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Journal

Annals of human genetics, 37(1)

ISSN

0003-4800

Authors

Smith, M
Hopkinson, DA
Harris, H

Publication Date

1973-07-01

DOI

10.1111/j.1469-1809.1973.tb01814.x

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Studies on the properties of the human alcohol dehydrogenase isozymes determined by the different loci ADH_1 , ADH_2 , ADH_3

By MOYRA SMITH, D. A. HOPKINSON AND HARRY HARRIS

*M.R.C. Human Biochemical Genetics Unit, Galton Laboratory,
University College London*

INTRODUCTION

Previous studies indicate that human alcohol dehydrogenase (ADH) is determined by three separate structural gene loci (ADH_1 , ADH_2 and ADH_3) and there is evidence for genetic polymorphism at the ADH_2 and ADH_3 loci (Smith, Hopkinson & Harris, 1971, 1972). Convincing evidence has been obtained from *in vitro* 'hybridization' experiments to support the hypothesis that the human ADH isozymes are dimers and that any one particular isozyme may be a homodimer consisting of two identical polypeptide subunits or a heterodimer consisting of two non-identical subunits coded by different gene loci or different alleles at the same locus (Schenker, Teeple & von Wartburg, 1971; Jörnvall & Pietruszko, 1972; Smith, Hopkinson & Harris, 1973). It has also been found that the isozymes are of similar molecular weight but differ according to their subunit composition in their relative electrophoretic mobilities on starch gels and in their relative activities with ethanol, *n*-amyl alcohol and butanol as substrates (Smith *et al.* 1972).

The present paper describes further investigations of the substrate specificities of the human ADH isozymes determined by the three different gene loci and also the results of studies on the inhibition characteristics, pH activity profiles and *in vitro* stabilities of the various isozymes.

Previous work using crude tissue homogenates and also purified preparations indicated that human liver ADH has a broad substrate specificity and is capable of catalysing the oxidation of a wide range of alcohols in the presence of NAD and the reduction of many corresponding aldehydes in the presence of NADH (von Wartburg, Bethune & Vallee, 1964; von Wartburg, Papenberg & Aebi, 1965; von Wartburg & Papenberg, 1966; von Wartburg, 1971). Several pharmacologically active alcohols such as Ronicol (β -pyridyl carbinol) and Myanesin (toloxy-1,2-propanediol) and aldehydes such as chloral hydrate and Acetaldol (β -hydroxybutyraldehyde) have also been shown to act as substrates for human liver ADH (von Wartburg & Schürch, 1968). The effects of inhibitors such as thiourea, pyrazole, several halogen derivatives of acetaldehyde and ethanol and various metal binding agents have also been described in the previous literature, particularly in connexion with comparisons of the 'usual' and 'atypical' forms of the enzyme (von Wartburg *et al.* 1964, 1965, 1966, 1968; Blair & Vallee, 1966).

The present work was planned as an extension of these earlier studies, to take into account the recently defined electrophoretic heterogeneity and genetic polymorphism of the human ADH isozymes and also to test further the validity of the genetic hypothesis outlined above that human ADH is determined by three separate structural gene loci, ADH_1 , ADH_2 and ADH_3 .

In presenting these results the nomenclature used in the recent publication (Smith *et al.* 1973) on the subunit structure will be adhered to. The loci ADH_1 , ADH_2 and ADH_3 are said to determine the polypeptide subunits α , β and γ respectively, which associate in pairs to form the

ADH isozymes. The isozymes may be homodimers, e.g. $\alpha\alpha$ and $\beta^1\beta^1$, characteristic of the ADH_1 locus and of the 'usual' allele (ADH_2^1) at the ADH_2 locus respectively. Or the isozymes may be heterodimers, e.g. $\alpha\beta$ and $\gamma^1\gamma^2$, consisting of subunits determined by different loci or different alleles at the same locus. The principal difficulties in nomenclature arise when discussing 'atypical' ADH since, although it seems that this variation is due to an allele ADH_2^3 at the ADH_2 locus, which determines a variant polypeptide β^2 , it is not yet clear whether the characteristic ' β -isozyme' of 'atypical' liver and lung samples usually represents a mixture of two isozymes $\beta^1\beta^2$ and $\beta^2\beta^2$ or only perhaps the $\beta^1\beta^2$ isozyme. Because of the uncertainty this component of 'atypical' ADH has been referred to as the 'atypical' β -isozyme. The 'atypical' heterodimeric isozymes $\alpha\beta^2$, $\beta^2\gamma^1$ and $\beta^2\gamma^2$ can, however, usually be identified in 'atypical' liver samples and are therefore referred to as such.

MATERIALS AND METHODS

Tissue extracts. Foetal material was obtained from therapeutic abortions and infant and adult tissues were obtained at autopsy. Crude tissue extracts were prepared using the methods described in previous studies (Smith *et al.* 1971, 1972) and partially purified preparations of the individual ADH isozymes were obtained by ion-exchange chromatography on CM cellulose (Smith *et al.* 1973).

