# LENS ALDEHYDE REDUCTASE AND CATARACT

A thesis submitted to the University of Oxford for the degree of Doctor of Philosophy

by

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Polyol dehydrogenase and aldehyde reductase B have been assayed in normal clear lenses of rat, rabbit, bovine, mouse and human. With DL-glyceraldehyde as substrate the percentage specific activitiesof aldehyde reductase are 60%, 16%, 6% and 1.6% in rabbit, human, bovine and mouse lenses respectively, taking the activity in rat lens as 100%. With D-glucose as substrate the specific activities followed a similar pattern. The specific activities of polyol dehydrogenase were 100%, 2.8% and 0, 0, 0 in human, bovine and rat, rabbit and mouse lens respectively. The properties of crude and of pure bovine lens aldehyde reductase B have been studied. Some properties of bovine polyol dehydrogenase have also been studied and compared to those of aldehyde reductase B.

Aldehyde reductase B activity was located in the β-crystalline fraction of bovine lens. The enzyme was purified to apparent homogeneity (on SDS gel) by use of a combination of gel-filtration and ion-exchange chromatography. The purification showed that glyceraldehyde and glucose reducing activities purified to different extent.

Chemical modification of bovine lens aldehyde reductase B showed that arginine and histidine are involved in catalytic function of the enzyme and are present at or near the NADPH binding site. A thiol group on the enzyme is necessary for acting

with glucose. Studies with sulindac and FPL 58665, the inhibitors of aldehyde reductase B, showed that the enzyme has a different site for its inhibitors.

Steady-state kinetic analysis showed that this enzyme obeys an ordered-Bi-Bi mechanism and NADPH is the first substrate to bind to the enzyme. Kinetic analysis showed that the binding of NADPH and glucose to the enzyme is 1:1 whereas with DL-glyceraldehyde the minimum degree of rate equation is 2:2.

NADPH-oxidizing activity was measured with DL-glyceraldehyde in human blood and this activity was found to be elevated in diabetic patients suffering from retinopathy with or without cataract. This activity was also found elevated in patients with senile cataract. A scheme has been proposed relating aldehyde reductase B to cataract and diabetic retinopathy.

Abbreviations used

NADPH = Nicotinamide adenine dinucleotide phosphate (reduced form)

$NADP^+$ = Nicotinamide adenine dinucleotide phosphate

NADH = Nicotinamide adenine dinucleotide (reduced form)

$NAD^+$ = Nicotinamide adenine dinucleotide

NEM = N-ethylmaleimide

p-CMB = p-Chloromercuribenzoate

CM Sephadex = Carboxymethyl Sephadex

cp = Centipoise

SDS = Sodium dodecyl sulphate

DEAE Sephadex = Diethylaminoethyl Sephadex

P = phosphate

K = Kelvin

KJ = Kilojoules

amp = Amperes

# C H A P T E R  1

## INTRODUCTION

### 1.1 The Anatomy of the Eye

The eye consists of three coverings enclosing the transparent media through which the light passes before reaching the retina: (1) The posterior five-sixths of the outermost coat is fibrous, protective in function, white and opaque and is called the sclera. The anterior part is the transparent cornea. (2) The middle coat is mainly vascular and nutritive in function. It consists of the choroid, ciliary body and iris. (3) The innermost covering is the retina, which contains nerve elements and forms the true reception position for visual impression(see Fig. 1.1).

### 1.2 Refractive Mechanism of the Eye

The refractive system of the eye consists of the transparent parts of the eye, the cornea, anterior chamber, the lens and the vitreous humor. The cornea is the first and most powerful refractive medium of the eye. The anterior chamber is the space bounded in front by the cornea and a small portion of the sclera and at the back by the iris, a part of the ciliary body and anterior surface of the lens. The anterior chamber is filled with

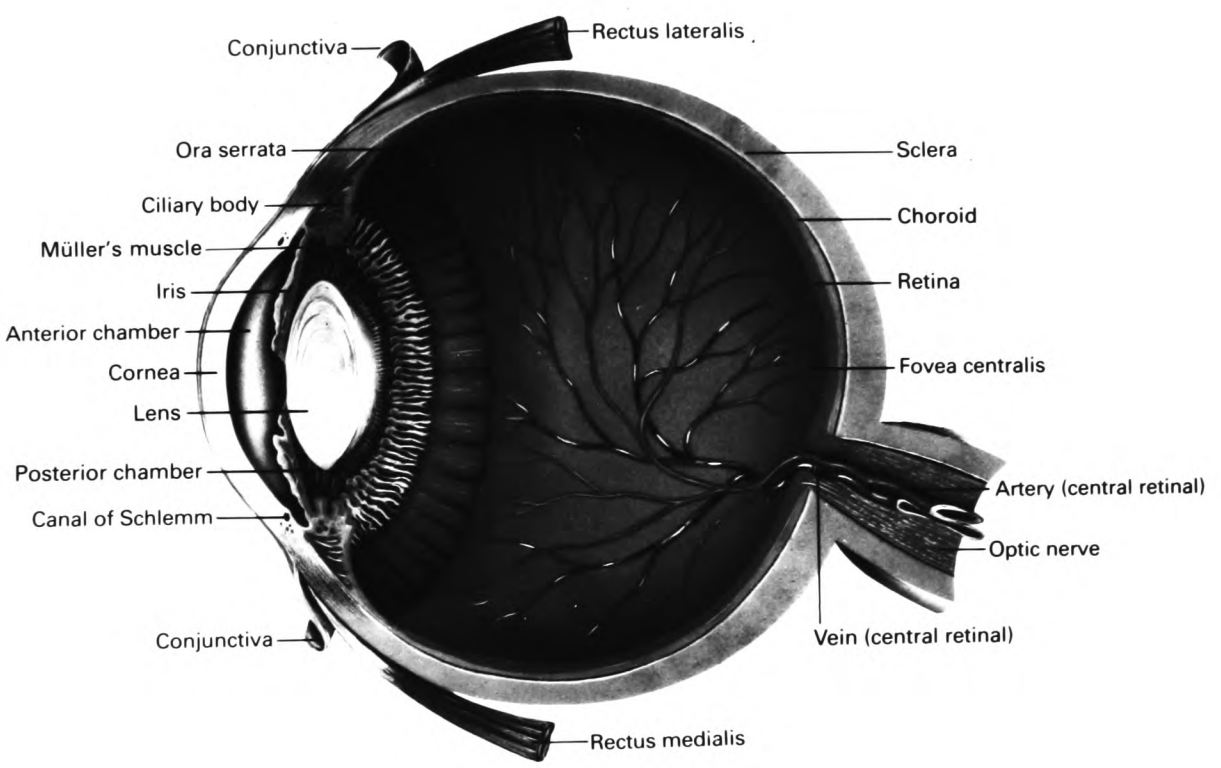



Fig. 1.1. A horizontal section of a human eye ( Calmic medical division, Wellcome Foundation Ltd., Crewe )

a transparent liquid called aqueous humor which also fills all of the posterior chamber. The aqueous humor is formed by the ciliary process. The cornea and the lens are fed by the aqueous humor. The aqueous is a slowly flowing stream into which surrounding tissues can discharge waste products of metabolism. By means of a steady aqueous formation and drainage, the intra-ocular pressure is maintained.

The vitreous is physically a hydrogel and it fills the posterior cavity of the eye. It forms a semi-solid support for the retina and allows light to pass through and fall on the retina. Collagen makes up the framework of the gel-like vitreous (Pirie et al., 1948) and hyaluronic acid forms the soluble part of the vitreous (Balazs et al., 1959).

## 1.3 The Lens

In the embryonic stage, two outgrowths from the forebrain form primary optic vesicle (Fig. 1.3a). The surface ectoderm near the optic vesicle becomes thickened as the cells become columnar (Fig. 1.3b) and forms the lens plate. The head ectoderm then forms an invagination into the optic vesicle (Fig. 1.3c). The lens is developed from this invaginated part of the ectoderm and the optic vesicle takes the shape of a cup called the optic cup (Fig. 1.3d-e). The anterior part of the

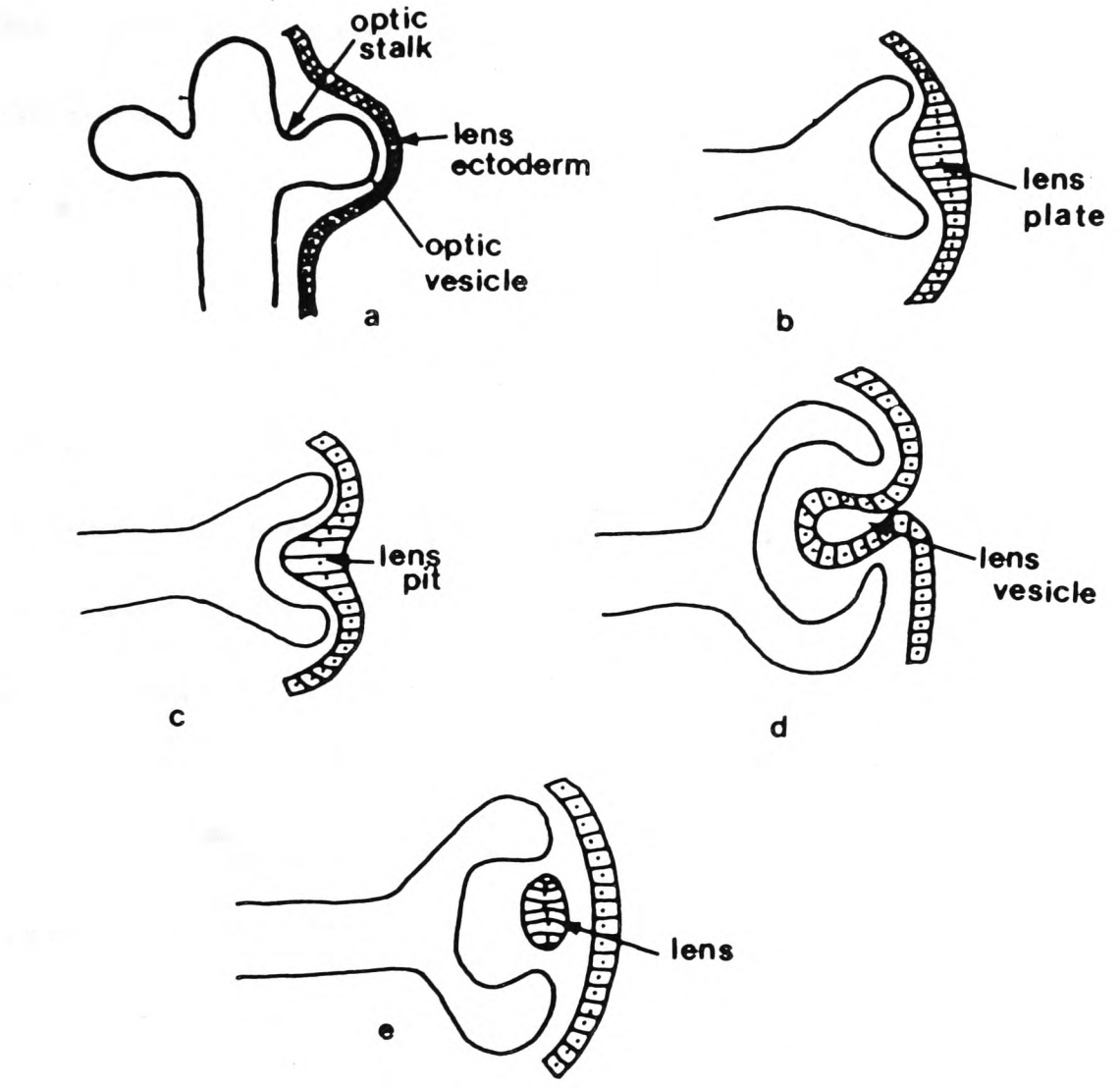



Fig. 1.3a-e Development of the lens.

lens vesicle becomes the anterior epithelium and the posterior part faces the developing retina.

The position of the lens with respect to the other parts of the eye is as follows. It is situated behind the iris and suspended from the ciliary process by the zonules. The lens has a shape of a flattened globe. It is covered by an acellular capsule and under the anterior capsule lie the epithelial cells (Fig. 1.4) Near the equator of the lens the epithelial cells start elongating and become fibres. The fibres are laid down on one another. The weight and thickness of the lens increase from the beginning of lens differentiation until death (Nordman et al., 1974).

The capsule of the lens is made up of collagen-like glycoprotein (Spiro and Fukushi, 1969). The capsule is formed by the lens epithelium at an embryological stage. The function of the capsule is to control permeability of different molecules into the lens (Friedenwald, 1930) and protect the lens from direct damage. An excess production of capsular protein has been reported in diabetes (Caird et al., 1969), in human mongo. cataract (Cogan and Kuwabara, 1962), congenital cataract in rats (Smith et al. 1969) and in mice (Pritchard et al., 1974).

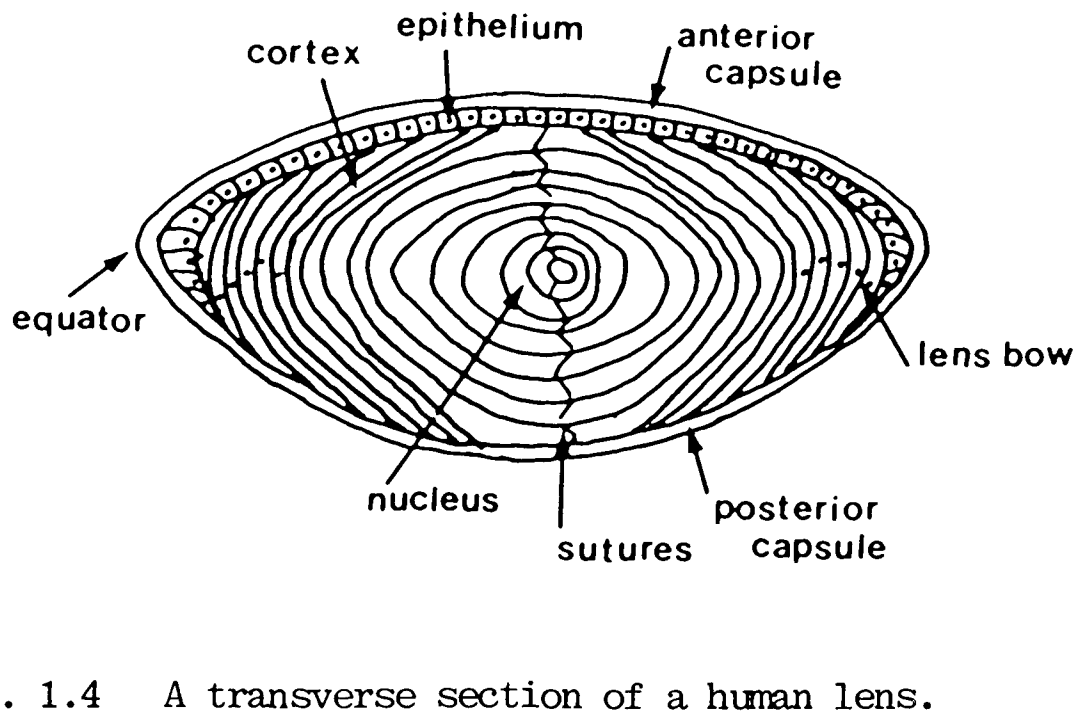



Fig. 1.4 A transverse section of a human lens.

## 1.4 Cataract

The most common and deleterious pathology of the lens is cataract.

Cataract may be simply defined as denoting any opacity in the lens which impairs vision (Duke-Elder, 1969). Basically the loss of transparency is due to a disturbance of the structure of the lens. The disturbance is of two types: (a) Swelling opacity - water is accumulated between the fibres or absorbed intracellularly into the protein molecules.
(b) Aggregation of protein which is irreversible.

### 1.4.1 Types of Cataract

Cataract can develop due to various causes. Newell and Ernest (1974) divided different types of cataract as follows:-

I Cataract in eye otherwise healthy and no systemic disease

Nearly all senile cataract and hereditary cataract fall under this class.

II Cataract combined with other ocular disorders but no systemic abnormalities

Cataract associated with aniridia and hetero-chromia, glaucoma or uveitis. It could develop due to

acquired defects, i.e. by toxic chemicals like naphthalene, triparanol, chlorpromazine, anti-cholinesterase, etc. Ocular trauma also can lead to cataract formation. Ocular trauma can develop due to the presence of foreign body, laceration or electro-magnetic radiation which include infrared, microwave or ionizing radiation, i.e. x-ray.

III Cataract and systemic disorders

Cataract can develop due to embryopathies like Marfan's syndrome, etc., due to cutaneous disorders like in atopic dermatitis, etc. Metabolic disorders, e.g. diabetes mellitus, galactosaemia, hypocalcaemia (with tetany) can cause cataract. Neurologic causes like hepatolenticular degeneration or muscular or bone disorders and also chromosomal abnormalities like that in Down's syndrome can give rise to cataract.

## 1.4.2 Classification of Cataract

The most widely used method of classification is that introduced by Pirie (1968) and van Heyningen (1972). This method is mainly based on the colour of the nucleus of the lens. Pirie-van Heyningen's method classified all cataract lenses into four types:

| Group I | Groups II, III and IV |
|---|---|
| Characterised by a pale uniform yellow colour; these lenses, according to the clinicians, belong to cortical and sub-capsular cataract lens. | Pale yellow cortex and visible nucleus in group II to a deep brown nucleus in group IV; these lenses represent various stages of nuclear cataract. |

There are certain characteristics of these lenses which follow a regular pattern as the nuclear colour deepens. The Table shown below contains a description of the components which vary according to the nuclear colour (Harding, 1981).

Table 1.4.2.1

| Component | Change | Group |
|---|---|---|
| Nuclear colour | increases | I to IV |
| Water, urea and urea-mercaptoethanol insoluble protein | increases | I to IV |
| Guanidino and guanidino-dithiothreitol insoluble protein | increases | II to IV |
| High molecular weight protein bonded by both -s-s and other covalent bonds | increases | I to IV |
| Protein fluorescence | increases | I to IV |
| Protein bound glutathione | increases | I to II |
| Methionine sulphoxide in lens protection | increases | I to IV |
| Aspartate racemization | increases | III to IV |
| $Ca^{+2}$ | increases | I to III |
| Wet weight | decreases | I to III |
| Dry weight | decreases | II to III |
| Inositol | decreases | I to III |
| Glutathione | decreases | I to III |
| $\gamma$-crystallin | decreases | I to III |
| Protein thiol | decreases | I to IV |
| Glutathione reductase | decreases | I to III |

This method does not take into account the minute differences between different cataract lenses but it is quick and inexpensive.

A recent method introduced by Chylack (1978) is used by Cooperative Cataract Research Group in the United States. This method insists on the photographic details of each and every lens. Compared to the classification system of Pirie-van Heyningen this method is expensive as it requires expensive equipment and is time-consuming.

## 1.5 Diabetes and the Eye

Ocular complications in diabetic patients pose a big problem to the clinicians as well as to the patients. Retinopathy, cataract and optical neuropathy are the ocular complications which can arise as a result of diabetes. Retinopathy is responsible for about 84% of blindness in diabetes (Liang and Goldberg, 1980). True diabetic cataract is rare and seen only in young diabetic patients with poorly controlled diabetes. Diabetic cataract starts as sub-cortical opacities inter-spaced with vacuoles and water clefts. Later, in mature stage it approaches nucleus. In a recent survey, 44% of cataract patients showed an abnormal glucose tolerance curve (Dugmore and Tun, 1980). Caird (1973) reported that diabetics were five times more likely to

have cataract than non-diabetics. Prchal et al (1980) showed that there was a positive association between duration of diabetes and posterior subcapsular cataract, though their data had the drawback of not having an adequate number of control patients.

To understand why diabetes, the main feature of which is hyperglycaemia, would give rise to cataract, let us consider the pathways through which glucose is metabolized in the lens (Fig. 1.5.1).

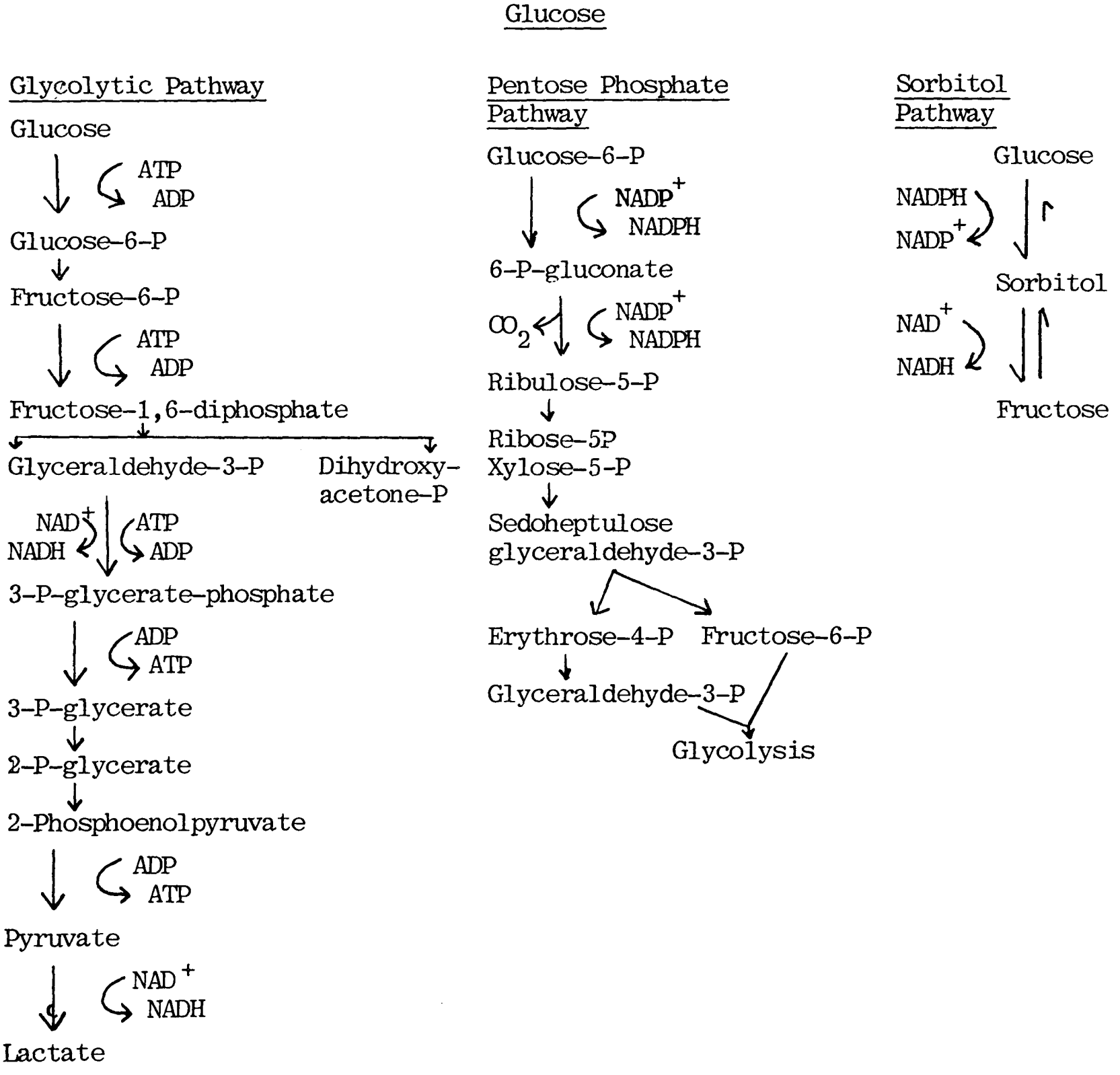



**Fig. 1.5.1 Pathways of glucose metabolism in the lens .**

The avascular lens derives most of its energy from metabolism of glucose (Kinoshita and Wachtl, 1958, Kinoshita, 1965b). Glucose transport, like in erythrocytes or in nerve tissue is independent of insulin (Farkas et al., 1960). Most of the enzymes of carbohydrate metabolism in the lens are located in the epithelium (Kaskel et al., 1978). Glucose metabolism in the lens occurs via the following pathways:

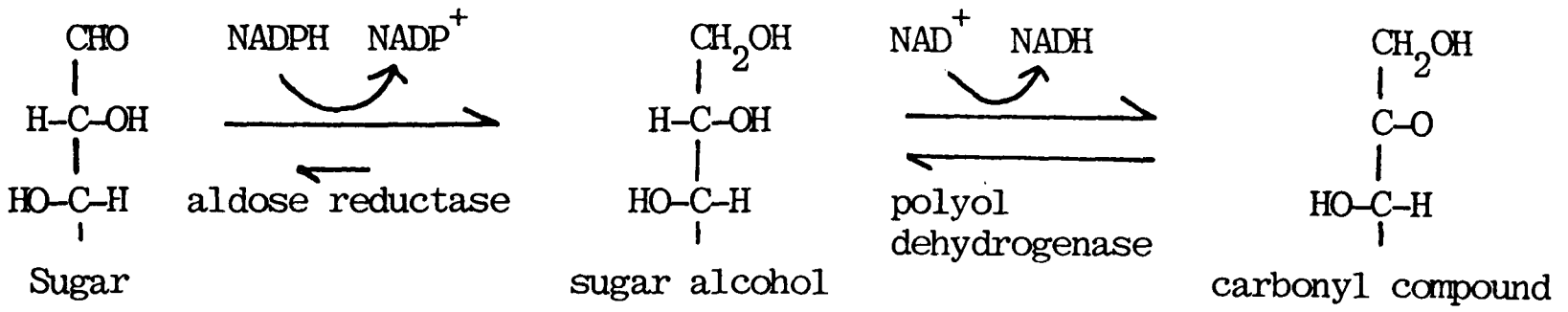
(a) Glycolytic pathway: Most of the glucose in the lens is metabolised via glycolytic pathway (Kinoshita and Wachtl, 1958; Kinoshita, 1965b).

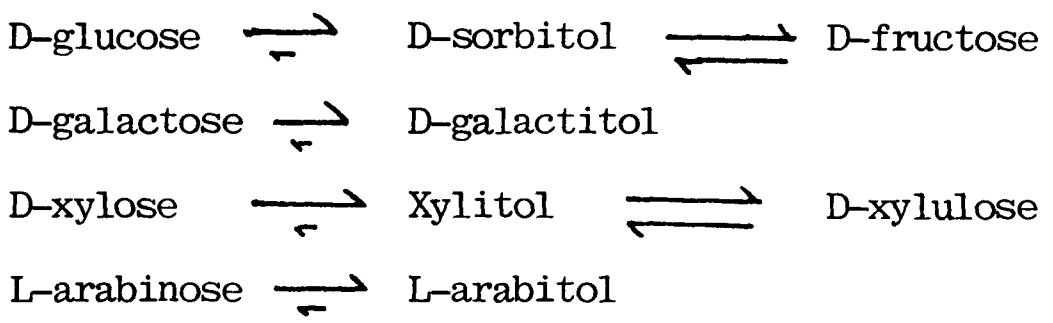
(b) The pentose phosphate pathway: This is the second major pathway of glucose metabolism in the lens and NADPH is produced via this pathway (Kinoshita, 1965b).

(c) The sorbitol pathway: This is a minor pathway for glucose metabolism in the lens (van Heyningen, 1959, 1976) as well as in other tissues like pancreas (Gabbay and Tze, 1972), nerve (Gabbay et al., 1966) and brain (Boghosian and McGuinness, 1981) etc.

## 1.6 The Sorbitol Pathway and Sugar Cataract

Sorbitol pathway is as follows (van Heyningen, 1962):




Aldose reductase (more appropriately called aldehyde reductase in the present study; see page 20 ) and polyol dehydrogenase are the two enzymes which constitute this pathway. Polyol dehydrogenase however does not act on galactitol or arabitol. The sorbitol pathway though supposed to be a minor pathway for glucose metabolism it has been thought to play an important role in "sugar cataract", the type of cataract induced by sugars like glucose, galactose, xylose and arabinose. van Heyningen (1959) first reported the presence of sugar alcohol in sugar cataract in rat. Sugar alcohols poorly penetrate cell membranes (Wick and Drury, 1951), so once they are formed they remain within the cell. Histopathological studies of sugar cataract showed that the swollen hydropic fibres are the first to appear in equatorial zone before opacity or vacuolation appears. These fibres eventually rupture and vacuolation follows (Friedenwald

and Rytel, 1955). The osmotic swelling of the lens in galactose cataract was demonstrated by Kinoshita (1965a) on the basis of the results of <u>in vitro</u> and <u>in vivo</u> experiments which is shown below:

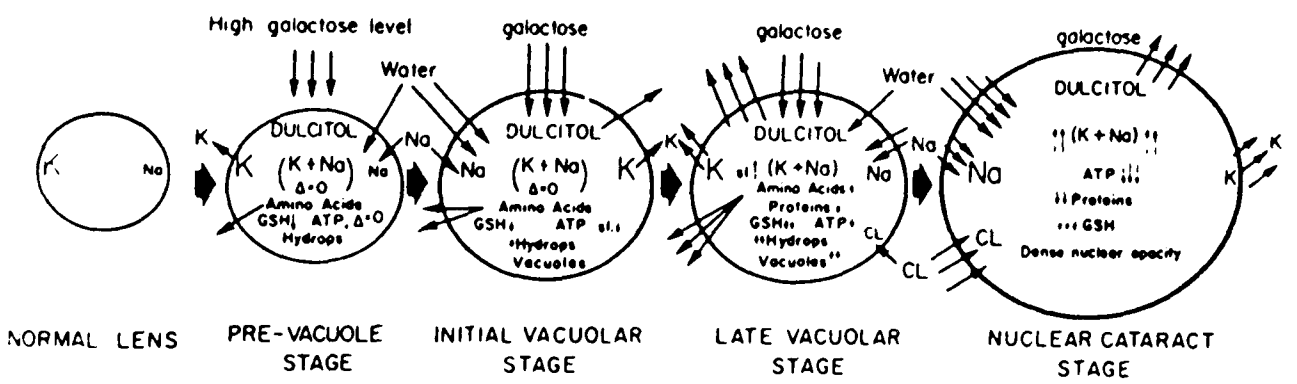
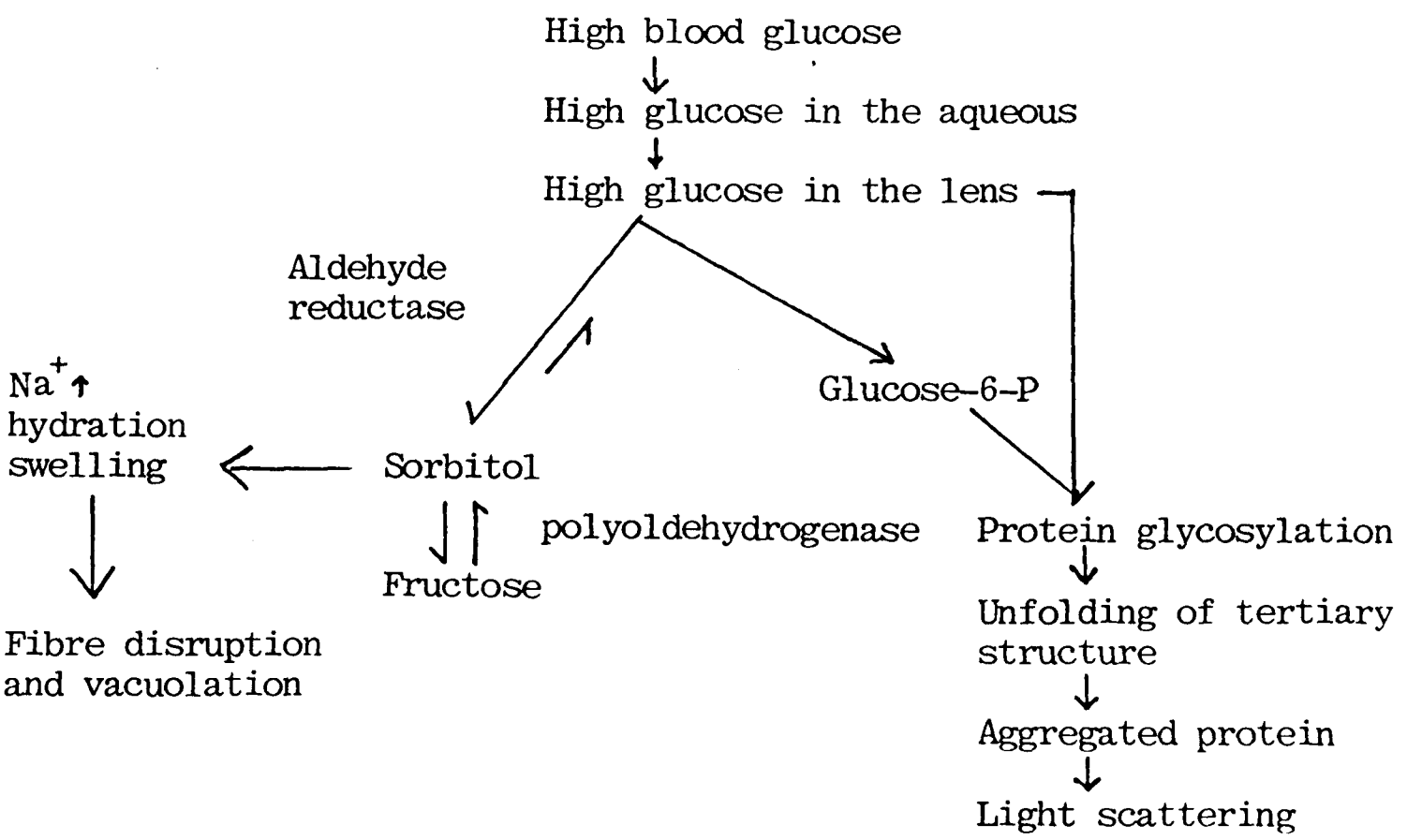



Fig. 1.6.1 Changes that occur during development of a galactose cataract.

