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Human Placenta Aldose Reductase

Forms Sensitive and Insensitive to Inhibition by Alrestatin

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SUMMARY

The inhibition of aldose reductase from a human source by alrestatin was studied. The enzyme from placenta was purified to apparent homogeneity by $(NH_4)_2SO_4$ precipitation, DEAE-cellulose chromatography, electrofocusing, and affinity chromatography. This enzyme from human or rat placenta at the $(NH_4)_2SO_4$ state of purification was relatively insensitive to alrestatin ($IC_{50} > 50$ μM). On purification by electrofocusing, however, human or rat placenta aldose reductase exhibited a marked increase in its sensitivity to alrestatin ($IC_{50} = 1.0$ μM). In contrast to human or rat placenta aldose reductase, rat lens aldose reductase was equally sensitive to alrestatin at the corresponding stages of purification ($IC_{50} = 1.0$ μM). Experiments in which the sensitive and insensitive forms of placenta aldose reductase were mixed revealed that the difference in susceptibility to alrestatin could not be attributed to nonspecific binding of alrestatin by proteins present in the $(NH_4)_2SO_4$ fraction. A heat-inactivated $(NH_4)_2SO_4$ fraction of human placenta aldose reductase added to the sensitive placenta enzyme from human or rats caused a time-dependent conversion to the insensitive form of aldose reductase. This suggested that a heat-stable dissociable factor, associated with placenta aldose reductase at the crude stage, may be responsible for the insensitivity to alrestatin. This insensitivity could be of pharmacological significance if it is relevant *in vivo* and it exists in tissues where aldose reductase plays a physiological role.

INTRODUCTION

Aldose reductase catalyzes the conversion of various aldoses to the corresponding alcohols. Interest in this enzyme has been generated because sorbitol is found in abnormally high levels in tissues bearing the brunt of diabetic complications (1). The accumulation of sorbitol provides pathogenic mechanisms by which hyperglycemia can alter the function and metabolism of certain tissues (1, 2).

Selective aldose reductase inhibitors are potential therapeutic agents for those diabetic complications in which the accumulation of sorbitol is thought to play a pathogenic role. Alrestatin and sorbinil are such inhibitors and are currently under clinical study (3, 4). In our search for such inhibitors, we used human placentas as a convenient source of the human enzyme in order to test its susceptibility to inhibitors developed against the rat lens or bovine lens enzyme (5). We observed that the placenta enzyme behaves differently from the lens aldose reductase with respect to its susceptibility to inhibition by alrestatin, depending on the stage of purification. This phenomenon was studied in some detail in this report.

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EXPERIMENTAL PROCEDURES

Materials. Rat lenses and placentas at day 20 of pregnancy were obtained from normal Charles River CD rats. Lenses were collected from 200- to 300-g male rats, pooled, and frozen at −20° until used. All of the materials used were obtained from commercially available sources.

Aldose reductase assay. Aldose reductase activity was measured on a Gilford spectrophotometer by monitoring NADPH oxidation at 340 nm for at least 3 min. Standard assay conditions were 0.067 M sodium phosphate (pH 6.5), 0.25 mM EDTA, 0.5 mM glyceraldehyde, 0.1 mM NADPH, and enzyme in a total volume of 1.5 ml. The reaction was started with addition of enzyme and followed at room temperature. Inhibitors were dissolved at 300 times final concentration in *N,N*-dimethylacetamide, and controls contained identical amounts of this vehicle. A unit of activity was defined as a change in absorbance of 0.001/min under the above conditions.

Enzyme purification. For each preparation, a single human placenta was obtained and processing started within 2 hr of childbirth. External blood clots were removed by washing with cold tap water. The vascular tissue was dissected from the connective tissue, weighed, and washed with 200–300 ml of buffer (Tris-phosphate, 0.05 M, pH 7.2 containing 0.2 M sucrose and 0.01 M mercaptoethanol). The tissue (250–350 g) was homogenized in 2 volumes of the buffer in a Waring blender for two 45-sec periods at maximal speed. The homogenate was centrifuged at

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16,000 × *g* for 20 min, and the sediment was discarded. Activity in the supernatant, $(NH_4)_2SO_4$, and DEAE fractions was measured after dialysis against the Tris-phosphate buffer and centrifugation for 1 hr at 105,000 × *g*.

Ammonium sulfate fractionation was carried out by adding solid $(NH_4)_2SO_4$ to obtain 0–30%, 30–42.5%, and 42.5–60% precipitates. The final precipitate (42.5–60%) was resuspended in 30 ml of Tris-phosphate buffer and dialyzed overnight against 2 liters of the same buffer. The procedure was carried to this point in 1 day.

