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Aldose reductase in the BB rat: isolation, immunological identification and localization in the retina and peripheral nerve

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Summary. Aldose reductase was purified from testis of non-diabetic BB rats using DEAE cellulose, hydroxylapatite and sephadex G-100 column chromatography. The molecular weight of the isolated enzyme was found to be 36,500 ± 1000. Antibody against the isolated enzyme was raised in rabbits. It was purified by affinity chromatography, characterised by double immunodiffusion and Western blot analysis and used to localize the enzyme in retina and in peripheral nerve of the BB rat. In the retina, aldose reductase immunoreactivity was seen in the ganglion cells, Müller cell processes, retinal pigment epithelium and in the pericytes and endothelial cells of retinal capillaries. In peripheral nerve, aldose reductase immunoreactivity was found in the paranodal cytoplasm of Schwann cells and in pericytes and endothelial cells of endoneurial capillaries.

Key words: Aldose reductase, BB rat, immunocytochemistry, retina, peripheral nerve.

Hyperglycaemia has been suggested as pivotal to the pathogenesis of chronic diabetic complications such as nephropathy, neuropathy and retinopathy. Glucose levels in these target tissues reflects that of extracellular fluids, since glucose entry is neither acutely regulated by insulin nor is it rate limiting for the metabolism of these tissues [1, 2]. Metabolic events related to abnormal glucose metabolism in these organs, such as non-enzymatic glycosylation of proteins [3] and increased polyol pathway activity [2], may therefore play important roles in the development of chronic complications.

The first class of enzymes in the polyol pathway, termed aldose reductases, are NADPH-requiring aldehyde reductases which convert glucose and other aldosugars to carbohydrate derived polyols. The second enzyme, sorbitol dehydrogenese, is an NAD^+ requiring dehydrogenase, which converts polyols to their corresponding keto-sugars [1]. Increased polyol pathway activity, subsequent to hyperglycaemia, has been invoked as an important pathogenetic factor in the development of several diabetic complications [2]. The osmotic effect of accumulated polyol is probably an important factor in the formation of diabetic cataract [1, 4, 5], whereas an osmotic basis for tissue damage is less well established in other target tissues. A further and probably more important consequence of increased polyol pathway activity is tissue depletion of *myo*-inositol, since aldose reductase inhibitors completely prevent the decrease in tissue *myo*-inositol in diabetic kidney [6], peripheral nerve [7] and blood vessel [8]. *Myo*-inositol depletion results in secondary changes in phosphoinositide metabolism that in turn lead to alteration in a series of membrane-bound enzymes including Na^+-K^+-ATPase [9].

In peripheral nerve the reduced Na^+-K^+-ATPase function is directly related to nerve conduction slowing that occurs in animal and human diabetes [10, 11] and to impaired protein synthesis in diabetic spinal ganglion cells [12]. The polyol and *myo*-inositol related Na^+-K^+-ATPase defect is also implicated in the early structural changes in rat peripheral nerve [11, 13]. A similar mechanism may be involved in the breakdown of the blood-retinal barrier in diabetes [14]. Hence, in peripheral nerve we can logically relate hyperglycaemia through a cascade of events involving polyol pathway activity and Na^+-K^+-ATPase function to defects in tissue structure and function.

Aldose reductase exhibits significant qualitative and quantitative variations among tissues and species, and its distribution remains controversial [15, 16]. Therefore, to examine further the correlations between structural events in specific cell components of diabetic target tissues and increased polyol pathway activity, immunohistochemical localization of aldose reductase was undertaken in the BB rat. It is assumed by inference that cells that normally demonstrate aldose reduc-

tase immunoreactivity may be succeptible to the consequences of increased polyol pathway activity in diabetes.

The spontaneously diabetic BB rat is an authentic animal model of human Type 1 (insulin-dependent) diabetes mellitus. Several diabetic complications develop in this model [17-20], and it therefore lends itself as an excellent model system to test the pathogenetic role of the polyol pathway. Since aldose reductase appears to be species specific [21], the enzyme was isolated from BB rat testis and antibody was raised in rabbits. The antibody was used for light microscopic immunocytochemical localization of the enzyme in the retina and peripheral nerve of the BB rat.

