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Journal

Annals of Human Genetics, 36(4)

ISSN

0003-4800

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Publication Date

1973-04-01

DOI

10.1111/j.1469-1809.1973.tb00604.x

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**Studies on the subunit structure and molecular
size of the human alcohol dehydrogenase isozymes determined
by the different loci, ADH_1 , ADH_2 , and ADH_3**

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

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It has been proposed (Smith, Hopkinson & Harris 1971, 1972) that human alcohol dehydrogenase (ADH) is determined by three separate structural gene loci, designated ADH_1 , ADH_2 and ADH_3 , which code for structurally different polypeptides α , β and γ respectively. Evidence has been obtained for the occurrence of two common alleles at each of the ADH_2 and ADH_3 loci, designated ADH_2^1 and ADH_2^2 and ADH_3^1 and ADH_3^2 , which code for structurally distinct forms of the corresponding polypeptides (β^1 , β^2 and γ^1 , γ^2 respectively). According to the hypothesis the isozymes of human ADH are dimers and any one particular isozyme may be homodimeric, consisting of two identical polypeptides (e.g. $\alpha\alpha$, $\beta^1\beta^1$, $\gamma^1\gamma^1$, etc.) coded by a specific allele at one of the loci, or heterodimeric, consisting of two non-identical polypeptides (e.g. $\alpha\beta^1$, $\alpha\gamma^1$, $\alpha\gamma^2$, etc.) coded by alleles at separate loci or heterodimeric but coded by different alleles at the same locus (e.g. $\gamma^1\gamma^2$).

The isozymes actually observed after electrophoresis of tissue extracts appear to depend on the relative activities of the three loci and they vary from tissue to tissue and also with development. Thus, in liver, the homodimeric $\alpha\alpha$ isozyme consisting of ADH_1 polypeptides predominates in early foetal life but later ADH_2 polypeptides appear and the heterodimeric $\alpha\beta$ and the homodimeric $\beta\beta$ isozymes can be detected. During the first year of post-natal life ADH_3 polypeptides also appear and a complex mixture of homodimeric ($\alpha\alpha$, $\beta\beta$ and $\gamma\gamma$) and heterodimeric ($\alpha\beta$, $\alpha\gamma$ and $\beta\gamma$) isozymes can be detected in liver from children and adults. In contrast, the isozyme pattern of lung tissue is simple in both foetal and adult samples and consists only of β polypeptides determined by the ADH_2 locus. In kidney and gastro-intestinal tract the ADH_3 polypeptides predominate in early foetal life and the homodimeric isozymes $\gamma^1\gamma^1$ and $\gamma^2\gamma^2$ can be readily seen in the respective homozygotes, while in the heterozygotes (ADH_3 2-1) they occur together with the hybrid heterodimeric $\gamma^1\gamma^2$ isozyme. In adult gastro-intestinal tract specimens the ADH isozymes determined by the ADH_3 locus also predominate but traces of the ADH_2 type isozymes are seen as well. In adult kidney it has now been found by careful homogenization of ice-cold post-mortem tissue that isozymes determined by the ADH_3 locus do occur, contrary to what was thought earlier (Smith *et al.* 1971) but most of the total ADH activity can be attributed to the ADH_2 locus.

The present paper is principally concerned with the results of *in vitro* hybridization experiments involving the dissociation and recombination of the postulated subunits of the different ADH isozymes. The findings support the hypothesis outlined above. In addition, we describe experiments using gel filtration column chromatography to determine the molecular size of the various isozymes and the separation of the individual isozymes by ion exchange chromatography on carboxymethyl (CM) cellulose.

MATERIALS AND METHODS

The sources of the tissues used in this study have been previously described (Smith *et al.* 1971, 1972).

Dissociation and recombination experiments. The method used in these experiments is simple and is based on the procedure devised by Hart (1971) for *in vitro* 'hybridization' experiments on wheat ADH. In this method dissociation and recombination of subunits is achieved by freezing and thawing samples in the presence of 1 M-NaCl, 0.4 M sucrose and 0.1 M 2-mercaptoethanol in 0.1 M sodium phosphate buffer, pH 7.0. The salt is then removed by dialysis. Experiments were carried out using crude tissue extracts prepared by homogenizing the tissue with an equal volume of the salt, sucrose, mercaptoethanol, phosphate mixture, pH 7.0, in a Silverson mixer emulsifier. Appropriate mixtures of the homogenates were prepared and were then left in a deep freeze at -20°C for about 17 hr. After thawing, dialysis was carried out for 4 hr. against the phosphate buffer, pH 7.0, containing sucrose (0.4 M) and mercaptoethanol (0.1 M) and for a further 4 hr. against 0.005 M tris-phosphate buffer, pH 7.0, containing 0.002 M NAD. The specimens were then examined by starch-gel electrophoresis immediately. A number of control samples, with and without salt, were prepared in parallel with each experiment, subjected to the same procedure and finally analysed by electrophoresis alongside the test material. Experiments were also done using the same technique with partially purified isozymes prepared by CM chromatography of liver extracts. For this purpose 50:50 mixtures of the sample and the salt-sucrose-mercaptoethanol phosphate buffer solution were made and then subjected to the freeze-thaw dialysis procedure described above.

Gel filtration chromatography. Experiments were carried out using Biogel P 150 (Calbiochem) in a water-jacketed column, 100×2.5 cm. The column and fraction collector were used in a cold room at 4°C .; the water circulating around the column was maintained at a constant 8°C . The Biogel P 150 was swollen in a large excess of sterile 0.005 M tris-phosphate buffer, pH 7.7, containing 0.1 M-NaCl, for 48 hr. at 4°C . The column was poured and allowed to settle overnight; then buffer was pumped in an upward direction through the column at a rate of about 20 ml./hr. for approximately 8 hr. During this period the sample was prepared by homogenizing 30–40 g tissue in 15–20 ml. of the column buffer (0.005 M tris-phosphate, pH 7.7). In some experiments pooled material of the same phenotype was used. The homogenate was centrifuged at 90,000 g for 45 min. and the supernatant then concentrated using Aquacide (Calbiochem) to about 3 ml. This was then applied to the bottom of the column, together with 10 mg. myoglobin, 20 mg. ovalbumin and 5 mg. Blue Dextran (Pharmacia Ltd.) as markers. The buffer was pumped through at about 20 ml./hr. and fractions were collected at 10 min. intervals. The fractions were tested for ADH activity by the spectrophotometric assay procedure described by Von Wartburg, Papenberg & Aebl (1965) as detailed by Smith *et al.* (1971) and active fractions were examined by starch-gel electrophoresis at pH 8.6 (Smith *et al.* 1971). The positions of the marker proteins, ovalbumin and myoglobin in the eluate were determined by spectrophotometric scanning at 280 m μ . and by starch-gel electrophoresis using the discontinuous buffer system of Poulik (1957). Several enzymes, peptidases A, B, C, D and S (Lewis & Harris, 1967; Rapley, Lewis & Harris, 1971), lactate dehydrogenase (Bergmeyer, Bernt & Hess, 1963) and nucleoside phosphorylase (Kalckar, 1947; Edwards, Hopkinson & Harris, 1971) present in the original extract, were used as markers and were located in the eluate by direct assay and electrophoresis.