The dialyzed 42.5–60% $(NH_4)_2SO_4$ fraction was passed through a DEAE-cellulose column (2 × 21 cm) and washed through with buffer, collecting 12-ml fractions. The dark red fractions were pooled and concentrated to 8.0 ml or less on an Amicon PM 10 membrane.

In order to lower the buffer concentration for electrofocusing, the concentrated material was diluted with 30 ml of 1% glycine containing 0.2 M sucrose and 0.01 M dithiothreitol, and concentrated to 10 ml using the same PM-10 Amicon membrane. It was then brought to 95 ml with 0.2 M sucrose containing 0.01 M dithiothreitol. To this solution, 5.0 ml of LKB ampholine (pH 5–7) and 4 g of LKB Ultradex were added, and the resulting gel was poured on a 109 × 244 mm plate and dried at room temperature under a fan to the evaporation limit of the gel. Drying required 60–180 min, depending on the humidity. It was then electrofocused at 6 W for 48–72 hr on an LKB Multiphor electrophoresis apparatus. The gel was sliced into 31 samples; each sample was eluted with 4 ml of 0.2 M sucrose containing 0.01 M dithiotheitol and washed with an additional 3 ml. Samples were analyzed for aldose reductase activity, and the pH was measured at 0°. The enzyme, aldose reductase, appeared as a one- or two-fraction peak between Fractions 15 and 20, with an isoelectric point of 6.05. The enzyme at this stage was stable for at least 2 months when stored at −20°.

Aldose reductase was further purified to apparent homogeneity by affinity chromatography. A column of Blue Sepharose CL-6B (1.5 × 10 cm) was prepared and equilibrated with 0.01 M sodium phosphate (pH 7.0) containing 0.2 M sucrose and 0.01 M dithiothreitol. The 5 ml of enzyme that were dialyzed into the same buffer were adsorbed to the column, and the column was washed with 50 ml of buffer. The enzyme was eluted with a 0–2 mM linear gradient of NADP in 140 ml of the same buffer.

Rat placenta enzyme was prepared from 95 g of rat placentas (204 placentas). They were washed free of external blood with Tris-phosphate buffer, and homogenized in the Waring blender for 1 min without further dissection. Subsequent processing was the same as for human placenta.

The rat lens enzyme was prepared by a modification on the procedure of Hayman and Kinoshita (6). Pooled lenses (20 g) were homogenized in 60 ml of water using a Duall glass tissue grinder. The homogenate was centrifuged at 14,500 × *g* for 15 min, and the supernatant was fractionated with $(NH_4)_2SO_4$ to obtain 0–40%, 40–50%, and 50–75% fractions. The 50–75% $(NH_4)_2SO_4$ precipitate was dissolved in a small volume of 0.05 M NaCl and dialyzed against 4 liters of 0.05 M NaCl at 2°. The precipitated proteins were centrifuged out, and the supernatant was sterilized by passage through a Millipore filter (Swinnex 25, 0.45 μm) and was stored at 0–4°. Freezing at −20° destroyed the enzyme, whereas storage at 0° caused a gradual loss in activity with a half-life of about 1 month.

RESULTS

Enzyme purification. Table 1 summarizes the results of purification of aldose reductase from a human placenta. The electrofocusing step (Fraction IV) yielded a 15-fold purification and a good separation of aldose reductase from glucuronate reductase. Fraction IV of the enzyme appeared as one band on SDS-acrylamide gel electrophoresis with an apparent molecular weight of 39,000. Fraction V of the enzyme was eluted as a single protein peak from a Blue Sepharose column and represented a 600-fold purification.

TABLE 1

Purification of human placenta aldose reductase

	Fraction	Protein	Total activity	Specific activity	Yield	Purification
		mg	*units*	*units/mg*	*%*	*fold*
I	105,000 × *g* supernatant	12,350	765,000	62	100	
II	42.5–60% $(NH_4)_2SO_4$	1,296	527,000	407	69	6.6
III	After adsorption of acidic proteins on DEAE-cellulose	357	527,000	1,480	69	24
IV	Electrofocused	11.7	262,000	22,500	34	362
V	Blue Sepharose	4.1	154,000	37,600	20	606

Effect of inhibitors. In the process of purifying human placenta aldose reductase, we observed that at the early stages of purification the enzyme was not susceptible to inhibition by alrestatin or sorbinil, which were developed against the lens enzyme (3, 4). This is shown in the *left panel* of Fig. 1, which is a Dixon plot for alrestatin inhibition at different stages of aldose reductase purification. At the early stages of purification (Fractions I–III of Table 1), the activity of the enzyme was little affected by the presence of up to 50 μM alrestatin. Fraction IV, however, became highly susceptible to alrestatin (IC_{50} = 1.0 μM). When Fraction IV was mixed with Fraction I, a biphasic effect was evident (Fig. 1, *bottom left*). This suggested that the difference in susceptibility was due to two different enzyme forms rather than nonspecific binding of the inhibitor by the proteins present in the supernatant fraction.