Materials and methods

Enzyme purification. Aldose reductase was purified using a modification of the method described by Ludvigson and Sorenson [22]. All steps were carried out at 4 °C in the presence of 5 mmol/l mercaptoethanol. Eighty grammes of fresh testis from non-diabetic BB rats (Courtesy Dr. A.A. Like, Department of Pathology, University of Massachusetts Medical School, Boston, Mass, USA) were homogenized with a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ontario, Canada) in 5 mmol/l tris buffer, pH 7.4, in a weight to volume ratio of 1:2. The homogenate was centrifuged at 45,000 *g* for 30 min and the supernatant was collected. Solid ammonium sulphate in the amount of 17.5 g/100 ml was slowly added to the supernatant under gentle strirring to produce a saturation of 30%. The solution was allowed to stand for 1 h and the precipitate was removed by centrifugation at 15,000 *g* for 30 min. Additional solid ammonium sulphate in the amount of 30 g/100 ml was added to the supernatant to yield a saturation of 75%. The solution was again allowed to stand for 1 h and pellets were collected following centrifugation at 15,000 *g* for 30 min [22]. They were dissolved in 40 ml of 5 mmol/l tris buffer, pH 7.4, containing 5 mmol/l mercaptoethanol. This solution was dialysed against 4 changes of each 800 ml of the same buffer using Spectropore no. 2 membrane (45 mm diameter, 12,000-14,000 mol. wt. cut off).

The dialysate was applied to a gravity packed DEAE cellulose column (2.5 × 30 cm) equilibrated with 5 mmol/l buffer (pH 7.4). The column was washed with the same buffer and eluted with a linear NaCl gradient (5-250 mmol/l); 7.5 ml fractions were then collected.

The pooled fractions containing aldose reductase activity were concentrated by pressure dialysis using an Amicon filter with a YM 10 membrane, 10,000 mol. wt. cut off (Amicon Corp., Danvers, Mass. USA) and applied to a gravity packed hydroxylapatite column (1.5 × 30 cm) equilibrated with 5 mmol/l Na-K phosphate buffer (pH 6.8). The column was subsequently washed with the same buffer and eluted with a linear 5-400 mmol/l Na-K phosphate buffer gradient; 7.5 ml fractions were again collected.

The fractions containing aldose reductase activity were pooled, concentrated by pressure dialysis and applied to a Sephadex G-100 column (2.5 cm × 100 cm) equilibrated with 5 mmol/l tris phosphate buffer (pH 7.4); 10 ml fractions were then collected. The pooled fractions containing enzyme activity were rechromatographed on a Sephadex G-100 column. The purified enzyme was dialysed to remove mercaptoethanol and stored frozen (−20 °C) in small aliquots. The aldose reductase peak from the 2nd Sephadex G-100 run was used for immunization of rabbits. Other fractions showing enzyme activity were pooled to be used as pre-absorption controls for future immunohistochemical studies. The molecular weight of the isolated enzyme was determined by comparing it with standard molecular weight protein markers on sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE).

Enzyme assay and units. Aldose reductase was assayed according to Gabbay and Kinoshita [23]. Enzyme activity was measured spectrophotometrically at 340 nm in glass cuvettes of 1 cm light path. Glyceraldehyde and glucoronate were routinely used as substrates. The final concentrations of the reagents in the assay were 1 mmol/l glyceraldehyde or 10 mmol/l glucoronate, 73 µmol/l of NADPH, 5 mmol/l of mercaptoethanol and 67 mmol/l of Na-K phosphate buffer (pH 6.2). The substrates were omitted from the control cuvettes. The reaction was started by adding substrate to the test cuvette and was followed on a LKB Ultrospec 4050 spectrophotometer (LKB Biochrom Ltd. Cambridge, England) at 25 °C. Aldose reductase activity was indicated by the loss of NADPH absorbancy at 340 nm.

One unit of enzyme activity was defined as the amount of enzyme that caused oxidation of 1 µmol NADPH per min. The specific activity was defined as units per mg protein. Protein concentrations were measured according to Lowry et al. [24].

Antibody preparation and identification. Antibodies against isolated aldose reductase were raised according to the following procedure. One milligramme of isolated enzyme in Freund's complete adjuvant (1:1, vol:vol) was injected subcutaneously in New Zealand rabbits. This was followed by the injection of two doses of 0.5 mg of the same enzyme in Freund's complete adjuvant at 2-week intervals; after 2 further weeks an intravenous booster of 0.5 mg of the enzyme was given. The rabbits were bled after one week following the intravenous booster injection. Serum was separated and the antibody was purified by affinity chromatography using CNBr activated sepharose. Purified protein was conjugated with sepharose and antiserum was added. Following incubation the antibody was eluted with glycine-HCl, neutralised with tris buffer and dialysed against phosphate buffered saline (PBS). Non-immune serum collected prior to the primary immunisation was used as control serum for immunohistochemical studies. The antiserum was subjected to double immunodiffusion on Ouchterlony plates against purified, semi-purified (hydroxylapatite peak), crude and heat inactivated (56 °C for 15 min) aldose reductase and also against the hexose dehydrogenase peak of the DEAE-cellulose elute. The specificity of the antibody was also judged by Western blot analysis on zeta-probe membrane against purified and crude enzyme. In vitro study for inhibition of enzyme activity was performed according to Gabbay and Cathcart [25]. Immune and non-immune serum absorbed with liver powder was used for this purpose. Ten µl of purified enzyme was incubated with increasing amounts of immune serum (10 µl to 320 µl) to a final volume of 400 µl. As control, non-immune serum was used. All samples contained 5 mmol/l of mercaptoethanol. The enzyme activity was assayed using glyceraldehyde as substrate after 20 min of incubation at room temperature.

