Immunohistochemical Localization of Human Liver Alcohol Dehydrogenase in Liver Tissue, Cultured Fibroblasts, and HeLa Cells

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Human liver alcohol dehydrogenase (ADH, EC 1.1.1.1) was purified by double ternary complex affinity chromatography on Sepharose-4-(3-[N-6 aminocaproyl]aminopropyl) pyrazole. The purified enzyme preparation still contains several isoenzymes reflecting the isoenzyme composition of the starting material. Antibodies against this mixture of isoenzymes were elicited in rabbits. The specificity of the antiserum was tested by double immunodiffusion, enzyme-linked immunosorbent assay, immunoprecipitation of ADH enzymatic activity, and adsorption to ADH, which was immobilized to Ultrogel AcA 44 by the use of glutardialdehyde as the coupling agent. Protein-A peroxidase with diaminobenzidine or amino ethyl carbazole as substrate, served to detect binding of anti-human liver ADH antibodies in human liver thin sections, cultured human skin and lung fibroblasts, and HeLa cells. Fluorescein-conjugated antibodies were also used in direct immunofluorescence on liver tissue. In the human liver, ADH was found to be localized in the cytoplasm of hepatocytes. Differences in the staining intensity of hepatocytes may reflect differences in ADH content. Strongly stained hepatocytes were localized mainly around the central veins. Perinuclear staining is often seen, especially in the more lightly stained cells. Human skin and lung fibroblasts, as well as HeLa cells, all exhibited positive staining for ADH. The pattern was identical to that found in hepatocytes, although the staining intensity was much weaker, indicating a lower ADH content. (Am J Pathol 1982, 108:89-99)

ALCOHOL DEHYDROGENASE (ADH, EC 1.1.1.1) is the first enzyme in the metabolism of ethanol. The enzyme catalyzes the oxidation of ethanol to the highly toxic acetaldehyde and therefore is thought to play a significant role in the pathology of alcohol intoxication.[1-3] During the oxidation of ethanol, large amounts of nicotinamide adenine dinucleotide (oxidized form; NAD^+) are reduced concomitantly, changing the cellular redox potential and thus leading to heavy disturbances of the cellular metabolism. Besides ethanol, numerous other xenobiotic and also naturally occurring alcohols are oxidized by ADH to their corresponding aldehydes, eg, β-pyridylcarbinol, 1,2-propanediol, acetaldol[4] or retinol, steroids, and ω-hydroxy fatty acids.[2] Since the pH-optimum for the alcohol oxidation reaction is around 10.5 and the optimum for the reverse reaction around 6, it is not unlikely that under physiologic conditions the reduction of aldehydes to the corresponding alcohols is favored. Although the function of ADH is thought to be the detoxication of ethanol normally present in the gastrointestinal tract,[5] the broad substrate specificity of this enzyme suggests other roles for ADH than only detoxication.

The exact localization of ADH in different tissues and cells, especially of organs that are affected most by high local concentrations of acetaldehyde or by the heavy disturbances of the cellular metabolism due to NAD^+ reduction, could give a basis for further elucidation of the role of ADH in alcohol intoxication. Present knowledge on the localization of ADH activity within organs and tissues only relies on data obtained with biochemical and histochemical techniques. In human tissue, ADH activity was found primarily in liver but also in the gastrointestinal tract, kidney, retina, pancreas, and prostate.[6] In rats additional ADH activity could be detected in brain and skeletal muscle.[7] Liver ADH in rats was mainly found in the parenchymal cells.[8] Light-microscopic histo-

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chemical studies visualizing ADH activity by the formation of formazan particles, have been confined to mice and rats.[9-13] With these techniques ADH was found exclusively in the cytoplasm, an observation that was confirmed by electron-microscopic histochemical studies of rat retina[14,15] and mouse hepatic tissue.[16] Histochemical detection of ADH lacks specificity, and the results are difficult to interpret, because the presence of various endogenous substrates as well as other dehydrogenases also leads to the formation of formazan particles.

To circumvent these difficulties, we used an immunohistochemical technique which, by its very nature, should be both specific and sensitive. So far, only one similar study, using anti-horse liver ADH antiserum and rat tissue, has been performed.[17]

This report describes the preparation and characterization of antibodies against human liver ADH purified by double ternary complex affinity chromatography on Sepharose-4-(3-[N-6 aminocaproyl] aminopropyl) pyrazole (CapGapp-Sepharose)[18] and the application of an immunocytochemical technique for the cellular localization of ADH in human cells and tissues.

Materials and Methods

Electrophoresis

Analytic sodium dodecyl sulfate (SDS) gel electrophoresis on slabs of 12% polyacrylamide was performed according to the method of Lämmli.[19]

Starch gel electrophoresis was performed according to Smith et al[20] in 100 mM Tris/phosphate buffer, pH 7.7 (electrode buffer) and 5 mM Tris/phosphate buffer, pH 7.7, containing 2.5 mM NAD^+ (gel buffer). The starch concentration was 10%. The starch was obtained from Electrostarch, Madison, Wisconsin.

Gels were stained for protein with Coomassie blue and for ADH activity by incubation in 25 mM Tris/Cl^-, pH 8.5, containing 0.5 mM nitrotetrazolium blue chloride (Fluka, Buchs, Switzerland), 0.2 mM phenazine methosulfate (Sigma, St. Louis, Missouri, USA), 1.3 mM NAD^+ (Grade AA-1, Sigma, St. Louis, Missouri, USA), and 100 mM ethanol.

Enzymatic Assay

Alcohol dehydrogenase activity was measured in 67 mM glycine/NaOH buffer, pH 10.5, according to Lutsdorf et al.[21]

Specific activity is defined in International Units (micro moles of substrate oxidized per minute) per milligram protein (IU/mg).