This phenomenon was also observed for sorbinil but it was not observed for quercetin (7), where at each successive purification step the enzyme became increasingly more sensitive to quercetin, probably because of the removal of the nonspecific quercetin-binding proteins (see *right* of Fig. 1).

In contrast to the placenta enzyme, rat lens aldose reductase Fractions II and IV were equally susceptible to alrestatin (IC_{50} = 1.0 μM). Furthermore, when rat lens aldose reductase was prepared by the same procedure as for human placenta aldose reductase, Fraction II showed the same IC_{50} (1 μM) for alrestatin as the standard preparation.

Mixing experiments. The inhibition in mixtures of the two enzyme forms was studied in order to investigate whether we were dealing with two forms of the human placenta enzyme rather than a nonspecific binding of the inhibitors to proteins present in the crude enzyme. As shown in Fig. 2, on addition of 10 μM alrestatin, the activity of Fraction IV was strongly inhibited at all enzyme levels. When the same determinations were done in the presence of approximately 100 units of Fraction II, only the activity of Fraction IV was blocked by alrestatin. The additional activity due to Fraction II was unaffected by alrestatin. An analogous experiment in which a fixed amount of Fraction IV was added to varying amounts of Fraction II gave similar results (data not shown).

Physical characteristics. The above experiments suggested that the enzyme in Fraction IV was in a form distinct from that in Fraction II with respect to their

