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PURIFICATION AND PROPERTIES OF CALF LIVER ALDOSE REDUCTASE*

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SUMMARY

Aldose reductase (alditol NADP$^+$ oxidoreductase, EC 1 1 1 21) from calf liver has been purified 340-fold using techniques which included molecular sieving and electrofocusing On polyacrylamide-disc gels the purified enzyme yielded two antigenically similar bands, with only the top band exhibiting enzymatic activity by the zymogram staining method Sodium dodecylsulphate gels indicated 30 500 as the M_r of the monomer The M_r of the active enzyme was 61 000 from Sephadex G-100 The active enzyme appeared to be a dimer composed of two 30 500 M_r monomers The isoelectric point was pH 8 25 NADPH and NADH were found to have apparent K_m values of 14 and 0 22 μM, respectively The enzyme was inhibited by increasing the ionic strength above 0 01, by sulfhydryl inhibitors, and by 5′-AMP At pH 7 0, the forward reaction was the essentially irreversible reduction of glyceraldehyde by NADPH

INTRODUCTION

The reaction catalyzed by aldose reductase (EC 1 1 1 21) can be represented as

aldose (or aldehyde) + NAD(P)H + H$^+$ $\rightleftharpoons$ alditol + NAD(P)$^+$

This enzyme together with polyol dehydrogenase (polyol NADP$^+$ oxidoreductase, EC 1 1 1 14) comprises the sorbitol pathway which has been found to be the means for conversion of glucose to fructose in seminal vesicles [1] Alditol formation has been implicated in cataract formation [2, 3] and also in clinical symptoms connected with diabetes [4, 5], as well as in insulin release [6] In *Rhodotorula* its presence enabled this yeast to grow on glyceraldehyde as the sole carbon source [7, 8] Studies in brain showed it was implicated in the metabolism of aldehydes formed from biogenic amines [9–11] It has been implicated in the metabolism of other tissues [12–26]

Abbreviation HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid

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This paper describes the isolation and purification of aldose reductase from calf liver and presents evidence of its functional subunit as a dimer Also, other general properties are presented

EXPERIMENTAL PROCEDURE

Materials

Dithiothreitol, NADPH, NADH, glutathione, and the adenosine monophosphates were purchased from Sigma DL-Glyceraldehyde was procured from Aldrich Trizma base ((Tris)hydroxymethylaminomethane) was obtained from Mann Calbiochem supplied the β-mercaptoethanol, iodoacetic acid, and the *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) Serum from a goat immunized with normal rabbit serum (goat anti-rabbit serum) and goat antiserum directed against the Fc fragment of rabbit IgG (goat anti-rabbit IgG (Fc)) were the kind gift of Dr Carey Hanly All other reagents were of the highest purity commercially available

Standard assay

The reaction was followed by measuring the decrease in absorbance of NADPH at 340 nm as previously described by Sheys et al [8] For the purification procedure, the assay mixture consisted of 100 nmoles of NADPH, 10 μmoles of DL-glyceraldehyde, and 10 mM Tris–HCl buffer (pH 7 0, 37 °C) Upon addition of the enzyme the total volume was 1 ml The initial velocities thus obtained were consistently reproducible and generally linear for the first 5 min An enzyme unit was defined as that quantity of enzyme which catalyzed the oxidation of 1 μmole of NADPH/min (37 °C) The specific activity was defined as the number of enzyme units/mg protein All other assays were performed in 10 mM HEPES buffer (pH 7), using 70 nmoles of NADPH and 10 μmoles of DL-glyceraldehyde

Protein determination

Protein determinations were made using the biuret method [27] except for the protein concentration from the final Sephadex G-100 column which was determined by the method of Lowry et al [28]

Enzyme purification

Livers from freshly slaughtered calves were kept on ice for 1–2 h and then stored frozen at −45 °C All purification steps were carried out at 4 °C The buffer used was 10 mM Tris–HCl buffer + 2 mM dithiothreitol [29] The pH was as indicated at 4 °C Unless otherwise specified all centrifugations were in a Sorvall RC-2B at 48 000 × *g* for 10 min