Protein Determination

Protein concentration was measured according to the method of Lowry et al,[22] with the use of bovine serum albumine (BSA; Sigma, St. Louis, Mo) as a standard.

Isolation of Human Liver Alcohol Dehydrogenase

Human livers were obtained from autopsies, frozen 10–20 hours after death, and stored at −20 C. For the isolation of ADH a slightly modified version of the procedure published by Lange et al[23] was used. The frozen livers were cut into small pieces and passed twice through a meat grinder. After the addition of cold water (2l/kg tissue) the slurry was extracted for 2 hours under continuous stirring, followed by centrifugation at 15,000g for 2 hours. The supernatant was filtered through cotton wool and chromatographed on diethylaminoethyl (DEAE)-cellulose (DE-52, Whatman Springfield Hill, Maidstone, Kent, England) according to Lange et al.[23] The ADH-containing fractions were dialyzed against 50 mM sodium phosphate buffer, pH 7.5, brought to 0.37 mM NAD^+, and applied to a 50 × 2-cm CapGapp-Sepharose affinity chromatography column, equilibrated with the same buffer. The column was washed with the same buffer until the protein absorption at 280 nm and the ADH activity of the effluent were zero (approximately 20 times the column volume). The column was then washed with half a column volume of 50 mM sodium phosphate buffer, pH 7.5, and the bound enzyme was finally eluted with the same buffer containing 500 mM ethanol. Fractions containing more than 1.5 IU/ml ADH activity were pooled, dialyzed extensively against 50 mM sodium phosphate buffer, pH 7.5, to remove the ethanol, concentrated in an Amicon ultrafiltration cell with a PM-10 membrane (Amicon, Oosterhout, Holland), sterile-filtered through a Millipore Millex HA filter (Millipore, Molsheim, France), and stored at 4 C. A total of 386 mg of homogeneous human liver ADH could be isolated from 520 g (wet weight) of human liver. The specific activity of this preparation was 1.71 IU/mg protein. SDS–polyacrylamide gel electrophoresis of pools before (left) and after (right) affinity chromatography on CapGapp–Sepharose are shown in Figure 1a. The purified human liver ADH exhibited only one band with an approximate subunit molecular weight of 43,000 ± 1000 daltons, characteristic for human liver ADH.[1,24] All other proteins present before affinity chromatography had disappeared. Figure 1b shows the pattern revealed by starch gel electrophoresis of purified human liver ADH after staining the gel for enzymatic activity. At

least five bands were detectable, indicating that the purified ADH consisted of several isoenzymes with identical molecular weights.

Immobilization of Human Liver Alcohol Dehydrogenase

Ultrogel AcA 44 (LKB, Bromma, Sweden) was activated with glutardialdehyde (Fluka, Buchs, Switzerland) according to Weston and Avrameas.[25] Activated Ultrogel in volumes of 8 ml was incubated with 12.8 mg purified human liver ADH in 10 ml 0.1 M sodium phosphate buffer, pH 7.4, under nitrogen, at 4 C, in the dark and under constant rotation of the reaction flask for 23 hours. The gel was then washed extensively with 0.05 M phosphate-buffered saline (PBS), pH 7.4. The effluent was collected for protein analysis. The gel was then incubated with 0.1 M lysine (Merck, Darmstadt, Germany) under the same conditions as above, followed by extensive washing with PBS. The immobilized ADH (ADH-Ultrogel) obtained in this way was stored at 4 C in PBS containing 0.02% sodium azide.

The amount of ADH coupled to the gel was determined by calculation of the difference between the amount of ADH present in solution before coupling and the amount of ADH that could be washed out of the gel with PBS; 8.2 mg of ADH could be coupled to 8 ml of settled Ultrogel. Therefore, 1 mg of protein was coupled to 1 ml of gel. The coupling efficiency was 64%; ie, 36% of the initial amount of ADH could be washed out of the gel with PBS.

Antiserum Preparation and Characterization

Antibodies were raised in rabbits by subcutaneous injection of purified human liver ADH (0.48 mg/animal) emulsified in Freund's complete adjuvant (Difco, Detroit, Mich) (1:1 vol/vol). After 30 days an additional series of three subcutaneous injections (0.2 mg ADH/animal) followed at weekly intervals. Ten days after the fourth injection the rabbits were bled and the antiserum collected. Preimmune serum samples collected immediately prior to the first injection served as controls.

The serum samples were sterile-filtered and stored in batches of 5 ml at −20 C. The antiserums from two animals treated identically had titers of 3.79 and 3.72 against ADH, as measured with an enzyme-linked immunosorbent assay (ELISA, *vide infra*). The antiserums were tested by double immunodiffusion in 1% agarose (Nordic No. 1, Biogenzia Lemania, Lausanne, Switzerland) according to Ouchterlony.[26] Because ADH activity was retained in immunoprecipitates, the gels could be stained for enzymatic activity in the same way as the starch gels used for electrophoresis, except that the plates were pressed and washed with 3% NaCl and water three times to remove unprecipitated proteins before incubation. To visualize precipitated proteins, we also stained the Ouchterlony plates for protein with 1% amido-black. The antiserums were also tested by ELISA (*vide infra*).