Immunocytochemical procedure. Retina and sciatic nerves from 5 non-diabetic BB rats were fixed overnight in Bouin's fixative and embedded in paraffin. Following deparaffinisation, the sections (15 from each tissue) were treated for 30 min with methanol containing 0.05% H_2O_2 to block endogenous peroxidase activity, washed in PBS (5 min × 3) and incubated with normal serum for 2 h. Trypsin digested preparations of the retina were prepared as previously described in detail [18] and were incubated with 0.1% trypsin inhibitor solution for 30 min and then processed following the same method. The sections were washed with PBS and incubated with primary antibody for 24 h at 4 °C. Liver powder absorbed antiserum in the dilution of 1:500 was used, since this dilution was found to produce optimum staining after trials with various dilutions ranging from 1:100 to 1:2000. Liver powder absorbed non-immune serum was used as control. Following rinsing in PBS the sections were incubated with goat anti-rabbit IgG for 2 h at room temperature. The sections were then rinsed again in PBS and treated with rabbit peroxidase-antiperoxidase (1 in 100) for 1 h at room temperature. The sections were again washed in PBS and incubated with freshly prepared 0.05% diaminobenzidine hydrochloride solution containing 0.01% H_2O_2 for 3 to 8 min, following which the sections were washed, dehydrated and mounted in Permount (Fisher Scientific Co., Fair Lawn, NJ, USA).

Glyceraldehyde, sodium-D-glucoronate, and NADPH were obtained from Sigma Chemical Co., St. Louis, Mo, USA. Hydroxylapa-

Table 1. Results of the various purification steps of aldose reductase in the BB rat

	Specific activity (units[a]/mg)	Total protein (mg)	Total activity	Yield (%)	Purification (-fold)
Crude	0.003	5950	17.85	100	1
Sample for DEAE cellulose	0.006	2061	12.36	69.24	2
Sample for H-apatite	0.052	113	5.87	32.88	17.33
Sample for S-G100 1st run	0.062	77.58	4.80	26.89	20.66
Sample for S-G100 2nd run	0.282	14.57	4.10	22.96	94
Ar pool	0.283	10.27	2.90	16.24	94.33
Ar peak	0.367	1.27	0.466	2.61	122.33

[a] One unit of enzyme activity was defined as the amount of enzyme that caused oxidation of 1 μmol NADPH per min. Ar: aldose reductase

tite and zeta-probe membrane were obtained from Bio-Rad Laboratories, Richmond, Calif, USA. Sephadex G-100 and DEAE-cellulose and CNBr activated sepharose were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Results

Enzyme purification

The purification procedure has been summarized in Table 1. Two enzyme activity peaks corresponding to two protein peaks were eluted from DEAE-cellulose column chromatography of ammonium sulphate precipitated fractions of rat testis (Fig. 1). The first peak eluted in the column wash had a glyceraldehyde/glucoronate reducing activity ratio of 1:4.2. The 2nd peak eluted after raising the NaCl gradient had a glyceraldehyde/glucoronate reducing activity ratio of 2.6:1. The first peak was identified as hexose dehydrogenase. The chromatography of the 2nd peak on hydroxylapatite column resulted in elution of two protein peaks, both appeared after raising the phosphate gradient. The enzyme activity was only found in the first peak with a glyceraldehyde/glucoronate reducing activity ratio of 3:1 (Fig. 2).

Repeated gel filtration chromatography on Sephadex G-100 was performed with concentrated hydroxylapatite column eluted fractions containing aldose reductase activity for further purification of the enzyme. The second Sephadex G-100 run resulted in a sharp protein peak with high enzyme activity (Fig. 3). The fractions in this protein peak were separated and used for immunization. The whole procedure was repeated several times to yield the desired amount of purified enzyme.