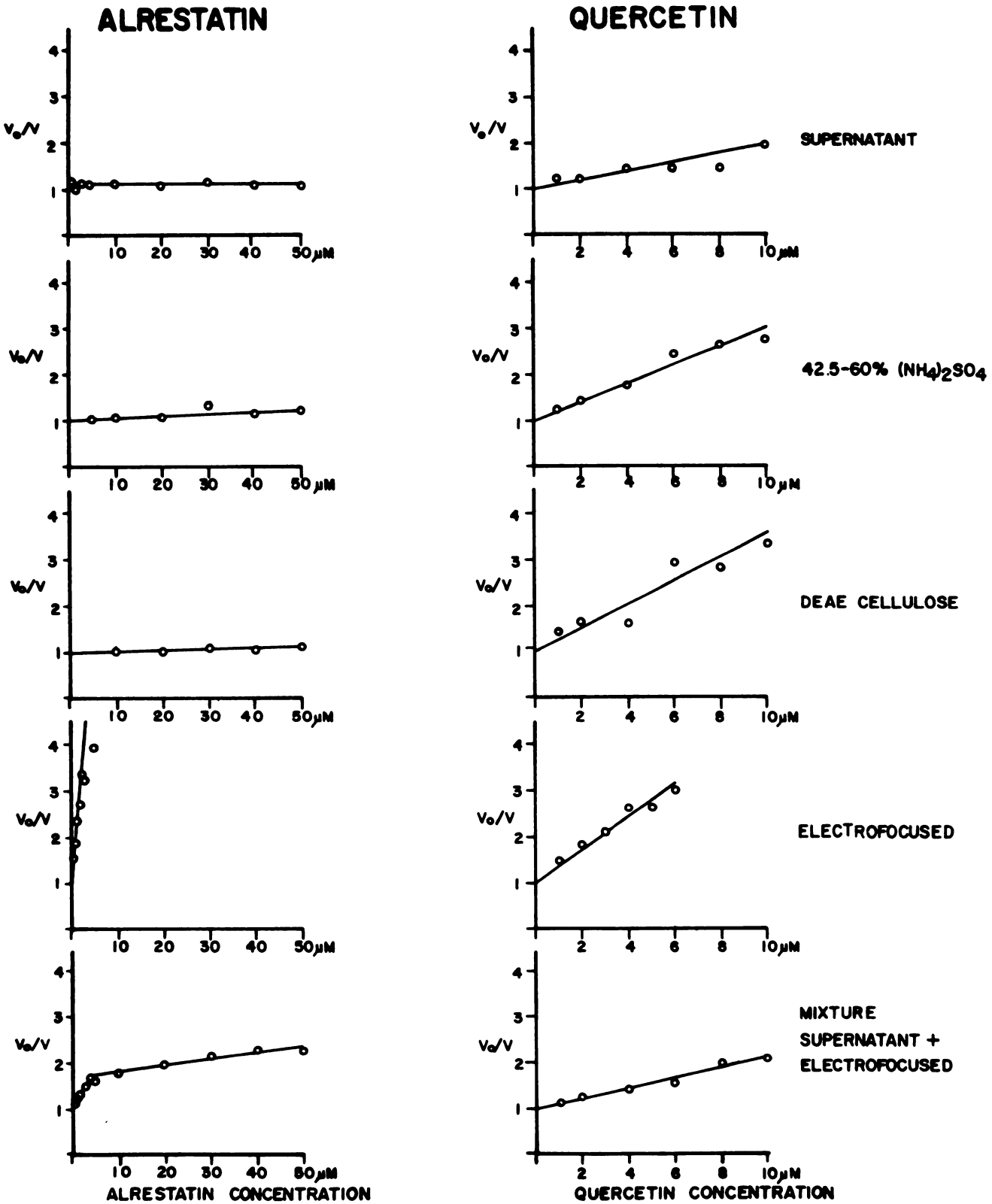

FIG. 1. *Dixon plots for inhibition of human placenta aldose reductase by alrestatin and quercetin at successive stages of purification*
V_0 is enzyme activity in the absence of inhibitor. Details of the assay are given under Experimental Procedures.

susceptibility to inhibition. Additional differences were observed in the kinetic parameters. The K_m values for glyceraldehyde were 62 μM and 24 μM, and the K_m values for NADPH were 0.55 μM and 0.42 μM for Fraction II and Fraction IV, respectively. Also, the pH optimum for Fraction IV was 5.0, whereas Fraction II had a pH optimum for activity of 5.4–6.0. The inhibition by 10 μM alrestatin of Fraction II was minimal at any pH, whereas