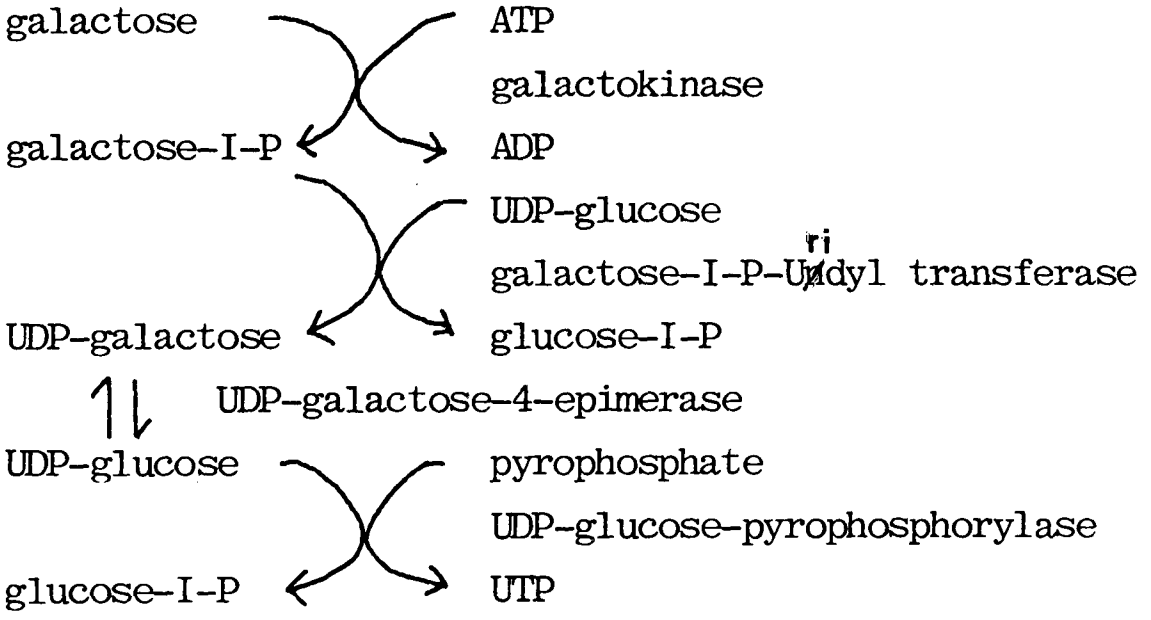
In addition to glucose and galactose, sugar cataract can also be produced in animals by xylose and by L-arabinose (van Heyningen 1962; Keller and Koch, 1978).

Formation of diabetic/sugar cataract can be schematically represented as follows:



Diabetes-induced cataractous process and galactose-induced cataract in animal are similar (Kinoshita et al., 1981) To learn more about diabetic cataract very often the study was made with galactosaemic animals (Kinoshita, 1965a). Galactosaemic model was found to be easier to study as galactose cataract developed more rapidly (Kinoshita et al., 1981) but this might, however, mean a different process was studied. A schematic diagram of pathway of galactose metabolism is given below:

Pathway of Galactose Metabolism




### 1.6.1 Galactose cataract

The production of cataract in rat by feeding them diet containing a high concentration of galactose was reported in 1935 (Mitchell and Dodge, 1935). Galactosaemic cataract was reported in human infants (Mellinkoff et al., 1945).

D-galactitol formed from D-galactose is a non-metabolizable sugar alcohol. The alcohol accumulates in the lens up to 0.1 mmole/g lens (van Heyningen, 1971). At this concentration galactitol exerts a strong osmotic effect and cataract develops (Kinoshita et al., 1962).

Galactose cataract in rat causes decrease in the synthesis of crystallin, but synthesis of non-crystallin protein remains unaffected (Kador et al., 1979). Recent studies with ultrastructural cytochemistry and spectrophotometric analysis showed that Na-K-ATP ase level decreased with the progress of galactose induced alteration in rat lens (Unakar and Tsui, 1980). The enzyme activity returned to normal level after withdrawal of galactose diet. Acid phosphatase activity increased in galactose cataract probably to repair the membrane damage (Unakar et al., 1982). Galactosaemic cataract has been recorded in kangaroos and wallabies (Stephens et al., 1975). Defective metabolism of galactose in these animals due to the depressed activity of the enzymes galactokinase and galactose-1-phosphate uridyl transferase in their erythrocytes gave rise to galactosaemic cataract (Stephens et al., 1975). In humans, a significant association between bilateral, pre-senile, subcapsular cataract formation and heterozygosity for galactokinase or galactose-1-phosphate uridyl transferase deficiency was found (Skalka et al., 1978).

It has been shown that systematically or topically applied corticosteroids may enhance the effect of several other experimental cataractogenesis such as galactose feeding (Bettman et al., 1964). In a recent report the glucocorticoids have been found to accelerate the development of galactose cataract in rat but a mineralocorticosteroid, desoxy-corticosterone was shown to retard the opacity (Koch et al., 1976). Synergestic effects of two or more insults together to the lens could lead to the development of cataract at an earlier time or could be cataractogenic at less than usual amount (Harding, 1983). The inhibitors of lens aldehyde reductase can delay the formation of galactose cataract and can limit the accumulation of galactitol (Dvornik et al., 1973; Varma and Kinoshita, 1976; Parmar and Ghosh, 1979). Galactose cataract did not develop in rats for 8 months when they were pre-treated with Sorbinil (Datiles et al, 1982). Restoration of a normal diet a few days before formation of a mature cataract does not prevent cataractogenesis, although the galactitol concentration in the lens falls rapidly, indicating probably galactitol is not directly responsible for the formation of mature cataract (Patterson and Patterson, 1965). Galactose cataract can, however, be prevented by early restoration of a normal diet in rat (Patterson and Patterson, 1965) by the simultaneous provision of ascorbic acid, yeast or cystine (Bellows, 1936) and both

galactose and diabetic cataract can be prevented by diet rich in protein or corn oil (Patterson et al., 1965).

Mouse lens did not develop cataract by feeding galactose or incubating it in 1.12M galactose (Kuck, 1970) probably because it contains little aldehyde reductase (aldose reductase) activity (Varma and Kinoshita, 1974; present work).

### 1.6.2 Xylose cataract

Xylose cataract does not afflict man but can be induced in young rat by feeding 35% xylose containing diet (van Heyningen, 1959a, b). The lens aldehyde reductase acts on xylose to form xylitol. In the lens of rat fed on 35% xylose, xylitol accumulates at 4 mg/g of lens. An extract of lens oxidized xylitol to xylulose in presence of $NAD^+$ but since this compound is not found in the lens, probably it was phosphorylated to xylulose-5-phosphate and removed by way of pentose phosphate pathway (van Heyningen, 1963). Xylose cataract shows some unique features which are not found in other sugar cataracts. Xylose cataract regresses even when the animal remains on a high xylose diet. Xylitol which accumulates in the lens remains at about the same level during the period when the visible vacuolation was regressing (van Heyningen, 1963). No marked imbibition of water in the lens in xylose cataract was observed by

van Heyningen (1963). But Obązawa et al. (1974) reported that the lens incubated in xylose medium accumulated high electrolytes and achieved highest hydration in the shortest period of time. In xylose cataract, hexose monophosphate shunt pathway is inhibited and level of ATP is decreased (Lerman et al., 1965). The hexose monophosphate shunt pathway is ten times more active in weanling rats than in mature ones (Lerman, 1964). This could explain why the young rats only develop xylose cataract (Lerman et al., 1965). Lerman and Heggeness (1961) reported that in xylose cataract no alteration in activities of either aldehyde reductase or polyol dehydrogenase were found. However, in lens culture, Obązawa et al. (1974) found that xylose-induced hydration and swelling of the lens could be prevented by AY-22,284 (1,2-dioxo-IH-benz-(de)-isoquinoline-2-(3H) acetic acid) a well-known lens aldehyde reductase (aldose reductase) inhibitor. More work is to be done to understand xylose cataract. This may answer the question whether polyol formation is really the key incident in sugar cataract.

### 1.6.3 Arabinose cataract

Arabinose cataract was produced in rat by both oral and intraperitoneal administration. The cataract had some similarities with galactose cataract but, contrary to galactose cataract, the opaque area in the equatorial

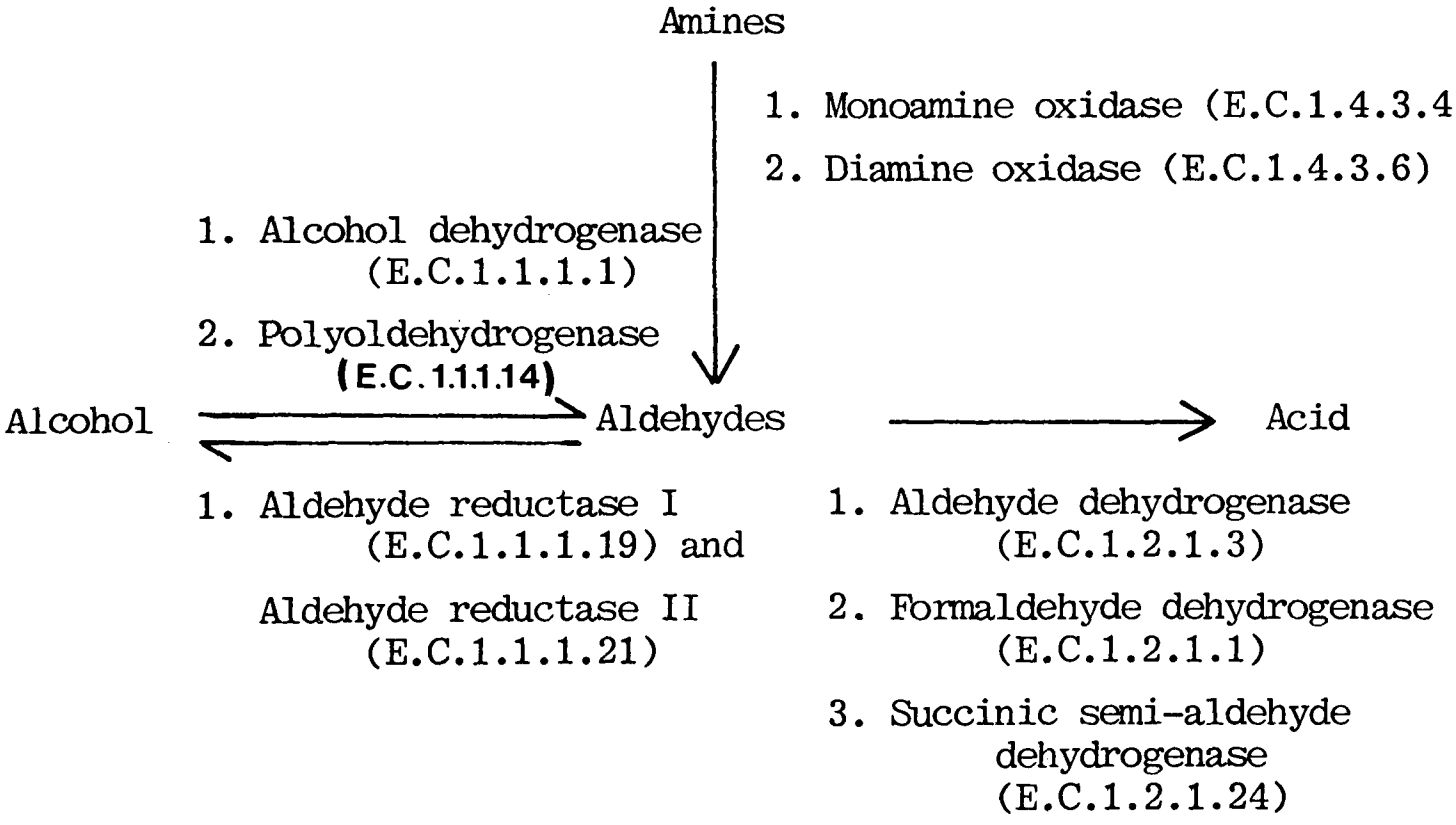
region did not progress to involve the whole nucleus but slowly regressed (Keller and Koch, 1978)

## 1.7 Sugar Cataract and Protein Glycosylation

A new approach to the formation of sugar cataract came from Cerami and his colleagues (1979). They suggested that in diabetic cataract or in galactosaemic cataract probably some other mechanism apart from the sorbitol pathway is involved. According to them, cataract formation in diabetes could be due to non-enzymatic glycosylation of lysine residues of crystallin protein by glucose or glucose-6-P as does occur with haemoglobin $A_{1c}$. The glycosylation possibly induces a change in conformation of lens protein and exposure of sulphydryl groups. This is supported by the report that in cataract lens, lens proteins show an increased sulphydryl group reactivity (Harding, 1972). Increased reactivity of sulphydryl groups will lead to disulphide bond formation and eventually form a high molecular weight aggregate to scatter light (Harding, 1973). Studies on normal human lens, senile and diabetic cataract lenses show that there is a small amount of non-enzymatic glycosylation in diabetic cataract lens (Pande et al., 1979; Ansari et al., 1980).

## 1.8 Aldehyde Reductases

In the brain, the biogenic aldehydes derived from biogenic amines are postulated to be related to sleep mechanism (Jouvet, 1969; Sabelli et al., 1969), maintenance of seizure susceptibility (Satoh et al., 1979; Fukumori et al., 1980) and body temperature (Barofsky and Feldstein, 1970; Gessner and Sable, 1973). The conversion of these aldehydes in the brain to the corresponding alcohols and acids are catalysed by a group of enzymes shown in the scheme below:




Aldehyde reductase activity has been found in other tissues like liver and kidney. These enzymes are found in the cytosol, they have broad substrate specificity, single subunit structure and higher affinity for NADPH than for NADH. Aldehyde reductases have similar molecular weight about 30 000 - 40 000 and similar amino-acid

composition. Mainly on the basis of susceptibility towards inhibition by valproate and barbiturates and substrate specificity these enzymes have been divided into two groups aldehyde reductase A and aldehyde reductase B (Crabbe et al., 1982) [Table 1.1], aldehyde reductase A being more susceptible to inhibition by barbiturates and valproate than aldehyde reductase B. Succinic-semi-aldehyde is a substrate for aldehyde reductase A (Kauffman et al., 1979; Rivett and Tipton, 1981) but a separate succinic-semi-aldehyde reductase has also been reported in brain (Hoffman et al., 1980; Tabakoff and von Wartburg, 1975). A ketose reductase has been found in rat liver which reduces daunorubicin, a cancer chemotherapeutic drug to the corresponding alcohol (Turner and Hick, 1976). Though aldehyde reductase can use daunorubicin as a substrate, this particular ketose reductase shows stereospecificity for the pro-4S hydrogen of NADPH (Felsted et al., 1978) whereas aldehyde reductases show stereospecificity for the pro-4R hydrogen of NADPH (Wermuth et al., 1979; Feldman et al., 1977). However, these monomeric oxidoreductases are interesting models for studying the interaction between pyridine nucleotide cofactor and the enzyme. These enzymes have remarkable similarities with monomeric dehydrogenase like octopine dehydrogenase (Olomucki, 1981; Gädi, 1980) and saccharopine dehydrogenase (Fujioka, 1981).

Table 1.1 Classification of Aldehyde Reductases

## Aldehyde Reductase A

Brain (human, pig, rat, ox)

(a) Hexonate dehydrogenase (Moonsammy and Stewart, 1967)

(b) 'High $K_m$' aldehyde reductase (Rivett et al., 1981)

(c) 'AR3' (Ris and von Wartburg, 1973; Hoffman et al., 1980)

(d) Succinate semi-aldehyde reductase (Kauffman et al., 1979)

Liver (human, pig, rat, mouse)

(a) Aldehyde reductase (Felsted et al., 1978; Tulsiani and Touster, 1977; Wermuth et al., 1979; Branlant and Biellman, 1980; Turner and Hryszko, 1980)

(b) Daunorubicin reductase (Turner and Hick, 1976)

(c) Xenobiotic carbonyl reductase (Felsted et al., 1980)

(d) Mevaldate reductase (Schlesinger and Coon, 1961)

Kidney (pig)

(a) Aldehyde reductase (Flynn et al., 1975; Morpeth and Dickinson, 1980)

Erythrocyte (human)

Hexonate dehydrogenase (Gabbay and Cathcart, 1974)

## Aldehyde Reductase B

Brain (human, pig, rat, ox)

(a) Aldose reductase (Moonsammy and Stewart, 1967; Clements et al., 1969; Dons and Doughty, 1976; O'Brien and Schofield, 1980; Boghosian and McGuinness, 1981)

(b) 'Low $K_m$' aldehyde reductase (Turner et al., 1974)

(c) 'AR2' (Hoffman et al., 1980)

Liver (pig)

Reductase II (Branland and Biellman, 1980)

Kidney (rat)

Aldose reductase (Corder et al., 1977)

Lens (human, rabbit, rat, ox, degu)

(van Heyningen, 1962; Hayman and Kinoshita, 1965; Scheaff and Doughty, 1976; Jedziniak et al., 1981)

Seminal vesicles (rat)

Hers (1957)

Placenta (human, rat)

(Kador et al., 1979)

Aorta (rat)

(Clements et al., 1969)

Sciatic nerve; pancreas; retina; optic nerve (ox, rat)

(Gabbay and Cathcart, 1974; Buzney et al., 1977)

Skeletal muscle (rabbit)

Glycerol dehydrogenase (Korman et al., 1972)

Aldose reductase (E.C.1.1.1.21) has been classified as aldehyde reductase B (Crabbe et al., 1982) on the basis of its properties. The latter name is used in the present work to denote aldose reductase. Aldehyde reductases have been found in a number of tissues, including lens (van Heyningen, 1959a,b;Hayman and Kinoshita, 1965; Jedziniak et al., 1981), sciatic nerve (Gabbay et al., 1966), retina (Buzney et al., 1977; Merola et al., 1982). It was also found in the corneal endothelium, in the ciliary body epithelium and its extension as the posterior surface of the iris (Ludvigson and Sorenson,1980). Lens aldehyde reductase or aldehyde reductaseBhas been implicated in ocular complications in diabetes mellitus. Corneal re-epithelialization in diabetic rats was found to be delayed and the topical administration of CP-45,634 (a spirohydantoin compound), a lens aldehyde reductase inhibitor, improved the regeneration time of the epithelium (Kinoshita et al., 1979). In diabetic retinopathy, the retinal mural cells are found to be selectively lost in diabetes (Cogan et al., 1961).

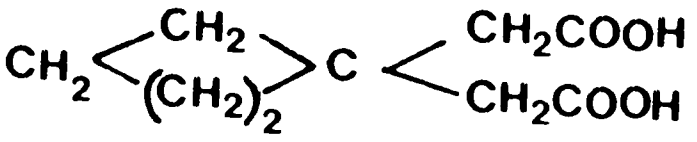
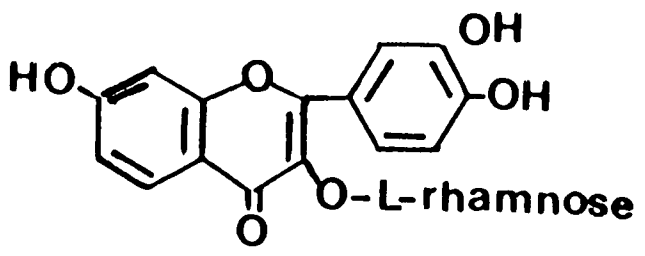
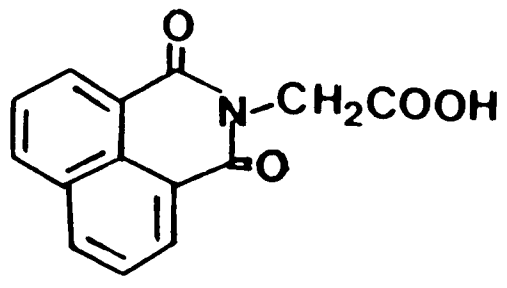
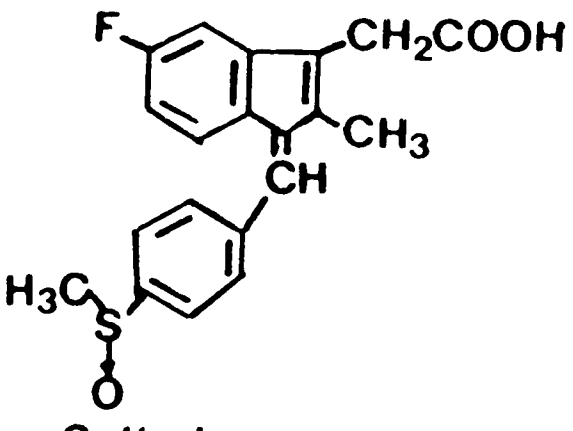
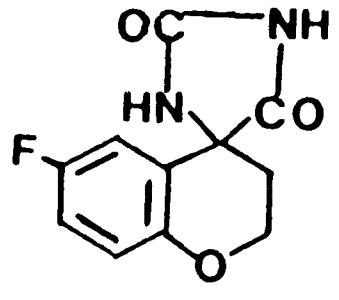
Polyneuropathy, another well-known complication of diabetes, may be caused by polyol formation in nerve tissue (Gabbay, 1973). An indirect evidence comes from the study in galactose fed rat in which an inverse relationship was found between galactitol accumulation and motor conduction velocities (Gabbay and Snider,

1972). The evidence though mostly indirect suggesting that aldehyde reductase B (aldose reductase) plays a key role in the development of some sugar cataract is very strong. Diabetic cataract cannot be induced in mouse, an animal which has little aldehyde reductase activity in the lens (Kuck, 1970; present work). A congenital strain of hyperglycemic mice did not develop lens opacities though their blood glucose level was very high (Varma and Kinoshita, 1974). Their lenses too showed little aldehyde reductase activity. The study with South-American rodents Octodon degu also supports the involvement of aldose reductase in sugar cataract. These animals have double thymus gland. Many of them developed cataract when placed on regular laboratory chow. The examination of these animals revealed that those with cataracts had mildly elevated blood sugar and further studies indicated the lens of an Octodon degus had about four times the level of aldehyde reductase as that of rat lens (Varma et al., 1975). The use of inhibitors which inhibited the formation of polyol in vivo or in vitro or inhibited the lens aldehyde reductase in vitro could delay the formation of cataract in animal models. This observation strengthens the argument in favour of aldehyde reductase playing an important role in the formation of sugar cataract in some animals. Tetramethyleneglutaric acid (Jedziniak and Kinoshita, 1971) and alrestatin (Sestanj et al., 1974) inhibited rat

lens aldehyde reductase. Alrestatin was also found effective in reducing polyneuropathy in diabetic animals as well as in humans (Gabbay et al., 1979; Fagius and Jameson, 1981; Culebras et al., 1981). Flavonoids have been found to be good inhibitors of lens aldehyde reductase (Varma and Kinoshita, 1976). These compounds are sparingly soluble in water. More soluble flavonoid compounds containing 4-oxo-4-H chromen ring system which can inhibit lens aldehyde reductase are now known (Kador and Sharpless, 1978). Sulindac, a non-steroid anti-inflammatory drug possessing analgesic and anti-pyretic activities has been found to be effective lens aldehyde reductase inhibitor in in vitro studies with animals (Duggan et al., 1977; Petchy et al., 1982; present work). Sorbinil (Pfizer) has been found to be the most effective drug for lens aldehyde reductase. It was given orally to diabetic and galactosaemic rats to prevent cataractous changes occurring in their lenses for up to 8 months (Datiles et al., 1982). The anti-rheumatic drugs, salicylate, indomethacine, sulindac, oxyphenbutazone are known to delay formation of senile cataract in humans. These drugs have recently been found to inhibit aldehyde reductase from cataractous humans and bovine lens (Sharma and Cotlier, 1982). Table 1.2 lists some of the inhibitors of lens aldehyde reductase.

Table 1.2

Some Inhibitors of Lens Aldehyde Reductase B

| Compound | Species | Degree of Inhibition in vitro | References |
|---|---|---|---|
|  Tetramethyleneglutaric acid | Bovine | 68% at $10^{-4}$M | Jedziniak and Kinoshita, 1971 |
|  Quercitrin | Bovine | 55% at $10^{-7}$M | Varma and Kinoshita, 1976 |
|  AY-22-285 | Bovine | 40% at $10^{-6}$M | Varma and Kinoshita, 1976 |
| | Rat | 50% at $10^{-4}$M | Dvornik et al, 1973 |
|  Sulindac | Calf | 69.1% at $10^{-6}$M | Sharma and Cotlier, 1982 |
| | Human cataract | 51% at $10^{-7}$M | Sharma and Cotlier, 1982 |
|  Sorbinil | Rat | 50% at $10^{-7}$M | Kador et al, 1980 |

## 1.9 Role of Aldehyde Reductase in Human Diabetic Cataract

Involvement of aldehyde reductase in human diabetic cataract is less clear.

The ratio $\frac{\text{aldehyde reductase activity}}{\text{polyol dehydrogenase activity}}$ in animal lens >>1 whereas in human lens the ratio is <<1 denoting that polyol dehydrogenase activity in normal human lens is much higher than that in the rat, rabbit or bovine lens (Jedziniak et al., 1981; present work).

Polyol dehydrogenase catalyzes the reversible reaction sorbitol $\rightleftharpoons$ fructose and $V_{max}$ for the enzyme in the fructose to sorbitol direction is ten times higher than that for sorbitol to fructose (Jedziniak et al., 1981). Sorbitol was found in the lens of diabetic patients (van Heyningen 1959b; Pirie and van Heyningen, 1964; Varma et al., 1979). This sorbitol could be formed by polyol dehydrogenase. Jedziniak et al (1981) also showed that polyol dehydrogenase activity remained persistently high in normal diabetic and non-diabetic cataract lens and remains so with increasing age, whereas aldehyde reductase activity decreased in all types of lenses with age.

Not much work has been done on polyol dehydrogenase either in animal or in human lens to find out what role this enzyme plays in the aetiology of diabetic cataract.

Although the evidence implicating the importance of aldehyde reductase in the development of sugar cataract in animals is very strong, it is not very convincing. Polyol theory alone cannot explain the xylose cataract where regression of cataract occurs even in the presence of xylitol.

Most of the inhibitors of lens aldehyde reductase attack other metabolic events. Alrestatin inhibited cyclic AMP accumulation (Lippman and Kobric, 1978), flavonoids inhibited calcium pumping, $Na^+$ $K^+$ ATP ase and erythrocyte adhesion in the red cell (Robbins, 1974; Wüthrich and Schatzmann, 1980). Alrestatin inhibits glucagon release induced by arginine and increased insulin release (Lippman and Kobric, 1978). Lens aldehyde reductase inhibitors have been found to inhibit glucose induced and tolbutamide induced release of insulin in the perfused rat pancreas (Gabbay and Tze, 1972).

The infererence that lens aldehyde reductase is involved in sugar cataract has been drawn from the results of experiments done in in vitro conditions. The in vitro experiments show that the enzyme has very low affinity for glucose, for bovine lens enzyme Km for glucose = 120 mM (present work) and for human lens Km = 200 mM (Jedziniak et al., 1981). It is unlikely,

therefore, that even in diabetic human lens where glucose concentration is 3-4.5 mM (Jedziniak et al., 1981) the enzyme will act on glucose and produce sufficient amount of sorbitol to exert osmotic stress.

## 1.10 Aims of the Present Work

So far, all the literature surveyed has dealt with the problems of sorbitol production from glucose by aldehyde reductase. Much effort has been concentrated on inhibiting this enzyme. It is worth remembering here, that the aldehyde reductase in pancreas is required to bring about glucose induced insulin release in response to hyperglycemic condition (Gabbay and Tze, 1972), hence inhibition of this enzyme could lead to disastrous effect. It is also rather odd that an enzyme should be present in the lens and nerve tissue solely for harmful effects. Probably the enzyme has some other physiological role to play which is yet undiscovered.

Recently, the aldehyde reductases from the brain (Turner and Tipton, 1972; Ris and von Wartburg, 1973; Rivett and Tipton, 1981; Hoffman et al., 1980), kidney (Davidson and Flynn, 1979a,b;Morpeth and Dickinson, 1980), liver (Turner and Hryszko, 1980; Branlant et al., 1981) of different mammalian species have been isolated and studied in great detail. Aldehyde reductase has

been purified from the pig brain and its kinetics has been studied (Boghosian and McGuinness, 1979; 1981). Aldehyde reductase has been purified from human placenta (Kador et al., 1979), rat lens (Kador et al., 1980) and bovine lens (Scheaff and Doughty, 1976). The lens enzyme has been subjected to different inhibitors in an attempt to find the best inhibitor for this enzyme. Some preliminary kinetics has been done on bovine lens aldehyde reductase (Scheaff and Doughty, 1976). Apart from that, not much work has been done on the mechanistic side of the aldehyde reductase action. The present work does not try to evaluate the importance of the enzyme in sugar cataract. The entire work has been devoted to the understanding of the mechanism of catalysis of lens aldehyde reductase, structure-function relationship and kinetics.

Aldehyde reductase from bovine lens was purified to apparent homogeneity using affinity and triazine dye columns, gel filtration and ion-exchange columns.

In order to understand the chemical nature of enzyme catalysis, the modification of aldehyde reductase was carried out with certain chemical reagents because the nature of the groups essential for catalytic activity of an enzyme can be deduced by using certain specific chemical reagents. The loss of activity of the

enzyme by such reagents would suggest the involvement of a particular residue in the catalytic process. However, the loss of activity by a specific reagent may not necessarily mean that particular residue is important for direct binding of the substrate to the enzyme; it may well mean that the particular group is important in maintaining the structure of the enzyme. Protection by substrate against inhibition would suggestthat the particular group is at or near the substrate binding site. Identification of catalytically essential groups helps to elucidate the mechanism of the enzyme. Many reagents are now available for modifying specific types of residues, for example, phenylglyoxal and butanedione modify the guanidino group of arginine, diethylpyrocarbonate modifies imidazole group of histidine, pyridoxal-5'-phosphate modifies the $\varepsilon$-$NH_2$ group of lysine and p-chloromercuribenzoate modifies the thiol groups of cysteine. These reagents have been used in the present study to identify catalytically important residues in aldehyde reductase. The findings of the present work suggest some possible physiological roles the enzyme could perform.

Crude polyol dehydrogenase was also studied but not in as much detail as aldehyde reductase. The properties of bovine lens polyol dehydrogenase, as found in this work, were compared to those of aldehyde reductase.

### 1.10.1 NADPH Oxidizing Activity in Blood

Lens, nerve and blood vessels are the tissues which manifest most of the long-term complications of diabetes. The red blood cell and the lens have many common features, like high glutathione content (Srivastava and Beutler, 1969; Giblin *et al.*, 1976), dependence on anaerobic glycolysis and pentose phosphate pathway as the main metabolic routes for glucose metabolism. There is indication that sorbitol pathway may be operative in the red blood cell. Travis *et al* (1971) found fructose and sorbitol in red blood cell by gas-liquid-chromatography technique. Sorbitol production could be inhibited by tetramethyleneglutaric acid, a lens aldehyde reductase inhibitor (Malone *et al.*, 1980). Malone *et al* (1980) also found a correlation between plasma glucose concentration and red cell sorbitol content in diabetic children. Hence the evidence for a lens aldehyde reductase type enzyme is indirect. However, Beutler and Guinto (1974) partially purified L-hexonate dehydrogenase (aldehyde reductase A) from red blood cell. Petrash *et al* (1982) purified an aldehyde reductase from red blood cell which did not act on glucose. These workers, however, worked with normal blood.

An initial study was undertaken with 104 patients to see if this blood aldehyde reductase in crude state showed any change in patients suffering from varying types of ocular complications. Oxidation of NADPH in blood samples with glyceraldehyde as substrate was followed to assay this activity.

# C H A P T E R 2

# MATERIALS AND METHODS

## 2.1 MATERIALS

The monobarium salt of diethylacetal glyceraldehyde-3-phosphate, diethyl pyrocarbonate, N-ethyl maleimide, lithium sulphate, blue dextran. D-sorbitol, phenazine methosulphate, nitro-blue tetrazolium succinic-semi-aldehyde and p-nitrobenzaldehyde were obtained from Sigma Chemical Company (Poole, Dorset, England).

β-NADPH (Type X) and β-$NADP^+$ (Sigma Grade) were from Sigma Chemical Company. β-$NAD^+$ (Grade 1) and β-NADH (Grade 1) were purchased from Boehringer Corporation Ltd. (Lewes, East Sussex). Glyceraldehyde-3-phosphate dehydrogenase and yeast alcohol dehydrogenase were also from Boehringer Corporation Ltd.

DL-glyceraldehyde, phenylglyloxal, tetranitromethane, bis-dimethylacetal ester of malondialdehyde were purchased from Aldrich Chemical Company (Gillingham, Kent, England).

D-glucose, p-chloromercuribenzoate, protein markers for sodium-dodecylsulphate polyacrylamide gel electrophoresis were obtained from British Drug House (Poole, Dorset, England).

