Purification of aldehyde dehydrogenase from rat liver mitochondria by α -cyanocinnamate affinity chromatography

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1. α -Cyano-4-hydroxycinnamate was coupled to Sepharose CL-4B activated with 1,2:3,4-bisepoxybutane.
2. The low- K_m rat liver mitochondrial aldehyde dehydrogenase was specifically bound to this affinity medium, and could subsequently be eluted with α -cyano-4-hydroxycinnamate.
3. The enzyme purified in this manner had a subunit molecular mass of 55 kDa and a pI of approx. 6.5. A minor component of approx. 57 kDa was also present and had a significantly higher pI value; this may be the precursor for aldehyde dehydrogenase.
4. α -Cyanocinnamate and some related compounds were found to be uncompetitive inhibitors of the enzyme.
5. No cytosolic aldehyde dehydrogenase was bound to the affinity column, but a protein from a rat liver post-mitochondrial supernatant with a molecular mass of approx. 25 kDa was bound, and could be eluted subsequently with α -cyano-4-hydroxycinnamate.

INTRODUCTION

Acetaldehyde, the toxic product of ethanol oxidation by alcohol dehydrogenase (EC 1.1.1.1), is converted into acetate by the enzyme aldehyde dehydrogenase (EC 1.2.1.3). On the basis of kinetic properties and subcellular distribution a number of isoenzymes have been identified in various mammalian species (Koivula & Koivusalo, 1975; Eckfeldt *et al.*, 1976; Greenfield & Pietruszko, 1977; Lindahl & Evces, 1984*a,c*). There are distinct cytoplasmic and microsomal isoenzymes, which have K_m values for acetaldehyde in the millimolar range. In addition, it has been shown that rat liver has inducible cytosolic aldehyde dehydrogenases that preferentially oxidize aromatic aldehydes (Lindahl & Evces, 1984*b*; Lindahl *et al.*, 1985). Mitochondria contain two isoenzymes of aldehyde dehydrogenase, one with a high K_m similar to the cytoplasmic and microsomal enzymes, and another with a K_m value for acetaldehyde in the micromolar range (Siew *et al.*, 1976; Lindahl & Evces, 1984*a*; Harrington *et al.*, 1987). The low- K_m mitochondrial aldehyde dehydrogenase is believed to provide the major contribution to acetaldehyde oxidation at the concentrations arising physiologically (Harada *et al.*, 1981; Lindahl & Evces, 1984*c*; Harrington *et al.*, 1988). Approx. 50% of orientals have a defective form of this enzyme and are susceptible to alcohol intoxication (Harada *et al.*, 1980; Ikawa *et al.*, 1983). Purifications of the low- K_m aldehyde dehydrogenase from a number of sources, including the livers of human (Greenfield & Pietruszko, 1977), horse (Eckfeldt *et al.*, 1976) and sheep (Hart & Dickinson, 1977), have been reported. A high- K_m enzyme has also been purified from rat liver mitochondria (Lindahl & Evces, 1984*a*; Senior & Tsai, 1988). However, the methods employed in the purification of these enzymes involve a number of steps, and have not always yielded homogeneous preparations.

During attempts to purify the mitochondrial pyruvate carrier, we decided to employ affinity chromatography with the potent transport inhibitor α -cyano-4-

hydroxycinnamate linked to Sepharose. This column failed to bind any detergent-solubilized rat liver mitochondrial membrane protein, but a soluble mitochondrial protein of subunit molecular mass 55 kDa was bound, and could be specifically eluted with α -cyano-4-hydroxycinnamate. A preliminary account of some of this work has been presented (Poole & Halestrap, 1988). In the present paper we provide evidence that this protein is the low- K_m rat liver mitochondrial aldehyde dehydrogenase. We also present data that show that α -cyanocinnamate and some related compounds are inhibitors of this enzyme. It is suggested that α -cyanocinnamate-Sepharose is a useful single-step isoenzyme-specific affinity purification of this aldehyde dehydrogenase.