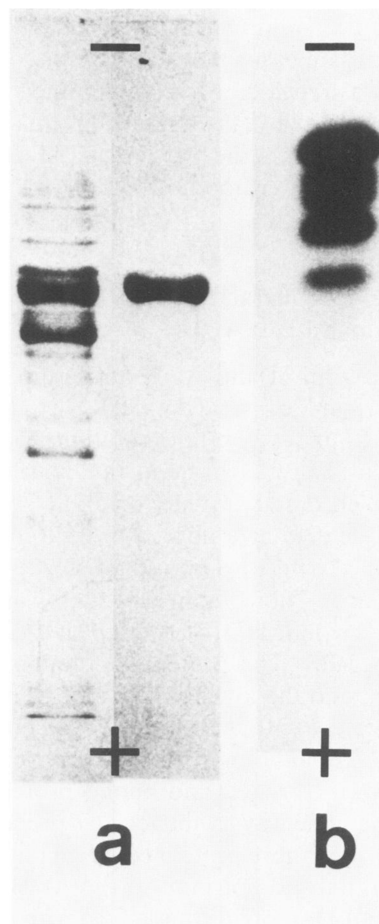

Figure 1a—SDS-gel electrophoresis (12%) of human liver ADH pools (see methods) before (*left*) and after (*right*) double ternary complex affinity chromatography. Approximately 20 μg of protein was loaded per track. The gel was stained for protein with Coomassie blue. **b**—Starch gel electrophoresis of purified human liver alcohol dehydrogenase at pH 7.7. The gel was stained for activity.

The ability of the antiserums to precipitate enzymatic activity was further tested under enzymatic assay conditions. Aliquots of ADH were incubated for 48 hours at 4 C with several dilutions of serum in PBS in a final volume of 0.5 ml. In most of the tubes containing antiserum a notable precipitate developed. At the end of the incubation period, enzymatic activity was measured in an aliquot of the suspension and of the supernatant after centrifugation. At two serum

dilutions the precipitate was carefully washed three times with PBS and resuspended. The activity of this suspension was measured as well. As a control, the procedure was repeated with preimmune serum.

We evaluated the data by plotting the amount of serum added against the percent of ADH activity, taking the sample without addition of serum as 100%.

Purification and Characterization of Antibodies Against Human Liver ADH

A total of 9 ml of anti-ADH antiserum was incubated with 8 ml of settled ADH-Ultrogel for 18 hours at 4 C under constant rotation of the incubation flask. The suspension was poured into a 20×0.9-cm chromatographic column, which was subsequently eluted at a flow rate of 60 ml/hr. The gel was washed with PBS until the OD 280 of the effluent was zero. The column was then washed with three column volumes of 0.1 M glycine/Cl^-, pH 3, followed by PBS. The protein-containing fractions were pooled, and the low pH fractions were neutralized immediately with 1 M Tris/Cl^-, pH 7.9. Fractions containing unbound proteins were reincubated with the ADH-Ultrogel, and bound proteins eluted in exactly the same way as above. This procedure was repeated until no more protein could be bound to the gel. Protein that did not bind to the ADH-Ultrogel (Ultrogel Pool 1), proteins that eluted with 0.1 M glycine/Cl^-, pH 3 (Ultrogel Pool 2), and proteins that eluted with the second PBS wash (Ultrogel Pool 3) were pooled separately, dialyzed against PBS, and concentrated in a Amicon ultrafiltration cell with a PM-10 membrane. The pools were stored in batches at −20 C.

During the above procedure the ADH-Ultrogel remains intact and can be used repeatedly to purify antibodies from anti-ADH serum.

Proteins in Ultrogel Pool 2 precipitated during dialysis and concentration, and only 14% of the total protein (based on OD 280 measurement) initially present in this pool could be recovered. Proteins in Pool 3 did not precipitate. It is interesting to note that these proteins could not be eluted with 0.1 M glycine/Cl^-, pH 3, but only with a second PBS wash and only when the column was washed with low pH buffer beforehand. Omission of the low pH buffer, ie, prolonged washing with PBS only, does not cause elution of any proteins. Avoiding low pH buffer, eg, eluting with 3 M isothiocyanate, pH 6, destroys the gel by dissolving the agarose.

We obtained a total of 5.3 mg anti-ADH antibodies in Ultrogel Pool 3 by extracting 9 ml of anti-ADH antiserum once with 8 ml settled ADH-Ultrogel. Since 1 mg ADH was coupled per milliliter of Ultrogel, this results in a binding capacity of 0.6 mg antibody per milligram ADH. A total of 1.3 mg anti-ADH antibodies per ml anti-ADH antiserum could thus be isolated. This compares with 8.5 mg total IgG per milliliter anti-ADH antiserum isolated by affinity chromatography on protein-A Sepharose. Therefore, 15% of the IgG present in the anti-ADH antiserum are antibodies specific against ADH. The titer of these specific anti-ADH antibodies was four times higher than that of the total IgG from the same antiserum (Figure 3).

Binding Assay (ELISA) for Anti-Human Liver ADH Antibodies

We carried out the procedure according to Berger et al,[27] using a 0.1 ml assay volume in flat-bottomed Immulon ELISA plates (Dynatech, Kloten, Switzerland) coated with 2 μg/ml of purified human liver ADH. A protein-A phosphatase conjugate was used to detect bound antibodies. After incubation with 200 μl *p*-nitrophenylphosphate (Merck, Darmstadt, Germany) as substrate, the optical density at 405 nm was measured. The negative logarithm of the dilution was plotted as a function of saturation (%). The titer of a particular antibody-containing solution is defined as the negative logarithm of the dilution at which 50% saturation was reached.

Cells and Tissue Sections

Cells were cultured on Cooke microprint stock slides (Dynatech, Kloten, Switzerland). Monolayers of lung fibroblasts were fixed in 1% formaldehyde for 2 minutes at room temperature, HeLa cells in ice-cold acetone for 30 minutes, and skin fibroblasts in 3.7% formaldehyde for 10 minutes at room temperature.