FPL 58665 a compound analogous to cromoglycate was provided by Fison Ltd., Pharmaceutical Division (Loughborough, Leics., England). Sulindac was obtained from Merck, Sharp and Dohme (Rahway, New Jersey, U.S.A.). Procion Red was from Imperial Chemical Industries Ltd. (Organic Division, Manchester, England).

5' AMP Sepharose 4B, Sepharose 4B, Sephadex G-25 (fine), Sephadex G-50 (fine) and Sephadex G-200 (fine), Carboxymethyl Sephadex C-50, Diethylaminoethyl Sephadex A-50 were purchased from Pharmacia Chemicals (Uppsala, Sweden). Reactive Red Agarose was from Sigma Chemicals, cyanogen bromide from Koch-Light Laboratory (Colbrook, Berks., England) and 6-amino-hexanoic acid was from British Drug House (Poole, Dorset, England).

All other chemicals used were obtained from British Drug House and were of the highest purity obtainable.

## 2.2 METHODS

### 2.2.1 Protein Estimation

Protein was estimated either by the method using Folin & Ciocalteu's phenol reagent (Lowry et al.,1951) or by protein-dye binding method of Bradford (1976) using Coomassie blue G-250. The latter method was followed when samples contained reductants usually mercaptoethanol, NADPH or NADH which interfered with

Folin & Ciocalteu's phenol reagent (Lowry et al, 1951). For these two methods a standard curve was constructed using known amount of bovine serum albumin against absorbance at 595 mμ, as described in the method of Bradford (1976), or at 750 mμ, as described in the method of Lowry et al. (1951). The amount of the unknown sample was calculated from the absorbance at the said wavelengths. For measurement of protein in fractions eluted from columns used in purification procedures (Section 2.2.6) the extinction at 280 mμ was measured (Warburg and Christian, 1941).

### 2.2.2 Preparation of Lens Homogenate and Red Blood Cell Haemolysate

#### 2.2.2.1 Preparation of Lens Homogenate

Bovine eyes were collected from the slaughterhouse (Alf Meade & Sons, Reading) on the day the animals were slaughtered. Lenses were extracted the same day and kept frozen until used. Lenses (average weight 1.9 ± 0.2 g) were homogenized at 4°C in 5 vols. of 50 mM potassium phosphate pH 7.2 buffer containing 2-mercaptoethanol (5 mM) using a mechanical homogenizer. The homogenate was then centrifuged at 10,000 x g for 20 min. at 4°C on a MSE centrifuge (Fison's Group, Crawley, Sussex, England). The pellet containing cellular debris was discarded. The supernatant was used as a source of enzyme where experiments were performed

with crude enzyme. All other lens homogenates, rat, mouse and human were prepared in the same way.

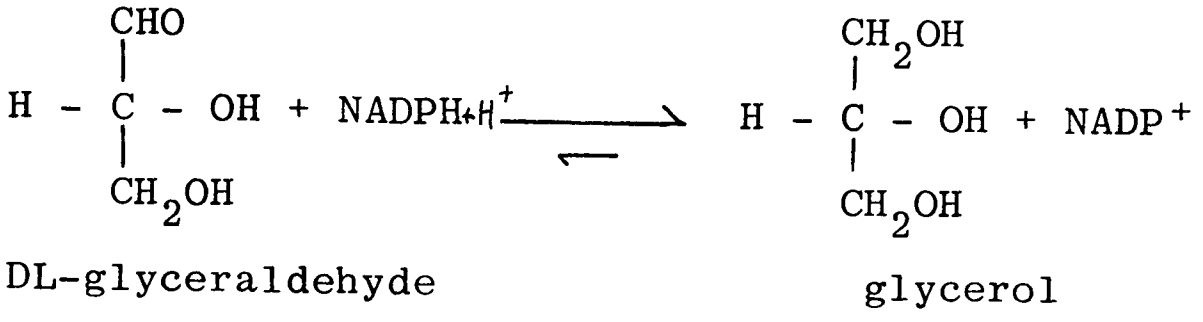
2.2.2.2 Preparation of Red Blood Cell Haemolysate

Blood samples were collected in heparinized bottles from the patients (n = 104) attending the Oxford Eye Hospital or the diabetic clinic at the John Radcliffe Hospital, Oxford. The blood samples were centrifuged on a small bench centrifuge at 15,000 r.p.m. for 10 min., the plasma decanted and erythrocytes were washed with 0.9% Nacl solution. Packed red cells were lysed by freezing and thawing. An equivalent volume of 100 mM potassium phosphate pH 6.2 buffer was then added and the suspension was diluted twenty-fold with distilled water. The pH was maintained constant at 6.2.

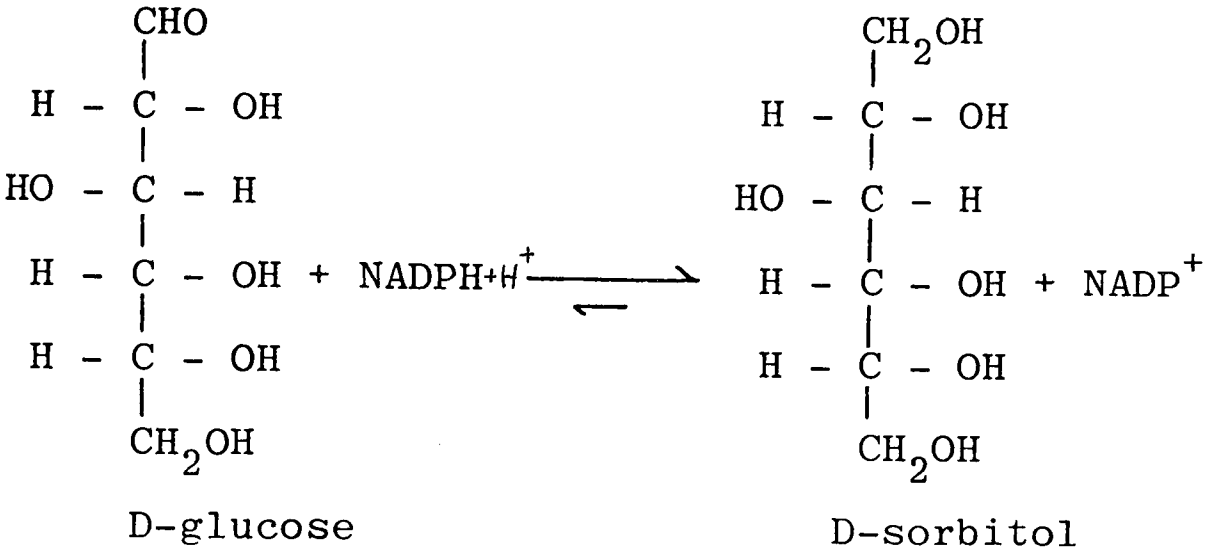
2.2.3 Enzyme Assay

All enzyme assays were carried out at 37°C (unless otherwise stated as described on page 36). Aldehyde reductase and polyol dehydrogenase activities in bovine lens and in crude haemolysate were measured on a Perkin-Elmer 550 UV-Vis double beam recording spectrophotometer fitted with an automatic cell changer, a Cecil double beam spectrophotometer and a Cary 119 double beam spectrophotometer. 1 ml or 3 ml quartz or glass cuvettes with 1 cm light path were used throughout. The Fin pipettes used had an accuracy of ± 3%.

2.2.3.1 Assay of Aldehyde Reductase B (E.C.1.1.1.21)



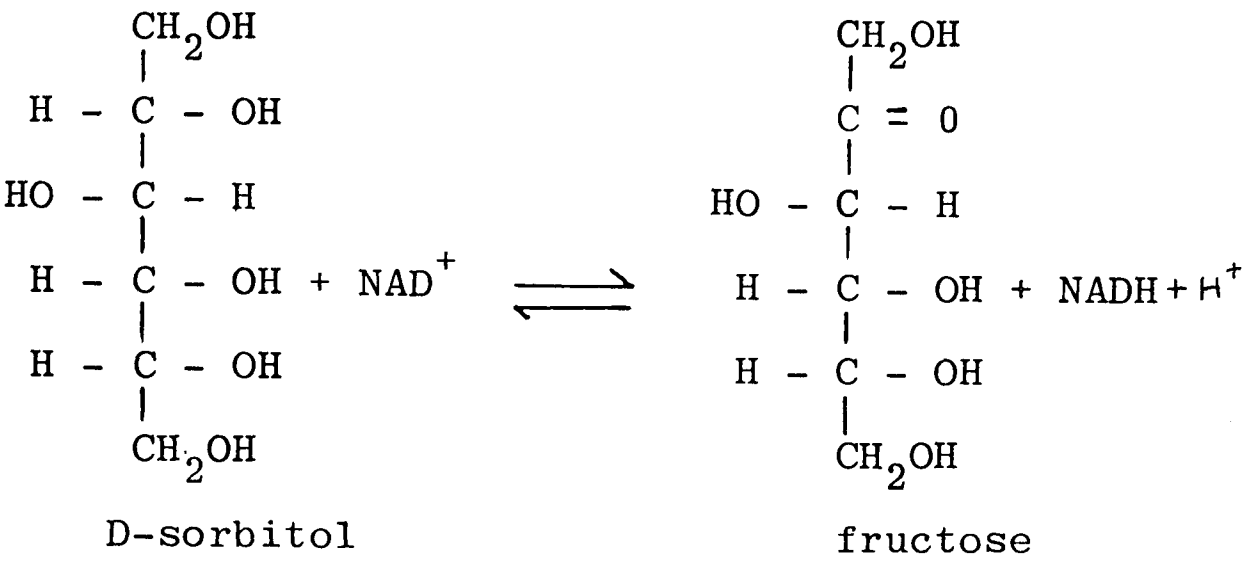

DL-glyceraldehyde glycerol




D-glucose D-sorbitol

Aldehyde reductase in rat, rabbit, bovine, human and mouse lens was assayed in 1 ml of either 100 mM potassium phosphate 6.2 buffer or 100 mM Tris-HCl pH 7.2 buffer as stated containing DL-glyceraldehyde (5 mM) or glucose (80 mM) and NADPH (50 μM) unless stated otherwise. Usually the assay was started by adding the enzyme to the test except in those cases where the enzyme was incubated with inhibitors or experiments were performed with crude extract. In the latter case the blank contained all but aldehyde substrates to correct for any non-specific changes due to inhibitor compound or endogenous substrates in the crude extract.

2.2.3.2 Assay of Polyol Dehydrogenase (E.C.1.1.1.14)




Polyol dehydrogenase in rat, rabbit, mouse, bovine and human lens was assayed in 1 ml of 100 mM Tris-HCl pH 8.9 buffer with sorbitol (40 mM) and $NAD^+$ (1.5 mM). The assay was started by adding the enzyme to both blank and test. The blank contained all reagents except sorbitol.

Where the effect of pH on enzyme activity was studied, buffers of different pH but of same ionic strength were used. The concentrations of substrates were varied for determination of kinetic parameters. Different concentrations of $(NH_4)_2SO_4$, $NH_4Cl$, KCl, $Li_2SO_4$ were included in the buffer to study the effect of ionic strength on both aldehyde reductase and polyol dehydrogenase in crude extracts. The inhibition of aldehyde reductase by sulindac, FPL 58665, Procion Red, 2'5' ADP, nicotinamide, sorbitol and $NADP^+$ was studied by including the

inhibitor in both blank and test. The enzyme rates for both aldehyde reductase and polyol dehydrogenase were linear for at least 8 min.

2.2.3.3 NADPH-oxidizing Activity and Polyol Dehydrogenase Activity in the Human Red Cell

The NADPH oxidizing activity in human blood samples (prepared as described in section 2.2.2.2) was measured with DL-glyceraldehyde (20 mM), NADPH (250 μM) in 1 ml of 100 mM potassium phosphate pH 6.2 buffer. The assay mixture for polyol dehydrogenase contained sorbitol (40 mM) and $NAD^+$ (300 μM) in 1 ml of 100 mM Tris-HCl pH 8.9 buffer. In both cases the blank contained all the reagents except DL-glyceraldehyde or D-sorbitol, as the case may be. The progress curves were linear for at least 8 min.

The decrease or increase in NADPH/$NAD^+$ absorbance at 340 mμ was measured. From this the initial velocity (denoted as V unless stated otherwise) was calculated as μmoles of substrate (NADPH/$NAD^+$) oxidized or reduced/min. One international unit of enzyme has been defined as the amount of enzyme catalyzing the oxidation of 1 μmole of NADPH or reduction of 1 μmole of $NAD^+$ per minute.

2.2.3.4 Statistical Tests

The rates shown in all figures and tables are the averages of 2-4 determinations with standard deviation. A linear regression analysis was performed to find the slope and intercept values and the best fit lines for all sets of initial velocity data obtained in the study of mechanism or in the study with inhibitors of aldehyde reductase B (including product inhibition study).

Both parametric (student'sttest) and non-parametric (Wilcoxon's rank sum test) methods were used to see if there was any significant difference between the NADPH-oxidizing activity in the control patients and that in the patients suffering from various ocular complications. Linear regression analysis was performed to see if there was any correlation between NADPH oxidizing activity and age, $HbA_{1c}$, height or weight or if there was any correlation between these parameters.

2.2.4 Choice of Buffer for Aldehyde Reductase B

In phosphate buffer the purified aldehyde reductase B showed pH optimum at 6.3-6.8 (Fig.3.7.1.1). The activity of the enzyme decreased beyond pH 7.0 with both DL-glyceraldehyde and D-glucose as aldehyde substrates. The decrease in activity was also noticed in

Tris-HCl buffer at above pH 7.4. Between pH 6-7, phosphate buffer offers good buffering capacity. The crude enzyme showed pH optimum at pH 6.2 in phosphate buffer which is close to that found with the pure enzyme. Hence 100 mM phosphate buffer pH 6.2 was used as an assay buffer in most of the experiments unless stated otherwise. But NADPH oxidation measured in terms of decrease in absorbence at 340 mμ is accelerated in phosphate buffer at around pH 6.8 (Lowry et al.,1961). The breakdown was compensated by including NADPH in both blank and test. The addition of DL-glyceraldehyde to NADPH in phosphate buffer did not stimulate the breakdown of NADPH for about 15 minutes. The addition of crude extract to the assay mixture containing NADPH and buffer was found to increase the NADPH oxidation by 10%, as measured by ΔA decrease at 340 mμ. But that rate was corrected when using crude enzyme by using Enzyme Blank ($E_B$) where the enzyme is added to both blank and test. NADPH, however, was not purified before use. But the molar extinction coefficient at 340 mμ ($E_{340}$) of the stock solution was checked and the observed ε value confirmed that it was 99% pure as suggested by the manufacturer (Sigma Chemical Company, Poole, Dorset). But a breakdown of 1.2%/hr. (measured in terms of ΔA 340) was noticed in phosphate buffer. NADPH solution was always made fresh and used up immediately.

### 2.2.5 Lens Crystallin Fraction Associated with Aldehyde Reductase Activity

Crude lens protein (55 mg) was loaded on to a Sephadex-G200 column (120 cm x 0.9 cm) pre-equilibrated with 50 mM Tris-HCl pH 7.5 containing 2-mercaptoethanol (10 mM), KCl (0.1 M), EDTA (5 mm) and sodium azide (0.02%). The flow rate was maintained at 9 ml/hr. Absorbance of every alternate fraction was measured at 280 mμ. Aldehyde reductase was assayed in all the crystallin fractions eluted from the column.

### 2.2.6 Purification of Aldehyde Reductase B

All the steps of purification of the enzyme were carried out at 4°C. The fractions were assayed for both glyceraldehyde and glucose reduction.

Step 1: Ammonium sulphate fractionation

The lens homogenate after centrifugation at 10,000 x g was brought to 0-20%, 20-40% and 40-60% saturation by adding solid ammonium sulphate. The addition was done slowly with constant stirring. After each ammonium sulphate fractionation, the fraction was allowed to settle for 1 hr and then centrifuged at 10,000 x g for 20 min. After centrifugation, 0-20%, 20-40% and 40-60% fractions were discarded and the supernatant from 40-60% fraction was brought to 60-85% saturation. The 60-85% ammonium sulphate fraction was

allowed to settle down for 1 hr and then centrifuged at 10,000 x g for 20 min. The precipitate was collected, dissolved in a minimum volume of 50 mM potassium phosphate pH 7.2 buffer containing 2-mercaptoethanol (5 mM) and dialysed overnight against 2 litres of the same buffer with one change. Aldehyde reductase was assayed with both glyceraldehyde and glucose in all the ammonium sulphate fractions after dissolving them in the buffer and dialysing them extensively to remove ammonium sulphate. The dialysis bagscontaining the dialysate were then put in sucrose in order to concentrate them and then tested for aldehyde reductase activity.

60-85% ammonium sulphate fraction after dialysis and concentration in sucrose was subjected to further purification procedures as described below.

2.2.6.1 Purification of Aldehyde Reductase B Using Gel-Filtration and Ion-Exchange Columns

The dialysate of 60-85% ammonium sulphate fraction (Step 1, Section 2.2.6) was concentrated in sucrose and passed through a Sephadex-G-25 column (24 cm x 3.0 cm) and eluted with the same buffer as used in dialysis to make sure that the enzyme was absolutely free from ammonium sulphate.

Step 2:

The enzyme eluted from Sephadex-G-25 was concentrated in sucrose and then loaded on to a Sephadex-G-200 column (90 cm x 1.2 cm) pre-equilibrated with 50 mM Tris-HCl pH 7.5 containing 2-mercaptoethanol (10 mM), KCl (0.1 M), EDTA (5mM) and sodium azide (0.02%). The flow rate of the column was 9 ml/hr. The elution was carried out with the same buffer. $A_{280}$ of every alternate fraction was measured and only those fractions which had very high enzyme activity were collected.

Step 3:

The pooled enzyme obtained from Step 2 was then dialysed overnight against 50 mM potassium phosphate buffer pH 5.9 containing 2-mercaptoethanol (10 mM) and sodium azide (0.02%). To make sure that the enzyme was in phosphate buffer and also KCl was completely removed, the enzyme was passed through Sephadex-G-25 column and eluted with the same phosphate buffer mentioned before.

The enzyme was then passed through a Carboxymethyl Sephadex-C-50 (50 cm x 2.5 cm) column pre-equilibrated with 50 mM potassium phosphate buffer pH 5.9 containing 2-mercaptoethanol (10 mM) and sodium azide (0.02%). The flow rate was maintained

at 10 ml/hr with the help of a peristaltic pump. The enzyme was eluted with the same buffer. The fractions containing enzyme activity were pooled and concentrated in sucrose (Step 1, Section 2.2.6).

Step 4:

The enzyme eluted from the Carboxymethyl Sephadex-C-50 column was then loaded on to a Diethyl-aminoethyl Sephadex-A-50 column (30 cm x 3 cm) pre-equilibrated with 50 mM potassium phosphate buffer pH 6.8 containing 2-mercaptoethanol (10 mM) and sodium azide (0.02%). The flow rate, as maintained with the help of a peristaltic pump, was 10 ml/hr. The enzyme from the column was eluted with a linear NaCl gradient (0-0.6 M) over a total volume of 300 ml. The fractionscontaining enzyme activity were pooled and extensively dialysed against 50 mM potassium phosphate buffer pH 6.8 containing 2-mercaptoethanol (10 mM) and sodium azide (0.02%). The enzyme was then passed through Sephadex-G-25 column for salt removal.

Step 5:

The enzyme collected from Sephadex-G-25 column was passed through a Sephadex-G-50 column (50 cm x 1.5 cm) pre-equilibrated with 50 mM potassium phosphate buffer pH 6.8 containing 2-mercaptoethanol (10 mM) and sodium azide (0.02%). The flow rate was

9 ml/hr and was maintained constant with the help of a peristaltic pump. The enzyme was eluted with the same buffer.

The enzyme eluted from the Sephadex-G-50 column was examined by SDS polyacrylamide gel electrophoresis (see Fig.3.3.1.6). The molecular weight of the enzyme was determined by both the gel-filtration method using a Sephadex-G-200 column and by SDS polyacrylamide gel electrophoresis.

### 2.2.6.2 Purification of Aldehyde Reductase B Using Affinity Columns

5' AMP Sepharose, Reactive Red Agarose (Procion Red HE3B attached to Sepharose matrix) and NADP Sepharose were tried with either salt or NADPH as eluent in order to make the purification of aldehyde reductase an easy and quick method.

#### 2.2.6.2.1 5' AMP Sepharose Column

5' AMP Sepharose was swollen for 2 hrs in 50 mM potassium phosphate pH 6.8 containing 2-mercaptoethanol (10 mM) and then poured into a glass tube (13 cm x 1 cm). The swollen gel contained 2 μmoles of nucleotide/ml of swollen gel (Pharmacia specification). The column was washed and equilibrated with the same buffer.

The dialysate of 60-85% ammonium sulphate fraction (Step 1, Section 2.2.6), before putting on to the 5' AMP Sepharose column, was passed through a Diethylaminoethyl Sephadex-A-50 column (50 cm x 3 cm). The flow rate was maintained constant (9 ml/hr) with the help of a peristaltic pump. The enzyme was eluted with a linear NaCl gradient (0-0.8 M) in 50 mM potassium phosphate pH 6.8 containing 2-mercaptoethanol (10 mM) over a total volume of 300 ml. The fractions containing enzyme activity were pooled, dialysed and concentrated in sucrose.

The concentrated enzyme was then loaded on to the 5' AMP Sepharose column, held there for ½ hour and then the column was washed with the 50 mM potassium phsophate buffer pH 6.8 containing 2-mercaptoethanol (10 mM) and 0.4 M NaCl until $A_{280}$ of the buffer was zero. The purpose of inclusion of NaCl was to remove any non-specifically adsorbed protein. The elution was then started with NADPH (200 µm). The fractions containing enzyme activity were pooled and dialysed against 50 mM potassium phosphate pH 6.8 containing 2-mercaptoethanol (10 mM) to remove NADPH. The dialysate was then concentrated using sucrose. SDS polyacrylamide gel electrophoresis was carried

out with the enzyme eluted from 5' AMP Sepharose according to Laemmli and Favre (1973).

2.2.6.2.2(a) Reactive Red Agarose Column - Elution with Salt

The Reactive Red Agarose from Sigma contained 1-4 μmoles Procion Red HE3B dye/ml of gel (Sigma specification) supplied as a suspended gel in NaCl solution (500 mM). It was poured into a glass tube (13 cm x 0.9 cm). The column was washed and equilibrated with 50 mM potassium phosphate buffer pH 6.8 containing 2-mercaptoethanol (10 mM) and the flow rate was 8 ml/hr.

About 10 mg of the enzyme protein eluted from the Diethylaminoethyl Sephadex-A-50 column, as described before, was loaded on to the Reactive Red Agarose column and held there for ½ hour and then the column was washed with the initial buffer until $A_{280}$ of the buffer was zero. The elution was then started with linear KCl gradient (0-1 M) over a total volume of 150 ml. The enzyme eluted at about 0.35 M KCl concentration. SDS polyacrylamide gel electrophoresis was performed with the enzyme eluted from the column.

2.2.6.2.2(b) Reactive Red Agarose Column - Elution with NADPH

To prevent non-specific adsorption to the column a further precaution was taken by washing the

column with 50 mM potassium phosphate pH 6.8 containing 2-mercaptoethanol (10 mM), sodium azide (0.02%) and glycine (1 M). The purpose of having glycine in the initial buffer was to attach glycine at the sites which might otherwise bind non-specific proteins, e.g. proteins other than aldehyde reductase or aldehyde reductase-like proteins.

The enzyme partially purified using a Sephadex-G-200 column followed by a Carboxymethyl Sephadex-C-50 Sephadex column was loaded on to the Reactive Red Agarose column. After ½ hour the column was washed with the initial buffer containing NaCl (200 mM) until $A_{280}$ of the buffer wash was zero. The elution was then begun with 10 ml of NADPH (5 mM). The eluent was then dialysed and concentrated and run on an SDS polyacrylamide gel.

2.2.6.2.3 NADP Sepharose Column

NADP Sepharose was prepared as follows (Mosbach et al., 1972). Sepharose 4B (50 ml) was activated by the cyanogen bromide method (Axén et al., 1967). 6-aminohexanoic acid (5 gm) dissolved in 100 mM $NaHCO_3$ (50 ml) was added and the final pH was adjusted to 8.5 with NaOH (4 M). After stirring the gel for 15 hrs at room temperature it was

extensively washed with $NaHCO_3$ (100 mM), HCl (100 mM), NaCl (500 mM) and water. The gel was then filtered off and 20 gm wet gel was washed with a large volume of aqueous 80% (v/v) pyridine. $NADP^+$ (200 mg) dissolved in water (6 ml) and dicyclohexyl-carbodi-imide (10 gm) dissolved in pyridine (24 ml) were added to the gel and the gel was then agitated on a rotary shaker for 10 days at room temperature. To remove formed dicyclohexyl urea, the gel was filtered off and washed successively with water, ethanol, warm (40°C) butan-I-ol, ethanol, water and aqueous pyridine 80% (v/v). The gel was then re-suspended in the first filter and additional carbo-di-imide (2.5 gm) was added. After one more week, the above washing procedure was repeated but with aqueous pyridine omitted and further washing was done according to the method of Mosbach et al. (1972) The gel contained approximately 15 μmoles of nucleotide/g wet gel. The final preparation was stored at 4°C.

A small column of size (15 cm x 0.6 cm) was prepared with the $NADP^+$ Sepharose thus made. The column was washed and equilibrated with 100 mM potassium phosphate buffer pH 6.2 containing 2-mercaptoethanol (10 mM) and sodium azide (0.02%). The flow rate of the column was 8 ml/hr. Ammonium

sulphate fraction (60-85%) [see 2.2.6] was dissolved in 100 mM potassium phosphate buffer pH 6.2 containing mercaptoethanol (10 mM) and sodium azide (0.02%) and dialysed extensively against the same buffer. The dialysate was concentrated in sucrose as described before. Approximately 50 mgm of enzyme protein was loaded on to the NADP-Sepharose column and held there for ½ hour. The column was then washed with the starting buffer containing 0.4 M NaCl until $A_{280}$ of the buffer wash was zero. The elution was then begun with NADPH (400 μM). The fractions containing enzyme activity were pooled and dialysed to remove NADPH. The enzyme was examined by SDS polyacrylamide gel electrophoresis.

### 2.2.7 Determination of the Molecular Weight of Lens Aldehyde Reductase B

Molecular weight of aldehyde reductase was determined by (a) SDS polyacrylamide gel electrophoresis, and by (b) Sephadex-G-200 column chromatography. The protein markers used for Sephadex-G-200 column chromatography were yeast alcohol dehydrogenase (140 000), bovine serum albumin (66 000), ovalbumin (45 000) and cytochrome C (12 500).

(a) Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Low molecular weight protein markers (14 300-71 500) were run along with the enzyme on 12% SDS polyacrylamide gel according to the method described by Laemmli and Favre (1973) using a current of 3 mamps/gel. A graph was constructed with mobility = $\left[\frac{\text{distance travelled by the band}}{\text{distance travelled by the dye front}}\right]$ of the protein marker bandsagainst their $\log_{10}$ molecular weight. The molecular weight of the enzyme eluted from Sephadex-G-50 column was determined from the graph from the relative migration of the band obtained with the enzyme.

(b) Sephadex-G-200 Column Chromatography

Blue dextran (1 mg/ml), alcohol dehydrogenase (0.2 ml of ammonium sulphate suspension from Boehringer Corporation Ltd.), bovine serum albumin (1 mg/ml), ovalbumin (1 mg/ml) and cytochrome C (1 mg/ml) along with the enzyme (0.3 mg/ml) in a total volume of 1 ml were passed through a Sephadex-G-200 column (120 cm x 0.9 cm) pre-equilibrated with 50 mM Tris-HCl pH 7.5 containing 2-mercaptoethanol (10 mM), KCl (100 mM), EDTA (5 mM) and sodium azide (0.02%) with a flow rate of 6 ml/hr. The elution was carried out with the same buffer. A graph was constructed with the elution volume of the protein markers against $\log_{10}$ of their

molecular weights. The molecular weight of the enzyme was determined from the graph from the elution volume of the enzyme.

### 2.2.8 Amino Acid Analysis of Aldehyde Reductase B

To determine amino acid composition of the enzyme obtained from Sephadex-G-50 (Step 5, Section 2.6.1) was hydrolysed in an evacuated sealed tube at 108°C for 18 hrs in 6N HCl at a protein concentration of 1 mg/ml. After hydrolysis the hydrolysate was rotary evaporated at 50°C, then dissolved in 0.2 ml of Citrate buffer pH 2.2 and analysed on an LKB 4400 amino acid analyser (LKB Biochem. Ltd., Cambridge, England). Amino acid mixture was passed through a cation exchange column and eluted with buffers of varying pH and varying ionic strength. The column effluent was mixed with ninhydrin reagent. The quantity of the coloured complex produced was directly proportional to the concentration of that particular amino acid present in the mixture. The reacted mixture was then passed through a two-channel photometer where the absorption of the coloured complex was measured at 440 nm (for imino acid like proline) and at 570 nm for other amino acids. The photometer output was linked to a two-channel recorder when a series of peaks representing the amino acids were recorded at two wavelengths mentioned above. The position of the peaks

on the chart represents the retention times for the individual amino acids and the measurement of the area under the peak gives quantitative value for each amino acid. The areas under the amino acid peaks were computed by an integrator (Tri Lab., Bedfordshire, U.K.) connected to the amino acid analyser using a programme written by Cronin et al. (1982).

The determination of cystine and cysteine is difficult by ordinary acid hydrolysis as they are rendered unstable in acid hydrolysis. Performic acid oxidation method of Schram et al. (1954) was followed prior to the acid hydrolysis of the protein whereby cystine and cysteine are converted into cysteic acid. Performic acid was removed by the use of HBr (Moore, 1963). The sample was then hydrolysed with 6N HCl in the usual way and analysed on amino acid analyser.

### 2.2.9 SDS Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate (SDS) binds to a wide variety of proteins on a basis of 1.4 gm/gm of protein when the equilibrium monomer concentration of SDS is greater than $5 \times 10^{-4}$M (Reynolds and Tanford, 1970) and confers negative charge on the SDS-protein complex swamping out the native charge of the protein molecule. Effectively, these complexes carry a constant negative charge/unit mass of protein. These complexes behave

like rod-shaped particles, the length of which is proportional to the molecular weight of the protein. A mixture of protein molecules dissolved in aqueous solution containing a high concentration of SDS (≒1%) have electrophoretic mobilities on polyacrylamide gels which are proportional to their polypeptide chain weights (Reynolds and Tanford, 1970).

SDS polyacrylamide gel electrophoresis was carried out according to the method of Laemmli and Favre (1973). A stacking gel (containing 3% polyacrylamide for disc gel and 6% for slab gel) and a separating gel (containing 12% polyacrylamide) were used at different pH values, 6.8 and 8.9 respectively, as described by Laemmli and Favre (1973). 0.25% Coomassie blue (R250) solution was used to stain protein bands.

2.2.10 Activity Staining on Polyacrylamide Gel

Polyacrylamide gels (7.5%) were prepared and run according to the method described by Scheaff and Doughty (1976). The gels were incubated in the dark in 10 ml of 100 mM sodium phosphate buffer pH 7.0 containing glycerol (10 mg/ml), $NADP^+$ (1 mg/ml), nitroblue tetrazolium chloride (0.5 mg/ml), phenazine methosulphate (0.02 mg/ml) for ½-1 hour.

Various combinations of gel buffer and running buffer were also tried with varying concentrations of substrates. The reason has been given in Chapters III and IV.

2.2.11 Chemical Modification of Aldehyde Reductase B

Experimental conditions for modification reactions were chosen in which both the enzyme and the inhibitors were stable over the periods of incubation under study.

For these experiments the enzyme eluted from CM-Sephadex-C-50 preceded by a Sephadex-G-200 column (Section 2.2.6.1) was used.

In all experiments the reagents were incubated at 25°C with the enzyme (800 nM) in a total volume of 100 µl. After incubation for different periods of time, the enzyme was assayed at 37°C in a total volume of 1 ml of either 100 mM potassium phosphate pH 6.2 or 100 mM Tris-HCl pH 7.2, depending upon the buffer used for incubation. The assay was performed with DL-glyceraldehyde or D-glucose as aldehyde substrate, as described before, unless stated otherwise.

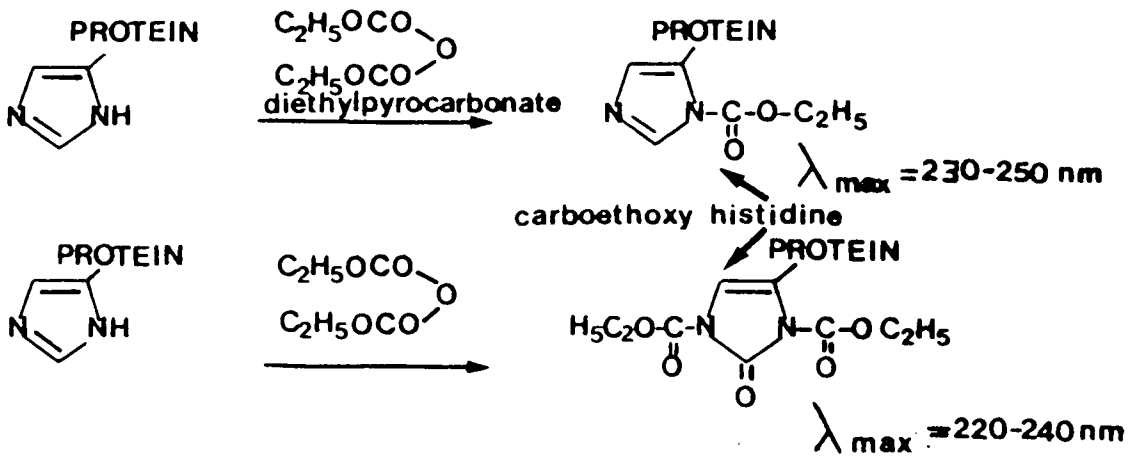
For substrate protection experiment the enzyme was incubated with the protecting substrate for ½ min

prior to the addition of the inhibitor. After adding the inhibitor the incubation was caried out for the time stated in individual sections.

