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IDENTIFICATION AND CHARACTERIZATION OF A NOVEL CLASS 3 ALDEHYDE DEHYDROGENASE OVEREXPRESSED IN A HUMAN BREAST ADENOCARCINOMA CELL LINE EXHIBITING OXAZAPHOSPHORINE-SPECIFIC ACQUIRED RESISTANCE*

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Abstract—Associated with the oxazaphosphorine-specific acquired resistance exhibited by a human breast adenocarcinoma subline growing in monolayer culture, viz. MCF-7/OAP, was the overexpression (>100-fold as compared with the very small amount expressed in the oxazaphosphorine-sensitive parent line) of a class 3 aldehyde dehydrogenase, viz. ALDH-3, judged to be so because it is a polymorphic enzyme (pI values *ca.* 6.0) present in the cytosol that is heat labile, is insensitive to inhibition by disulfiram (25 μM), much prefers benzaldehyde to acetaldehyde as a substrate and, at concentrations of 4 mM, prefers NADP to NAD as a cofactor. No other aldehyde dehydrogenases were found in these cells. As compared with those of the prototypical class 3 human ALDH-3, viz. constitutive human stomach mucosa ALDH-3, the physical and catalytic properties of the MCF-7/OAP enzyme differed somewhat with regard to pI values, native M_r, subunit M_r, recognition of the subunit by anti-stomach ALDH-3 IgY, pH stability, cofactor influence on catalytic activity, and the ability to catalyze, albeit poorly, the oxidation of an oxazaphosphorine, viz. aldophosphamide. Hence, the MCF-7/OAP ALDH-3 was judged to be a novel class 3 aldehyde dehydrogenase. Small amounts of a seemingly identical enzyme are also present in normal pre- and post-menopausal breast tissue. None could be detected in human liver, kidney or placenta, suggesting that it may be a tissue-specific enzyme.

Mafosfamide, 4-hydroperoxycyclophosphamide and cyclophosphamide are antineoplastic agents collectively referred to as oxazaphosphorines [1]. Each is a prodrug, i.e. *per se*, without therapeutic (cytotoxic) activity. Salient aspects of their metabolism are presented in Fig. 1. Most pertinent to the present investigation is the irreversible detoxification that occurs when NAD(P)-dependent aldehyde dehydrogenases catalyze the oxidation of a pivotal metabolite, viz. aldophosphamide, to carboxyphosphamide. Class 1 aldehyde dehydrogenases, e.g. human ALDH‡-1, are particularly important in this regard [2, 3]. At least two other human "aldehyde" dehydrogenases, viz. ALDH-2 (a class 2 aldehyde dehydrogenase) and succinic semialdehyde dehydrogenase (SSDH), also catalyze the reaction albeit less well; still others, viz. ALDH-4 (glutamic γ-semialdehyde dehydrogenase), ALDH-5 (a microsomal class 3 aldehyde dehydrogenase) and betaine aldehyde dehydrogenase (BADH), do not catalyze it at all [3]. Not known is whether a cytosolic class 3 aldehyde dehydrogenase, viz. ALDH-3, catalyzes the reaction but a putative homolog of it, viz. mouse AHD-4, is known to do so [2]; large amounts of ALDH-3 are found in human stomach mucosa though not in the liver [4].

Relatively elevated levels of a relevant aldehyde dehydrogenase would be the basis of a relatively decreased cellular sensitivity to the oxazaphosphorines [5]. Indeed, relatively elevated levels of the mouse homolog of ALDH-1, viz. AHD-2, account for the acquired resistance exhibited by two mouse lymphocytic leukemia sublines, viz. L1210/OAP and P388/CLA [1, 5–10]. Resistance was oxazaphosphorine-specific as would be expected since AHD-2 is not known to catalyze the detoxification of any of the other widely used antineoplastic agents nor of phosphoramide mustard, the oxazaphosphorine metabolite that effects the cytotoxic action of these agents.

* Descriptions of parts of this investigation have appeared in abstract form (Sreerama L and Sladek NE, *Proc Am Assoc Cancer Res* **32**: 352, 1991; Sreerama L and Sladek NE, *Proc Am Assoc Cancer Res* **33**: 447, 1992).

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‡ Abbreviations: ALDH, human aldehyde dehydrogenase; SSDH, succinic semialdehyde dehydrogenase; BADH, betaine aldehyde dehydrogenase; AHD, mouse aldehyde dehydrogenase; BSA, bovine serum albumin; PAGE, polyacrylamide gel eletrophoresis; SDS, sodium dodecyl sulfate; pI, isoelectric point; and mIU, milli-International Unit of enzyme activity (nmol NAD(P)H formed/min as used herein).

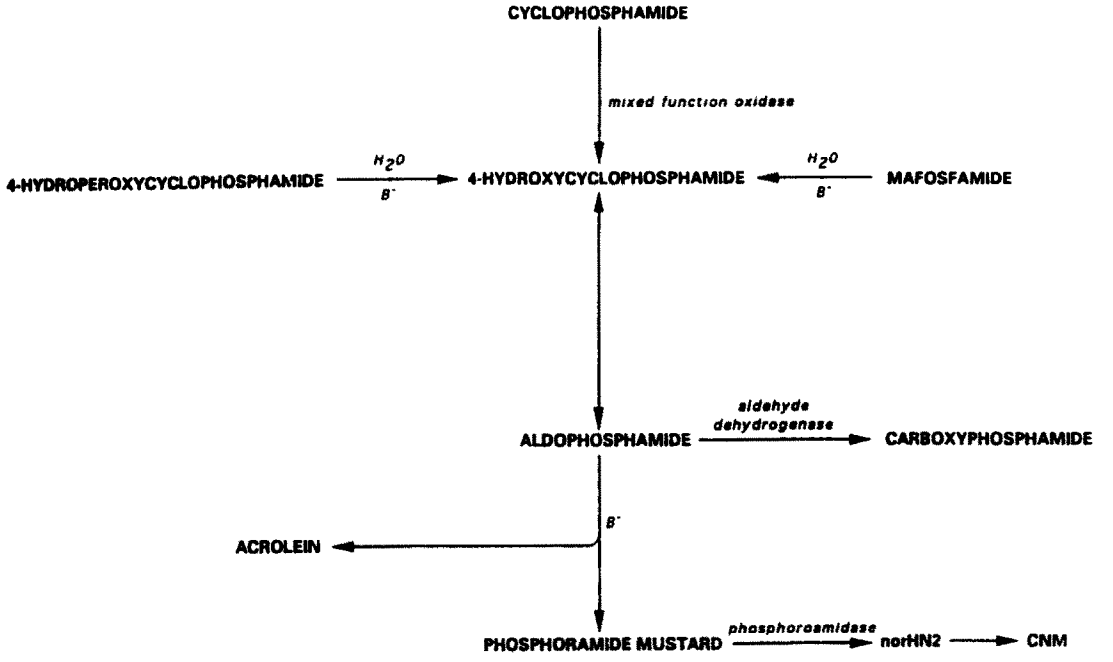

Fig. 1. Salient features of oxazaphosphorine metabolism. The prodrugs, cyclophosphamide, mafosfamide and 4-hydroperoxycyclophosphamide, each give rise to 4-hydroxycyclophosphamide which exists in equilibrium with its ring-opened tautomer, aldophosphamide. 4-Hydroxycyclophosphamide and aldophosphamide are, themselves, also without cytotoxic activity. However, aldophosphamide gives rise to acrolein and phosphoramide mustard, each of which is cytotoxic; the latter effects the bulk of the therapeutic action effected by the oxazaphosphorines [1]. Alternatively, aldophosphamide can be further oxidized to carboxyphosphamide by certain aldehyde dehydrogenases [1–3]. Carboxyphosphamide is without cytotoxic activity nor does it give rise to a cytotoxic metabolite. Aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide to carboxyphosphamide is, therefore, properly viewed as an enzyme-catalyzed detoxification of the oxazaphosphorines. Key: norHN2, bis-(2-chloroethyl)-amine; and CNM, 3-(2-chloroethyl)-1,3-oxazolidine-2-one.

Frei and associates [11] have developed a human breast adenocarcinoma subline (termed MCF-7/OAP herein) that also exhibits oxazaphosphorine-specific acquired resistance. The expectation was that overexpression of, most probably, ALDH-1 or, alternatively, one of the other human aldehyde dehydrogenases known to catalyze the oxidation of aldophosphamide, viz. ALDH-2 or SSDH, would account for the oxazaphosphorine-specific resistance exhibited by these cells. Early on it became apparent that this expectation would not be realized. Instead, markedly elevated levels of a cytosolic class 3 enzyme that appeared to be similar, but not identical, to human stomach mucosa ALDH-3 were found. The investigations reported herein describe the isolation, physical and kinetic characterization, and identification of this enzyme.

MATERIALS AND METHODS

Materials. Mafosfamide and 4-hydroperoxycyclophosphamide were provided by Dr. J. Pöhl, Asta-Werke AG, Bielefeld, Germany. Phosphoramide mustard·cyclohexylamine was supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. 4-Hydroperoxyifosfamide was prepared for us from ifosfamide by Dr. Kathleen Getman, University of Minnesota, according to the protocol described by Peter *et al.* [12]. Melphalan-HCl was supplied by Dr. G. M. Lyon, Jr., Burroughs Wellcome & Co., Research Triangle Park, NC. Actinomycin D was purchased from Calbiochem, Los Angeles, CA. Benzaldehyde, 4-pyridinecarboxaldehyde, octanal, propionaldehyde, acetaldehyde, acrolein (99+%), bis-(2-chloroethyl)-amine and methyl sulfide (99+%) were purchased from the Aldrich Chemical Co., Milwaukee, WI. NAD, NADP, NADH, NADPH, glutathione (reduced form), pyrazole, betaine aldehyde, succinic semialdehyde, γ-aminobutyraldehyde diethyl acetal (90%), DL-Δ^1-pyrroline-5-carboxylate-2,4-dinitrophenylhydrazone-HCl, glyceraldehyde 3-phosphate diethyl acetal monobarium salt, glyceraldehyde 3-phosphate dehydrogenase, bovine serum albumin (BSA; Fraction V), *p*-nitrophenyl acetate, Lubrol®, L-glutamine, Coomassie Brilliant Blue R-250, nitroblue tetrazolium, phenazine methosulfate, 2-(*N*-morpholino)ethane sulfonic acid, dithiothreitol, Freund's complete adjuvant, Freund's incomplete adjuvant, 1-chloro-2,4-dinitrobenzene, *p*-chloromercuribenzoic acid, disulfiram, menadione, ethacrynic acid, Reactive Blue 2-Sepharose CL 6B, Sephacryl S-200, standard protein marker kits for gel permeation, sodium dodecyl sulfate–

polyacrylamide gel electrophoresis (SDS–PAGE), gradient PAGE, and anti-chicken IgG alkaline phosphatase conjugate were purchased from the Sigma Chemical Co., St. Louis, MO. Fetal bovine serum was obtained from Hyclone Laboratories, Logan, UT. Ultrafiltration membranes (YM-30) were purchased from Amicon Division, W. R. Grace & Co., Danvers, MA. DEAE-Sephacel, CM-Sepharose CL 6B, PD-10 (Sephadex G-25) columns, Ampholine PAGplates® (pH 3.5–9.5) and an isoelectrofocusing marker kit were products of Pharmacia-LKB Biotechnology, Piscataway, NJ. Protein assay dye reagent concentrate, acrylamide and *N,N'*-methylene-bis-acrylamide were obtained from Bio-Rad Laboratories, Richmond, CA. Immobilon-PVDF transfer membrane was purchased from the Millipore Corp., Bedford, MA. Gentamicin (50 mg/mL), trypsin 2.5% (lyophilized powder; 10X) and Dulbecco's modified Eagle medium (powder; low glucose) were purchased from Gibco Laboratories, Grand Island, NY. Chloral hydrate and doxorubicin-HCl were purchased from the University of Minnesota Hospital Pharmacy, Minneapolis, MN. All other chemicals and reagents were of analytical grade.

