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A CHEMICAL AND ENZYMOLOGICAL ACCOUNT OF THE MULTIPLE FORMS OF HUMAN LIVER ALDEHYDE DEHYDROGENASE

IMPLICATIONS FOR ETHNIC DIFFERENCES IN ALCOHOL METABOLISM

GRAHAM LLOYD JONES * and Y.-S. TENG

Department of Human Biology, The John Curtin School of Medical Research, P.O. Box 334, Canberra City, ACT 2601 (Australia)

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Three major low-p*I* zones of aldehyde dehydrogenase (aldehyde: NAD^+ oxidoreductase, EC 1.2.1.3) may be visualized with specific histochemical staining after starch gel electrophoresis at pH 7.4 of Caucasian human liver extracts, whereas about 50% of Chinese human liver extracts show only two such zones. The three zones of activity were purified to apparent homogeneity from Caucasian liver. The substrate specificity of each form was investigated by double reciprocal plots using 13 aldehydes of various chemistries. The acetaldehyde-preferring isozyme I lacking in 50% of Chinese livers had a slightly lower native and subunit molecular weight than the 'universal' isozymes IIa and IIb. All forms were highly sensitive to disulfiram inhibition. This inhibition could be protected against, or reversed, by dithiothreitol. 2,2′-Dithiodipyridine was a slower inhibitor of isoenzyme I. All three purified forms of the enzyme, as well as crude extracts of normal and isozyme I-deficient Chinese livers, showed positive immunoreactivity to antibodies prepared in rabbits against type I enzyme. Tryptic peptide maps of forms IIa and IIb were almost identical, whereas that of form I, although showing some similarities, was clearly different. These results provide a consistent explanation for the acetaldehyde-mediated extreme sensitivity to moderate alcohol ingestion shown normally by about 50% of oriental subjects and during disulfiram (Antabuse) therapy by all subjects.

Introduction

Studies in animal and human tissues have established that the first product of ethyl alcohol metabolism, acetaldehyde, is metabolized mainly by NAD^+-dependent aldehyde dehydrogenases (EC 1.2.1.3), which occur in highest concentration in the liver [1,2]. Aldehyde dehydrogenase was first partially purified from human liver separately by Kraemer and Dietrich [2] and Blair and Bodley [4].

In 1977 Greenfield and Pietruszko [5] purified two human liver isozymes, E_1 and E_2, to homogeneity using AMP-Sepharose affinity chromatography, and E_2 was shown subsequently [6] to be the same enzyme previously described separately by Kraemer and Dietrich and Blair and Bodley. Tryptic peptide maps and cyanogen bromide digests suggest that the E_1 and E_2 isozymes differ in their primary structure [7]. E_2 has been reported to be a low-K_m isozyme which is relatively insensitive to disulfiram inhibition, whereas E_1 has a higher K_m and is highly sensitive to disulfiram inhibition [5]. Although reliable subcellular fractionation studies in combination with electrophoretic analysis have not been performed for the human en-

* To whom correspondence should be sent at (present address): Department of Human Biology, Faculty of Medicine, The University of Papua New Guinea, P.O. Box 5623, Boroko, Papua New Guinea.

zyme, the isoenzyme E_2 may be analogous to the mitochondrial enzyme of the horse, whereas E_1 may be analogous to the cytoplasmic horse enzyme [8].

Recently electrophoretic studies have indicated a widespread lack of a low-p*I* form of aldehyde dehydrogenase in liver samples from vaious oriental populations [9–13]. Studies on hair root lysates [14] have shown that the lack of the low-p*I* isozyme is directly associated with distinctive flushing reactions and greatly elevated blood acetaldehyde levels in these individuals after a single dose of alcohol [13]. Based on the low K_m for acetaldehyde, the native molecular weight of 235000 and the apparent insensitivity to disulfiram, Harada et al. [11] claim identity between their partially purified isoenzyme ALDH I and the enzyme referred to by Greenfield and Pietrusko as E_2 or just 2. Harada et al. [11] have shown that 50% of oriental liver samples lack this most acidic (p*I* = 4.7–4.8) ALDH I, whereas the other more basic forms of the enzyme (ALDH II, III, IV) are universal in distribution.

Given the apparent importance of this enzyme in determining ethnic [9–13] and individual differences in reactions to alcohol which may be involved in a predisposition or otherwise to alcoholism [15], we saw the necessity for a thorough re-investigation of the isoenzyme status of purified human liver aldehyde dehydrogenase. Using standardized conditions throughout and by extending the range of enzymological, chemical and immunological criteria for comparisons between the individual forms, we have arrived at a meaningful biochemical explanation for the observed physiological differences in acetaldehyde metabolism.

Materials and Methods

Small pieces of Chinese liver were obtained at autopsy from the General Hospital, Kuala Lumpur, Malaysia, and Caucasian liver from the Royal Canberra Hospital, Canberra, Australia, quick-frozen in solid CO_2 and stored at −20°C until needed. For the large-scale purification, an entire healthy liver was removed with permission at about 6 h post mortem from a 35-year-old male Caucasian who had died from gunshot wounds to the head, and stored frozen at −20°C for 2 weeks before the enzyme purification. Crude liver homogenates were prepared by grinding one part tissue with two parts buffer A, which was used in the initial stages of the purification. The homogenate was centrifuged and the clear supernatant used for electrophoresis.

Formaldehyde, acetaldehyde and furfuraldehyde were obtained from BDH (Poole, U.K.), *n*-butyraldehyde from Ajax Chemicals (Sydney, Australia) and propionaldehyde and isobutyl aldehyde from Fluka, A.G. (Switzerland). NAD, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide, phenazine methosulphate, disulfiram, 2,2′-dithiodipyridine and the other aldehydes were obtained from Sigma Chemical Co. (St. Louis, MO). Hydrolysed starch was from Connaught Diagnostics (Willowdale, Canada).

Electrophoresis. Horizontal starch gel electrophoresis was carried out with cooling for 16 h at 5 V/cm using a bridge buffer consisting of 0.1 M Tris/0.1 M maleic anhydride/0.01 M KCl/0.01 M EDTA at pH 7.4. The gel buffer was a 1/15 dilution of the bridge buffer. After electrophoresis the gels were sliced and stained with 30 ml of a 1% agar overlay containing 20 mM acetaldehyde or propionaldehyde, 10 mg NAD^+, 16 mg of the above tetrazolium bromide, 4 mg phenazine methosulphate, 30 mg sodium pyruvate buffered with 0.05 M Tris-HCl at pH 9.0. The gels were incubated at 37°C until the blue formazan bands formed.