EXPERIMENTAL

Materials

The sources of all chemicals and biochemicals were as given by Thomas & Halestrap (1981), except where stated below. Acetaldehyde and $\text{Na}_4\text{P}_2\text{O}_7$ were obtained from BDH Chemicals, Poole, Dorset, U.K. DL-Glyceraldehyde, NaBH_4 , benzamidine, Sepharose CL-4B and phenylpyruvate were obtained from Sigma Chemical Co., Poole, Dorset, U.K. 1,2:3,4-Bisepoxybutane was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Methods

Coupling of α -cyano-4-hydroxycinnamate to Sepharose CL-4B. Sepharose CL-4B was activated with 1,2:3,4-bisepoxybutane by using the method of Sundberg & Porath (1974). α -Cyano-4-hydroxycinnamate was coupled to the activated support through its phenolic hydroxy group as described below. A 10 ml portion of the activated gel was taken and mixed with 15 ml of 500 mM- α -cyano-4-hydroxycinnamate in 20 mM-sodium phosphate buffer, pH 10.0. This suspension was gently

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stirred for 24 h at room temperature. Any remaining activated sites on the support were blocked with 100 mM-ethanolamine, pH 8.0. The α -cyanocinnamate-Sepharose prepared as outlined above was stored at 4 °C in 0.02% NaN_3 and remained effective in purifying aldehyde dehydrogenase 12 months after preparation. The u.v.-absorption spectrum of a suspension of the coupled ligand in 100 mM-Mops buffer, neutralized to pH 8.0 with NaOH, was measured in a custom-built split-beam spectrophotometer (Halestrap, 1982). A suspension of Sepharose CL-4B was present in the reference cuvette and both cuvettes were constantly stirred.

Purification of aldehyde dehydrogenase from rat liver mitochondria. Rat liver mitochondria were prepared as described by Halestrap (1978). A fraction containing soluble mitochondrial proteins was obtained by disruption, and then removing the membranes, as follows. Mitochondria, in 300 mM-sucrose/2 mM-EGTA/10 mM-Tris/HCl buffer, pH 7.2, were mixed with an equal volume of ice-cold water containing 2 mM-benzamidine before sonication. Membranes were then removed by centrifugation at 150 000 *g* for 45 min at 4 °C. The soluble mitochondrial protein was applied to an α -cyanocinnamate-Sepharose column equilibrated in 50 mM-NaCl/2 mM-benzamidine/1 mM-EDTA/0.1 mM-dithiothreitol/20 mM-Mops neutralized to pH 7.4 with NaOH. The column was washed free of unbound protein before elution with buffer containing 2 mM- α -cyano-4-hydroxycinnamate. Before further use the column was washed in the buffer as above but with the inclusion of 1 M-NaCl, in order to remove any proteins bound in an unspecific manner. Aldehyde dehydrogenase prepared in this way was either used directly or dialysed overnight against the column buffer to remove α -cyano-4-hydroxycinnamate. For long-term storage the enzyme was kept at -20 °C with the addition of 30% (v/v) glycerol.

A fraction containing soluble cytoplasmic proteins was prepared from the supernatant obtained from the mitochondrial preparation, which was subsequently centrifuged at 100 000 *g* for 45 min at 4 °C to remove microsomal and plasma membranes.

Activity assay and measurement of kinetic parameters. Aldehyde dehydrogenase activity was monitored spectrophotometrically at 340 nm at 30 °C in a Pye-Unicam SP8-100 split-beam spectrophotometer. Measurement of the activity of the low- K_m isoenzyme was performed in 100 mM-sodium pyrophosphate buffer, pH 9.0, containing 1 mM- NAD^+ and 100 μM -acetaldehyde. High- K_m isoenzymes were assayed by raising the concentration of acetaldehyde to 10 mM and measuring the increase in the rate of NAD^+ reduction. One unit of activity is defined as the amount of enzyme that produces 1 μmol of NADH/min under these conditions. In experiments where the kinetics of α -cyanocinnamate inhibition of the purified enzyme were investigated, DL-glyceraldehyde was used as the substrate, since it has a relatively high K_m value (Feldman & Weiner, 1972). In these experiments buffer containing both NAD^+ and glyceraldehyde was present in a reference cuvette to correct for significant NAD^+ reduction in the absence of enzyme. Solutions of DL-glyceraldehyde and α -cyanocinnamate were prepared fresh each day. Kinetic