Electrophoresis and isozyme staining. Starch-gel electrophoresis was carried out at pH 8.6 (Tris-HCl buffer) or pH 7.7 (Tris-phosphate buffer) and the ADH isozymes were located in the starch gels using methods given previously (Smith *et al.* 1971, 1972). In experiments designed to compare the substrate specificity of the ADH isozymes the gels were usually cut into three slices; one was stained with ethanol (0.6%, v/v) as substrate, one was stained with the alcohol under test as substrate (0.6%, v/v) and the third slice was incubated with the same volume of staining mixture but contained no substrate. The latter control was necessary since the human ADH isozymes do exhibit some activity even in the absence of substrate. This is the so-called 'nothing dehydrogenase' reaction.

ADH isozyme activity using different aldehydes as substrate was tested by applying to the gels a mixture containing 10 mg. NADH and 0.1 ml. of the aldehyde being tested in 25 ml. 0.05 M tris-phosphate buffer, pH 7.0, together with 25 ml 2% aqueous agar. The gels were incubated at 37° C. for up to $\frac{1}{2}$ hr. and the ADH isozymes were detected in u.v. light as dark zones (due to NAD) on a fluorescent background (due to NADH). Chloral hydrate (0.05 M) was also tested as a substrate in the same way.

Assays. Spectrophotometric assays of ADH activity were carried out using ethanol as substrate by the method previously described (Smith *et al.* 1971). Assays using aldehydes as substrate were carried out at 25° C. in a Gilford spectrophotometer. The assay mixtures consisted of the sample under analysis, NADH (1.6×10^{-4} M) and phosphate buffer (3.3×10^{-2} M) at pH 6.5 together with the aldehyde being tested, usually at final concentrations between 8.3×10^{-2} and 1.6×10^{-2} M. The reaction rate was followed at 340 m μ against a blank solution containing sample, NADH and buffer but no aldehyde.

pH activity curves. Similar assay mixtures were used when pH activity curves were constructed but different buffer solutions were employed. Sodium phosphate (pH 6.0–8.0), sodium pyrophosphate (pH 8.0–9.0) and sodium glycine (pH 9.0–12.0) buffers at a final concentration

Table 1. *Relative activities of the ADH isozymes with various alcohols as substrates*(Assessments based on isozyme staining after starch-gel electrophoresis.
Substrate concentration 0.6% (v/v).)

Alcohol	M.W.	Formula	ADH locus and presumed polypeptide			
			ADH ₁ (α)	ADH ₂		ADH ₃ (γ^1, γ^2)
				'Usual' (β^1)	'Atypical' (β^2)	
Methanol	32	CH ₃ .OH	+	+	+	+
Ethanol	46	CH ₃ .CH ₂ .OH	++++	++++	++++	++++
Propanol	60	CH ₃ .CH ₂ .CH ₂ .OH	+++	++	+++	+++
Butanol	74	CH ₃ .(CH ₂) ₂ .CH ₂ .OH	++	++++	+++	+++++
Amyl	88	CH ₃ .(CH ₂) ₃ .CH ₂ .OH	++	+++	+++	+++++
Hexanol	102	CH ₃ .(CH ₂) ₄ .CH ₂ .OH	++	+++	+++	+++++
Heptanol	116	CH ₃ .(CH ₂) ₅ .CH ₂ .OH	++	+++	+++	+++++
Octanol	130	CH ₃ .(CH ₂) ₆ .CH ₂ .OH	++	++++	++++	+++++
Allyl	58	CH ₂ .CH.CH ₂ OH	++++	+++	+++	+++
Sec-propanol	60	CH ₃ .CHOH.CH ₃	+++	++	++	++
Sec-butanol	74	CH ₃ .CH ₂ .CHOH.CH ₃	+++	+++	+++	+++
Sec-amyl	88	CH ₃ .(CH ₂) ₂ .CHOH.CH ₃	++	+++	+++	+++
Sec-octanol	130	CH ₃ .(CH ₂) ₅ .CHOH.CH ₃	++	++	++	++
Tert-amyl	88	(CH ₃) ₂ .COH.CH ₂ .CH ₃	++	++	++	++
Cyclohexanol	100	C ₆ H ₁₁ .OH	+++	+++	++	++
Benzyl alcohol	108	C ₆ H ₅ .CH ₂ OH	++	+++	++	++

of 3.3×10^{-2} M were used for the assays with ethanol as substrate. Phosphate (pH 4.5–8.0) and barbitone (pH 8.0–9.0) buffers were used for the assays with acetaldehyde as substrate.

Inhibitors. The effects of inhibitors on ADH activity were investigated in two ways: qualitative analysis was carried out by adding inhibitors to the gel staining mixture and in some cases to the samples and the buffer solutions prior to electrophoresis; quantitative studies were carried out spectrophotometrically by comparing the ADH activity of samples with and without inhibitors.

In vitro stability. Assessments of the relative heat-stabilities of the ADH isozymes were obtained by either heating samples prior to or after electrophoresis using the method of Mc-Alpine, Hopkinson & Harris (1970). The relative stabilities of the isozymes to freezing and thawing or storage at 10° C. were also investigated.

RESULTS

Substrate specificity

(α) *Alcohols.* Sixteen different alcohols have been tested as substrates for the ADH isozymes after starch-gel electrophoresis. These included primary, secondary, tertiary and cyclic alcohols. The primary alcohols tested ranged from methanol through to octanol. The secondary alcohols used were *sec*-propanol, *sec*-butanol and *sec*-pentanol (secondary amyl alcohol). Tertiary amyl alcohol was tested and the cyclic alcohols included cyclohexanol and benzyl alcohol. Allyl alcohol, an unsaturated alcohol, was also used.