Whole liver tissue was fixed by immersion in buffered (pH 7.5) 4% formaldehyde, embedded in paraffin, and sectioned at a thickness of 5 μ. Mounted sections were deparaffinized and rehydrated in a graded series of ethanol and incubated in 0.1% trypsin (bovine; Merck, Darmstadt, Germany) in 0.05 M Tris-buffered saline, pH 7.6, at 37 C for 10 minutes. The reaction was stopped by immersion of the slides in ice-cold PBS.

Fluorescence Microscopy

The Ig-fraction (sodium sulfate precipitate) of nonpurified anti-ADH antiserum was labeled with

fluorescein-isothiocyanate (FITC; Calbiochem, Lucerne, Switzerland) at a molar FITC:protein ratio of 2.9 and a protein concentration of 2.8 mg/ml. Paraffin sections of formalin-fixed human liver were treated with 0.2% trypsin (10 minutes at room temperature), washed, and incubated with FITC-labeled anti-ADH (30 minutes at room temperature). The slides were evaluated in a fluorescence microscope with incident light (Leitz Laborlux 12 with Ploemopak; 100 W mercury lamp; narrow filter excitation at 490 nm, barrier at 510 nm).

Immunohistochemical Staining

The staining procedure was identical for cell cultures and tissue sections. Prior to applying the antibodies, the slides were washed in PBS twice for 15 minutes. They were then incubated in an appropriate working dilution of antibodies for 1 hour at room temperature, washed twice with PBS, incubated with protein-A peroxidase for 1 hour, and washed twice with PBS. We detected peroxidase activity by incubating the slides with 0.005% diaminobenzidine (Serva, Heidelberg, Germany) in 50 mM Tris/Cl^-, pH 7.6, containing 0.02% H_2O_2 (skin fibroblasts, liver tissue thin sections) or by incubation in 0.02% amino ethyl carbazole (Sigma, St. Louis, Mo) in 50 mM sodium acetate, pH 5, containing 0.01% H_2O_2 (HeLa cells, lung fibroblasts) for 5 to 20 minutes at room temperature. The reaction was stopped by washing the slides in several changes of PBS. The slides were evaluated microscopically immediately after they were mounted with glycerol/PBS (9:1 vol/vol).

The following controls were used routinely: preimmune serum; immunoadsorbed antiserum, ie, anti-ADH serum which was passed through an ADH–Ultrogel affinity chromatography column to remove the anti-ADH antibodies; or anti-ADH serum which was immunoprecipitated with ADH by incubation of appropriate amounts of anti-ADH serum with ADH for 48 hours at 4 C and removal of the precipitate by centrifugation.

Results

Characterization of Anti-ADH Antiserum and Purified Anti-ADH Antibodies

In a double immunodiffusion assay of purified ADH with anti-ADH antiserum a single precipitation line developed. The precipitate could be stained for protein as well as for enzymatic activity, and there was no other precipitation line visible that stained only for protein (Figure 2a and b). The same result was obtained when crude homogenate was tested against anti-ADH antiserum as well as against ADH–Ultrogel affinity-purified anti-ADH antibodies (not shown). In this assay, identical precipitation lines were obtained with ADH preparations from different human livers containing different isoenzyme compositions, while purified horse liver ADH exhibited only partial identity. When anti-ADH antiserum was preincubated with purified ADH and the resulting precipitate was removed by centrifugation, the antiserum depleted by precipitation failed to react with ADH (Figure 2c). The more sensitive ELISA revealed, however, that precipitation depleted anti-ADH antiserum still contained about 2% residual antibodies. Double immunodiffusion with antibodies obtained by elution from the ADH–Ultrogel column produced a single precipitation line with ADH, and no precipitation reaction was observed with the nonbinding fraction (Figure 2d). The precipitation line between ADH and ADH–Ultrogel purified antibodies was identical to the one formed by ADH and purified IgG from anti-ADH antiserum (Figure 2d).

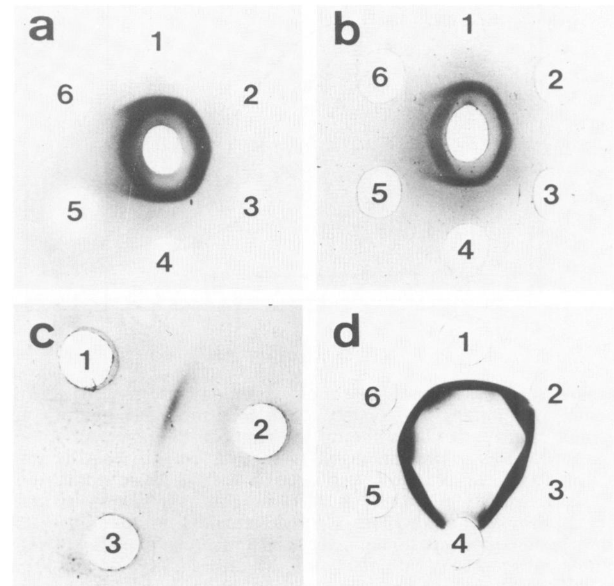

Figure 2a and **b**—Double immunodiffusion of rabbit anti-human liver ADH antiserum (center well) against purified human liver and horse liver ADH. Comparison of protein staining (**a**) with ADH-activity staining (**b**). Wells 1–4: 4 different purified liver ADH samples, representing 4 different isoenzyme compositions. Well 5: horse liver ADH isoenzyme EE. Well 6: horse liver ADH isoenzyme ES. For more details see text. **c**—Double immunodiffusion of rabbit anti-human liver ADH antiserum (Well 2) and immunoprecipitated rabbit anti-human liver ADH antiserum (Well 3) against purified human liver ADH (Well 1). The plate was stained for ADH activity. For details, see text. **d**—Double immunodiffusion of purified human liver ADH (center well) against anti-human liver ADH antiserum (Well 1), protein-A-Sepharose-affinity-purified total anti-human liver ADH IgG (Wells 2 and 5), ADH-Ultrogel-affinity-purified anti-human liver ADH antibodies (Wells 3 and 6), and immunoadsorbed anti-human liver ADH antiserum (Well 4). The plate was stained for activity. For details see text.