Gradipore electrophoresis was carried out using a commercial gel (Townsend and Mercer Co., Sydney, Australia) with an acrylamide concentration from 4 to 30%. The electrophoresis buffer was Tris/boric acid/EDTA, pH 8.3. The gel was prerun for 30 min before application of sample and then run at 250 V for 5 h. After electrophoresis gels were stained for activity using the same cocktail as for the starch gel overlay without the agar, and for protein using 0.2% Coomassie blue in methanol/acetic acid/water (5 : 1 : 5).

SDS-acrylamide electrophoresis was performed on samples denatured in SDS/mercaptoethanol/glycerol in vertical slabs using a 4% stacking gel and a 12% running gel essentially according to O'Farrell [16]. Gels were run at 25 mA for 3 h, fixed in 10% trichloroacetic acid, stained with 0.2%

Coomassie blue in ethanol/acetic acid/water (5 : 2 : 13) and destained in the same solution without the Coomassie blue.

Activity assay. The routine assay mixture for enzyme purification contained 5 mM acetaldehyde or propionaldehyde, 2 mM NAD^+ and 0.1 M Tris-HCl at pH 9.0. The reduction to $NADH_2$ was followed at 340 nm on a Varian Superscan 3 recording spectrophotometer. For the determination of Michaelis-Menten constants for various aldehydes the NAD^+ concentration was held constant at 2 mM and aldehyde concentrations varied from 1 μM to as high as solubilities would permit. The aldehydes not readily soluble in water were dissolved in acetone. The inhibitor disulfiram was also dissolved in acetone. In all cases blank cuvettes had an equal volume of acetone added. One I.U. of enzyme activity is defined as that amount producing 1 μmol $NADH_2$ per min. Activity was also measured in a 0.1 M sodium glycine buffer at pH 9.5 and 0.1 M sodium phosphate buffer at pH 7.0.

Protein assay. Protein was assayed according to the method of Lowry et al. [17] using bovine serum albumin as standard.

Enzyme purification. 500 g of liver were sliced into small slivers while still frozen and homogenized with 1 litre of chilled 30 mM sodium phosphate, pH 6.0, containing 0.1% mercaptoethanol/1 mM EDTA/1 mM ϵ-aminocaproic acid as protease inhibitor (buffer A). After centrifugation and overnight dialysis against buffer A, 400 ml of packed swollen CM-Sephadex (pre-equilibrated against buffer A) were added to the supernatant and the mixture was stirred for 5 h at 4°C. The mixture was filtered at the pump. The aldehyde dehydrogenase activity was recovered in the filtrate. 400 ml of packed pre-equilibrated (buffer A) DEAE-cellulose were now added to the CM-Sephadex filtrate, the mixture was stirred for 5 h and filtered. The aldehyde dehydrogenase activity was again recovered in the filtrate. Ammonium sulphate was now added to the DEAE-cellulose filtrate. Both 50% and 65% pellets were redissolved, dialysed against buffer A and applied separately to an AMP-Sepharose column. The affinity column was washed successively with buffer A, with buffer A raised to pH 7.6 with NaOH, with buffer A raised to pH 7.6 and 50 mM KCl, with buffer A raised to pH 7.6 and 100 mM KCl and, finally, with buffer A raised to pH 7.6, 100 mM KCl and 2 mM $NADH_2$. Enzyme activity was eluted in the final two washes, separately pooled, concentrated in an Amicon unit and dialysed against buffer A.

The three concentrated dialysed pools of activity from the AMP-Sepharose column were further purified by column isoelectric focussing using 1% pH 4–6 ampholines. Focussing was allowed to proceed for 50 h at 1200 V. Active fractions were pooled, dialysed against buffer A and stored at 4°C with 10% glycerol added. Prior to peptide mapping, samples of the isoelectrically resolved isozymes I, IIa and IIb were chromatographed in 0.5% ammonium bicarbonate on a 60×2.5 cm column of Bio-Gel A-1.5.

Effects of disulfiram and 2,2′-dithiodipyridine. NAD^+ (2 mM) and acetaldehyde or propionaldehyde (5 mM) were added in 0.1 M Tris-HCl buffer, pH 9.0, to a total volume of 1 ml in a 1-ml spectrophotometric cuvette. Disulfiram dissolved in acetone was added to the cuvette to a final concentration of 1–10 μM. The reaction was initiated by the addition of enzyme I at a final concentration of $3.6 \cdot 10^{-8}$ M or enzymes IIa, IIb at a final concentration of $8.5 \cdot 10^{-7}$ M. Dithiothreitol was added at 5 mM both prior to and after addition of enzyme. 2,2′-Dithiopyridine, at concentrations from 1–50 μM, was added to the cuvette prior to initiation of the reaction with enzyme I.

Immunological studies. 1 mg of purified human liver ALDH I in 0.5 ml 30 mM phosphate buffer, pH 6.0, was emulsified with an equal volume of complete Freund's adjuvant and injected at multiple sites beneath the skin on the stomach of a rabbit. This procedure was twice repeated at fortnightly intervals.

9 days after the third injection of antigen the rabbit was bled from the ear. The blood was allowed to clot and the antisera were collected by centrifugation.

Double diffusion analysis [19] was performed in wells cut in 1% agar gels made up with phosphate-buffered saline (pH 7.4).

Peptide mapping. Heat denaturation, proteolytic digestion and peptide mapping techniques were adaptations of those reported by Katz et al. [20] and Chernoff and Liu [21]. Prior to the chemical

studies the purified separated heteromorphs of human liver aldehyde dehydrogenase were dialysed extensively against 0.5% ammonium bicarbonate, pH 8.0, and concentrated (Amicon UM-10) to approx. 5 mg/ml.

These enzyme solutions were denatured by heating at 100°C for 3–4 min. The denatured protein precipitated and was washed twice with 0.5% ammonium bicarbonate before being suspended in 1 ml of the same buffer and digested with 2% (w/w) trypsin at 37°C for 3 h. After this digestion the insoluble fraction (tryptic 'core') was removed by centrifugation and washed twice with ammonium bicarbonate. The soluble digest and washings were combined and lyophilized.