Aldophosphamide was generated in aqueous solution by chemical reduction of 4-hydroperoxycyclophosphamide using methyl sulfide (99+%) as the reducing agent [3]. Glutamic-γ-semialdehyde was prepared from DL-Δ^1-pyrroline-5-carboxylate-2,4-dinitrophenylhydrazone-HCl according to the method of Mezl and Knox [13]. γ-Aminobutyraldehyde was prepared from γ-aminobutyraldehyde diethyl acetal by acid hydrolysis according to the method of Ambroziak and Pietruszko [14]. Glyceraldehyde 3-phosphate was prepared by acid hydrolysis of glyceraldehyde 3-phosphate diethyl acetal monobarium salt, and its concentration was determined using glyceraldehyde 3-phosphate dehydrogenase according to the protocol provided by the manufacturer. Homogenization medium was 1.15% (w/v) KCl and 1 mM EDTA in aqueous solution, pH 7.4. Drug-exposure medium was fetal bovine serum (10%) in phosphate-buffered saline-based solution, pH 7.4, prepared as previously described [8]. Growth medium was fetal bovine serum (10%) in Dulbecco's modified Eagle medium supplemented with L-glutamine (2 mM), sodium bicarbonate (3.7 g/L) and gentamicin (50 mg/L). Buffer A was 25 mM 2-(*N*-morpholino)ethane sulfonic acid, pH 6.5, supplemented with 1 mM EDTA and 0.05% dithiothreitol. Buffer B was 25 mM sodium phosphate, pH 7.5, supplemented with 1 mM EDTA and 0.05% dithiothreitol. Buffer C was 25 mM Tris–HCl in normal saline, pH 7.5.

Human MCF-7 breast adenocarcinoma cells sensitive (MCF-7/0) and resistant (MCF-7/OAP) to oxazaphosphorines, and growing in monolayer culture, were obtained originally from Dr. B. Teicher, Dana-Farber Cancer Institute, Boston, MA. They were propagated at 37° in dishes/flasks containing growth medium; the atmosphere of 5% CO_2 in air was fully humidified. Mean population-doubling times were approximately 25 (MCF-7/0) and 30 (MCF-7/OAP) hr. Mean plating efficiencies were approximately 50% in each case.

Cultured tumor cells in asynchronous exponential growth were submitted to trypsinization (0.25%) and then harvested by low-speed centrifugation (500 *g* for 10 min). After washing once with drug-exposure medium, they were resuspended in drug-exposure medium and checked for viability (usually greater than 95% as judged by trypan blue exclusion; preparations exhibiting less than 85% viability were discarded). This was the preparation used in the colony-forming assays. Cells were further handled in several different ways when enzyme activity in cell-free fractions was to be quantified or when enzymes were to be extracted and purified. Usually the cells were again harvested by low speed centrifugation, resuspended (1×10^7 cells/mL) in homogenization medium, and then used. Infrequently, this suspension was stored at −20° until used. Sometimes the harvested cells were stored at −20° as a pellet overlaid with homogenization medium until used. Enzyme activity was essentially unaffected by freezing and storage at −20°.

Except for liver, all human tissues were obtained from the Tissue Procurement Facility, University of Alabama Comprehensive Cancer Center, Birmingham, AL, through the Cooperative Human Tissue Network, Midwestern Division, Columbus, OH. Liver was obtained through the Liver Procurement and Distribution System, University of Minnesota, Minneapolis, MN. The suppliers certified all samples to be nonpathological. Breast samples were from a 19-, a 51-, a 59- and a 61-year-old Caucasian female; each was frozen at −70° within 3–5 hr of surgical removal and was stored at this temperature until used. Stomach, lung and kidney were from a 47-year-old Caucasian male who died of severe coronary heart disease, a 16-year-old Caucasian male who died of injuries sustained in a motor vehicle accident, and a 4½-month-old Caucasian female who died of sudden infant death syndrome, respectively. Each was surgically removed 4–6 hr post-mortem and was immediately frozen and stored at −70° until used. Liver was from a 22-year-old Caucasian male who died of injuries sustained in a motor vehicle accident. It was kept at 0–4° and delivered to us within 12 hr of donation; upon delivery it was frozen and stored at −70° until used. Placenta was kept at 0–4° and delivered to us within 24 hr of donation; it was assayed immediately for enzyme activity, i.e. without ever being frozen.

Purified ALDH-1, ALDH-2 and SSDH [3] were provided by Dr. P. A. Dockham.

Drug exposure and colony-forming assay. Freshly harvested cells were diluted with drug-exposure medium to a concentration of 1×10^5 cells/mL and were exposed to various drugs or the appropriate vehicle for 30 min at pH 7.4 and 37° in air. Except for ethacrynic acid, 1-chloro-2,4-dinitrobenzene, actinomycin D and menadione, all of the test drugs were dissolved in double-deionized water. Ethacrynic acid and 1-chloro-2,4-dinitrobenzene were dissolved in 50% ethanol. Actinomycin D and menadione were dissolved in absolute ethanol. All of the drug solutions were sterilized by passage through 0.22 μm Millipore filters; all were used within 1 hr of preparation and were kept at approximately 4° prior to their use. Drug solutions were added to tumor

cell suspensions in a volume of 0.1 mL; the final volume of the tumor cell suspension was 5 mL. Ethanol, at the concentrations used, was not toxic to tumor cells. At the end of the 30-min incubation periods, the cells were placed in an ice-bath and allowed to chill for 5 min. They were then harvested by low-speed centrifugation, washed with drug-free growth medium, and resuspended in growth medium at concentrations that allowed the transfer, in triplicate, of 10,000, 1000 and 100 cells, each in a volume of 1 mL, to 60 × 15 mm petri dishes containing 4 mL of growth medium. The cells were allowed to grow at 37° in a fully humidified 5% CO_2 in air atmosphere for 15 days after which time the medium was poured off, cells were stained with methylene blue dye, and colonies (≥50 cells) were counted.

Preparation of Lubrol®-treated whole homogenates. MCF-7/0 and MCF-7/OAP cells suspended in homogenization medium were lysed in an ice-bath by submitting them to sonication (Artek Dismembrator model 300; setting of 30) for a total period of 10 sec (divided into 3 bursts). The homogenate was then adjusted to 0.3% Lubrol®, vortexed, and centrifuged at 105,000 *g* and 4° for 1 hr. The resultant supernatant fraction was used for enzyme activity assay.

Lubrol®-treated 10% (w/v) liver whole homogenates were prepared as previously described [3]. Kidney Lubrol®-treated 10% (w/v) whole homogenates were prepared in an identical manner except that the homogenizing medium was Buffer B devoid of dithiothreitol. These preparations were used when enzyme activity was to be determined.

All preparations were transferred into Buffer B with the aid of Pharmacia PD-10 columns when electrophoresis was to be effected.

Preparation of subcellular fractions. Subcellular fractions of MCF-7/0 and MCF-7/OAP cells were prepared essentially as described before [15] except that 0.3% Lubrol®, rather than 0.3% deoxycholate, was used to solubilize the particulate fraction. Briefly, a Dounce homogenizer was used to homogenize cells in ice-cold homogenization medium and the homogenate was centrifuged at 105,000 *g* and 4° for 1 hr. The resultant supernatant (soluble) fraction was saved for enzyme activity assay; it was transferred into Buffer B or Buffer A with the aid of Pharmacia PD-10 columns when electrophoresis or chromatography, respectively, was to be effected. The pellet was washed once with homogenization medium and resuspended in homogenization medium containing 0.3% Lubrol®. This preparation was centrifuged as above and the resultant supernatant (solubilized particulate) fraction was saved for enzyme activity assay; it was transferred into Buffer B as above when electrophoresis was to be effected.

Soluble (105,000 *g* supernatant) fractions of lung, placenta and stomach mucosa (scraped away from the serosa with a scalpel) were prepared by homogenizing these tissues in Buffer B devoid of dithiothreitol, and submitting the 10% (w/v) homogenates to centrifugation at 105,000 *g* and 4° for 1 hr. The soluble (105,000 *g* supernatant) fraction of the breast tissue was prepared in an identical manner except that this tissue was first submitted to disruption in a Waring blender for 1 min before it [50% (w/v) homogenate] was submitted to further homogenization in a Dounce homogenizer. These preparations were used when enzyme activity was to be determined. All of the preparations were transferred into Buffer B with the aid of Pharmacia PD-10 columns when electrophoresis was to be effected or when the preparation, viz. stomach mucosa soluble fraction, was to undergo chromatography.

Enzyme assays. Aldehyde dehydrogenase activity was quantified spectrophotometrically, essentially as previously described [2, 3]. The reaction mixture (1 mL, pH 8.1) contained substrate (aldehyde), NAD (1 or 4 mM) or NADP (4 mM), pyrazole (0.1 mM), glutathione (5 mM), EDTA (1 mM), tetrasodium pyrophosphate (32 mM), crude fraction or (semi)purified aldehyde dehydrogenase, and, in some experiments, a potential inhibitor/modulator of the enzyme activity. Some of the substrates/inhibitors/modulators, viz. octanal, propionaldehyde and disulfiram, were dissolved in methanol and added to the reaction mixture in a volume not exceeding 50 μL. Aldehyde dehydrogenase activity was unaffected by this amount of methanol in the reaction mixture. The reaction was initiated by the addition of aldehyde and was followed at 37° by monitoring the appearance of NAD(P)H at 340 nm in a Beckman DU-70 automated recording spectrophotometer. All rates were determined in duplicate.

Esterase activity was determined by monitoring the increase in absorbance at 400 nm due to *p*-nitrophenol production from *p*-nitrophenyl acetate [16].

Protein determination. Protein concentrations were estimated by the method of Bradford [17], using commercially available Bio-Rad protein assay regaent and BSA as the standard.

Chromatographic purification of aldehyde dehydrogenases. DEAE-Sephacel, CM-Sepharose CL 6B and Reactive Blue 2-Sepharose CL 6B column chromatography was performed at 4–6°. All buffers were degassed prior to use. Linear flow rates were 30, 30 and 20 mL/hr, respectively. Concentration of samples was with an Amicon Diaflo concentrator fitted with a YM-30 membrane and pressurized with nitrogen. Protein concentrations of samples loaded onto columns never exceeded 15 mg/mL and typically were much less. Benzaldehyde (4 mM) and NAD (1 mM) were used as the substrate and cofactor, respectively, to monitor aldehyde dehydrogenase activity in column eluates. Protein was monitored at 280 nm with an ISCO UA-5 absorbance monitor.

Analytical and preparative non-denaturing PAGE. Non-denaturing PAGE was carried out essentially as described by Davis [18] to monitor enzyme purification. A discontinuous gel system, 8% separating gel (pH 8.8) and 4% stacking gel (pH 6.8), was used for this purpose. Slab gels were 70 × 80 × 0.5 mm. Samples (1 mg protein/mL) were prepared in an aqueous solution of 0.5 M Tris–HCl, pH 6.8, containing 10% glycerol or 20% sucrose, and 2 μL 0.05% bromophenol blue. Aliquots (25 μL) were electrophoresed at 4° with the aid of a Bio-Rad Protein-II (mini) vertical slab gel electrophoretic

system by applying a constant voltage (200 V) until the tracking dye reached the lower tip of the gel.

Preparative PAGE was performed as above except that the gels were 200 × 160 × 1.5 mm in size and electrophoresis was performed in a Bio-Rad Protein-II system at low constant voltage (75 V).

Isoelectric focusing. Isoelectric focusing was as described by Manthey *et al.* [2], except that commercially available Ampholine PAGplates® (250 × 115 × 1 mm) containing 10% glycerol and 2% ampholite (pH 3.5 to 9.5), and a constant power output of 20 W for 3000 V-hr, were used for this purpose. Samples (50 μL) were loaded onto the gel surface with the aid of sample applicators and electrophoresis was effected at 4–6°. A broad range standard protein isoelectric point (pI) marker kit was used to assign the pI values to aldehyde dehydrogenases.

