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STEREOSPECIFICITY OF THE HYDROGEN TRANSFER CATALYZED BY HUMAN PLACENTAL ALDOSE REDUCTASE

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Summary

Placental aldose reductase (EC 1.1.1.21) was incubated with glucose in the presence of [*4A*-^{2}H]NADPH prepared in the oxidation of [2-^{2}H]isocitrate by isocitrate dehydrogenase (EC 1.1.1.42) or [*4B*-^{2}H]NADPH prepared in the oxidation of [1-^{2}H]glucose by glucose-6-phosphate dehydrogenase (EC 1.1.1.49). The sorbitol formed from [*4A*-^{2}H]NADPH contained deuterium and from [*4B*-^{2}H]NADPH it did not. Therefore, aldose reductase in an A-type enzyme.

Introduction

Enzymatically catalyzed oxidation and reductions of aldehydes by pyridine nucleotides occur with the direct and stereospecific addition and removal of hydrogen from the para position of the nicotinamide ring of the nucleotide.

The formation of sorbitol is via reduction by NADPH of glucose, catalyzed by aldose reductase (EC 1.1.1.21). Increased attention has been directed to this reaction in part because of the possible involvement of the polyol pathway in the development of complications in the diabetic [1]. This reaction and the oxidation of the sorbitol to fructose, catalyzed by sorbitol dehydrogenase (EC 1.1.1.14) constitute the polyol pathway. Using enzymes with known stereospecificity [2] as reference enzymes, the stereospecificity of the hydrogen transfer in the aldose reductase catalyzed reaction has been determined. Human placental aldose reductase was coupled with isocitrate dehydrogenase (EC 1.1.1.42), an NADP-dependent A-type enzyme, and with glucose-6-*P* dehydrogenase (EC 1.1.1.49), an NADP-dependent B-type enzyme.

Experimental procedure

Materials: Pig heart isocitrate dehydrogenase, yeast hexokinase (EC 2.7.1.1) and NADP were purchased from Sigma Chemical Company, St. Louis, Mo. Glucose-6-*P* dehydrogenase was purchased from Boehringer Mannheim Corporation, New York, N.Y. D-[1-^{2}H]glucose and sodium borodeuteride, each with a reported minimum isotopic purity of 98%, were purchased from Merck and Company, Inc. St. Louis, Mo. Aldose reductase was prepared from fresh human placenta as described by Clements and Winegrad [3], except that purification did not include isoelectric focusing.

Isocitrate dehydrogenase as reference enzyme. The preparation of [*4A*-^{2}H]-NADPH was as described by Freudenthal et al. [4] except that sodium borodeuteride was used in the reduction of triethyloxalosuccinate. 400 μmol of the deuterated isocitrate preparation was incubated at 37°C in 9 ml of Tris sulfate buffer, pH 7.5, containing 110 μmol of NADP, 100 μmol of $MnSO_4$ and 50 units of isocitrate dehydrogenase. After 10 min of incubation, when 70 μmol of NADPH were formed as measured by optical absorbance, the reaction mixture was heated at 90°C for 2 min. It was centrifuged and 3 units of aldose reductase and sufficient glucose to achieve a concentration of 800 μmol/ml were added to the supernatant. This reaction mixture was incubated for 105 min by which time 50 μmol of NADPH has disappeared. The reaction was then terminated by the addition of 1 ml of 0.3 M $Ba(OH)_2$ and 1 ml of 5% $ZnSO_4$ [5] and the mixture centrifuged. The supernatant was deionized by passage through a mixed-bed ion-exchange resin (Amberlite MB-3, Mallinkrodt Chemical Works, St. Louis, Mo.). The effluent was concentrated and incubated with glucose oxidase in pH 5.6 phosphate buffer. This reaction mixture was deproteinized with $Ba(OH)_2$ and $ZnSO_4$, centrifuged, and the supernatant passed through another mixed-bed ion-exchange column to remove gluconate formed from the glucose. The effluent and washings were concentrated and the treatment with glucose oxidase and deionization repeated. The effluent was concentrated and applied to Whatman preparative 3MM paper, and the chromatography performed using an *n*-butanol/glacial acetic acid/water system (by volume 50 : 25 : 25) [6]. The sorbitol area, identified by guide spots, was eluted with water and an aliquot was assayed for sorbitol [7] and the remainder was lyophilized to dryness and the sorbitol residue analyzed for deuterium content as described below.