Ion-exchange chromatography. Chromatography was carried out in a cold room at 4° C. using carboxymethyl (CM) cellulose (Whatman CM 52), in glass columns 40 × 2.5 cm., and the starting buffer was 0.005 M tris-phosphate, pH 7.7, containing 2-mercaptoethanol (2 mM) and NAD (1 mM). These additives were found to improve the recovery of ADH activity and the resolution of the isozymes. Thirty to forty g. of liver was homogenized in 30–40 ml. starting buffer and the debris was spun down at 45,000 *g* for 2 hr. The clear supernatant was dialysed against an excess of the same buffer and centrifuged again at 45,000 *g* for 1 hr. This material was applied to the column, followed by 300–400 ml. of the starting buffer at a flow rate of about 30 ml./hr. Five ml. fractions were collected and assayed for ADH activity but no ADH was detected during this phase. Suitable conditions for elution were devised by experimentation. At first a linear saline gradient (0–0.5 M-NaCl in 500 ml. starting buffer) was used but in later experiments the conditions were modified until eventually it was found that a two-stage procedure gave the best separation of the ADH isozymes. The first stage of elution was carried out with 0.008 M-NaCl and the second stage was done with 0.02 M-NaCl in the starting buffer. Five ml. fractions were collected during each phase and the ADH activity was assayed spectrophotometrically (Von Wartburg *et al.* 1965; Smith *et al.* 1971). The active fractions were also examined by starch-gel electrophoresis (Smith *et al.* 1971). Each step in the elution phase was continued until after the ADH activity in the fractions had subsided to zero. Application of a final higher salt concentration (up to 0.04 M) did not result in the elution of further activity.

RESULTS

Molecular-weight determinations

A representative selection of tissues was examined by gel filtration chromatography on Biogel P 150. This included infant and adult liver, infant kidney and adult gastric mucosa extracts.

In each experiment ADH activity was detected in the eluted fractions as a single symmetrical peak, with a molecular weight of about 80,000. Analysis of the individual fractions by starch gel electrophoresis showed that all the isozymes were eluted together and are therefore of similar molecular weight, irrespective of their subunit composition. The results are summarized in Table 1.

In some experiments with liver extracts it was noticed that the $\alpha\alpha$ isozyme, though prominent in the original extract, was often very weak and sometimes not detected in the eluate from the column, though the α containing heterodimeric isozymes, $\alpha\beta$ and $\alpha\gamma$ remained relatively strong in staining intensity. This suggests that the $\alpha\alpha$ isozyme is relatively less stable under the conditions used for chromatography than most other ADH isozymes; a conclusion which is supported by direct studies on the relative thermal stabilities of the ADH isozymes (Smith *et al.* in preparation). Another interesting observation concerning the relative stabilities was made in an experiment using a liver extract of the atypical pH ratio phenotype. Here the total ADH activity recovered in the fractions was only 25 % of the original, compared with recoveries of between 90 % and 75 % from experiments carried out with livers of the usual pH ratio phenotype. This suggests that the isozymes containing the polypeptide determined by the variant ADH_2^2 ('atypical') allele are less stable than the others.

Table 1. *Molecular-weight estimates of ADH isozymes in various human tissues*

(Isozymes shown in parentheses were relatively weak in starting material.)

Tissue	Isozymes	Estimated mol. wt.
Liver: infant	$\alpha\alpha, \alpha\beta^1 (\beta^1\beta^1)$	79,000
Liver Adult, usual pH ratio ADH ₃ 2	$\alpha\alpha, \alpha\beta^1, \beta^1\beta^1$ $\gamma^2\gamma^2, \alpha\gamma^2, \beta^1\gamma^2$	83,000
Liver Adult, atypical pH ratio ADH ₃ 1	$\alpha\alpha, \alpha\beta^1, \alpha\beta^2$ $\gamma^1\gamma^1, \alpha\gamma^1, \beta^1\gamma^1, \beta^2\gamma^1$ and the 'atypical' β isozyme	83,000
Gastric mucosa: pooled sample (all ADH ₃ 2-1)	$\gamma^1\gamma^1, \gamma^1\gamma^2, \gamma^2\gamma^2$	82,000
Mixture of infant tissues: liver and kidney (ADH ₃ 1)	$\alpha\alpha, \alpha\beta^1, (\beta^1\beta^1)$ $\gamma^1\gamma^1, \alpha\gamma^1, \beta^1\gamma^1$	80,000

CM cellulose chromatography

All these experiments were carried out using liver extracts.

(i) *Foetal and infant liver, usual pH ratio.* Three isozymes ($\alpha\alpha, \alpha\beta^1, \beta^1\beta^1$) characteristic of these liver extracts were cleanly separated by CM cellulose chromatography. Using an infant liver extract, for example, three individual peaks of activity were detected in the eluate (Fig. 1*a*) which corresponded with $\alpha\alpha, \alpha\beta^1$ and $\beta^1\beta^1$ respectively (Fig. 1*b*). The order of elution of the isozymes was that expected from starch-gel electrophoresis at pH 8.6: $\alpha\alpha$ followed by $\alpha\beta^1$ then $\beta^1\beta^1$. Comparison of the elution profiles obtained in a series of experiments in which liver from different infants and foetuses was used showed that in early foetuses the first peak ($\alpha\alpha$) was relatively much larger than the second ($\alpha\beta^1$) and third ($\beta^1\beta^1$) peaks but in older foetuses and young infants the second and third peaks were relatively more prominent.

(ii) *Adult liver, usual pH ratio, ADH₃ 2.* A more complex elution profile was obtained on CM cellulose chromatography of adult liver samples of the ADH₃ 2 phenotype. Up to six separate peaks of activity were obtained which corresponded with maximum elution of the isozymes $\gamma^2\gamma^2, \alpha\gamma^2, \beta^1\gamma^2, \alpha\alpha, \alpha\beta^1$ and $\beta^1\beta^1$ respectively. Results from a representative experiment are shown in Fig. 2*(a)* and *(b)*. It will be seen that in addition to the isozymes mentioned above, several other minor zones were detected in the fractions. These minor bands were most prominent in the fractions exhibiting $\alpha\gamma^2, \beta^1\gamma^2$ and $\alpha\beta^1$ activity and are believed to be 'secondary' isozymes. They correspond in electrophoretic mobilities with similar minor isozymes demonstrable after electrophoresis of crude liver extracts, particularly in samples which have been stored for 48 hr. or longer at 4° C. Similar minor bands will be noted in the other photographs shown in this paper and it seems that secondary isozymes can be formed from almost all of the main ADH components. One easily recognized minor zone is seen in crude liver homogenates cathodal to, and believed to be derived from, the $\beta\beta$ zone. However, this particular component does not seem to occur in samples containing 2-mercaptoethanol and this reagent was therefore added routinely to the buffers used for chromatography. Another confusing extra isozyme often seen in crude liver extracts (e.g. in Fig. 2*b*) can be attributed to the LDH 5 isozyme, since this is active in the presence of NAD even when the stain mixture does not contain the lactate substrate.

