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

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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 $ADH_1$ 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 ($\beta$-pyridyl carbinol) and Myanesin (toloxy-1,2-propanediol) and aldehydes such as chloral hydrate and Acetaldol ($\beta$-hydroxybutyaldehyde) have also been shown to act as substrates for human liver ADH (von Wartburg & Schürich, 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 $\alpha$, $\beta$ and $\gamma$ 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 $ADH_2^2$ at the $ADH_2$ locus, which determines a variant polypeptide $\beta^2$, it is not yet clear whether the characteristic 'beta-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' $\beta$-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 1/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 x 10⁻⁴ M) and phosphate buffer (3.3 x 10⁻² M) at pH 6-5 together with the aldehyde being tested, usually at final concentrations between 8.3 x 10⁻² and 1.6 x 10⁻² M. The reaction rate was followed at 340 mμ 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
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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  | \(ADH_1\) (a) | 'Usual' (\(\beta^1\)) | 'Atypical' (\(\beta^2\)) | \(ADH_2\) (c) |
|------------|--------|----------|---------------|------------------------|--------------------------|---------------|
| Methanol   | 32     | \(\text{CH}_3\cdot\text{OH}\) | +              | +                      | +                        | +             |
| Ethanol    | 46     | \(\text{CH}_3\cdot\text{CH}_2\cdot\text{OH}\) | +              | + ++                   | ++                      | +++           |
| Propanol   | 60     | \(\text{CH}_3\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{OH}\) | +              | + ++                   | ++                      | ++ +          |
| Butanol    | 74     | \(\text{CH}_3\cdot(\text{CH}_2)_2\cdot\text{CH}_2\cdot\text{OH}\) | +              | + ++ ++                | +++                     | + + +         |
| Amyl       | 88     | \(\text{CH}_3\cdot(\text{CH}_2)_3\cdot\text{CH}_2\cdot\text{OH}\) | +              | + ++ ++                | +++                     | +++ +         |
| Hexanol    | 102    | \(\text{CH}_3\cdot(\text{CH}_2)_4\cdot\text{CH}_2\cdot\text{OH}\) | +              | + ++ ++                | +++                     | +++ +         |
| Heptanol   | 116    | \(\text{CH}_3\cdot(\text{CH}_2)_5\cdot\text{CH}_2\cdot\text{OH}\) | +              | + ++ ++                | +++                     | + + + +       |
| Octanol    | 130    | \(\text{CH}_3\cdot(\text{CH}_2)_6\cdot\text{CH}_2\cdot\text{OH}\) | +              | + ++ ++ ++             | +++                     | + + + +       |
| Allyl      | 58     | \(\text{CH}_3\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{OH}\) | +              | + ++ ++ + +            | +++ +                   | ++ + +        |
| Sec-propanol| 60    | \(\text{CH}_3\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{CH}_3\) | +              | + ++ +                 | + +                     | + + + +       |
| Sec-butanol| 74     | \(\text{CH}_3\cdot\text{CH}_2\cdot\text{CHOH}\cdot\text{CH}_3\) | +              | + ++ +                 | + +                     | + + + +       |
| Sec-amyl   | 88     | \(\text{CH}_3\cdot(\text{CH}_2)_2\cdot\text{CHOH}\cdot\text{CH}_3\) | +              | + ++ +                 | + +                     | + + + +       |
| Sec-octanol| 130    | \(\text{CH}_3\cdot(\text{CH}_2)_3\cdot\text{CHOH}\cdot\text{CH}_3\) | +              | + ++ +                 | + +                     | + + + +       |
| Tert-amyl  | 88     | \(\text{CH}_3\cdot\text{CH}_2\cdot\text{COH}\cdot\text{CH}_2\cdot\text{CH}_3\) | +              | + +                    | + +                     | ++           |
| Cyclohexanol| 100   | \(\text{C}_6\text{H}_{11}\cdot\text{OH}\) | +              | + ++ +                 | + +                     | ++           |
| Benzyl alcohol | 108 | \(\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{OH}\) | +              | + ++ +                 | + +                     | ++           |

Substrate specificity

(a) 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-
Ethanol (a) and Amyl alcohol (b) substrates were used to assess the relative staining intensities of ADH isozymes in infant liver and kidneys. The photographs (Fig. 1) show the staining intensities of the isozymes. The activities of certain isozymes, particularly the ADH components, were assessed from the relative staining intensities of the 'hybrid' heterodimeric isozymes. 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 I.