2.2.11.1 Treatment with Diethylpyrocarbonate

Diethylpyrocarbonate reacts with histidine residues in model systems (Ovádi et al., 1967; Mühlrád et al., 1967) and in proteins (Ovádi and Keleti, 1969).

The reaction between diethylpyrocarbonate and imidazole group of histidine is as follows (Miles, 1972):




Nucleophils are reactive in their unprotonated farms and pka of imidazole group is 6.95 (Holbrook and Ingram, 1973) so modification with diethylpyrocarbonate should be carried out at around pH 6.0 (Ovádi et al., 1967). Studies at

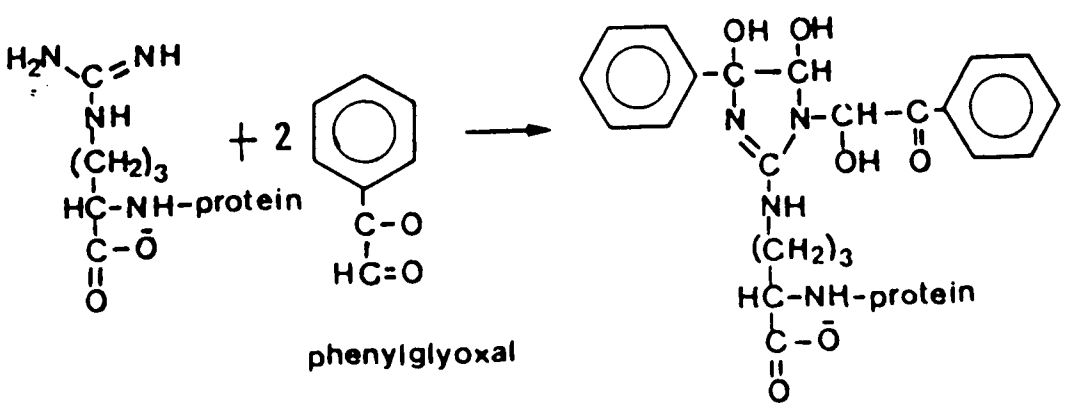
pH 6.0 is also advantageous as N-carboethoxy-histidyl residue is most stable at this pH.

In the present study, the stock diethyl-pyrocarbonate reagent was diluted with 95% ethanol. The incubation was carried out in 100 mM potassium phosphate buffer pH 6.1 for 1-30 min (Fig.3.10.1a) and the enzyme was assayed in 100 mM potassium phosphate pH 6.2 buffer.

2.2.11.2 Treatment with Butanedione and Phenylglyoxal

Modification of arginine using 2,3 butane-dione under mild condition was first introduced by Yankeelov et al. (1966). They proposed that it is the trimeric form of the reagent $(C_4H_6O_2)_3$ which is actually reactive.

Phenylglyoxal, another $\alpha,\alpha$-dicarbonyl compound, was shown to be specifically modifying arginine group under mild condition (Takahashi, 1968). According to Takahashi, two molecules of phenyl-glyoxal react with one molecule of arginine as follows:



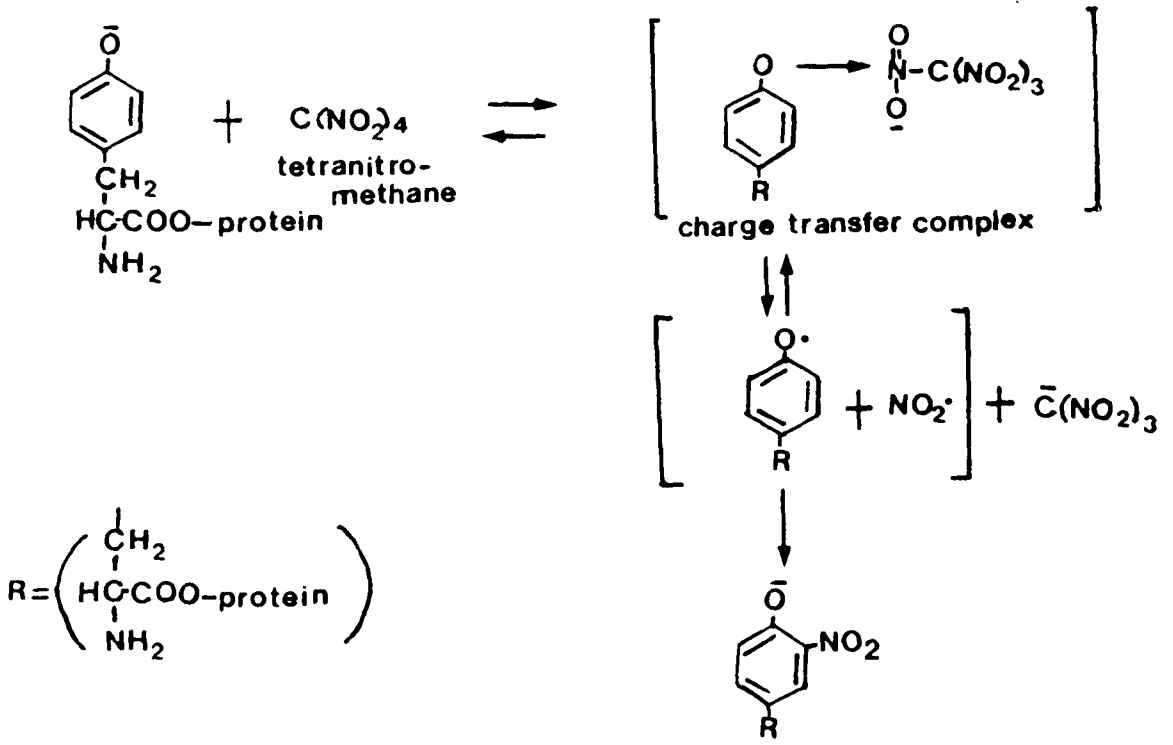
Both butanedione and phenylglyoxal have been used to investigate the role of arginine in a number of enzymes, e.g. alkaline phosphatase (Daemn and Riordan, 1974), D(-) β hydroxy-dehydrogenase (Phelps and Hatefi, 1981) etc.

For the present work butanedione stock solution was made up by diluting the reagent with 20 mM potassium phosphate buffer pH 7.0. The incubation with the enzyme was performed in the same buffer for about 10 min. The enzyme was assayed, as described before, with DL-glyceral-dehyde (5 mM) as the aldehyde substrate in 100 mM phosphate buffer pH 6.2. Phenylglyoxal was recrystallized from water to remove insoluble materials, if any, and dissolved in 100 mM phosphate buffer pH 7.0. The enzyme was incubated with phenylglyoxal in the same buffer for up to 20 min.(Fig.3.10.2.1)and assay was carried out using both D-glucose and DL-glyceraldehyde as aldehyde substrates in 100 mM phosphate buffer pH 6.2, as before.

2.2.11.3 Treatment with Tetranitromethane

Tetranitromethane has been reported to be very specific for nitration of tyrosyl residues in protein (Riordan et al., 1967; Sokolovsky et

al., 1966). By chromatography the product of nitration was found to be 3-nitrotyrosine (Sokolovsky et al., 1966). However, tetranitromethane can also modify tryptophan group (Sokolovsky et al., 1970). The reaction of phenolate ion with tetranitromethane has been investigated in detail by Bruice et al. (1968) who have suggested the following mechanism for nitration of tyrosyl residue:




As nitration of phenolate ion is pH dependent with nearly no reaction at pH 6.0 and a rapid modification at pH 9.0, prior to incubation of the enzyme with this reagent, the enzyme was passed through a Sephadex-G-25 column for buffer

exchange from 50 mM potassium phosphate to 50 mM Tris-HCl pH 8.0. A 1% tetranitromethane stock solution was made by diluting the reagent with 95% ethanol. The incubation was then carried out with 20 µl of 1% tetranitromethane in a total volume of 100 µl for up to 1 hr and then the enzyme was assayed in 100 mM Tris-HCl pH 7.2 buffer as before.

2.2.11.4 Treatment with Pyridoxal-5'-phosphate and Potassium Cyanate

To test the involvement of amino group at the substrate binding site the modification of the enzyme was carried out with two amino group specific reagents.

2.2.11.4.1 Potassium Cyanate

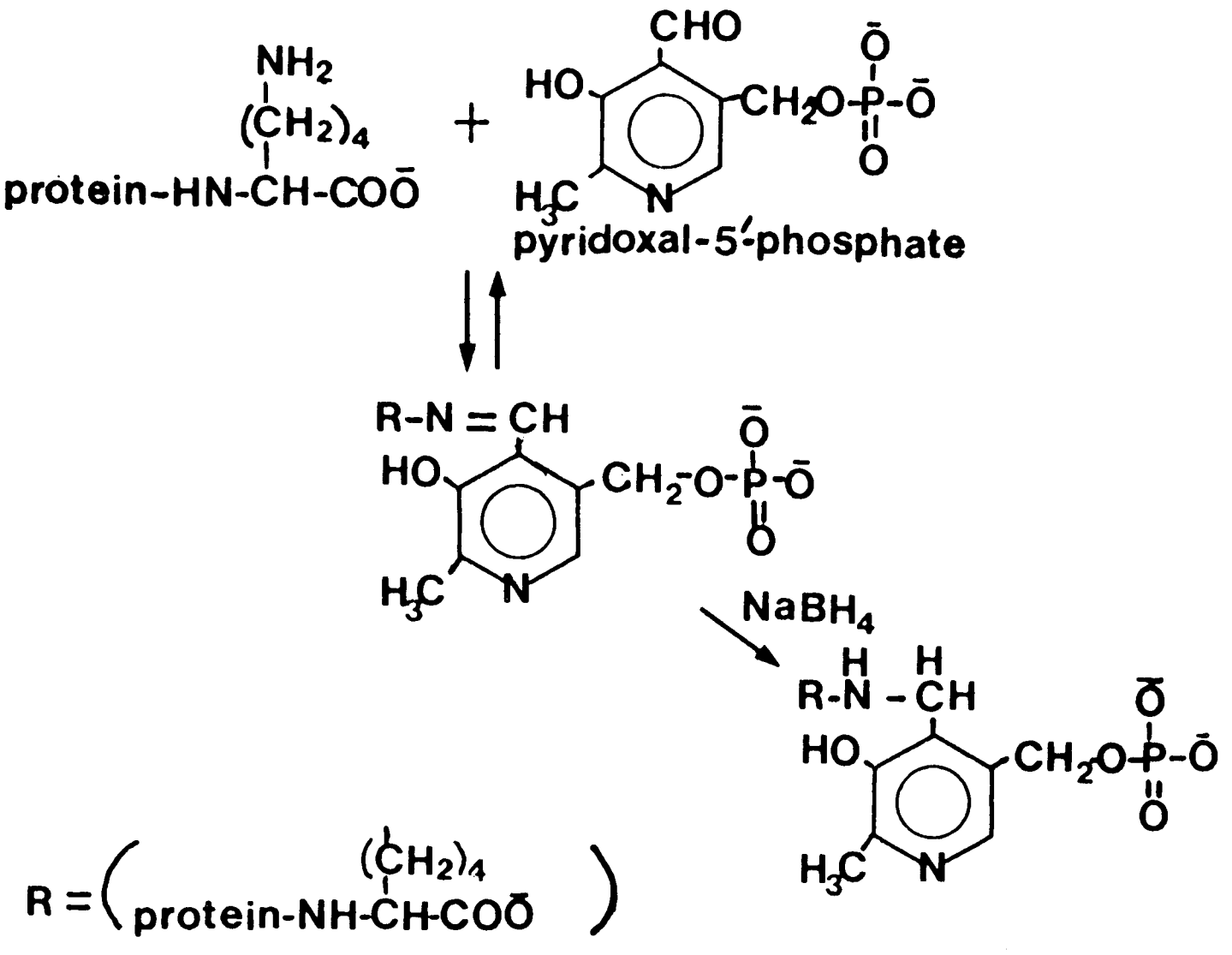
Though cyanate is capable of reacting with amino, sulphydryl, carboxyl, imidazole (Stark, 1964; 1965a; 1965b), phenolic hydroxyl (Smyth, 1967) groups in protein to yield carbamoyl derivative, at pH 7.0 the reagent is fairly specific for amino group (Stark, 1965a). The reaction with amino group with cyanate is described below:

$$\text{Protein} - NH_2 + HNCO \longrightarrow \text{Protein} - NH - \overset{\overset{O}{\|}}{C} - NH_2$$

For the present work, potassium cyanate was re-crystallized from 50% ethanol at 50°C to remove insoluble materials. It was then dissolved in 100 mM potassium phosphate pH 7.0 buffer. The incubation was carried out with potassium cyanate (100 mM and 500 mM) in the same buffer for up to 5 hrs. The enzyme was then assayed in 100 mM potassium phosphate pH 6.2 buffer.

2.2.11.4.2 Treatment with Pyridoxal-5'-phosphate

Pyridoxal-5'-phosphate has been found to be a highly specific reagent for the $\varepsilon$-$NH_2$ group of lysine residue when used alone, as was used in the experiment to inactivate rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Ronchi et al., 1969) or with sodium borohydride as has been used for rabbit muscle phosphoglucose isomerase (Schnackerz and Noltzmann, 1971). The function of sodium borohydride is to stabilize the Schiff's base formed between $\varepsilon$-$NH_2$ group of lysine and aldehyde group of pyridoxal-5'-phosphate. The reaction is shown as follows:

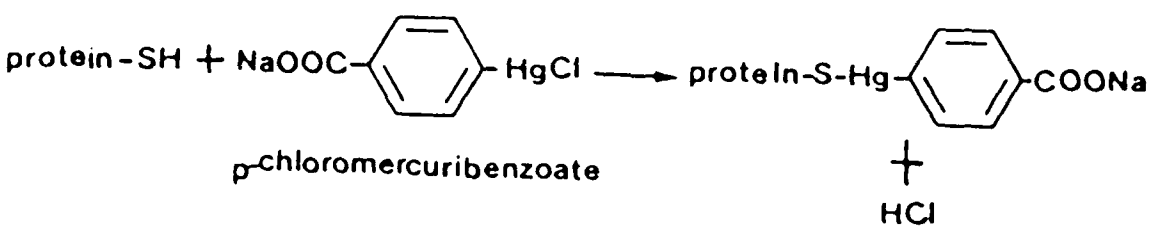



Pyridoxal-5'-phosphate solution was made up in 100 mM Tris-HCl pH 7.2 buffer and the incubation with the enzyme was carried out with the reagent (10 μm - 1 mM) for up to 30 min in the same buffer in the dark, as the reagent was photo-sensitive. The enzyme was assayed using 100 mM Tris-HCl pH 7.2 as assay buffer.

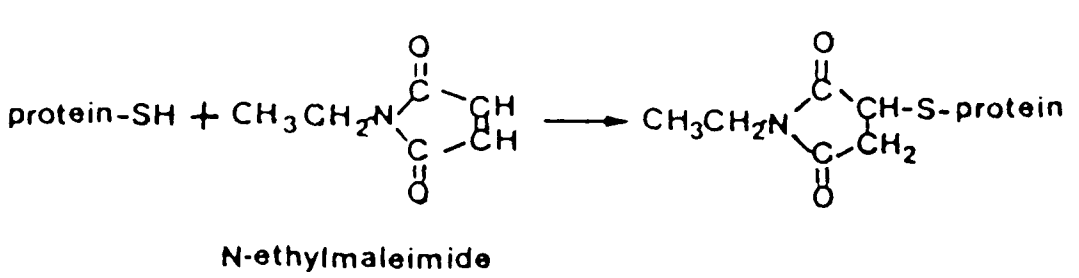
2.2.11.5 Treatment with Thiol-group Reagents, p-chloro-mercuribenzoate and N-ethylmaleimide

Both p-chloromercuribenzoate and N-ethyl-maleimide can react with thiol group selectively and rapidly.

The organic mercurial, p-chloromercuribenzoate reacts with thiol group as follows:




Reaction with N-ethylmaleimide with thiol group is shown below:




Before incubation with thiol reagents, the enzyme eluted from Carboxymethyl Sephadex-C-50 column was passed through a Sephadex-G-25 column to remove any residual 2-mercaptoethanol.

2.2.11.5.1 Modification with p-chloromercuribenzoate

1mM p-chloromercuribenzoate was prepared by dissolving first in NaOH (200 mM) and then neutralized to pH 8.2 with dilute HCl. Stock solution thus made was diluted with Tris-HCl pH 7.2 buffer to the required strength. The enzyme was incubated

with different concentrations of the reagent for 90 sec and then assayed in 100 mM Tris-HCl pH 7.2 buffer with both aldehyde substrates, as described before.

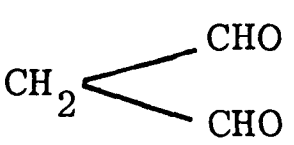
2.2.11.5.2 Modification with N-ethylmaleimide

N-ethylmaleimide was dissolved in 100 mM potassium phosphate pH 7.0 buffer and incubation with the enzyme was carried out in the same buffer for 2-15 min. The enzyme was assayed with both aldehyde substrates, as described before in phosphate pH 6.2 buffer.

2.2.12 Preparation of Glyceraldehyde-3-phosphate (GAP) from Diethylacetal (Monobarium) Salt

GAP was prepared from the salt by treating with Dowex-50 Hydrogen Form Resin according to the method suggested by Sigma Chemical Company. The enzymatic assay of D-isomer of GAP was carried out with GAP dehydrogenase and $NAD^+$ according to the method provided by Sigma Chemical Company.

2.2.13 Preparation of Malondialdehyde



Malondialdehyde

Malondialdehyde was prepared from its bis-dimethyl acetal) ester by the method described by

Kikugawa *et al.*(1980). Malondialdehyde bis (dimethyl acetal) ester (1.64 gm) was mixed with 1 N HCl (0.9 ml) and the heterogeneous mixture was shaken at 40°C until it became homogenous. The solution was made up to 10 ml with water and the acidic solution was incubated at 37°C for 1 hr, and then the pH was adjusted to 6.2 to use as a substrate for aldehyde reductase assay.

### 2.2.14 Fluorescence Spectroscopy

Fluorescence emission spectra of NADPH (1 μM), NADPH (1 μM) + enzyme (0.2 mgm/ml) were obtained using a Perkin-Elmer single beam spectrofluorimeter. The slit width of excitation and emission bands were 5 nm each, and the excitation wavelength used was 340 nm.

### 2.2.15 Determination of Viscosity

Viscosity of Sorbitol at 500 mM and 1 M concentrations was determined with respect to water using an Ostwald's Capillary Viscometer in a water bath, temperature of which was kept constant at 25°C.

### 2.2.16 Computing

$K_m$ and $V_{max}$ for each substrate were obtained by feeding the initial velocity data against substrate concentration into a Pet 16K Microcomputer (Commodore Electronics) using a Non-linear Regression Programme

(Crabbe, 1982) based on the equation $v = \frac{V_{max}\ [S]}{K_m + [S]}$.

The programme was a part of an elaborate programme on enzyme kinetics. For substrate showing non-linear kinetics like DL-glyceraldehyde (see Fig. 3.12.3) to find out the minimum degree of rate equation, the initial data at each substrate concentration were computed using a BMDP3R Non-Linear Regression Programme on an ICL 2988 computer. The programme used the following function for computing the data:

$$\frac{\alpha_0 + \alpha_1 x + \alpha_2 x^2 + \ \ldots\ldots\ + \alpha_m x^{\frac{m-1}{2}}}{1 + \beta_1 x + \beta_2 x^2 + \ \ldots\ldots\ + \beta_{m-1} x^{m-1}} \quad \text{if m is odd}$$

or

$$\frac{\alpha_0 + \alpha_1 x + \alpha_2 x^2 + \ \ldots\ldots\ + \alpha_{m-1} x^{\frac{m}{2}-1}}{1 + \beta_1 x + \beta_2 x^2 + \ \ldots\ldots\ + \beta_m x^{\frac{m}{2}}} \quad \text{if m is even}$$

where $\alpha$, $\beta$ ..... are the parameters or the kinetic constants and m = degree of rate equation.

The programme is attached in the appendices.

# C H A P T E R 3

## RESULTS

### 3.1 Polyol Pathway Enzymes in Rat, Rabbit, Mouse, Bovine and Human Lens

Polyol pathway enzymes, i.e. aldehyde reductase B and polyol dehydrogenase, were assayed in mouse (5 weeks old), rat (5 weeks old), rabbit (5 months old), bovine (2-4 years old) and human (50-60 years old) lenses. Aldehyde reductase B was measured with both of its aldehyde substrates, DL-glyceraldehyde and D-glucose. Fig. 3.1.1 shows the variation of activities of these enzymes in these lenses. It shows that both rat (n = 4) and rabbit (n = 2) lenses have high specific activities of aldehyde reductase. Taking aldehyde reductase activity in rat as 100% with DL-glyceraldehyde as aldehyde substrate, the percentage activity in rabbit is 60%, in bovine lens 6% (n = 4), in human lens (n = 4) 16% and in the mouse lens (n = 4) it is approximately 2%. With D-glucose as the aldehyde substrate, with respect to rat lens the percentage activities are 23%, 5%, 15%, 1'6% in rabbit, bovine, human and mouse lens respectively. Sorbitol dehydrogenase activity is highest in the human lens and with respect to the human lens the percentage activities in bovine, rat, rabbit and mouse lenses are 2'8%, 0,0,0 respectively. The total activity of aldehyde reductase with DL-glyceraldehyde was highest in the rabbit lens (100%), the

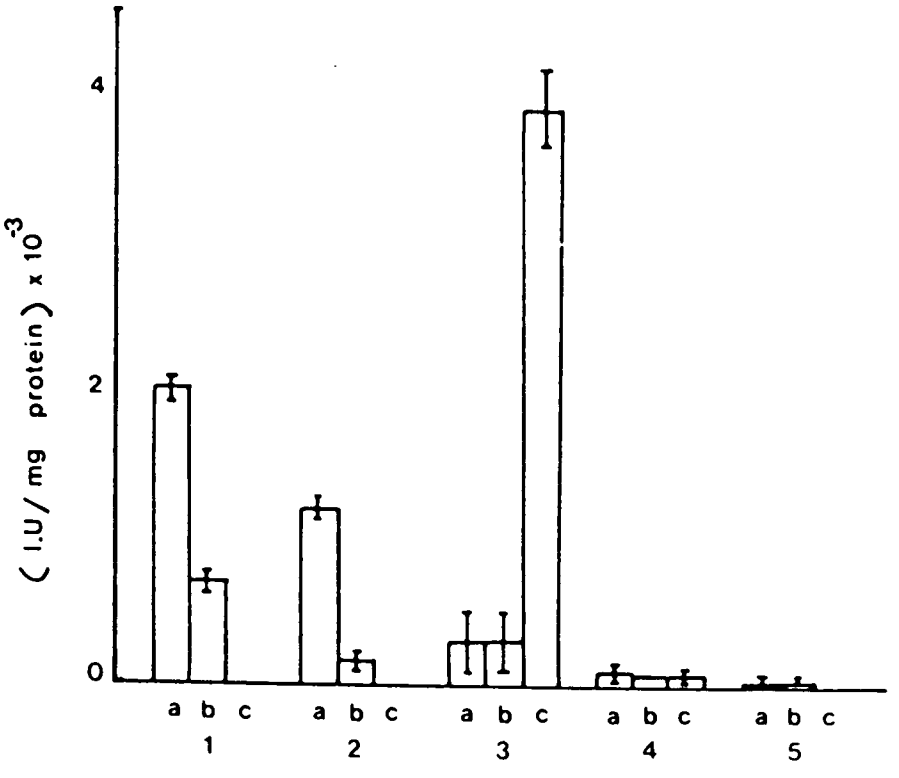
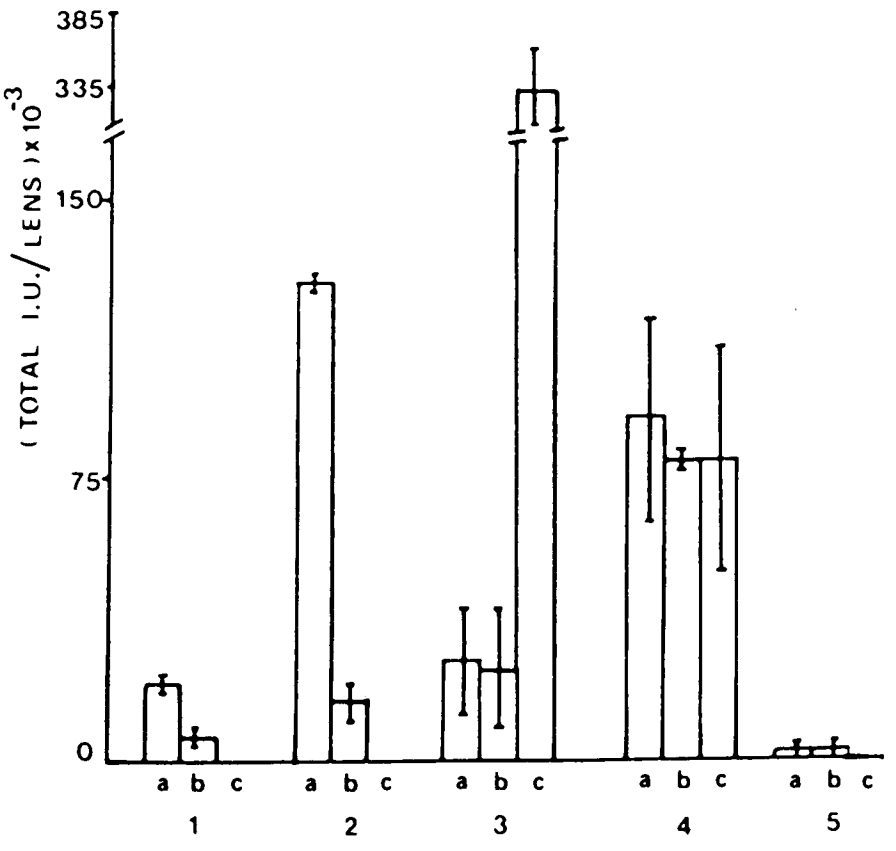



Fig. 3.1.1 Specific activity of the polyol pathway enzymes in (1) rat, (2) rabbit, (3) human, (4) bovine and (5) mouse lens. a = aldehyde reductase activity with DL-glyceraldehyde, b = aldehyde reductase activity with D-glucose, and c = polyol dehydrogenase activity. Aldehyde reductase was assayed in Tris-HCl pH 7.2 buffer and polyol dehydrogenase was assayed in Tris-HCl pH 8.9 buffer (see Section 2.2.3 for details).




ig. 3.1.2 Total activity of the polyol pathway enzymes in (1) rat, (2) rabbit, (3) human, (4) bovine, and (5) mouse lens. a = aldehyde reductase activity with DL-glyceraldehyde as substrate, b = aldehyde reductase activity with D-glucose as substrate, c = polyol dehydrogenase activity.

bovine lens has 72%, both the rat and human lens contain 16% and the mouse lens contains negligible amount of activity (Fig. 3.1.2). With D-glucose as substrate, however, the bovine lens appears to contain the highest activity (100%), and the activities in the rabbit, human, rat are 21%, 24%, 8% respectively. Polyol dehydrogenase activity with sorbitol as substrate is highest in the human lens (100%) and the bovine lens has 26% activity.

## 3.2 Aldehyde Reductase Activity B Associated with β-crystallin Fraction

The lens protein (55 mg) was loaded on to a Sephadex-G-200 (120 cm x 0.9 cm) column and the separation of crystallins is shown in Fig. 3.2.1. The aldehyde reductase activity with both DL-glyceraldehyde and D-glucose as aldehyde substrates was found to be located in the shoulder of $\beta_L$ peak (> 52,000). Only 15% of the activity in the crude extract was recovered in the $\beta_L$ fraction. This suggests that the enzyme protein is associated with β-crystallin which when separated from other crystallins may have formed aggregate with the enzyme that affected its activity.

## 3.3 Purification of Bovine Lens Aldehyde Reductase B

### 3.3.1 Purification using gel-filtration and ion-exchange columns

Both glyceraldehyde and glucose reducing activities were located in the 60-85% ammonium sulphate fraction

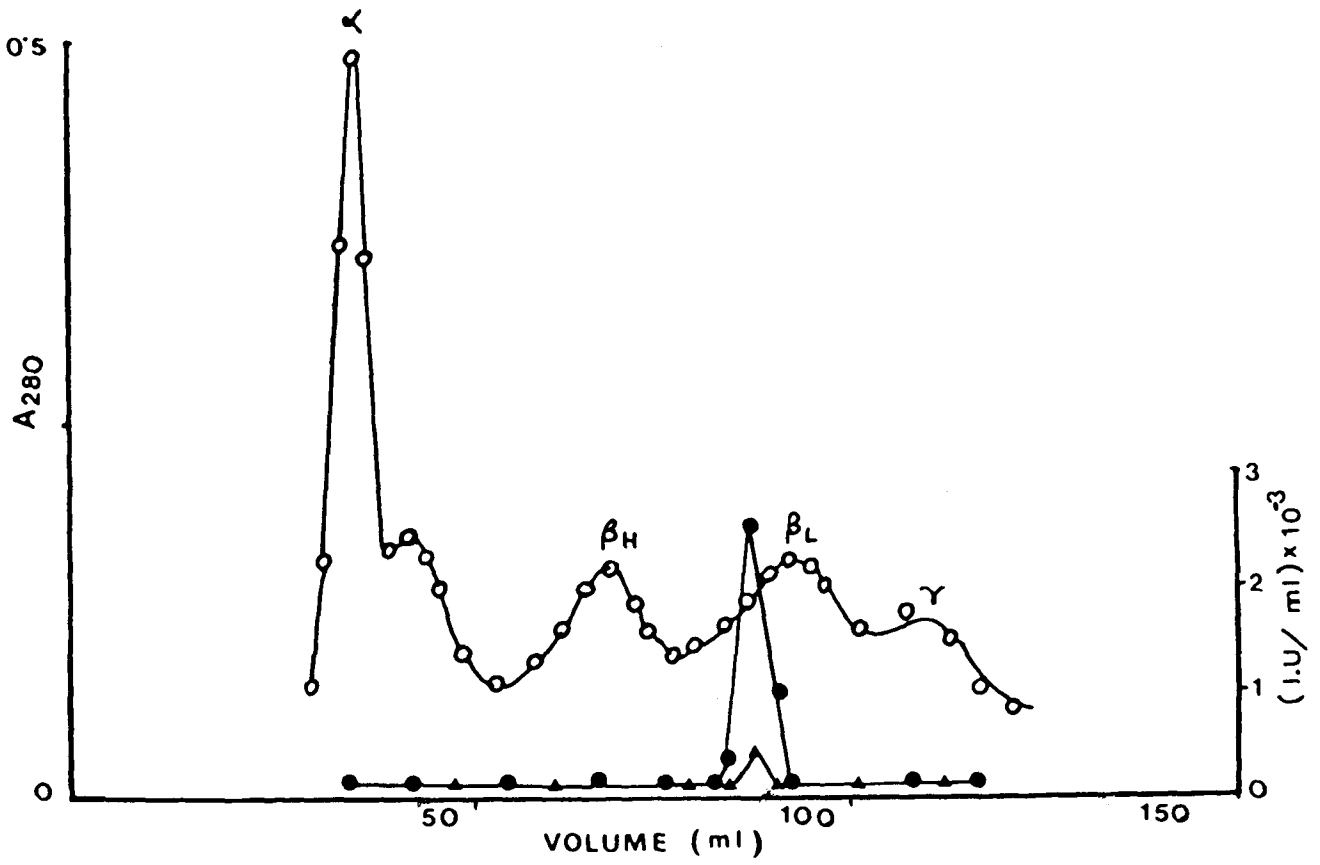



Fig. 3.2.1 Crystallin separation on a Sephadex-G-200 column (120 cm x 0.9 cm). o $A_{280}$ of fractions. Aldehyde reductase activity (●) with DL-glyceraldehyde (▲) with D-glucose. The enzyme was assayed in phosphate buffer pH 6.2 (see Section 2.2.3 for details).