Gels were stained for both aldehyde dehydrogenase activity and protein. They were immersed in an aqueous solution containing 32 mM sodium pyrophosphate, pH 8.1, 1 mM nitroblue tetrazolium, 130 μM phenazine methosulfate, 1 mM pyrazole, 4 mM NAD(P) and aldehyde substrate for 20–45 min at 37° to visualize aldehyde dehydrogenases. Staining solutions lacking either NAD(P) or aldehyde were used to develop control gels, i.e. to distinguish the enzymes of interest from other enzymes that directly or indirectly transfer electrons to nitroblue tetrazolium when aldehyde or NAD(P) alone is present. Coomassie Brilliant Blue R-250 (0.05%) was used to visualize proteins. The gels were destained by slow leaching in a destaining solution containing water:methanol:acetic acid (6:3:1).

Molecular weight determinations. Native molecular weights of the purified enzymes were determined by gel filtration on a Sephacryl S-200 column (1 × 50 cm) equilibrated with Buffer B. They were also determined by linear gradient gel electrophoresis as described by Margolis and Kenrick [19].

Subunit molecular weights of the two purified aldehyde dehydrogenases were determined on SDS–polyacrylamide gels (12%) essentially according to the method of Laemmli [20].

Preparation of antibodies. Antibodies against stomach mucosa ALDH-3 were obtained by immunization of egg-laying hens (White Leghorn) essentially according to the method of Gassmann *et al.* [21]. Briefly, stomach mucosa ALDH-3 (350 μg in 0.5 mL of a phosphate-buffered saline solution, pH 7.4) was mixed with an equal volume of Freund's complete adjuvant and emulsified. One-half of the emulsion was then injected subcutaneously into the pectoral muscle (two sites) of each of two birds. A booster injection (100 μg of enzyme in phosphate-buffered saline solution, pH 7.4, and Freund's incomplete adjuvant) was given 3 weeks later. Eggs were collected daily, marked and stored at 4° until used. Antibodies (IgY) were isolated from egg yolk by polyethylene glycol precipitation and were partially purified by DEAE-Sephacel chromatography. As judged by ELISA (1 μg purified enzyme) and by SDS–PAGE/immunoblot assay (5 μg purified enzyme), anti-stomach mucosa ALDH-3 IgY prepared in this manner was not cross-reactive with ALDH-1 or ALDH-2.

Immunoblot analysis. Purified stomach mucosa and MCF-7/OAP ALDH-3s were electrophoresed on a 12% SDS–polyacrylamide gel and electrotransferred onto Immobilon-PVDF transfer membrane using a Bio-Rad semidry blotter [22]. The transfer membrane was processed at room temperature by, first, shaking it for 2 hr in a blocking solution of 5% (w/v) instant nonfat milk in Buffer C; second, shaking it for 2 hr with partially purified anti-stomach ALDH-3 IgY suspended (1:500) in the blocking solution; third, vigorously shaking it three times, 10 min each time, in Buffer C; fourth, incubating it for 2 hr with a secondary antibody (anti-chicken IgG, 1:1000) coupled to alkaline phosphatase; fifth, vigorously shaking it three times, 10 min each time, in Buffer C; and lastly, staining it for alkaline phosphatase activity.

Purified stomach mucosa and MCF-7/OAP ALDH-3s were also electrophoresed on Ampholine PAGplates® and electrotransferred onto transfer membranes essentially as described by Dunn [23]. Thus, the gel was removed from the backing, rinsed with an aqueous solution, pH 9.9, containing 10 mM $NaHCO_3$ and 3 mM $NaCO_3$ but devoid of methanol, and electrotransferred with the same solution and a Bio-Rad semidry blotter. Further processing of the membrane was as described above except that the blocking solution was 5% BSA (w/v), rather than 5% instant nonfat milk, in Buffer C.

Data analysis. Double-reciprocal plots of initial rates versus substrate concentrations were used to estimate all K_m and V_{max} values. Initial rates were determined in duplicate with each of five to twelve substrate concentrations to generate each value.

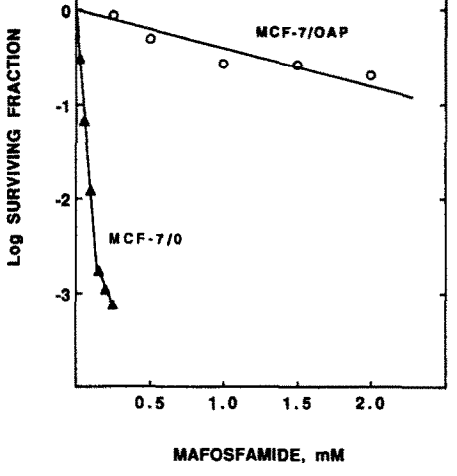

Fig. 2. Sensitivity of cultured MCF-7/0 and MCF-7/OAP cells to mafosfamide. Cultured oxazaphosphorine-sensitive MCF-7/0 (▲), and oxazaphosphorine-resistant MCF-7/OAP (○), cells were incubated with mafosfamide for 30 min at 37°. The cells were then harvested and grown in a drug-free medium. The colony-forming assay described in Materials and Methods was used to determine surviving fractions. Each point is the mean of measurements on triplicate cultures.

Table 1. Sensitivity of MCF-7/0 and MCF-7/OAP cells to mafosfamide and other cytotoxic agents*

Drug	Exposure time (min)	LC_{90} (μM) MCF-7/0	LC_{90} (μM) MCF-7/OAP	Ratio
Mafosfamide	30	50	>2,000	>40
4-Hydroperoxyifosfamide	30	90	>1,000	>11
Phosphoramide mustard	30	800	1,530	1.9
Acrolein	30	34	50	1.5
Bis-(2-chloroethyl)-amine	30	134	420	3.1
Melphalan	30	4	5	1.3
Doxorubicin	30	1.5	2.2	1.5
Actinomycin D	30	1.0	1.7	1.7
Menadione	30	32	30	1.0
1-Chloro-2,4-dinitrobenzene	35	12	12	1.0
Ethacrynic acid	35	312	378	1.2

* Cultured oxazaphosphorine-sensitive MCF-7/0, or oxazaphosphorine-resistant MCF-7/OAP, cells were exposed at 37° to five to seven concentrations of each of the cytotoxic agents for the time periods indicated. The cells were then harvested and grown in a drug-free medium. The colony-forming assay described in Materials and Methods was used to determine surviving fractions. The resultant data i.e. the means of measurements on triplicate cultures at each concentration, were plotted as illustrated in Fig. 2. The LC_{90} (concentration of drug required to effect a 90% cell kill) values were obtained from such plots.

Wilkinson weighted linear regression analysis [24] was used to fit lines to the double-reciprocal plot values.

Computer-assisted unweighted regression analysis was carried out using the STATView® (Brainpower, Inc., Calabas, CA) statistical program to generate all other straight line functions.

RESULTS

Frei and associates [11] developed a subline of the human breast adenocarcinoma MCF-7/0 cell line, viz. MCF-7/OAP, that is relatively insensitive to 4-hydroperoxycyclophosphamide by growing the parent line in the presence of increasing amounts of 4-hydroperoxycyclophosphamide over a period of several months. The subline had not lost sensitivity to several other agents, viz. mechlorethamine, melphalan, thioTEPA, mitomycin C, *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) and cisplatin, that are chemically and/or pharmacologically related but that are not oxazaphosphorines. In our hands, MCF-7/OAP cells exhibited a cross-resistance to two other oxazaphosphorines, viz. mafosfamide and 4-hydroperoxyifosfamide, but not to any of several oxazaphosphorine metabolites, including phosphoramide mustard, that are not oxazaphosphorines, or to several other agents, some of which are used as antineoplastic agents, but none of which are oxazaphosphorines (Fig. 2 and Table 1). Thus, MCF-7/OAP cells exhibit an oxazaphosphorine-specific acquired resistance.

Table 2. Aldehyde dehydrogenase activity in the soluble (105,000 *g* supernatant) fractions of MCF-7/0 and MCF-7/OAP cells*

Substrate (concentration)	Cofactor (4 mM)	Aldehyde dehydrogenase activity (mIU/10^7 cells) MCF-7/0	Aldehyde dehydrogenase activity (mIU/10^7 cells) MCF-7/OAP
Aldophosphamide (160 μM)	NAD	0†	2.8 ± 0.6
	NADP	0	0
Acetaldehyde (4 mM)	NAD	1.4	6.6
	NADP	0	0
Benzaldehyde (4 mM)	NAD	1.7 ± 0.3	110 ± 6.6
	NADP	1.9 ± 0.3	254 ± 15.8

* Soluble fractions were freshly prepared from tumor cells in exponential growth, and aldehyde dehydrogenase activity in such fractions obtained from 2×10^5 to 1×10^7 cells was determined as described in Materials and Methods. Each value is the mean of duplicate determinations on each of one to three samples. Standard errors of these values are presented in those cases where determinations were on three samples.

† Subsequently, it will be shown that the MCF-7/0 cell line does contain very small amounts (below detectable levels in the present experiment) of an aldehyde dehydrogenase, seemingly Type-2 ALDH-3, that catalyzes the NAD-dependent oxidation of aldophosphamide to carboxyphosphamide.

The expectation was that NAD(P)-dependent enzyme-catalyzed detoxification of the oxazaphosphorines would be elevated markedly in the MCF-7/OAP cells, thereby accounting for the oxazaphosphorine-specific acquired resistance. NAD(P)-dependent enzyme-catalyzed oxidation of aldophosphamide to carboxyphosphamide was elevated in the resistant subline (Table 2), but the activity was of such a small magnitude that any notion that increased oxidation of aldophosphamide to carboxyphosphamide accounts for oxazaphosphorine-specific acquired resistance is highly problematical. Enzyme-catalyzed oxidation of acetaldehyde was also minimal in these cells, but that of benzaldehyde was elevated markedly and of a large magnitude regardless of whether NAD or NADP was used as the cofactor. Pyridine nucleotide-dependent oxidation of glutamic-γ-semialdehyde, succinic semialdehyde, betaine aldehyde or glyceraldehyde 3-phosphate was not catalyzed by either subcellular (soluble and particulate) fractions or Lubrol®-solubilized whole homogenates of the two cell lines (data not presented). Aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide, acetaldehyde and/or benzaldehyde, was confined to the cytosol, more accurately, the soluble (105,000 *g* supernatant) fraction in each cell line, i.e. it was not detected in particulate (105,000 *g* pellet) fractions (data not presented). Disulfiram (50 μM) and chloral hydrate (100 μM) did not inhibit the catalysis of aldehyde (acetaldehyde, benzaldehyde and octanal) oxidation by the soluble (105,000 *g* supernatant) fraction obtained from MCF-7/OAP cells (data not presented).