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' $\beta$ 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 $\gamma$-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' $\beta$-isozyme characteristic of liver and lung samples of the 'atypical' pH ratio...
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**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, 1) using (a) ethanol and (b) butanol as substrate.

The activity of the ADH isozymes was also examined using the substituted alcohol Ronicol (β-pyridylicarbinol) 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’ $\beta^3\beta^4$ 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 ADH₁ 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) | ADH₁ | ‘Usual’ | ‘Atypical’ | ADH₃ |
|-----------------------------------|------|---------|------------|------|
|                                   | αα   | ββ      | γγ         | γγ   |
| 46                                | 86   | 48      | 23         | 1    |
| 16                                | 68   | 32      | 3          | 1    |

(either γ¹γ¹ or γ²γ²) 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
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 $\beta^+$' 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$_1$ isozyme ($\gamma\gamma$) activity, were almost identical. The pH optimum was about 6-5. No differences were detected among ADH$_1$, ADH$_2$ 2–1 and ADH$_2$ 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$_2$ 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.
In adult livers, however, a single pH optimum was observed at pH 6.0, presumably a reflection of the relatively high activity of the ADH$_2$ 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 $\beta\beta\beta$ 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

| Thiourea | Liver |  |  | Lung |  |  | ADH4 |  |  |
|----------|------|---|---|------|---|---|------|---|---|
|          | ‘Usual’ | ‘Atypical’ | ‘Usual’ | ‘Atypical’ | ‘Usual’ | ‘Atypical’ | ‘Usual’ | ‘Atypical’ |
| Nil      | 100  | 100 |  | 100  | 100 | 100 | 100  | 100 |
| 0·1 M    | 116  | 81  |  | 112  | 71  |  | 98   | 95  |
| 0·2 M    | 112  | 71  |  | 98   | .   |  | 100  | 82  |
| 0·3 M    | 100  | 55  |  | 100  | 37  |  | 100  | 59  |
| 0·5 M    | 100  | 55  |  | 100  | 37  |  | 100  | 59  |
| 0·6 M    | 100  | 55  |  | 100  | 37  |  | 100  | 59  |

which the ‘atypical’ allele occurs. The ADH in liver extracts in contrast is very heterogeneous and consists of a mixture of αα, ββ and γγ isozymes and the heterodimeric αβ, αγ and βγ 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 (β1β1). Similarly a partially purified preparation of the ‘atypical’ αβ2 isozyme was found to have a pH optimum of 7·5 compared with 5·5–6·0 for the ‘usual’ αβ1 isozyme. The relatively low pH optimum obtained in ‘atypical’ as well as ‘usual’ adult liver homogenates with acetaldehyde as substrate is probably a reflection of the relatively high activity of the ADH4 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 αα isozyme activity was not affected, the ADH activity of the ‘usual’ β1β1 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 ADH4 (ββ) 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   | ADH₁       | ADH₂       |
|------------|------------|------------|
|            | αα         | ββ         | '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 ADH1 phenotype. Gel stained with ethanol as substrate. Pyrazole added to samples, gel buffers and staining mixture. Note the marked inhibition of the 'atypical' $\beta$ 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 $\gamma\gamma$ and $\gamma^2\gamma^2$ isozymes and the $\gamma$ containing heterodimeric isozymes $\alpha\gamma$ and $\beta\gamma^2$.