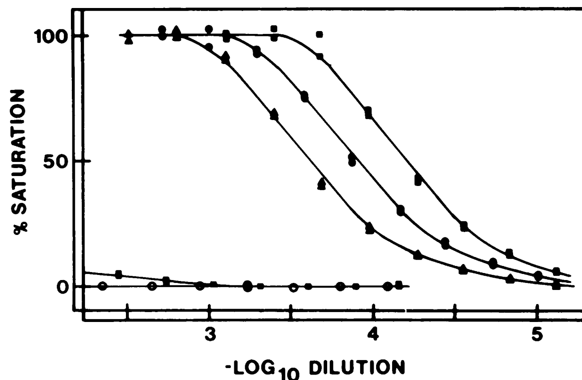

Figure 3—ELISA for the detection of anti-human liver ADH antibodies. Antibodies (%) bound to immobilized ADH are plotted versus the negative logarithm of the antibody dilutions. All points were measured in duplicates. ●——●, anti-human liver ADH antiserum; ▲——▲, protein-A-Sepharose-affinity-purified total anti-human liver ADH IgG; ■——■, ADH-Ultrogel-affinity-purified anti-human liver ADH antibodies; ○——○, preimmuneserum; □——□, immunoadsorbed anti-human liver ADH antiserum (depleted of anti-human liver ADH antibodies by affinity chromatography on ADH-Ultrogel and at the same concentration as the original antiserum). For more details see text.

In contrast to precipitation-depleted anti-ADH, the breakthrough fraction of affinity chromatography on ADH-Ultrogel contained only 0.1% anti-ADH activity as revealed by ELISA (Figure 3).

Enzymatic ADH activity could almost be quantitatively precipitated by the addition of ADH antiserum (Figure 4). Upon removal of the enzymatically active precipitate (about 80% of the original activity), about 6% of the initial ADH activity was detectable in the supernatant. Therefore, anti-ADH exhibited an inhibiting effect on the enzymatic activity of ADH. On the other hand, the addition of preimmune serum had a stabilizing effect. After the addition of 100 μl of preimmune serum to the reaction mixture, ADH activity was 120% of that of the serum-free control.

Immunohistochemical Localization of ADH

As is evident from Figure 5a, strong but uneven (Figure 5b) staining of the cytoplasm of the hepatocytes was found when sections of normal human liver were incubated with anti-ADH antiserum and peroxidase-labeled protein-A. Cells which stained strongly for ADH were localized mainly around the central veins of the liver lobules, while cells in the periphery, on the average, appeared much fainter (Figure 6). This finding indicates an uneven distribution of ADH within the lobules of the human liver as well as among individual hepatocytes. Perinuclear staining for ADH was observed in some lightly stained cells.

An identical pattern was obtained with nonpurified anti-ADH antiserum and affinity-column-purified anti-ADH antibodies. Staining of hepatocytic cytoplasm was absent when sections were incubated either with the effluent from the affinity columns (Figures 5c and 6b) or with preimmune serum. No endogenous peroxidase could be detected in hepatocytes. The use of direct immunofluorescence with paraffin sections of human liver yielded results comparable to those obtained with the protein-A peroxidase method (Figure 5d).

ADH could be detected also in human skin fibroblasts, human lung fibroblasts, and HeLa cells (Figure 7). Corresponding to the finding in hepatocytes, ADH was localized in the cytoplasm. All cells exhibited a granular deposition of the peroxidase reaction product in the cytoplasm in addition to frequently observed perinuclear staining (Figure 7a, b, and c). No difference was observed between non-purified anti-ADH antiserum and affinity-column-purified anti-ADH antibodies (not shown). Control assays (Figure 7d) were negative. Compared with the hepatocytes, fibroblasts and HeLa cells stain less for ADH, indicating that these cells contain much less ADH.

While similar results were obtained with amino ethyl carbazole or diaminobenzidine as peroxidase substrates, the latter appeared to yield a denser staining after the same incubation period. In both cases the developed granular deposit faded during storage, especially when light was not excluded.

Discussion

Human liver ADH was purified by double ternary complex chromatography on CapGapp-sepharose.[18]

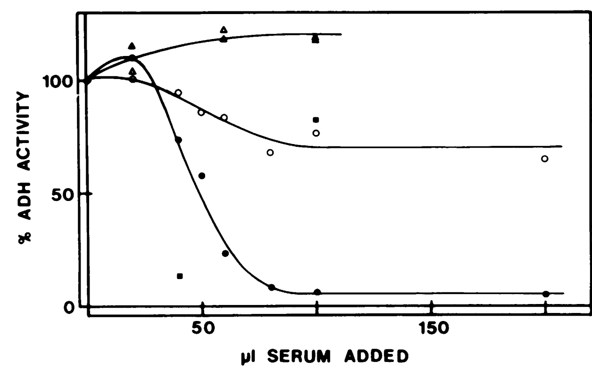

Figure 4—Titration of purified human liver ADH with increasing amounts of anti-human liver ADH antiserum under enzymatic activity conditions. ADH-activity (%) remaining after a 48-hour incubation period is plotted versus μl of antiserum added. ○——○, precipitate not removed; ●——●, precipitate removed by centrifugation; △——△, control (preimmuneserum) precipitate not removed; ▲——▲, control precipitate removed. ■, ADH activity of the washed precipitate at two different antiserum concentrations. The points are average values of 2-4 different measurements. For more details see text.