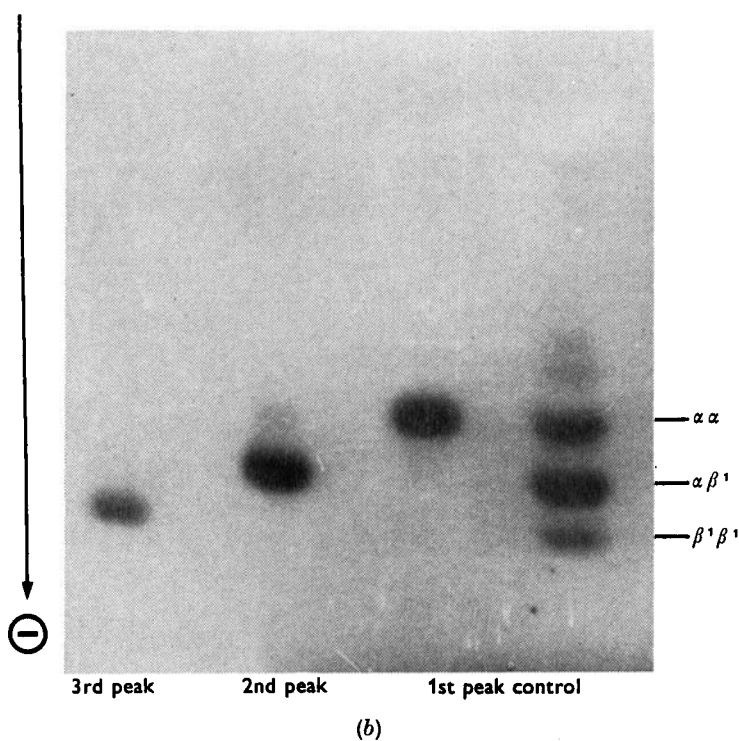
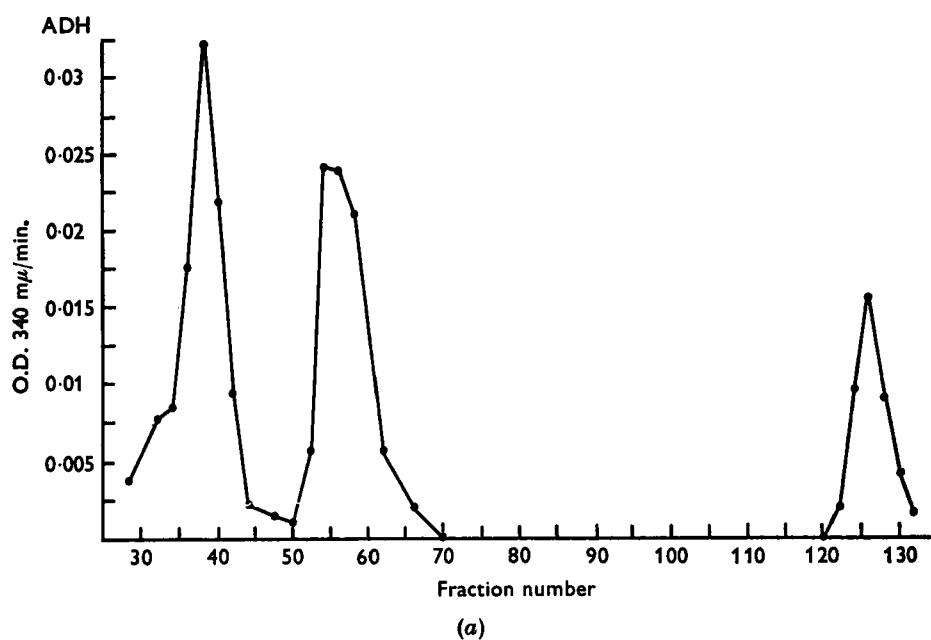


Fig. 1. (a) Elution of ADH activity from a CM cellulose column (source of enzyme: infant liver). (b) Photograph of starch gel showing ADH isozymes present in fractions of the three peaks of ADH activity which are obtained from CM chromatography of an infant liver (Fig. 1a). Electrophoresis at pH 7.7.

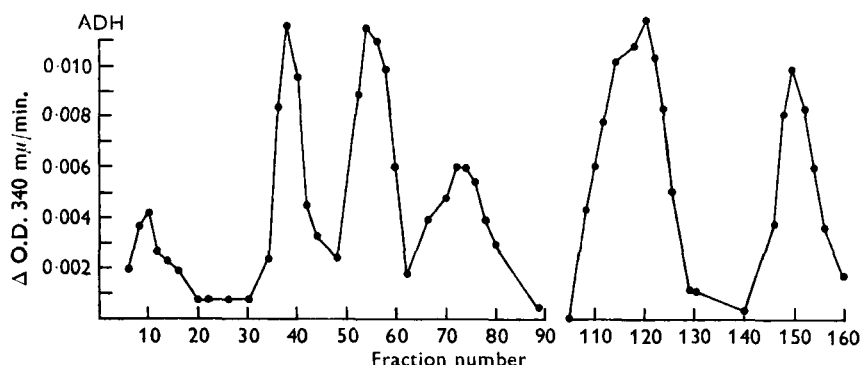


Fig. 2(a). Elution of ADH activity from a CM cellulose column. Source of enzyme: adult liver of the ADH_3 2 phenotype, usual pH ratio.

(iii) *Adult liver, usual pH ratio, ADH_3 1.* Only three peaks of ADH activity were detected in the eluate from experiments using samples of the ADH_3 1 phenotype (Fig. 3a). Electrophoresis (Fig. 3b) showed that the first peak contained a mixture of the $\gamma^1\gamma^1$, $\alpha\gamma^1$ and $\alpha\alpha$ isozymes and the second peak contained both the $\beta^1\gamma^1$ and the $\alpha\beta^1$ isozymes. However, the third peak was homogeneous and displayed only $\beta^1\beta^1$ isozyme activity. The relative sizes of the three peaks varied considerably in different experiments according to the relative contributions of the various isozymes to the total activity of the liver sample used.

(iv) *Adult liver, atypical pH ratio, ADH_3 1.* Three peaks of ADH activity were also detected in the eluate obtained from chromatography of atypical pH ratio phenotype liver samples of the ADH_3 1 type but the shape of the elution curve was quite distinctive (Fig. 4). The third peak (fractions 140–170 in Fig. 4) was relatively much larger than the corresponding peak of activity obtained from chromatography of the usual pH ratio phenotype liver samples of the ADH_3 1 type.