Each substrate was assessed by examining the activities of the ADH isozymes in liver, lung, kidney and gastro-intestinal tract homogenates from adult, new-born and foetal material. The assessments given are inevitably somewhat subjective and take into account not only the rela-

Fig. 1. Photographs of starch gels showing the relative staining intensities of the ADH isozymes in infant liver and kidneys using (a) ethanol and (b) amyl alcohol as substrate.

tive staining intensities of the isozymes but also the speed at which staining occurs. Also in some tissues, notably liver, the activities of certain isozymes, in particular the ADH_3 components, were assessed from the relative staining intensities of the 'hybrid' heterodimeric isozymes. As far as one could judge, the activities of heterodimeric isozymes such as $\alpha\gamma$ and $\beta\gamma$ were intermediate between the activities of the corresponding homodimeric isozymes but potentiation of the ADH activity of one type of subunit in combination with another subunit is a possibility which could not be excluded. The results are summarized in Table 1.

In general, the secondary and tertiary alcohols appear to be poorer substrates for ADH than the corresponding primary alcohols, but there are exceptions. For example, *sec*-propanol and *sec*-butanol are better substrates for the ADH_1 isozymes than propanol or butanol respectively and methanol is a poor substrate for all the ADH isozymes. The cyclic alcohols were found to be poorer substrates than ethanol for all of the ADH isozymes, except the ADH_1 isozymes which were equally active with ethanol and cyclohexanol.

Comparing the relative activities of the products of the three ADH loci, it was found that the $\alpha\alpha$ isozyme, determined by ADH_1 , is more active with ethanol, allyl alcohol, *sec*-propanol and cyclohexanol than with the other alcohols tested. The 'usual' β isozyme ($\beta^1\beta^1$) determined by ADH_2 , and characteristic of liver and lung samples of the 'usual' pH ratio phenotype, was more active with ethanol, butanol, octanol and *sec*-butanol than with the other alcohols tested. The $\gamma\gamma$ isozyme ($\gamma^1\gamma^1$, $\gamma^2\gamma^2$ or $\gamma^1\gamma^2$) determined by ADH_3 and also the γ -containing heterodimeric isozymes ($\alpha\gamma$ and $\beta\gamma$) were relatively more active with the longer straight-chain alcohols than with ethanol (Figs. 1, 2). This phenomenon was noted previously (Smith *et al.* 1972) and utilized in the elucidation of the ADH isozyme patterns in adult liver samples.

The 'atypical' β -isozyme characteristic of liver and lung samples of the 'atypical' pH ratio

Fig. 2. Photographs of starch gels showing the relative staining intensities of the ADH isozymes in infant and adult liver samples and gastric mucosa (ADH_3 1) using (a) ethanol and (b) butanol as substrate.

phenotype and presumed to be due to allelic variation at the ADH_2 locus was found to be most active with ethanol and octanol and was also slightly more active with propanol than the 'usual' β isozyme. When the gels obtained with 'atypical' liver samples were stained with butanol, *sec*-butanol or the cyclic alcohols, however, the 'atypical' β isozyme was much less prominent. The 'atypical' $\alpha\beta^2$ and $\beta^2\gamma^1$ or $\beta^2\gamma^2$ isozymes (depending on the ADH_3 phenotype) were also rather less prominent in their staining intensities with butanol as substrate. Fig. 3(a) shows this effect in a liver sample of the 'atypical' pH ratio phenotype, ADH_3 type 2.

The activity of the ADH isozymes was also examined using the substituted alcohol Ronicol (β -pyridylcarbinol) since von Wartburg & Schürch (1968) had previously reported rather low activity in liver samples of the 'atypical' pH-ratio phenotype using this substrate compared with ethanol. The isozymes characteristic of the 'usual' pH-ratio phenotype liver samples showed about the same activity with Ronicol as they do with ethanol and this was found to be so irrespective of the ADH_3 phenotype. The isozymes characteristic of the 'atypical' pH ratio liver samples, namely the 'atypical' β isozyme and also $\alpha\beta^2$ and $\beta^2\gamma^1$ or $\beta^2\gamma^2$ were, however, much more weakly stained with Ronicol as substrate than with ethanol (Fig. 3b).

(b) *Aldehydes*. Acetaldehyde, formaldehyde, butyraldehyde, glyceraldehyde and glyceraldehyde-3-phosphate were tested as substrates for the ADH isozymes after starch-gel electrophoresis. In each case the ADH isozyme pattern was similar to that obtained using ethanol as substrate and the relative staining intensities of the various isozymes were not noticeably different from those obtained using ethanol.

Significant differences in activity between the ADH isozymes were, however, found using chloral hydrate as substrate. The initial observations were made on the relative staining intensities of the ADH isozymes separated by starch-gel electrophoresis and were confirmed by direct assay on some partially purified preparations (Table 2). The $\alpha\alpha$ isozyme was found to exhibit relatively high activity, the 'usual' $\beta^1\beta^1$ isozyme moderate activity and the $\gamma\gamma$ isozyme

Fig. 3. Photographs of starch gels showing the relative staining intensities of the ADH isozymes in 'usual' and 'atypical' adult liver samples, both ADH_3 2 phenotype, using (a) butanol and (b) Ronicol as substrate.