Glucose-6-P dehydrogenase as reference enzyme. The preparation of the [*4B*-^{2}H]NADPH was essentially as described by Stern and Vennesland [8]. 10 ml of 0.1 M potassium phosphate buffer, pH 8.0, containing 300 μmol of [1-^{2}H]glucose, 380 mg of ATP, 168 μmol of NADP, 120 μmol of $MgSO_4$, 400 units of hexokinase, and 27 units of glucose-6-*P* dehydrogenase were incubated for 1 h at 30°C, at which time 150 μmol of NADPH had been formed as measured by optical absorbance. The reaction mixture was boiled for 2 min and the pH adjusted to 6.2. 2 units of aldose reductase were dissolved in this reaction mixture and an additional 3300 μmol of unlabeled glucose were added. Incubation was then continued for 3 h at 30°C, at which time 93% of the NADPH had been oxidized. The incubation was terminated and the sorbitol isolated as described above.

In a second preparation the procedure was identical except that the reaction mixture, after incubation with glucose-6-*P* dehydrogenase, was centrifuged and the resulting supernatant was applied to a Bio-Gel-P-2 column and eluted with potassium phosphate buffer pH 9.1 [4]. The fractions containing the $NADP^2H$, 114 μmol by optical absorbance, were combined and had a total volume of 35 ml. The pH of this solution was adjusted to 6.5 with 1 M H_3PO_4, the aldose reductase dissolved in the solution and unlabeled glucose added to a concentration of 800 μmol per ml. Incubation was for 30 min at which time 64% of the NADPH had been oxidized. The reaction was then terminated and sorbitol isolated as described above.

Another experiment was carried out twice using glucose-6-*P* dehydrogenase. In it, the NADP regenerated by aldose reductase was reduced, and utilized in a second aldose reductase catalyzed reduction. For this [1-^{2}H]glucose was incubated with the dehydrogenase as described above on a larger scale, yielding 309 μmol of NADPH. After the reaction mixture was heated and centrifuged, the supernatant was desalted by passage through a Sephadex G-10 column (Pharmacia Fine Chemicals, Uppsala, Sweden). The effluent, containing 215 μmol of NADPH, was applied to a DEAE cellulose column (Whatman DE 52, W. and B. Balston Ltd., Maidstone, Kent, U.K.) in the bicarbonate form and the NADPH separated from unreacted NADP by discontinuous ammonium bicarbonate elution [9]. The fraction, containing 179 μmol of NADPH, was lyophilized and to the resulting NADPH residue was added 6 ml of 0.06 M, pH 6.2, potassium phosphate buffer, 648 mg of glucose and 3.5 units of aldose reductase. After 105 min of incubation when 48 μmol of NADPH remained, the reaction mixture was heated for 2 min, centrifuged, and the supernatant applied to another DEAE cellulose column. On elution with ammonium bicarbonate the initial eluate contained the unreacted glucose, as determined using glucose oxidase, and from this fraction sorbitol was isolated as described above. A subsequent fraction containing 53 μmol of NADP, and devoid of NADPH as determined by absorbance, was lyophilized. This NADP was reduced to NADPH by incubation with glucose-6-dehydrogenase as described above, but using unlabeled glucose rather than [1-^{2}H]glucose. The reaction was terminated after 36 μmol of NADPH formed. The pH of the reaction mixture was adjusted to 6.2, glucose to a concentration of 600 μmol/ml and aldose reductase was added and after 1 h incubation 12 μmol of NADPH remained. Sorbitol was then isolated from this reaction mixture.

Deuterium analyses. Deuterium content of the sorbitols was determined using a gas chromatograph-mass spectrometer-accelerating voltage alteration system, previously described by Klein et al. [10]. Sorbitol was converted to its hexaacetate [11] prior to gas chromatography on a 1 mm × 122 cm glass column packed with 1% SP-525 on 100/200 mesh Supelcoport. Column temperature was 180° C; injection and manifold temperatures were 250° C. The ion source was operated at 150° C, with the electron energy at 70 eV. The mass spectrum exhibits a base peak at *m/e* 115 ($C_5H_7O_3$) (Fig. 1). [1-^{2}H]sorbitol, prepared both by (a) reduction of unlabeled glucose with sodium borodeuteride, and (b) reduction of [1-^{2}H]glucose with sodium borohydride [12] was used for reference. Abbreviated spectra of [1-^{2}H]sorbitol and unlabeled sorbitol are shown in Fig. 2. Incorporation of deuterium was measured as the

