Title
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Peer reviewed

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In man, alcohol dehydrogenase (alcohol:NAD oxidoreductase E.C. 1.1.1.1) occurs principally in liver, though low levels of activity have also been found in lung, kidney and the gastrointestinal tract (Moser, Papenberg & von Wartburg, 1968). Evidence for at least three distinct isozymes has been obtained by chromatography of liver extracts on CM cellulose (Blair & Vallee, 1966) and also by electrophoresis (Moser *et al.* 1968; Pikkarainen & Räihä, 1969; Murray & Motulsky, 1970).

Von Wartburg, Papenberg & Aebi (1965) reported that certain individuals have an atypical form of alcohol dehydrogenase associated with an increased level of activity. The usual and atypical forms of the enzyme were shown to differ markedly in pH activity curves with ethanol as substrate. The pH optimum for the usual form was found to be pH 10·8 and for the atypical form pH 8·5. The enzymes also differed in the relative rates at which they oxidized various other alcohols, and in the degree of inhibition produced by various metal binding agents. On the other hand no significant differences were observed in Michaelis constants for the substrates ethanol or acetaldehyde or for the corresponding coenzymes NAD or NADH. Also the pH activity curve with acetaldehyde as substrate was essentially the same for both enzymes, having an optimum at pH 6·0–6·5.

A simple screening test to distinguish the usual from the atypical enzyme in crude liver homogenates was designed (von Wartburg *et al.* 1965). This involves determining the ratio of the activity at pH 11·0 to that at pH 8·8 with ethanol as substrate under standard conditions. The usual enzyme gives a value for this ratio greater than 1·0, and the atypical enzyme less than 1·0. In a survey of 59 liver samples from different individuals in Switzerland, 12 were found to have the atypical alcohol dehydrogenase, and in another series of 50 individuals from London, 2 were found to be atypical (von Wartburg & Schürch, 1968). The atypical enzyme occurred in individuals varying from 16 to 82 years of age.

Pikkarainen & Räihä (1967) reported that alcohol dehydrogenase activity in liver is low during foetal life and reaches adult levels about 5 years after birth. Changes in electrophoretic pattern have also been noted during development (Pikkarainen & Räihä, 1969; Murray & Motulsky, 1970). In the earliest stages only a single isozyme is observed but later further isozymes appear. In adult liver individual variations in the relative contribution of the different isozymes to the total activity have been noted (von Wartburg & Schürch, 1968), but no clear electrophoretic differences between the usual and atypical alcohol dehydrogenases as determined by the ratio of activity at pH 11·0 and pH 8·8 were detected.

The present paper is concerned with a study of human alcohol dehydrogenase in which liver, lung, kidney and intestinal material from foetuses, infants and adults has been examined. The enzyme has been investigated both by spectrophotometric assay at different pH's and by starch-gel

electrophoresis. The findings suggest that at least three distinct gene loci may be concerned in determining the structure of the various alcohol dehydrogenase isozymes in man, and that they are active to markedly different degrees in different stages of development in different tissues.

<center>MATERIALS AND METHODS</center>

Foetal samples (9–22 weeks gestation) were obtained from therapeutic abortions. Other samples were obtained from autopsies, and were mainly from adults but some came from premature infants and a few from young children. The autopsy samples were generally taken between 12 and 48 hr. after death, the body having been refrigerated in the meanwhile. The samples if not examined immediately were stored at –20° C. Care was taken to avoid repeated freezing and thawing of samples since this was found to lead to a rapid decay of alcohol dehydrogenase activity. The tissues examined in detail were liver, lung, kidney and intestine. Although traces of alcohol dehydrogenase activity probably occur in certain other tissues, the activity was found to be much too low to be investigated by the techniques used here.

Assay of alcohol dehydrogenase was carried out by the method described by von Wartburg *et al.* (1965) using ethanol as substrate. In each case an assay was carried out at pH 8·8 and also at pH 11·0. For assays of the enzyme in liver, the tissue was homogenized in 9 volumes of 0·1 M phosphate buffer pH 7·0 and the debris removed by centrifugation at 3000 rev./min. for 15 min.; 0·2 ml. of the supernatant was added to 2·8 ml. of the reaction mixture, giving a final volume of 3 ml. which contained $1·6 \times 10^{-3}$ M NAD and $1·6 \times 10^{-2}$ M ethanol in $3·3 \times 10^{-2}$ M sodium pyrophosphate buffer pH 8·8 *or* $3·3 \times 10^{-2}$ M glycine/NaOH buffer pH 11·0. The reaction was followed in a Gilford spectrophotometer at 340 mμ and at 25° C. A blank reaction without ethanol was also run. The enzyme activity was expressed as the change in optical density per minute per gram of tissue. The assays on lung tissue required more concentrated extracts, so here the tissue was homogenized in 2 vol. of phosphate buffer and after centrifugation 0·2 ml. of the supernatant was used in the reaction mixture.

For electrophoresis the tissue was homogenized with an equal volume of water in the case of liver. For lung, intestine and kidney the samples were generally homogenized directly without any addition of water. After centrifugation the supernatant was subjected to horizontal starch-gel electrophoresis the inserts being made with filter paper (Whatman no. 17). The gel buffer was 0·025 M Tris/HCl at pH 8·6 and contained 4×10^{-3} M NAD. The bridge buffer was 0·3 M Tris/HCl pH 8·6. Suitably insulated metal cooling plates through which chilled water (5–10° C.) circulated were used to control the gel temperature during electrophoresis. Electrophoresis was either carried out for 4–5 hr. at 12 V./cm. or for 16 hr. at 5 V./cm.

Following electrophoresis the gels were sliced and stained using an agar overlay. Alcohol dehydrogenase was detected with a reaction mixture containing 20 mg. NAD, 10 mg. tetrazolium salt MTT, 2 mg. phenazine methosulphate and 0·1 ml. ethanol in 25 ml. 0·05 M Tris/HCl buffer at pH 8·6 mixed with 25 ml. 2 % aqueous agar at 55° C. The gels were incubated at 37° C. for 1–2 hr.

<center>RESULTS</center>

(a) Activity determinations <center>*Liver*</center>

Alcohol dehydrogenase was assayed at both pH 8·8 and pH 11·0 in 222 different liver samples using ethanol as substrate. The samples came from individuals of both sexes and covered a

wide range of ages: 56 were from early foetuses (9–22 weeks gestation), 37 from premature infants and infants less than 1 year old, and the remainder were adults over 20 years of age.

Fig. 1 shows a plot of activity at pH 11·0 against activity at pH 8·8, each point representing a separate individual. Because of the very wide range of activities observed at both pH's a log. scale has been used. The points appear to be distributed into two distinct groups. In the larger group the activity at pH 11·0 is greater than at pH 8·8. In the smaller group the opposite is the case. Within each group there is a good correlation between activity at pH 8·8 and activity at pH 11.
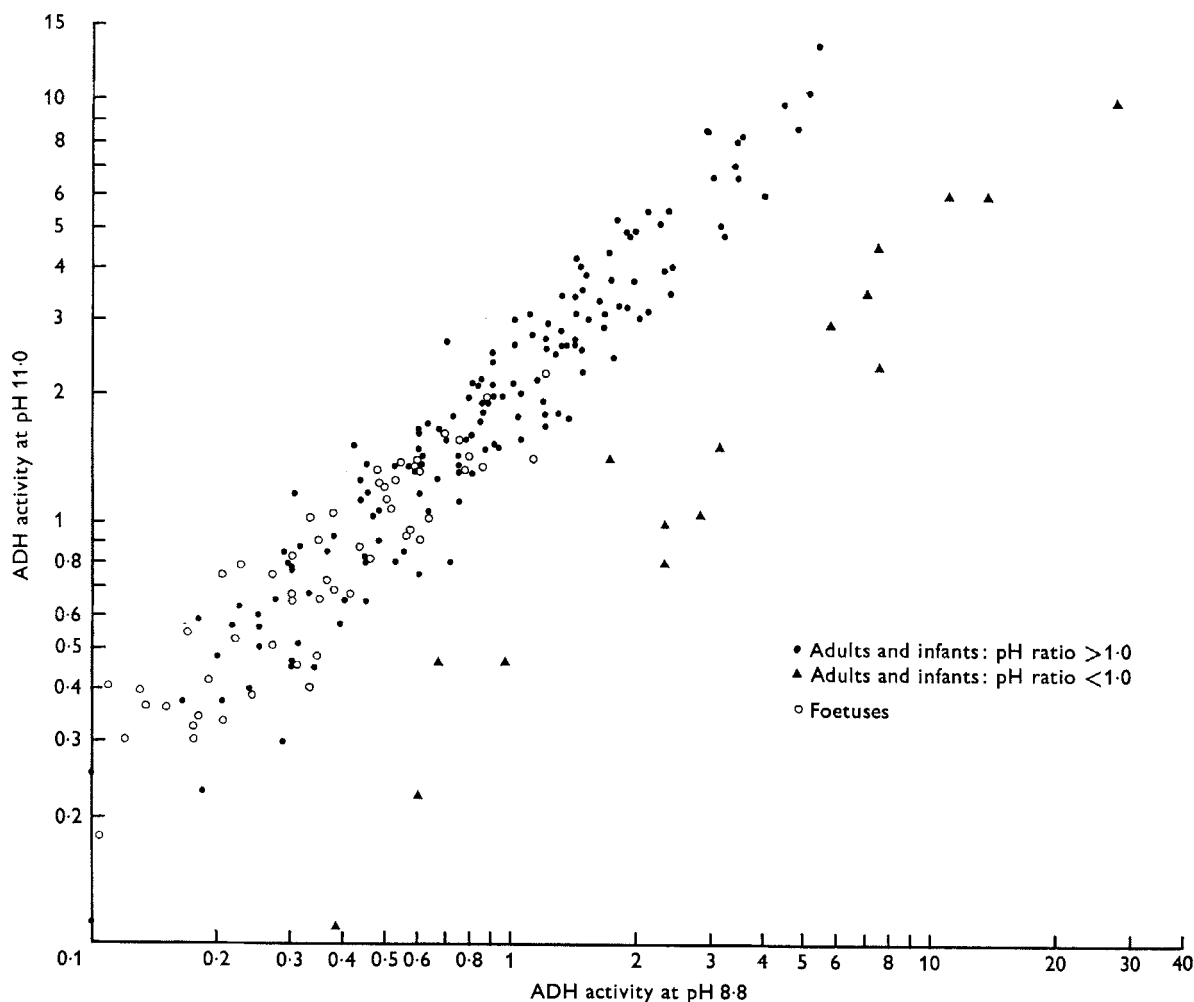