About 3-mg amounts of the lyophilized soluble tryptic peptides of isoenzymes I, IIa and IIb were taken up in a minimum of buffer and applied to Whatman 3MM paper. Electrophoresis was performed in pyridine/acetic acid/water (25 : 25 : 950, v/v), pH 4.7, at 40 V/cm for 90 min. The strips containing the peptides were sewn into fresh sheets of Whatman 3MM paper and ascending chromatography was performed overnight in the second dimension using *n*-butanol/acetic acid/water/pyridine (15 : 3 : 12 : 10, v/v). The sheets were dried and the peptides containing tryptophan were evident when viewed under ultraviolet light, due to their natural fluorescence. The majority of peptides were stained using Fluram (2 mg/litre acetone containing 0.05% pyridine, ultraviolet visualization) or, for photographic purposes, ninhydrin (0.02% in acetone).

Results

Purification of three forms of human liver aldehyde dehydrogenase

The results of a typical purification are shown in Table I. We found that the level of aldehyde dehydrogenase varied considerably from one liver to another. We only proceeded with the purification if the activity was greater than 15 I.U.$^{-3}$/mg protein. In preliminary experiments we established that the forms of the enzyme were differentially precipitated by ammonium sulphate and this has been exploited in the purification. The activity precipitating at 50% saturation was shown by starch gel electrophoresis to be mainly isoenzyme I with some form IIa, whereas most of the activity in the 50–65% cut is IIb type. As previously demonstrated [5], the key to the purification lies in the AMP-Sepharose step. All forms of the enzyme bind to the resin at pH 6.0 but, as shown by Fig. 1, the forms do elute differentially and, once again, this has been exploited in the purification procedure.

Most of the activity in the 50% ammonium

TABLE I

PURIFICATION OF HUMAN LIVER ALDEHYDE DEHYDROGENASES

AS, ammonium sulphate.

	Volume (ml)	Total protein (g)	Total units I.U.$^{-3}$ (Acetaldehyde)	Total units I.U.$^{-3}$ (Propionaldehyde)	Spec. act. (acet.) (I.U.$^{-3}$/mg)	Spec act. (propion.) (I.U.$^{-3}$/mg)
1. 30 mM phosphate pH 6.0 extract (2 : 1)	1175	25.25	750942	604655	21.30	17.15
2. CM-Sephadex pH 6.0 batch eluate	1500	9.0	771900	610050	85.80	67.8
3. DEAE-cellulose pH 6.0 batch eluate	1500	4.5	560250	570000	124.5	126.6
4. Ammonium sulphate 50% saturation	150	1.5	242775	240285	162.0	160.2
5. Ammonium sulphate 50–65% cut	75	2.25	14469	24500	6.43	10.9
6. AMP-Sepharose						
(i) KCl eluate 50% AS	8.5	0.144	164516	115162	1144	801
(ii) $NADH_2$ eluate 50% AS	3.5	0.068	872	–	12.8	–
(iii) $NADH_2$ eluate 50–65% AS	4.5	0.043	11576	15555	269	361
7. Isoelectric focussing						
(i) KCl eluate 50% AS	8.0	0.064	128617	70740	1610	1105
(ii) $NADH_2$ eluate 50% AS	3.5	0.005	535	731	107	146
(iii) $NADH_2$ eluate 50–65% AS	4.0	0.008	965	1222	121	153

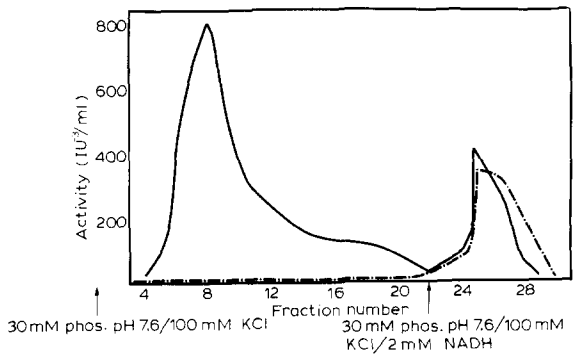

Fig. 1. Elution profile of aldehyde dehydrogenase from AMP-Sepharose affinity column. ———, profile for 50% ammonium sulphate precipitate; ·–·–·; profile for 65% ammonium sulphate precipitate of 50% supernatant. The vertical arrows show the points of addition of the different eluting buffers.

sulphate pellet is eluted by 30 mM sodium phosphate (pH 7.6) 100 mM KCl, as shown by the first vertical arrow in Fig. 1. This material was shown by starch gel electrophoresis to be isozyme I and Table I shows that this material has a very high activity towards acetaldehyde. Some activity is also eluted by the 30 mM sodium phosphate (pH 6.0)/100 mM KCl/2 mM $NADH_2$ buffer. This is IIa type enzyme and proved to be rather unstable. With the 50–65% ammonium sulphate pellet all activity is recovered in the NADH wash. This activity proved to be IIb type enzyme and, as shown by Table I, shows a preference for propionaldehyde as substrate.

Fig. 2(a) shows the column isoelectric focussing profile of the KCl AMP-Sepharose eluate of the 50% ammonium sulphate pellet, whereas Fig. 2(b) shows the profile for the $NADH_2$ eluate of the 50–65% ammonium sulphate cut. Substantial further purification was clearly effected in this step. The isoelectric points derived from these profiles and a similar one for the $NADH_2$ eluate of the 50% ammonium sulphate pellet are presented in Table II. Active fractions from the isoelectric focussing were concentrated and dialysed against buffer A. Table I shows that although isoenzyme I is recovered in good yield, isoenzyme IIb proved to be quite unstable, perhaps due to its extreme lability in the absence of thiol reagent.

We found that isozyme I, but not IIa or IIb, also bound to Cibachrome blue-Sepharose, and we were able to purify this isoenzyme by an alternate route using this resin in place of AMP-Sepharose.

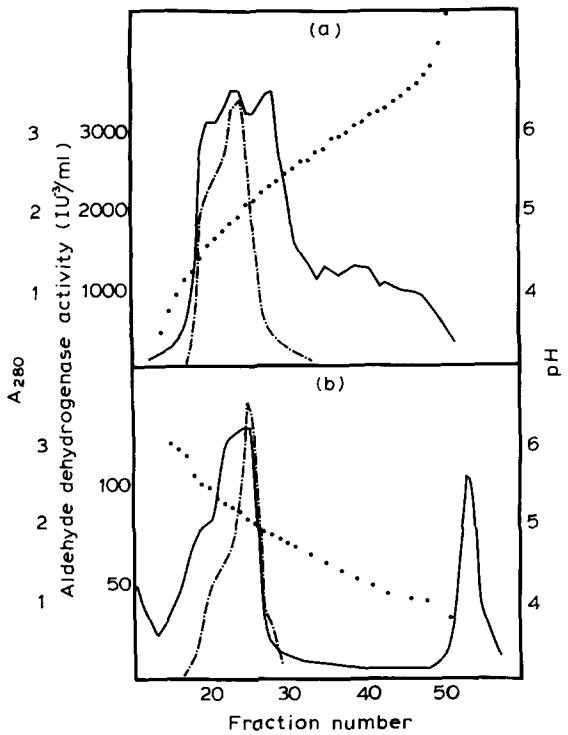

Fig. 2. Column isoelectric focussing profile of pools of aldehyde dehydrogenase activity differentially eluted from AMP-Sepharose column. The conditions for the separation are given in the methods section. ———, A_{280}; ·–·–·; activity; ●●●●, pH. (a) isoenzyme I; (b) isoenzyme IIb.