Fig. 1. Absorption spectra of α -cyanocinnamate-Sepharose

U.v.-absorption spectra were obtained as described in the Experimental section. The results shown are for 200 μl of α -cyanocinnamate-Sepharose in 3 ml of 100 mM-Mops/NaOH buffer, pH 8.0 (—), and for 50 μM - α -cyano-4-hydroxycinnamate (----) in the same buffer. Spectra were recorded in the absence (A and D) or in the presence of 10 mM-2-mercaptoethanol (B) or 50 mM-2-mercaptoethanol (C and E).

data were fitted by non-linear least-squares regression analysis to the equations for competitive, non-competitive, uncompetitive and mixed inhibition. Some assays were performed at pH 7.4 in a buffer containing 20 mM-Mops/NaOH, 1 mM- NAD^+ and the appropriate substrate as above.

Analytical procedures. Protein was assayed by the method of Bradford (1976), with bovine serum albumin as standard. SDS/polyacrylamide-gel electrophoresis was performed by using the method of Laemmli (1970). Protein was precipitated with 10% (w/v) trichloroacetic acid, and washed with 0.2 M- Na_2HPO_4 before solubilization in 62 mM-Tris/HCl buffer, pH 6.8, containing 20% (w/v) sucrose, 5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.02% (w/v) Bromophenol Blue. Two-dimensional polyacrylamide-gel electrophoresis was carried out essentially as described by O'Farrell (1975), but with modifications to the sample preparation in accordance with Garrison (1983). Gels were stained with Coomassie Blue G. Sequencing was carried out on an Applied Biosystems 477A pulsed liquid-protein sequencer. Purified aldehyde dehydrogenase was prepared for sequencing by extensive dialysis against water at 4 °C.

RESULTS

Coupling of α -cyano-4-hydroxycinnamate to Sepharose

α -Cyanocinnamate and its derivatives react with thiol groups with an accompanying spectral change (Halestrap, 1976). The reaction is believed to be a reversible addition across the α - β double bond with the formation of an adduct. Fig. 1 shows u.v.-absorption spectra of α -cyanocinnamate-Sepharose. It is clear that the coupled ligand had a similar absorption spectrum to α -cyano-4-hydroxycinnamate with the same λ_{max} , and

Fig. 2. Electrophoretic analysis of α -cyanocinnamate-Sepharose column fractions

Electrophoretic separations and α -cyanocinnamate-Sepharose affinity chromatography were performed as described in the Experimental section. In (a) we show the results of SDS/polyacrylamide-gel electrophoresis on fractions obtained during purification of aldehyde dehydrogenase from rat liver mitochondria. The lanes were loaded with (i), a crude soluble mitochondrial fraction, (ii), a flow-through fraction from α -cyanocinnamate-Sepharose containing no low- K_m aldehyde dehydrogenase activity, and (iii), the α -cyano-4-hydroxycinnamate eluate. In (b) two-dimensional electrophoretic analysis of this α -cyano-4-hydroxycinnamate eluate is shown. SDS/polyacrylamide-gel electrophoresis on fractions obtained from α -cyanocinnamate-Sepharose affinity chromatography of a post-mitochondrial supernatant is shown in (c). The lanes were loaded with (i), unfractionated post-mitochondrial supernatant, and (ii) and (iii), the first two fractions of the α -cyano-4-hydroxycinnamate eluate. The molecular-mass standards shown are β -galactosidase (116 kDa), bovine serum albumin (67 kDa), glutamate dehydrogenase (56 kDa), fructose-bisphosphate aldolase (39 kDa) and chymotrypsinogen A (25.6 kDa).

that it retained reactivity towards 2-mercaptoethanol. α -Cyano-4-hydroxycinnamate itself has an additional absorption peak at higher wavelength that is not seen in the coupled ligand. This is probably related to ionization of the phenolic hydroxy group of the free reagent, which forms part of the linkage of the ligand to the support in the coupled ligand.