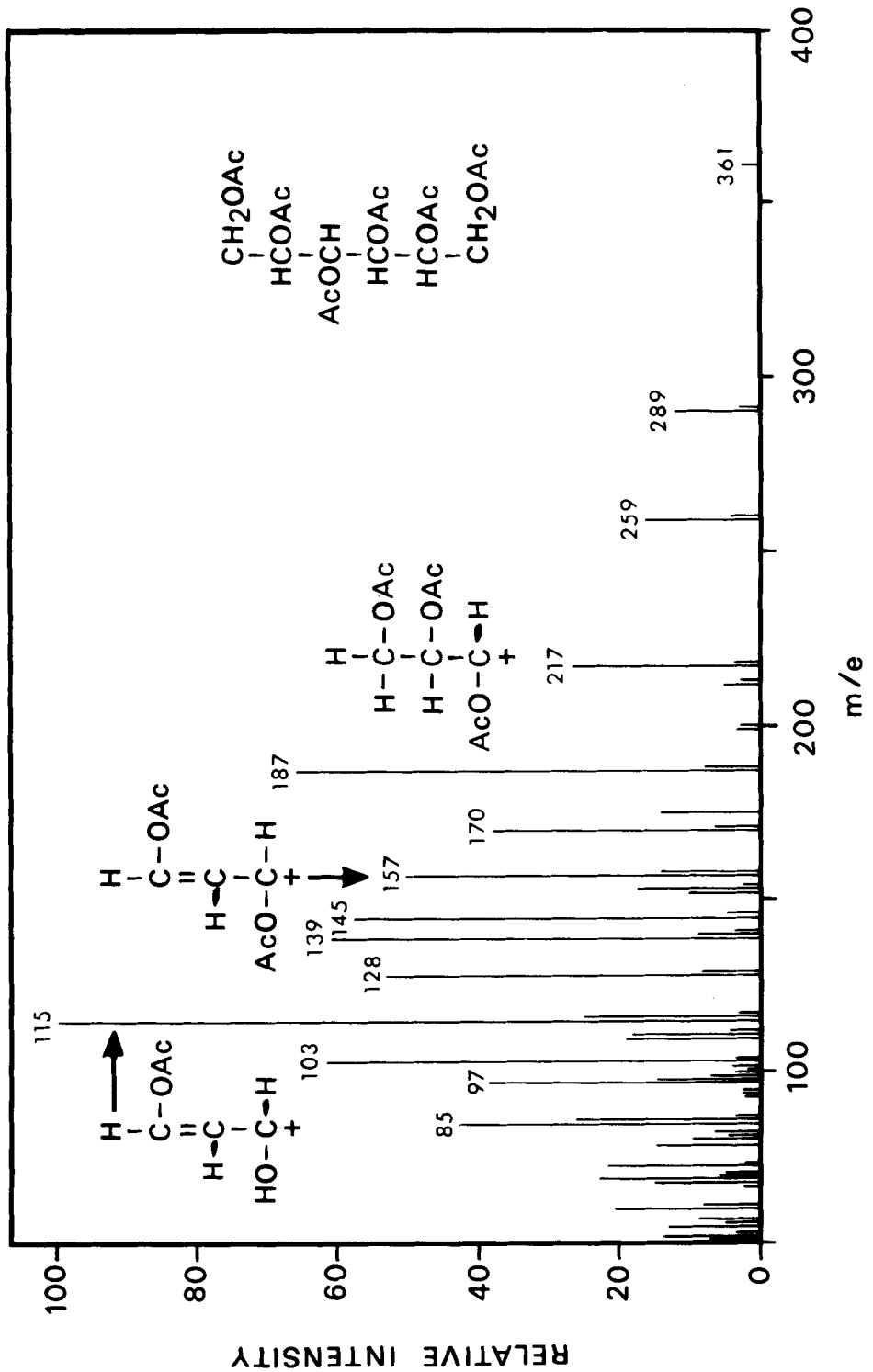

Fig. 1. Mass spectrum of sorbitol hexaacetate.

