450 system from the Mongolian gerbil. Lipids 22 (1987) 987-993.

Miyauchi, K., Masaki, R., Taketani, S., Yamamoto, A., Akayama, M. and Tashiro, Y.: Molecular cloning, sequencing, and expression of cDNA for rat liver microsomal aldehyde dehydrogenase. J. Biol. Chem. 266 (1991) 19536-19542.

Moreau, R.A. and Huang, A.H.C.: Oxidation of fatty alcohol in the cotyledons of jojoba seedlings. Arch. Biochem. Biophys. 194 (1979) 422-430.

Morinan, A. and Garratt, H.M.: An improved fluorimetric assay for brain monoamine oxidase. J. Pharmac. Meth. 13 (1985) 213-223.

Muralidharan, F.N. and Muralidharan, V.B.: In vitro conversion of phytol to phytanic acid in rat liver: Subcellular distribution of activity and chemical characterization of intermediates using a new bromination technique. Biochim. Biophys. Acta 835 (1985) 36-40.

Muralidharan, F.N. and Muralidharan, V.B.: Characterization of phytol-phytanate conversion activity in rat liver. Biochim. Biophys. Acta 883 (1986) 54-62.

Naccarato, W.F., Gelman, R.A., Kawalek, J.C. and Bilbertson, J.R.: Characterization and metabolism of free fatty alcohols from Escherichia coli. Lipids 7 (1972) 275-281.

Nakayasu, H., Mihara, K. and Sato, R.: Purification and properties of a membrane-bound aldehyde dehydrogenase from rat liver microsomes. Biochem. Biophys. Res. Commun. 83 (1978) 697-703.

Natarajan, V. and Schmid, H.H.O.: Biosynthesis and utilization of long-chain alcohols in rat brain: Aspects of chain length specificity. Arch. Biochem. Biophys. 187 (1978) 215-222.

Nevenzel, J.C.: Occurrence, Function and biosynthesis of wax esters in marine organisms. Lipids 5 (1970) 308-317.

Patton, S. and Benson, A.A.: Phytol metabolism in the bovine. Biochim. Biophys. Acta 125 (1966) 22-32.

Pietruszko, R.: Aldehyde dehydrogenase isozymes. In: Rattazi, M.C., Scandalios, J.G. and Whitt, G.S. (eds.), Isozymes: Current topics in biological and medical research, Vol. 8. Alan R. Liss, Inc., New York, 1983, pp. 195-217.

Pisani, D. and Cacchione, A.: Frenastenia e dermatosi. Riv. sper. Freniat. 58 (1935) 722-736.

Prunonosa, J., Sagrista, M.L. and Bozal, J.: Inactivation mechanism of low-K_m rat liver mitochondrial aldehyde dehydrogenase by cyanamide in vitro. Drug Metab. Disposition 19 (1991) 787-792.

Prydz, K., Kase, B.F., Björkhem, I. and Pedersen, J.I.: Subcellular localization of 3α, 7α-dihydroxy- and 3α, 7α, 12α-trihydroxy-5ß-cholestanoyl-coenzyme A ligase(s) in rat liver. J. Lipid Res. 29 (1988) 997-1004.

Reddy, T.S., Sprecher, H. and Bazan, N.G.: Long-chain acyl-coenzyme A synthetase from rat brain microsomes. Eur. J. Biochem. 145 (1984) 21-29.

Riendeau, D. and Meighen, E.: Enzymatic reduction of fatty acids and acyl-CoAs to long chain aldehydes and alcohols. Experientia 41 (1985) 707-713.

Rizzo, W.B.: Sjögren-Larsson syndrome. Sem. Dermatol. 12 (1993) 210-218.

Rizzo, W.B. and Craft, D.A.: Sjögren-Larsson syndrome. Deficient activity of the fatty aldehyde dehydrogenase component of fatty alcohol:NAD^+ oxidoreductase in cultured fibroblasts. J. Clin. Invest. 88 (1991) 1643-1648.

Rizzo, W.B., Dammann, A.L., Craft, D.A. and Phillips, M.W.: Fatty alcohol metabolism in cultured human fibroblasts. Evidence for a fatty alcohol cycle. J. Biol. Chem. 262 (1987) 17412-17419.

Rizzo, W.B., Dammann, A.L. and Craft, D.A.: Sjögren-Larsson syndrome: Impaired fatty alcohol oxidation in cultured fibroblasts due to deficient fatty alcohol:nicotinamide adenine dinucleotide oxidoreductase activity. J. Clin. Invest. 81 (1988) 738-744.