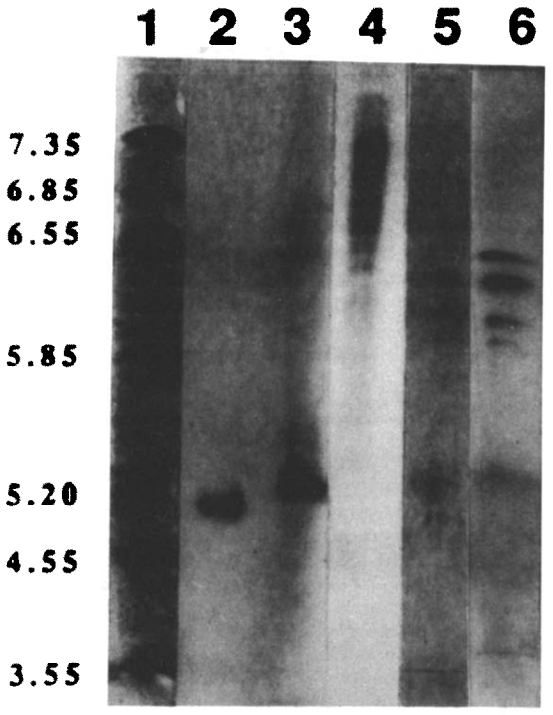

Fig. 3. Isoelectric focusing of human aldehyde dehydrogenases known to catalyze the oxidation of aldophosphamide to carboxyphosphamide, and whole homogenates of MCF-7/0 and MCF-7/OAP cells. Human aldehyde dehydrogenases known to catalyze the oxidation of aldophosphamide to carboxyphosphamide, viz. ALDH-2 (lane 2), ALDH-1 (lane 3) and SSDH (lane 4), Lubrol®-treated whole cell homogenates of MCF-7/0 (lane 5) and MCF-7/OAP (lane 6), and pI standards (lane 1) were subjected to isoelectric focusing as described in Materials and Methods. The amount of each purified enzyme, i.e. ALDH-1, ALDH-2 and SSDH, loaded onto the gel was sufficient to generate 1.5 to 2.0 nmol NADH/min as determined by spectrophotometric assay and the substrate with which the enzyme was ultimately stained. MCF-7/0 and MCF-7/OAP Lubrol®-treated whole homogenates loaded onto the gel were from 1×10^7 and 2.5×10^5 cells, respectively. The nitroblue tetrazolium-coupled enzyme activity stain described in Materials and Methods was used to visualize aldehyde dehydrogenases in lanes 2–6. Substrates were acetaldehyde (4 mM) for ALDH-1 and ALDH-2, succinic semialdehyde (100 μM) for SSDH, and benzaldehyde (4 mM) for aldehyde dehydrogenases present in tumor cell whole homogenates; the cofactor was NAD (4 mM). Coomassie Brilliant Blue R-250 was used to stain the pI standards. The relatively dark background in lane 5 is because MCF-7/0 cells contain comparatively very little of the enzyme necessitating that this part of the gel be left in the staining solution for a relatively much longer time period in order to visualize the enzyme. In a separate experiment (not shown) ALDH-1 or ALDH-2 activity was also not detectable in Lubrol®-treated whole homogenates of MCF-7/0 and MCF-7/OAP cells when acetaldehyde (4 mM) was used as the substrate to assay for such activity.

ALDH-3 is a cytosolic class 3 aldehyde dehydrogenase that utilizes both NAD and NADP as cofactors, prefers aromatic and long-chain aliphatic aldehydes to short-chain aliphatic aldehydes as substrates, and is relatively insensitive to disulfiram inhibition [4]. Moreover, pI values (*ca.* 5.7 to 6.4) for this enzyme are substantially different from other known aldehyde dehydrogenases. It is found in several, but not all, human organs/tissues/cells [4, 25], and an enzyme with physical and catalytic properties similar to the human enzyme has been found in various organs/tissues/cells of a number of mammalian species [25].

Electrofocusing experiments (Fig. 3) revealed that an aldehyde dehydrogenase exhibiting pI values and an electrofocusing pattern very similar, if not identical, to those reported for ALDH-3 was present in both MCF-7/0 and MCF-7/OAP cells, that the amount of this enzyme was substantially greater in the MCF-7/OAP cell line, and that detectable amounts of other aldehyde dehydrogenases, including those known to catalyze the oxidation of aldophosphamide, viz. ALDH-1, ALDH-2 and SSDH, were not present in either cell line.

The only well-characterized human cytosolic ALDH-3 is the one constitutively present in stomach mucosa [26, 27]. However, although limited, available data indicate that the ALDH-3s identified thus far in other human tissues are identical to the stomach mucosa enzyme [4]. Side-by-side electrophoresis of the crude fractions prepared from stomach mucosa and MCF-7/OAP cells revealed that, while the MCF-7/OAP enzyme isoelectric-focused in a manner very similar to stomach mucosa ALDH-3, it did not do so in an identical manner (Fig. 4) suggesting that, whereas the MCF-7/OAP enzyme was indeed a cytosolic class 3 aldehyde dehydrogenase, it was different from the ALDH-3

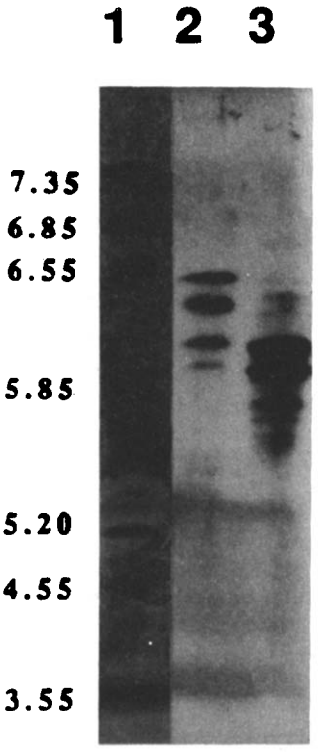

Fig. 4. Isoelectric focusing of aldehyde dehydrogenases present in the soluble (105,000 *g* supernatant) fractions of human stomach mucosa and Lubrol®-treated whole homogenates of MCF-7/OAP cells. An aliquot of a human stomach mucosa soluble fraction sufficient to generate 5.0 nmol NADH/min when benzaldehyde was the substrate, a Lubrol®-treated whole homogenate obtained from 2.5×10^5 MCF-7/OAP cells, and pI standards were loaded onto the gel and electrofocused as described in Materials and Methods. Lane 1 (pI standards) was stained for protein using Coomassie Brilliant Blue R-250. Lanes 2 (MCF-7/OAP whole cell homogenate) and 3 (human stomach mucosa soluble fraction) were stained for aldehyde dehydrogenase activity as described in Materials and Methods; benzaldehyde (4 mM) was the substrate and NAD (4 mM) was the cofactor.

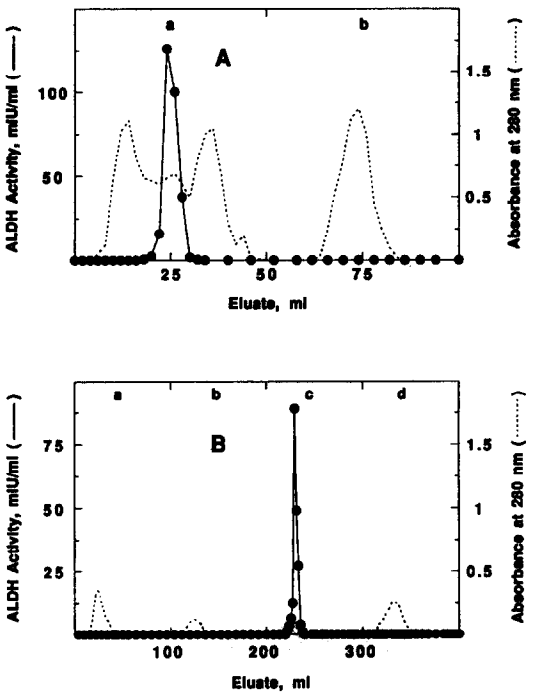

Fig. 5. Chromatographic purification of a class 3 aldehyde dehydrogenase present in MCF-7/OAP cells. (A) A concentrated (1.5 mL) soluble (105,000 *g* supernatant) fraction of MCF-7/OAP cells (5×10^7) was prepared as described in Materials and Methods and was then loaded onto a CM-Sepharose CL 6B column (1.5 × 20 cm) equilibrated with Buffer A. Elution was with (a) 50 mL Buffer A, followed by (b) 50 mL Buffer A supplemented with 700 mM NaCl. (B) CM-Sepharose CL 6B column eluates exhibiting aldehyde dehydrogenase activity were pooled (15 mL), concentrated (1 mL) as described in Materials and Methods, and loaded onto a Reactive Blue 2-Sepharose CL 6B column (1.5 × 20 cm) equilibrated with Buffer A. The loaded column was successively eluted with (a) 100 mL Buffer A, (b) 100 mL Buffer B, (c) 100 mL Buffer B supplemented with 5 mM NAD, and (d) 100 mL Buffer B supplemented with 700 mM NaCl. Eluates were collected in 2-mL fractions. Benzaldehyde (4 mM) and NAD (1 mM) were used to monitor aldehyde dehydrogenase activity. Enzyme activities, yields, and fold-purifications are tabulated in Table 3.

Table 3. Purification of a cytosolic class 3 aldehyde dehydrogenase from MCF-7/OAP cells*

Purification step	Total activity† (mIU)	Yield (%)	Total protein (mg)	Specific activity (mIU/mg)	Fold-purification
Soluble (105,000 *g* supernatant) fraction	600	100	23.7	25	1
CM-Sepharose CL 6B chromatography	560	93	1.8	311	12
Reactive Blue 2-Sepharose CL 6B affinity chromatography	364	61	0.027	13,481	539
Preparative PAGE/ Electroelution	250	42	0.015	16,667	667

* Purification was as described in Fig. 5 and the text.

† Benzaldehyde (4 mM) and NAD (1 mM) were used as substrate and cofactor, respectively, to quantify aldehyde dehydrogenase activity.

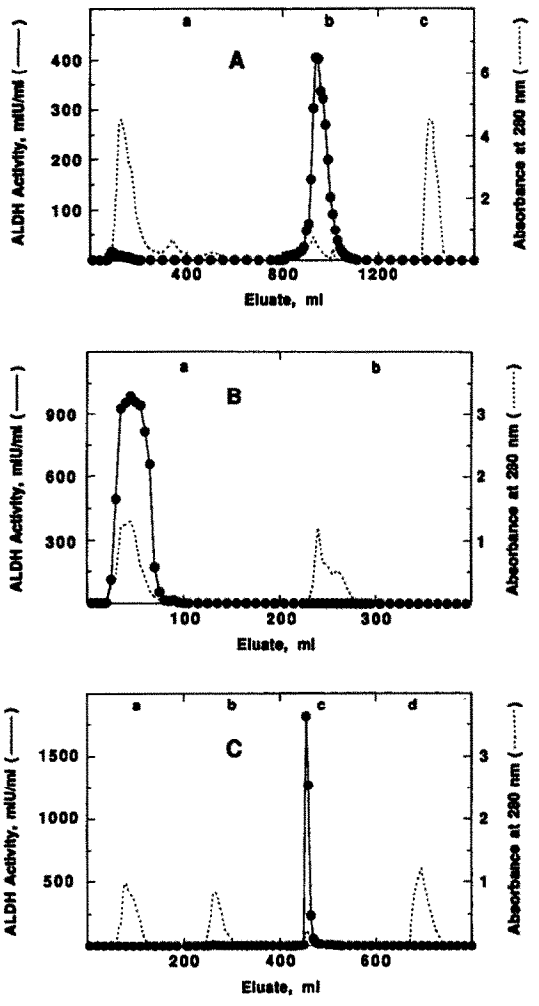

Fig. 6. Chromatographic purification of a class 3 aldehyde dehydrogenase present in human stomach mucosa. (A) A (50 mL) soluble (105,000 *g* supernatant) fraction of human stomach mucosa (4 g) was prepared as described in Materials and Methods and was then loaded onto a DEAE-Sephacel column (2.5 × 25 cm) equilibrated with Buffer B. The loaded column was successively eluted with (a) 750 mL Buffer B, (b) 400 mL Buffer B supplemented with 100 mM NaCl, and (c) 400 mL Buffer B supplemented with 700 mM NaCl. (B) DEAE-Sephacel column eluates exhibiting aldehyde dehydrogenase activity were pooled (150 mL), concentrated (5 mL) as described in Materials and Methods, transferred into Buffer A with the aid of Pharmacia PD-10 columns, and loaded onto a CM-Sepharose CL 6B column (1.5 × 35 cm) equilibrated with Buffer A. Elution was with (a) 195 mL Buffer A, followed by (b) 200 mL Buffer A supplemented with 700 mM NaCl. (C) CM-Sepharose CL 6B column eluates exhibiting aldehyde dehydrogenase activity were pooled (65 mL), concentrated (5 mL) as described in Materials and Methods, and loaded onto a Reactive Blue 2-Sepharose CL 6B column (1.5 × 35 cm) equilibrated with Buffer A. The loaded column was successively eluted with (a) 195 mL Buffer A, (b) 200 mL Buffer B, (c) 200 mL Buffer B containing 5 mM NAD, and (d) 200 mL Buffer B supplemented with 700 mM NaCl. Eluates were collected in fractions of 5–10 mL. Benzaldehyde (4 mM) and NAD (1 mM) were used to monitor aldehyde dehydrogenase activity. Enzyme activities, yields, and fold-purifications are tabulated in Table 4.

constitutively present in human stomach and other tissues. This notion was pursued in the next series of experiments. Purified enzymes were used for this purpose.