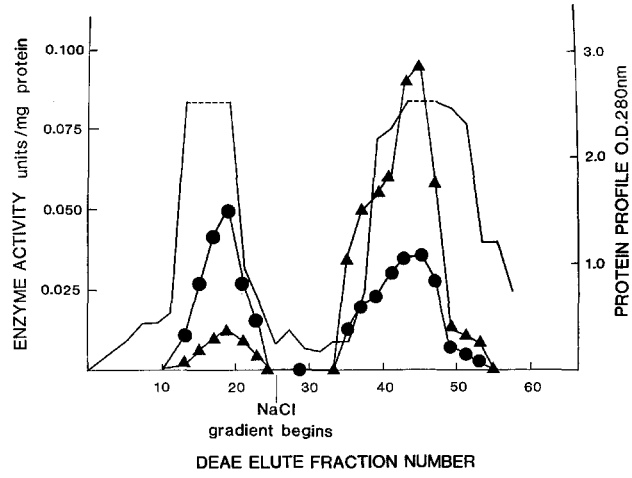


Fig. 1. DEAE-cellulose column chromatography of dialysed ammonium sulphate precipitated fraction of testis homogenate. – Protein profile as measured spectrophotometrically at 280 nm. ●—● Enzyme activity with glucoronate as substrate. ▲—▲ Enzyme activity with glyceraldehyde as substrate. O.D. = optical density

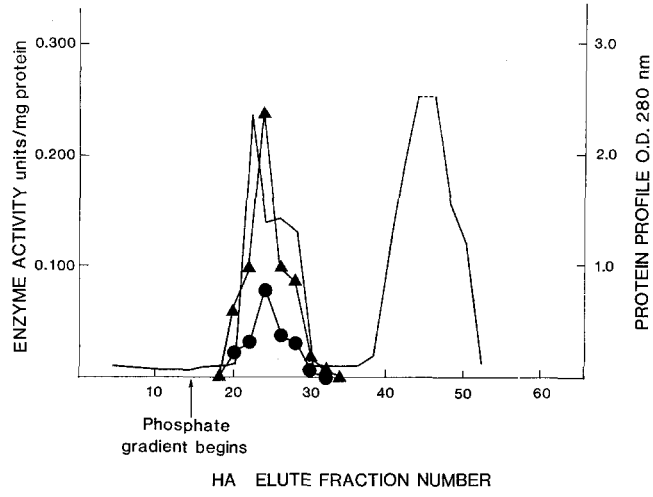


Fig. 2. Hydroxylapatite column chromatography of the pooled DEAE-cellulose column eluted fractions containing aldose reductase activity (see Figure 1 for legend)

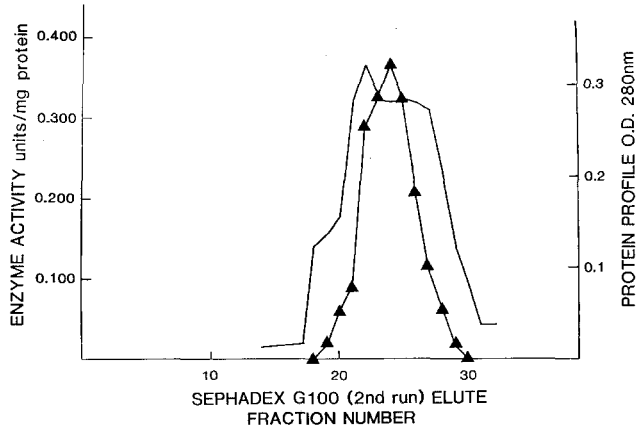


Fig. 3. Repeated Sephadex G-100 column chromatography of the pooled fractions containing aldose reductase activity from the 1st Sephadex G-100 column elute (see Figure 1 for legend)

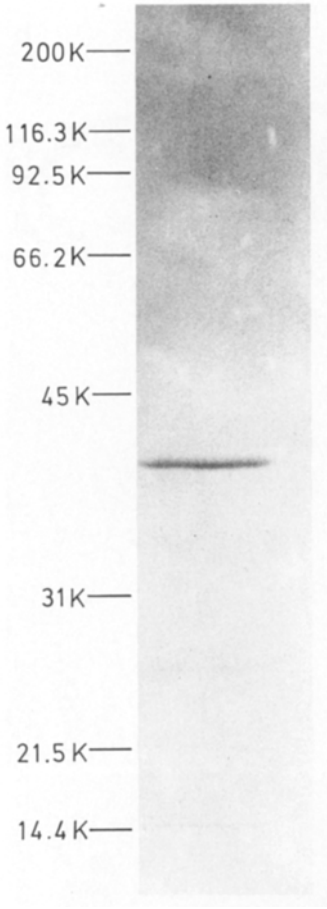


Fig. 4. SDS-PAGE electrophoresis from the aldose reductase peak of the repeated Sephadex G-100 column elute