Fig. 1. Plot on log. scale of alcohol dehydrogenase activity ($o.d._{340}$/min./G tissue) at pH 11 against activity at pH 8·8 in liver samples from 129 adults, 37 premature infants and infants between term and one year and 56 foetuses. Each point represents a separate individual.

The samples with greater activity at pH 8·8 presumably represent those which have the 'atypical' alcohol dehydrogenase described by von Wartburg *et al.* (1965). This interpretation has been confirmed in several cases by determining the full pH activity curves. 'Atypical' samples with higher activities at pH 8·8 were found to have pH optima close to this value whereas other samples were found to have pH optima close to pH 11·0.

The pH activity ratio (activity at pH 11·0/activity at pH 8·8) which was suggested by von Wartburg as a simple discriminant between the two types of enzyme has been calculated for each sample. The distribution of values of this ratio in the series of samples is shown in Fig. 2. Fig. 2(a) shows the distribution of the values of this ratio in 166 samples obtained from adults
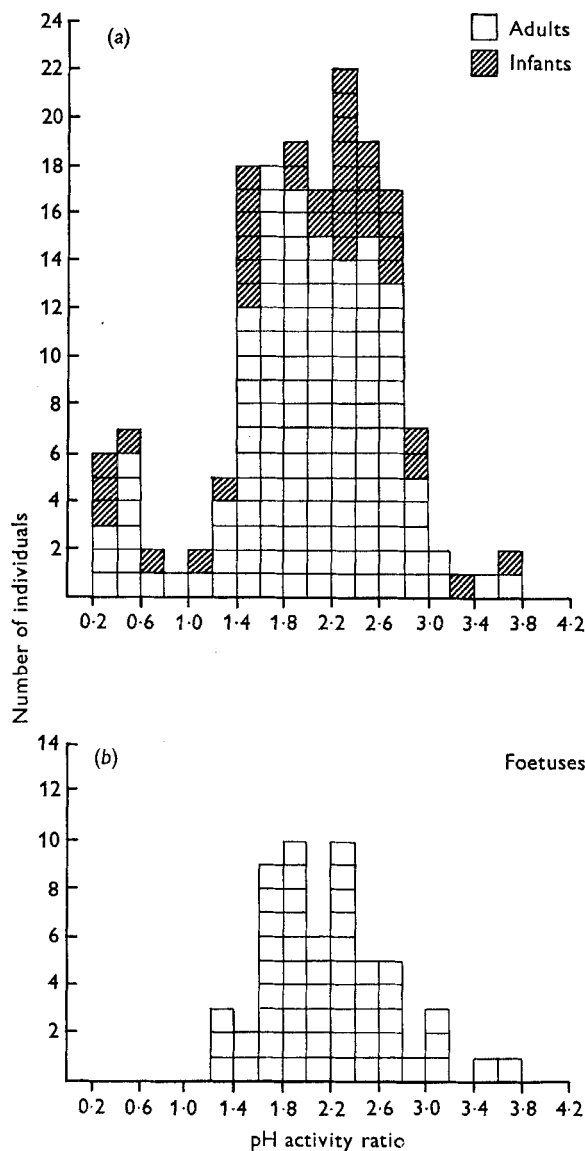

Fig. 2. Distribution of alcohol dehydrogenase pH activity ratios (activity at pH 11/activity at pH 8·8) in liver samples from (a) 129 adults and 37 premature infants and infants between term and one year of age, (b) 56 foetuses. Each square represents one individual.

and infants. It is clearly bimodal. Sixteen samples can be seen to have the atypical pH ratio phenotype, and 150 to have the usual pH ratio phenotype. The atypical phenotype was found in both sexes and over a wide range of ages. The youngest was in a premature infant of about 28 weeks gestation, and the oldest in an individual of 90 years.

Fig. 2(b) shows the distribution of pH ratios in fifty-six samples from young foetuses (9–22 weeks gestation). Here all the values fall into the range of the usual pH ratio phenotype. The

absence of the atypical phenotype in this series of samples is probably not a chance finding, and its significance will be considered later in relation to the electrophoretic results.

Table 1 gives the means and standard deviations of the activities at pH 8·8 and pH 11·0 in the various sets of samples. Although there is a considerable variation within each set of samples, it is apparent that on average the activity both at pH 8·8 and at pH 11·0 increases during foetal life and infancy. Another point of importance which emerges from Table 1 is that the mean activity at pH 8·8 is very much higher in the atypical phenotype than in the usual phenotype, whereas their mean activities at pH 11·0 are quite similar. Thus the low pH ratio of the atypical phenotype appears to derive mainly from enhanced activity at pH 8·8.

Table 1. *Alcohol dehydrogenase activity at pH* 8·8 *and pH* 11·0 *in liver samples obtained from* 56 *foetuses,* 37 *infants and* 129 *adults*

	Foetuses (9–22 weeks gestation)	Infants (premature and 0–1 yr.)	Adults (over 20 yr. of age)
Usual pH ratio phenotype			
No. observed	56	32	118
Mean activity ± S.D.			
pH 8·8	0·419 ± 0·244	0·603 ± 0·634	1·338 ± 1·045
pH 11·0	0·869 ± 0·464	1·284 ± 1·413	2·812 ± 2·262
Ratio pH 11/pH 8·8	2·162 ± 0·531	2·225 ± 0·595	2·115 ± 0·499
Atypical pH ratio phenotype			
No. observed	—	5	11
Mean activity + S.D.			
pH 8·8	—	1·482 ± 1·028	7·966 ± 7·650
pH 11·0	—	0·603 ± 0·325	3·582 ± 2·887
Ratio pH 11/pH 8·8	—	0·447 ± 0·142	0·489 ± 0·163

The activities are expressed as Δ O.D.$_{340}$/G. tissue/min. and also as a ratio (activity pH 11·0/activity pH 8·8). On the basis of this ratio the samples are classified as usual (pH ratio > 1·0) or atypical (pH ratio < 1·0).

When the data tabulated in Table 1 were broken down by sex, no significant differences were found between the sexes in the mean values for the activities or ratios in the separate age groups and the separate phenotypes.

(b) Electrophoretic findings

In liver samples from very early foetuses a single alcohol dehydrogenase isozyme band migrating cathodally was observed. In later foetuses two additional isozymes migrating more cathodally were seen (Fig. 3). The isozymes will be referred to as a, b and c, in order of their appearance during development. Their relative mobilities towards the cathode are $c > b > a$.

The progressive appearance of these isozymes during early development is illustrated in Table 2, in which the average crown–rump length of foetuses in which only isozyme a was detected in the liver is compared with the average lengths of foetuses in whose livers isozymes a and b, or a, b and c were detected.

Livers from premature infants generally showed all the three isozymes, but their relative activities appeared to change progressively with increasing gestational age as is illustrated in Table 3.