Purification of the MCF-7/OAP enzyme was as shown in Fig. 5. Small amounts of nonspecific proteins were still present in the enzyme preparation after Reactive Blue 2-Sepharose CL 6B chromatography. Therefore, it was submitted to further fractionation on preparative PAGE. Specific activity of the final product was 16,667 mIU/mg protein (Table 3). Purification of the stomach mucosa enzyme was as shown in Fig. 6. Specific activity of the final product was 32,951 mIU/mg protein (Table 4). The specific activity exhibited by the final product obtained by Eckey *et al.* [27] was much lower; that obtained by Wang *et al.* [26] was very similar. As judged by isoelectric focusing, non-denaturing linear gradient PAGE, and SDS–PAGE (Figs. 7–9, respectively) each of the enzymes had been purified to homogeneity.

As judged by a number of criteria, the purified enzymes, while resembling each other, were not identical entities. Four bands of catalytic activity, two major and two minor, were observed when the MCF-7/OAP enzyme was subjected to isoelectric focusing (Fig. 7); pI values for the major and minor bands were 6.35 and 6.45, and 6.0 and 6.25, respectively. In contrast, five bands of catalytic activity, two major and three minor, were observed when the stomach mucosa enzyme was isoelectric-focused; pI values for the major and minor bands were 6.0 and 6.25, and 5.75, 5.85 and 6.35, respectively. As determined by non-denaturing linear gradient PAGE, the relative molecular mass of each of the native enzymes was 110 kDa (Fig. 8), but relative molecular masses of 125 and 108 kDa were obtained for the MCF-7/OAP and stomach mucosa enzymes, respectively, when Sephacryl S-200 gel permeation column chromatography was utilized to make these determinations (Fig. 10). Subunit relative molecular masses for the MCF-7/OAP and stomach mucosa enzyme were 40 and 54.5 kDa, respectively (Fig. 9). Anti-stomach mucosa ALDH-3 IgY recognized native, but not denatured, MCF-7/OAP ALDH-3 (Figs. 11 and 12, respectively). Least recognized of the native MCF-7/OAP enzyme were the two major bands (pI = 6.35 and 6.45). Failure to recognize the MCF-7/OAP enzyme subunit may be due to the loss of an epitopic recognition site on denaturation of the native enzyme. According to this scenario, the epitopic recognition site would also be lost by the stomach mucosa enzyme upon denaturation but some part of the primary structure of this enzyme would still be recognized by the antibody. Further, the primary structure of this part of the MCF-7/OAP enzyme subunit would differ to the extent that it would not be recognized by the antibody.

The pI and apparent molecular weight values reported herein for the native and denatured stomach mucosa enzyme are in close agreement with those reported by others [26, 27]. The latter suggest that the native enzyme is a dimer [4, 26, 27]. With the very large caveat that molecular weights estimated by gel permeation chromatography or linear

Table 4. Purification of a cytosolic class 3 aldehyde dehydrogenase from human stomach mucosa*

Purification step	Total activity† (mIU)	Yield (%)	Total protein (mg)	Specific activity (mIU/mg)	Fold-purification
Soluble (105,000 *g* supernatant) fraction	33,500	100	430	78	1
DEAE-Sephacel chromatography	28,475	85	26.1	1,092	14
CM-Sepharose CL 6B chromatography	25,125	75	16.3	1.542	20
Reactive Blue 2-Sepharose CL 6B affinity chromatography	20,100	60	0.61	32,951	422

* Purification was as described in Fig. 6 and the text.
† Benzaldehyde (4 mM) and NAD (1 mM) were used as substrate and cofactor, respectively, to quantify aldehyde dehydrogenase activity.

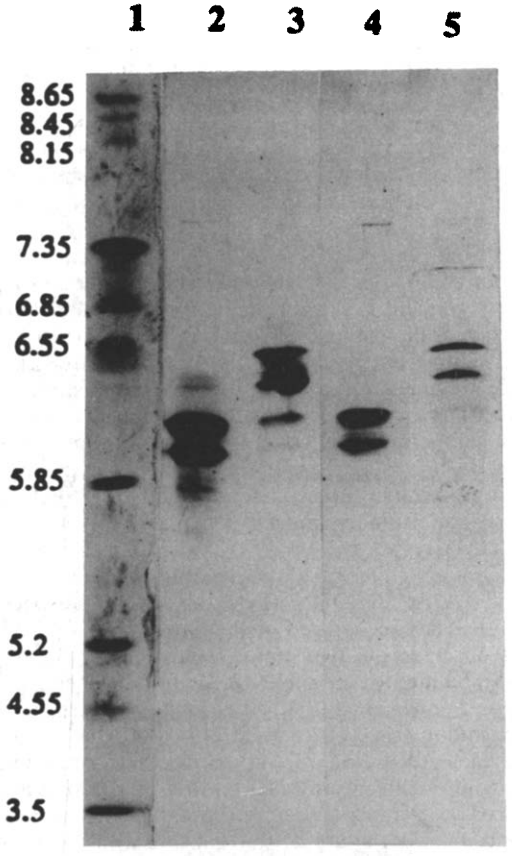

Fig. 7. Isoelectric focusing of purified stomach mucosa and MCF-7/OAP aldehyde dehydrogenases. Purified enzymes (10 μg each; lanes 2–5) and pI standards (lane 1) were electrofocused as described in Materials and Methods. Lanes 1 (pI standards), 4 (stomach mucosa aldehyde dehydrogenase) and 5 (MCF-7/OAP aldehyde dehydrogenase) were stained with Coomassie Brilliant Blue R-250 for the presence of proteins. Lanes 2 (stomach mucosa aldehyde dehydrogenase) and 3 (MCF-7/OAP aldehyde dehydrogenase) were stained for enzyme activity as described in Materials and Methods; benzaldehyde (4 mM) was the substrate and NAD (4 mM) was the cofactor.

gradient gel electrophoresis actually estimate relative molecular volumes (Stokes radii) and thus, for any given molecule, are accurate only to the extent that the molecular volume accurately reflects the molecular weight, the apparent molecular weight that we obtained for the MCF-7/OAP enzyme suggests that it may be a trimer when in its native form.

Temperature and pH optimums for catalytic activity are shown in Figs. 13 and 14, respectively. Each of the enzymes was optimally active at 37°. Energy of activation values, as determined by Arrhenius plots, were 15 and 14 kcals/mol for the MCF-7/OAP and stomach mucosa enzymes, respectively. Catalysis of benzaldehyde oxidation by the MCF-7/OAP enzyme (as well as that of aldophosphamide—data not shown) was optimum in the pH range approximately 8.0–8.5. Catalysis of benzaldehyde oxidation by the stomach mucosa enzyme was optimum over a slightly broader pH range, viz, approximately 8.0–9.0.

The MCF-7/OAP and stomach mucosa enzymes were each heat labile with the latter being slightly more so (Fig. 15). Crucial to the determination of meaningful kinetic constants, *vide infra*, full enzyme activity was retained by both enzymes for at least 10 min when they were exposed to a temperature of 37°. Retained enzyme activity as a function of storage (4°; 24 hr) pH is shown in Fig. 16. The MCF-7/OAP enzyme was optimally stable in the pH range 6.5–7.5. The stomach mucosa enzyme was optimally stable in the pH range 7.5–9.5.

The ability of the purified MCF-7/OAP enzyme to catalyze the oxidation of benzaldehyde was completely lost within 15 days when it was kept at 4° in Buffer B, pH 7.5, ± 10% glycerol, whereas about 25% of the catalytic activity remained when the stomach mucosa enzyme was kept under identical conditions for 3 months (data not presented). Full catalytic activity was retained for at least 6 months by both enzymes when they were placed in Buffer B, pH 7.5, and stored frozen at −20° (data not presented). However, freezing and thawing of the MCF-7/OAP enzyme more than once caused a complete loss of catalytic activity whereas the stomach mucosa enzyme retained approximately

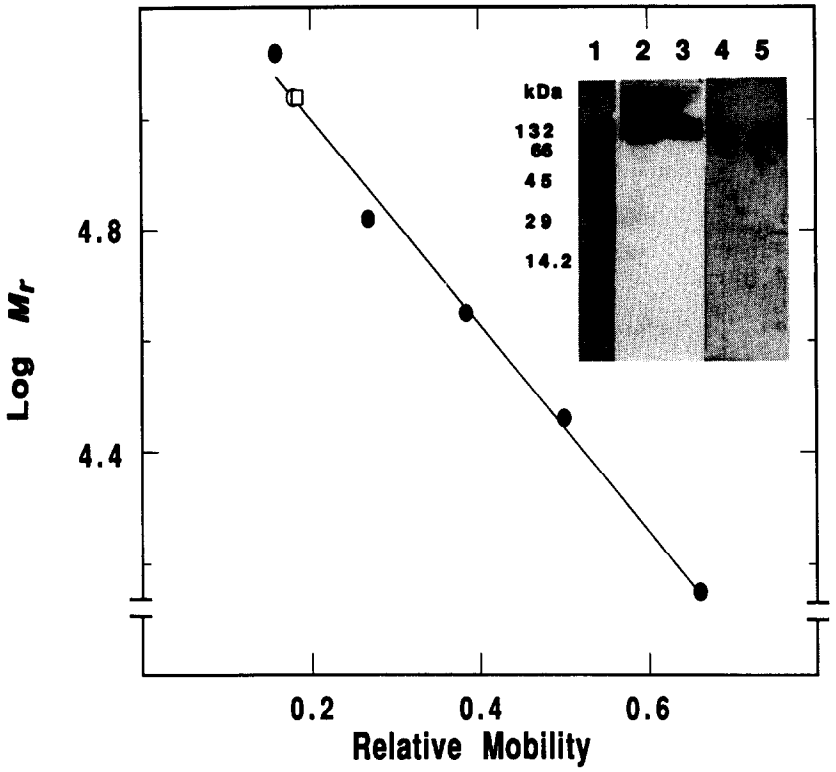

Fig. 8. Molecular weight determination of class 3 aldehyde dehydrogenases by non-denaturing linear gradient PAGE. Native molecular weights of the purified enzymes were determined as described in Materials and Methods using 5–20% non-denaturing linear gradient polyacrylamide gels. Molecular weight markers (●) were lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA monomer (66 kDa) and BSA dimer (132 kDa). Estimates of MCF-7/OAP (□) and stomach mucosa (○) aldehyde dehydrogenase molecular weights were made from the plot shown. Inset: Photograph of the original, developed gel. Lanes 1 (marker proteins), 4 (stomach mucosa aldehyde dehydrogenase) and 5 (MCF-7/OAP aldehyde dehydrogenase) were visualized for proteins by staining with Coomassie Brilliant Blue R-250. Lane 2 (stomach mucosa aldehyde dehydrogenase) and lane 3 (MCF-7/OAP aldehyde dehydrogenase) were stained for enzyme activity as described in Materials and Methods. Placed on gels were 10 μg of each purified protein.

Table 5. Kinetic properties of purified class 3 aldehyde dehydrogenases originally present in human MCF-7/OAP cells and stomach mucosa*

Substrate (mM)	Cofactor	MCF-7/OAP K_m (μM)	MCF-7/OAP V_{max} or v (mIU/mg)	MCF-7/OAP V_{max}/K_m (mIU/mg/μM)	Stomach mucosa K_m (μM)	Stomach mucosa V_{max} (mIU/mg)	Stomach mucosa V_{max}/K_m (mIU/mg/μM)
Benzaldehyde	NAD	640	16,900†	26	505	32,000	63
(0.1–4.0)	NADP	640	29,600†	46	486	51,190	105
4-Pyridinecarboxaldehyde	NAD	91	5,333†	59	190	21,250	112
(0.02–4.0)	NADP	91	8,886†	98	190	49,019	258
Octanal	NAD	67	7,207†	108	113	15,226	135
(0.01–0.5)	NADP	67	9,195†	137	104	19,545	188
Propionaldehyde	NAD	ND‡	545§		19,060	16,388	0.9
(4.0–60)	NADP	ND	573§		19,000	23,290	1.2
Acetaldehyde	NAD	ND	229§		80,000	20,500	0.3
(4.0–200)	NADP	ND	0§		81,000	26,660	0.3
Aldophosphamide	NAD	640	573†	0.9		0	
(0.16–1.12)	NADP		0†			0	

* Kinetic constants were determined as described in Materials and Methods. Stock purified enzyme preparations were in Buffer B and were added to the reaction mixture in a volume of 100 μL. NAD and NADP concentrations were 1.0 and 4.0 mM, respectively. Each value is the mean of three determinations.