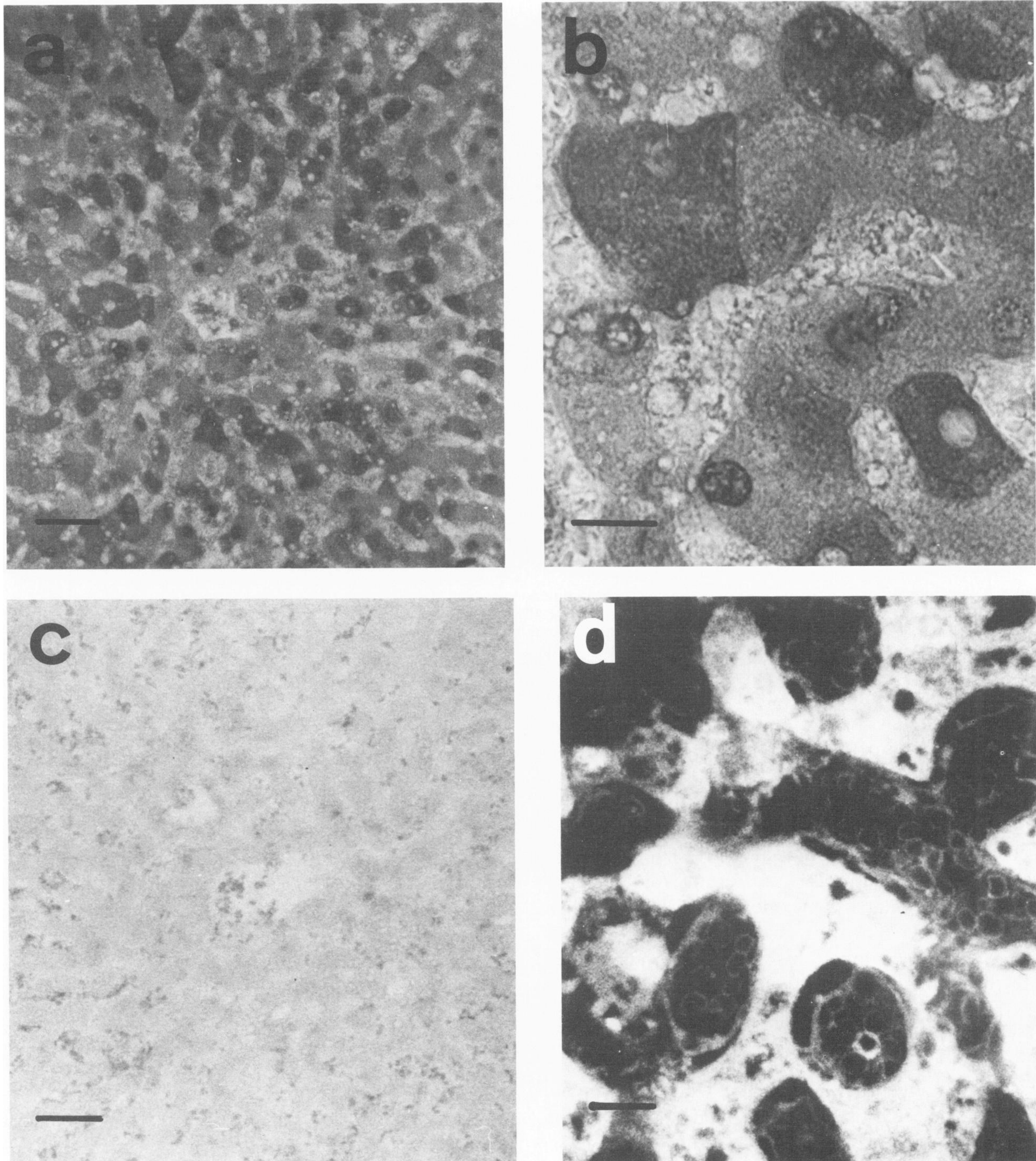

Figure 5—Immunohistochemical staining of ADH in sections of human liver. **a**—Sections were incubated with anti-human liver ADH antiserum and the bound antibodies detected with protein-A peroxidase followed by incubation with diaminobenzidine for 10 minutes as described in Materials and Methods. *Bar*, 50 μ. **b**—Higher magnification of **a**. **c**—Immunoadsorbed anti-human liver ADH antiserum (depleted of anti-human liver ADH antibodies by affinity chromatography on ADH-Ultrogel). *Bar*, 50 μ. **d**—Direct immunofluorescence of a human liver section using FITC-labeled anti-human liver ADH antibodies. *Bar*, 50 μ. For more details see text.

We obtained an enzyme preparation of comparable purity and characteristics to that described by Lange et al,[23] and the enzyme proved suitable for working with immunologic methods. The preparation contained a mixture of isoenzymes, thus reflecting the isoenzyme composition of the starting tissue. Antibodies against this mixture of isoenzymes were used to demonstrate the presence of ADH in tissues, in-