At term isozyme b is usually the most active, but in postnatal life isozyme c appears to
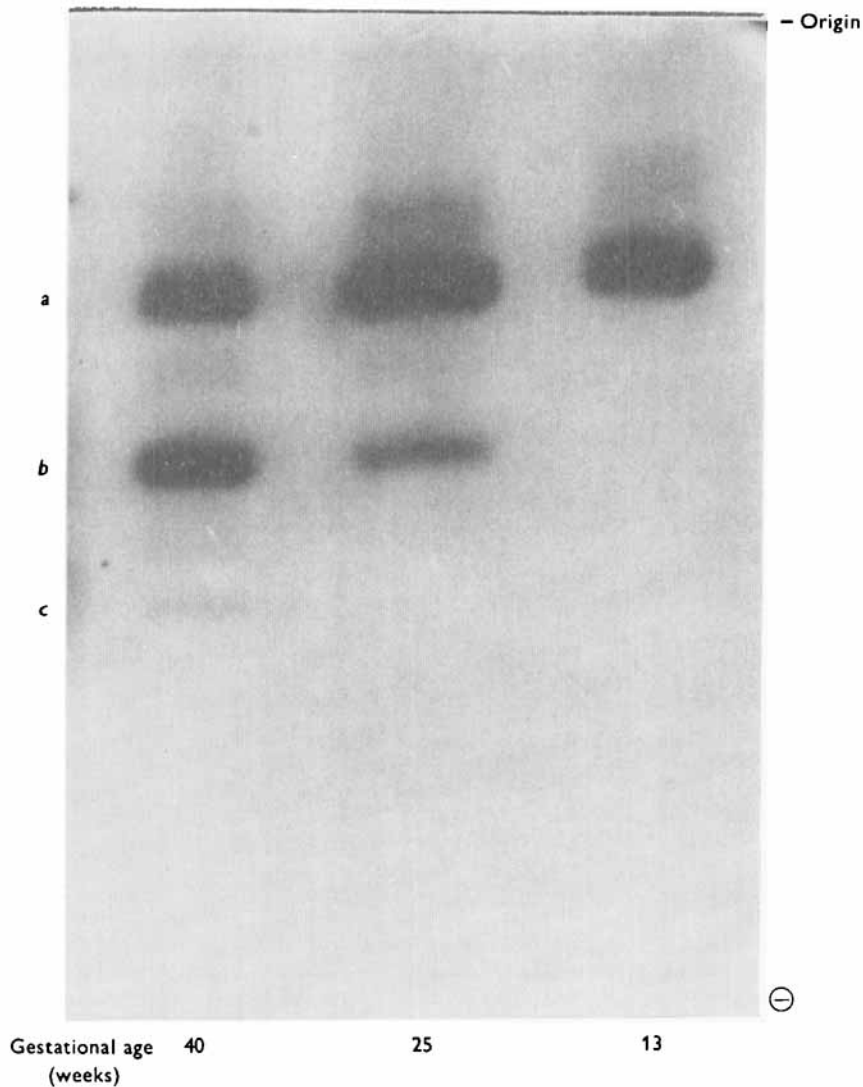
Fig. 3. Photograph of starch gel illustrating liver alcohol dehydrogenase isozyme patterns at various stages of foetal development. The positions of the isozymes a, b and c (see text) are indicated. The minor zones occurring anodal to the isozyme a are thought to represent lactate dehydrogenase (LDH 5) also present in the samples.

increase in relative activity, and isozyme a to decrease. These progressive changes in electrophoretic pattern appear in general to be correlated with an increasing total level of ADH activity. In adults isozymes b and c consistently account for most of the total activity, but their relative activities vary somewhat from person to person. In addition other isozymes with mobilities between b and c and between a and b are also seen. They also vary widely in activity from person to person. They are generally less active than isozymes b and c, though often they may be as active or more active than a. It does not appear that their occurrence can be accounted for in terms of the time after death when the samples are taken, or the length of time or conditions of storage. Quite possibly their variations reflect real individual differences. Typical examples of ADH isozyme patterns in liver samples from two adults and one premature infant, aged 36 weeks, are shown in Fig. 4. All these samples are of the usual pH ratio phenotype.

Table 2. *Foetal size in relation to presence or absence of*
ADH isozymes a, b *and* c *in liver*

ADH isozymes detected	No. of foetuses	Mean crown–rump length (mm)
a only	48	79
a and *b*	50	143
a, *b* and *c*	19	166

Table 3. *Comparison of liver alcohol dehydrogenase isozyme patterns and*
gestational age in a group of thirty-nine premature infants

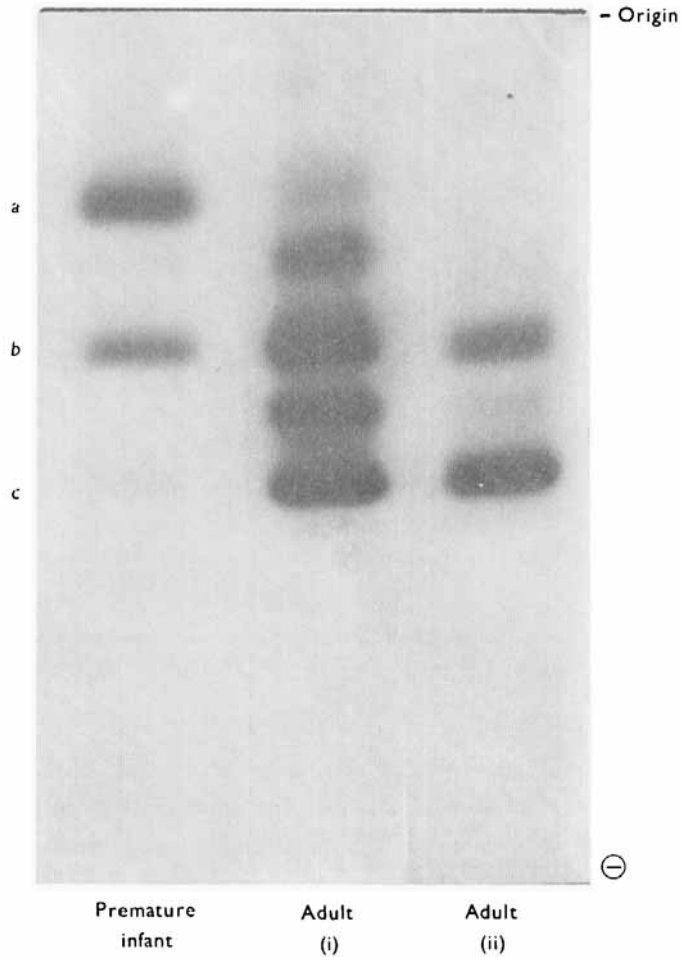| ADH isozyme pattern	No. of premature infants observed	Mean age (weeks gestation)
$a > b > c$	27	28·1
$a = b > c$	10	30·3
$a < b > c$	2	35·5

Fig. 4. Photograph of starch gel showing the liver alcohol dehydrogenase isozyme patterns in a premature infant (36 weeks gestation) and in two different adults, (i) and (ii). Both kinds of adult pattern are frequently observed. All three samples are of the usual pH ratio phenotype.

Prolonged storage of samples, particularly if associated with repeated freezing and thawing, results in a progressive decline in activity. However, it has not been found to lead to any marked systematic alterations in the relative activities of the various isozymes, though minor changes may well occur, for example a weak storage band sometimes appears just cathodal to the main c isozymes in adult livers.
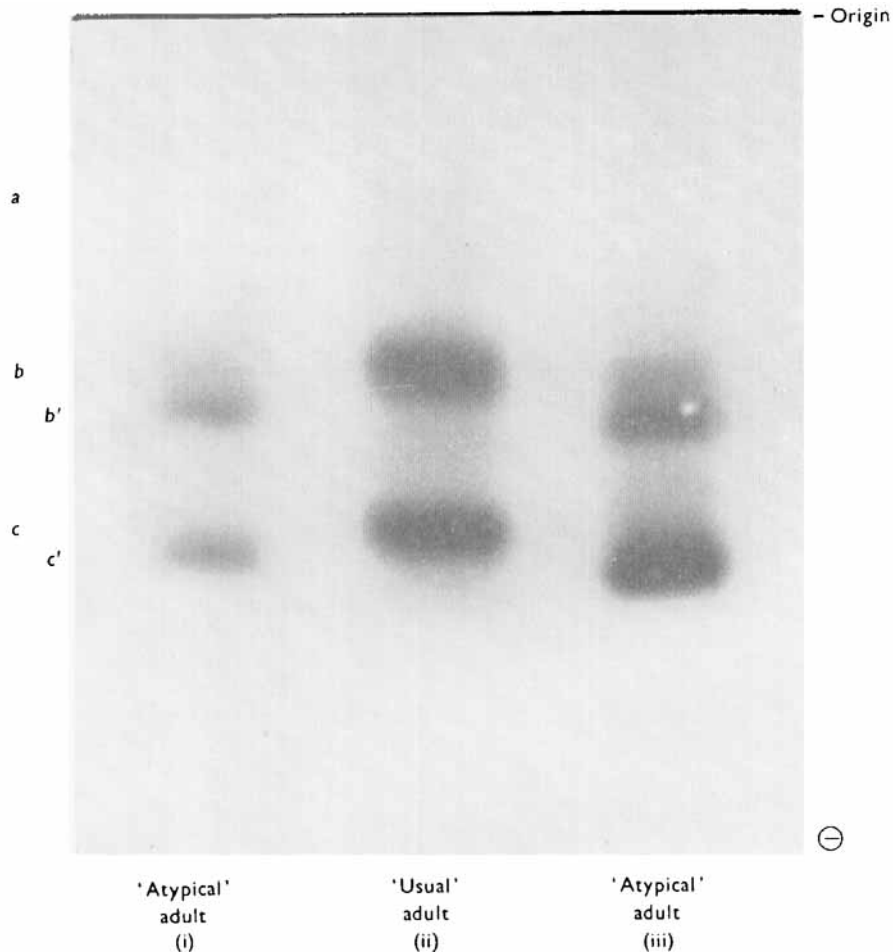

Fig. 5. Photograph of starch gel showing alcohol dehydrogenase isozyme patterns in livers from individuals with usual and atypical pH activity ratios. Sample (i) is a fivefold dilution of sample (iii).