† V_{max}.

‡ ND: not determined.

§ v; the substrate concentration was 4.0 mM.

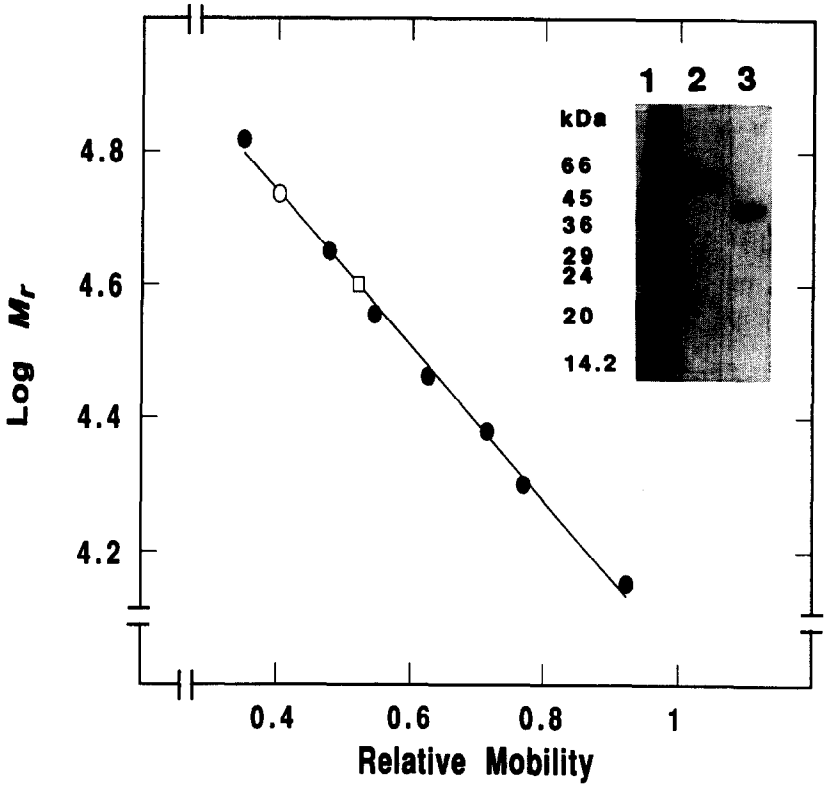

Fig. 9. Subunit molecular weight determination of class 3 aldehyde dehydrogenases by SDS–PAGE. Subunit molecular weights of the purified enzymes were determined as described in Materials and Methods. Molecular weight markers (●) were lactalbumin (14.2 kDa), trypsin inhibitor (20 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa) and BSA monomer (66 kDa). Estimates of MCF-7/OAP (□) and stomach mucosa (○) aldehyde dehydrogenase subunit molecular weights were made from the plot shown. Inset: Photograph of the original, developed gel. Proteins were visualized by staining with Coomassie Brilliant Blue R-250. Lane 1, marker proteins; lane 2, stomach mucosa aldehyde dehydrogenase (25 μg); and lane 3, MCF-7/OAP aldehyde dehydrogenase (25 μg).

40% of its catalytic activity even after being frozen and thawed eight times (data not presented). Regardless of the storage conditions and the amount of enzyme activity lost, new enzyme activity bands were not observed with either enzyme after isoelectric focusing (data not presented).

The MCF-7/OAP and stomach mucosa enzymes each catalyzed the oxidation of a variety of aliphatic and aromatic aldehydes (Table 5). In each case, short-chain aliphatic aldehydes were, as compared with long-chain aliphatic and aromatic aldehydes, poor substrates. The stomach enzyme did not catalyze the oxidation of aldophosphamide to carboxyphosphamide, but, with all other substrates, the specific activity of this enzyme exceeded that of the MCF-7/OAP enzyme even though K_m values

Table 6. Cofactor preferences of purified class 3 aldehyde dehydrogenases originally present in human MCF-7/OAP cells and stomach mucosa*

	MCF-7/OAP			Stomach mucosa		
Cofactor (mM)	K_m (μM)	V_{max} (mIU/mg)	V_{max}/K_m (mIU/mg/μM)	K_m (μM)	V_{max} (mIU/mg)	V_{max}/K_m (mIU/mg/μM)
NAD (0.02–1.0)	550	22,297	41	54	26,594	492
NADP (0.1–4.0)	940	37,133	40	1,000	80,160	80

* Kinetic constants were determined as described in Materials and Methods. Stock purified enzyme preparations were in Buffer B and were added to the reaction mixture in a volume of 100 μL. Benzaldehyde (4 mM) was the substrate. Each value is the mean of three determinations.

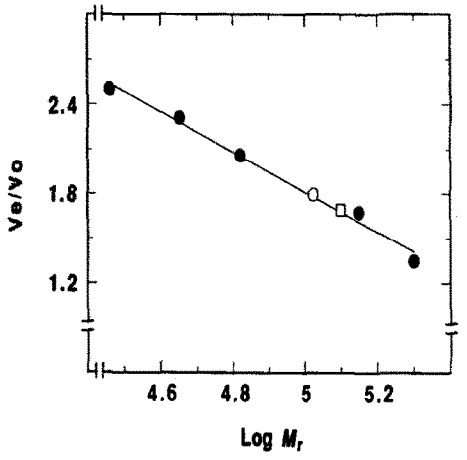

Fig. 10. Molecular weight determination of class 3 aldehyde dehydrogenases by gel permeation chromatography on Sephacryl S-200. Native molecular weights of the two enzymes were determined by gel permeation chromatography as described in Materials and Methods. Molecular weight markers (●) were carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA monomer (66 kDa), alcohol dehydrogenase (141 kDa) and β-amylase (200 kDa). Marker proteins and aldehyde dehydrogenases (100 μg of each) were placed on the gel and elution was monitored at 280 nm. Elution of MCF-7/OAP (□) and stomach mucosa (○) aldehyde dehydrogenases was also monitored by measuring enzyme activity in the eluate fractions; substrate and cofactor were benzaldehyde (4 mM) and NADP (4 mM), respectively. V_e/V_0 = elution volume/void volume.

Fig. 11. Isoelectric focusing and immunoblot visualization of native stomach mucosa and MCF-7/OAP class 3 aldehyde dehydrogenases with anti-stomach mucosa ALDH-3 IgY. Anti-stomach mucosa ALDH-3 IgY was generated and used as described in Materials and Methods to visualize the purified stomach mucosa (lane 1) and MCF-7/OAP (lane 2) enzymes after they had been electrophoresed under non-denaturing conditions and electrotransferred onto Immobilon-PVDF transfer membranes. Placed on gels were 10 μg of each purified enzyme.

did not differ appreciably. The K_m value that we obtained for NAD-dependent stomach mucosa enzyme-catalyzed oxidation of benzaldehyde is similar to those reported by others [26, 27]. The MCF-7/OAP enzyme did catalyze the oxidation of aldophosphamide to carboxyphosphamide, albeit relatively poorly and then only when NAD was used as the cofactor. Glutamic-γ-semialdehyde (500 μM), succinic semialdehyde (100 μM), betaine aldehyde (100 μM), glyceraldehyde 3-phosphate (10 μM) and γ-aminobutyraldehyde (100 μM) were not substrates for either enzyme (data not presented).

As judged by K_m values, each of the enzymes preferred NAD as cofactor (Table 6). The K_m values reported herein for NAD and NADP (stomach mucosa enzyme-catalyzed oxidation of benzaldehyde) are not markedly different from those reported by others [26, 27]. Striking was the low K_m value, viz. 54 μM, obtained for NAD-dependent catalysis of benzaldehyde by the stomach mucosa enzyme as compared to that, viz. 550 μM, obtained for NAD-dependent catalysis of this substrate by the MCF-7/OAP enzyme. Cofactor inhibition was observed in the case of the MCF-7/OAP enzyme when the NAD concentration exceeded 1.0 mM. In contrast, cofactor "activation" was observed in the case of stomach mucosa enzyme when this concentration of NAD was exceeded (Fig. 17). High concentrations of NADP did not inhibit or "activate" either enzyme.

Each of the enzymes also exhibited esterolytic activity (Table 7). In each case, catalysis of *p*-nitrophenyl acetate hydrolysis was enhanced by 20 μM NAD and partially inhibited by 100 μM NAD. Inhibition of rat liver cytosol class 3 aldehyde dehydrogenase-catalyzed hydrolysis of *p*-nitrophenyl acetate by 100 μM NAD has been reported previously [28]. However, these investigators did not observe enhancement or inhibition of the reaction by 20 μM NAD. In contrast, Takahashi and Weiner [16] observed an enhancement of horse liver class 2 aldehyde dehydrogenase-catalyzed hydrolysis of *p*-nitrophenyl acetate by concentrations of NAD ranging from 120 to 200 μM.

The influence of agents known to enhance or inhibit the catalytic activity of various aldehyde dehydrogenases, on the ability of the two enzymes purified in this investigation to catalyze the oxidation of benzaldehyde is summarized in Table 8. Takahashi and Weiner [29] have shown that Mg^{2+} (250 and 500 μM) enhances the catalytic activity of a horse liver class 2 aldehyde dehydrogenase. Others have reported that these concentrations of Mg^{2+} do not enhance the catalytic activity of either a rat class 3

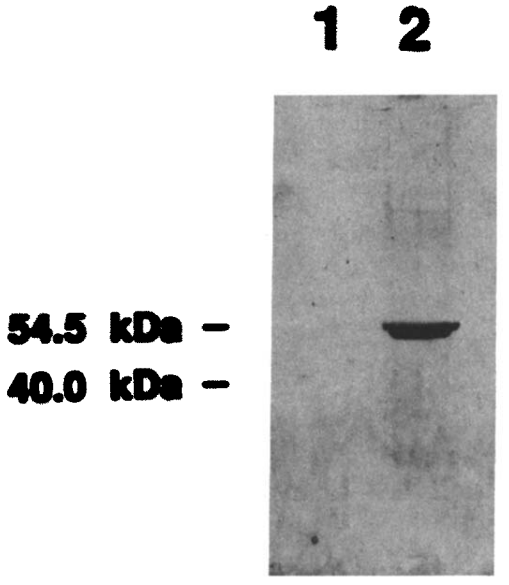

Fig. 12. SDS–PAGE and attempted immunoblot visualization of denatured stomach mucosa and MCF-7/OAP class 3 aldehyde dehydrogenases with anti-stomach mucosa ALDH-3 IgY. Anti-stomach mucosa ALDH-3 IgY was generated and used as described in Materials and Methods in an attempt to visualize the purified MCF-7/OAP (lane 1) and stomach mucosa (lane 2) enzymes after they had been electrophoresed under denaturing conditions and electrotransferred onto Immobilon-PVDF transfer membranes. Placed on the gels were 25 μg of each purified enzyme.

aldehyde dehydrogenase [28] or human stomach mucosa ALDH-3 [26]. In the present investigation, Mg^{2+} (250 or 500 μM) did not enhance the catalytic activity of either the human stomach mucosa, or the MCF-7/OAP, enzyme. Both enzymes were inhibited by *p*-chloromercuribenzoate; enzymes with a cysteine

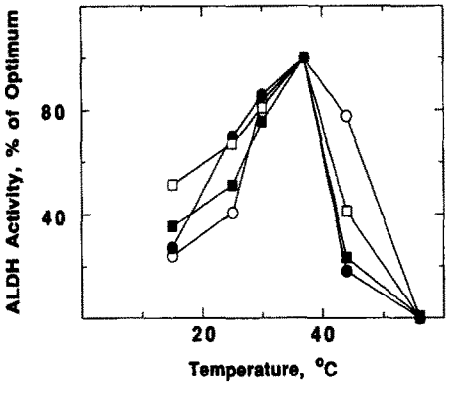

Fig. 13. Effect of temperature on the catalytic activity of purified stomach mucosa and MCF-7/OAP class 3 aldehyde dehydrogenases. Enzyme activity was determined as described in Materials and Methods using benzaldehyde (4 mM) as the substrate and NAD (1 mM) or NADP (4 mM) as cofactor. Optimal rates were at 37° and were 31 (NAD, ■) and 62 (NADP, □) nmol/min for stomach ALDH-3, and 2.7 (NAD, ●) and 5.9 (NADP, ○) nmol/min for MCF-7/OAP ALDH-3.