Interaction of α -cyanocinnamate-Sepharose with mitochondrial and cytosolic proteins

In Fig. 2(a) we show gel-electrophoretic patterns of fractions obtained during purification of rat liver mitochondrial aldehyde dehydrogenase with α -cyanocinnamate-Sepharose. The α -cyano-4-hydroxycinnamate eluate contained only one major band, of 55 kDa. However, when a high protein concentration was run on the gel, as in Fig. 2(a), a minor component at 57 kDa was also visible. Under these conditions variable quantities of other contaminating proteins of higher molecular mass were visible, but these never exceeded 2% of total protein. The 55 kDa protein was also found to be present in mitochondria prepared from rat heart and both white and brown adipose tissue. Elution of this protein could also be achieved with 15 mM-dithiothreitol, which is able to modify the bound ligand as outlined above (results not shown). In Fig. 2(b) we show two-dimensional electrophoretic analysis (O'Farrell, 1975) of the α -cyano-4-hydroxycinnamate eluate. Two distinct spots of similar intensity were present at 55 kDa, these having pI values of approx. 6.4 and 6.6. In addition a minor spot at about

57 kDa was observed, with a considerably higher pI value. In Fig. 2(c) we show the gel-electrophoretic pattern of both applied protein and α -cyano-4-hydroxycinnamate eluate from an α -cyanocinnamate-Sepharose column loaded with a rat liver cytosolic fraction. It is clear that only trace amounts of the 55 kDa protein were present in the cytoplasm, but that a protein of about 25 kDa, as judged by SDS/polyacrylamide-gel electrophoresis, does interact with the affinity matrix. Preliminary sequencing data suggested that the 55 kDa soluble mitochondrial protein might be aldehyde dehydrogenase, and this was subsequently confirmed by assay of the enzyme.

Aldehyde dehydrogenase activities in rat liver mitochondria and α -cyanocinnamate-Sepharose column fractions

Unfractionated soluble mitochondrial protein had considerable aldehyde dehydrogenase activity with 100 μ M-acetaldehyde as substrate, although raising the concentration to 10 mM caused an increase in the rate of NAD⁺ reduction. In contrast, although the α -cyano-4-hydroxycinnamate eluate from the affinity column had aldehyde dehydrogenase activity in the presence of 100 μ M-acetaldehyde, no further increase in the rate of NAD⁺ reduction was observed upon increasing the acetaldehyde concentration in this range. This observation clearly indicates that the affinity purification is specific for the low- K_m mitochondrial aldehyde dehydrogenase, and that the increase in the rate of

Fig. 3. Fractionation of aldehyde dehydrogenase activities from rat liver mitochondria on α -cyanocinnamate-Sepharose

A 4 ml α -cyanocinnamate-Sepharose column was loaded with a soluble mitochondrial extract, washed free of unbound protein and subsequently eluted with α -cyano-4-hydroxycinnamate (α CHC) as described in the Experimental section. Fractions (1.3 ml) were collected and assayed for protein (●) and both low- K_m (■) and high- K_m (▲) aldehyde dehydrogenase activities as described in the Experimental section.

NAD⁺ reduction upon raising the acetaldehyde concentration from 100 μ M to 10 mM with unfractionated protein is a reflection of the presence of an additional high- K_m enzyme in rat liver mitochondria (Siew *et al.*, 1976; Lindahl & Evces, 1984a). Fig. 3 shows elution profiles of aldehyde dehydrogenase activity and protein for a typical purification of the low- K_m mitochondrial aldehyde dehydrogenase. The rate of NAD⁺ reduction at 100 μ M-acetaldehyde was taken as a measure of the activity of the low- K_m isoenzyme, and the difference between this and the rate at 10 mM-acetaldehyde as a measure of the activity of the high- K_m mitochondrial isoenzyme. The Figure shows that the high- K_m isoenzyme appeared exclusively in the flow-through column fractions. Most of the low- K_m activity was bound to the column and eluted with α -cyano-4-hydroxycinnamate, although some activity could be detected in the flow-through fractions. This became a larger proportion of the total when the quantity of protein applied was increased, which suggests overloading under these conditions. It appears that this activity measured in the flow-through fractions was not due to the high- K_m isoenzyme, since assaying at 50 μ M- rather than 100 μ M-acetaldehyde did not decrease the rate of NAD⁺ reduction (results not shown). For five separate preparations the specific activity of the purified enzyme at 100 μ M-acetaldehyde was 1.46 ± 0.07 units/mg of protein; this compares with a value of 0.045 ± 0.004 unit/mg of protein for the unfractionated extract (values expressed as means $\pm$ S.E.M.) and represents a 33-fold purification. There are no previous reports in the literature of the specific activity of the low- K_m aldehyde dehydrogenase of rat liver mitochondria. However, the enzyme from human and horse liver, assayed with propionaldehyde as substrate at 25 °C at pH 9, has a specific activity of