Livers with the atypical pH ratio phenotype were consistently found to have an electrophoretic pattern different from those with the usual phenotype (Fig. 5). In each case the most cathodal band (c') had a slightly greater mobility towards the cathode than the c isozyme of the usual pH ratio phenotype. Similarly the intermediate isozyme (b') in the atypical phenotype moves slightly more cathodally than the b isozyme of the usual phenotype. In some cases a band corresponding to the b isozyme also appears to be present in the atypical phenotype but this is relatively much weaker in activity than the slightly more cathodal variant. The a isozyme is also usually seen in the atypical phenotype, but is very weak. It has the same mobility as the a

isozyme of the usual phenotype. In general the enhanced total activity of the atypical pheno-type is seen from the electrophoretic pattern as largely arising from a relatively much greater activity of the variant component c'.
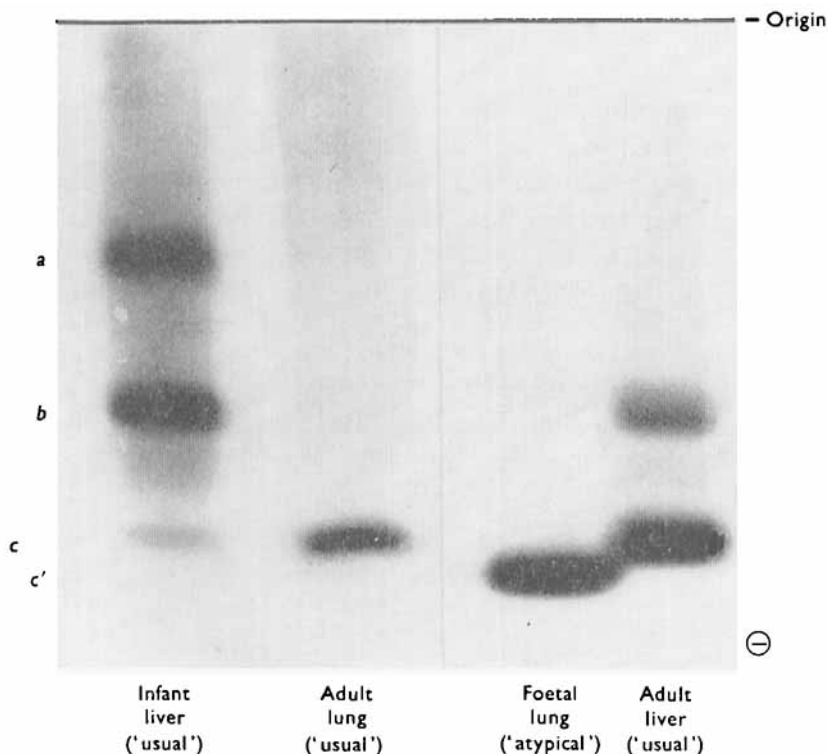

Fig. 6. Photograph of starch gel showing the alcohol dehydrogenase isozyme patterns in an adult lung of the usual pH ratio phenotype and a foetal lung of the atypical pH ratio phenotype compared with adult and infant liver ADH isozymes of the usual pH ratio phenotype.

Lung

Electrophoresis of alcohol dehydrogenase activity from lung tissue revealed no consistent differences between young foetuses and adults. Virtually all the activity occurs in a single isozyme, which in most cases has the same electrophoretic mobility as the c isozyme of liver (Fig. 6). In the other cases its mobility is slightly more cathodal and corresponds to the variant isozyme c' of livers with atypical pH activity ratios. In a few lung samples a very weak band corresponding to the b isozyme of liver is also seen.

Fifty-four samples of lung tissue obtained from foetuses of between 9 and 22 weeks gesta-tional age were tested. In 48 the main isozyme corresponded in mobility to the c isozyme of livers of the usual pH ratio phenotype. In six samples the main isozyme corresponded in mobility to the variant isozyme c' of livers with atypical pH activity ratios. Assays at pH 8·8 and at pH 11·0 were carried out on 17 foetal lung samples with the 'usual' c isozyme and on four samples with the 'atypical' c' isozyme. The results are summarized in Table 4. The seventeen samples with the 'usual' c isozyme all had pH activity ratios within the range of the usual phenotype of liver. The other samples with the slightly more cathodal isozyme had pH ratios characteristic of the atypical phenotype due to enhanced activity at pH 8·8. It will also be noted that the average level of activity in foetal lung is only about 1/20th of that in foetal liver.

The correspondence between the liver and lung phenotypes was also checked in two adults in which the liver had been found to have an atypical pH ratio. In each case the lung showed the slightly more cathodal isozyme c' and the atypical pH activity ratio. A further correspondence was noted in the older group of foetuses in which it was generally possible to detect the a and b isozymes and sometimes the $a, b,$ and c isozymes in liver. In foetuses where the lung had been found to exhibit the usual isozyme pattern, the liver b isozyme and, when present, the c isozyme, had exactly the same electrophoretic mobility as the b and c isozymes of the usual liver pattern. On the other hand where the lung was found to be of the atypical pH ratio phenotype, the liver was also atypical, in that the 'b' isozyme and, where present, the 'c' isozyme, were cathodally displaced and corresponded in mobility to the b' and c' isozymes found in the atypical adult liver (Fig. 7). However, these 'atypical' foetal livers fell in the usual pH activity ratio range, and this was thought to be probably due to the preponderance of the 'a' isozyme fraction.

Table 4. *Alcohol dehydrogenase activity at pH* 8·8 *and pH* 11 *in* 21 *different foetal lung samples*

(The activities are expressed as Δ O.D.$_{340}$/G. tissue/min. ($\pm$ S.D.) and also as a ratio (activity pH 11·0/activity pH 8·8 $\pm$ S.D.). The classification into 'usual' and 'atypical' phenotypes was based on the ADH isozyme patterns obtained by electrophoresis.)

	'Usual' phenotype	'Atypical' phenotype
No. tested	17	4
Mean activity at pH 8·8	0·024 ± 0·013	0·132 ± 0·107
Mean activity at pH 11·0	0·041 ± 0·029	0·029 ± 0·018
pH activity ratio (pH 11·0/pH 8·8)	1·646 ± 0·606	0·263 ± 0·144

Kidney and intestine

The activity of alcohol dehydrogenase in kidney and intestine is generally very low both in foetuses and in adults, and satisfactory assays could not be carried out using ethanol as substrate. However, clear electrophoretic patterns were obtained.

In foetuses and premature infants the isozyme patterns in kidney and intestine differ from the patterns found in the liver and lung. Furthermore it was found that they can be classified into three clearly distinct phenotypes. These are shown in Fig. 8 and referred to as ADH$_3$ 1, ADH$_3$ 2–1 and ADH$_3$ 2. The reason for this terminology will become apparent later. In any single individual the kidney and intestinal sample always showed the same phenotype.

In phenotype ADH$_3$ 1 the main isozyme present has a mobility somewhat less cathodal than isozyme c of liver, and in phenotype ADH$_3$ 2 the main isozyme has a mobility somewhat less cathodal than isozyme b of liver (Fig. 9). In ADH$_3$ 2–1 three main isozymes are seen, two of which correspond to the main isozymes of ADH$_3$ 1 and ADH$_3$ 2, while the other is exactly intermediate in mobility and also relatively the most active. Thus there appears to be a typical enzyme polymorphism. In addition to these main isozymes weak bands corresponding in mobility to isozymes a, b and c of liver are also often seen, the a isozyme in the younger foetal samples and the b and c isozymes in the samples from premature infants.

Among 117 different samples from foetuses and premature infants, 49 showed phenotype ADH$_3$ 1, 49 phenotype ADH$_3$ 2–1 and 19 phenotype ADH$_3$ 2. The isozymes characteristic of these three phenotypes were easily recognized in all the samples from foetuses and premature infants.