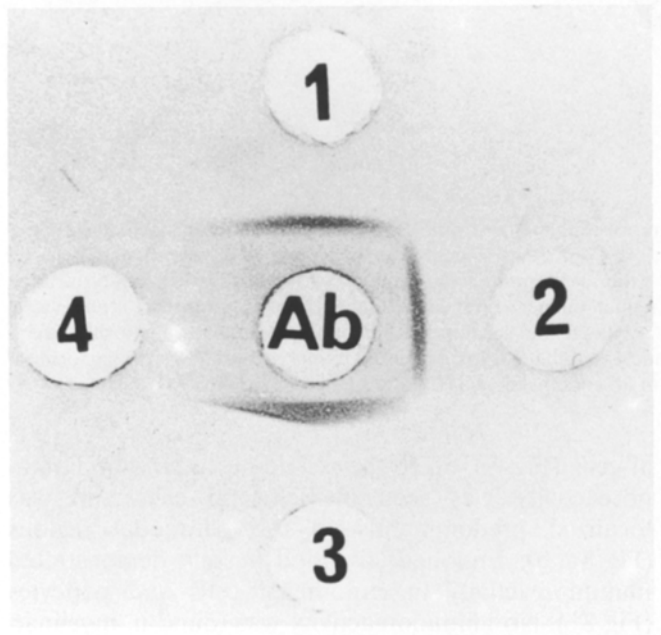
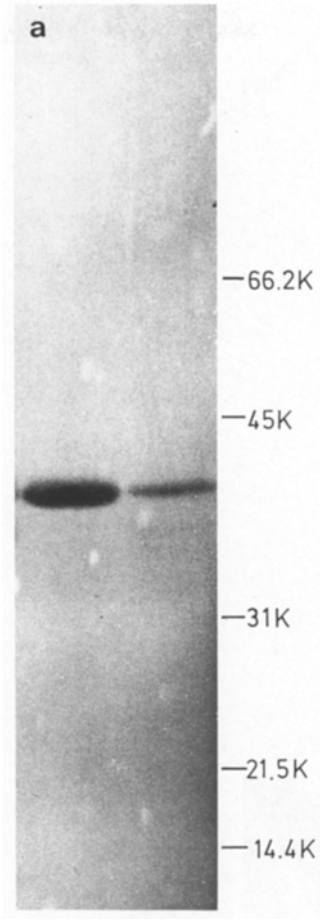


Fig. 5. Ouchterlony plate showing immunological identity of aldose reductase. Central well (Ab) - Antibody to aldose reductase. (1) Purified aldose reductase; (2) hydroxylapatite column eluted peak of aldose reductase; (3) crude extract from the testis of BB rat; (4) hexose dehydrogenase peak of DEAE-cellulose column elute

The isolated enzyme peak was subjected to SDS-PAGE electrophoresis to determine purity and molecular weight by comparing with standard molecular



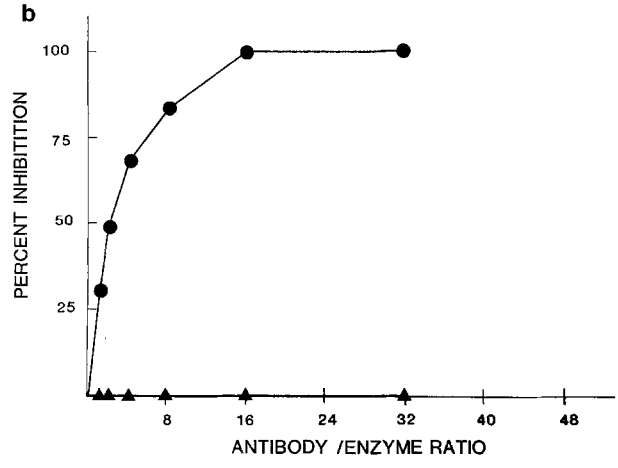


Fig. 6. **a** Western blot analysis showing specificity of rabbit anti-rat aldose reductase antibody. Antibody reacting with pure enzyme (left), and with crude enzyme (right). **b** Inhibition of aldose reductase activity with increasing amount of immune serum (●—●). No inhibition of enzyme activity was noted when incubated with non-immune serum (▲—▲)

weight protein markers. The enzyme showed a single sharply defined protein band with a molecular weight of 36,500 ± 1000 (Fig. 4).