(Fig. 3.3.1.1). The dialysate of 60-85% fraction (see 2.2.6) was loaded on to a Sephadex-G-200 column (90 cm x 1.2 cm). The elution profile is shown in Fig. 3.3.1.2. The enzyme activity with both DL-glyceraldehyde and D-glucose was confined to certain fractions of the huge protein peak eluted from the Sephadex-G-200 column. The enzyme eluted from this column was loaded on to a Carboxymethyl-Sephadex-C-50 column. The enzyme came out in the first small protein peak (Fig. 3.3.1.3). The enzyme eluted from CM-Sephadex-C-50 column was then loaded on to the DEAE-Sephadex-A-50 column (Fig. 3.3.1.4) and eluted with salt gradient. The enzyme came off at approximately 0.35 M NaCl concentration. The final purification was achieved by the use of Sephadex-G-50 column (Fig. 3.3.1.5). Table 3.3.1 gives the extent of purification (3,951-fold) of the enzyme obtained with this 4-column procedure. SDS polyacrylamide gel electrophoresis picture is shown in Fig. 3.3.1.7a. Fig. 3.3.1.6 shows the purification of the enzyme at various stages of purification. The gel scan of the band obtained with the enzyme eluted from Sephadex-G-50 is shown in Fig. 3.3.1.7b.

### 3.3.2 Purification of aldehyde reductase B using affinity columns

Purification using 5'AMP Sepharose, Reactive Red column and NADP-Sepharose column will be called affinity

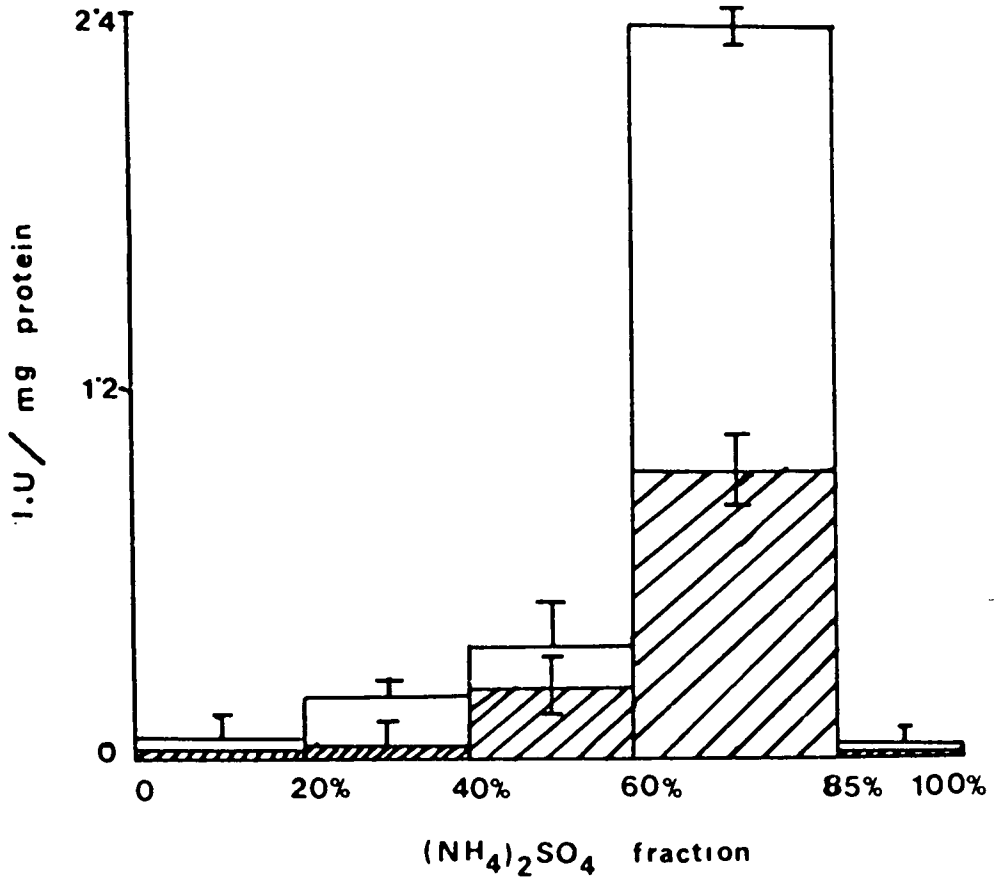



Fig. 3.3.1.1 Aldehyde reductase activity in different ammonium sulphate fractions. ☐ Activity with DL-glyceraldehyde. ▨ Activity with D-glucose. The enzyme was assayed in phosphate buffer pH 6.2 (see Section 2.2.3 for details).

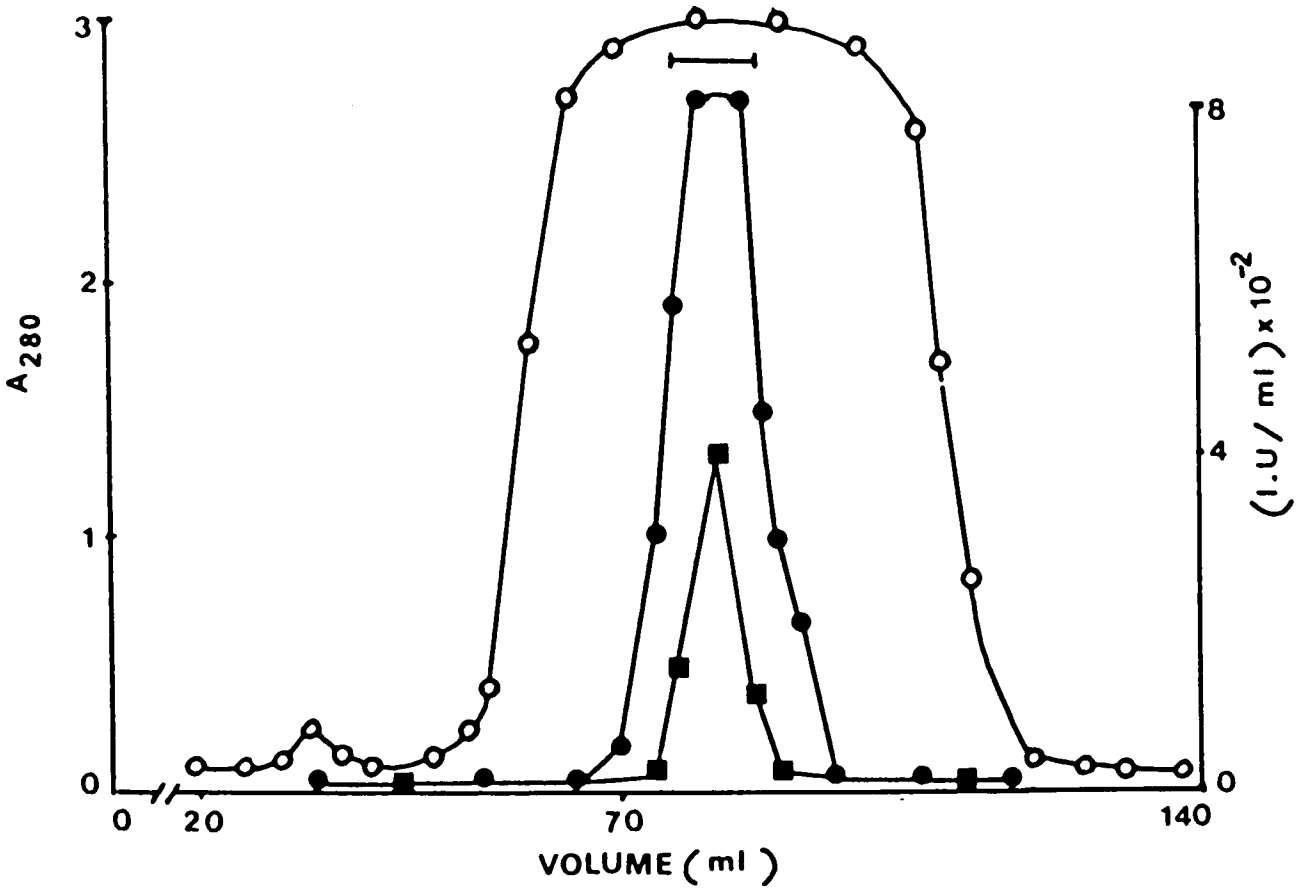



Fig. 3.3.1.2 Elution of enzyme from Sephadex-G-200 (90 cm x 1.2 cm). o $A_{280}$ of fraction, ● enzyme activity with DL-glyceraldehyde, ■ enzyme activity with glucose. Horizontal bar indicates the active fraction pooled.

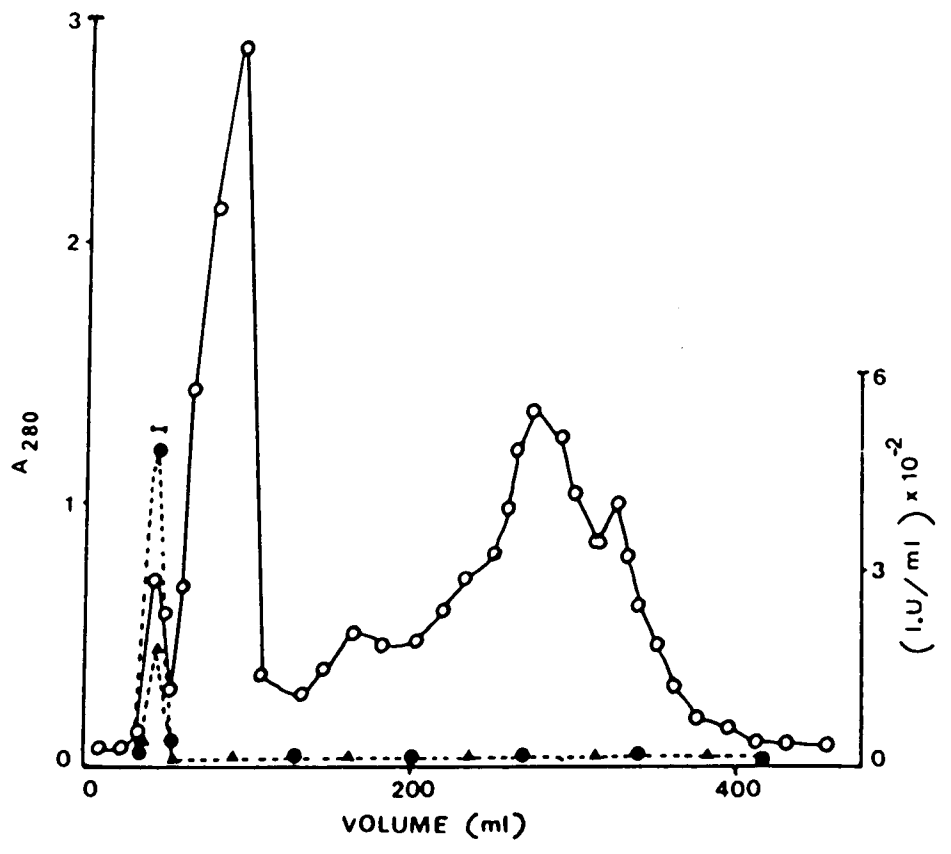



Fig. 3.3.1.3 Elution of enzyme from CM-Sephadex-C-50 (50 cm x 2.5 cm). o $A_{280}$ of fractions, ● enzyme activity with DL-glyceraldehyde, ▲ enzyme activity of D-glucose. Horizontal bar indicates the active fractions pooled.

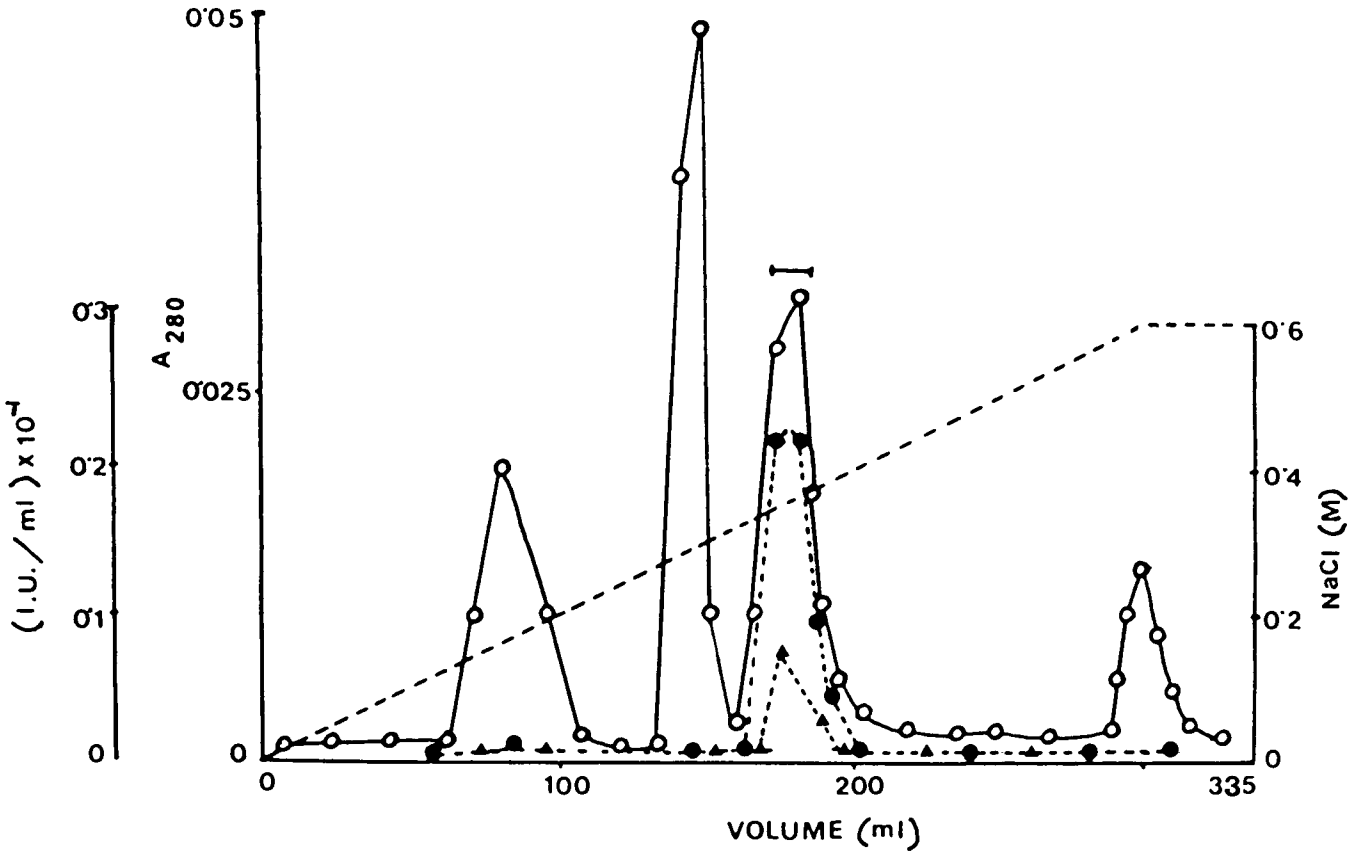



Fig. 3.3.1.4 Elution of enzyme with salt from DEAE Sephadex-A-50 (30 cm x 3 cm). o $A_{280}$ of the fractions, ● enzyme activity with DL-glyceraldehyde, ▲ activity with D-glucose. Horizontal bar indicates the active fractions collected; - - - - salt gradient.

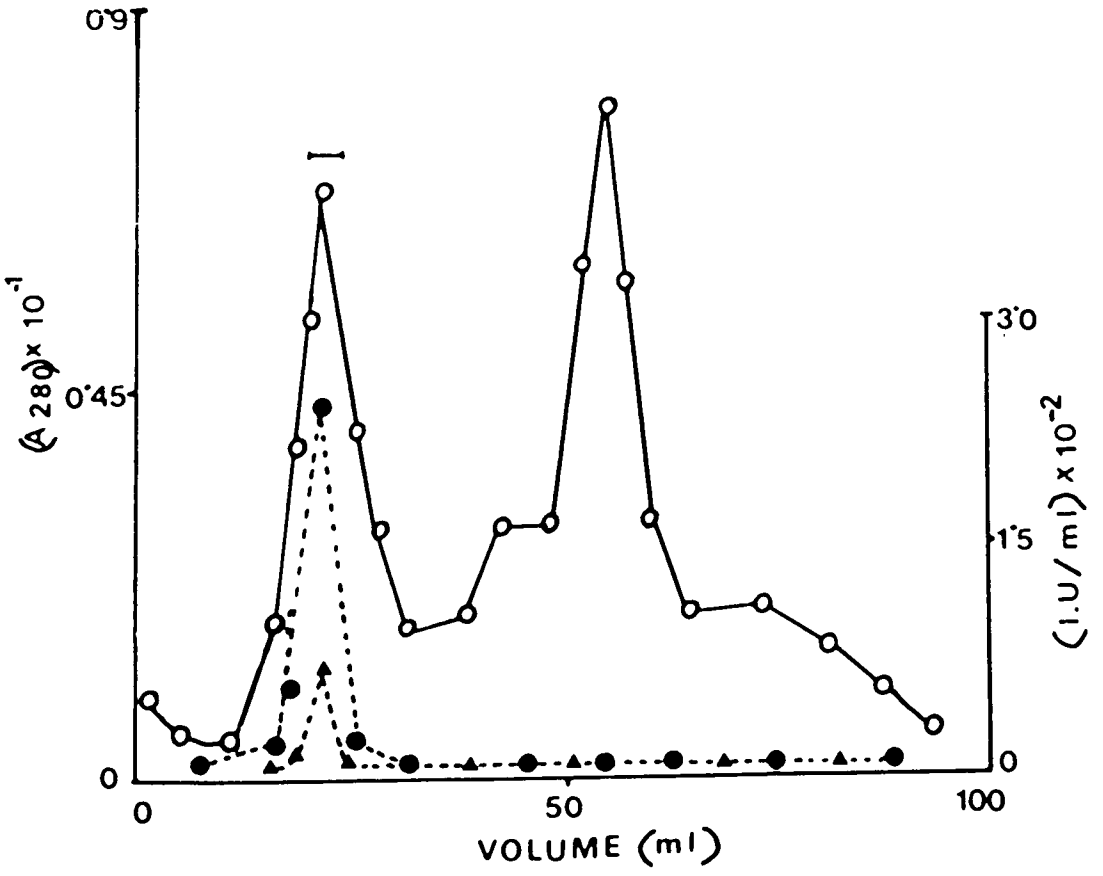



Fig. 3.3.1.5 Elution of enzyme from Sephadex-G-50 (50 cm x 1.5 cm). o $A_{280}$, ● enzyme activity with DL-glyceraldehyde and ▲ enzyme activity with D-glucose. Horizontal bar indicates the active fractions pooled.

Table 3.3.1 Purification of Aldehyde Reductase B by Gel Filtration and Ion-Exchange Columns (from 30 Lenses)

| | Total protein (mg) | Total activity (unit) | Specific activity x $10^{-4}$ (unit/mg protein) | Purification factor | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 42900 | 4.8 (1)<br>3 (2) | 1.1 (1)<br>0.69 (2) | 1 (1)<br>1 (2) | 100 (1)<br>100 (2) |
| 60-85% $[NH_4]_2SO_4$ | 2118 | 1.5 (1)<br>0.46 (2) | 7.1 (1)<br>2.1 (2) | 6.45 (1)<br>3.04 (2) | 31 (1)<br>15 (1) |
| Sephadex-G-200 | 300 | 0.36 (1)<br>0.15 (2) | 12.0 (1)<br>5 (2) | 10.9 (1)<br>7.2 (2) | 7.5 (1)<br>5.0 (2) |
| CM-Sephadex-C-50 | 1.36 | 0.14 (1)<br>0.04 (2) | 1029 (1)<br>294 (2) | 935.4 (1)<br>426 (2) | 2.9 (1)<br>1.3 (2) |
| DEAE Sephadex A-50 | 0.398 | 0.08 (1)<br>0.02 (2) | 2010 (1)<br>502 (2) | 1827.2 (1)<br>727.5 (2) | 1.6 (1)<br>0.6 (2) |
| Sephadex-G-50 | 0.138 | 0.06 (1)<br>0.01 (2) | 4347 (1)<br>724 (2) | 3951.8 (1)<br>1049.2 (2) | 1.2 (1)<br>0.3 (2) |

Values (1) represent the purification obtained with DL-glyceraldehyde and values (2) represent the purification with D-glucose as substrate. One unit is defined as the amount of enzyme which catalyzes the oxidation of 1 µmole of NADPH/min at 37°C.

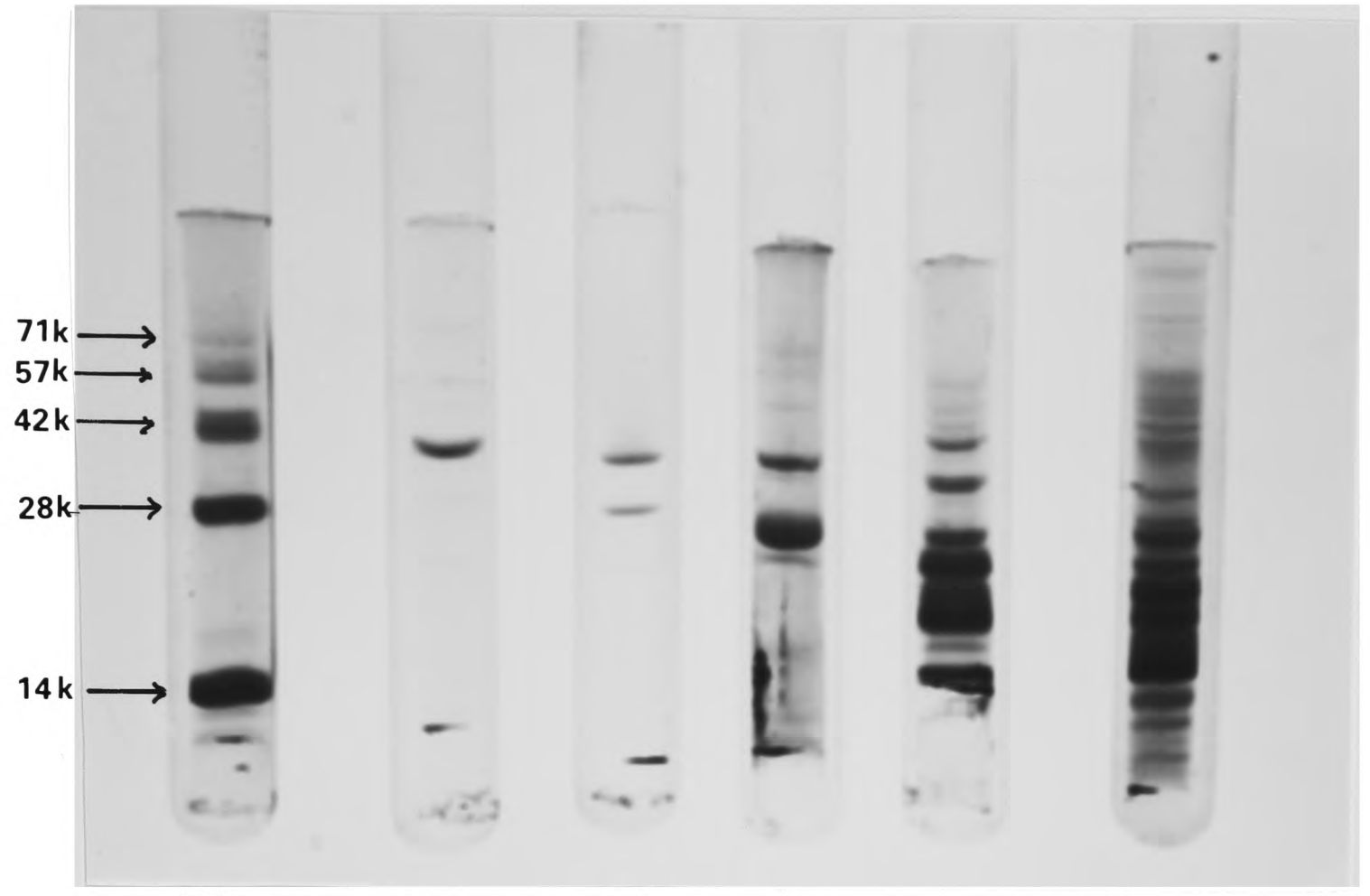



Fig. 3.3.1.6 SDS gel electrophoresis of the lens aldehyde reductase at various stages of purification: (a) crude homogenate, (b) 60-85% $(NH_4)_2SO_4$ fraction, enzyme fraction eluted from (c) CM-Sephadex, (d) DEAE Sephadex, (e) Sephadex-G-50 (50 µg), (f) protein markers.

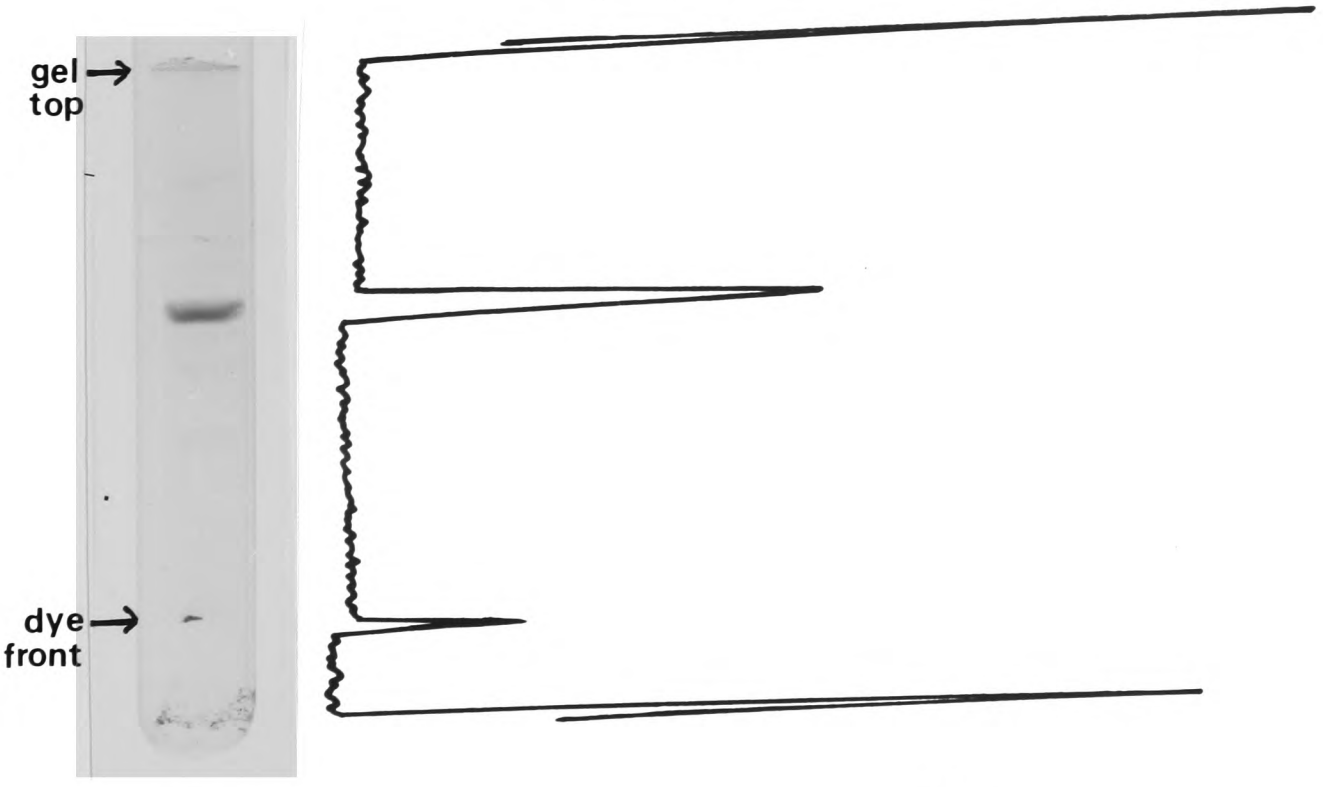



Fig. 3.3.1.7 (a) SDS gel electrophoresis of the lens aldehyde reductase (50 µg) purified by gel-filtration and ion-exchange column chromotography.

(b) Gel scan of the band.

purification because aldehyde reductase could be eluted from these columns by use of NADPH. As both DL-glyceraldehyde and D-glucose reducing activities were obtained in the same fraction in the purification described before (3.3.1) only DL-glyceraldehyde was used as an aldehyde substrate to assay the fractions eluted from these columns but the fractions were occasionally checked for D-glucose reducing activity.

Purification using 5'AMP Sepharose

Fig. 3.3.2.1 shows the elution profile for 5'AMP Sepharose preceded by DEAE Sephadex-A-50. The procedure gave purification of 750-fold with a yield of 2.9%. The SDS polyacrylamide gel electrophoresis picture of the enzyme eluted from 5'AMP Sepharose column is shown in Fig. 3.3.2.2.

Purification using Reactive Red Agarose column

Salt elution from Reactive Red agarose column is shown in Fig. 3.3.2.3. A purification of 667-fold with a yield of 0.7% was achieved by this method. Nearly 98% of the enzyme activity came out in the buffer wash. The enzyme which was loaded on Reactive Red column was partially purified by salt elution from a DEAE Sephadex column and probably the enzyme preparation still had salt with it in spite of extensive dialysis.

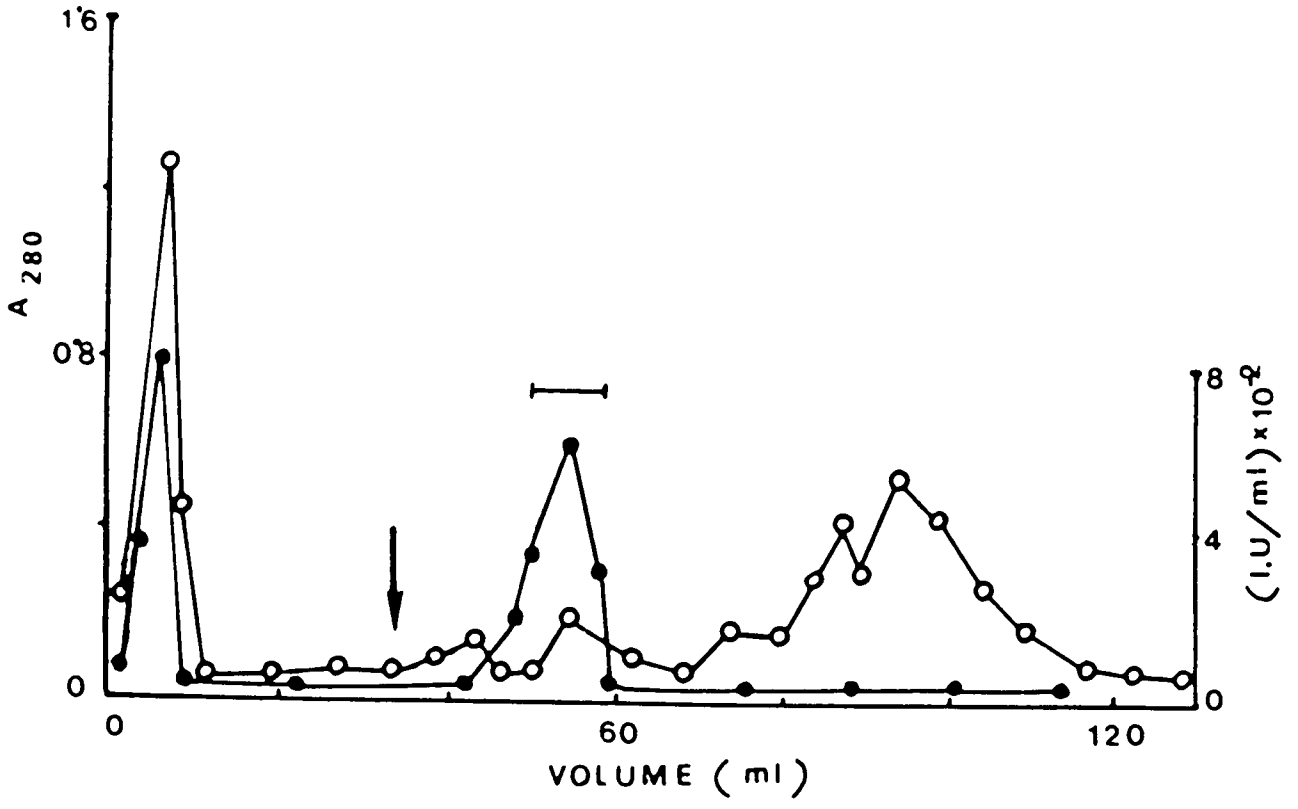



Fig. 3.3.2.1 Elution profile for 5' AMP Sepharose. o $A_{280}$ of the fractions, ● enzyme activity. Arrow mark indicates the beginning of elution with NADPH (200 μM). The horizontal bar indicates the active fractions pooled.