Table 2. Activities of partially purified ADH isozymes with chloral hydrate (46 and 16 mM) as substrate, expressed in each case as a percentage of activity with acetaldehyde as substrate at the same concentration

Chloral hydrate concentration (mM)	ADH isozymes			
	ADH_2			ADH_3 $\gamma\gamma$
	ADH_1 $\alpha\alpha$	'Usual' $\beta^1\beta^1$	'Atypical'	
46	86	48	23	1
16	68	32	3	1

(either $\gamma^1\gamma^1$ or $\gamma^2\gamma^2$) showed virtually no activity with chloral hydrate. This aldehyde was also found to be a relatively poor substrate for the 'atypical' β isozyme, which agrees with the previous observation of von Wartburg & Schürch (1968) that chloral hydrate is reduced more slowly by the ADH from livers of the 'atypical' pH ratio than livers of the 'usual' pH ratio phenotype.

pH activity profiles

(a) *Ethanol*. The pH optimum for ethanol oxidation by the ADH in crude homogenates of adult liver samples of the 'usual' pH ratio phenotype was found to be about 11.0. The pH optimum for foetal and newborn liver extracts under identical conditions was about the same or slightly higher (Fig. 4). Assays on partially purified preparations of the ADH isozymes showed

Fig. 4. pH activity curves of ADH in homogenates of 'usual' and 'atypical' adult liver and foetal liver using ethanol as substrate.

that $\alpha\alpha$, $\alpha\beta^1$, $\beta^1\beta^1$ and $\alpha\gamma^1$ exhibited similar pH activity curves. It was not possible to obtain enough material to do pH curves on the $\alpha\gamma^2$, $\gamma^2\gamma^2$ or $\gamma^1\gamma^1$ isozymes.

The 'atypical' pH-ratio liver samples exhibited a pH optimum with ethanol at about pH 8.8, confirming the original observations of von Wartburg *et al.* (1965). Using partially purified preparations the 'atypical β ' isozyme and the 'atypical $\alpha\beta$ ' isozyme ($\alpha\beta^2$) were also found to be most active at around pH 8.8.

(b) *Acetaldehyde*. The pH activity profiles obtained using acetaldehyde as the substrate for ADH activity were more complicated than those obtained using ethanol and varied according to the type of tissue being tested, whether the sample was from an adult, a newborn or a foetus and whether the individual was of the 'usual' or 'atypical' pH ratio phenotype. Fig. 5 shows representative pH profiles.

The pH activity curves exhibited by the ADH in homogenates of foetal intestine and kidney, which consists entirely of ADH_3 isozyme ($\gamma\gamma$) activity, were almost identical. The pH optimum was about 6.5. No differences were detected among ADH_3 1, ADH_3 2-1 and ADH_3 2 phenotype samples. Extracts of lung tissue from individuals of the 'usual' pH ratio phenotype, in which the 'usual' ADH_2 isozyme ($\beta^1\beta^1$) predominates, showed a similar low pH optimum with acetaldehyde as substrate at about pH 6.0. Extracts of foetal liver in which the ADH_1 isozyme ($\alpha\alpha$) was predominant showed a different pH activity profile, however, with an optimum at about pH 8.5.

The results of experiments with partially purified preparations of the $\alpha\alpha$, $\beta^1\beta^1$ and a mixture of the $\gamma^1\gamma^1$, $\gamma^1\gamma^2$ and $\gamma^2\gamma^2$ isozymes were consistent with the results obtained using whole tissue extracts. With acetaldehyde as substrate the pH optima for $\alpha\alpha$, $\beta^1\beta^1$ and $\gamma\gamma$ were found to be about 8.0, 5.0-5.5 and 5.5-6.0 respectively. Preparations of the $\alpha\beta^1$ isozyme were found to exhibit a pH activity curve which was intermediate between the $\alpha\alpha$ and $\beta^1\beta^1$ pH curves.

Results in keeping with these observations were obtained when acetaldehyde assays were carried out with homogenates of newborn and adult liver samples of the 'usual' pH ratio phenotype (Fig. 5). In the newborn livers in which the ADH_1 and ADH_2 isozymes are about equally active and ADH_3 isozyme activity is relatively low, biphasic pH curves were obtained. One peak was at about pH 6.5 and presumably represented the $\alpha\beta^1$ and $\beta^1\beta^1$ isozyme peak activity, the other peak was at pH 8.5 and presumably represented the $\alpha\alpha$ isozyme peak activity.

Fig. 5. pH activity curves of ADH in homogenates of (a) foetal kidney, intestine, lung and liver and (b) infant and adult liver using acetaldehyde as substrate.

In adult livers, however, a single pH optimum was observed at pH 6.0, presumably a reflexion of the relatively high activity of the ADH_3 isozymes and the low activity of the ADH_1 isozymes in adult liver samples compared with the newborn.