Step 1 Preparation of the cell-free extract Approx 300 g of liver were sliced into thin sections after partial thawing The preparation of a 20% homogenate (pH 7 0) was accomplished by use of a Potter–Elvejhem homogenizer The resulting suspension was centrifuged and the lipid layer removed and discarded The enzyme was found to be in the supernatant fluid or cell-free extract

Step 2 Lipid removal and protamine sulfate treatment The cell-free extract was divided into aliquots of 100–150 ml and then equilibrated to 30 °C for 1–2 min in a water bath The solution was then centrifuged after cooling The lipid layer was again removed and discarded A 1% solution of protamine sulfate was added to the extract

while stirring to give a final concentration of 0 5 mg of protamine sulfate per ml of extract After equilibrating for 15 min with the pH maintained at 7, the solution was centrifuged, and the precipitate was discarded

Step 3 Acid treatment and concentration The pH of the supernatant fluid was lowered to 5 5 with 1 M HCl The solution was then adjusted to pH 7 0 with 1 M NaOH The precipitate formed was removed by centrifugation The supernatant solution was concentrated by ultrafiltration The ultrafiltration device consisted of 0 25-inch dialyzing membrane connected to a receiving funnel, the stem of which had been placed through a rubber stopper The membrane was placed in a vacuum flask, filled with the solution to be concentrated and then the flask was evacuated The vacuum was released when the solution was concentrated to the desired volume The concentrated preparations (maximal total volume of 20 ml) were centrifuged in a Beckman Model L-2 Ultracentrifuge for 90 min at 100 000 $\times$ *g* (39 000 rev /min) using a SW-40 rotor The resulting supernatant fluid was dialyzed overnight against 200 vol of buffer (pH 7 2)

Step 4 DEAE-Sephadex column The dialyzed sample was then placed on a DEAE-50 Sephadex column (2 5 cm $\times$ 60 cm) which was equilibrated and eluted with buffer at pH 7 2 The appropriate fractions were pooled, the pH was adjusted to 7 0, and the sample was then concentrated to a maximal volume of 10 ml by ultrafiltration

Step 5 Sephadex G-100 column The sample was divided in half and each half was then placed on a Sephadex G-100 column (2 5 cm $\times$ 55 cm) which was equilibrated and eluted with buffer (pH 6 8–7 0) The appropriate fractions from both columns were pooled and concentrated to less than 20 ml by ultrafiltration The sample was then dialyzed against 200 vol of buffer (pH 6 8–7 0) for two intervals of 2 h each

Step 6 Electrofocusing column (pH 7–9) The electrofocusing column (LKB 1800) was prepared according to the manufacturer's instructions using pH 7–9 Ampholine in a final concentration of 1% The gradient was formed by use of a gradient maker and both the dense and light solutions were 2 mM in dithiothreitol The column was focused without sample for 48 h The dialyzed sample from the Sephadex G-100 columns was mixed with sucrose and layered at an isodense position on the column Electrofocusing of the sample was accomplished in 18–20 h using 0 45 W The fractions containing activity were pooled, the pH was adjusted to 7 0, and after dilution with an equal volume of buffer (pH 7 0), the pooled fractions were concentrated to 5 ml by ultrafiltration The concentrated sample was then placed on a Sephadex G-100 column equilibrated and eluted as previously described The appropriate fractions were pooled and concentrated to 15 ml by ultrafiltration

Step 7 Electrofocusing column (pH 7 5–8 5) A second electrofocusing column was prepared as previously described, except that the Ampholine concentration was increased to 2% After focusing the column for 48 h, it was drained and all the fractions between pH 7 5 and 8 5 were pooled and used according to the manufacturer's instructions to prepare a column with an expanded gradient of pH 7 5–8 5 This column was focused (without sample) for 48 h and then the sample was applied and focused as previously described After concentration to 5 ml, the electrofocused sample was placed on a Sephadex G-100 column equilibrated and eluted as previously described The fractions containing activity were pooled and ultrafiltered until the protein concentration was 500 μg/ml or greater (approx 15 ml) The purified enzyme was then stored at -45 °C