Electrophoretic analysis revealed that the first peak (fractions 10–40) exhibited the $\alpha\gamma^1$ isozyme only, no $\alpha\alpha$ or $\gamma^1\gamma^1$ isozymes were detected; the second peak (fractions 40–90) showed the two isozymes thought to represent $\alpha\beta^2$ and $\beta^2\gamma^1$ but the $\alpha\beta^1$ and $\beta^1\gamma^1$ isozymes usually seen in electrophoresis of whole liver extracts of this phenotype could not be detected; the third peak exhibited a single isozyme with the same mobility as the most cathodal component of the atypical ADH_3 1 livers. The subunit structure of this component, which is characteristic of the atypical pH ratio phenotype, is still uncertain but is possibly $\beta^1\beta^2$.

Dissociation and recombination experiments

The aim of these experiments was to cause dissociation of the isozymes into their respective subunits and then allow the subunits to recombine at random to see whether new isozymes were formed. Thus mixtures of postulated homodimeric isozymes – for example, $\alpha\alpha$ and $\beta^1\beta^1$ – treated appropriately would be expected to generate an additional heterodimeric isozyme, in this example $\alpha\beta^1$. Vice versa, a single heterodimeric isozyme should be capable of generating the two corresponding homodimeric isozymes.

(i) *Isozymes $\alpha\alpha$, $\alpha\beta^1$, $\beta^1\beta^1$.* A number of experiments were carried out with crude tissue extracts, using early foetal liver as a source of the $\alpha\alpha$ isozyme and foetal lung as a source of $\beta^1\beta^1$. Treatment of a mixture of these two tissues in the manner described in the methods section led to the

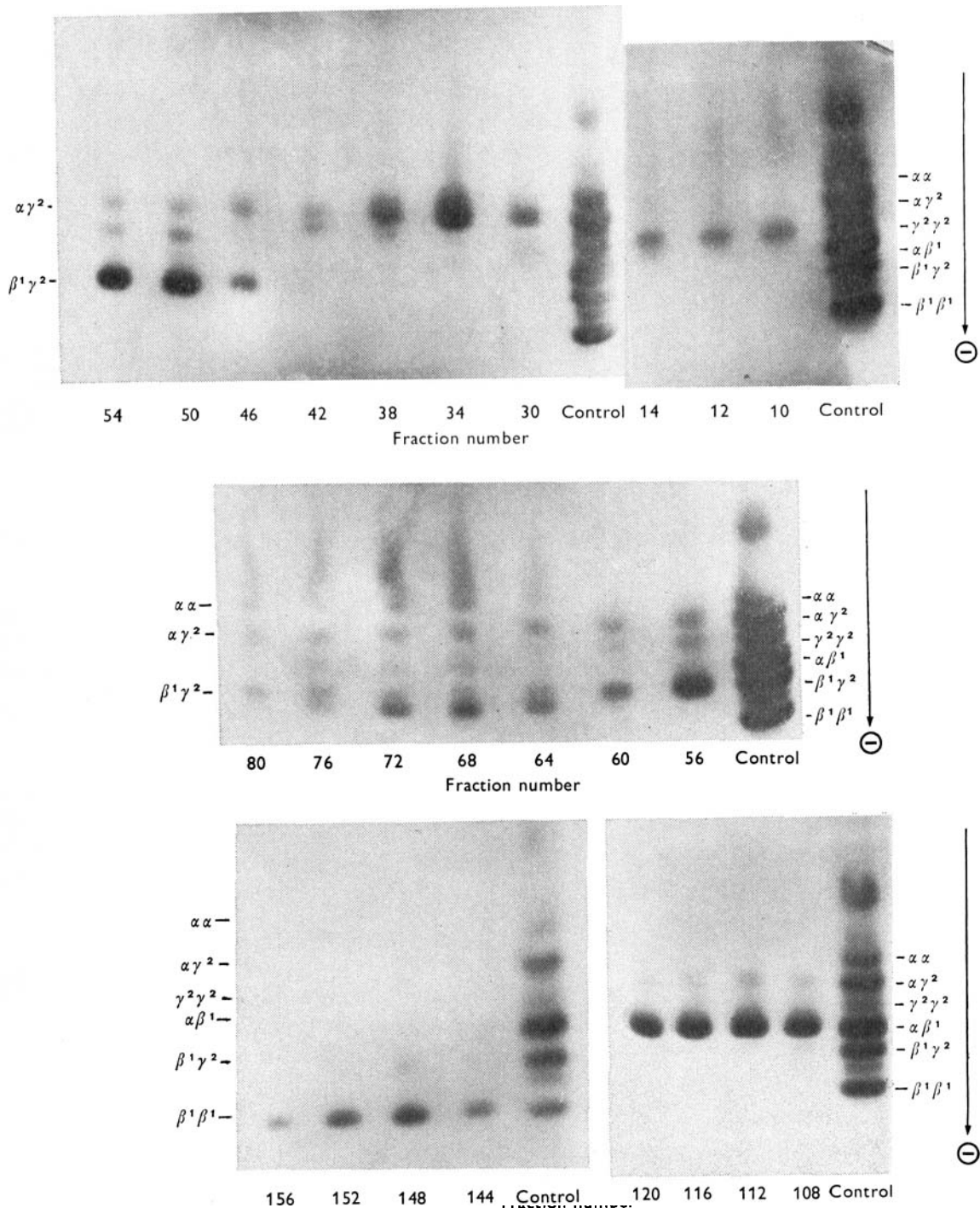


Fig. 2(b). Photographs of starch gels showing ADH isozymes present in the fractions obtained on CM chromatography of an adult liver of the ADH₂ phenotype, usual pH ratio (Fig. 2a). Electrophoresis at pH 8.6.