Identification and specificity of the antibody

The double immunodiffusion test on Ouchterlony plates with antiserum produced a single band of precipitation with purified enzyme. Single precipitation bands were also observed when the antibody was allowed to react with crude extract and the hydroxylapatite column eluted enzyme peak. No precipitation band was seen between the antibody and the hexose

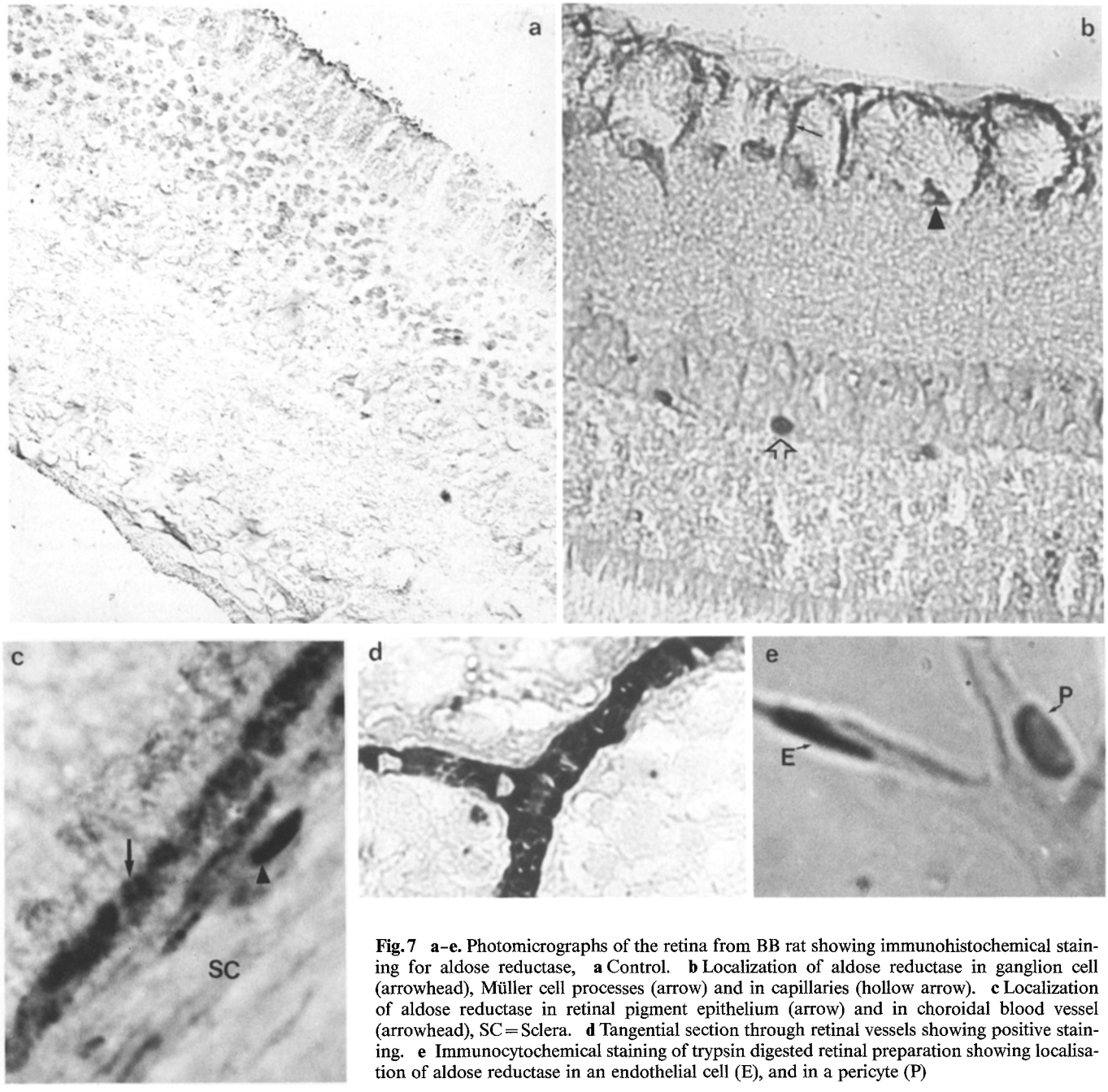


Fig. 7 a–e. Photomicrographs of the retina from BB rat showing immunohistochemical staining for aldose reductase, **a** Control. **b** Localization of aldose reductase in ganglion cell (arrowhead), Müller cell processes (arrow) and in capillaries (hollow arrow). **c** Localization of aldose reductase in retinal pigment epithelium (arrow) and in choroidal blood vessel (arrowhead), SC = Sclera. **d** Tangential section through retinal vessels showing positive staining. **e** Immunocytochemical staining of trypsin digested retinal preparation showing localisation of aldose reductase in an endothelial cell (E), and in a pericyte (P)

dehydrogenase peak of DEAE elute (Fig. 5). When the enzyme was heated to 56 °C for 15 min there was no precipitation band formation. The specificity of the antibody was also confirmed by Western blot analysis, which showed a single band with both crude and purified enzyme, and by the inhibition of enzyme activity when incubated with increasing amounts of immune serum but not with non-immune serum (Fig. 6 a, b).