Polyacrylamide-disc gel electrophoresis

The use of a 7% gel in a pH 9 5 system [30] was found to be the most reproducible Gels (0 5 cm × 7 cm) were run for 2–3 h at a current of 2 5 mA/gel Bromphenol blue (0 05%) was used as the dye front The gels were stained for 1 h with 0 25% Coomassie blue in 45% methanol and 9% acetic acid, destained electrophoretically, and stored in 7 5% acetic acid and 5% methanol

Zymogram staining method

Electrophoresis was accomplished as previously described Enzymatic activity was determined by employing the staining method described by Kormann et al [19], substituting NAD^+ for $NADP^+$ A positive reaction was indicated by the presence of a vibrant pink band

Immunization procedure

Two female white rabbits were each immunized with 200 μg of aldose reductase (Step 7) Five injections were administered at intervals of 2 weeks Serum was obtained and stored at −45 °C

Ouchterlony double diffusion technique [31]

Slides were covered with 1 5% Noble agar in 0 2 M borate–0 15 M NaCl buffer (pH 8) The wells were filled, the slide was placed in a humidity chamber at room temperature, and diffusion was allowed to take place for 18–24 h After diffusion the slide was read and then placed in 0 15 M NaCl for 48 h with intermittent readings The final reading was taken after washing in distilled water for 24 h Staining of dried slides was carried out in 0 12% Coomassie blue in 0 12% methanol and 4% acetic acid for 1–1 5 h Slides were destained in 3% acetic acid and 5% glycerol for 18 h

Purification of IgG [32, 33]

Sera were pooled to comprise two separate samples (1) pre-immune and (2) anti-enzyme Each of these samples was separately treated with a 25% sodium sulfate solution to give successive final concentrations of 18% and 14% The samples were dialyzed overnight against 10 vol of cold 0 2 M borate–0 15 M NaCl buffer (pH 8) between sodium sulfate precipitations, and the final precipitate was dialyzed against 10 mM phosphate buffer (pH 7 5) Each of the samples was then placed on a DEAE-cellulose column which had been equilibrated and was eluted with 10 mM phosphate buffer (pH 7 5) The IgG was eluted with this buffer and then ultrafiltered and dialyzed against NaCl–borate buffer (pH 8)

Immunoelectrophoresis procedure [34]

In order to determine the purity of the various fractions, immunoelectrophoresis using 1 5% Noble agar in 0 05 ionic strength barbital buffer (pH 8 6) was performed The same barbital buffer was used in the buffer chambers The plates were electrophoresed for 1 25–1 5 h while maintaining a voltage of 4–4 6 V/cm After electrophoresing, troughs were cut, filled with antisera, and the plates were placed in a humidity chamber Usually precipitin bands could be discerned after diffusion for 24–48 h for the enzyme preparations and after 18–24 h for the IgG preparations

TABLE I

PURIFICATION OF LIVER ALDOSE REDUCTASE

Pooled four purifications (Steps 1–3) and then proceeded with Steps 4–7 Results are given for four combined preprarations Procedures are described in the text

Step	Total enzyme units	Total protein (mg)	Spec act (units/mg)	Total purification	Recovery (%)
1 Cell-free extract	1830	55 000	0 033	1 00	100
2 Protamine sulfate supernatant	1790	46 000	0 039	1 18	99
3 Acid treatment and concentration of the supernatant	1290	31 000	0 041	1 24	72
4 DEAE-Sephadex column eluate	1280	1 840	0 71	22	69
5 Sephadex G-100 column eluate	970	560	1 7	52	53
6 Electrofocusing (pH 7–9) column eluate	460	135	3 4	103	25
7 Electrofocusing (pH 7 5–8 5) column eluate	180	16 0	11 2	340	10