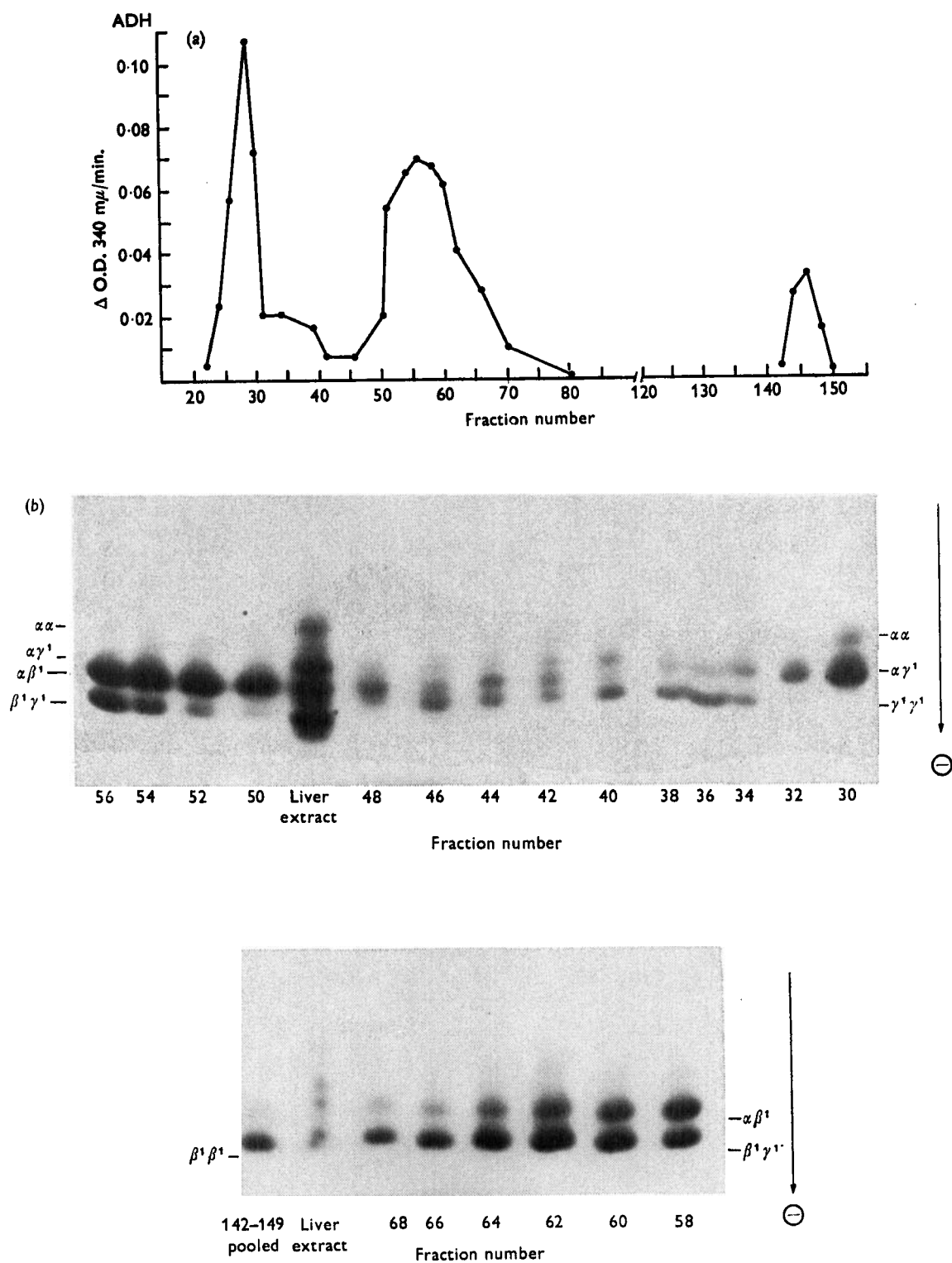


Fig. 3. (a) Elution of ADH activity from a CM cellulose column. Source of enzyme: adult liver of the ADH_3 1 phenotype, usual pH ratio. (b) Photographs of starch gels showing ADH isozymes present in the fractions obtained on CM chromatography of an adult liver of the ADH_3 1 phenotype, usual pH ratio (Fig. 3a). Electrophoresis at pH 7.7.

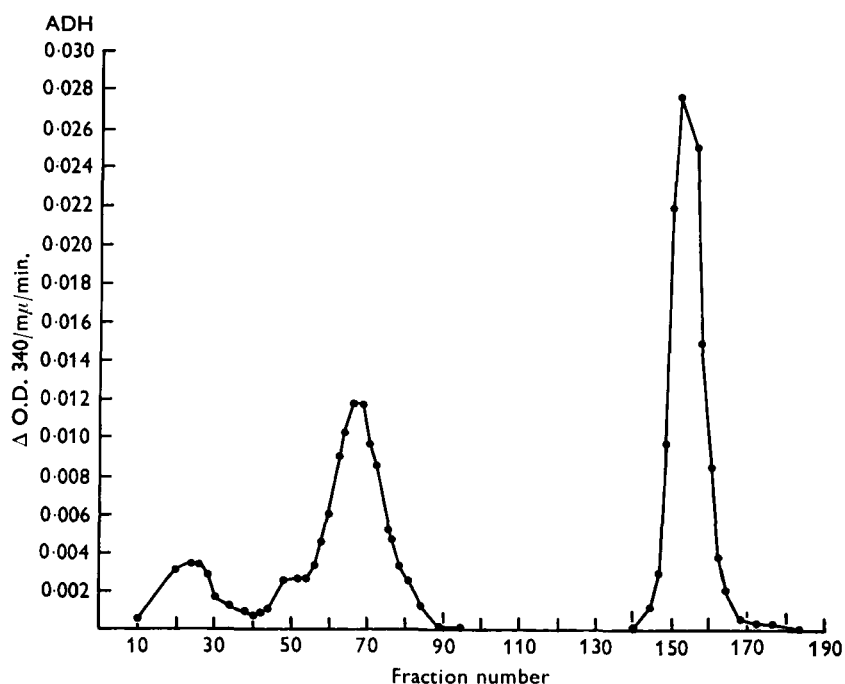


Fig. 4. Elution of ADH activity from a CM cellulose column. Source of enzyme: adult liver of the ADH₃ 1 phenotype, atypical pH ratio.

appearance of a new isozyme with the same electrophoretic mobility as the $\alpha\beta^1$ isozyme of infant liver. Similarly, experiments with mixtures of $\alpha\alpha$ and $\beta^1\beta^1$ prepared from CM cellulose chromatography led to the production of the postulated heterodimeric $\alpha\beta^1$ isozyme (Fig. 5a). A noticeable feature in all these experiments, however, in keeping with the observations made during the gel filtration of foetal liver samples and in direct heat stability studies (Smith *et al.* in preparation), was the apparent lability and ready inactivation of the $\alpha\alpha$ isozyme. Thus, to be certain of detecting the $\alpha\alpha$ isozyme in the hybridization experiments, an excess of $\alpha\alpha$ was used in the starting material whenever possible.

In the reciprocal experiments, preparations of the $\alpha\beta^1$ isozyme were shown to generate the two homodimeric isozymes $\alpha\alpha$ and $\beta^1\beta^1$ (Fig. 5b).

(ii) *Isozymes $\alpha\alpha$, $\alpha\gamma^1$, $\gamma^1\gamma^1$ and $\alpha\alpha$, $\alpha\gamma^2$, $\gamma^2\gamma^2$.* Experiments were carried out using mixtures of foetal liver ($\alpha\alpha$) and infant kidney of ADH₃ type 1 ($\gamma^1\gamma^1$) or ADH₃ type 2 ($\gamma^2\gamma^2$). In both cases heterodimeric ($\alpha\gamma^1$ or $\alpha\gamma^2$) isozymes were generated from the homodimeric forms by the salt freeze-thaw-dialysed treatment. The reciprocal experiment using $\alpha\gamma^1$ or $\alpha\gamma^2$ as the starting material to generate $\alpha\alpha$ and $\gamma^1\gamma^1$ or $\alpha\alpha$ and $\gamma^2\gamma^2$ respectively was not attempted due to lack of suitable purified material with high specific activity.