The results obtained from acetaldehyde assays at different pHs using samples of tissue from individuals of the 'atypical' pH ratio phenotype were also interesting. Von Wartburg and his colleagues (1965) found that the pH optimum for acetaldehyde reduction by the ADH in 'usual' and 'atypical' livers was the same, about pH 6.0–6.5, and the phenotypes could not be distinguished. We confirmed this observation in our experiments with crude liver extracts – both 'usual' and 'atypical' samples were most active at pH 6.0–6.5 with acetaldehyde as substrate.

'Atypical' lung samples, however, were found to exhibit a different optimum, at pH 7.0–7.5, in contrast to the more acid pH optimum (pH 6.0) shown by 'usual' pH-ratio lung samples. These results suggest that the 'usual' and 'atypical' forms of ADH do in fact have different pH optima with acetaldehyde as substrate since the ADH in both foetal and adult lung tissue consists almost entirely of $\beta\beta$ isozymes determined by the ADH_2 locus, which is the locus at

Table 3. *Relative activities of ADH in crude homogenates and partially purified preparations with ethanol as substrate at different concentrations of thiourea*

(Activities are expressed as percentages of control assays done in absence of thiourea and are means of duplicate estimations done on crude homogenates prepared from tissues of ten different individuals and on partially purified ADH isozymes prepared from livers of three different individuals.)

Thiourea	Crude homogenates				Partially purified ADH isozymes		
	Liver		Lung		ADH_1 , $\alpha\alpha$	ADH_2	
	'Usual'	'Atypical'	'Usual'	'Atypical'		'Usual', $\beta^1\beta^1$	'Atypical'
Nil	100	100	100	100	100	100	100
0.1 M	.	.	.	.	100	112	100
0.2 M	116	81	.	.	98	.	95
0.3 M	112	71	.	.	100	118	82
0.4 M	.	.	.	.	98	138	72
0.5 M	100	55	.	.	98	.	61
0.6 M	100	37	116	45	100	158	59

which the 'atypical' allele occurs. The ADH in liver extracts in contrast is very heterogeneous and consists of a mixture of $\alpha\alpha$, $\beta\beta$ and $\gamma\gamma$ isozymes and the heterodimeric $\alpha\beta$, $\alpha\gamma$ and $\beta\gamma$ isozymes.

This difference between 'atypical' and 'usual' ADH was confirmed by studying partially purified preparations of the 'atypical' β isozyme from liver which were found to have a pH optimum of 7.0–7.5 with acetaldehyde as substrate compared with an optimum of 5.0–5.5 for a similar preparation of the 'usual' β isozyme ($\beta^1\beta^1$). Similarly a partially purified preparation of the 'atypical' $\alpha\beta^2$ isozyme was found to have a pH optimum of 7.5 compared with 5.5–6.0 for the 'usual' $\alpha\beta^1$ isozyme. The relatively low pH optimum obtained in 'atypical' as well as 'usual' adult liver homogenates with acetaldehyde as substrate is probably a reflexion of the relatively high activity of the ADH_3 isozymes (pH optimum 6.0) in the liver samples tested.

It is of interest to note that while with ethanol as substrate 'atypical' ADH has a lower pH optimum than the 'usual' form, with acetaldehyde as substrate it appears to have a somewhat higher pH optimum than the 'usual' form.

Inhibitors

Thiourea. The effects of thiourea (0.1–0.6 M) on ADH activity were investigated using crude tissue homogenates and partially purified preparations of the individual isozymes obtained by CM cellulose chromatography with ethanol as substrate at pH 8.8. The results are summarized in Table 3.

Little or no effect was observed on the ADH activity of homogenates from livers of the 'usual' pH ratio phenotype; in contrast marked inhibition was observed using livers of the 'atypical' pH ratio phenotype. Different results were obtained with partially purified material: the $\alpha\alpha$ isozyme activity was not affected, the ADH activity of the 'usual' $\beta^1\beta^1$ isozyme was enhanced and the 'atypical' β isozyme was inhibited by the addition of thiourea (Table 3). Comparable results were obtained using crude extracts of lung tissue, which exhibit only ADH_2 ($\beta\beta$) isozyme activity; the 'usual' lung ADH was slightly more active in the presence of 0.6 M thiourea but the 'atypical' lung ADH was much less active.

Fig. 6. ADH activity in two adult liver homogenates of the 'usual' and in two of the 'atypical' pH ratio phenotype, with ethanol as substrate at different concentrations of pyrazole.

Table 4. *Relative activities of partially purified ADH isozymes with ethanol as substrate at different concentrations of pyrazole*

(Activity expressed as a percentage of control assay done without pyrazole.)

Pyrazole	ADH isozymes		
	ADH ₁ <i>αα</i>	ADH ₂	
		'Usual' <i>β¹β¹</i>	'Atypical'
Nil (control)	100	100	100
0.003 mM	100	93	86
0.006 mM	100	80	73
0.010 mM	100	78	64
0.016 mM	100	68	50
0.066 mM	85	57	16
0.130 mM	75	38	11

These results are consistent with those previously obtained by von Wartburg *et al.* (1964) when it was found that thiourea acts as a strong inhibitor of 'atypical' ADH in crude liver homogenates and in purified material and as an activator of 'usual' ADH in purified material but not in crude homogenates.