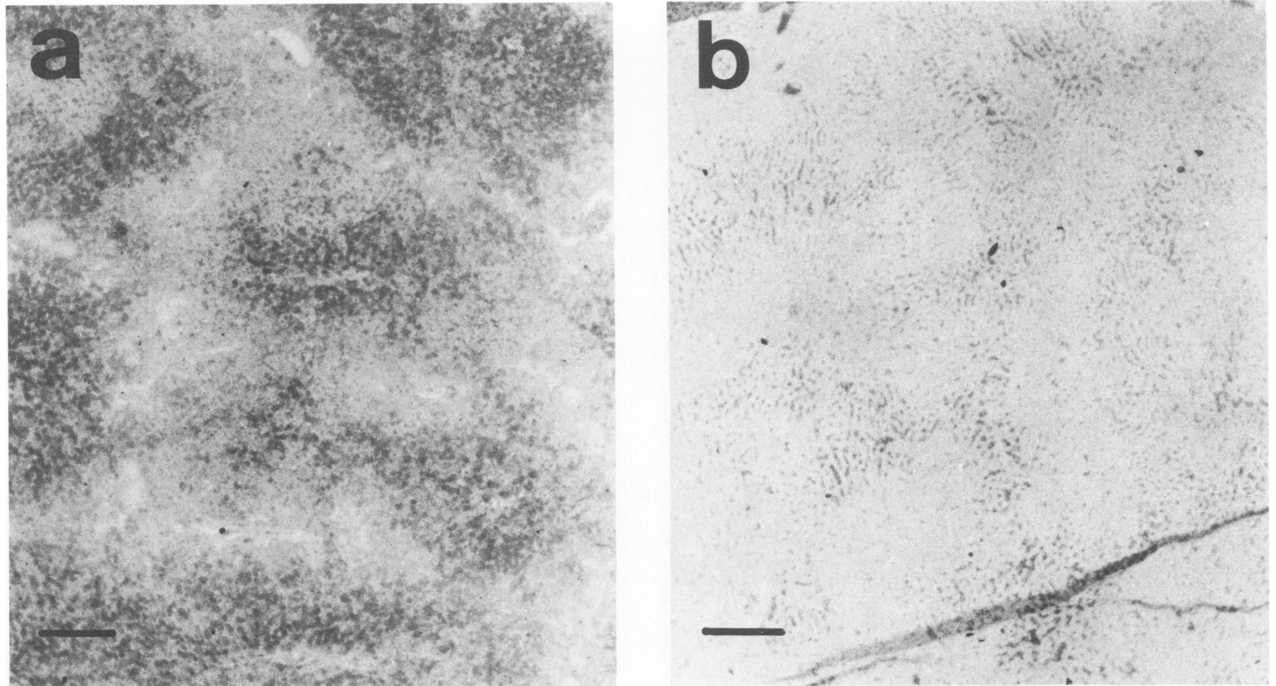

Figure 6—Immunohistochemical staining of ADH in sections of human liver. Low magnification to visualize centrilobular distribution of ADH. *Bars*, 300 μ. **a**—The section was incubated with anti-human liver ADH antiserum and bound antibodies detected with protein-A peroxidase followed by incubation with diaminobenzidine. **b**—Control: immunoadsorbed anti-human liver ADH antiserum. For more details see legend to Figure 5 and text.

cluding those that might contain only one particular isoenzyme.

The specificity of the nonpurified anti-ADH antiserum against human liver ADH was tested by four different methods: double immunodiffusion, ELISA, immunoprecipitation of enzymatic activity, and specific binding to immobilized ADH. The results indicated that our anti-ADH antiserum is not specific for one particular isoenzyme but also for other human ADH isoenzymes as well as partially for horse liver ADH isoenzymes. This finding is in agreement with the results of Adinolfi et al,[28] who reported on crossreactivity of all human liver ADH isoenzymes with an antiserum raised against one particular isoenzyme. Interspecies cross-reactivity was also demonstrated by Fuller and Marucci,[29] who tested anti-horse liver ADH antiserum against various vertebrate liver enzymes.

Immunohistochemical analysis of ADH localization was carried out on sections of human liver tissue, which exhibits high ADH activity,[6] and on cultured fibroblasts and HeLa cells with supposedly very little ADH activity.[30] With histochemical methods ADH activity could be localized in the cytoplasm of various cells in rats and mice.[13,31] Watabiki used ultracytochemical methods to demonstrate that ADH within rat hepatocytes is in close connection to rough endoplasmatic reticulum, but also in a diffuse distribution within the cytoplasmic matrix.[16] Our data confirm and extend these observations. The finding of an uneven staining of the hepatocytes is in parallel with results on the distribution of albumin in the liver[32]: similar to the distribution pattern of ADH, not all hepatocytes contained identical amounts of albumin, but while albumin-containing hepatocytes were distributed uniformly within the liver lobule, clustering of ADH-containing hepatocytes was found near the central veins. This finding is in agreement with the results of Morrison and Brock,[33] who observed a two-fifths higher ADH activity in the centrilobular quarter of the liver lobule, compared with the periportal quarter of both rats and man by using a quantitative microchemical method in conjunction with microdissection. A similar centrilobular localization was observed for glutamate dehydrogenase, isocitrate dehydrogenase, and epoxide hydratase.[34,35]

Histologic analysis of liver tissue from ethanol-intoxicated rats and man indicates that initial hepatocyte death is confined to the centrilobular zone, a finding which was tentatively related to a hypermetabolic state in an area with decreased oxygen tension.[36,37] If hepatocytes with a high ADH content are preferentially found in the vicinity of the central veins, as our experiments suggest, then one of the reasons for a pronounced lack of oxygen may be a