(iii) *Isozymes $\beta^1\beta^1$, $\beta^1\gamma^1$, $\gamma^1\gamma^1$ and $\beta^1\beta^1$, $\beta^1\gamma^2$, $\gamma^2\gamma^2$.* Mixtures of foetal lung extracts ($\beta^1\beta^1$) and foetal kidney or intestinal extracts of the ADH₃ 1 ($\gamma^1\gamma^1$) or ADH₃ 2 ($\gamma^2\gamma^2$) phenotype subjected to the dissociation-recombination procedure were found to generate a new isozyme ($\beta^1\gamma^1$ or $\beta^1\gamma^2$ respectively) with intermediate electrophoretic mobility. Also a preparation of partially purified $\beta^1\gamma^2$ isozyme was treated and shown to generate $\beta^1\beta^1$ and $\gamma^2\gamma^2$ isozymes. A clean preparation of the $\beta^1\gamma^1$ isozyme was not available for study but a mixture of the $\alpha\beta^1$ and $\beta^1\gamma^1$ isozymes obtained from CM cellulose chromatography of an adult liver ADH₃ 1 sample was available.

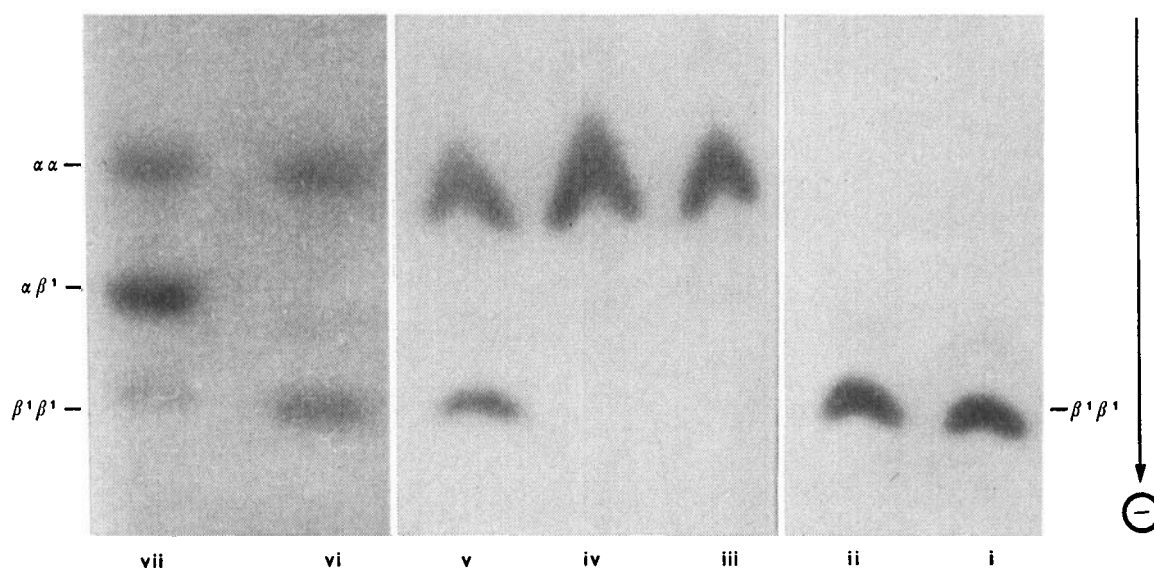


Fig. 5(a). Photograph of starch gel showing the generation of a new isozyme with postulated sub-unit structure $\alpha\beta^1$ from two homodimeric isozymes, $\alpha\alpha$ and $\beta^1\beta^1$ isolated from an infant liver by means of ion exchange chromatography.

	Treatment			New isozyme
	PSM	NaCl	FTD	
(i) Column fraction containing $\beta^1\beta^1$ isozyme	+	—	—	—
(ii) Column fraction containing $\beta^1\beta^1$ isozyme	+	—	+	—
(iii) Column fraction containing $\alpha\alpha$ isozyme	+	—	—	—
(iv) Column fraction containing $\alpha\alpha$ isozyme	+	—	+	—
(v) Mixture of fractions containing $\alpha\alpha$, $\beta^1\beta^1$	+	—	+	—
(vi) Mixture of fractions containing $\alpha\alpha$, $\beta^1\beta^1$	+	+	—	—
(vii) Mixture of fractions containing $\alpha\alpha$, $\beta^1\beta^1$	+	+	+	$\alpha\beta^1$

It will be noted that the background of the photograph showing (vi) and (vii) is darker than that showing (i)–(v). This difference is due to the fact that the portion of the gel which contained (vi) and (vii) was incubated for a longer period in order to detect all isozymes present in these samples. PSM = sodium phosphate, sucrose, mercaptoethanol; FTD = frozen, thawed and then dialysed.

The dissociation treatment was seen to lead to the formation of three out of the four additional isozymes expected from such a mixture viz., $\alpha\alpha$, $\beta^1\beta^1$, and $\alpha\gamma^1$ (Fig. 6). The $\gamma^1\gamma^1$ isozyme was not detected.

(iv) *Isozymes $\gamma^1\gamma^1$, $\gamma^1\gamma^2$, $\gamma^2\gamma^2$* . A number of attempts were made to produce the heterodimeric $\gamma^1\gamma^2$ isozyme from a mixture of the homodimeric $\gamma^1\gamma^1$ and $\gamma^2\gamma^2$ isozymes prepared with crude extracts of foetal intestine or foetal kidney of ADH₃ 1 and ADH₃ 2 phenotypes. Quite often no activity was detected in the final product of the experiment, probably because of the low concentration of ADH relative to other proteins in these tissues. However, in some experiments successful hybridization was accomplished and the $\gamma^1\gamma^2$ isozyme was detected. The reciprocal experiment ($\gamma^1\gamma^2 \rightarrow \gamma^1\gamma^1$ and $\gamma^2\gamma^2$) was not attempted.