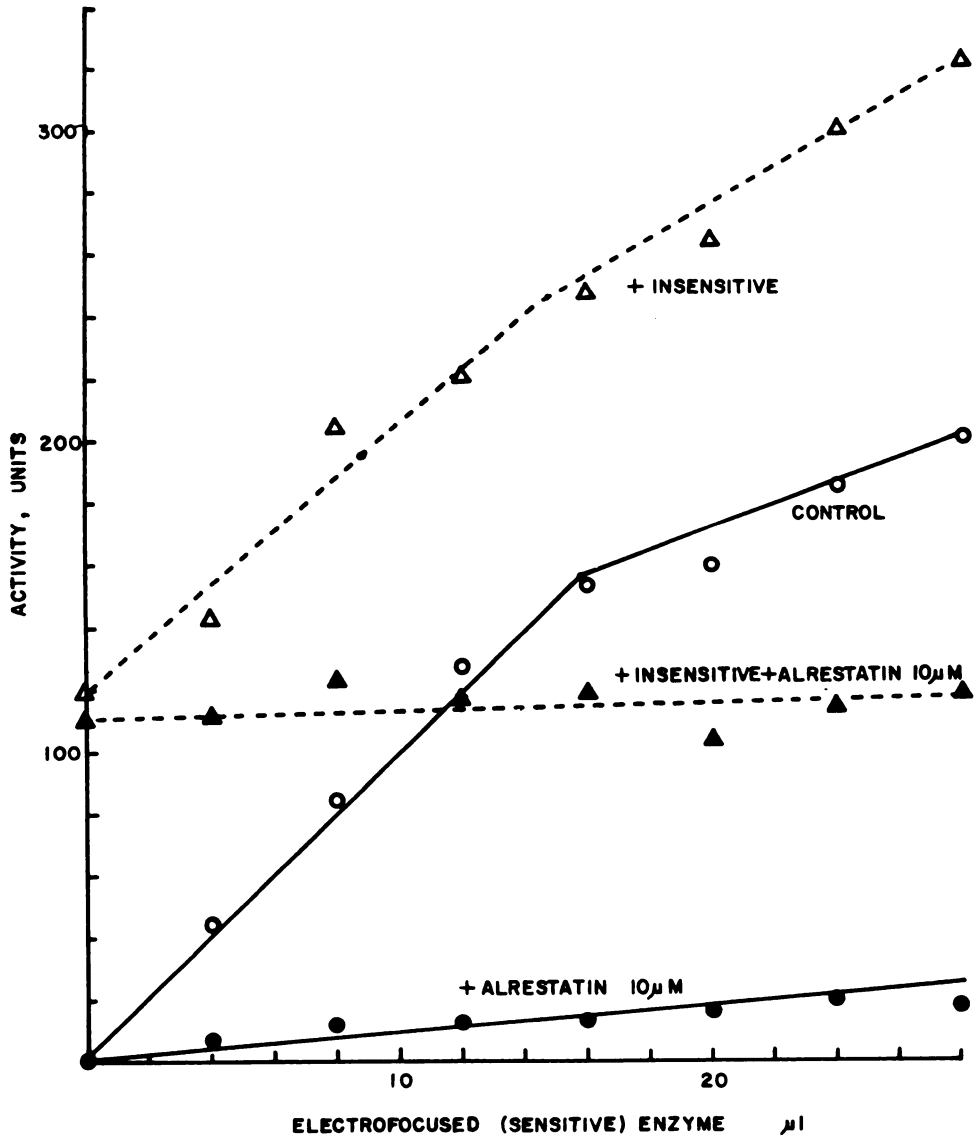

FIG. 2. *Activity of varied amounts of Fraction IV from human placenta aldose reductase ± 10 μM alrestatin (solid lines), and varied amounts of Fraction IV + 100 μl of Fraction II of human placenta aldose reductase ± 10 μM alrestatin (broken lines)*

Fraction II contained 2.85 mg of protein/ml, and Fraction IV 0.45 mg/ml. The reaction was started by adding Fraction II, mixing, and then immediately adding Fraction IV of the enzyme.

Fraction IV showed greater inhibition at lower pH (data not shown).

Fractions II and IV were indistinguishable when examined for apparent molecular weight on Sephadex G-75. Both appeared as single activity peaks with an apparent molecular weight of 39,000. The same results were obtained on disc gel electrophoresis under nondenaturing conditions, followed by iodonitrotetrazolium violet staining to localize the enzyme activity (8). The major band of activity in Fraction II was identical with that of Fraction IV. Thus, no major differences in the molecular size or charge could be seen between the two enzyme forms, from the aforementioned experiments. It should be noted, however, that electrophoresis may have converted the insensitive form of the enzyme to the sensitive form by removing a bound factor, and in that case differences in charge could not be detected.

Rat placenta enzyme compared with rat lens and human placenta. In experiments similar to the ones shown in Fig. 2, it was shown that rat placenta Fraction II exhibited the same insensitivity to alrestatin as human placenta Fraction II. This was evident under conditions where varying amounts of human placenta Fraction IV or rat lens enzyme were totally inhibited by 10 μM alrestatin (data not shown). From these experiments it seems that the observed differences between lens and placenta enzyme is tissue-specific rather than species-specific.

Reconstitution of insensitivity removed by electrofocusing. The method of obtaining the sensitive form of placenta aldose reductase suggested that there might have been a factor in the crude enzyme which was dissociated from the enzyme during the electrofocusing process, and that this factor, when associated with aldose reductase,

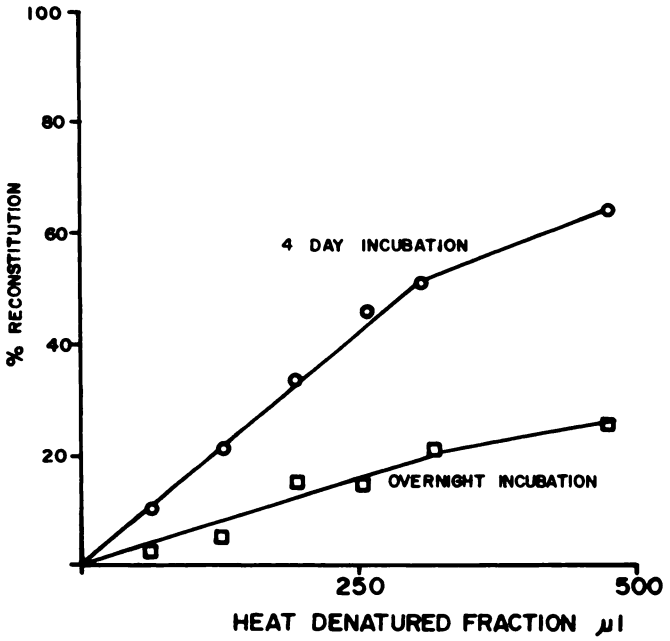

FIG. 3. *Reconstitution of insensitive form from Fraction IV of human placenta by incubation with heat-inactivated Fraction II*