RESULTS

Purification

The results of 340-fold purification of the enzyme are contained in Table I The cell-free extract (spec act 0 033) obtained from 300 g of liver contained 1830 enzyme units The purified enzyme (spec act 11 2) was recovered in 10% yield All of the experiments employed this purified preparation unless otherwise indicated To prevent

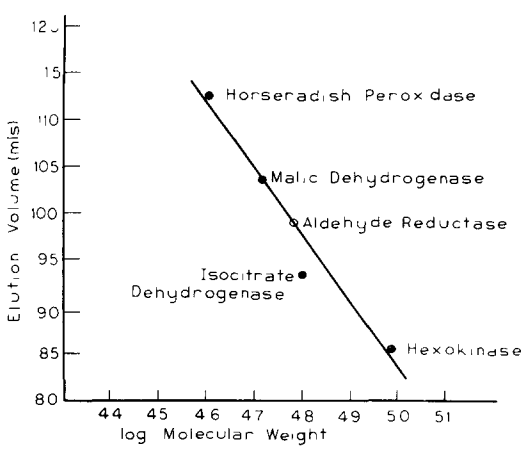

Fig 1 Determination of the molecular weight of the enzyme using Sephadex G-100 The gel filtration method as developed by Andrews [35] was used The Sephadex G-100 column (0 9 cm × 48 cm) was both equilibrated and eluted with 10 mM Tris–HCl buffer + 2 mM dithiothreitol (pH 7 0) Enzyme from Step 7 (500 μg) was placed on the column along with 50 μl of a 1% Blue Dextran 2000 solution and four marker enzymes 0 3 mg yeast hexokinase (M_r 96 000) (36), 0 1 mg pig-heart isocitrate dehydrogenase (M_r 64 000) (37), 0 5 mg pig-heart malate dehydrogenase (M_r 52 000) (38), and 0 3 mg horseradish peroxidase (M_r 40 000) (39) The enzymatic activity of aldose reductase and each of the marker enzymes was measured in the eluant

activity loss, it was essential to add 2 mM dithiothreitol The purified enzyme (Step 7) could be stored frozen (−45 °C) for at least 1 year and retain activity

Subunit structure

After electrophoresis on polyacrylamide disc gels and staining with Coomassie blue, the purified enzyme was resolved into two bands wich migrated approx 0 5 and 1 cm into the gel When the two bands were stained by the zymogram staining method adapted by Kormann et al [19] for staining $NADP^+$-glycerol dehydrogenase, only the top band exhibited enzymatic activity

The enzyme was recovered in 95% yield in the 61 000 M_r fraction from a Sephadex G-100 column (Fig 1) and no activity was found in the 31 000 M_r or any other region In order to exclude the possibility that the M_r was being altered during the purification procedure, enzyme from Step 3 was placed on a similar Sephadex G-100 column Both the crude and purified enzyme had a M_r of 61 000 (in the absence or presence of 2 mM dithiothreitol)

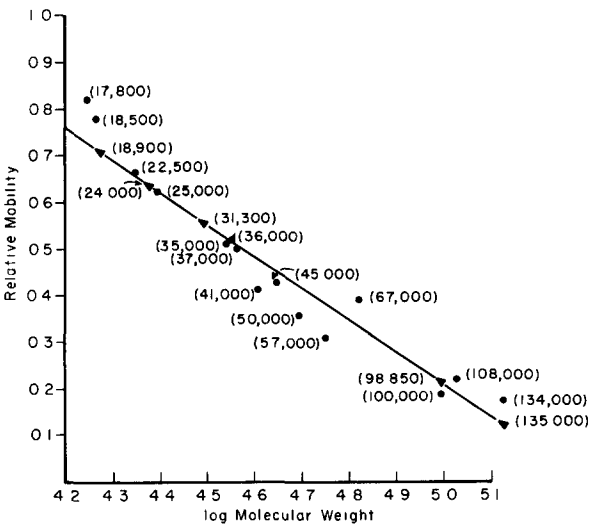

Fig 2 Determination of the molecular weight using sodium dodecylsulphate gels Nine marker proteins of known M_r were used according to the procedure described by Weber and Osborn [40] Gels of 10% were run at 37 °C for 2–3 h using 8 mA/gel The gels were stained with Coomassie blue as described in the text The following markers were used