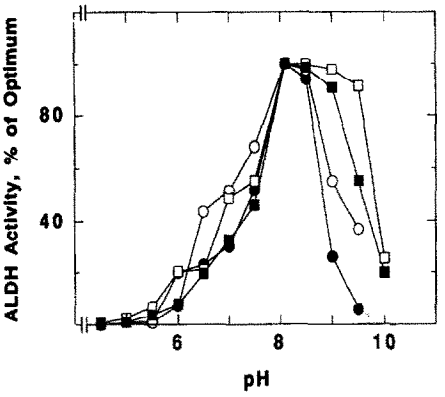

Fig. 14. Effect of pH on the catalytic activity of purified stomach mucosa and MCF-7/OAP class 3 aldehyde dehydrogenases. Enzyme activity was determined as described in Materials and Methods using buffers of different pH, benzaldehyde (4 mM) as substrate, and NAD (1 mM) or NADP (4 mM) as cofactor. Buffers were 32 mM sodium acetate (pH 4.5 to 5.5), 32 mM sodium pyrophosphate (pH 6.0 to 8.1) and 32 mM Tris–HCl (pH 8.5 to 10). Optimal rates were at pH 8.1 and were 33 (NAD, ■) and 62 (NADP, □) nmol/min for stomach ALDH-3, and 4.3 (NAD, ●) and 8.4 (NADP, ○) nmol/min for MCF-7/OAP ALDH-3.

residue at the catalytic site are typically sensitive to inhibition by this agent. The MCF-7/OAP enzyme was unaffected by 25 or 50 μM disulfiram; the stomach enzyme was inhibited slightly by these concentrations of disulfiram. Neither enzyme was affected by chloral hydrate.

ALDH-3 was also found in normal breast tissue obtained from either pre- or post-menopausal women (Fig. 18). In agreement with the findings of others [4], it was also detected in lung tissue but not in liver, kidney or placenta. The normal breast enzyme isoelectric-focused as did the MCF-7/OAP enzyme; the lung enzyme isoelectric-focused as did the stomach mucosa enzyme.

Quantitatively, and as expected given the foregoing observations, the ability of normal tissue to catalyze the oxidation of benzaldehyde was highest in stomach mucosa and was relatively high in lung (Table 9). It was, however, even higher in the malignant MCF-7/OAP cells. Assuming that 1×10^9 cells weigh 1 g, it can be calculated from the data presented in Table 2 that NAD- and NADP-dependent aldehyde dehydrogenase-catalyzed oxidation of benzaldehyde occurred at rates of 11,000 and 25,400 mIU/g, respectively. This type of a calculation reveals that, of the tissues examined, MCF-7/OAP cells were second only to the liver in being able to catalyze the oxidation of aldophosphamide to carboxyphosphamide, the calculated rate for MCF-7/OAP cells being 280 mIU/g.

DISCUSSION

The data reported herein clearly demonstrated that *associated* with the oxazaphosphorine-specific acquired resistance exhibited by the MCF-7/OAP cell line is a markedly elevated level of ALDH-3,

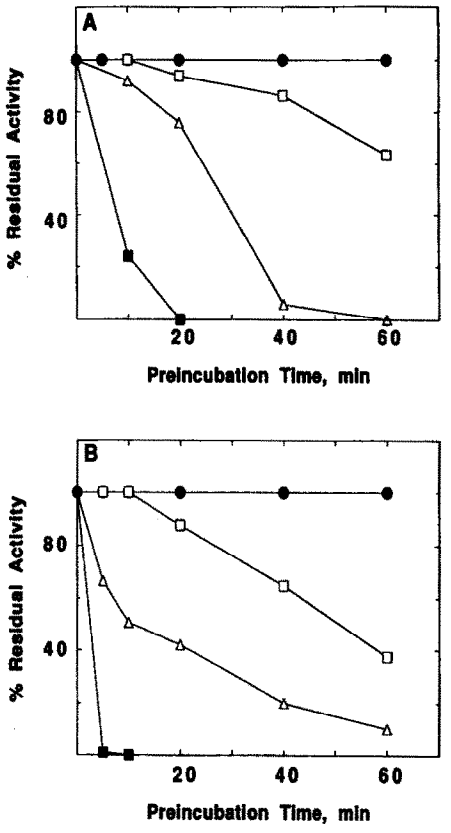

Fig. 15. Thermal stability of purified stomach mucosa and MCF-7/OAP class 3 aldehyde dehydrogenases. Enzymes were placed in Buffer B and were preincubated at 25° (●), 37° (□), 44° (△) or 56°(■) for the time periods indicated. At the end of the preincubation period, enzyme suspensions were rapidly cooled in an ice-bath, and enzyme activity was determined as described in Materials and Methods using benzaldehyde (4 mM) as substrate and NADP (4 mM) as cofactor. Control rates were 70 and 6.5 nmol/min for the stomach mucosa and MCF-7/OAP enzymes, respectively. Panel A, the MCF-7/OAP enzyme. Panel B, the stomach mucosa enzyme.

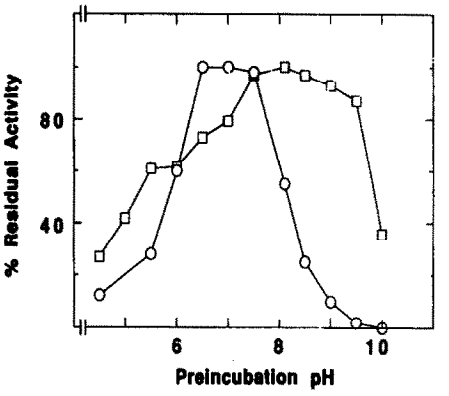

Fig. 16. Effect of pH on the stability of purified stomach mucosa and MCF-7/OAP class 3 aldehyde dehydrogenases. The purified stomach mucosa (□) and MCF-7/OAP (○) enzymes were preincubated at 4° for 24 hr in buffers adjusted to pHs ranging from 4.5 to 10. Buffers were 32 mM sodium acetate (pH 4.5 to 5.5), 32 mM sodium pyrophosphate (pH 6.0 to 8.1) and 32 mM Tris–HCl (pH 8.5 to 10). At the end of the 24-hr preincubation, pHs were readjusted to 8.1, and enzyme activity was determined as described in Materials and Methods using benzaldehyde (4 mM) as substrate and NADP (4 mM) as cofactor. Control rates were 52 and 7 nmol/min for the stomach mucosa and MCF-7/OAP enzymes, respectively.

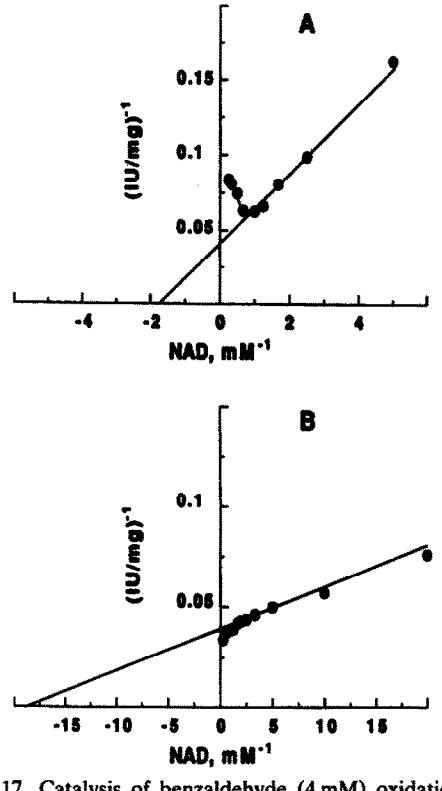

Fig. 17. Catalysis of benzaldehyde (4 mM) oxidation by purified MCF-7/OAP (A), and human stomach mucosa (B), ALDH-3 as a function of NAD concentration: Lineweaver–Burk kinetic analysis. Initial rates were determined as described in Materials and Methods. K_m and V_{max} values were calculated from x- and y-intercepts, respectively, and are given in Table 6. Each point is the mean of triplicate determinations.

and, in aggregate, led us to conclude that whereas the MCF-7/OAP enzyme, as well as the MCF-7/0 and normal breast counterparts, is a cytosolic class 3 aldehyde dehydrogenase, it is somewhat different from the previously characterized stomach mucosa ALDH-3 and hence a novel ALDH-3. Thus, the breast enzyme is referred to hereafter in this paper as Type-2 ALDH-3 to distinguish it from the stomach mucosa enzyme referred to hereafter in this paper as Type-1 ALDH-3. The molecular basis for the overexpression of Type-2 ALDH-3 by MCF-7/OAP cells is not known but is being investigated currently in our laboratory.

Type-2 ALDH-3 catalyzed the oxidation of aldophosphamide, albeit poorly, whereas Type-1 ALDH-3 did not. AHD-4, the class 3 aldehyde dehydrogenase found in the cytosol of mouse stomach mucosa may be, somewhat unexpectedly, the mouse homolog of human breast Type-2 ALDH-

Table 7. Esterase activity of purified class 3 aldehyde dehydrogenases present in human MCF-7/OAP cells and stomach mucosa*

NAD (μM)	v (mIU/mg)	
	MCF-7/OAP	Stomach mucosa
0	3,350†	9,800†
20	4,500	11,000
100	2,500	8,500

* The rate at which purified class 3 aldehyde dehydrogenases catalyzed the hydrolysis of *p*-nitrophenyl acetate (500 μM) to *p*-nitrophenol was determined as described in Materials and Methods. Stock purified enzyme preparations were in Buffer B and were added to the reaction mixture (final volume = 3 mL) in a volume of 200 μL. Each value is the mean of three determinations, each made in duplicate.

† NADP-dependent (4 mM) MCF-7/OAP and stomach mucosa ALDH-3-catalyzed oxidation of benzaldehyde (V_{max} values, Table 5) was about nine and five times faster, respectively.

3 rather than of human stomach mucosa Type-1 ALDH-3 since it also catalyzes the oxidation of aldophosphamide [2]. Mouse hepatic AHD-7 has also been putatively identified as a cytosolic class 3 aldehyde dehydrogenase [2, 30]. It does not catalyze the oxidation of aldophosphamide and may be the mouse homolog of human Type-1 ALDH-3.

Given that aldophosphamide is a relatively poor substrate for Type-2 ALDH-3, the notion that increased Type-2 ALDH-3-catalyzed oxidation of aldophosphamide accounts for the oxazaphosphorine-specific acquired resistance exhibited by the MCF-7/OAP cell line remains highly problematical. Perhaps Type-2 ALDH-3 accounts for this resistance in some other, as yet unidentified or even anticipated, way. Strongly supporting the notion that Type-2 ALDH-3 in some way accounts for the oxazaphosphorine-specific acquired resistance exhibited by MCF-7/OAP cells are the observations that inclusion of aldehydes that are good substrates for this enzyme, viz. benzaldehyde, 4-pyridinecarboxaldehyde or octanal, in the drug-exposure medium largely restored the sensitivity of the MCF-7/OAP cells to mafosfamide whereas inclusion of a poor substrate, viz. acetaldehyde, did not, that inclusion of benzaldehyde, 4-pyridinecarboxaldehyde or octanal in the drug-exposure medium only slightly increased the sensitivity of MCF-7/0 cells to mafosfamide, and that sensitivity to phosphoramide mustard on the part of either MCF-7/0 or MCF-7/OAP cells was largely unaffected by any of these aldehydes.*

It follows that Type-1 ALDH-3 may also be an important determinant of cellular sensitivity to the

* Sladek NE and Sreerama L, Manuscript in preparation.