Fig. 4. Kinetics of α -cyanocinnamate inhibition of the low- K_m mitochondrial aldehyde dehydrogenase

Data are shown for the rates of DL-glyceraldehyde oxidation by purified dialysed aldehyde dehydrogenase (1.65 μ g/assay) at pH 9 at 30 °C in the absence (●) or in the presence of 100 μ M- (▲), 250 μ M- (■) and 500 μ M- (▼) α -cyanocinnamate at the concentrations of substrate shown. Each data point represents the mean of duplicate determinations, and the lines drawn through these points are those obtained by fitting the data by non-linear least-squares regression analysis to the equation for uncompetitive inhibition, as is described in the text. The derived K_m value for DL-glyceraldehyde and K_i for α -cyanocinnamate are also given in the text.

between 0.8 and 1.0 unit/mg of protein (Feldman & Weiner, 1972; Eckfeldt *et al.*, 1976; Greenfield & Pietruszko, 1977). The yields of enzyme depended on the quantity of protein applied to the column as outlined above, but provided that overloading was prevented yields of approx. 80 % were possible. We have also found that no cytosolic aldehyde dehydrogenase was bound to an α -cyanocinnamate-Sepharose column; these experiments were performed with a rabbit liver cytosolic fraction, since rat liver appears to have no such cytosolic aldehyde dehydrogenase activity (Lindahl & Evces, 1984c; R. C. Poole & A. P. Halestrap, unpublished work).

Nature of the interaction between α -cyanocinnamate-Sepharose and the low- K_m mitochondrial aldehyde dehydrogenase

In an attempt to investigate the interaction between α -cyanocinnamate-Sepharose and aldehyde dehydrogenase, we have tried to elute the enzyme from the affinity column with both substrates and compounds related to α -cyano-4-hydroxycinnamate. Such studies have shown that 2 mM-acetaldehyde will not elute the enzyme from α -cyanocinnamate-Sepharose, but that 5 mM-phenylpyruvate is capable of doing so. Phenylpyruvate has certain structural similarities to α -cyanocinnamate derivatives, and like these compounds it is an inhibitor of both mitochondrial pyruvate transport and plasma-membrane lactate transport (Halestrap *et al.*, 1974). Having found α -cyano-4-hydroxycinnamate and phenylpyruvate to elute aldehyde dehydrogenase from the affinity column, we have investigated the effect of these compounds on enzyme activity. For such studies

we have used α -cyanocinnamate rather than the 4-hydroxy derivative, since at pH 9.0 the latter compound absorbs strongly at 340 nm. α -Cyanocinnamate was found to be an effective inhibitor of the purified low- K_m mitochondrial aldehyde dehydrogenase; in Fig. 4 we show Lineweaver–Burk plots at various concentrations of inhibitor that suggest that the inhibition is uncompetitive. The data were fitted by non-linear least-squares regression analysis to the equation for mixed inhibition and gave the following values ($\pm$ S.E.): V_{max} , $3.38 \pm 0.11 \mu\text{mol/min per mg of protein}$, K_m , $185 \pm 19 \mu\text{M}$, $K_{i,1}$, $1703 \pm 1819 \mu\text{M}$, $K_{i,2}$, $232 \pm 23 \mu\text{M}$. The derived K_i values are again indicative of uncompetitive inhibition, and when the data were re-analysed with the use of this model the fit was as good as for mixed inhibition at a 5% probability level. The calculated values ($\pm$ S.E.M.) for the V_{max} , K_m and K_i were now $3.42 \pm 0.10 \mu\text{mol/min per mg of protein}$, $196 \pm 17 \mu\text{M}$ and $219 \pm 16 \mu\text{M}$ respectively. This K_m value for glyceraldehyde agrees closely with that reported by Feldman & Weiner (1972) for the horse liver enzyme. α -Cyanocinnamate was found to be equally potent at pH 7.4 (results not shown). Similarly, phenylpyruvate was also found to be an inhibitor of the enzyme, and again the equation for uncompetitive inhibition was the best fit for the kinetic data and gave a K_i value ($\pm$ S.E.) of $1.28 \pm 0.06 \text{ mM}$. In addition to the compounds mentioned above, α -fluorocinnamate was found to inhibit aldehyde dehydrogenase with a similar potency to α -cyanocinnamate (results not shown).