Marker	M_r on gel (main band first)
Bovine serum albumin	67 000 + 134 000
Pyruvate kinase	57 000
Human γ-globulin	50 000, 100 000 + 25 000
Ovalbumin	45 000 + 22 500
Enolase	41 000
Yeast alcohol dehydrogenase	37 000 + 18 500
Glyceraldehyde phosphate dehydrogenase	36 000 + 108 000
Pepsin	35 000
Myoglobin	17 800

On sodium dodecylsulphate gels also only one major band was obtained, but this was at M_r 30 500 with no detectable band at M_r 61 000 (Fig 2) When 0 1% β-mercaptoethanol was included, the same results were obtained Only trace bands appeared at M_r 136 000, 97 000, 23 000, and 18 500

Immunological properties

Antibodies to the purified enzyme were used to determine whether the two bands obtained on the disc gels showed antigenic similarity Purified anti-enzyme and pre-immune IgG preparations were obtained from rabbit sera Enzymatic activity was inhibited (30%) in vitro by the anti-enzyme IgG fraction When the anti-enzyme fraction was tested against purified enzyme by Ouchterlony double diffusion in agar gel, only one precipitin band resulted No precipitin reaction was observed between the pre-immune IgG fraction and the enzyme

In order to demonstrate the immunochemical similarity of the 61 000 and 30 500 M_r components, immunoelectrophoretic studies were pursued The anti-enzyme preparation recognized two separate but antigenically related components as indicated by two precipitin arcs which showed partial coalesence

Enzyme samples of 37 5 and 75 μg protein were electrophoresed on separate disc gels, and then these gels were used as the antigen source The gels were placed on glass slides, covered with 1 5% Noble agar, and antibody was placed in troughs cut parallel to the gel After 96 h of diffusion, no distinct precipitin arcs were detectable To enhance the visibility of the insoluble antigen–antibody complexes, an indirect method was used, i e the immunodiffusion plates were soaked in 0 15 M NaCl for 48 h to remove soluble proteins and then soaked in goat anti-rabbit IgG (Fc) for 24 h at 4 °C The plates were then soaked in saline for 48 h and then in water for 24 h After this treatment, two precipitin arcs which showed cross reactivity were present The position of the precipitin arcs corresponded to the position of the two protein bands on disc gels

Effects of pH and ionic strength

The isoelectric point of liver aldose reductase was determined to be 8 25 on an electrofocusing column, using 1% Ampholine with a pH range of 7–9 and was confirmed again using Ampholine pH range 7 5–8 5 The pH optimum was 6 7 in 0 01 M HEPES buffer At 0 01 ionic strength, it was 6 6 in Tris–HCl buffer and 6 25 in sodium phosphate buffer

At pH 7 0, only the forward reaction of the reduction of glyceraldehyde by NADPH or NADH could be measured However, as the pH was raised above 8, the reverse reaction increased to a measurable rate The pH optimum of the reverse reaction was 9 7–10 4 in 10 mM Tris–HCl buffer with 75 nmoles NAD^+ and 100 μmoles glycerol No reverse reaction could be detected with $NADP^+$ (75 –225 nmoles) and glycerol (100–300 μmoles) The velocity of the reverse reaction at pH 9 7 with NAD^+ was about 10% of the velocity for the forward reaction at pH 7 0 with NADH

Raising the ionic strength from 0 01 to 0 03 caused a decrease in enzymatic activity of at least 35% This effect was obtainable upon the addition of any of the following salts NaCl, KCl, NH_4Cl, LiCl, $MgCl_2$, $CaCl_2$, $(NH_4)_2SO_4$, NaH_2PO_4, Na_2HPO_4, Tris–HCl, NH_4Br, KBr, KF, NaF, and sodium acetate