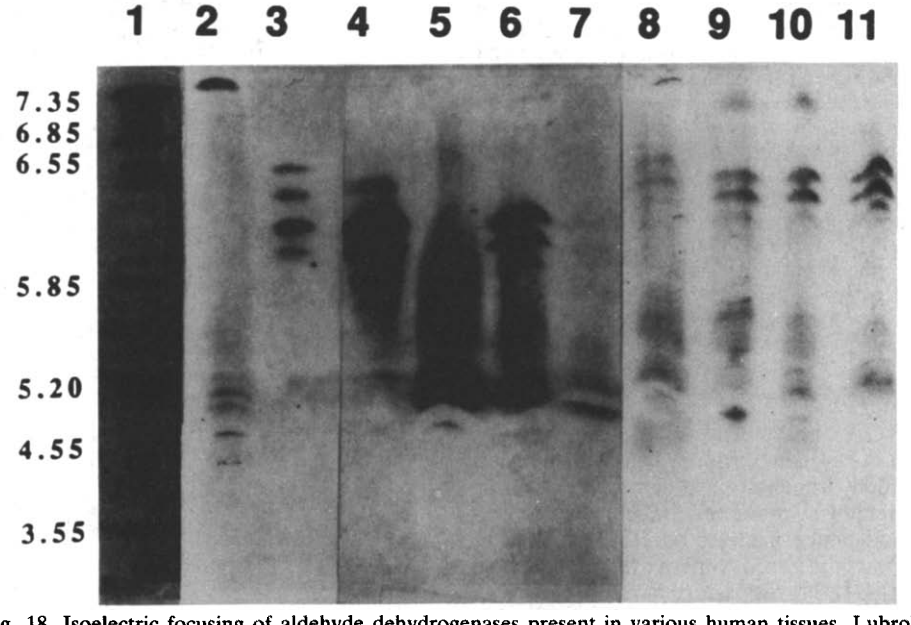

Fig. 18. Isoelectric focusing of aldehyde dehydrogenases present in various human tissues. Lubrol®-treated whole homogenates of liver, kidney, and 2.5×10^5 MCF-7/OAP cells, and soluble (105,000 *g* supernatant) fractions of stomach mucosa, lung, placenta and 1 g of normal breast, were electrofocused as described in Materials and Methods. Placed on the gel were the amounts of tissue preparation indicated above or an amount of tissue preparation sufficient to generate 5.0 nmol NADH/min when 4 mM acetaldehyde (liver, placenta) or 4 mM benzaldehyde (kidney, stomach mucosa, lung) was used as the substrate. Lane 1, pI standards; lane 2, liver; lane 3, MCF-7/OAP cells; lane 4, stomach mucosa; lane 5, kidney; lane 6, lung; lane 7, placenta; lane 8, breast from a 61-year-old female; lane 9, breast from a 59-year-old female; lane 10, breast from a 51-year-old female; and lane 11, breast from a 19-year-old female. Lane 1 was stained for protein with Coomassie Brilliant Blue R-250, and lanes 2–11 were stained for aldehyde dehydrogenase activity using benzaldehyde (4 mM) as substrate and NAD (4 mM) as cofactor, as described in Materials and Methods.

Table 8. Influence of various effectors on the catalytic activity of human class 3 aldehyde dehydrogenases isolated from MCF-7/OAP cells and stomach mucosa*

Effector	Cofactor	Concentration of effector (μM)	% Control aldehyde dehydrogenase activity	
			MCF-7/OAP	Stomach mucosa
Disulfiram	NAD	25	98	75
		50	99	70
	NADP	25	100	80
		50	98	70
p-Chloromercuribenzoate	NAD	10	87	89
		25	4	7
	NADP	10	75	91
		25	2	3
Chloral hydrate	NAD	50	99	100
		1000	96	93
	NADP	50	100	98
		1000	95	96
Mg^{2+}	NAD	250	96	100
		500	90	99
	NADP	250	87	98
		500	87	89

* Purified enzymes were transferred from Buffer B into 25 mM sodium phosphate buffer, pH 7.5, with the aid of a PD-10 column prior to assay, and aldehyde dehydrogenase activities were measured as described in Materials and Methods except that the complete reaction mixture, minus the substrate, was preincubated at 37° for 5 min. Benzaldehyde (4 mM) was the substrate and NAD (1 mM) or NADP (4 mM) was the cofactor. Glutathione was omitted from the reaction mixture when disulfiram, *p*-chloromercuribenzoate and chloral hydrate were tested; EDTA was left out when Mg^{2+} was tested. Control rates ranged from 5.0 to 5.5 (NAD) and 8.5 to 9.0 (NADP) nmol/min for MCF-7/OAP aldehyde dehydrogenase, and from 26 to 27 (NAD) and 58 to 59 (NADP) nmol/min for stomach mucosa aldehyde dehydrogenase. Values are the means of triplicate determinations.

oxazaphosphorines although this possibility is even more remote since Type-1 ALDH-3 does not catalyze the oxidation of aldophosphamide, and an inverse relationship between Type-1 ALDH-3 levels and cellular sensitivity to oxazaphosphorines has yet to be reported. Nevertheless, the idea is attractive, especially since the Type-1 enzyme may have a much wider tissue distribution than does the Type-2 enzyme [4]. Indeed, at this time, the possibility that the Type-2 enzyme is a tissue-specific, viz. breast, enzyme cannot be dismissed. Relevant also is that Type-1 ALDH-3 is not found in all normal tissue nor is it found in all malignant tissue [4, 25]. Whether cells that ordinarily do not express ALDH-3 (even at low levels) can be caused to do so on a permanent basis by exposing them to a mutagen such as one

Table 9. Aldehyde dehydrogenase activity in selected human tissues*

Substrate (mM)	Cofactor (4 mM)	Aldehyde dehydrogenase activity (mIU/g tissue)					
		Breast	Stomach mucosa	Lung	Placenta	Kidney	Liver
Benzaldehyde	NAD	24	4615	1495	8	360	610
(4.0)	NADP	6	8750	3270	1	34	567
Acetaldehyde	NAD	68	2406	407	26	197	6642
(4.0)	NADP	0	2150	344	0	0	1417
Aldophosphamide	NAD	36	63	44	11	52	1295
(0.16)	NADP	0	0	0	0	0	ND†
Glutamic-γ-semialdehyde	NAD	0	0	0	0	1880	701
(0.5)	NADP	0	0	0	0	0	ND

* Preparation of soluble (105,000 *g* supernatant) fractions from breast, stomach mucosa, lung and placenta, and Lubrol®-treated whole homogenates of kidney and liver, and determination of the rate at which these preparations catalyzed the oxidation of various aldehydes to their corresponding acids were as described in Materials and Methods. Except in the case of breast tissue, each value is the mean of duplicate determinations made three different times on the same tissue sample. The mean of duplicate determinations made once on each of four tissue samples is reported for breast tissue.

† ND: not determined.

of the oxazaphosphorines, also remains to be determined.

Seemingly at odds with any suggestion that increases in ALDH-3 account for decreases in sensitivity to the oxazaphosphorines is the lack of any inverse correlation between sensitivity to the oxazaphosphorines on the part of several hepatoma cell lines and expression of a cytosolic class 3 aldehyde dehydrogenase by these cell lines reported by Lin and Lindahl [31]. A shortcoming of these experiments in that regard, however, is that the relative sensitivity of each of these cell lines to non-oxazaphosphorine cross-linking agents was not determined so the values quantifying oxazaphosphorine-sensitivity could not be normalized for nonspecific determinants of cellular sensitivity to the oxazaphosphorines, i.e. values quantifying sensitivity to the oxazaphosphorines were influenced by both oxazaphosphorine-specific, e.g. metabolism, and -nonspecific, e.g. nonspecific binding sites, determinants of sensitivity.

The foregoing deliberations are potentially of substantial clinical significance in view of the extensive use of cyclophosphamide as part of the therapeutic protocol in the treatment of breast cancer and the unacceptably high relapse (resistance) rate that neoplasms treated with such protocols exhibit.

Multiple bands were observed when either Type-1 or Type-2 ALDH-3 was isoelectric-focused; pI values for three of the bands obtained with the Type-2 enzyme were identical with the pI values, viz. 6.0, 6.25 and 6.35, obtained with the Type-1 enzyme. However, one of the bands (pI = 6.45) seen with the Type-2 enzyme was not seen with the Type-1 enzyme, and two of the bands (pI = 5.75 and 5.85) seen with the Type-1 enzyme were not seen with the Type-2 enzyme. Moreover, the relative amounts of the common bands differed substantially.

It has been established that a single gene codes for the synthesis of human stomach mucosa ALDH-3 [32]. This gene is found in chromosome 17 [33], is about 8 kb in length, and is interspersed with 10 exons. Whether the polymorphism exhibited by human stomach mucosa ALDH-3 is due to transcriptional, translational, and/or *in vivo* or *in vitro* post-translational events, e.g. proteolytic digestion [34], is unknown. Similarly, the basis for the unique polymorphism exhibited by the MCF-7/OAP enzyme is unknown. The presence of a protease inhibitor during enzyme purification did not alter the isoelectric focusing banding patterns of either enzyme; "new" bands of catalytic activity did not appear when either of the two enzymes was stored under conditions that allowed for some loss of gross catalytic activity.

ALDH-3 is one of at least six "drug-metabolizing enzymes" that are coded for by genes that are members of the so-called polycyclic aromatic hydrocarbon-responsive gene battery [25]. The others are cytochrome P450s IA1 and IA2, glutathione *S*-transferase, NAD(P)H:menadione oxidoreductase (DT-diaphorase) and UDP-glucuronyl transferase. Activation of the gene battery is believed to involve a series of receptor-mediated events initiated by the binding of an appropriate agonist (ligand) to a cytosolic protein termed the Ah receptor [25]. Known agonists for the Ah receptor include the polycyclic aromatic hydrocarbons, 3-methylcholanthrene, benzpyrene, 9,10-dimethyl-1,2-benzanthracene and, especially, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [25]. One or more of these agents have been shown to induce ALDH-3 in rat and human hepatocytes and hepatoma cells [35–37]. Moreover, polycyclic aromatic hydrocarbons are known to induce cytochrome P450 IA1 in MCF-7 cells [38]. Thus, it seemed likely that these agents would also induce the expression of ALDH-3 in these cells. This expectation has been realized [39].

An endogenous substrate for ALDH-3 has yet to be identified and its biological role is unknown. Perhaps its *raison d'etre* is to detoxify xenobiotics. Supporting this notion are the observations that tissues which come in direct contact with xenobiotics present in food, water and air, e.g. stomach mucosa, intestinal mucosa and lungs, contain large amounts of this enzyme [4]. Apparently inconsistent with this notion is the high level of ALDH-3 in the cornea although a similar role, viz. protection against UV-light-induced cellular damage, has been proposed for ALDH-3 in this tissue [40]. Alternatively, ALDH-3 levels, as well as the levels of other products of the Ah receptor/agonist-activated gene battery, may be high in tissues that constitute "ports of entry" because they come in direct contact with relevant inducing agents that are present in the environment. The elevated ALDH-3 levels may then serve to protect such cells from any further damage by these agents. Along these lines, Yin *et al.* [41] reported that ALDH-3 could not be found in 56% of lung tissue samples obtained from Chinese adults, but low levels were found in some (34%) and relatively high levels were found in the remainder (10%). Attractive is the notion that lung tissue ALDH-3 levels reflect the exposure of this tissue to polycyclic aromatic hydrocarbons, e.g. those in cigarette smoke.

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