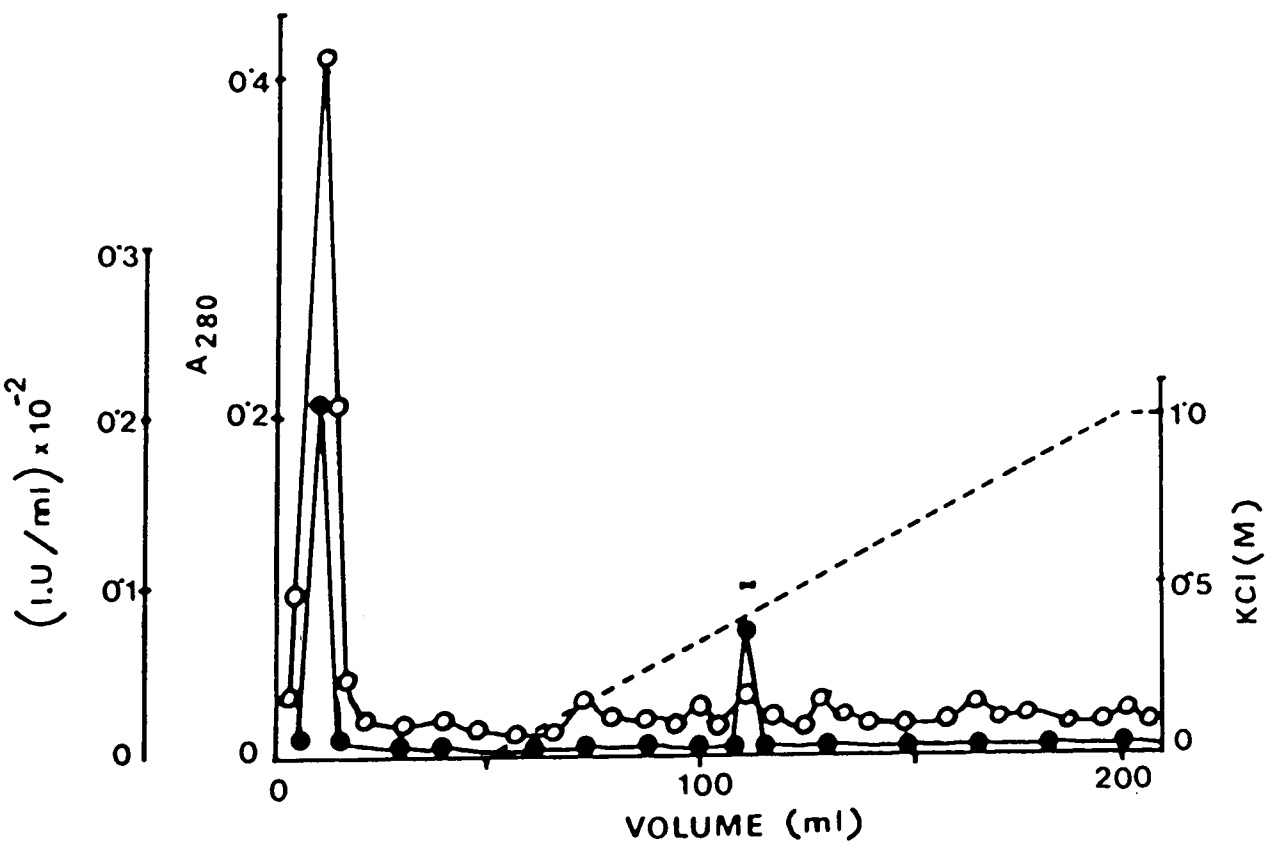



Fig. 3.3.2.3 Salt elution from Reactive Red column. o $A_{280}$ of the fractions, ● enzyme activity. The horizontal bar indicates the pooled fractions containing enzyme activity, - - - - salt gradient.

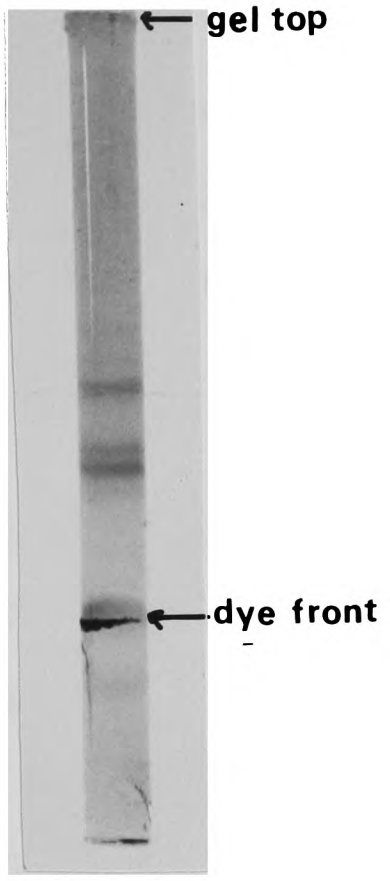



Fig. 3.3.2.2 SDS gel electrophoresis of the aldehyde reductase eluted from 5'AMP Sepharose (see Section 2.2.6.2b).

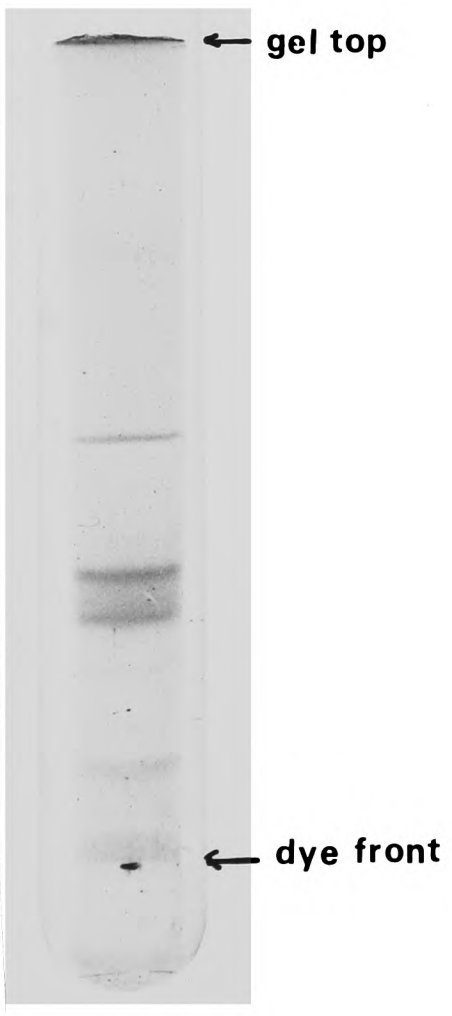



Fig. 3.3.2.4 SDS gel electrophoresis of the aldehyde reductase eluted with salt from Reactive Red column (see Section 2.2.6.2.2a).

This can explain 98% activity in buffer wash. The SDS gel picture (Fig. 3.3.2.4) shows the protein bands obtained with the enzyme eluted from Reactive Red column.

A purification of 2,461-fold (Table 3.3.2.1) was achieved using NADPH as eluent (Fig. 3.3.2.5) with Reactive Red column (prepared as described in Chapter 2, Section 2.2.6.2.2b) preceded by CM-Sephadex-C-50 and Sephadex-G-200 column. A yield of 2.75% was obtained by this 3-column procedure. The SDS poly-acrylamide gel picture (Fig. 3.3.2.6) shows an apparently homogenous band obtained with the enzyme eluted from Reactive Red column.

Purification using NADP Sepharose

Using NADPH as eluent with NADP Sepharose column (Fig. 3.3.2.7) a purification of 1,333-fold (Table 3.3.2.2) was obtained in this 1-column procedure. SDS polyacrylamide gel picture (Fig. 3.3.2.8a) shows an apparently homogenous band obtained with the enzyme eluted from NADP Sepharose column. A gel scan picture (Fig. 3.3.2.8b) of the band accompanies the SDS gel picture.

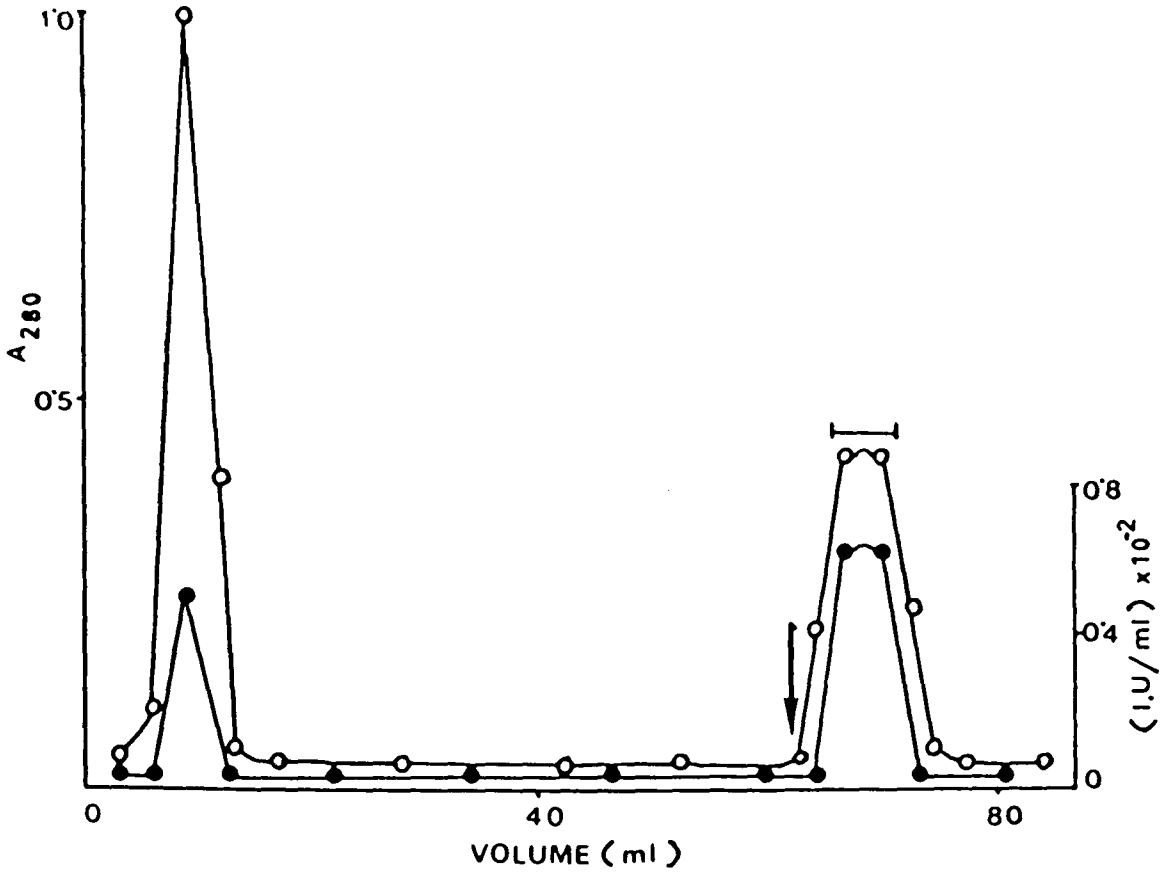



Fig. 3.3.2.5 Elution with NADPH from Reactive Red column. o $A_{280}$ of the fractions, ● enzyme activity with DL-glyceraldehyde as substrate. The arrow mark indicates the beginning of elution with NADPH (5 mM). The horizontal bar indicates the active fractions pooled.

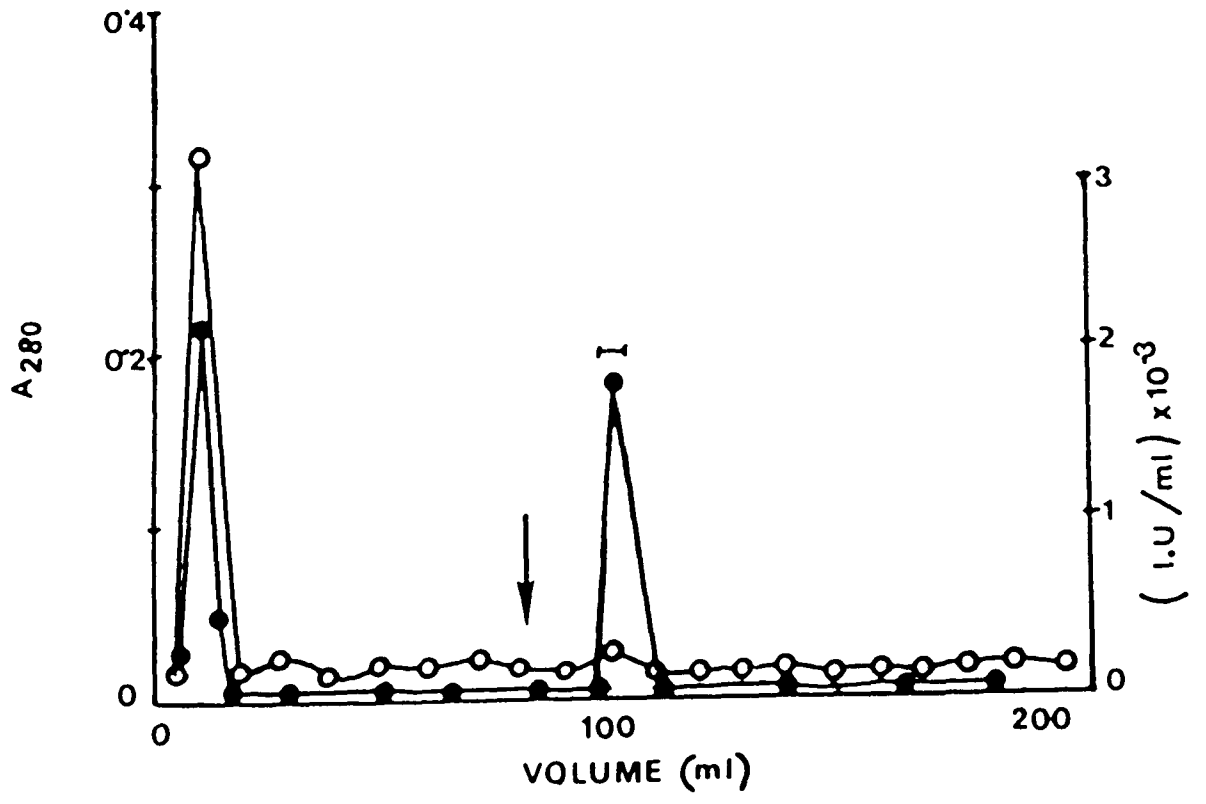



Fig. 3.3.2.7 Elution profile for NADP-Sepharose column. o $A_{280}$ of the fractions, ● enzyme activity with DL-glyceraldehyde as substrate. The arrow mark indicates the start of elution with NADPH (400 μM). The horizontal bar indicates the active fractions pooled.

Table 3.3.2.1 Purification of Aldehyde Reductase B Using Reactive Red Agarose Column and NADPH as Eluent (from 30 Lenses)

| | Total protein (mg) | Total activity (unit) | Specific activity x $10^{-4}$ (unit/mg protein) | Purification factor | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 42900 | 4.8 | 1.1 | 1 | 100 |
| 60-85% $[NH_4]_2SO_4$ | 2118 | 1.548 | 7.3 | 6.6 | 32 |
| Sephadex-G-200 | 300 | 0.36 | 12.0 | 11.0 | 7 |
| CM-Sephadex-G-50 | 1.35 | 0.19 | 1407.0 | 1279.1 | 3.9 |
| Reactive Red Agarose | 0.48 | 0.13 | 2708.0 | 2461.8 | 2.7 |

Unit of enzyme has been defined in Table 3.3.1.

Table 3.3.2.2 Purification of Aldehyde Reductase B Using NADP-Sepharose Column (from 5 Lenses)

| | Total protein (mg) | Total activity (unit) | Specific activity x $10^{-4}$ (unit/mg protein) | Purification factor | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 7140 | 1.1 | 1.5 | 1 | 100 |
| 60-85% $[NH_4]_2SO_4$ | 300 | 0.5 | 16.6 | 11 | 45 |
| NADP Sepharose | 0.2 | 0.04 | 2000 | 1333 | 50* |

Unit of enzyme is the same as described in Table 3.3.1.

*50 mg of 60-85% $[NH_4]_2SO_4$ fraction was loaded on to the NADP Sepharose column. The final yield was determined on the basis of 60-86% $[NH_4]_2SO_4$ step.

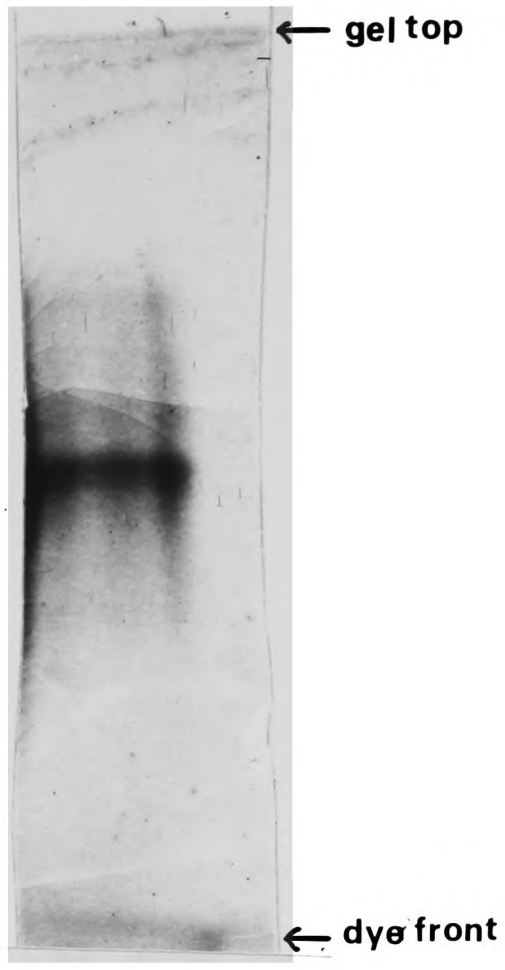
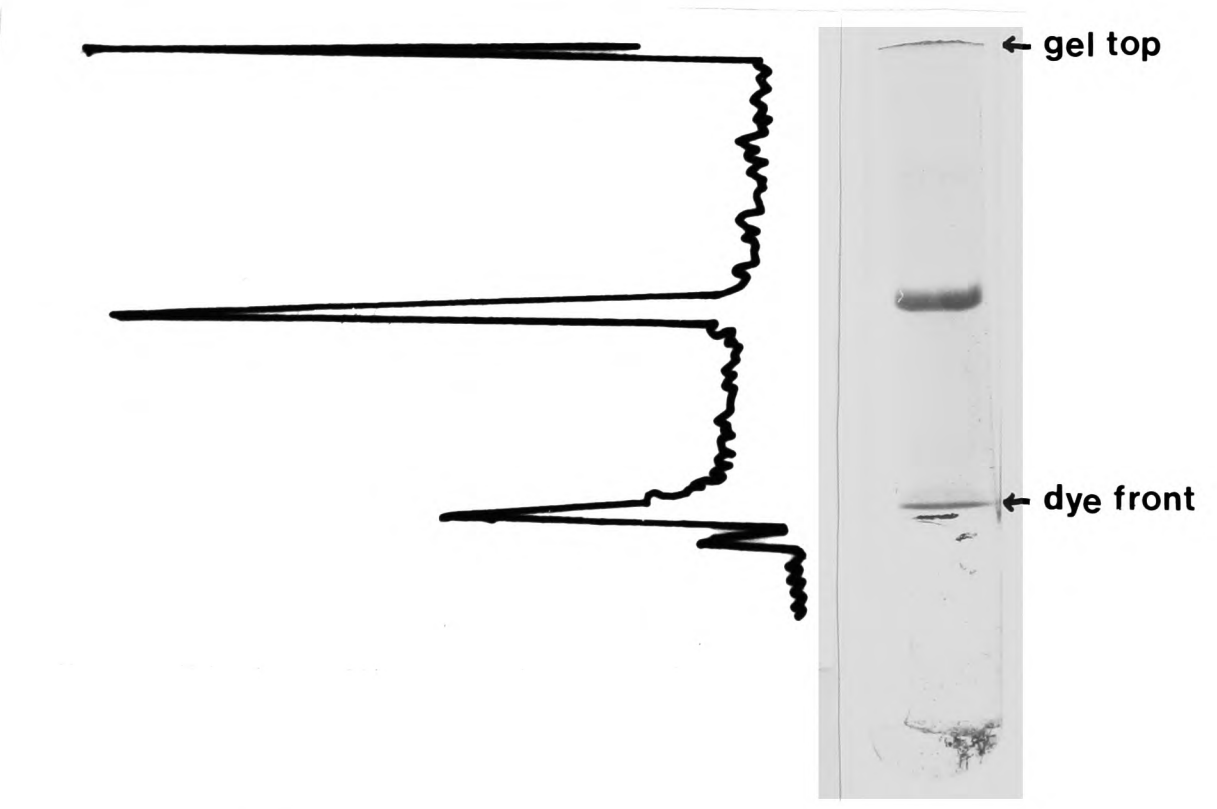



Fig. 3.3.2.6 SDS gel electrophoresis of the aldehyde reductase eluted with NADPH from Reactive Red column (see 2.2.6.2.2b).




(b) (a)

Fig. 3.3.2.8 (a) SDS gel electrophoresis of the aldehyde reductase eluted from NADP Sepharose.
(b) Gel scan of the band.

## 3.4 Molecular Weight Determination

The molecular weight of aldehyde reductase B purified using gel-filtration and ion-exchange columns was determined by both SDS polyacrylamide gel electrophoresis (Fig. 3.4.1) and gel-filtration using a Sephadex-G-200 column (120 cm x 0.9 cm) (Fig. 3.4.2). The molecular weight by SDS polyacrylamide gel electrophoresis was found to be 40 000 ± 2 819 (n = 3) and by gel-filtration 32 000 (n = 2). The same type of discrepancy was reported by Scheaff and Doughty (1976). The bovine lens aldehyde reductase was found to have a molecular weight 34 000 by gel filtration and 37 000 by SDS polyacrylamide gel electrophoresis and by ultra-centrifugation study.

## 3.5 Activity Staining on Polyacrylamide Gel

No activity stained band on the polyacrylamide gel was obtained with the enzyme purified using gel-filtration and ion-exchange columns following the method described by Scheaff and Doughty (1976) using both glycerol (concentration tried up to 1 M) and sorbitol (1 M, 2 M) as alcohol substrates or by using various combinations of gel and running buffers. No significant rate was found with the enzyme using the reverse reaction

$$\text{glycerol (/sorbitol)} + NADP^{+} \longrightarrow \text{glyceraldehyde (/glucose)} + NADPH$$

at pH 7.0 or at pH 9.0. This could be the reason why no

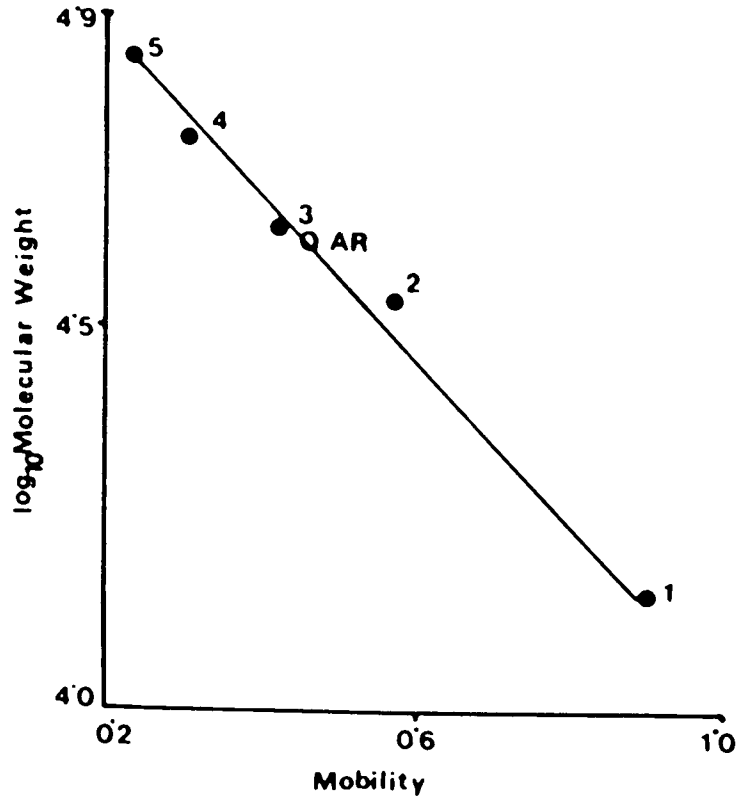



Fig. 3.4.1 Molecular weight determination by SDS polyacrylamide gel electrophoresis. 1, 2, 3, 4 and 5 are the multiples of monomeric protein markers (BDH). 1 = 14 300, 2 = 28 600, 3 = 42 900, 4 = 57 200, 5 = 71 500. o represents the position of pure aldehyde reductase.

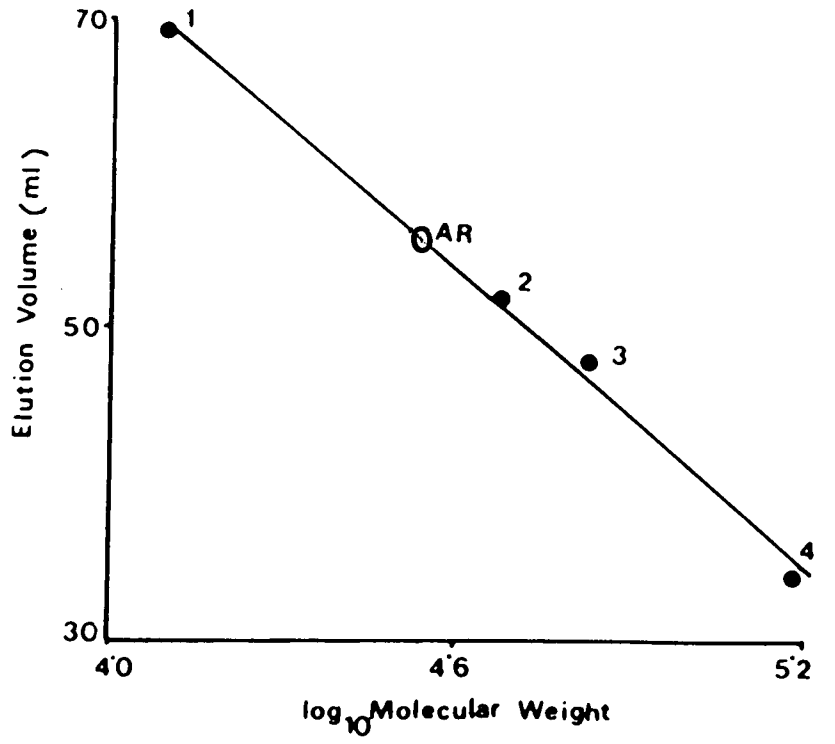



Fig. 3.4.2 Molecular weight determination by gel filtration. o represents standard protein markers, 1 = cytochrome C (12 500), 2 = hen albumin (45 000), 3 = bovine serum albumin (66 000), 4 = yeast alcohol dehydrogenase (140 000). o represents the position of pure aldehyde reductase.

activity stained band was obtained as the activity staining reaction requires the formation of NADPH to reduce tetrazolium salt in the reaction

$$\text{oxidoreductase} \longrightarrow NAD^{+}/NADP^{+} \longrightarrow \text{tetrazolium salt}$$

## 3.6 Amino-acid Composition of Bovine Lens Aldehyde Reductase B

Amino-acid composition of aldehyde reductase (10 µg) purified using gel-filtration and ion-exchange columns was determined by the method described in Chapter 2 (2.2.8) and is given in Table 3.6.1. From the result obtained it seems that high content of aspartic and glutamic acid and relatively lower amount of lysine, arginine and histidine are the characteristic features of this enzyme. The result, however, shows a higher value of glycine and serine which may indicate fingerprint contamination though no ornithin was obtained. Other values are in close agreement with those for other aldehyde reductases (Crabbe et al., 1982). Cysteine content determined by performic acid oxidation method (Schram et al., 1954; Moore, 1963) was 8/1,000 residues.

## 3.7 Effect of pH and Ionic Strength on the Polyol Pathway Enzymes

### 3.7.1 Effect of pH

Buffers of constant ionic strength (I = 0.1) and of different pH were used to study the effect of pH on pure aldehyde reductase (Fig. 3.7.1.1). Potassium phosphate

Table 3.6.1 Amino Acid Composition of Bovine Lens Aldehyde Reductase Purified by Gel Filtration and Ion-Exchange Chromatography

| | Residue/1000 |
|---|---|
| Aspartic acid | 101 |
| Threonine | 34 |
| Serine | 103 |
| Glutamic acid | 124 |
| Proline | 60 |
| Glycine | 111 |
| Alanine | 84 |
| Valine | 52 |
| Methionine | 8 |
| Isoleucine | 37 |
| Leucine | 71 |
| Tyrosine | 31 |
| Phenylalanine | 30 |
| Histidine | 23 |
| Arginine | 41 |
| Lysine | 83 |

Cysteine content (corrected) was determined by performic acid oxidation and the value (residue/1000) was found to be 8.

buffer was used in the range 5.9-7.8, and pyrophosphate buffer in the pH range 7.8-9.3. The studies were done with both DL-glyceraldehyde and D-glucose as aldehyde substrates. With glyceraldehyde the pH optimum was relatively broad, pH 6.3-6.7. There was a sharp fall in activity above pH 6.8. With D-glucose the pH optimum was relatively sharper and was at pH 6.4. The fall in activity above pH 6.8 was not so abrupt as with DL-glyceraldehyde, although the overall pH profile looked similar for both glyceraldehyde and glucose.

All the buffers, citrate, acetate, succinate and cacodylate which were tried below pH 5.9 were found to be strong inhibitors of the enzyme with both glyceraldehyde and glucose as aldehyde substrates.

A preliminary investigative study was done on the effect of pH variation on crude polyol dehydrogenase (Fig. 3.7.1.2). Potassium phosphate buffer (I = 0.4M) was used in the pH range 6.4-6.8, Tris-HCl (I = 0.1M) in the range 7.0-8.9 and glycine-NaOH (I = 0.1M) in the range 9.2-10.4. In the Tris-HCl buffer the enzyme showed optimum activity at pH 8.9. In glycine-NaOH buffer a little hump was observed at around pH 9.6. As no single buffer was used in the pH range 8.6-9.4 the effect of pH on the initial velocity of polyol dehydrogenase remains somewhat inconclusive but it is clear

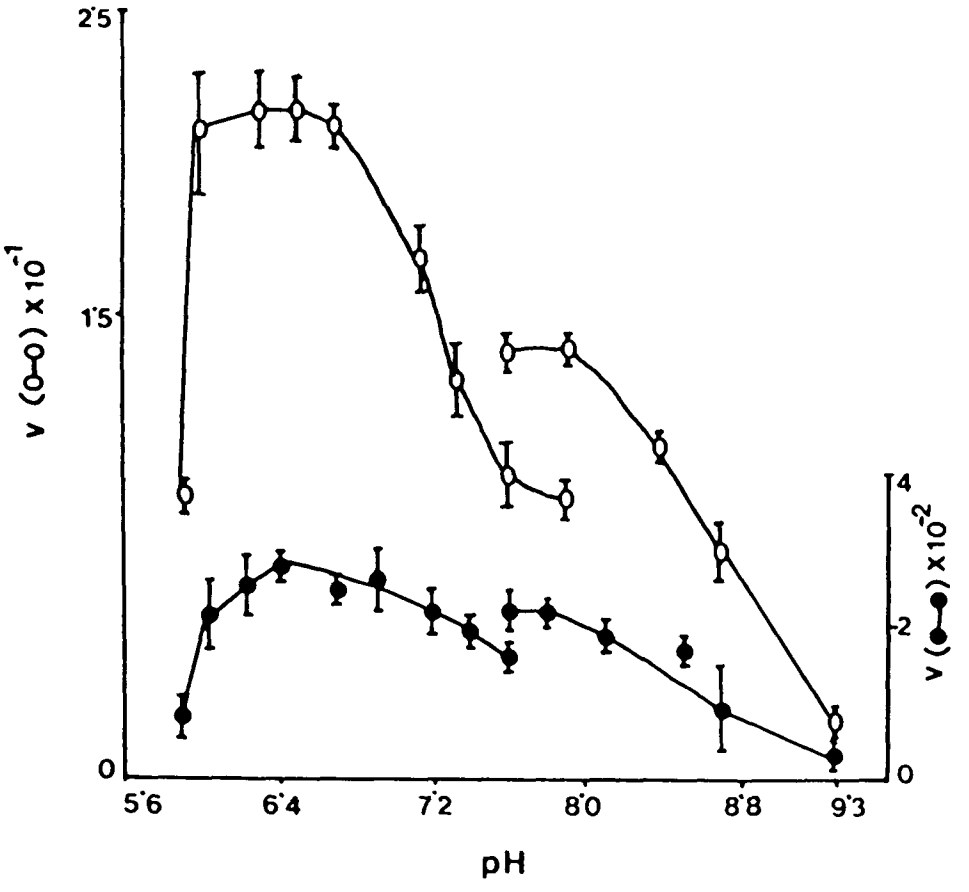



Fig. 3.7.1.1 Effect of pH on aldehyde reductase activity with (o) DL-glyceraldehyde and (●) D-glucose as aldehyde substrates (see Section 3.7.1 for details).