Substrate requirements

Both NADPH and NADH were compared as the pyridine nucleotide for the reaction at pH 7 0 The maximal velocity with NADH was about 50% of that obtained with NADPH The relative apparent K_m values were 14 μM for NADPH and 0 22 μM for NADH

Both the D- and L-enantiomers of glyceraldehyde were capable of serving as substrates Both isomers had the same maximal velocity of 1 4 μmoles/min per mg protein and close apparent K_m values of 3 0 and 2 1 mM for the DL-racemic mixture and the D-isomer, respectively

Substrate profile

Aldehydes and aldoses of varying concentrations were tested as substrates (Table II)

The enzyme was found to reduce short chain (2–5 carbon) aliphatic aldehydes giving the greatest velocity for *n*-butyraldehyde (spec act 26) *p*-Nitrobenzaldehyde, benzaldehyde, and DL-glyceraldehyde were reduced at about half the rate obtained for *n*-butyraldehyde

TABLE II

SUBSTRATE PROFILE

The assay cuvette contained 100 nmoles of NADPH and substrate in the concentration indicated in 10 mM Tris–HCl buffer (pH 7 0, 37 °C)

Substrate	Concn	Spec act (units/mg)	Relative activity* (%)
D-Glucose	21 mM	0 010	0 08
D-Frucose	21 mM	0 018	0 15
D-Galactose	21 mM	0 018	0 15
D-Ribose	21 mM	0 018	0 15
D-Xylose	21 mM	0 026	0 22
Dihydroxyacetone	21 mM	8 8	73 0
DL-Glyceraldehyde	10 mM	12 0	100 0
p-Nitrobenzaldehyde	250 μM	14 4	120 0
p-Hydroxybenzaldehyde	650 μM	0 27	2 3
Benzaldehyde	650 μM	14 3	119 0
Acetaldehyde	650 μM	16 3	136 0
Propionaldehyde	650 μM	23 3	194 0
n-Butyraldehyde	650 μM	26 1	218 0
n-Valeraldehyde	650 μM	4 3	36 0

* The activity obtained with DL-glyceraldehyde was chosen as 100%

Sulfhydryl compounds

In order to purify the enzyme, it was found necessary to add a sulfhydryl compound The enzymatic activity was stabilized by 2 mM dithiothreitol Other sulfhydryl compounds such as β-mercaptoethanol, glutathione, and cysteine were less effective

Dialysis of the enzyme against buffer without dithiothreitol caused a loss of at least 35% of the activity However, most of this activity was recoverable upon equilibration with dithiothreitol (2 mM)

Dithiothreitol was a competitive inhibitor when either glyceraldehyde or NADPH was the variable substrate When glyceraldehyde was the variable substrate, the K_i was 4 8 10^{-4} M for the dialyzed enzyme (i e enzyme–dithiothreitol) When NADPH was the variable substrate, the K_i was 6 3 10^{-4} M

β-Mercaptoethanol was also a competitive inhibitor when NADPH was the variable substrate The K_i was 4 2 10^{-4} M for the dialyzed enzyme

Sulfhydryl blocking reagents

The effect of various sulfhydryl blocking reagents was examined $HgCl_2$ (10 nmoles) precipitated the enzyme *p*-Hydroxymercuribenzoate inhibited the enzyme to the extent of 75% when present in a concentration of 10 nmoles In the presence of 20 nmoles, the enzymatic activity was 90% inhibited Iodoacetic acid (500 nmoles) also inhibited the enzyme about 50%

Other inditors

In the present study, both sulfate and pyrophosphate were competitive inhibitors when NADPH was the variable substrate The K_i was 2 0 10^{-4} M for $(NH_4)_2SO_4$ and 12 4 10^{-4} M for $Na_4P_2O_7$

Adenosine monophosphates were competitive inhibitors when NADPH was the variable substrate The inhibition constants were 2′-AMP, 370 10^{-6} M, 3′-AMP, 910 10^{-6} M, 5′-AMP, 2 9 10^{-6} M, and cyclic 3′, 5′-AMP, 83 10^{-6} M