Fig. 7. Photographs of starch gel showing the effects of 0.3 mM pyrazole on the staining intensities of the ADH isozymes present in 'usual' and 'atypical' adult liver samples, both ADH₃ 1 phenotype. Gel stained with ethanol as substrate. Pyrazole added to samples, gel buffers and staining mixture. Note the marked inhibition of the 'atypical' β and $\alpha\beta^2$ isozymes.

Fig. 8. Photograph of starch gels showing the effects of 0.3 M isobutyramide on the staining intensities of the ADH isozymes in infant liver, lung and kidney and adult liver. Gel stained with butanol as substrate. Isobutyramide added to the staining mixture. Note the marked inhibition of the homodimeric $\gamma^1\gamma^1$ and $\gamma^2\gamma^2$ isozymes and the γ containing heterodimeric isozymes $\alpha\gamma^2$ and $\beta^1\gamma^2$.

Pyrazole. The effects of pyrazole on ADH activity were examined by assay and by gel experiments with ethanol as the substrate.

Concentrations of pyrazole ranging from 0.03 to 0.6 mM were found to inhibit ADH in crude liver extracts but the inhibition was more marked in the 'atypical' pH ratio samples than in the 'usual' pH ratio samples (Fig. 6). Similar results were obtained when the effects of pyrazole on the ADH activity of the isolated 'atypical' β isozyme and the 'usual' β ($\beta^1\beta^1$) isozyme were

Fig. 9. Photograph of starch gels showing the effects of 0.06 M trichloroethanol on the staining intensities of the ADH isozymes in foetal and adult liver. Gel stained with ethanol as substrate, trichloroethanol added to staining mixture. Note the inhibition of the $\alpha\alpha$ isozyme and the α containing heterodimeric isozymes $\alpha\gamma^2$ and $\alpha\beta^1$.

compared (Table 4). The ADH in the $\alpha\alpha$ isozyme preparation was found to be somewhat less susceptible to pyrazole than either the 'usual' ($\beta^1\beta^1$) or the 'atypical' β isozymes.

These observations were substantiated by the results obtained from gel electrophoresis. Fig. 7 shows the effects of pyrazole (0.3 mM), added to the gel and bridge buffers and the liver homogenates prior to electrophoresis and to the staining mixture, on the relative staining intensities of the ADH isozymes in adult liver samples of the 'usual' and 'atypical' pH ratio phenotypes. The 'atypical' β isozyme and the 'atypical' $\alpha\beta$ isozyme ($\alpha\beta^2$) are very much less active in the presence of pyrazole than in the control sample. The staining intensity of the 'usual' β isozyme ($\beta^1\beta^1$) is also reduced but the $\alpha\alpha$ isozyme is hardly affected.

Isobutyramide. Fig. 8 is a photograph of a starch gel showing the effects of isobutyramide at a concentration of 0.3 M in the ADH staining mixture with ethanol as the substrate. Comparison with the control sample shows that the ADH_3 (γ) containing isozymes are relatively less active in the presence of isobutyramide than the ADH_2 (β)- and ADH_1 (α) containing isozymes.

Trichloroethanol. This reagent was also found to be a selective inhibitor of human ADH but with most effect on the ADH_1 ($\alpha\alpha$) isozyme and the heterodimeric α containing isozymes ($\alpha\beta$ and $\alpha\gamma$). Fig. 9 shows a result obtained using ethanol as substrate and 0.06 M trichloroethanol in the staining mixture.

In vitro stability

Experiments were carried out using the method of McAlpine *et al.* (1970) to compare the relative heat-stabilities of the ADH isozymes. Fig. 10 shows the result of an experiment in which the thermal stabilities of the $\alpha\alpha$, $\alpha\beta^1$, $\beta^1\beta^1$ and $\gamma^1\gamma^1$ isozymes were compared. It will be

Fig. 10. Photograph of starch gel showing the ADH isozyme activity in (i) newborn kidney, ADH_3 1 and (ii) newborn liver after heating the isozymes *in situ* in the gel at 75° C. for 0, 5 and 10 min. Note the labile nature of the $\alpha\alpha$ isozyme.

seen that the $\alpha\alpha$ isozyme is relatively much less stable than any of the other isozymes, the $\alpha\beta^1$ is of intermediate stability and the $\beta^1\beta^1$ and $\gamma^1\gamma^1$ isozymes are of similar stabilities in this experiment. Comparable results were obtained in similar experiments with different tissue samples and also in experiments where the samples were treated prior to electrophoresis. It was concluded that the $\alpha\alpha$ isozyme characteristic of the ADH_1 locus is more thermolabile than the $\beta^1\beta^1$ isozyme determined by ADH_2 and the $\gamma\gamma$ isozyme ($\gamma^1\gamma^1$ or $\gamma^2\gamma^2$) determined by ADH_3 . The 'usual' ADH_2 ($\beta^1\beta^1$) and ADH_3 ($\gamma\gamma$) isozymes appeared to be similar in their relative stabilities though the overall impression from several experiments was that perhaps the ADH_3 isozymes ($\gamma^1\gamma^1$ and $\gamma^2\gamma^2$) were slightly more stable than the 'usual' ADH_2 ($\beta^1\beta^1$) isozyme.