Rizzo, W.B., Dammann, A.L., Craft, D.A., Black, S.H., Henderson Tilton, A., Africk, D., Chaves-Carballo, E., Holmgren, G. and Jagell, S.: Sjögren-Larsson syndrome: Inherited defect in the fatty alcohol cycle. J. Pediatr. 115 (1989) 228-234.

Ryzlak, M.T. and Pietruszko, R.: Purification and characterization of aldehyde dehydrogenase from human brain. Arch. Biochem. Biophys. 255 (1987) 409-418.

Ryzlak, M.T. and Pietruszko, R.: Human brain glyceraldehyde-3-phosphate dehydrogenase, succinic semialdehyde dehydrogenase and aldehyde dehydrogenase isozymes: Substrate specificity and sensitivity to disulfiram. Alcoholism: Clin. Exp. Res. 13 (1989) 755-761.

Sanny, C.G. and Weiner, H.: Inactivation of horse liver mitochondrial aldehyde dehydrogenase by disulfiram. Biochem. J. 242 (1987) 499-503.

Santisteban, I., Povey, S., West, L.F., Parrington, J.M. and Hopkinson, D.A.: Chromosome assignment, biochemical and immunological studies on a human aldehyde dehydrogenase, ALDH3. Ann. Hum. Genet. 49 (1985) 87-100.

Shapiro, L.J.: Steroid sulfatase deficiency and X-linked ichthyosis. In: Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds.), The metabolic basis of inherited disease II, 6th ed., Vol. 2. McGraw-Hill Information Services Company, New York, 1989, pp. 1945-1964.

Siegenthaler, G., Saurat, J.-H. and Ponec, M.: Retinol and retinal metabolism: Relationship to the state of differentiation of cultured human keratinocytes. Biochem. J. 268 (1990) 371-378.

Singer, M.E. and Finnerty, W.R.: Alcohol dehydrogenases in Acinetobacter sp. strain H01-N: Role in hexadecane and hexadecanol metabolism. J. Bacteriol. 164 (1985) 1017-1024.

Singh, H. and Poulos, A.: Distinct long chain and very long chain fatty acyl CoA synthetases in rat liver peroxisomes and microsomes. Arch. Biochem. Biophys. 266 (1988) 486-495.

Singh, H., Beckman, K. and Poulos, A.: Exclusive localization in peroxisomes of dihydroxyacetone phosphate acyltransferase and alkyl-dihydroxyacetone phosphate synthase in rat liver. J. Lipid Res. 34 (1993) 467-477.

Singh, I., Singh, R., Bhushan, A. and Singh, A.K.: Lignoceroyl-CoA ligase activity in rat brain microsomal fraction: Topographical localization and effects of detergents and alpha-cyclodextrin. Arch. Biochem. Biophys. 236 (1985) 418-426.

Sjögren, T. and Larsson, T.: Oligophrenia in combination with congenital ichthyosis and spastic disorders. Acta Psychiatr. Neurol. Scand. 32(Suppl. 113) (1957) 1-113.

Snyder, F.: Metabolism, regulation, and function of ether-linked glycerolipids. In: Vance, D.E. and Vance, J.E. (eds.), Biochemistry of lipids and membranes, 1st ed., Vol. 1. The Benjamin/Cummings Publishing Co., Inc., Menlo Park, CA, 1985, pp. 271-298.

Soderhjelm, A.L. and Enell, H.: Iktyos, spastisk diplegi i nedre extremiteterna och oligofreni - Ett sarskilt syndrom. Nord. Med. 25 (1957) 624-625.

Soodsma, J.F., Piantadosi, C. and Snyder, F.: Partial characterization of the alkylglycerol cleavage enzyme system of rat liver. J. Biol. Chem. 247 (1972) 3923-3929.

Steinberg, D.: Refsum disease. In: Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds.), The metabolic basis of inherited disease II, 6th ed., Vol. 2. McGraw-Hill Information Services Company, New York, 1989, pp. 1533-1550.

Steinberg, D., Avigan, J., Mize, C., Eldjarn, L., Try, K. and Refsum, S.: Conversion of U-C14-phytol to phytanic acid and its oxidation in heredopathia atactica polyneuritiformis. Biochem. Biophys. Res. Commun. 19 (1965) 783-789.