Immunocytochemical localization. In the retina aldose reductase immunoreactivity was found to be localized in Müller cell processes, ganglion cells, retinal pigment epithelium and in the walls of the blood vessels. Trypsin digested preparations of the retina showed immunoreactive localization in both pericytes and endothelial cells (Fig. 7 a–e). In the sciatic nerve enzyme immunoreactivity was seen in Schwann cells and was localized predominantly in the paranodal regions (Fig. 8 a, b). Endoneurial blood vessels demonstrated immunoreactivity in endothelial cells and pericytes (Fig. 8 c). No immunoreactivity was found in myelinated axons.

Discussion

Aldose reductase is present in the Sertoli cells and in mature spermatids of the rat testis [22]. In this study we have demonstrated purification of this enzyme from

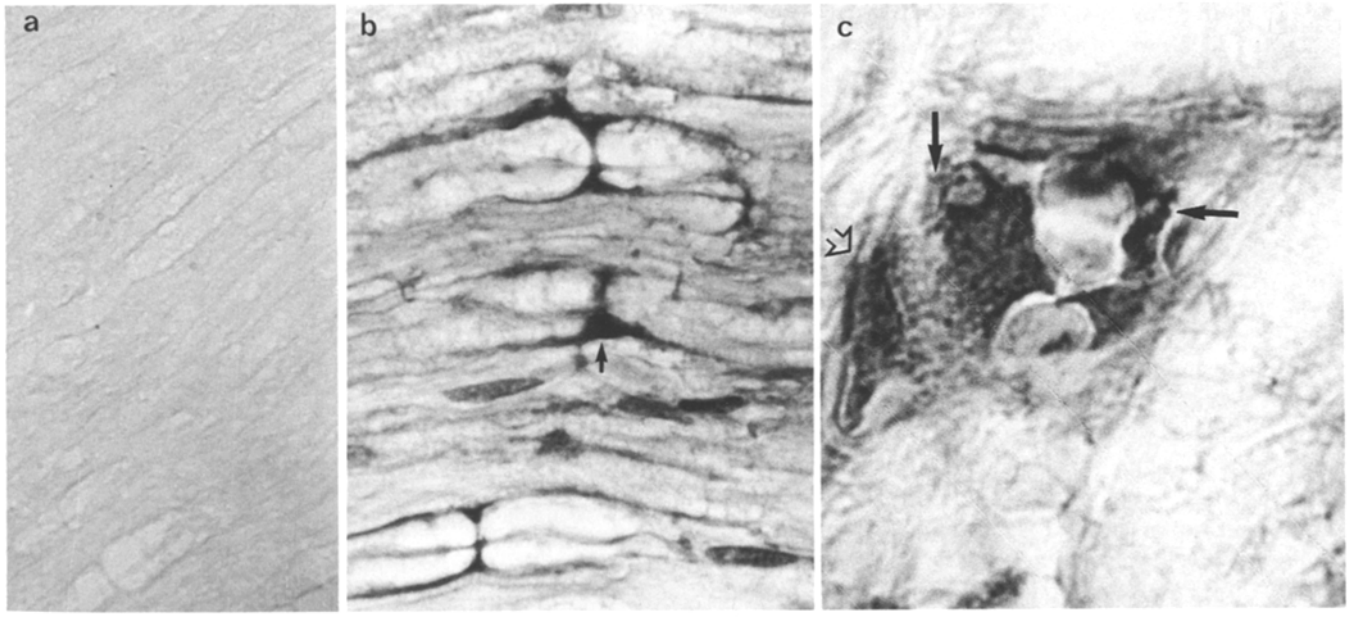


Fig. 8 **a–c.** Photomicrographs from sciatic nerve of a BB rat showing immunohistochemical staining for aldose reductase. **a** Control **b** Localization of aldose reductase in the paranodal regions of the Schwan cells (arrow) (The preparation was counterstained with haematoxylin, evident in Schwann cell nuclei) **c** Endoneurial capillary showing positive staining for aldose reductase in endothelial cells (arrows) and in a pericyte (hollow arrow)

BB rat testis to a degree similar to that obtained by other investigators [22, 23]. Further purification was not undertaken because of possible loss of enzyme yield and stability [22]. The glyceraldehyde/glucoronate reducing ratio is a simple and useful criterion for the separation of aldose reductase from hexose dehydrogenase in the DEAE-cellulose column elute [25]. Unlike Gabbay and Kinoshita [23], who found separation of two isoenzymes on hydroxylapatite column chromatography of bovine aldose reductase, no isoenzymes were separated in this study. Our findings are therefore similar to the observations made by Ludvigson and Sorenson on rat aldose reductase [22]. Sephadex G-100 column chromatography led to an increased purification yield with the removal of unwanted protein.