Stability properties

The enzyme was inactivated at 50 °C after 10 min Repeated freeze-thawing resulted in loss of activity At any stage of purification the enzyme could be stored at −45 °C with little or no loss of activity

DISCUSSION

The active liver aldose reductase is believed to be a dimer This was supported by the quantitative recovery of aldose reductase in the 61 000 M_r fraction of Sephadex G-100 as well as the positive enzyme zymogram staining of only one of the two bands obtained on disc gels, although both bands were antigenically related On dodecyl-sulphate gels, the data suggested the nearly quantitative conversion to the 30 500 M_r fraction Although a number of aldose reductases have been described from mammalian and microbial sources, incomplete data was available to indicate a functional subunit structure

The isoelectric point of liver aldose reductase was found to be pH 8 25, as compared to pH 5 05 for aldose reductase from *Rhodotorula* [8] and pH 4 9 for aldose reductase from calf lens [24]

Both D- and L-glyceraldehyde were substrates for the reductase Similar results had previously been reported for aldose reductase from *Rhodotorula* [8] Even though a greater max velocity was attained with NADPH as compared to NADH, the apparent K_m for NADPH was over 60 times higher than for NADH Similar results were found for the retinol reductase from rat intestine [16] The enzyme was found to reduce short chain aliphatic aldehydes as well as cyclic aldehydes and some aldoses The relative activity was found highest for several non-aldose aldehydes including *n*-

butyraldehyde and propionaldehyde From the results of the substrate profile in Table II, it was evident that this enzyme could best be classified as an aldose reductase (alditol $NADP^+$ oxidoreductase, EC 1 1 1 21)

$(NH_4)_2SO_4$ and $Na_4P_2O_7$ were competitive inhibitors when NADPH was the variable substrate Similar inhibition has been reported for the aldose reductase from *Rhodotorula* [8] and for the glycerol dehydrogenase from rabbit skeletal muscle [19] However, the aldose reductase from calf lens [24] was activated by the addition of sulfate

Dithiothreitol was found to stabilize the enzyme and also was a competitive inhibitor for both substrates The fact that this compound exhibited this inhibition pattern could be due to structural resemblances to both substrates It is also possible that dithiothreitol was only a partially competitive inhibitor and that its inhibitory effects were due solely to alterations in the affinity of the enzyme for the substrates rather than actually acting at the active site Under these conditions, dithiothreitol would be altering the conformation of the enzyme Although the exact nature of the competitive inhibition has not yet been determined, considering the fact that dithiotheitol stabilizes the enzyme, it seems most likely that the compound was a partially competitive inhibitor

The adenosine monophosphates were competitive inhibitors when NADPH was the variable substrate The K_i for the 5′-AMP was about 30 times lower than for the cyclic compound and 120 times lower than for the 2′-AMP isomer The order of these inhibition constants along with the fact that either NADPH or NADH were effective as cofactors indicated that the 5′-phosphate moiety was the most necessary phosphate group for binding to the enzyme Since the 2′-AMP isomer was a competitive inhibitor, and with NADH as cofactor the reaction reached only half of the velocity reached with NADPH as the cofactor, it was possible that the additional 2′-phosphate moiety of NADPH enabled it to be bound at the active site less tightly since the apparent K_m values for NADPH and NADH were found to be 14 and 0 22 μM, respectively Thus the binding at the 2′-site could have caused a change in the conformation of the active site such that the binding at the 5′-site was now not as tight as it would have been if there was no phosphate moiety bound at the 2′-site This could account for the increased maximum velocity obtained with NADPH as the cofactor

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REFERENCES

1 Hastein, T and Velle, W (1969) Biochim Biophys Acta 178, 1–10
2 Kinoshita, J H , Dvornik, D , Kraml, M and Gabbay, K H (1968) Biochim Biophys Acta 158, 472–475
3 Chylack, L T and Kinoshita, J H (1969) Invest Ophthalmol 8, 401–408