Stoffel, W., Sticht, G. and Lekim, D.: Degradation in vitro of dihydrosphingosine and dihydrosphingosine phosphate to palmitaldehyde and ethanolamine phosphate. Hoppe-Seyler's Z. Physiol. Chem. 348 (1968) 1745-1748.

Storrie, B. and Madden, E.A.: Isolation of subcellular organelles. In: Deutscher, M.P. (ed.), Guide to protein purification, 1st ed., Vol. 182. Academic Press, Inc., New York, 1990, pp. 203-224.

Sutyak, J., Austen, K.F. and Soberman, R.J.: Identification of an aldehyde dehydrogenase in the microsomes of human polymorphonuclear leukocytes that metabolizes 20-aldehyde Leukotriene B_4. J. Biol. Chem. 264 (1989) 14818-14823.

Sylvester, P.E.: Pathological findings in Sjögren-Larsson syndrome. J. Mental Defic. Res. 13 (1969) 267-275.

Tabsh, K., Rizzo, W.B., Holbrook, K. and Theroux, N.: Sjögren-Larsson syndrome: Technique and timing of prenatal diagnosis. Obstet. Gynecol. 82 (1993) 700-703.

Takagi, Y., Ito, A. and Omura, T.: Biogenesis of microsomal aldehyde dehydrogenase in rat liver. J. Biochem. 98 (1985) 1647-1652.

Takahashi, K. and Weiner, H.: Magnesium stimulation of catalytic activity of horse liver aldehyde dehydrogenase. J. Biol. Chem. 255 (1980) 8206-8209.

Takahashi, T. and Schmid, H.H.O.: Long-chain alcohols in mammalian tissues. Chem. Phys. Lipids 4 (1970) 243-246.

Tanaka, T., Hosaka, K. and Numa, S.: Long-chain acyl-CoA synthetase from rat liver. In: Lowenstein, J.M. (ed.), Methods in enzymology, 1st ed., Vol. 71. Academic Press, Inc., New York, 1981, pp. 334-341.

Tanaka, T., Hosaka, T., Hoshimaru, M. and Numa, S.: Purification and properties of long-chain acyl-coenzyme A synthetase from rat liver. Eur. J. Biochem. 98 (1979) 165-172.

Theile, U.: Sjögren-Larsson syndrome. Oligophrenia-ichthyosis-di/tetraplegia. Humangenetik 22 (1974) 91-118.

Tietz, A., Lindberg, M. and Kennedy, E.P.: A new pteridine-requiring enzyme system for the oxidation of glyceryl ethers. J. Biol. Chem. 239 (1964) 4081-4090.

Tu, G.-C. and Weiner, H.: Identification of the cysteine residue in the active site of horse liver mitochondrial aldehyde dehydrogenase. J. Biol. Chem. 263 (1988) 1212-1217.

Tulloch, A.P.: The composition of beeswax and other waxes secreted by insects. Lipids 5 (1970) 247-258.

Tuma, D.J., Jennett, R.B. and Sorrell, M.F.: The interaction of acetaldehyde with tubulin. Ann. N.Y. Acad. Sci. 492 (1987) 277-286.

Vallari, R.C. and Pietruszko, R.: Human aldehyde dehydrogenase: Mechanism of inhibition by disulfiram. Science 216 (1982) 637-639.

Venteicher, R., Mope, L. and Yonetani, T.: Metal ion effectors of horse liver aldehyde dehydrogenase. In: Thurman, R.G., Williamson, J.R., Drott, H.R. and Chance, B. (eds.), Alcohol and aldehyde metabolizing systems, 1st ed., Vol. 2. Academic Press, Inc., New York, 1977, pp. 157-166.

Weiner, H.: Aldehyde oxidizing enzymes. In: Jakoby, W.B. (ed.), Enzymatic basis of detoxication, Vol. 1. Academic Press, Inc., New York, 1980, pp. 261-280.

Wester, P., Bergstrom, U., Brun, A., Jagell, S., Karlsson, B. and Eriksson, A.: Monoaminergic dysfunction in Sjögren-Larsson syndrome. Mol. Chem. Neuropathol. 15 (1991) 13-28.

Williams, M.L.: Generalized disorders of cornification: The ichthyoses. In: Sams, J., Wm. and Lynch, P.L. (eds.), Principles and practice of dermatology, 1st ed. Church Livingstone, New York, 1990, pp. 341-355.

Williams, M.L.: Lipids in normal and pathological desquamation. In: Elias, P.M., Havel, R.J. and Small, D.M. (eds.), Advances in lipid research, Vol. 24. Academic Press, Inc., New York, 1991, pp. 211-262.