Fraction II (5 ml) (28.5 mg of protein/ml, 10.500 units/ml) were diluted to 50 ml with 0.067 M phosphate buffer (pH 6.5) containing 0.25 mM EDTA. The mixture was brought to 55° for 20 min and the denatured protein was removed by centrifuging at 105,000 × *g* for 45 min. The heat-treatment inactivated more than 95% of the enzyme activity, so that the activity of Fraction IV could be followed without large corrections for the activity of Fraction II. Fraction IV and varying amounts of heat-inactivated Fraction II were mixed and allowed to stand at 2° for 24–96 hr. The mixtures were assayed for enzyme activity with ±10 μM alrestatin. Total activity was unchanged after 96 hr. Results are expressed as percentage of the activity in Fraction IV that was originally inhibited by alrestatin to that which was no longer inhibited after incubation.

had prevented alrestatin from blocking the activity of the enzyme. The possibility that adding back such a factor could convert the sensitive form of aldose reductase to the insensitive enzyme was investigated. The heat-inactivated Fraction II was used as a source of this factor, and it was prepared as detailed in the legend to Fig. 3. When Fraction IV was stored with heat-inactivated Fraction II at 2°, the former became insensitive to alrestatin to an extent which depended on the time of incubation and the amount of heat-inactivated Fraction II present (Fig. 3).

DISCUSSION

Human placenta aldose reductase was first isolated by Clements and Winegrad (9). Our procedure was developed to yield a stable enzyme for evaluation of aldose reductase inhibitors.

The enzyme, when purified through the electrofocusing stage or further through Blue Sepharose chromatography, was stable for several days at 2° and was stable to repeated freezing (−20°) and thawing. Both total activity and inhibition constants were reproducible for several different placenta preparations.

We noted, however, that human placenta aldose reductase at the supernatant and $(NH_4)_2SO_4$ stages of purification showed almost no susceptibility to alrestatin. The effect seemed too great to be explained by impurities alone, so experiments were designed to test the possibility of two different forms of enzyme.

The possibility was unlikely that the crude enzyme contains another enzyme with aldose reductase activity which was responsible for the results observed. In Fraction II some glucuronate reductase (hexanoate dehydrogenase) was present. However, the activity ratios observed using glucuronate as a substrate instead of glyceraldehyde indicate that less than 15% of the activity in Fraction II was glucuronate reductase. The 50% yield of sensitive aldose reductase on electrofocusing, coupled with the observation that sensitive enzyme was fully active in the presence of the insensitive fraction (Fig. 2) indicates that at least 50% of the activity in Fraction II was aldose reductase. If the enzyme in Fraction II was in a form indentical with that of the enzyme in Fraction IV, at least 50% inhibition should be observed in Fraction II, but the actual inhibition was barely detectable. Finally, the reconstitution of insensitive from sensitive enzyme provides further indications that there are two forms of one enzyme, rather than two separate enzymes.

Electrofocusing was not the only way to prepare sensitive enzyme from insensitive enzyme. It was also possible to prepare sensitive enzyme from Fraction II by either adsorption to DEAE-cellulose and gradient salt elution or direct adsorption to Blue Sepharose and elution with NADP (data not shown).

It is not uncommon for enzymes to have different behavior in the crude and purified stages because of competing reactions or competitive bindings. However, we are not aware that a difference had been previously observed in susceptibility to an inhibitor due to different forms of the enzyme at the crude and purified stage. A possible explanation may be a bound factor to aldose reductase at the crude stage that prevents binding of the inhibitor.

Aldose reductase inhibitors have attracted interest as potential therapeutic agents for those diabetic complications in which hyperglycemia and the resulting accumulation of sorbitol play a pathogenic role. On the other hand, the physiological role of aldose reductase has not been established, although it is known to be widely distributed in many tissues such as placenta, brain, and aorta (10, 11). It is conceivable that aldose reductase plays a detoxifying role by reducing harmful aldehydes in several tissues (10). Placenta aldose reductase may serve as a barrier to harmful endogenous or exogenous aldehydes, preventing them from reaching the fetus. If there is a physiological role for aldose reductase in placenta or other tissues, selective inhibition of aldose reductase in the target organs for which the therapeutic or prophylactic effects are required would be highly desirable. In that light, the phenomenon we studied in this report with the placenta enzyme may be of therapeutic significance.

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