In adult kidney the isozyme patterns were found to be more like those encountered in adult liver. In the majority of samples the most prominent kidney isozyme had an electrophoretic mobility corresponding to that of the c isozyme of liver (Fig. 10). However, in four kidneys from individuals in whom the atypical pH ratio phenotype has been demonstrated in liver, the main kidney ADH isozyme was found to correspond in mobility to that of the variant c' isozyme of liver.
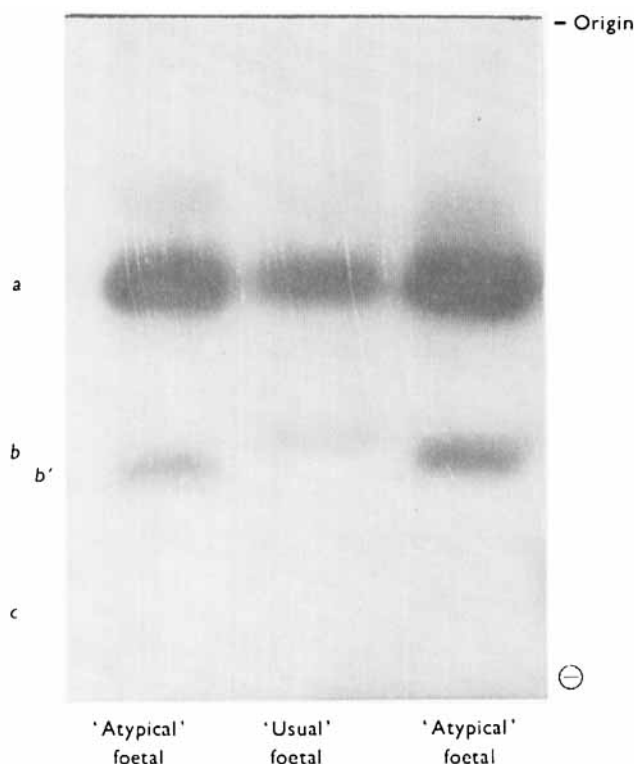

Fig. 7. Photograph of starch gel showing the liver alcohol dehydrogenase isozyme patterns in one foetus in which the lung was found to be of the usual pH ratio phenotype (ii) and in another in which the lung was of the atypical pH ratio phenotype (i) and (iii).

Adult intestinal ADH isozyme patterns have not been examined so far due to a lack of suitable material.

SUMMARY OF ELECTROPHORETIC FINDINGS IN DIFFERENT TISSUES

The main features of the isozyme patterns in different tissues and at different stages of development are illustrated diagrammatically in Fig. 11. Marked developmental changes occur in liver (i–vii) and in kidney (x–xiii), but not in lung (viii + ix). Two phenotypes (usual and atypical) can be clearly distinguished in livers from adults and premature infants and also in lung. Three distinct phenotypes (ADH_3 1, ADH_3 2-1 and ADH_3 2) can be distinguished in kidney and intestine from foetuses and premature infants. Variations in the adult liver pattern (Fig. 11 (v) and (vi)) are also seen and may represent true individual differences.
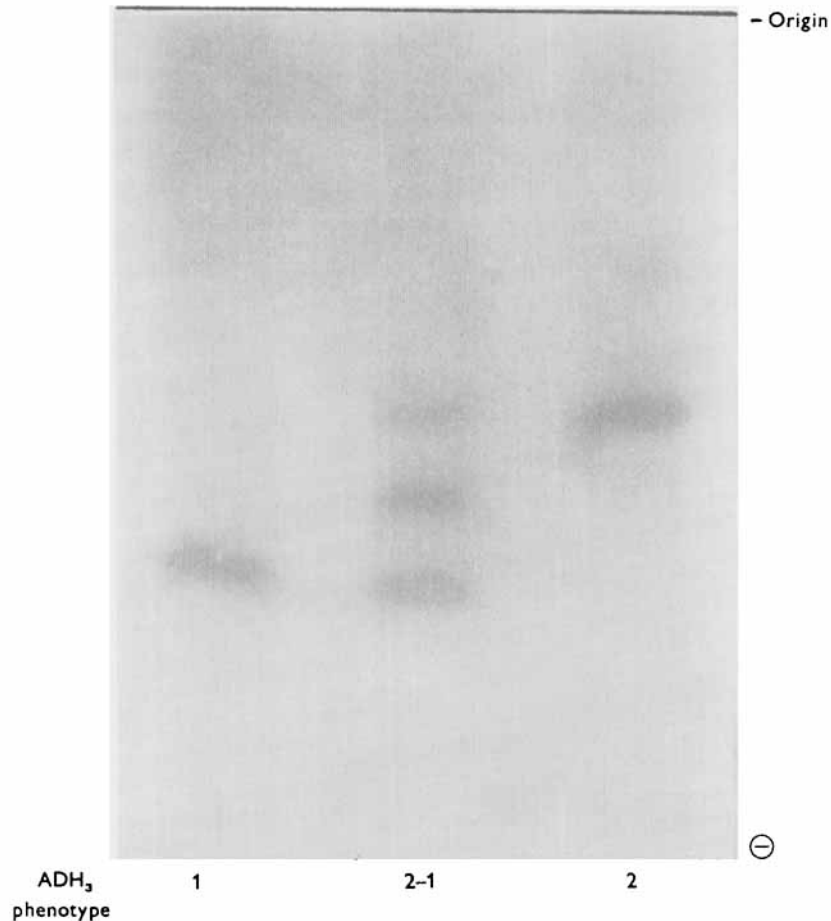
Fig. 8. Photograph of starch gel showing alcohol dehydrogenase isozyme patterns of the three ADH₃
phenotypes—ADH₃ 1, ADH₃ 2-1, and ADH₃ 2—in foetal intestine.

DISCUSSION

In general the findings indicate that there are significant qualitative differences in the alcohol
dehydrogenase which is present at different stages of foetal development in different human
tissues and in different individuals. However, a major difficulty in attempting to analyse the
possible genetical basis of these variations is that appreciable levels of activity have only been
found in tissues such as liver, lung, kidney and intestine, which are unsuitable for carrying out
detailed family studies. Also, in practice the enzyme can only be examined in any extensive
series of different individuals by using autopsy material, or material from therapeutic abortions
carried out in early pregnancy.

Nevertheless, using such materials it has been possible to define certain distinctive tissue
differences, developmental changes and individual differences which cannot be readily accounted
for in terms of post-mortem changes or storage artefacts, and which may reasonably be regarded
as reflecting the true *in vivo* situation. In the present discussion we put forward a genetical
hypothesis which appears to account economically for several of the major variations which
have been established. It will perhaps serve as a useful working hypothesis for further studies.

The most detailed investigations of alcohol dehydrogenase in higher organisms have been

carried out with horse liver as the source of the enzyme. The main horse liver isozyme appears to have a molecular weight of about 80,000 and to be dimeric in structure (Theorell & Bonnichsen, 1951; Theorell, 1967; Jörnvall & Harris, 1970). Evidence has also been obtained which suggests that adult human-liver alcohol dehydrogenase has about the same molecular size as horse-liver alcohol dehydrogenase (von Wartburg, Bethune & Vallee, 1964; von Wartburg et al. 1965) and is also probably dimeric (von Wartburg et al. 1964). The electrophoretic patterns observed in the present work in human liver at various stages of foetal development and in human foetal kidney and intestine are also consistent with the different isozymes each being dimeric.
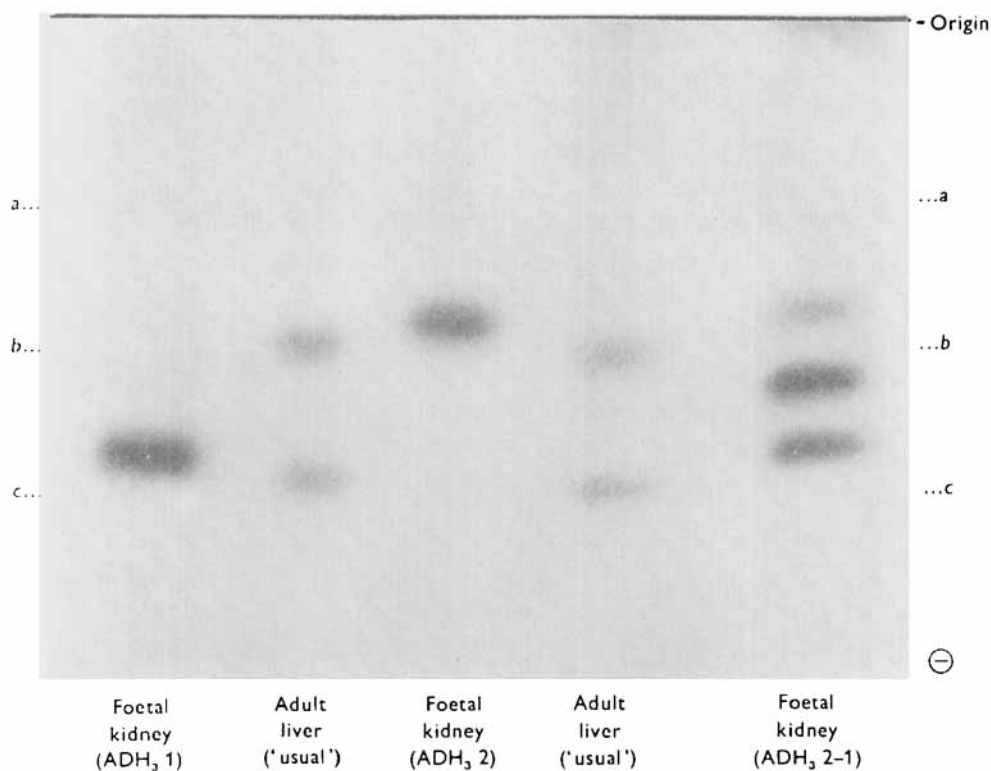

Fig. 9. Photograph of starch gel showing alcohol dehydrogenase isozyme patterns of the three ADH₃ phenotypes ADH₃ 1, ADH₃ 2 and ADH₃ 2-1 in foetal kidney, side by side with a diluted adult liver sample of the usual pH ratio phenotype.