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’ $\beta$ isozyme and the ‘usual’ $\beta$ ($\beta^1\beta^1$) isozyme were
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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 \( \alpha \) 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’ \( \beta \) 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’ \( \beta \) 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’ \( \beta \) 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\(_3\) (\( \gamma \)) containing isozymes are relatively less active in the presence of isobutyramide than the ADH\(_4\) (\( \beta \)-) and ADH\(_1\) (\( \alpha \)) 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 \( \alpha \) 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
seen that the αα isozyme is relatively much less stable than any of the other isozymes, the αβ\(^1\) is of intermediate stability and the β\(^1\)β\(^1\) and γ\(^1\)γ\(^1\) 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 αα isozyme characteristic of the \(ADH_1\) locus is more thermolabile than the β\(^1\)β\(^1\) isozyme determined by \(ADH_2\) and the γγ isozyme (γ\(^1\)γ\(^1\) or γ\(^2\)γ\(^2\)) determined by \(ADH_3\).

The 'usual' \(ADH_3\) (β\(^1\)β\(^1\)) and \(ADH_2\) (γγ) isozymes appeared to be similar in their relative stabilities though the overall impression from several experiments was that perhaps the \(ADH_3\) isozymes (γ\(^1\)γ\(^1\) and γ\(^2\)γ\(^2\)) were slightly more stable than the 'usual' \(ADH_2\) (β\(^1\)β\(^1\)) 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' β\(^1\)β\(^1\) isozyme; in liver homogenates the same difference was observed (Fig. 11b) and the 'atypical' heterodimeric isozymes αβ\(^2\), β\(^2\)γ\(^1\) and β\(^2\)γ\(^2\) were also shown to be much less heat-stable than the corresponding 'usual' isozymes.

The γ\(^1\)γ\(^1\) and γ\(^2\)γ\(^2\) 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.
Human alcohol dehydrogenase 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 ADH, 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.
Human alcohol dehydrogenase isozymes

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 ADH₁, ADH₂ and ADH₃ isozymes. The ADH₁ isozymes for example were found to be most active with ethanol, allyl alcohol, sec-propanol and cyclohexanol; ADH₂ isozymes characteristic of the ‘usual’ pH ratio phenotype were most active with ethanol, butanol, octanol and sec-butanol; whereas the ADH₃ 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 γ¹ and γ² at the ADH₃ locus but striking differences were observed between the ‘usual’ (β¹) and ‘atypical’ (β²) subunits characteristic of alleles at the ADH₁ 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 ADH₁, ADH₂ and ADH₃ 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 ADH₃ 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 ADH₁, ‘usual’ ADH₂ and the ADH₃ 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 ADH₁, ‘usual’ ADH₂ and ADH₃ 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’ ADH₂ has, with acetaldehyde as substrate, a pH optimum of about 6·0 whereas ‘atypical’ ADH₂ 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 ADH₁ isozymes; isobutyramide an inhibitor of the ADH₂ isozymes; and thiourea and pyrazole as previously reported (von Wartburg & Schürch, 1968) have most effect on the ‘atypical’ ADH₂ isozymes. It became apparent, however, that the effects of thiourea are complicated since it inhibits ‘atypical’ ADH₂ isozyme activity, enhances ‘usual’ ADH₂ but has apparently no effect on ADH₁ isozyme activity. Unfortunately it was not possible to establish whether thiourea affects ADH₃ 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’ ADH₄ 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’ ADH₄ 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’ ADH₄ 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’ ADH₄ isozymes was about pH 8-8. With acetaldehyde as substrate the pH optima for the ADH₁, ‘usual’ ADH₂, ‘atypical’ ADH₄ 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’ ADH₄ isozymes.

(5) *In vitro stability.* The ADH₁ isozymes appeared to be relatively less stable than the ‘usual’ ADH₂ and ADH₃ isozymes. The ‘atypical’ ADH₄ isozymes were found to be relatively very labile and particularly susceptible to freezing and thawing or storage at 10°C.

(6) The ADH₁ and ADH₃ isozymes were not demonstrably different in the properties tested.
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