As was noted above, dithiothreitol was able to elute the low- K_m mitochondrial aldehyde dehydrogenase from α -cyanocinnamate–Sephacrose. We found that an excess of dithiothreitol was also capable of reversing α -cyanocinnamate inhibition of this enzyme (results not shown). In contrast, dithiothreitol failed to reverse the inhibition caused by α -fluorocinnamate, which, unlike cyanocinnamate derivatives, does not react with thiols.

DISCUSSION

We have shown that the low- K_m aldehyde dehydrogenase from rat liver mitochondria can be purified on an α -cyanocinnamate–Sephacrose column. The enzyme purified in this way has a subunit molecular mass of 55 kDa, which agrees closely with the values reported by other workers for the enzyme from rat (Farrés *et al.*, 1988) and other species (Eckfeldt *et al.*, 1976; Greenfield & Pietruszko, 1977; Ikawa *et al.*, 1983). We have attempted to sequence the *N*-terminus of the protein (see the Experimental section) and found the results to be entirely consistent with the cDNA sequence reported by Farrés *et al.* (1988) for the first ten amino acid residues of the enzyme, i.e. Ser-Ala-Ala-Ala-Thr-Ser-Ala-Val-Pro-Ala. However, our data also indicated that there is some *N*-terminal heterogeneity, with two major starting positions, namely Ser-1 and Ala-2. Such *N*-terminal heterogeneity has been noted for the enzyme from both human and horse liver (Hempel *et al.*, 1985; Johansson *et al.*, 1988). Two-dimensional electrophoresis shows two major spots at 55 kDa and a minor spot at 57 kDa with a higher pI. The observation of two spots at 55 kDa could be due to some *N*-terminal acetylation, perhaps on serine as suggested by Farrés *et al.* (1988). The purified enzyme preparation contains a minor 57 kDa component with a somewhat higher pI than the major 55 kDa

protein. It is of note that Braun *et al.* (1987) and Farrés *et al.* (1988) have reported the mitochondrial aldehyde dehydrogenase precursor protein to have a molecular mass of 57 kDa. Like other mitochondrial targeting sequences, the *N*-terminal extension has been found to contain a large number of basic residues (Farrés *et al.*, 1988), and will therefore increase the pI of the protein. Hence it would appear that our data are consistent with the 57 kDa protein present in the affinity-purified aldehyde dehydrogenase being uncleaved precursor for the enzyme. The identity of the rat liver cytosolic protein of approx. 25 kDa that interacts with α -cyanocinnamate–Sephacrose remains unclear.

Kinetic analysis of α -cyanocinnamate interaction with purified aldehyde dehydrogenase showed the compound to be an uncompetitive inhibitor of the enzyme with glyceraldehyde as substrate. Inhibition does not seem to involve a thiol group on the protein, since the related compounds α -fluorocinnamate and phenylpyruvate, neither of which will react with dithiothreitol, are also effective inhibitors of the same enzyme. However, reduction of α -cyanocinnamate with an excess of dithiothreitol produced an adduct (Halestrap, 1976) that did not interact with the enzyme. This explains how dithiothreitol elutes the enzyme from α -cyanocinnamate–Sephacrose. The tight binding of aldehyde dehydrogenase to the α -cyanocinnamate–Sephacrose column would not have been predicted from the K_i value for α -cyanocinnamate inhibition of the enzyme of about $200 \mu\text{M}$. One explanation for this paradox may be that the enzyme is a tetramer (Feldman & Weiner, 1972; Eckfeldt *et al.*, 1976; Greenfield & Pietruszko, 1977) and this provides the potential for four simultaneous binding interactions with the column.

In conclusion, we suggest that α -cyanocinnamate affinity chromatography will be a useful rapid isolation procedure for the low- K_m mitochondrial aldehyde dehydrogenase and its precursor protein. It is possible that this purification procedure may also be of use in purifying the mutant enzyme present in the 50% of orientals who are alcohol-intolerant.

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