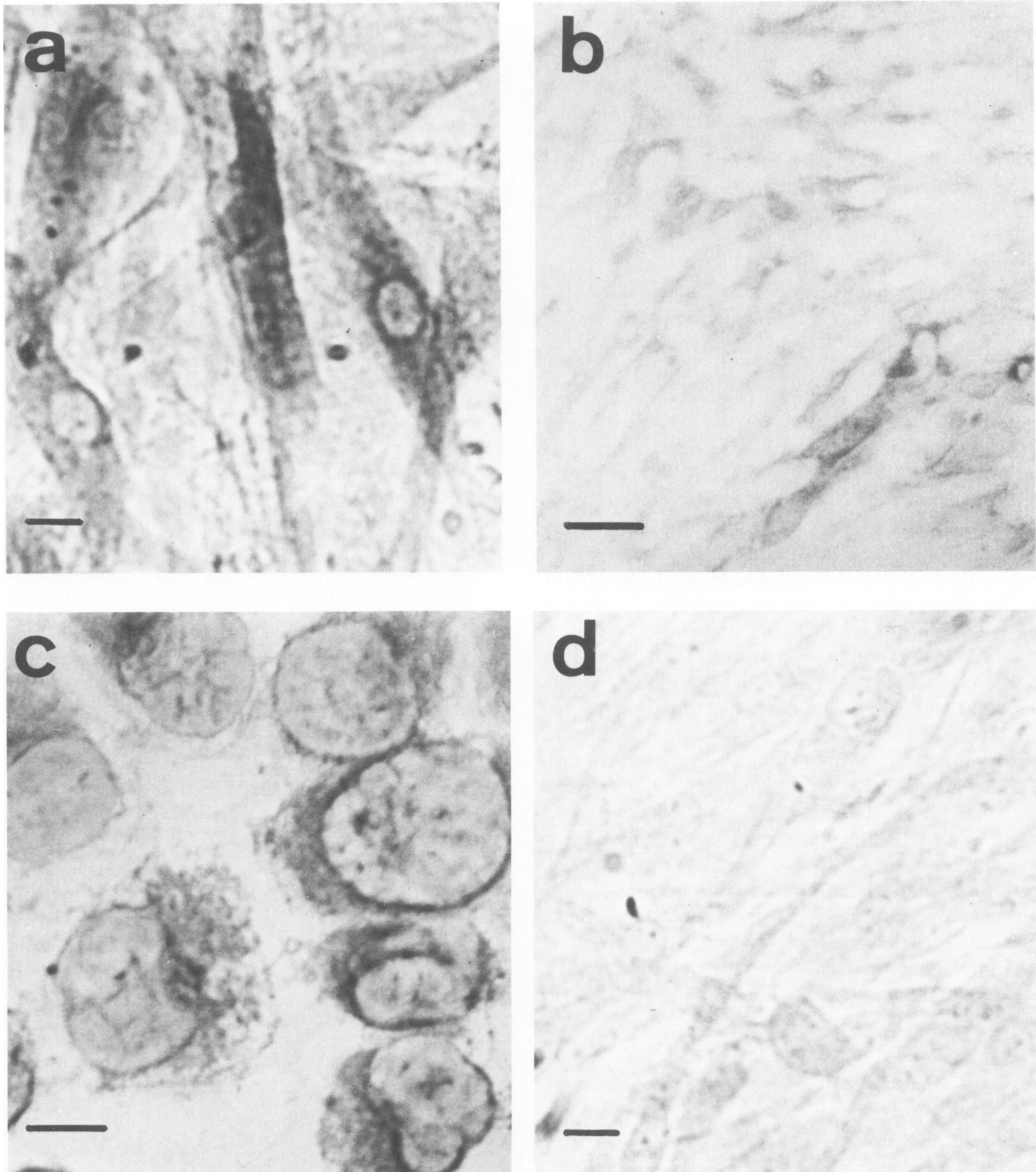

Figure 7—Immunohistochemical detection of ADH in human cell cultures. **a**—Formaldehyde-fixed confluent human skin fibroblasts. *Bar*, 10 μ. **b**—Formaldehyde-fixed confluent human lung fibroblasts. *Bar*, 40 μ. **c**—Acetone-fixed confluent HeLa cells. *Bar*, 10 μ. **d**—Control to **a** at the same magnification. Incubation time with peroxidase substrate was 20 minutes. In **a** and **b** diaminobenzidine and in **b** and **c** amino ethyl carbazole were used as peroxidase substrate. For more details see legend to Figure 5 and text.

high production of nicotine amide adenine dinucleotide (reduced form; NADH) in this area with an even higher oxygen consumption during alcohol intoxication. If this hypothesis is correct, a strong shift in the redox potential in centrilobular hepatocytes should precede necrosis. Indeed, heterogeneity of the redox state of liver cell populations has been observed in rats[38,39]; and during chronic treatment with ethanol,

oxygen is depleted in pericentral areas of the liver lobule while the oxygen tension in periportal areas remains unchanged.[40]

Our immunohistochemical results suggest that ADH is a common component of several human cells. It can be demonstrated in as diverse cells as liver cells, fibroblasts of different origin, and HeLa cells. Preliminary investigations with sections of organs and tissues other than liver confirm this observation. There seemingly is a wide variation in the amount of ADH per single cell. This is especially true for cells within the same tissue; which probably exhibit the same isoenzyme composition. The difference in staining intensity between hepatocytes and fibroblasts or HeLa cells incubated with anti-ADH antiserum may not be entirely due to different ADH content but also could reflect differences in the isoenzyme composition. The antiserum used does not differentiate between ADH isoenzymes, but because of the broad spectrum of isoenzyme specificity, our anti-ADH preparation should also be useful in detecting single isoenzymes. Peterson et al demonstrated by isoelectric focusing that the isoenzyme composition of fibroblasts at least in part is very similar to that obtained from liver of the same individual and that these fibroblasts exhibit very little ADH activity.[30] We may conclude, therefore, that the staining intensity correlates with the amount of cytoplasmic ADH.

Antibodies against ADH are a powerful tool for the detection and localization of very low quantities of ADH. The possibility of demonstrating this enzyme in tissue culture either by immunocytochemistry or by an ELISA for the detection of antigen (presently under investigation in our laboratory) indeed could lead to a major step forward in elucidating the molecular mechanism of alcohol intoxication. Such investigations were until now restricted exclusively to experimental animals and often led to misinterpretations of events in man.[41]

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