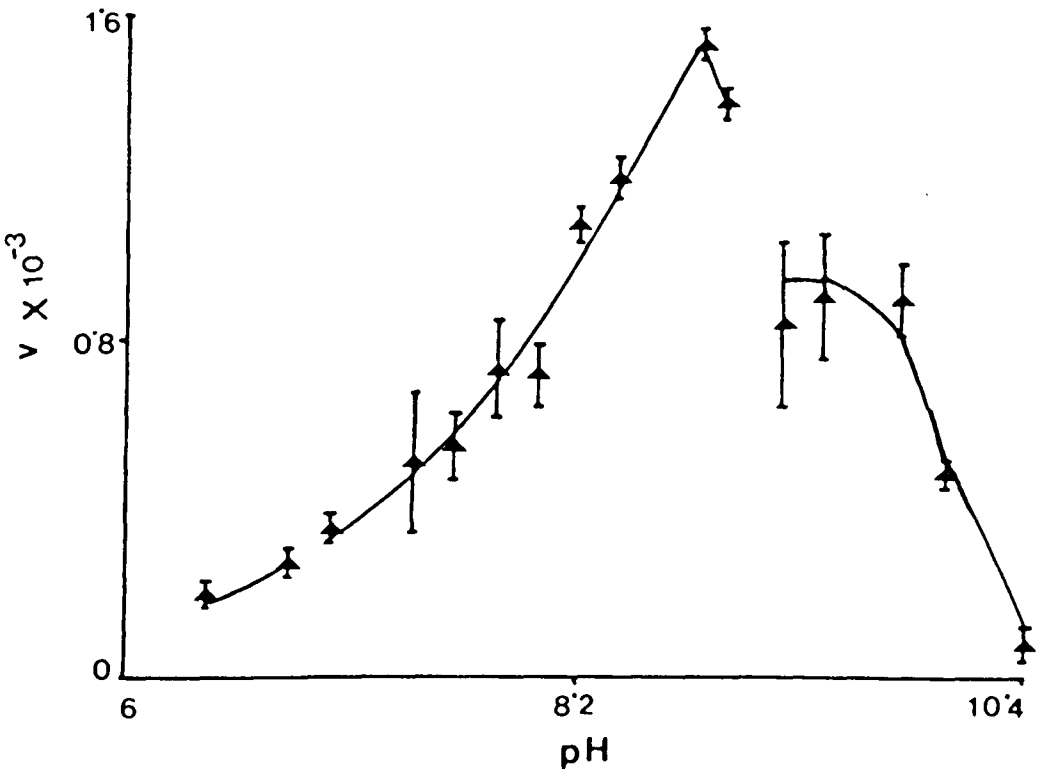



Fig. 3.7.1.2 Effect of pH on polyol dehydrogenase activity with sorbitol as substrate (see Section 3.7.1 for details).

that the pH optimum of of polyol dehydrogenase is higher than that of aldehyde reductase.

### 3.7.2 Effect of ionic strength on aldehyde reductase B and polyol dehydrogenase activity

The purpose of this study was to investigate how these two enzymes in crude state respond to the change in ionic strength as well as to the presence of a particular ion in the assay mixture. As the ionic concentration used in this study was mostly above physiological ionic strength (> 0.16) Brönsted plots were drawn. Fig. 3.7.2.1 shows the effect of $[NH_4]_2SO_4$, $NH_4Cl$, $Li_2SO_4$ and KCl on the aldehyde reductase activity. With both $[NH_4]_2SO_4$ and $Li_2SO_4$ the enzyme activity increased 1.8-fold at around I = 0.6 M, the activating effect declined above I = 0.6 and then the activity slowly resumed its original value at I = 0.85 M. With $NH_4Cl$ there was little (with glucose as substrate) or no activating effect (with glyceraldehyde as substrate) on the enzyme. At above I = 0.2 M, $NH_4Cl$ exerted inhibitory effect on the enzyme. With KCl, there was a slight activating effect on the glyceraldehyde reducing activity up to about I = 0.4 M, the activity then gradually reverted to its original value with further increase in I. With both glyceraldehyde and glucose, the effect of ions was very similar. With polyol dehydrogenase, however, the picture (Fig. 3.7.2.2) was exactly opposite.

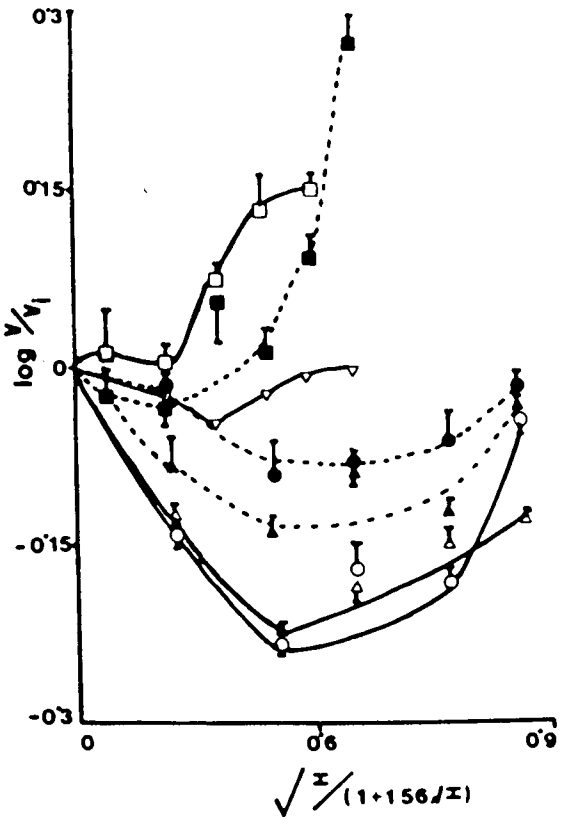



Fig. 3.7.2.1 Brönsted plot for aldehyde reductase with (o) $[NH_4]_2SO_4$, (Δ) $Li_2SO_4$, (□) $NH_4Cl$, (▽) KCl. Open symbols represent the activity with DL-glyceraldehyde and the closed symbols represent activity with D-glucose. V and $V_i$ are the initial velocities without and with ions, respectively. The enzyme was assayed in Tris-HCl pH 7.2 buffer (see Section 3.7.2 for details).

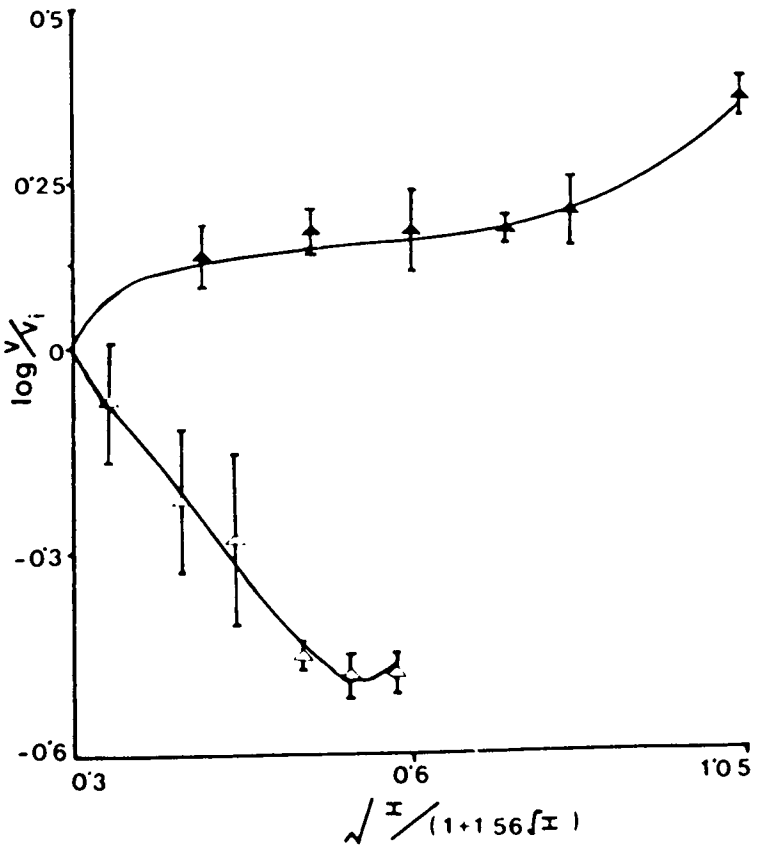



Fig. 3.7.2.2 Brönsted plot for polyol dehydrogenase with (Δ) KCl and (▲) $[NH_4]_2SO_4$ (see Section 2.2.3 for details of assay).

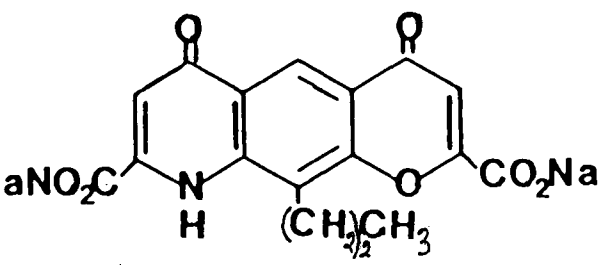
$[NH_4]_2SO_4$ exerted slightly inhibitory effect up to about I = 0.8 M and the effect became more prominent above I = 0.8 M. KCl clearly showed activating effect on polyol dehydrogenase. There was a sharp increase up to I = 0.5 M and then it levelled off.

## 3.8 Effect of Temperature on Aldehyde Reductase B Activity

Arrhenius plots for crude aldehyde reductase with both glyceraldehyde and glucose as aldehyde substrates are very similar (Fig. 3.8.1). Both the plots show a sharp bend at a point corresponding to 42°C. The activation energy calculated from the linear portions of the plots for both glyceraldehyde and glucose are 58.84 and 64.74

## 3.9 Effect of Inhibitors on Aldehyde Reductase B

3.9.1 Sulindac (see Table 1.2 for structure) is an anti-inflammatory drug which has been reported to have inhibited calf and human lens aldehyde reductase (Sharma and Cotlier, 1982).



FPL 58665

The compound (FPL 58665) which is analogous to cromoglycate is a product of Fisons (U.K.). This compound

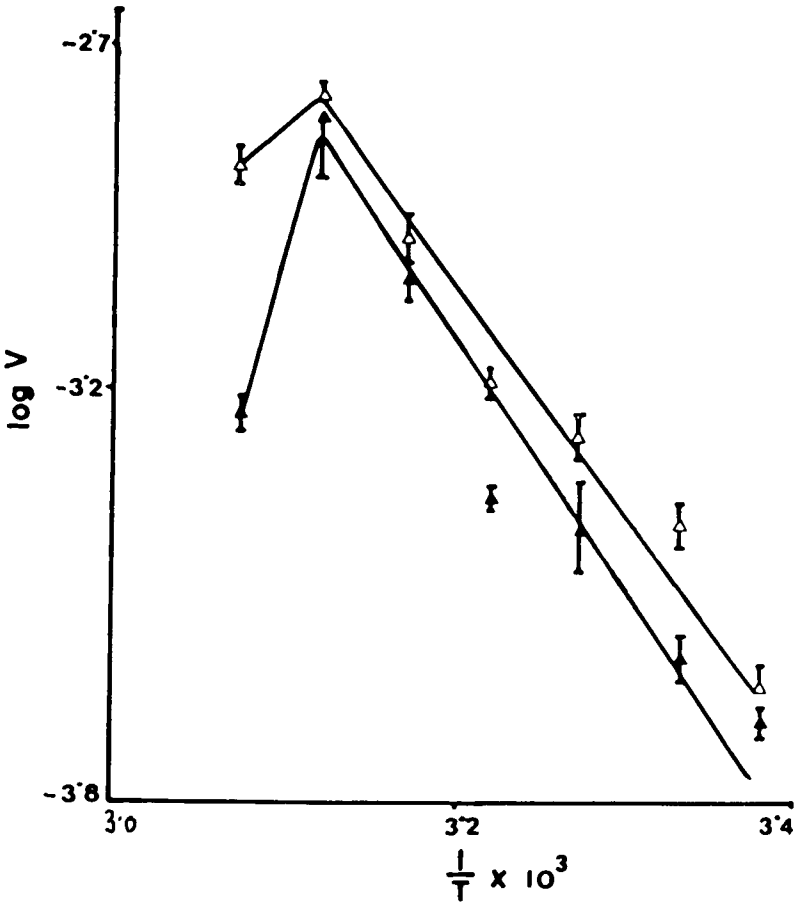
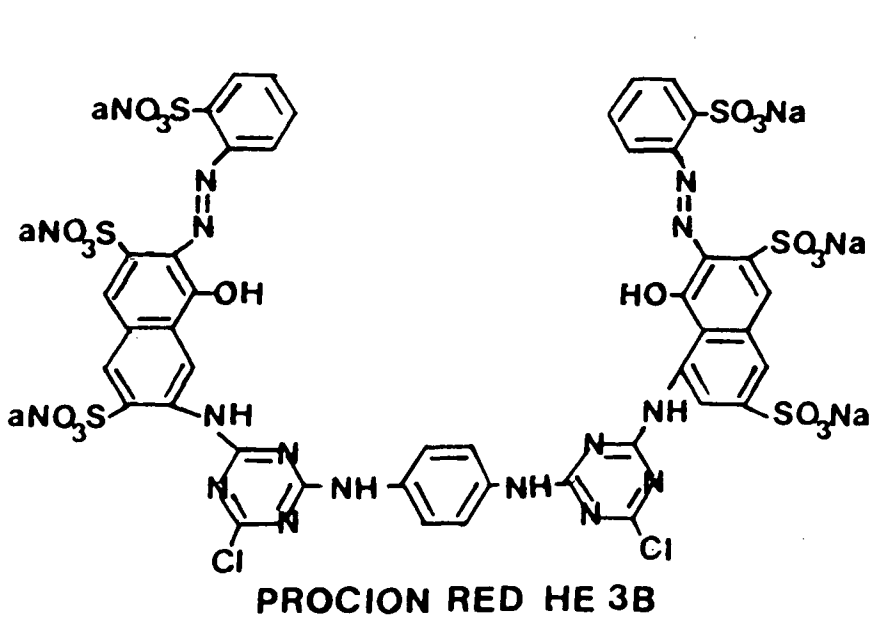


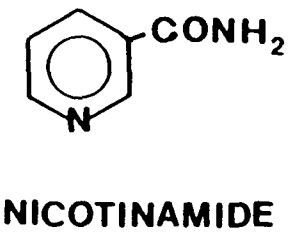

Fig. 3.8.1 Effect of temperature on aldehyde reductase B with (Δ) DL-glyceraldehyde and (▲) D-glucose. V represents the initial velocity and T is the temperature on Kelvin scale. The enzyme was assayed in phosphate buffer pH 6.2 (see Section 2.2.3 for details).

(99% pure according to Fisons specifications) along with sulindac were used as inhibitors of crude aldehyde reductase. The effect of these inhibitors were studied with all the three substrates, DL-glyceraldehyde, D-glucose and NADPH in phosphate buffer pH 6.2. The double reciprocal plots of initial velocities against all the three substrates at different concentrations of sulindac (Figs. 3.9.1.1a,b,c) suggest that the inhibition is non-competitive in nature. The nature of double reciprocal plots with FPL 58665 (Figs. 3.9.1.2a,b,c) too indicates that the compound acts as a non-competitive inhibitor of aldehyde reductase. The insets are the replots of slopes and intercepts plotted against inhibitor concentrations, the Ki and Kis values for all these inhibitors calculated from these replots are listed in Table 3.9.1. The result is discussed in detail in Chapter 4.

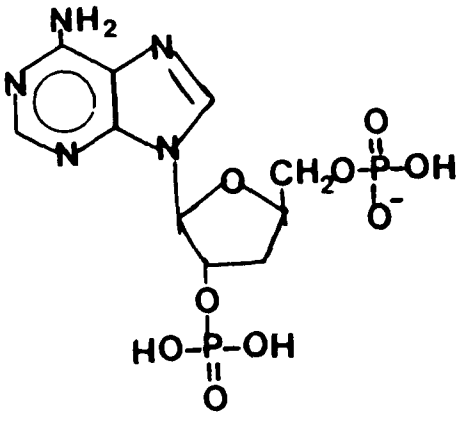
3.9.2 Procion Red, Nictotinamide and 2'5'ADP as inhibitors of aldehyde reductase B




PROCION RED HE 3B




NICOTINAMIDE




2',5'ADP

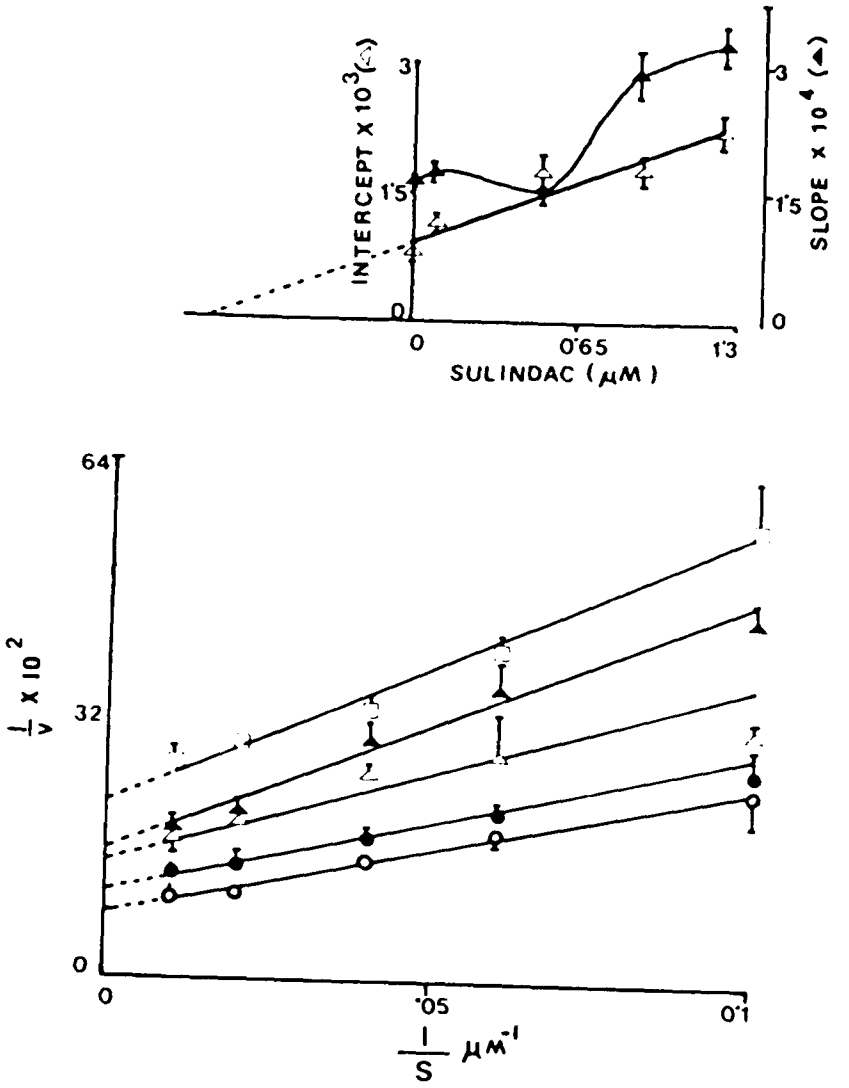
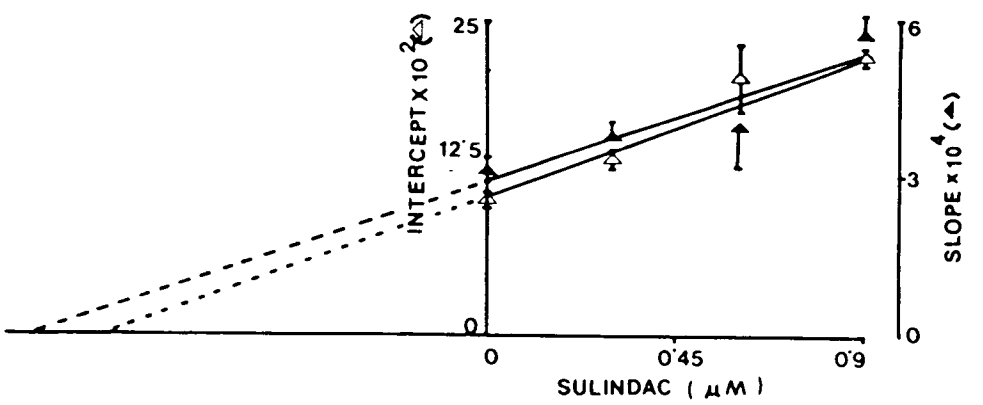



Fig. 3.9.1.1a Double reciprocal plot of initial velocity (v) against DL-glyceraldehyde as variable substrate with sulindac as inhibitor. The concentration of NADPH was held constant at 50 μM. o 0, ● 0.1 μM, Δ 0.5 μM, ▲ 0.9 μM, □ 1.2 μM sulindac, respectively. The inset shows the replot of primary slope and intercept against sulindac concentration.



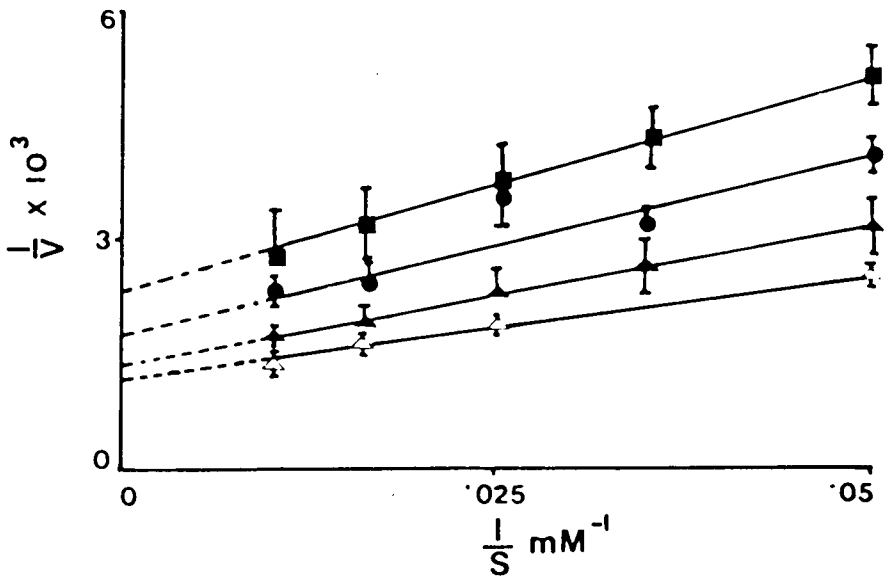
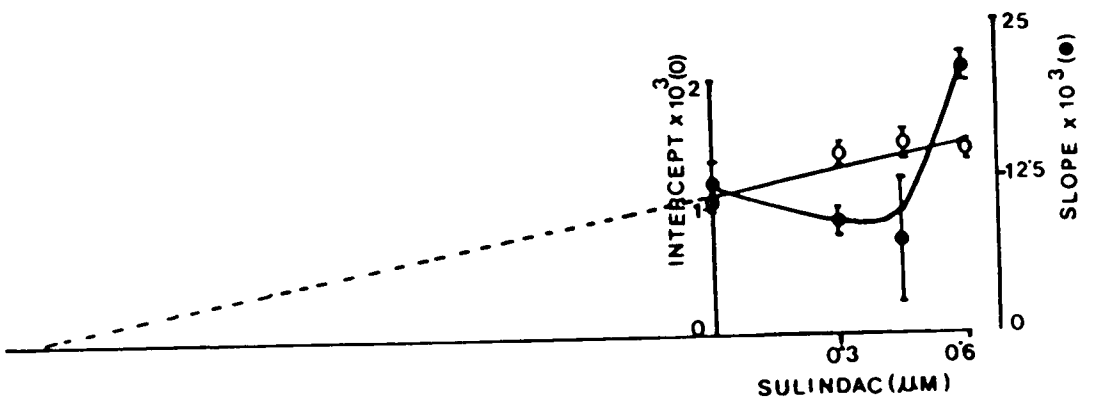




Fig. 3.9.1.1b Double reciprocal plot of initial velocity (v) against D-glucose as variable substrate with sulindac as inhibitor. The concentration of NADPH was held constant at 50 μM. △ 0, ▲ 0.3 μM, ● 0.5 μM, ■ 0.9 μM sulindac, respectively. The inset shows the replot of primary slope and intercept against sulindac concentration.



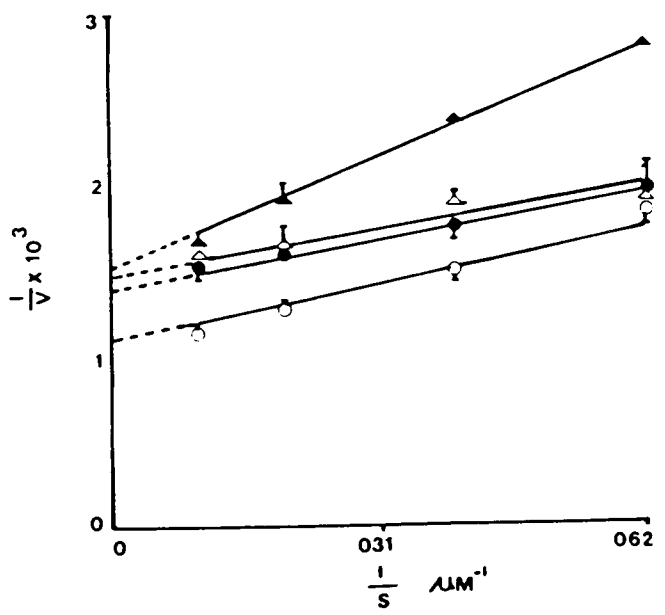
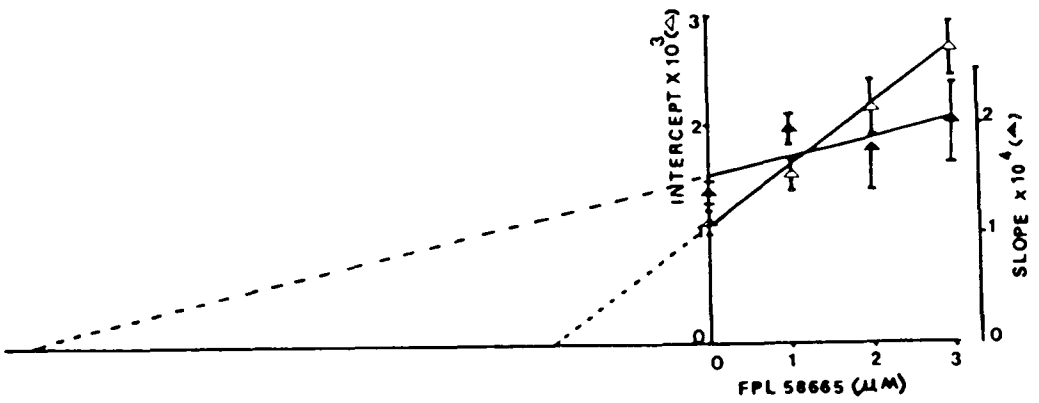




Fig. 3.9.1.1c Double reciprocal plot of initial velocity (v) against NADPH as variable substrate with sulindac as inhibitor. The concentration of DL-glyceraldehyde was held constant at 5 mM. ○ 0, ● 0.3 μM, Δ 0.45 μM, ▲ 0.6 μM sulindac, respectively. The inset shows the replot of primary slope and intercept against sulindac concentration.



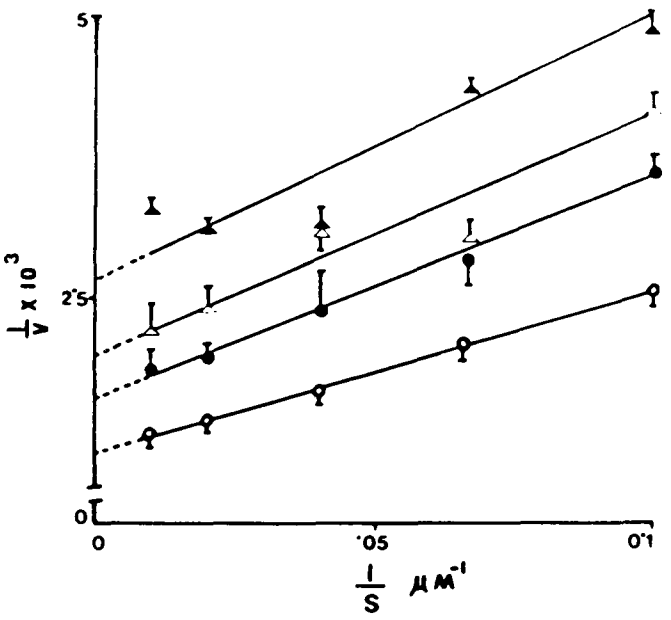




Fig. 3.9.1.2a. Double reciprocal plot of initial velocity (v) against DL-glyceraldehyde as variable substrate with FPL 58665 as inhibitor. The concentration of NADPH was held constant at 50 μM. o 0, ● 1 μM, Δ 2 μM and ▲ 3 μM FPL 58665, respectively. The inset shows the replot of intercept and slope against FPL 58665 concentration.

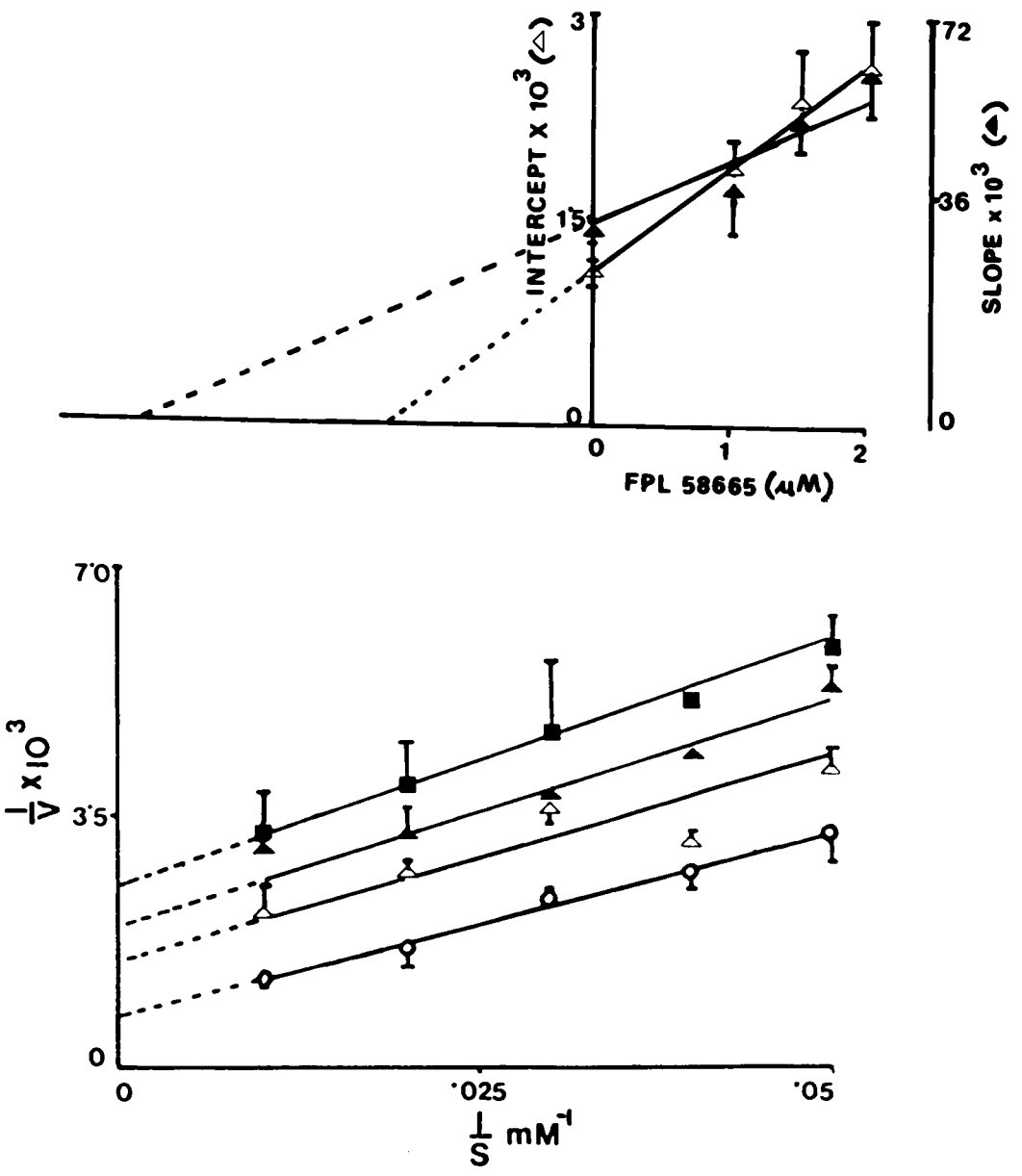
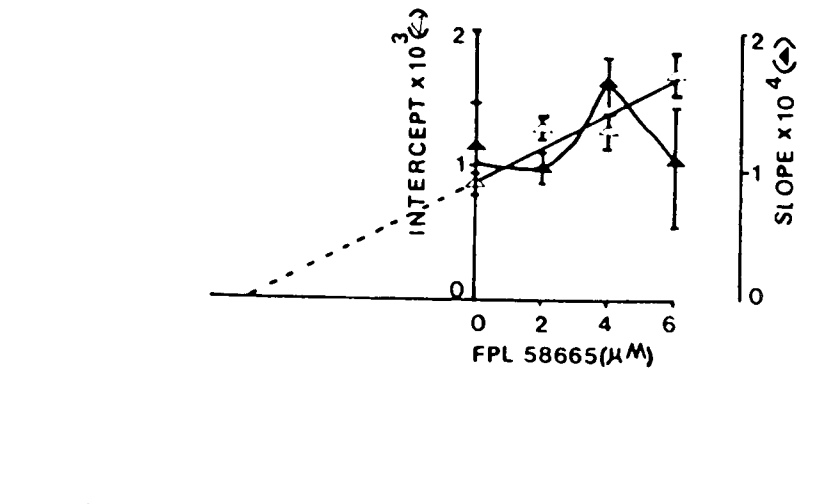



Fig. 3.9.1.2b Double reciprocal plot of initial velocity (v) against D-glucose as variable substrate. The concentration of NADPH was held constant at 50 μM. o 0, Δ 1 μM, ▲ 1.5 μM, ■ 2 μM FPL 58665, respectively. The inset shows the replot of primary slope and intercept against FPL 58665 concentration.