Starch gel electrophoresis of purified and crude extracts of human liver aldehyde dehydrogenase

Fig. 3 shows the separation of aldehyde dehydrogenase after starch gel electrophoresis of vari-

TABLE II

COMPARATIVE PHYSICOCHEMICAL PROPERTIES OF HUMAN LIVER ALDEHYDE DEHYDROGENASES

Native molecular weights were obtained by extrapolation from a graph of log molecular weight vs. R_F value on gradipore gel electrophoresis of standard molecular weight marker proteins as shown in Fig. 5. Subunit molecular weights were obtained by extrapolation from a graph of log molecular weight vs. R_F value for standard marker proteins on SDS denaturing electrophoresis as shown in Fig. 6. Isoelectric points were obtained by column isoelectric focussing as shown in Fig. 2.

Isozyme	Native M_r	Subunit M_r	p*I*
Enzyme I	235000	51000	4.9
Enzyme IIa	248000	53000	5.05
Enzyme IIb	248000	53000	5.15

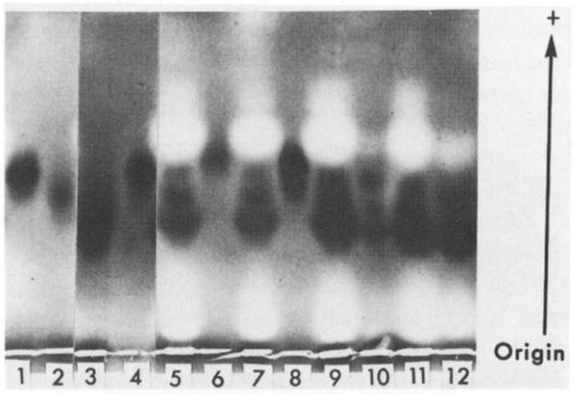

Fig. 3. Starch gel electrophoresis of human liver aldehyde dehydrogenases. Conditions for the separation and histochemical stain are noted in the methods section. 1, 2 and 3, purified isoenzymes I, IIa and IIb, respectively, from isoelectric focussing; 4, as in 1, different preparation; 5, crude extract of Caucasian liver; 6, purified isoenzyme I from Cibachrome blue-Sepharose; 7, as in 5, different individual; 8, KCl eluate of AMP-Sepharose column; 9, as in 5 and 7, different individual; 10, supernatant after batch absorption with CM-Sephadex and DEAE-cellulose; 11, as in 5, 7 and 9, different individual; 12, 65% ammonium sulphate precipitate of 50% supernatant.

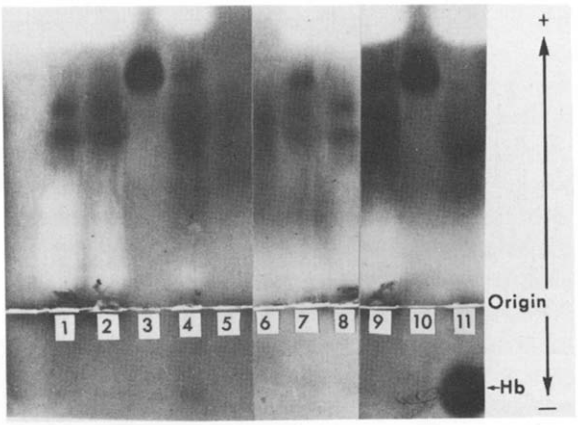

Fig. 4. Starch gel electrophoretic comparison of crude extracts of different Chinese aldehyde dehydrogenase phenotypes with purified aldehyde dehydrogenase from Caucasian liver. Conditions for separation and histochemical stain are noted in the methods section. 1 and 2, 'deficient' phenotype Chinese liver; 3, purified isoenzyme I; 4, 'non-deficient' phenotype Chinese liver; 5 and 6, purified isoenzymes IIa and IIb, respectively; 7, as in 4; 8, as in 1 and 2; 9, crude extract of Caucasian liver used in large-scale purification; 10, as in 3; 11, crude haemolysate.

ous purified and crude activity fractions. Slots 1 and 4 show that the purified low-p*I* (4.9) enzyme I is the most anodal of the three major forms seen in crude liver extracts from various individual Caucasian livers (slots 5, 7, 9, 11). Slot 8 shows the pattern obtained for the KCl eluate of the AMP-Sepharose resin before isoelectric focussing. Slot 6 shows the enzyme I purified by Cibachrome blue-Sepharose affinity chromatography. The separated purified isozymes IIa and IIb are shown in slots 2 and 3. Slot 10 shows the pattern obtained after the initial CM-Sephadex and DEAE-cellulose batch absorptions. Slot 12 shows, as previously mentioned, that the 50–65% ammonium sulphate cut contains isoenzyme IIb activity.

In order to demonstrate the relationship between purified forms of the enzyme from Caucasian livers and the widespread 'deficiency' polymorphism in Chinese liver [9–11], our purified fractions were subjected to electrophoresis on the same gel as crude samples of various Chinese and Caucasian livers, and the results are shown in Fig. 4. Slots 1, 2 and 8 show the pattern of aldehyde dehydrogenase forms in these individual Chinese 'deficient'-type liver samples, whereas slots 4 and 7 show the pattern in 'non-deficient'-type Chinese liver samples. In a survey of more than 100 separate Chinese livers about 50% showed the deficient-type two-banded phenotype shown in slots 1, 2 and 8, whereas the remainder showed the three-banded phenotype shown in slots 4 and 7. All Caucasian liver samples showed the three-banded pattern shown in slot 9. Slots 3 and 10 show the purified isoenzymes I. Clearly it is the low-p*I* highly active enzyme I which is missing in the deficient samples. Human erythrocyte lysates (slot 11) show predominantly IIb activity with some IIa activity but no detectable type I activity. Recently a survey of 930 individual haemolysates representing all major human population groups has shown that this pattern of erythrocyte aldehyde dehydrogenase is invariant (Jones and Pate, unpublished data).