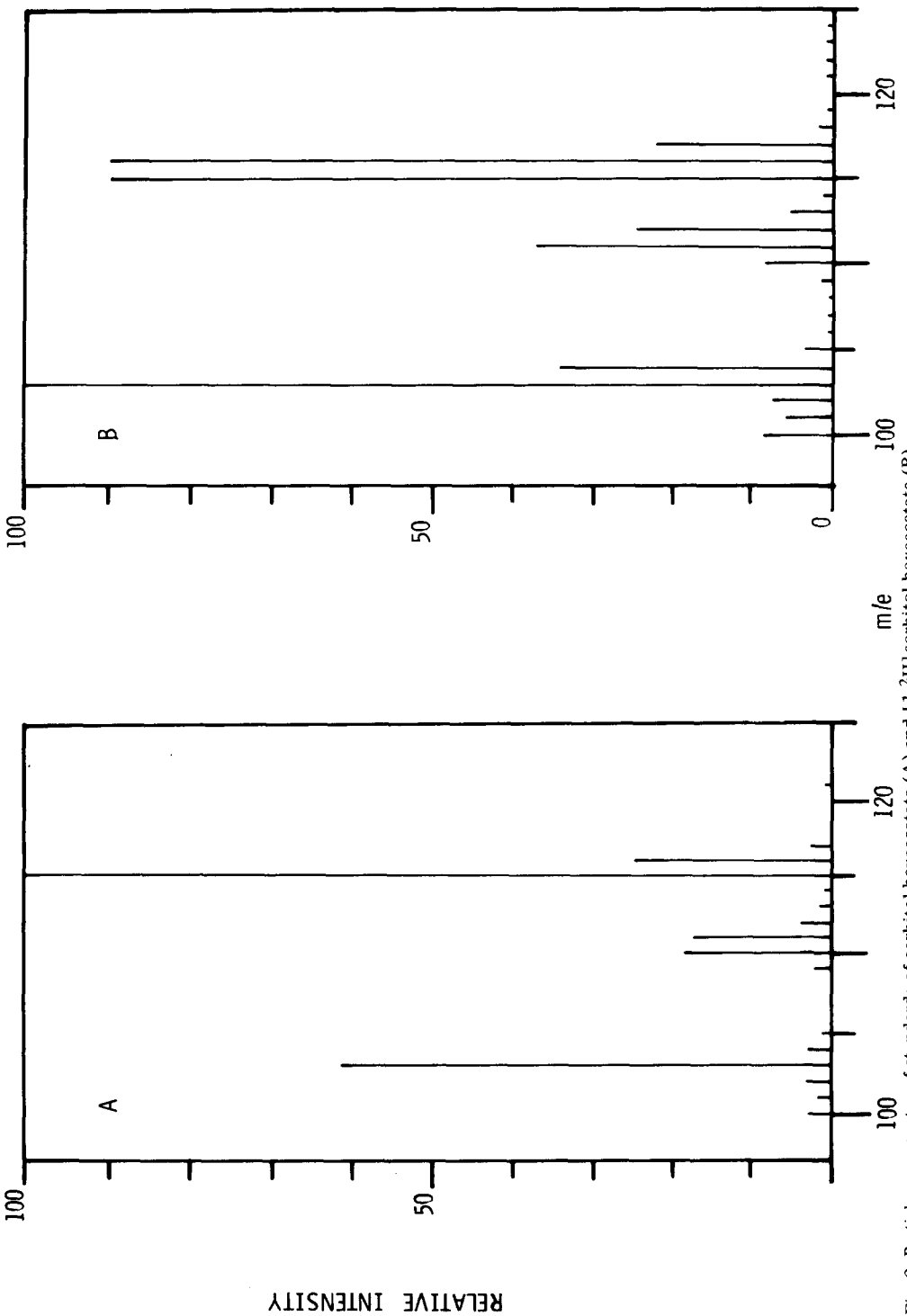

Fig. 2. Partial mass spectra of standards of sorbitol hexaacetate (A) and [1-^{2}H]sorbitol hexaacetate (B).

increase in the ratio of ions at *m/e* 116/115.