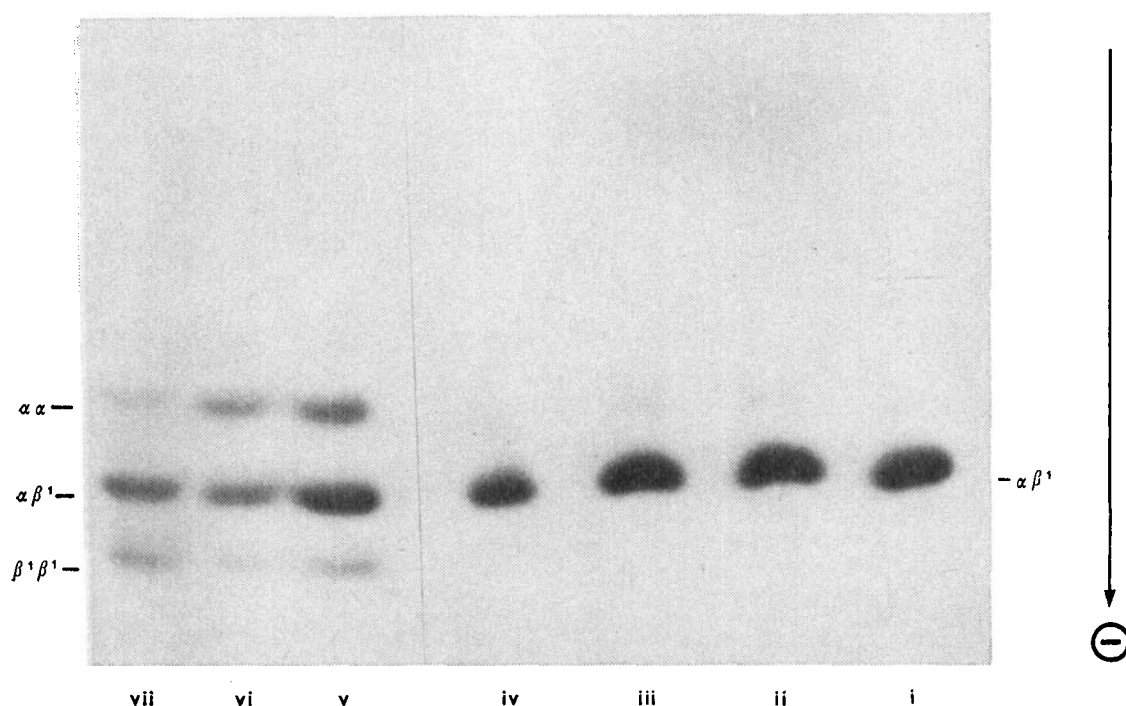


Fig. 5(b). Photograph of starch gel showing the generation of two new isozymes with postulated sub-unit structure $\alpha\alpha$ and $\beta^1\beta^1$ from a heterodimeric isozyme $\alpha\beta^1$ obtained on CM chromatography of an infant liver.

	Treatment			New isozymes
	PSM	NaCl	FTD	
(i) Column fraction containing $\alpha\beta^1$ isozyme	—	—	—	—
(ii) Column fraction containing $\alpha\beta^1$ isozyme	+	—	—	—
(iii) Column fraction containing $\alpha\beta^1$ isozyme	+	—	+	—
(iv) Column fraction containing $\alpha\beta^1$ isozyme	+	+	—	—
(v) Column fraction containing $\alpha\beta^1$ isozyme	+	+	+	$\beta^1\beta^1, \alpha\alpha$
(vi) Column fraction containing $\alpha\beta^1$ isozyme	+	+	+	$\beta^1\beta^1, \alpha\alpha$
(vii) Control infant liver extract in H_2O	—	—	—	—

PSM = sodium phosphate, sucrose, mercaptoethanol; FTD = frozen, thawed and then dialysed.

CONCLUSIONS

Previous studies (Von Wartburg, Bethune & Vallee, 1964; Schenker, Teeple & von Wartburg, 1971; Jörnvall & Pietruszko, 1972) have shown that human liver alcohol dehydrogenase is a dimeric enzyme with a molecular weight of about 80,000, indicating that the molecular weight of the constituent polypeptides is in the region of 40,000. Our experiments support these conclusions since the ADH in newborn liver, adult liver, gastric mucosa and foetal kidney extracts showed a similar elution profile on Biogel P 150 and hence gave a similar estimate of molecular weight (c. 80,000) in each experiment. Furthermore, electrophoretic analysis of the individual fractions selected from different areas revealed no asymmetry in the elution profile, thus indicating that all the isozymes, irrespective of their constitution are of similar molecular weight. It may be concluded therefore that the ADH polypeptides α , β and γ determined by ADH_1 , ADH_2 and ADH_3 respectively have about the same molecular weight (c. 40,000). Also the

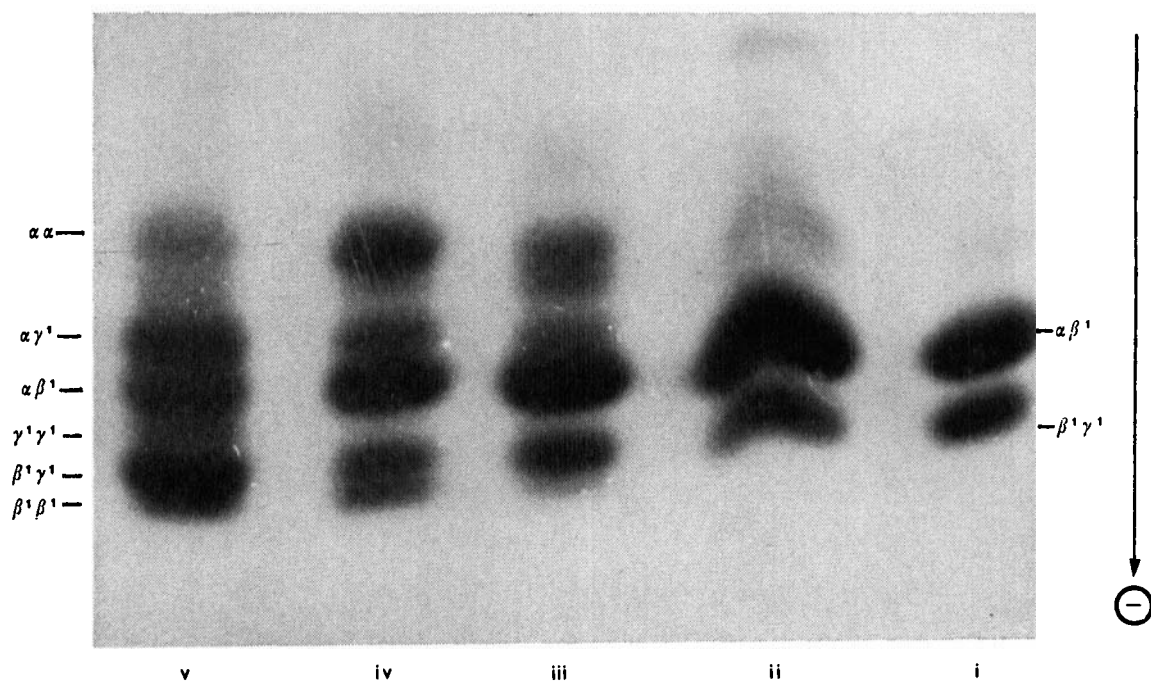


Fig. 6. Photograph of starch gel showing the generation of three new isozymes with postulated sub-unit structure $\alpha\alpha$, $\alpha\gamma^1$, and $\beta^1\beta^1$ from a mixture of $\alpha\beta^1$ and $\beta^1\gamma^1$ isozymes obtained from CM chromatography of an adult liver of the ADH_3 1 usual pH ratio phenotype.