Native molecular weight of the different aldehyde dehydrogenase forms

Fig. 5 shows the pattern of aldehyde dehydrogenase (Fig. 5a) and protein (Fig. 5b) obtained after gradipore electrohoresis. Crude extracts of deficient-type Chinese livers (Fig. 5a, slot 4) show

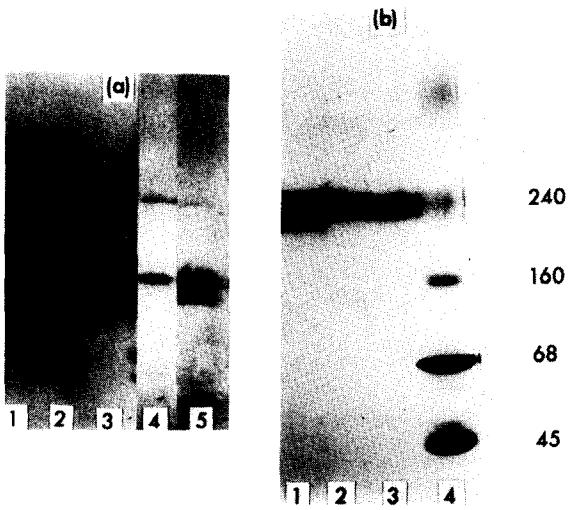

Fig. 5. Gradipore electrophoresis of purified and crude liver aldehyde dehydrogenase. Conditions for the separation and histochemical stain are noted in the methods section. (a) Stained for aldehyde dehydrogenase activity. 1, 2 and 3, purified isoenzymes IIa, IIb and I, respectively; 4, crude extract of 'deficient' Chinese liver; 5, crude extract of 'non-deficient' Chinese liver; (b) Stained for protein. 1, 2 and 3, purified isoenzymes I, IIb and IIa, respectively; 4, molecular weight standards (catalase, 240000; aldolase, 160000; bovine serum albumin, 68000; ovalbumin, 45000).

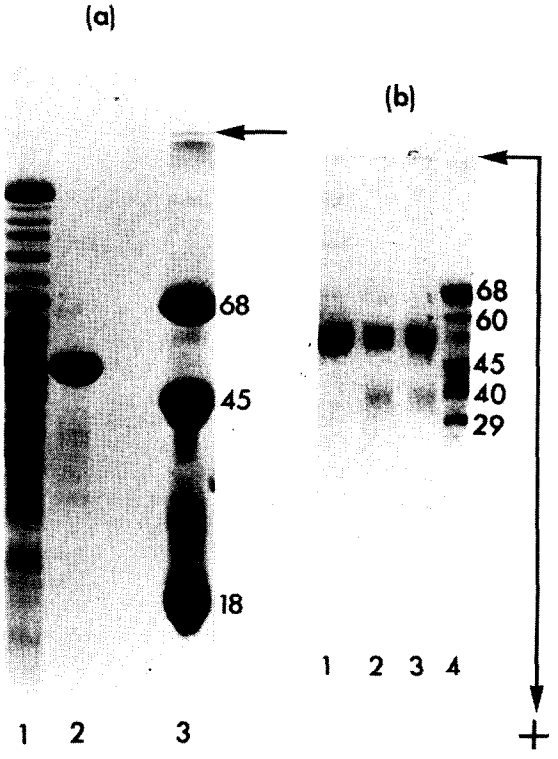

Fig. 6. SDS denaturing electrophoresis of aldehyde dehydrogenase. Conditions for the separation and staining are noted in the methods section. The horizontal arrows show the interface between stacking and running gels. (a) 1, ammonium sulphate precipitate before application to AMP-Sepharose; 2, concentrated KCl eluate from AMP-Sepharose; 3, molecular weight standards. (b) 1, 2 and 3, purified isoenzymes I, IIa and IIb, respectively; 4, molecular weight standards (bovine serum albumin, 68000; catalase subunit, 60000; ovalbumin 45000; aldolase subunit 40000; carbonic anhydrase I, 29000).

only one zone of activity, corresponding to the zone shown by purified aldehyde dehydrogenase IIb (Fig 5a, slot 2), whereas crude extracts of 'non-deficient'-type Chinese livers (Fig. 5a, slot 5) show two zones of activity; one of which corresponds to the purified isoenzyme IIb and the purified isoenzyme I (Fig. 5a, slot 3). The protein stain reveals that all preparations are fairly pure, although Fig. 5b, slot 1 shows that the purified isoenzyme I is slightly contaminated with IIa or b. Clearly, isoenzyme I has a lower molecular weight than IIa or IIb (Fig. 5b, slots 3 and 2, respectively). In slot 4 molecular weight standards have been run alongside the purified aldehyde dehydrogenase fractions and allow the extrapolation of the native molecular weights. These are presented in Table II.

Subunit molecular weights of the different forms of aldehyde dehydrogenase

Fig. 6(b) shows the pattern obtained after SDS electrophoresis of denatured samples of the three purified isoenzyme forms. Again, the subunit molecular weight of form I (Fig. 6b, slot 1) is slightly lower than that of either IIa (slot 2) or IIb (slot 3). The minor zone at about 35000 in forms IIa and IIb is probably a degradation product, since its intensity increases with the time of storage of the enzymes. In other experiments not shown here, this zone was cut from the gel along with the major zone and subjected to in situ proteolysis (in the sample well of another gel) with the *S. aureus* V8 protease. There were no cleavage fragments which did not correspond to those generated by the major 53000 peptide. Molecular weight standards were run on the same gel (Fig. 6(b), slot 4). The subunit molecular weights of isoenzymes I, IIa and IIb obtained by extrapola-

tion are presented in Table II. The results of the native and subunit molecular weights indicate that each isozyme consists of four identical subunits and that these subunits are slightly smaller for the type I enzyme than for the IIa or IIb enzymes. Fig. 6(a) demonstrates the efficacy of the AMP-Sepharose purification of isoenzyme I. Slot I shows the ammonium sulphate pellet applied to the resin, whereas slot 2 shows the concentrated KCl eluate from the AMP-Sepharose.