The ADH isozymes of 'usual' and 'atypical' pH ratio samples were also compared and marked differences were detected between the two phenotypes. Using lung samples, for example (Fig. 11a), the 'atypical' β isozyme was found to be much more heat-labile than the 'usual' $\beta^1\beta^1$ isozyme; in liver homogenates the same difference was observed (Fig. 11b) and the 'atypical' heterodimeric isozymes $\alpha\beta^2$, $\beta^2\gamma^1$ and $\beta^2\gamma^2$ were also shown to be much less heat-stable than the corresponding 'usual' isozymes.

The $\gamma^1\gamma^1$ and $\gamma^2\gamma^2$ isozymes were found to be similar in their relative heat-stabilities.

Most of the gel heating experiments were carried out at pH 8.6 but some experiments were also done at pH 7.7. All the ADH isozymes appeared to be more stable at this lower pH. However, the relative differences in thermostabilities between the isozymes observed at pH 8.6 were also demonstrable at pH 7.7.

The relative stabilities of the ADH isozymes were also compared in a series of short-term storage experiments which involved keeping homogenates in a refrigerator for 17 hr. at 10° C. The effect on ADH isozyme activity of freezing and thawing homogenates was also examined.

Fig. 11. Photograph of starch gel showing the ADH isozyme activity in (a) lung and (b) liver samples of the 'usual' and 'atypical' pH ratio phenotype after heating the isozymes *in situ* in the gels at 70° and 75° C. respectively for up to 25 min. Note the labile nature of the 'atypical' β and the $\alpha\beta^2$ isozymes.

The results of these experiments were similar to those obtained with the heating experiments and in particular emphasized the extremely unstable nature of the 'atypical' ADH isozymes. The activity of 'atypical' β isozyme can be almost completely abolished by freezing and thawing liver homogenates (Fig. 12a) or by simply keeping the homogenates at 10° C. for about 18 hr. (Fig. 12b), while the 'usual' ADH isozymes are not seriously affected by these treatments.

CONCLUSIONS

We have observed significant differences in the properties of the various ADH isozymes of human tissues with regard to their substrate specificities, pH optima, inhibition characteristics and *in vitro* stabilities. These differences can be related to the subunit composition of each individual isozyme and in turn attributed to differences in the enzymic properties of the three different forms of human ADH, determined by the postulated structural loci ADH_1 , ADH_2 and

Fig. 12. Photographs of starch gels showing the relative staining intensities of the ADH isozymes in 'usual' and 'atypical' liver samples, both ADH₃ 2 phenotype, after (a) freezing and thawing once and (b) at 10° C. for 24 hr. Note the labile nature of the 'atypical' β, αβ² and β²γ² isozymes.

*ADH*₃. The results are entirely consistent with the three locus hypothesis and they are also consistent with the previous studies on human liver ADH.

The experiments with different alcohols as substrate agree with the previous reports (von Wartburg *et al.* 1964, 1965, 1966, 1968; Blair & Vallee, 1966; von Wartburg, 1971) that human ADH has a wide and varied substrate specificity, but they also revealed marked differences in specificity among the *ADH*₁, *ADH*₂ and *ADH*₃ isozymes. The *ADH*₁ isozymes for example were found to be most active with ethanol, allyl alcohol, *sec*-propanol and cyclohexanol; *ADH*₂ isozymes characteristic of the 'usual' pH ratio phenotype were most active with ethanol, butanol, octanol and *sec*-butanol; whereas the *ADH*₃ isozymes showed relatively very high activities with the longer chained alcohols, butanol, amyl alcohol, heptanol and octanol. No differences were observed between the alternative subunits γ^1 and γ^2 at the *ADH*₃ locus but striking differences were observed between the 'usual' (β^1) and 'atypical' (β^2) subunits characteristic of alleles at the *ADH*₂ locus. From the starch-gel zymogram assessments, made using liver and lung extracts, the 'atypical' isozymes were found to be less active than the 'usual' isozymes with butanol, *sec*-butanol, cyclohexanol, benzyl alcohol and Ronicol. These observations agree with the quantitative data of von Wartburg & Schürch (1968) obtained with ADH from 'usual' and 'atypical' human liver samples.

Several aldehydes were found to be suitable substrates for ADH and it is interesting to note that glyceraldehyde-3-phosphate, a potential physiological substrate, was apparently reduced by all the ADH isozymes. No significant differences were observed between the *ADH*₁, *ADH*₂ and *ADH*₃ isozymes in their relative activities with various aldehydes as substrates except for chloral hydrate. This was only slowly reduced by the 'atypical' isozymes, confirming the original report of von Wartburg & Schürch (1968). The *ADH*₃ isozymes were also found to show relatively very low activity with chloral hydrate as substrates.

The pH activity studies were interesting since they showed that the *ADH*₁, 'usual' *ADH*₂ and the *ADH*₃ isozymes have a similar pH optimum (*c.* 11.5) with ethanol as substrate but different pH optima (*c.* pH 8.8, 6.0 and 6.5 for *ADH*₁, 'usual' *ADH*₂ and *ADH*₃ respectively) with acetaldehyde as substrate. The hybrid heterodimeric isozymes were found to have pH optima intermediate between those of the corresponding homodimeric isozymes. This series of experiments also showed that the 'usual' and 'atypical' ADH isozymes differ in their pH optima not only with ethanol as substrate, as demonstrated originally by von Wartburg *et al.* (1964) but also with acetaldehyde as substrate. 'Usual' *ADH*₂ has, with acetaldehyde as substrate, a pH optimum of about 6.0 whereas 'atypical' *ADH*₂ has an optimum around pH 7.0–7.5.