	Treatment			New isozymes
	PSM	NaCl	FTD	
(i) Fraction containing $\alpha\beta^1$ and $\beta^1\gamma^1$ isozymes	—	—	—	—
(ii) Fraction containing $\alpha\beta^1$ and $\beta^1\gamma^1$ isozymes	+	—	+	—
(iii) Fraction containing $\alpha\beta^1$ and $\beta^1\gamma^1$ isozymes	+	+	+	$\alpha\alpha$, $\alpha\gamma^1$, $\beta^1\beta^1$
(iv) Fraction containing $\alpha\beta^1$ and $\beta^1\gamma^1$ isozymes	+	+	+	$\alpha\alpha$, $\alpha\gamma^1$, $\beta^1\beta^1$
(v) Control of the ADH_3 1 phenotype	—	—	—	—

PSM = sodium phosphate, sucrose, mercaptoethanol; FTD = frozen, thawed and then dialysed.

alternative polypeptides (β^1 and β^2 from ADH_2 and γ^1 and γ^2 from ADH_3) appear to be of the same size.

The similarity of the ADH isozymes in molecular size indicates that their separation by starch-gel electrophoresis is mainly dependent on charge differences between the isozymes. The results obtained from ion-exchange chromatography of human liver reported here and in previous studies (Von Wartburg *et al.* 1964; Blair & Vallee, 1966; Von Wartburg & Schürch, 1968; Schenker *et al.* 1971; Jörnvall & Pietruszko, 1972) support this view. Our most successful experiments with CM cellulose were those carried out on newborn liver samples, which exhibit the simplest isozyme patterns. In such cases it was possible to obtain a clear separation of the three principal isozymes $\alpha\alpha$, $\alpha\beta^1$ and $\beta^1\beta^1$ and the order of their elution from the ion exchanger was as expected from their relative electrophoretic mobilities on starch gels. Furthermore, the preparations of the individual isozymes thus obtained were found to be suitable for the investigation of the subunit structure by *in vitro* hybridization and also for studying enzymic properties of the individual isozymes.

More complicated elution profiles but less clear-cut separations of the individual isozymes were obtained from ion exchange chromatography of adult liver extracts. Different results were obtained according to the ADH₂ and ADH₃ phenotypes of the samples being analysed. For example, using liver extracts of the usual pH ratio phenotype ($ADH_2^1 ADH_2^1$) and the ADH₃ 2 phenotype ($ADH_3^2 ADH_3^2$) six peaks of ADH activity were detected in the eluate which corresponded with the principal isozymes characteristic of this type of sample: the γ^2 containing isozymes $\gamma^2\gamma^2$, $\alpha\gamma^2$ and $\beta\gamma^2$ were eluted first and in that order, followed by $\alpha\alpha$, $\alpha\beta^1$ and $\beta^1\beta^1$ although absolute separation of the individual isozymes was not obtained under these conditions. Using adult liver of the usual pH ratio ($ADH_2^1 ADH_2^1$) and the ADH₃ 1 phenotype ($ADH_3^1 ADH_3^1$) less good separations of the individual isozymes were obtained, however, since only three peaks of ADH activity were usually detected in the eluate. The $\gamma^1\gamma^1$ isozyme was eluted first but together with $\alpha\gamma^1$ and $\alpha\alpha$ as the first peak, the $\beta^1\gamma^1$ and $\alpha\beta^1$ isozymes came off together as the second peak and the $\beta^1\beta^1$ isozyme was eluted as the third peak of ADH activity.

Ion-exchange experiments with adult livers of the atypical pH ratio phenotype showed elution profiles quite different from those obtained using livers of the usual pH ratio phenotype. The outstanding feature was the very large peak of ADH activity containing the characteristic 'atypical' β isozyme.

These results obtained by ion-exchange chromatography of human liver of the usual and of the atypical pH ratio ADH phenotype are comparable with the observations made by Von Wartburg and his colleagues using similar techniques (1964, 1968; Schenker *et al.* 1971). However, exact comparison with this previous work is difficult since the methods used were not exactly the same for either the chromatographic or the electrophoretic separation of the isozymes. Furthermore, the phenotype of their starting material had not been assessed in the same way as ours by starch-gel electrophoresis. However, there appear to be no major inconsistencies between the present work and that previously reported, and the ion-exchange technique is clearly very suitable for obtaining partially purified preparations of some of the individual isozymes of human ADH which occur in crude liver homogenates.

The *in vitro* hybridization experiments carried out using crude tissue extracts and partially purified material from CM cellulose chromatography were very successful and provide convincing evidence in support of the postulated subunit composition of the various ADH isozymes. Experiments were designed to show the formation of 'hybrid' heterodimeric isozymes starting with mixtures of two different homodimeric isozymes and vice versa and the results obtained were exactly as expected. Also the results are consistent with the *in vitro* hybridization experiments reported on purified preparations of different human liver ADH isozymes separated by ion exchange chromatography (Schenker *et al.* 1971).

The subunit structure of the most cathodal β isozyme characteristic of the atypical pH ratio phenotype still remains uncertain. In one experiment in which hybridization of this isozyme, in an extract of atypical lung, was carried out with a foetal liver sample, containing principally the $\alpha\alpha$ isozyme, two additional isozymes were generated having electrophoretic mobilities which would correspond to $\alpha\beta^1$ and $\alpha\beta^2$. This suggests that the structure of the 'atypical' β isozyme may be $\beta^1\beta^2$. However, more work will be required on this problem.

SUMMARY

1. *In vitro* hybridization experiments, involving the dissociation and recombination of the postulated subunits of different human ADH isozymes, have been carried out using crude tissue homogenates and partially purified preparations of individual ADH isozymes obtained by ion exchange chromatography on CM cellulose.

2. The hybridization technique and the CM cellulose chromatography technique are reported in detail.

3. Using the hybridization technique, mixtures of postulated homodimeric isozymes were shown to generate the expected hybrid heterodimeric isozymes and in the reciprocal experiments single isozymes, believed to be heterodimeric, were shown to generate the two homodimeric isozymes expected from the proposed subunit structure.

The results are fully consistent with the previously described hypothesis (Smith *et al.* 1971, 1972) that human ADH is determined by three separate structural gene loci, *ADH*₁, *ADH*₂ and *ADH*₃.

4. Molecular-size estimates have been made by gel filtration on Biogel P 150. All the ADH isozymes appear to be of about the same molecular weight (c. 80,000), suggesting that the ADH polypeptides determined by *ADH*₁, *ADH*₂ and *ADH*₃ have the same molecular weight (c. 40,000).

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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