Substrate specificity of the purified forms of aldehyde dehydrogenase

Table III shows a comparison of Michaelis-Menten constants for the different forms derived from double reciprocal plots of activity vs. substrate concentration for 13 different aldehydes. Using a 100 mM Tris-HCl buffer at pH 9.0 the range of K_m values for the different substrates is quite striking. The short-chain aliphatic aldehydes do not show substrate saturation until quite high concentrations are reached, whereas the aromatic aldehydes, where the aldehyde function group is directly attached to the ring, all saturate at much lower concentrations. Significantly, in the case of phenylacetaldehyde, where the aldehyde group is not directly attached to the ring, the K_m value is of the same order as that observed for the short-chain aliphatic aldehydes. With the substrates, benzaldehyde, 3,4,5-trimethoxybenzaldehyde, *p*-nitrobenzaldehyde, pyruvic aldehyde and furfuraldehyde, substrate inhibition was observed above 0.5 mM. With the exception of furfuraldehyde, this substrate inhibition was abolished by addition of 5 mM acetaldehyde to the cuvette, presumably in direct competition for the active-site region. Furfuraldehyde may well contain a contaminant which is an irreversible inhibitor of the enzyme.

The very high K_m value of 5 mM obtained for isoenzyme I with acetaldehyde as substrate using the 100 mM Tris-HCl, pH 9.0, buffer stands in stark contrast with the μmolar values reported by other authors [5,11] for what appears (from a comparison of the previously mentioned physiochemical properties) to be the same ezyme. Indeed isoenzyme I has often been called the low-K_m isozyme [5,11] to distinguish it from other forms. Other authors have used pyrophosphate buffer at pH 9.0 [5,11] and sodium glycine buffer at pH 9.5 [11]. Table IV shows the Michaelis-Menten con-

TABLE III

KINETIC PARAMETERS OF HUMAN LIVER ALDEHYDE DEHYDROGENASES WITH VARIOUS SUBSTRATES AT pH 9.0

Reactions were followed by observing the increase in A_{340} due to $NADH_2$ formation in 1-ml cuvettes. NAD^+ concentration was held constant at 2 mM and 0.1 M Tris-HCl buffer, pH 9.0, was used throughout. K_m and V_{max} values were obtained from double reciprocal plots. All assays were done in duplicate. Final enzyme concentrations: ALDH I, $3.6 \cdot 10^{-8}$ M; ALDH IIa and IIb, $8.5 \cdot 10^{-8}$ M.

Substrate	Enzyme I		Enzyme IIa		Enzyme IIb	
	K_m (mM)	V_{max} (I.U.$^{-3}$/mg)	K_m (mM)	V_{max} (I.U.$^{-3}$/mg)	K_m (mM)	V_{max} (I.U.$^{-3}$/mg)
Formaldehyde	27.0	3030	27.0	167	2.56	75
Acetaldehyde	5.0	5000	0.22	71	0.14	74
Proprionaldehyde	1.0	3334	0.40	400	0.50	444
n-Butyraldehyde	0.106	820	0.019	200	0.035	118
Isobutyraldehyde	2.10	4000	0.250	333	0.400	500
Benzaldehyde	0.031	1026	0.0028	167	0.0056	125
3,4,5-Trimethoxybenzaldehyde	0.071	1538	–	–	0.071	357
p-Nitrobenzaldehyde	0.028	1695	–	–	0.0059	118
Phenylacetaldehyde	1.49	3334	–	–	–	–
Furfuraldehyde	0.027	455	0.057	218	0.063	166
Pyruvic aldehyde	0.380	333	0.330	100	0.140	81
(±)-Glyceraldehyde	1.300	2667	0.073	128	0.270	118
Glutaraldehyde	2.500	143	0.190	65	0.160	80

TABLE IV

KINETIC PARAMETERS OF HUMAN LIVER ALDEHYDE DEHYDROGENASES WITH TWO SUBSTRATES AT pH 9.5 (GLYCINE, SODIUM SALT)

Conditions were the same as those employed in Table III except that 0.1 M sodium glycine buffer pH 9.5 was used.

Substrate	Enzyme I		Enzyme IIa		Enzyme IIb	
	K_m (mM)	V_{max} (I.U.$^{-3}$/mg)	K_m (mM)	V_{max} (I.U.$^{-3}$/mg)	K_m (mM)	V_{max} (I.U.$^{-3}$/mg)
Acetaldehyde	0.056	5024	0.230	192	0.190	217
Propionaldehyde	0.060	4823	0.108	250	0.091	238

stants obtained by double reciprocal plots of aldehyde concentration vs. reaction rate in a 100 mM sodium glycine buffer at pH 9.5. Using this buffer the K_m value for isozyme I with acetaldehyde is in the range observed by other authors [5,11] and much lower than that observed for isozyme IIa or IIb. Using a 100 mM sodium phosphate buffer at pH 7.0 a very low K_m value was again observed for form I although, at this pH, the V_{max} value is only about 5% of the value observed at pH 9.0 (Tris-HCl) or 9.5 (sodium glycine).

The most profound difference between isoenzymes I and IIa or IIb lies in the reaction rates with different substrates (Table III). Using the 100 mM Tris-HCl, pH 9.0, buffer, acetaldehyde is clearly the best substrate for form I (V_{max} = 5000 I.U.$^{-3}$/mg), whereas for forms IIa or IIb it is a very poor substrate. Isoenzyme I proved to be much more active than either IIa or IIb with all the substrates examined. In part, the extremely low activity of these two enzymes may be explained by their greater lability in the absence of reducing thiols. Table V shows that IIb is activated more than 2-fold towards acetaldehyde by addition of 5 mM dithiothreitol to the cuvette, whereas form I is not activated. Forms IIa and IIb show a remarkably similar profile of K_m and V_{max} towards the different aldehydes; both showing much greater activity towards propionaldehyde and isobutyraldehyde than towards acetaldehyde (Table III). Even allowing for the differential effects of reducing thiols it is apparent that isoenzyme I is by far the most important of the three forms in the oxidation of acetaldehyde. Isoenzyme I is also effective with formaldehyde but only at very high substrate concentration and with propionaldehyde, isobutyraldehyde, phenylacetaldehyde and (±)-glyceraldehyde at moderate concentrations. The aromatic aldehydes, including furfuraldehyde, are poorer substrates, saturating and eventually inhibiting, as mentioned previously, at quite low concentration. Pyruvic aldehyde and glutaraldehyde are extremely poor substrates by comparison with acetaldehyde for form I, whereas all appear to be equally poor substrates for forms IIa and IIb.

Inhibition by disulfiram and 2,2'-dithiodipyridine

Table V shows the effects of various concentrations of disulfiram on the activity of aldehyde

TABLE V

THE EFFECT OF DITHIOTHREITOL ON THE ACTIVITY OF HUMAN LIVER ALDEHYDE DEHYDROGENASE ISOENZYMES I AND IIb BEFORE AND AFTER DISULFIRAM INHIBITION WITH ACETALDEHYDE AS SUBSTRATE

Results are given as percentage of control (no dithiothreitol) values.