4 Gabbay, K and Snider, J J (1972) Diabetes 21, 295–300
5 Gabbay, K and O'Sullivan, J B (1968) Diabetes 17, 300
6 Gabbay, K and Tze, W J (1972) Proc Natl Acad Sci U S 69, 1435–1439
7 Watson, J A , Hayashi, J A , Schuytema, E and Doughty, C C (1969) J Bacteriol 100, 110–116
8 Sheys, G H , Arnold, W J , Watson, J A , Hayashi, J A and Doughty, C C (1971) J Biol Chem 246, 3824–3827
9 Breese, R G , Chase, T N and Kopin, I J (1969) Biochem Pharmacol 18, 863–869
10 Eccleston, D , Moir, A T B , Reading, H W and Ritchie, I M (1966) Br J Pharmacol Chemother 28, 367–377
11 Rutledge, C O and Jonason, J (1967) J Pharmacol Exp Ther 157, 493–502
12 Bosron, W F and Prairie, R L (1973) Arch Biochem Biophys 154, 166–172
13 Bronaugh, R L and Erwin, V G (1973) J Neurochem 21, 809–815
14 Clements, Jr, R S , Weaver, J P and Winegrad, A (1969) Biochem Biophys Res Commun 37, 347–353
15 Erwin, V G , Heston, W D W and Tabakoff, B (1972) J Neurochem 19, 2269–2278
16 Fidge, N and Goodman, D S (1968) J Biol Chem 243, 4372–4379
17 Gabbay, K (1972) Isr J Med Sci 8, 1626–1629
18 Hayman, S and Kinoshita, J (1965) J Biol Chem 240, 877–882
19 Kormann, A W , Hurst, R O and Flynn, T G (1972) Biochim Biophys Acta 258, 40–55
20 Mano, Y , Suzuki, K , Yamada, K and Shimazono, N (1961) J Biochem Tokyo 49, 618–634
21 Moonsammy, G I and Stewart, M A (1967) J Neurochem 14, 1187–1193
22 Moore, B W (1959) Comm to the ed J Am Chem Soc 81, 5837–5838
23 Tabakoff, B and Erwin, V G (1970) J Biol Chem 245, 3263–3268
24 Thrash, C (1971) M S Thesis, Univ of Illinois at the Medical Center
25 Toews, C J (1966) Biochem. J 98, 27C–29C
26 Turner, A J and Tipton, K F (1972) Biochem J 130, 765–772
27 Gornall, A G , Bardawill, C J and David, M M (1949) J Biol Chem 177, 751
28 Lowry, O H , Rosebrough, N J , Farr, A L and Randall, R J (1951) J Biol Chem 193, 265–275
29 Cleland, W W (1964) Biochemistry 3, 480–482
30 Zweig, G and Whitaker, J R (1967) in Paper Chromatography and Electrophoresis, pp 105–172, Academic Press, New York
31 Dray, S , Young, G and Gerald, L (1963) J Immunol 91, 403–415
32 Kekwick, R A (1940) Biochem J 34, 1248–1257
33 Fahey, J L and Horbett, A P (1959) J Biol Chem 234, 2645–2651
34 Grabar, P and Williams, Jr, C A (1955) Biochim Biophys Acta 17, 67–74
35 Andrews, P (1964) Biochem J 91, 222–223
36 Slein, M W (1965) in Methods of Enzymatic Analysis (Bergmeyer, H U , ed), pp 117–123, Academic Press, New York
37 Siebert, G (1965) in Methods of Enzymatic Analysis (Bergmeyer, H U , ed), pp 318–323, Academic Press, New York
38 Hohorst, H J (1965) in Methods of Enzymatic Analysis (Bergmeyer, H U , ed), pp 328–332, Academic Press, New York
39 Bernt, E and Bergmeyer, H U (1965) in Methods of Enzymatic Analysis (Bergmeyer, H U , ed), pp 633–635, Academic Press, New York
40 Weber, K and Osborn, M (1969) J Biol Chem 244, 4406–4412