We will therefore assume in the present discussion that each of the main isozymes observed in different human tissues is a dimer, and that each may consist of two identical polypeptide subunits coded by a single allele at a given gene locus, *or* of two non-identical subunits coded by different alleles at the same gene locus, *or* of two non-identical subunits coded by genes at two different loci.

The progressive appearance of the isozymes *a*, *b* and *c* in liver during foetal development can perhaps by analogy with what is thought to occur with certain other enzymes such as lactate dehydrogenase (Fine, Kaplan & Kuftinec, 1963) and aldolase (Rutter et al. 1968), be most simply accounted for by assuming that two different gene loci whose relative activities vary at different stages of development are involved. We will suppose that one of these which we call ADH_1 codes for the amino acid sequence of a polypeptide α, and the other ADH_2 codes for the amino acid

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sequence of a different polypeptide β, such that the isozymes a, b and c have subunit structures α_2, $\alpha\beta$ and β_2 respectively. The relative electrophoretic mobilities of a, b and c are consistent with this.
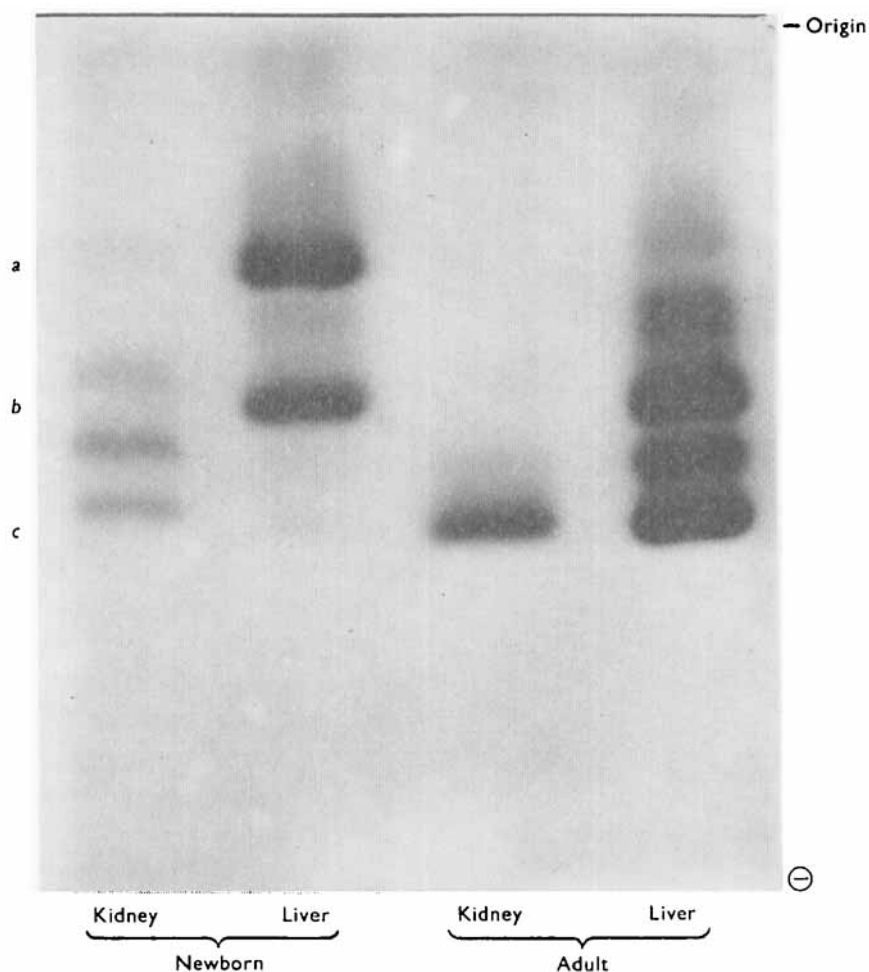

Fig. 10. Photograph of a starch gel showing alcohol dehydrogenase isozyme patterns in liver and kidney samples from an adult and from a premature infant at 36 weeks gestation. Both liver samples were of the usual pH ratio phenotype. The newborn kidney exhibits the ADH_3 2–1 isozyme pattern.

With these assumptions we can infer from the observed electrophoretic patterns that in very early foetal life ADH_1 contributes virtually all the polypeptide subunits present in liver cells, but as development proceeds polypeptide subunits coded by ADH_2 appear in increasing amounts, so that in the course of foetal life the isozyme pattern progressively changes. At the beginning only isozyme a (i.e. α_2) is seen. But later isozyme b ($\alpha\beta$) appears, and subsequently isozyme c (β_2). The successive changes in the relative amounts of the three isozymes in the course of development may in these terms be written as follows:

$$\alpha_2 \text{ only}; \quad \alpha_2 > \alpha\beta; \quad \alpha_2 = \alpha\beta > \beta_2;$$
$$\alpha_2 < \alpha\beta > \beta_2; \quad \alpha_2 < \alpha\beta = \beta_2; \quad \alpha_2 < \alpha\beta < \beta_2.$$

The changes are accompanied by a progressive increase in total activity, and probably go on into the first few years of postnatal life until what can be regarded as the adult pattern of isozyme

activity is established. By this time the *a* isozyme shows very much less activity than either the *b* or *c* isozymes. Thus the systematic changes that occur may be mainly attributable to a progressive increase in the rate of β-chain synthesis, which by the end of the process and then throughout adult life outstrips α chain synthesis. However, the differences in relative activities of the three isozymes may also in part be due to differences in stability or to differences in specific activity.
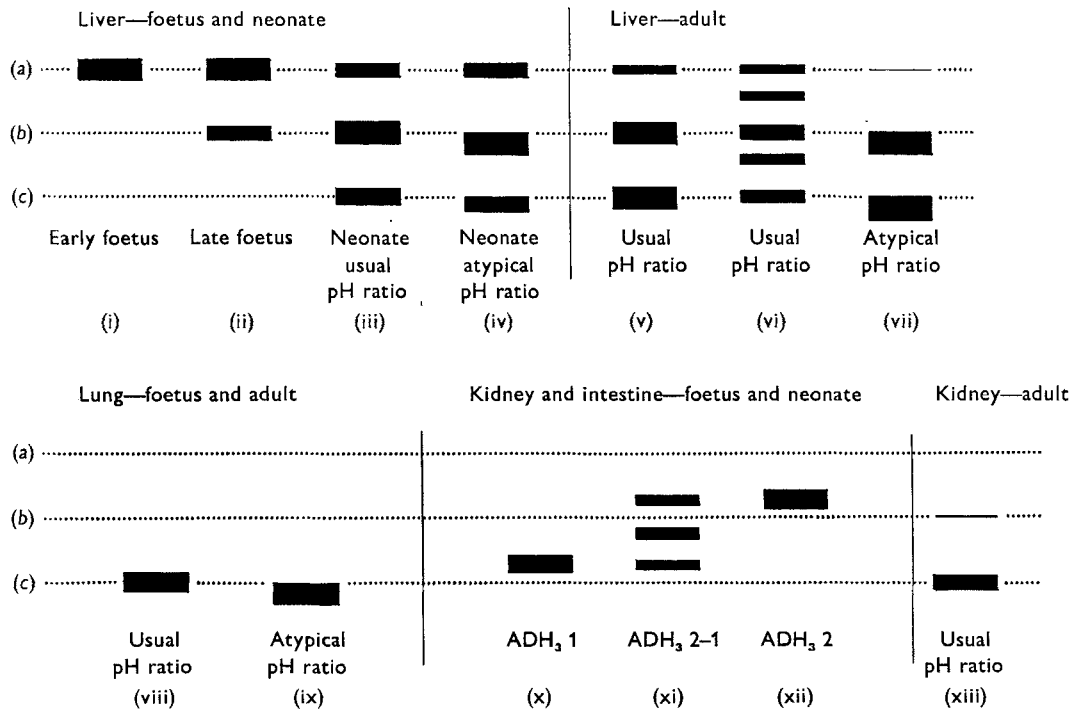

Fig. 11. Diagrammatic representation of the main alcohol dehydrogenase isozymes in liver, kidney, intestine and lung from human foetuses, infants and adults.