Disulfiram concentration (μM)	−Dithiothreitol		+5 mM dithiothreitol	
	Isoenzyme I	Isoenzyme IIb	Isoenzyme I	Isoenzyme IIb
Control	100	100	95	220
1.2	45	–	90	–
1.5	40	–	95	–
1.7	35	–	90	–
2.0	25	65	95	230
5.0	–	41	–	192
0.0	–	19	–	215

dehydrogenases I and IIb in the presence and absence of 5 mM dithiothreitol with acetaldehyde as substrate. Propionaldehyde was also used as substrate in similar inhibition studies. Using the Tris-HCl buffer system, isoenzyme I is clearly very sensitive to disulfiram inhibition. The inhibition is more marked with acetaldehyde as substrate (I_{50} = 0.98 μM) than with propionaldehyde (I_{50} = 4.2 μM). This result is in contrast to previous results [5,11] which claim that this isozyme is relatively insensitive to disulfiram. The enzyme is completely protected from inhibition by 5 mM dithiothreitol. In these experiments the final concentration of mercaptoethanol in the reaction mixture is 8 μM and the enzyme concentration $3.6 \cdot 10^{-8}$ M. When all mercaptoethanol is removed by dialysis and the enzyme I at $3.6 \cdot 10^{-5}$ M in 0.5% NH_4HCO_3 (pH 8.0) treated with disulfiram in the absence of

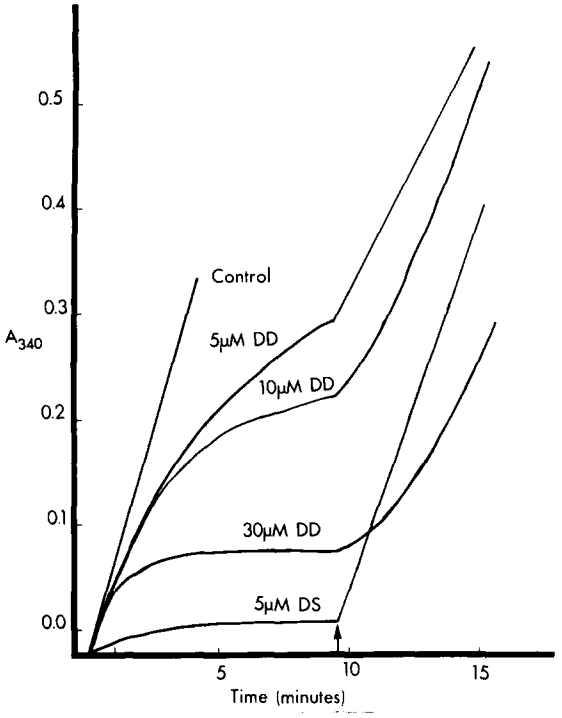

Fig. 7. Inhibition of aldehyde dehydrogenase I by disulfiram and 2,2'-dithiodipyridine. Reaction was initiated by addition of $3.6 \cdot 10^{-8}$ M isoenzyme I to the reaction cuvette containing acetaldehyde, NAD^+ and varying amounts of inhibitor buffered with 0.1 M Tris-HCl, pH 9.0. The vertical arrow shows the point of addition of 5 mM dithiothreitol. The figure is a direct trace from the spectrophotometric chart. DD, dithiodipyridine; DS, disulfiram.

NAD^+ or substrate the I_{50} value is 250 μM. A thorough investigation of the effects of pH, buffer constituents and sulphydryl status of the enzyme on the behaviour towards disulfiram is indicated, although this is beyond the scope of this paper. Under our conditions both isoenzymes I and IIb are very sensitive to disulfiram inhibition, although form I is more sensitive than form IIb when acetaldehyde is used as substrate (I_{50} of 0.98 μM and 3.6 μM, respectively) but not when propionaldehyde is used as substrate (I_{50} of 4.2 μM and 4.1 μM, respectively). It should be noted here that forms IIa and IIb both lose more than 50% of their activity per month on storage at 4°C. The activity may be regenerated by addition of 5 mM dithiothreitol.

Fig. 7 shows that 2,2-dithiodipyridine is also an inhibitor of human aldehyde dehydrogenase I, although the inhibition is not so effective or rapid as that noted with disulfiram. An earlier report shows that 2,2'-dithiodipyridine activated the sheep enzyme and protected it against inhibition by disulfiram [22].

The vertical arrow at about 9 min. (Fig. 7) shows the point of addition of 5 mM dithiothrei-

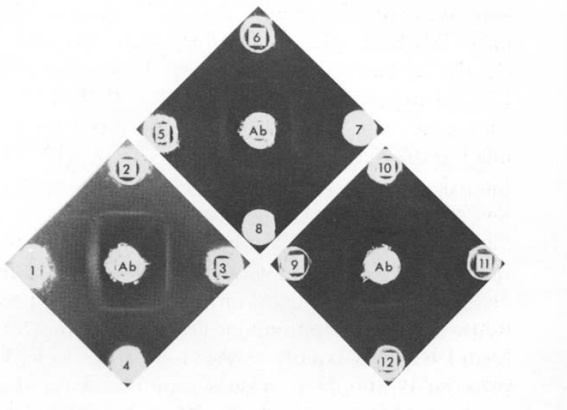

Fig. 8. Double immunodiffusion analysis of purified and crude aldehyde dehydrogenase. Ab, antibody in rabbits against the purified isoenzyme I only. 1 and 2, purified isoenzymes I and IIb, respectively; 3, Caucasian crude liver extract; 4, Chinese 'deficient'-type crude liver extract; 5, as in 1; 6, purified isoenzyme IIa; 7, 50% ammonium sulphate precipitate; 8, 65% ammonium sulphate precipitate from 50% supernatant; 9, 'non-deficient' Chinese crude liver extract; 10, 'deficient' Chinese crude liver extract; 11, as in 9; 12, as in 10.