Since aldose reductase probably acts upon the aldehyde form of D-glucose in vivo, the discrepancy between in vivo activity of the enzyme and the low Km reported for D-glucose in purified enzyme preparations [16, 26] raises the possibility of some alteration of the enzyme during purification. In the present study, however, a satisfactory purification was achieved as demonstrated by a single band on SDS-PAGE electrophoresis as opposed to previous reports of triplet bands [22]. The estimated molecular weight of the enzyme was $36{,}500 \pm 1000$, which is similar to that previously reported: 36,500 in rat seminal vesicle [22], 39,000 in human placenta [27], 37,000 in bovine lens [28] and 32,000 in bovine kidney [25].

The formation of a single immunoprecipitation band after double immunodiffusion of the antibody with purified, semi-purified and crude enzyme, and no immunoprecipitation band with hexose dehydrogenase, demonstrate the specificity of the obtained antibody. This is further supported by non-immunoreactivity of heat inactivated enzyme, single bands on Western blot analysis with both crude and purified enzyme, and inhibition of enzyme activity by incubation with immune serum [25].

It is widely held that metabolic factors, such as activation of the polyol pathway conditioned by hyperglycaemia, contribute to the characteristic structural alterations in diabetic retinopathy and neuropathy [5, 7].

The finding of aldose reductase immunoreactivity in ganglion cells and in Müller cell processes of the retina is similar to the localization of this enzyme in the human retina [29]. In addition we have demonstrated immunoreactivity in the pigment epithelium and in endothelial cells and pericytes of retinal capillaries. Although there is no previous report on the localization of aldose reductase in retinal capillaries in paraffin embedded sections, enzyme immunoreactivity has been demonstrated in endothelial cells of rat aorta and muscular arteries [22] and in cultured bovine retinal and cerebral microvessels [30]. Isolated canine microvessels were also found to contain hexitol producing activity [31]. In the present study trypsin digested preparations showed positive immunoreactivity both in endothelial cells and in pericytes, whereas similar studies in humans showed localization of the enzyme in pericytes only [32]. Aldose reductase immunoreactivity in pigment epithelium of the BB rat may be relevant in explaining the pigment epitheliopathy in the diabetic BB rat retina [19]. The localization of the enzyme to retinal pericytes and endothelial cells in the BB rat

may explain previously described hydropic and degenerative changes of these cells, and may be a factor responsible for subsequent retinal thrombosis in this model [18].

An activated polyol pathway has been linked to the characteristic *myo*-inositol depletion and related Na^+-K^+-ATPase defect in diabetic peripheral nerve [10, 33]. These metabolic defects not only explain the rapidly reversible slowing of nerve conduction velocity in the acutely diabetic BB rat [11], but have also been invoked to explain paranodal swelling and subsequent axo-glial dysjunction. The latter is directly related to the less readily reversible nerve conduction defect in chronic diabetes [34]. These axonal swellings precede the loss of axo-glial dysjunctions in both the BB rat and man, and are ameloriated in the BB rat by the administration of an aldose reductase inhibitor and *myo*-inositol supplimentation, which prevent the Na^+-K^+-ATPase defect [13, 35]. Since aldose reductase inhibitors but not *myo*-inositol supplimentation decrease nerve sorbitol levels, *myo*-inositol depletion and the associated Na^+-K^+-ATPase deficiency rather than sorbitol accumulation appears to underlie paranodal axonal swelling.

Aldose reductase immunoreactivity in endoneurial endothelium has not been previously demonstrated. This finding may be of importance in linking increased polyol pathway activity with endoneurial capillary closure in diabetic peripheral nerve and in explaining a presumed ischemic pathogenetic influence on diabetic neuropathy [35-37].

Since naturally occuring proteins other than aldose reductase may share homologous sequences and therefore may cross-react with antibodies raised against aldose reductase, the possibility of false positive immunostaining exists and needs further clarification. On the other hand, failure of immunostaining of a particular cell type such as the myelinated axon, however, does not exclude the possibility of an increased polyol pathway activity in such tissues in diabetes. However, findings that ligated diabetic sciatic nerve retains its ability to oxidize NADPH in the presence of glyceraldehyde suggests that no significant amount of aldose reductase is present in or transported by the axon [38]. Ultrastructural immunocytochemical localization and quantification of aldose reductase in various cells of normal and diabetic rats is currently being performed in our laboratory.

In summary, we have isolated and characterized a hexitol producing enzyme, aldose reductase, in the BB rat and demonstrated the immunoreactive distribution of the enzyme in the retina and peripheral nerve in this model. The present findings will serve as a basis for future studies in linking metabolic abnormalities with structural changes occurring in the target tissue of secondary diabetic complications.

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