Besides the isozymes *a*, *b* and *c* other isozymes with mobilities intermediate between *a* and *b*, or *b* and *c* are also seen in the fully developed liver pattern. They have as a rule less activity than the main isozymes *b* and *c* but often show as much activity or more than isozyme *a*. Also they vary quite considerably in activity from one sample to another, and this may reflect real individual differences. Their occurrence cannot be accounted for simply, without some extension of the general hypothesis. Since they are not seen in early foetal livers when *a* is the principal isozyme present, it is possible that they arise by secondary modifications of the *b* and *c* isozymes. Such secondary *in vivo* changes are thought to occur not uncommonly in other isozyme systems (Harris, 1969). However, they may also represent the polypeptide product of yet another gene locus in hybrid combination with the α and the β polypeptides proposed earlier, the isozyme occurring between *a* and *b* being a dimer containing the additional subunit together with α, and the isozyme occurring between *b* and *c* the additional subunit together with β.

The variant form of liver alcohol dehydrogenase which is characterized by an atypical pH activity ratio has been found to exhibit characteristic electrophoretic alterations of isozymes

b and c but not of a. This suggests that it is due to a variant form of the β subunit presumably determined by a second allele at the ADH_2 locus. If so the alteration in structure must be such as to cause dimers containing the variant subunit to have a slightly greater cathodal electrophoretic mobility than the corresponding dimers containing the usual β subunit. Also the structural difference must lead to a marked alteration of the pH activity profile with considerably enhanced activity at pH 8·8. It may be noted that since in the early foetus most of the alcohol dehydrogenase activity is presumed to be attributable to the α subunit determined by the ADH_1 locus, one would not expect to find early foetal liver samples with characteristic atypical pH activity ratios. In fact out of fifty-six early foetal liver samples in which pH activity ratios were determined, none were found to have a characteristic atypical ratio, although this was seen in 10 % of liver samples from older individuals.

In lung tissue no obvious changes in the qualitative characteristics of alcohol dehydrogenase appear to occur during development. In both foetuses and adults the main electrophoretic zone of activity in the majority of cases was found to have the same electrophoretic mobility as isozyme c of liver, and in the other cases as the variant form of isozyme c present in livers with atypical pH activity ratios. This suggests that in lung tissue the ADH_2 locus predominates throughout life.

There is clearly a very marked difference in early foetuses between the characteristics of the alcohol dehydrogenase in lung and liver. If our interpretation of this in terms of the relative activities of the two loci ADH_1 and ADH_2 is correct, and if as has been suggested earlier the atypical pH activity variant is due to a second allele at the ADH_2 locus, then one would expect that the incidence of the variant in lung samples from young foetuses would be similar to the incidence of the variant found in liver samples from older individuals. In 54 samples of lung from early foetuses 6 were classified as having the variant. This is in reasonable agreement with the finding of 16 examples of the variant in 166 liver samples from older individuals. Combining the two sets of data gives a population frequency for the variant of 0·10 (22 out of 220 samples).

If the atypical pH ratio variant is indeed due to a second allele at the ADH_2 locus, then the question arises as to whether individuals with the phenotype are usually homozygous or heterozygous for this allele. We will call the common allele ADH_2^1 and the allele determining the variant ADH_2^2, and since the incidence of the atypical pH ratio phenotype does not appear to differ significantly between the two sexes we may assume that the locus is autosomal. If the atypical phenotype represents the homozygous genotype $ADH_2^2 ADH_2^2$ then the usual pH ratio phenotype will include both homozygotes $ADH_2^1 ADH_2^1$ and heterozygotes $ADH_2^1 ADH_2^2$. Taking the population incidence of the atypical phenotype as 0·10, the expected allele frequencies would be ADH_2^1 0·68 and ADH_2^2 0·32, and the incidence of genotype $ADH_2^1 ADH_2^1$ 0·46, and of genotype $ADH_2^1 ADH_2^2$ 0·44. Thus nearly half the individuals with the usual phenotype should be heterozygotes.

The usual phenotype in liver has been defined as having a ratio of activity at pH 11·0 to that at pH 8·8 as greater than 1·0. If it is composed of these two genotypes one might expect that the homozygotes $ADH_2^1 ADH_2^1$ would have on average higher pH activity ratios than the heterozygotes $ADH_2^1 ADH_2^2$ even though no clear bimodality may be apparent in the combined distribution. Furthermore, since the low pH ratio in the atypical phenotype is apparently largely due to enhanced activity at pH 8·8 (see Table 1) one would expect that within the usual phenotype samples with lower pH ratios would tend to show on average higher activities at pH 8·8

than samples with higher pH ratios. In other words, within the usual phenotype one would expect to find a negative correlation between pH ratio and activity at pH 8·8.

In Table 5 the average activity of pH 8·8 is shown for a series of liver samples from adults with the usual phenotype grouped according to their pH activity ratios. There appears to be no tendency for the average activity levels to decrease with increasing pH ratio. The correlation coefficient between activity at pH 8·8 and pH ratio was estimated to be $-0·01 \pm 0·09$. Thus the data do not support the idea that the usual phenotype includes both homozygotes and heterozygotes. It might also have been expected that the two genotypes within the usual phenotype

Table 5. *Alcohol dehydrogenase activities at pH* 8·8 *in liver samples obtained from* 118 *adults over* 20 *years old, grouped according to pH activity ratio*

(Only samples classified as having the usual pH ratio phenotype (i.e. pH ratio $> 1·0$) are included.)

pH activity ratio (activity at pH 11/ activity at pH 8·8)	No. of samples	Mean activity at pH 8·8 ± S.D.
1·00–1·49	10	1·143 ± 0·999
1·50–1·99	42	1·381 ± 1·013
2·00–2·49	36	1·467 ± 1·124
2·50–2·99	26	1·245 ± 1·077
3·00–3·49	2	1·087 ± 0·901
3·50–3·99	2	0·560 ± 0·197
Total	118	1·338 ± 1·045

would at least to some extent be distinguishable on the basis of their electrophoretic patterns in the regions of the c and b isozymes, but no indication of such a distinction has in fact been observed.

If, on the other hand, the usual phenotype is composed entirely of homozygotes $ADH_2^1 ADH_2^1$, the atypical phenotype would consist mainly of heterozygotes $ADH_2^1 ADH_2^2$. In this case the frequency of the allele ADH_2^2 would be close to 0·05 and the expected incidence of homozygotes $ADH_2^2 ADH_2^2$ would be roughly 1 in 400 individuals. On this basis only about 1 in 40 of individuals classified as having the atypical phenotype would be homozygotes $ADH_2^2 ADH_2^2$. Such homozygotes might be expected to have an average level of activity nearly twice that found in the atypical phenotype as a whole. They would also be expected to show on average lower pH activity ratios. Rough calculations based on the data given in Table 1 suggest that the pH activity ratio in such homozygotes would be in the region 0·15–0·20. No liver sample clearly exhibiting these characteristics has in fact been found among the atypical phenotype samples so far identified. However, in view of the wide range of activities observed both at pH 8·8 and pH 11·0 among samples with the atypical phenotype, it would clearly be difficult to identify such a homozygote with confidence by these criteria. Furthermore, since its predicted incidence in the general population is only about 1 in 400 it is quite likely that no such sample occurred in the present survey.

If, however, individuals with the atypical phenotype are usually heterozygous ($ADH_2^1 ADH_2^2$) this should be reflected in the electrophoretic pattern. If we call the enzyme subunit presumed to be determined by ADH_2^1, β^1, and the variant enzyme subunit determined by ADH_2^2, β^2, then we would expect that the single c isozyme ($\beta^1 \beta^1$) of the usual phenotype would be replaced in the atypical phenotype in liver and in lung by the three isozymes $\beta^1 \beta^1$, $\beta^1 \beta^2$ and $\beta^2 \beta^2$. Furthermore

since β^2 is evidently associated with enhanced activity at pH 8·8, one would expect that $\beta^2\beta^2$ would show considerably more activity than $\beta^1\beta^1$ (possibly as much as a tenfold increase), and that $\beta^1\beta^2$ would be intermediate in activity as well as in electrophoretic mobility. One would also expect in the atypical phenotype in liver to find the usual b isozyme ($\alpha\beta^1$) replaced by two isozymes $\alpha\beta^1$ and $\alpha\beta^2$, the latter having more activity than the former. However, in the most cathodal position only a single zone of activity moving slightly more cathodally than the 'usual' isozyme c is seen in liver and in lung with the atypical phenotype. In liver in the isozyme 'b' position one also sees only a single zone moving slightly more cathodally than the 'usual' isozyme b, although in occasional samples resolution into two zones with the expected relative mobilities and activities can occasionally be observed.