Williams, M.L. and Elias, P.M.: The ichthyoses. In: Thiers, B.H. and Dobson, R.L. (eds.), Pathogenesis of skin disease. Churchill Livingstone, New York, 1986, pp. 519-551.

Woenckhaus, C., Bieber, E. and Jeck, R.: Studies on the inactivation of aldehyde dehydrogenase (ALDH). In: Weiner, H. and Flynn, T.G. (eds.), Enzymology and molecular biology of carbonyl metabolism: Aldehyde dehydrogenase, Aldo-keto reductase, and alcohol dehydrogenase, Vol. 1. Alan R. Liss, Inc., New York, 1987, pp. 53-65.

Wykle, R.L., Malone, B. and Snyder, F.: Acyl-CoA reductase specificity and synthesis of wax esters in mouse preputial gland tumors. J. Lipid Res. 20 (1979) 890-896.

Yin, S.-J., Liao, C.-S., Wang, S.L., Chen, Y.J. and Wu, C.W.: Kinetic evidence for human liver and stomach aldehyde dehydrogenase-3 representing an unique class of isozymes. Biochem. Genet. 27 (1989) 321-331.

Yoshida, A., Hsu, L.C. and Yasunami, M.: Genetics of human alcohol-metabolizing enzymes. Prog. Nucleic Acid Res. Mol. Biol. 40 (1991) 255-287.

Yoshida, A., Hsu, L.C. and Dave, V.: Retinal oxidation activity and biological role of human cytosolic aldehyde dehydrogenase. Enzyme 46 (1992) 239-244.

Yourick, J.J. and Faiman, M.D.: Disulfiram metabolism as a requirement for the inhibition of rat liver mitochondrial low K_m aldehyde dehydrogenase. Biochem. Pharmacol. 42 (1991) 1361-1366.

APPENDIX

FAO and FALDH enzyme activities assayed in cultured skin fibroblasts (passage 2-12) from normal controls, SLS heterozygotes, and SLS homozygotes using 18-carbon substrates.

Name	Status[a]	FAO[b]	FALDH[b]
Brian	nl	73.9	7649
Drucker	nl	NT[a]	8399
Eaton	nl	66.8	8610
Gendreau	nl	107.0	NT
Harty	nl	81.8	9576
Kelson	nl	70.9	7720
Nonie	nl	66.0	6950
Rall	nl	69.8	8768
Rizzo	nl	90.7	8867
Sam	nl	61.0	9975
Sorg	nl	67.4	NT
Taylor	nl	93.4	7008
Trent	nl	74.1	NT
Wilde	nl	67.0	8197
Zenger	nl	62.0	10772
BBledsoe	hetero	76.3	4948
TBledsoe	hetero	47.3	5188
AmBledsoe	homo	5.3	1054
AnBledsoe	homo	5.1	212
ILarsonneau	hetero	44.6	3284
YLarsonneau	homo	7.9	1167
ESmithSr	hetero	58.5	6916
CSmith	hetero	57.0	6427
ESmithJr	homo	6.3	853
DZahr	hetero	60.6	3370
PZahr	hetero	46.9	4069
AZahr	homo	4.1	736
CBrown	homo	1.6	591
ECooper	homo	6.2	545
ACross	homo	5.8	787
CEerden	homo	5.0	NT
DEerden	homo	7.1	283
BHanita	homo	9.1	137
NWafa	homo	6.1	325

[a] nl = normal control, hetero = SLS heterozygote, homo = SLS homozygote, NT = not tested

[b] enzyme activity expressed as picomoles/min/mg protein

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FAO and FALDH enzyme activities assayed in cultured skin fibroblasts (passage 2-12) from normal controls, SLS heterozygotes, and SLS homozygotes using 18-carbon substrates.

Name[a]	Status[b]	FAO[c]	FALDH[c]
HAlevik	hetero	59.0	4517
AAlevik	homo	5.1	996
MrJohansson	hetero	32.3	6962
MlJohansson	homo	4.2	510
ENystrom	hetero	57.2	4673
LNystrom	homo	7.3	213
BSandgren	hetero	64.3	5967
RSandgren	homo	7.3	1078
ELovgren	homo	7.0	1461
EN	homo	4.1	319
TJ	homo	3.6	1196

[a] swedish families, presumably descended from a common ancestor
[b] hetero = SLS heterozygote, homo = SLS homozygote
[c] enzyme activity expressed as picomoles/min/mg protein

Vita