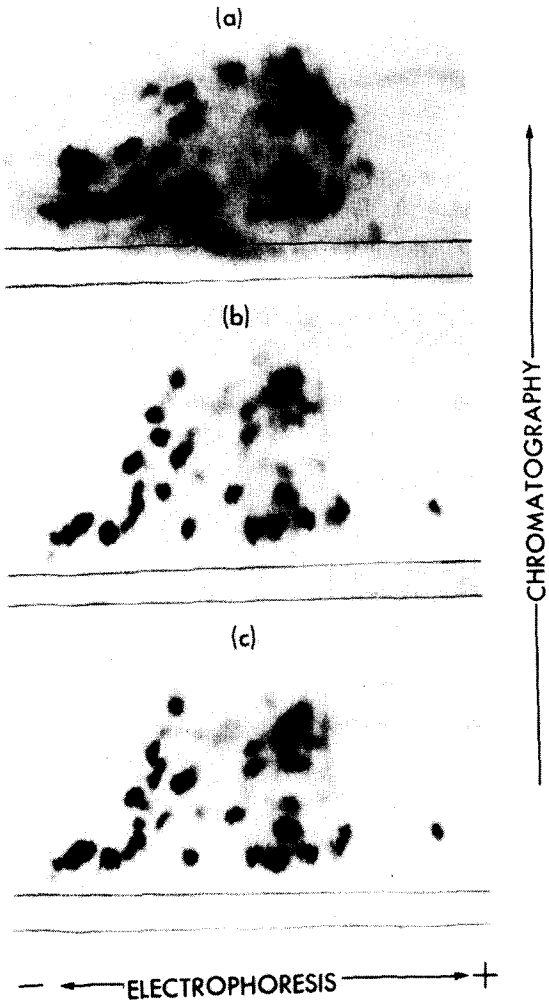

Fig. 9. Tryptic peptide maps of different forms of human liver aldehyde dehydrogenase. Conditions of electrophoresis and chromatography are noted in the methods section. (a), (b) and (c), purified isoenzymes I, IIa and IIb, respectively.

tol. As well as protecting aldehyde dehydrogenase I from disulfiram inhibition (Table V), it is also clear that this inhibition, once established, may be completely and rapidly reversed. This agrees with previous reports [23] and has significance for the mechanism of inhibition by disulfiram. In other experiments not shown here we demonstrated that the inhibition of isoenzymes IIa and IIb may also be reversed by 5 mM dithiothreitol. The inhibition of isoenzyme I noted with 2,2′-dithiodipyridine (Fig. 7) is also reversed by addition of 5 mM dithiothreitol, although not as rapidly as disulfiram inhibition.

Immunological relationships of the different aldehyde dehydrogenase forms

Fig. 8 shows that all the forms of human liver aldehyde dehydrogenase show immunological reaction to antibody prepared against isoenzyme I only. In addition, crude extracts of Chinese liver showing the deficiency of form I phenotype (wells 4, 10, 12) show a positive reaction. Although these forms of the enzyme are kinetically quite distinct they must share antigenically active structural regions.

Tryptic peptide mapping of the purified aldehyde dehydrogenase forms

Fig. 9 shows a comparison of tryptic peptide maps of the three purified forms of human liver aldehyde dehydrogenase. It is apparent that form IIa (Fig. 9(b)) and IIb (Fig. 9(c)) show patterns which are nearly identical, whereas the pattern for isoenzyme I (Fig. 9(a)), although showing some similarities, is quite distinct.

This agrees with previous results on the so-called E_1 (IIa or IIb) and E_2 (I) [7]. The near identity of the maps for the isoenzymes IIa and IIb taken in conjunction with their very similar substrate specificities (Table III) is strong evidence to suggest that they are minor modifications of the one gene product, whereas the many apparent differences seen with ALDH I and its strikingly different substrate specificity suggests that this form of the enzyme which is lacking in 50% of Chinese liver samples is, in fact, the product of a separate genetic locus.

Discussion

In recent years much emphasis has been placed on the importance of acetaldehyde metabolism in determining individual and ethnic differences in alcohol use and abuse. Though the results are somewhat clouded by methodological uncertainties, several authors have reported moderately elevated levels of acetaldehyde in alcoholics [4–26] and non-alcoholic relatives of alcoholics [26]. Although no definitive evidence is forthcoming, a good deal of indirect evidence and speculation has linked alcohol addiction with acetaldehyde-mediated formation of morphine-like tetrahydropapaveroline and salsolinol [27–29] and with modified enkephalin activities [29,31].

In this context, then, one of the most outstanding problems in alcohol metabolism is the identity of the enzyme responsible for acetaldehyde metabolism in vivo. Eckfeldt et al. [8] suggest that, in the horse, the cytoplasmic enzyme (Fl) is responsible. More recent physiological and population genetic studies have cast considerable light on the more important human situation. Oriental subjects, typed by analysis of hair root lysates as isoenzyme I-negative, show blood acetaldehyde levels about 20-fold higher than isoenzyme I-positive individuals after a single dose of ethanol [13]. There is a direct correlation between lack of isoenzyme 1, elevated blood acetaldehyde and the characteristic acetaldehyde-mediated flushing response in those individuals, although the other aldehyde dehydrogenase isozymes appear at a normal level. In isoenzyme I-positive individuals such high levels of acetaldehyde and the attendent ill-effects may be observed if alcohol is given after treatment with the alcoholic aversive drug, disulfiram (Antabuse) [32].

In this report we have detailed the properties of three purified major acid-p*I* forms of aldehyde dehydrogenase. Although in previous reports only two such isozymes have been noted [9,10], similar three-banded patterns have recently been observed by other authors [33] using the same starch gel system. Using a 100 mM Tris-HCl buffer at pH 9.0, we have shown that isoenzyme I is the most important acetaldehyde-metabolizing enzyme and furthermore that this enzyme, in contradiction with previous reports [5,11], is very sensitive to in vitro disulfiram inhibition. We have shown that, although closely related antigenically, isoenzyme I is most probably a product of a genetic locus separate from that of forms IIa and IIb, which appear to be minor modifications of the same peptide chain. The widespread lack of active acetaldehyde-preferring isoenzyme I in oriental populations, by contrast with its universal occurrence in Caucasian populations [9–13], may reflect differential socially applied selection pressures with respect to alcohol consumption in the different groups.

Our results provide a biochemical explanation for the hypersensitivity to alcohol observed in 50% of oriental populations and in all individuals undergoing disulfiram treatment. More intensive studies should resolve the disparity between these and former results [5,11]. We would also like to study the relationship between levels of form I activity and alcohol addiction. Clearly, a total lack of isoenzyme I activity militates against addiction through accumulation of unpleasantly high levels of acetaldehyde in a similar manner to disulfiram treatment [34], but a heterozygous individual may be expected to manifest a mildly elevated acetaldehyde level which, as previously mentioned, has been associated with alcoholism. Indeed, recently Agarwal et al. [35] have observed lower than normal levels of isoenzyme I in all liver specimens taken from alcoholics.

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