Table 6. *Distribution of alcohol dehydrogenase (ADH_3) phenotypes in kidney and intestine from* 117 *foetuses and premature infants*

Phenotype	No. observed	No. expected
ADH$_3$ 1	49	46·4
ADH$_3$ 2–1	49	54·6
ADH$_3$ 2	19	16·0
Total	117	117·0

(The expected numbers are calculated assuming a Hardy–Weinberg equilibrium and allele frequencies: $ADH_3^1 = 0.63$ and $ADH_3^2 = 0.37$.)

It is possible that the failure to identify the three postulated isozymes in the c position may be due to their differing so slightly in their relative mobilities that they are not resolved into separate bands by the electrophoretic system used. The marked differences in relative activity expected would also tend to make their resolution very difficult. Similarly the failure to observe a consistent separation of the postulated two isozymes in the b region of the electrophoretic pattern may also be due to inadequate resolving power of the electrophoretic system.

The alcohol dehydrogenase isozymes found in kidney and intestine of foetuses and premature infants differ in a characteristic manner from the isozymes in liver and in lung. Furthermore the samples from different individuals fall quite clearly into three distinct phenotypes. The findings are most simply accounted for by postulating a third alcohol dehydrogenase locus ADH_3, at which two relatively common alleles ADH_3^1 and ADH_3^2 occur. The three phenotypes have been referred to as ADH$_3$ 1, ADH$_3$ 2–1 and ADH$_3$ 2. If we assume that the allele ADH_3^1 determines an enzyme subunit γ^1, and ADH_3^2 a structurally different subunit γ^2; then the single isozyme seen in ADH$_3$ 1 will have the subunit structure $\gamma^1\gamma^1$, the single isozyme seen in ADH$_3$ 2 the subunit structure $\gamma^2\gamma^2$ and the three isozymes seen in ADH$_3$ 2–1 the structures $\gamma^1\gamma^1$, $\gamma^1\gamma^2$ and $\gamma^2\gamma^2$ respectively.

Among 117 different foetuses or premature infants in which kidney and/or intestine were examined electrophoretically, phenotype ADH$_3$ 1 was found in 49 cases, phenotype ADH$_3$ 2–1 in 49 cases, and phenotype ADH$_3$ 2 in 19 cases. In any one individual the kidney and intestinal samples always showed the same phenotype. From these phenotype frequencies a direct estimate of the frequencies of the postulated alleles can be obtained. They are for ADH_3^1 0·63 and for ADH_3^2 0·37. It will be seen from Table 6 that these allele frequencies give a reasonable fit between

the observed incidence of the three phenotypes and the expected incidence assuming a Hardy–Weinberg equilibrium.

In some of the samples of foetal kidney and intestine very weak bands corresponding in mobility to isozyme *a* (in young foetuses) or to isozymes *b* and *c* (in premature infants) were also detected in addition to the principal isozymes characteristic of the three phenotypes discussed above. But they were relatively much less prominent. Thus locus ADH_3 activity appears to predominate in kidney and intestine in foetal life. However, after birth the isozymes attributed to ADH_3 diminish, at least in kidney, and in the adult kidney the electrophoretic patterns resemble those found in liver, though the activity level is usually extremely low.

In those kidney and intestinal samples from foetuses and premature infants in which the ADH_3 isozymes were seen along with small amounts of isozymes *a*, *b* or *c*, there was no indication in the electrophoretic patterns of isozymes with mobilities to be expected from hybrid forms such as $\alpha\gamma$ or $\beta\gamma$. This suggests that *either* the polypeptide products of ADH_3 (γ^1 and γ^2) do not combine to form hybrid isozymes with the α or β products of ADH_1 and ADH_2, *or* that locus ADH_3 is active in different cells from ADH_1 and ADH_2 in kidney and intestine, so that the products of the loci cannot associate to give dimers.

Thus in essence the hypothesis proposes that there are at least three different loci coding for the polypeptide sequences of alcohol dehydrogenases in man. In liver ADH_1 is the most active in early foetal life, but ADH_2 progressively increases in activity during development so that it becomes relatively more prominent in late foetal life, and is eventually very much more active than ADH_1. In lung ADH_2 predominates both in early foetal life and later. In kidney ADH_3 predominates before birth, but in postnatal life it appears to be much less active and the alcohol dehydrogenase present is derived mainly from ADH_2. In foetal intestine ADH_3 predominates; however, it is not yet clear whether ADH_3 persists after birth. At both the ADH_2 and ADH_3 loci two common alleles are present in European populations so that person to person differences in the corresponding isozymes occur.

Detailed studies on the structural characteristics and other properties of the various isozymes should enable us to test many of the facets of the hypothesis, despite the difficulties imposed by the absence of family data and by not being able to follow single individuals through different stages of development.

SUMMARY

1. Human alcohol dehydrogenase (ADH) has been investigated by spectrophotometric assay and by starch-gel electrophoresis.

2. Assays were carried out at pH 8·8 and pH 11·0 on liver samples obtained *post mortem* from 129 adults over the age of 20, 37 premature infants and infants less than one year old and 56 foetuses. Sixteen cases of the previously described atypical pH ratio phenotype were identified among the 166 adults and infants tested. No examples of the atypical pH ratio phenotype were encountered among the foetuses. On average the foetal liver ADH activity was less than in adults and it appeared to increase with increasing gestational age.

3. Electrophoretic analyses of ADH in liver samples obtained from 117 foetuses of various gestational ages, 62 premature infants and infants less than a year old and a group of more than 200 adults over the age of 20, indicate that developmental changes occur during intra-uterine life.

4. The atypical pH ratio phenotype liver ADH isozyme pattern was found to be electrophoretically different from that of the usual pH ratio phenotype.

5. The ADH isozyme pattern in lung tissue was the same in adults, infants and foetuses. The overall activity was low and mainly concentrated in a single isozyme which was electrophoretically indistinguishable from the main ADH isozyme of adult liver. Usual and atypical pH ratio phenotypes were identified, both by assay and by starch-gel electrophoresis, in foetal, infant and adult lung specimens.

6. The ADH activity of kidney and intestine was too weak for assay with ethanol as substrate. In adults the isozyme patterns in kidney were similar to those found in adult liver. In foetal intestine and kidney, however, the ADH isozymes were quite different from those of adult liver and also foetal liver. Three distinct phenotypes, designated ADH_3 1, ADH_3 2-1 and ADH_3 2, were recognized in foetal kidney and intestine, occurring with frequencies of 0·42, 0·42, and 0·16 respectively in a survey of 117 specimens.

7. The appearance of the ADH isozyme patterns in liver and in foetal kidney and intestine is consistent with the hypothesis that ADH has a dimeric subunit structure.

8. The findings suggest that at least three autosomal gene loci may be concerned in determining the structure of alcohol dehydrogenase in man.

(a) Locus ADH_1 – primarily active in the liver in early foetal life, becoming less active during gestation and only weakly active during adult life.

(b) Locus ADH_2 (i) expressed in lung in early foetal life and remaining active in this tissue throughout life, (ii) active in liver after about the first trimester and gradually becoming more active so that in adults this locus is responsible for most of the liver ADH activity, (iii) also active in adult kidney, (iv) the atypical pH ratio phenotype is probably determined by a variant allele at the ADH_2 locus.

(c) Locus ADH_3 – active during foetal and early post-natal life in intestine and kidney. The variant phenotypes ADH_3 1, ADH_3 2-1 and ADH_3 2 are thought to represent the genotypes $ADH_3^1 ADH_3^1$, $ADH_3^1 ADH_3^2$ and $ADH_3^2 ADH_3^2$ respectively, where ADH_3^1 and ADH_3^2 are alleles at the ADH_3 locus. The gene-frequency estimate of ADH_3^1 is 0·63 and of ADH_3^2 is 0·37.

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Note added in proof

Recently we have been able to study ADH in homogenates of fresh post mortem adult stomach. Prominent ADH_3 activity was detected and examples of the three ADH_3 phenotypes (1, 2–1 and 2) were encountered with about the same frequencies as in the foetal intestinal and kidney samples. ADH_1 and ADH_2 activity was also often detected in the stomach but these isozymes were generally much weaker than the ADH_3 isozymes.

The liver corresponding with each stomach has also been examined and detailed analysis and comparison of the liver and stomach isozyme patterns suggests that the ADH_3 locus is in fact active in adult liver. However the ADH_3 activity appears to be demonstrable mainly in the form of hybrid isozymes, containing a polypeptide derived from ADH_3 with a polypeptide derived from either ADH_2 or ADH_1.

The occurrence of ADH_3 activity in adult liver can account for the individual variation in the adult liver ADH isozyme patterns which was described briefly in the text and illustrated in figures 4 and 11, since the occurrence of liver isozymes presumed to represent ADH_1/ADH_3 and ADH_2/ADH_3 hybrids is precisely what would be expected from the ADH_3 phenotypes observed in the corresponding stomachs.