The atoms percent excess in a sorbitol sample was calculated from the ratio of ions in the sample and that in the unlabeled standard [13]:

$$\text{Atoms percent excess} = \frac{R}{100 + R} \times 100,$$

where R = ratio (sample) — ratio (unlabeled standard)

The observed atomic percent excess has been multiplied by 2 to reflect cleavage into two identical fragments and doubled again to reflect the loss of one of the two hydrogens in the formation of the measured ion. The calculated atomic percent excess of the [1-^{2}H] sorbitol reference standard, prepared either way, was 122—123%. Several factors, such as mass discrimination during fragmentation, could account for the greater than 100% labeling. The effect of the factor(s) is amplified by the four-fold multiplication. The data are reported as % incorporation, where % incorporation is (atomic percent excess of the sorbitol sample divided the atom percent excess of the standard) × 100 as well as being reported as atoms percent excess.

Results

Results of the incubations are recorded in Table I. Deuterium incorporated into NADPH by isocitrate dehydrogenase was incorporated into the sorbitol formed by the reduction by aldose reductase of glucose (Exp. 1). There was negligible deuterium incorporation into sorbitol from [1-^{2}H] glucose, incorporated via NADPH by glucose-6-*P* dehydrogenase (Expts. 2, 3, 4a and 5a). The greatest incorporation was in Exp. 2, but in contrast to the other three experiments, in this experiment NADPH was not separated from the unutilized [1-^{2}H] glucose before incubation with unlabeled glucose and aldose reductase. The deuterium in the NADP remaining after the reductions of experiment 4a and 5a was incorporated into sorbitol if the NADP was first reduced to NADPH using unlabeled glucose and glucose-6-*P* dehydrogenase (Expts. 4b and 5b). The quantity of sorbitol isolated in each experiment is also recorded.

TABLE I

DEUTERIUM CONTENT OF SORBITOLS FORMED BY ALDOSE REDUCTASE-CATALYZED REDUCTION OF GLUCOSE

Expt. No.	Source of NADPH		Atom per cent excess	% incorpo-tion	Sorbitol isolated (mg)
	Substrate	Enzyme			
1	[2-^{2}H] Isocitrate	Isocitrate dehydrogenase	64.7	52.7	2
2	[1-^{2}H] Glucose	Glucose-6-*P* dehydrogenase	13.7	11.2	5
3*	[1-^{2}H] Glucose	Glucose-6-*P* dehydrogenase	6.7	5.4	5
4a	[1-^{2}H] Glucose	Glucose-6-*P* dehydrogenase	2.3	1.8	4
4b**	Glucose	Glucose-6-*P* dehydrogenase	89.8	73.2	2
5a	[1-^{2}H] Glucose	Glucose-6-*P* dehydrogenase	1.4	1.1	6
5b**	Glucose	Glucose-6-*P* dehydrogenase	66.7	54.3	3

* NADPH was separated by column before incubation with aldose reductase.

** NADP was recovered from 4a and used in 4b and from 5a and used in 5b.

Discussion

The stereochemistry of the aldose reductase catalyzed reaction has been established by the standard method of referral to reference enzymes. Since isocitrate dehydrogenase is an A-type enzyme the results with this enzyme indicate aldose reductase is also an A-type enzyme, that is, the hydrogen transfered by the isocitric dehydrogenase in the formation of NADPH was then transfered by aldose reductase during its catalysis. The experiments with glucose-6-*P* dehydrogenase confirm this stereochemistry since this labels the B face of NADPH. Therefore, the sorbitol formed by the aldose reductase was essentially unlabeled when the deuterated NADPH formed from [1-^{2}H]glucose by glucose-6-*P* dehydrogenase was used because the deuterium was in the B position of the NADPH. When the deuterated NADP that remained after formation of the sorbitol was reduced with unlabeled glucose + glucose-6-P dehydrogenase, the B position was then unlabeled and the A position contained the deuterium. In accord with this, aldose reductase then transfered the A-positioned hydrogen-forming deuterated sorbitol. The quantities of sorbitol formed relative to the glucose present in the incubations were small. High concentrations of glucose were used because of the high Km of aldose reductase.

Sorbitol dehydrogenase, the other enzyme of the polyol pathway, is also the A-type. The biochemical importance, if any, of both these enzymes being of the A-type is unknown. Other aldehyde reductases have been reported to be of the A-type [14], but the relationship, if any, of these enzymes to the aldose reductase of placenta is also not established.

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