The experiments with thiourea, pyrazole, isobutyramide and trichloroethanol confirmed previous studies that these substances inhibit ADH activity (von Wartburg *et al.* 1965, 1968; Lester & Benson, 1970; Blair & Vallee, 1966) but each reagent was found to have a relatively selective action. For example, trichloroethanol was found to be a potent inhibitor of the *ADH*₁ isozymes; isobutyramide an inhibitor of the *ADH*₃ isozymes; and thiourea and pyrazole as previously reported (von Wartburg & Schürch, 1968) have most effect on the 'atypical' *ADH*₂ isozymes. It became apparent, however, that the effects of thiourea are complicated since it inhibits 'atypical' *ADH*₂ isozyme activity, enhances 'usual' *ADH*₂ but has apparently no effect on *ADH*₁ isozyme activity. Unfortunately it was not possible to establish whether thiourea affects *ADH*₃ isozyme activity.

Our experiments on the *in vitro* stability of human ADH isozymes really commenced at the very beginning of our work on the electrophoresis of this enzyme when it became apparent from the rapid decline of ADH activity in crude tissue homogenates and changing isozyme patterns on storage that ADH is a relatively unstable enzyme. In order to obtain reproducible and reliable results we found that post-mortem tissues must be frozen as soon as possible after autopsy; that thawing and refreezing during storage must be avoided; that homogenization must be done with care to avoid even a moderate rise in temperature; and that electrophoresis must be carried out as soon as possible after homogenization, preferably in the presence of relatively large amounts of coenzyme NAD and with cooling plates to avoid temperature changes during the separation of the ADH isozymes. Otherwise much of the ADH activity originally present in the tissue sample will be lost by the time the isozymes are stained.

The necessity for these stringent precautions has been confirmed by the results obtained from the deliberate heat-stability tests and storage experiments described in the present paper. The ADH₁ isozymes were found to be relatively more labile than the 'usual' ADH₂ and the ADH₃ isozymes. Also the 'atypical' ADH₂ isozyme appears to be very much less stable than any of the other ADH isozymes.

SUMMARY

The substrate specificity, pH activity curves, inhibition characteristics and *in vitro* stabilities of the human ADH isozymes characteristic of the structural loci, ADH₁, ADH₂ and ADH₃, have been investigated using crude tissue extracts and partially purified material.

(1) *Alcohol substrates*. Seventeen different alcohols were tested. The products of the three loci showed differences in their relative activities with the different substrates. Thus ADH₁ isozymes were most active with ethanol, allyl alcohol, *sec*-propanol and cyclohexanol; the 'usual' ADH₂ were most active with ethanol, butanol, octanol and *sec*-butanol; the 'atypical' ADH₂ isozymes were most active with ethanol and octanol, but showed relatively low activity with butanol and Ronicol; the ADH₃ isozymes were relatively very active with long straight-chain primary alcohols.

(2) *Aldehyde substrates*. Six different aldehydes were tested. No significant differences between the isozyme products of the three loci were detected except in the case of chloral hydrate. The ADH₁ and 'usual' ADH₂ isozymes showed activity with chloral hydrate but this was a very poor substrate for the ADH₃ and 'atypical' ADH₂ isozymes.

(3) *pH activity profiles*. With ethanol as substrate the pH optimum for the ADH₁, 'usual' ADH₂ and the ADH₃ isozymes was around pH 11.5 and for the 'atypical' ADH₂ isozymes was about pH 8.8. With acetaldehyde as substrate the pH optima for the ADH₁, 'usual' ADH₂, 'atypical' ADH₂ and ADH₃ isozymes were about pH 8.8, 6.0, 7.0–7.5 and 6.5 respectively.

(4) *Inhibitors*. Trichloroethanol was found to be a potent inhibitor of the ADH₁ isozymes; isobutyramide an inhibitor of ADH₃; and pyrazole and thiourea were shown to be powerful inhibitors of the 'atypical' ADH₂ isozymes.

(5) *In vitro stability*. The ADH₁ isozymes appeared to be relatively less stable than the 'usual' ADH₂ and ADH₃ isozymes. The 'atypical' ADH₂ isozymes were found to be relatively very labile and particularly susceptible to freezing and thawing or storage at 10° C.

(6) The ADH₁¹ and ADH₃² isozymes were not demonstrably different in the properties tested.

We thank Dr D. J. de Sa', Department of Pathology, Radcliffe Infirmary, Oxford, for supplying adult and infant post-mortem tissues; Dr A. H. Cameron, Department of Pathology, The Children's Hospital, Birmingham, for supplying infant post-mortem tissues; Staff of the Obstetric Department, University College Hospital, and the MRC tissue bank of the Royal Marsden Hospital for supplying foetal tissue. We are also grateful for the assistance of Mrs J. Slee in the collection of tissue samples and Mrs J. Vegoda for her skilled technical help.

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