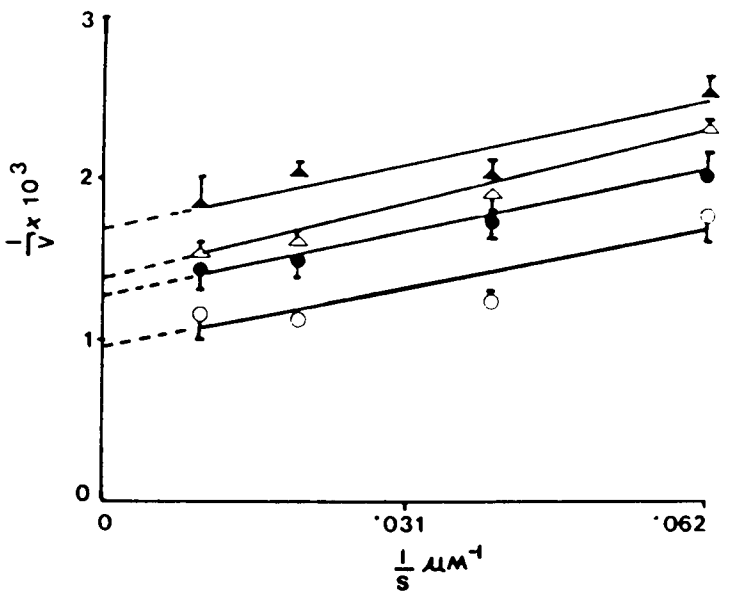




Fig. 3.9.1.2c Double reciprocal plot with initial velocity (v) against NADPH as variable substrate. The concentration of DL-glyceraldehyde was held constant at 5 mM. o 0, ● 2 μM, Δ 4 μM, ▲ 6 μM FPL 58665, respectively. The inset shows the replot of primary slope and intercept against FPL 58665 concentration.

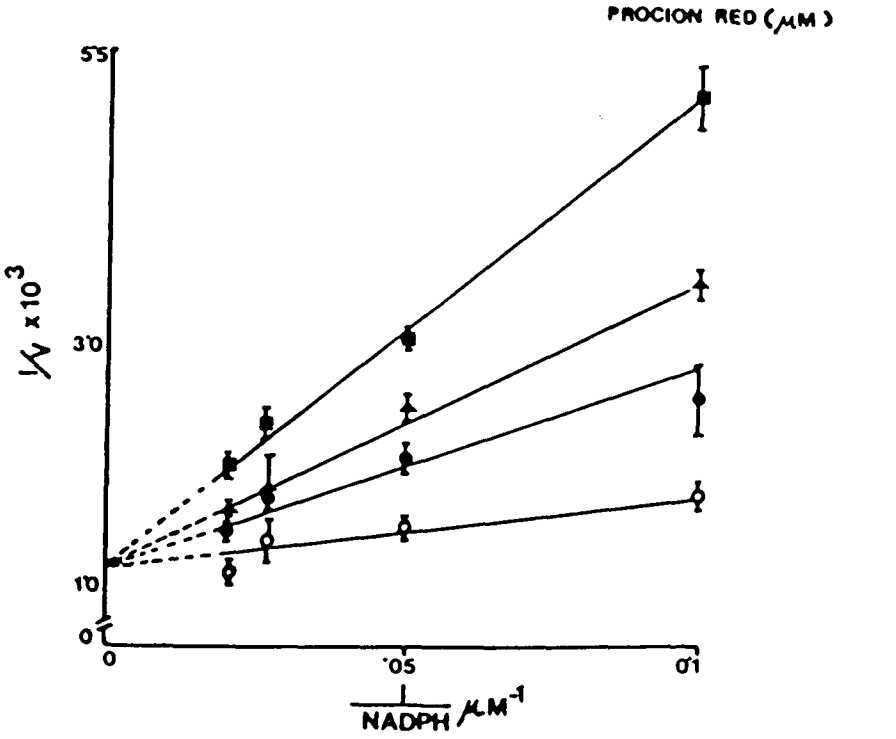
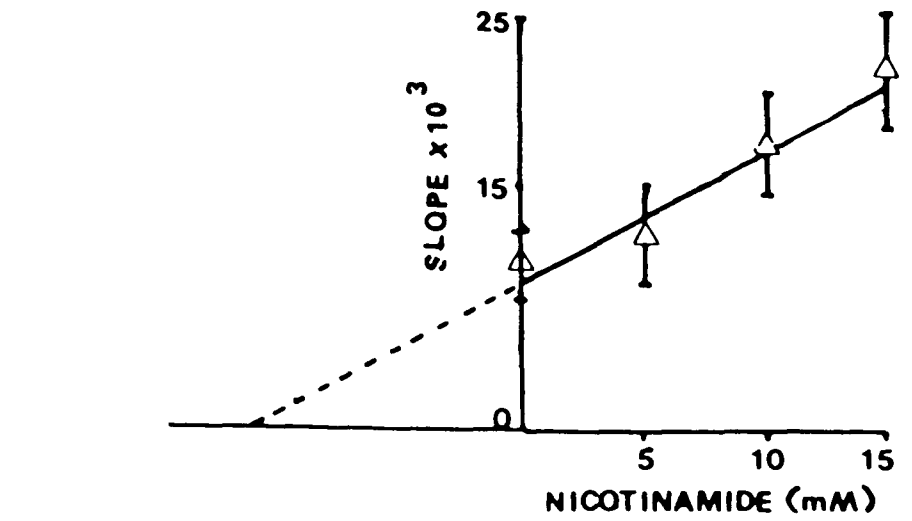



Fig. 3.9.2.1 Double reciprocal plot of initial velocity (v) against NADPH as variable substrate with Procion Red as inhibitor. The concentration of DL-glyceraldehyde was held constant at 5 mM. o 0, ● 10 μM, ▲ 20 μM, ■ 30 μM Procion Red, respectively. The inset shows the replot of primary slope against Procion Red concentration.



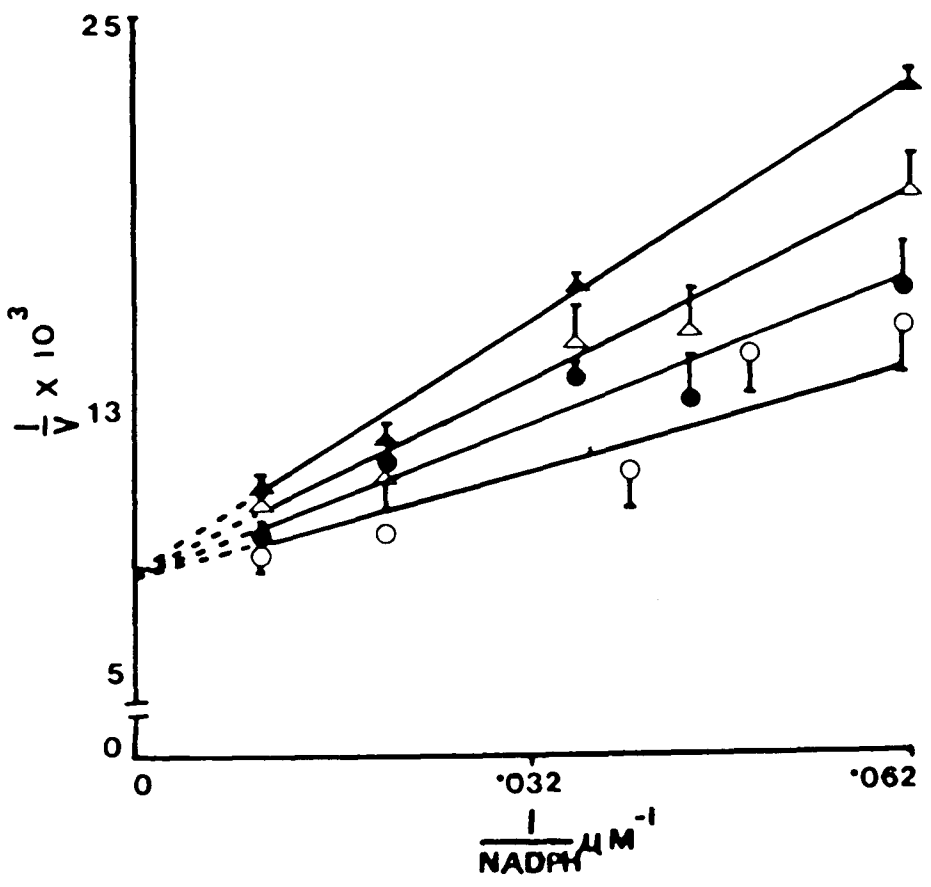




Fig. 3.9.2.2 Double reciprocal plot of initial velocity (v) against NADPH as variable substrate with nicotinamide as inhibitor. The concentration of DL-glyceraldehyde was held constant at 5 mM. o 0, ● 5 mM, Δ 10 mM, ▲ 15 mM nicotinamide, respectively. The inset is the replot of primary slope against nicotinamide concentration.

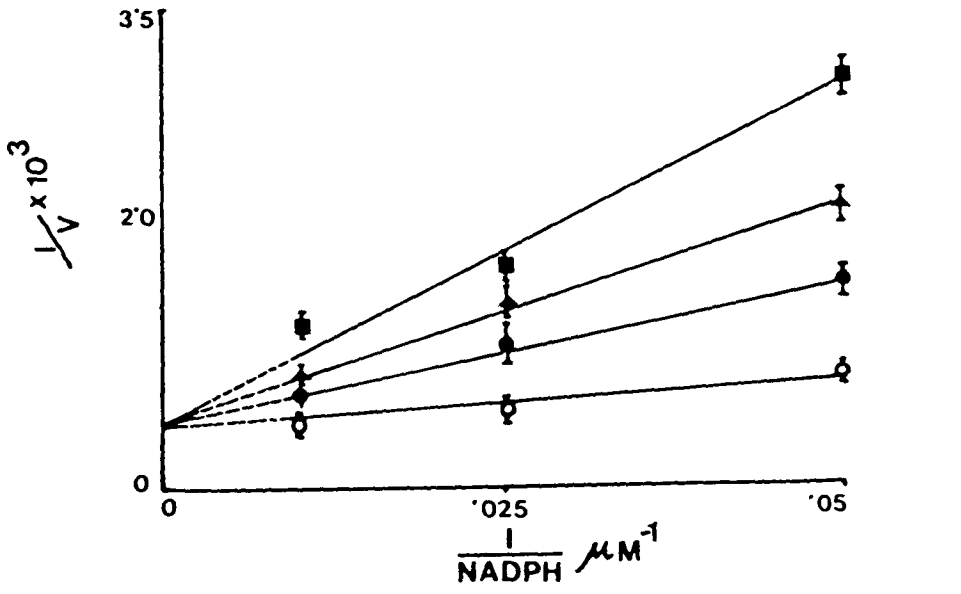



Fig. 3.9.2.3 Double reciprocal plot of initial velocity (v) against NADPH as variable substrate with 2'5' ADP as inhibitor. The concentration of DL-glyceraldehyde was held constant at 5 mM. o 0, ● 25 μM, ▲ 50 μM, ■ 100 μM 2'5' respectively. The inset shows the replot of primary slope against 2'5' ADP concentration.

Table 3.9.1 Inhibition Constants for Sulindac, FPL 58665, Procion Red, Nicotinamide, 2'5' ADP

| Inhibitor | Variable [s] | $K_{ii}$ | $K_{is}$ |
|---|---|---|---|
| Sulindac | DL-glyceraldehyde | 0.38 μM | - |
| | D-glucose | 0.9 μM | 1 .05 μM |
| | NADPH | 1.5 μM | - |
| FPL 58665 | DL-glyceraldehyde | 2 μM | 8 μM |
| | D-glucose | 1.5 μM | 3.2 μM |
| | NADPH | 6.6 μM | - |
| Procion Red | NADPH | - | 3.5 μM |
| 2'5' ADP | NADPH | - | 20 μM |
| Nicotinamide | NADPH | - | 10 mM |

All of these compounds were tried for their inhibitory effect on the enzyme with NADPH as the variable substrate. Procion Red (Fig. 3.9.2.1), nicotinamide (Fig. 3.9.2.2) and 2'5'ADP (Fig. 3.9.2.3) all showed competitive inhibition pattern with NADPH as the variable substrate. The Kis values for these inhibitors calculated from the insets are listed in Table 3.9.1. Sodium barbitone at 5 mM concentration did not inhibit partially purified aldehyde reductase using either DL-glyceraldehyde or D-glucose as its substrate.

## 3.10 Chemical Modification of Aldehyde Reductase B

The enzyme eluted from Carboxymethyl Sephadex-C-50 preceded by Sephadex-G-200 was used for this study.

### 3.10.1 Effect of diethyl pyrocarbonate

The enzyme was inactivated by diethyl pyrocarbonate in a time dependent fashion when assayed with both DL-glyceraldehyde and D-glucose as substrates (Fig. 3.10.1a). The pseudo-first order rate constants for glyceraldehyde at 5 mM and 10 mM diethyl pyrocarbonate concentrations are $0.87 \times 10^{-3}$ $sec^{-1}$ and $1.9 \times 10^{-3}$ $sec^{-1}$ respectively. For glucose at 10 mM concentrations the rate constant was $2.8 \times 10^{-3}$ $sec^{-1}$. Both 2 mM NADPH and 2 mM NADH offered protection against inhibition of glyceraldehyde and glucose reduction (Table 3.10.1) suggesting that histidine probably is at

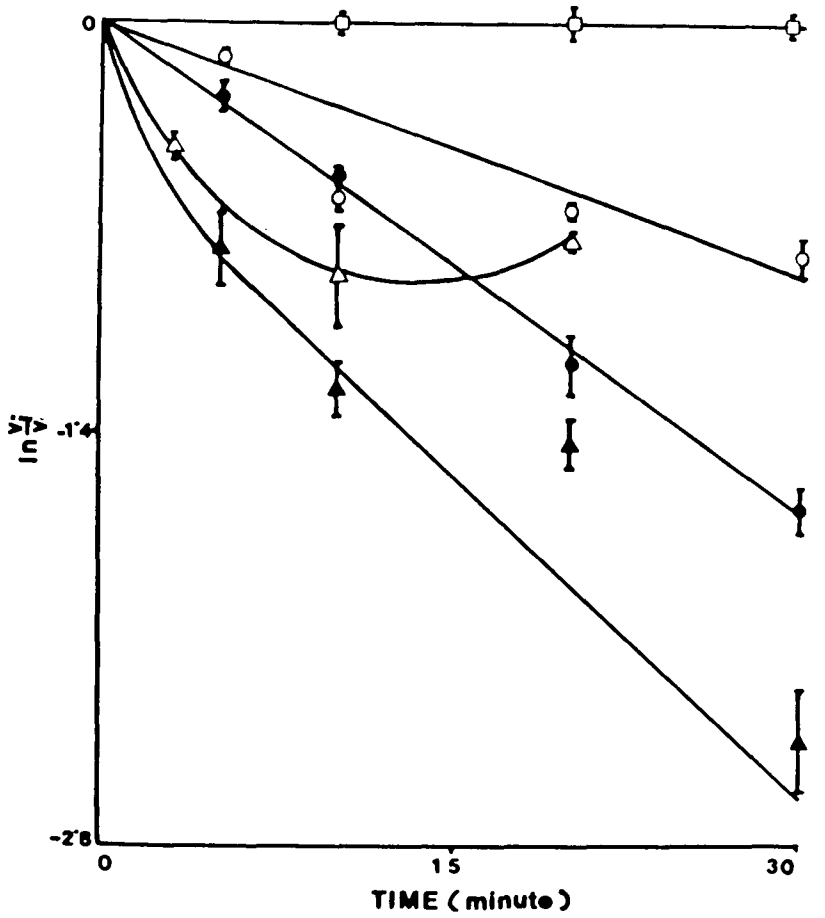



Fig. 3.10.1a Modification with diethyl pyrocarbonate, □ control activity without diethyl pyrocarbonate with both DL-glyceraldehyde and D-glucose as aldehyde substrates, o DL-glyceraldehyde, 5 mM diethyl pyrocarbonate, ● DL-glyceraldehyde, 10 mM diethyl pyrocarbonate, Δ D-glucose, 5 mM diethyl pyrocarbonate, ▲ D-glucose, 10 mM diethyl pyrocarbonate. V and $V_i$ are the initial velocities without and with inhibitor, respectively (see Section 2.2.11.1 for details).

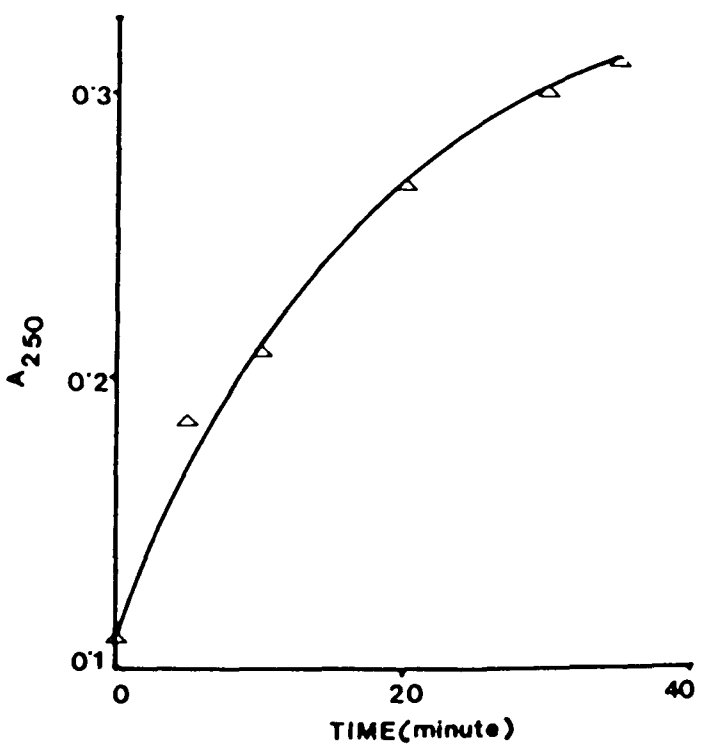



Fig. 3.10.1b Increase in absorbance with time at 250 nM of diethyl-pyrocarbonate modified enzyme (see Section 3.10.1).

or near the NADPH binding site. Absorbance measurement at 250 nM of the enzyme and 5 mM diethyl pyrocarbonate with the blank containing buffer and the reagent increased with time (Fig. 3.10.1b). Carboethoxyhistidyl derivative shows an absorbance maxima at 240-250 nM and the absorbance at 242 mM increased with time (Ovádi et al., 1967) and it was found that the histidine residue was attacked by the reagent. Hence the increased interaction with the enzyme and the reagent with time as evidenced by an increase in absorbance at 250 mM and the corresponding loss of activity of the enzyme may be correlated.

### 3.10.2 Effect of phenylglyoxal and butanedione

Phenylglyoxal too inhibited both DL-glyceraldehyde and D-glucose reducing activities in a time dependent fashion (Fig. 3.10.2.1). Neither glyceraldehyde nor glucose protected the enzyme against inhibition by phenylglyoxal. NADPH (2 mM) partly and NADH (2 mM) offered full protection (Table 3.10.1). Modification with butanedione was carried out in potassium phosphate (20 mM) buffer pH 7.0. The inhibition of glyceraldehyde reducing activity with butanedione shows time dependence but a reactivation phenomenon was observed after 8th minute (Fig. 3.10.2.2). The incubation with butanedione in borate buffer showed the same pattern.

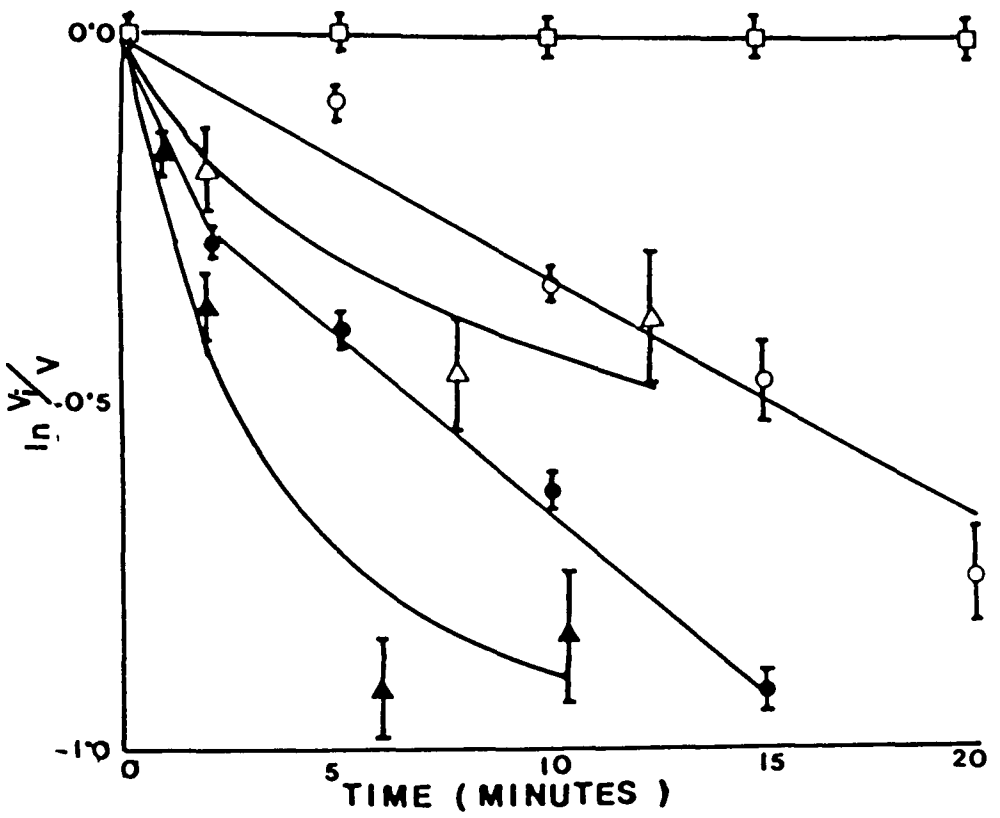



Fig. 3.10.2.1 Modification with phenylglyoxal. □ control without phenylglyoxal with both DL-glyceraldehyde and D-glucose, o DL-glyceraldehyde, 0.2 mM phenylglyoxal, ● DL-glyceraldehyde, 1 mM phenylglyoxal, Δ D-glucose, 0.1 mM phenylglyoxal, ▲ D-glucose, 0.7 mM phenylglyoxal. V and $V_i$ represents the initial velocities without and with inhibitor, respectively (see Section 2.2.11.2 for details).

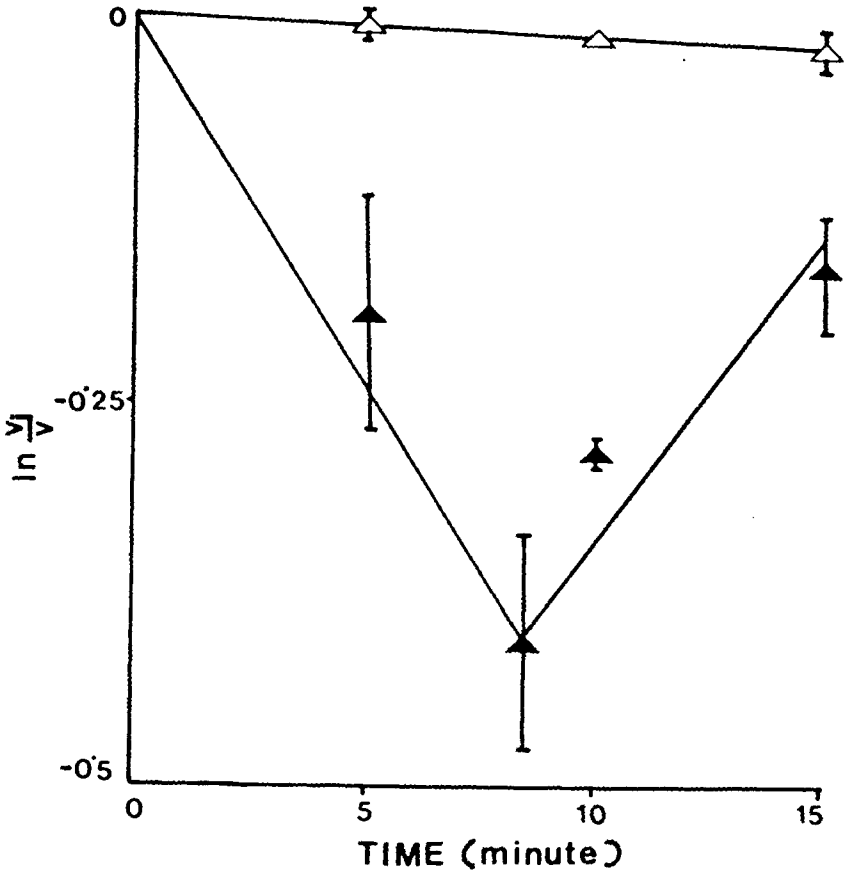



Fig. 3.10.2.2 Modification with butanedione, Δ control with DL-glyceraldehyde as aldehyde substrate without butanedione, ▲ DL-glyceraldehyde, 20 mM butanedione. V and $V_i$ are the initial velocities without and with inhibitor, respectively (see Section 2.2.11.2 for details).

### 3.10.3 Effect of N-ethylmaleimide and p-chloromercuribenzoate

DL-glyceraldehyde reducing activity was not affected by either of the two sulphydryl group reagents tried in the present work. D-glucose reducing activity was inhibited by both N-ethylmaleimide and p-chloromercuribenzoate (Fig. 3.10.3.1 and 3.10.3.2). With N-ethylmaleimide, however, the glucose reduction showed time dependent inhibition but at 60 μM NEM, after 5 min a reactivation phenomenon was again observed. A possible explanation has been given in Chapter 4. pCMB showed a concentration dependent inhibition (Fig. 3.10.3.2). Further evidence to the finding that glucose reducing activity is somehow dependent on sulphydryl group is that after passing through a Sephadex-G-25 column to remove residual mercaptoethanol, glyceraldehyde reducing activity remained unaffected but 75% of the glucose reducing activity was lost. After incubating with 5 mM 2-mercaptoethanol 60% of the activity was recovered. Glucose protected the enzyme from NEM inactivation whereas glucose and NADPH both offered protection against p-CMB inactivation. p-CMB, probably because of its large molecular size, occupies both NADPH and glucose binding site. Hence both substrates, NADPH and glucose protected against p-CMB inhibition.

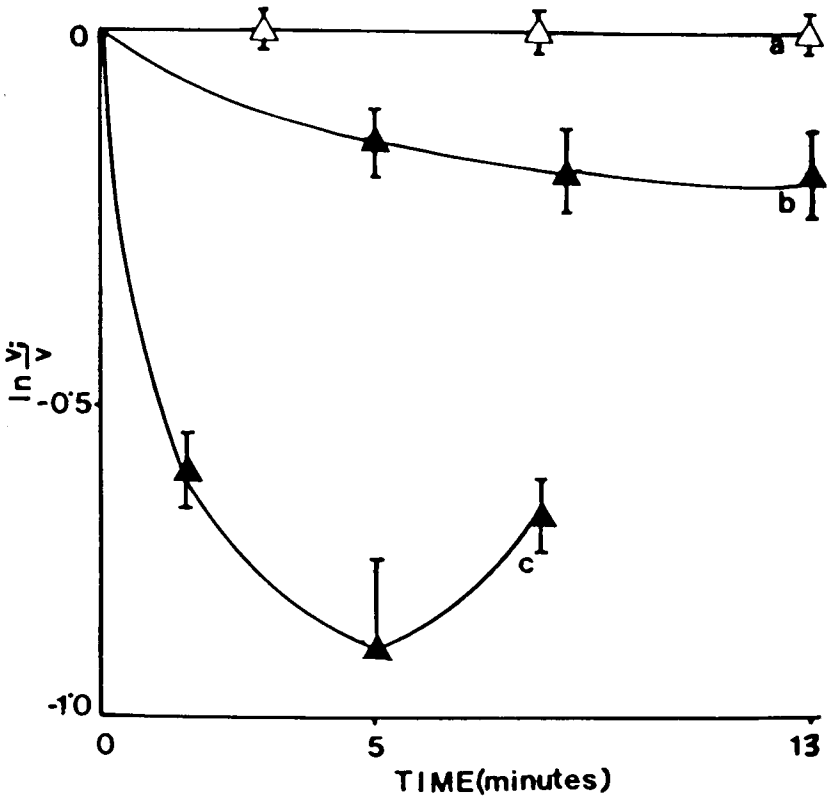



Fig. 3.10.3.1 Modification with NEM. (a) Control without inhibitor. (b) D-glucose, 6 μM N-ethylmaleimide. (c) D-glucose, 60 μM NEM. (The assay is described in Section 2.2.3.)

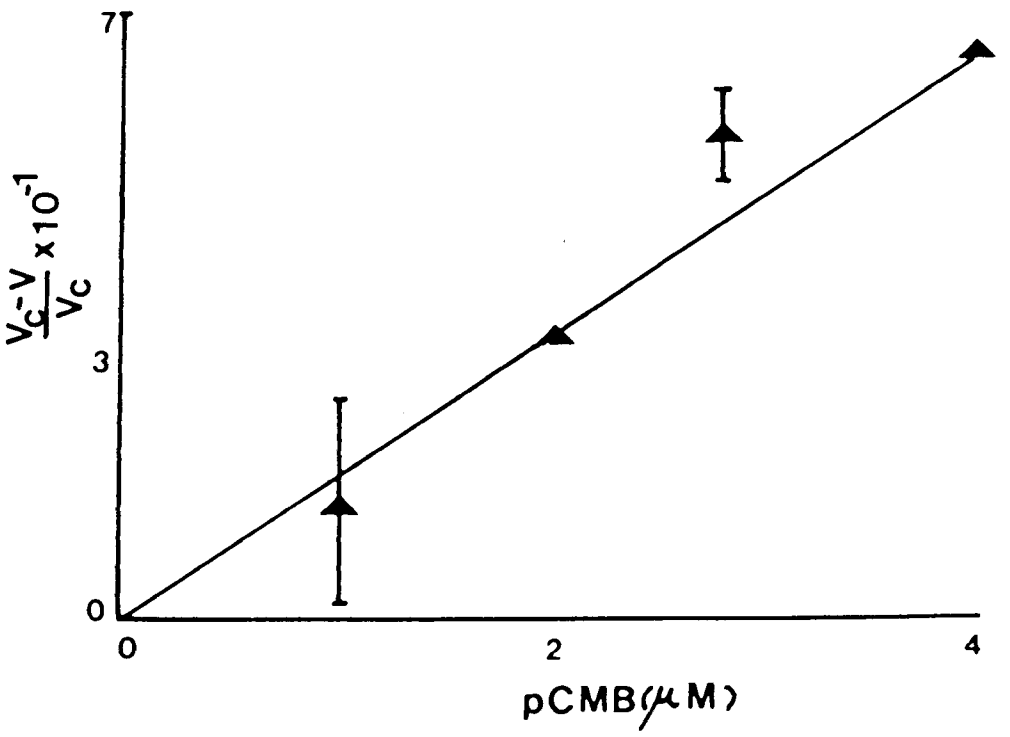



Fig. 3.10.3.2 Modification with p-CMB. The enzyme was incubated with p-CMB at the said concentrationsfor 90 sec and then assayed with D-glucose (see Section 2.2.11.5.1).
$V_c$ = Velocity without inhibitor
V = Velocity with inhibitor

### 3.10.4 Effect of KNCO and Pyridoxal-5'-phosphate

Neither DL-glyceraldehyde nor D-glucose reduction was inhibited by KNCO and pyridoxal-5'-phosphate which would react with amino-group under the experimental conditions (see 2.2.11.4). Hence it could be assumed that amino-group does not play any important role in the catalytic process.

### 3.10.5 Effect of Tetranitromethane

The activity of aldehyde reductase was not affected by 0.2% tetranitromethane even after incubating for 1 hr (see 2.2.11.3). At 1% tetranitromethane, the enzyme solution showed phase separation. The condition under which the incubation was carried out, i.e. pH 8.0 (Riordan and Vallee, 1972) tetranitromethane should have reacted with tyrosine, but the concentration of tetranitromethane may be too low to inhibit the enzyme.

### 3.10.6 Substrate protection experiment

The results of substrate protection experiments are listed in Table 3.10.1. These substrate protection experiments were carried out according to the methos described by Poulose and Kolattukudy (1980) and their result on substrate protection experiment is given in Table 3.10.2.

Table 3.10.1 Substrate Protection Experiment

| Inhibitor | Period of incubation (min) | Substrate | Residual activity (%) | Protecting substrate | Residual activity (%) |
|---|---|---|---|---|---|
| Diethylpyro-carbonate (5 mM) | 5 | DL-glyceral-dehyde | 44 ± 4 | NADPH (2 mM) | 55 ± 2 |
| | | | | NADH (2 mM) | 56 ± 8 |
| | | | | DL-glyceral-dehyde (10 mM) | 33 ± 4 |
| | | D-glucose | 53 ± 2 | NADPH (2 mM) | 60 ± 4 |
| | | | | NADH (2 mM) | 63 ± 0 |
| | | | | D-glucose (80 mM) | 50 ± 1 |
| Phenyl-glyoxal (100 µM) | 10 | DL-glyceral-dehyde | 74 ± 1 | NADPH (2 mM) | 85 ± 0 |
| | | | | NADH (2 mM) | 100 ± 0 |
| | | | | DL-glyceral-dehyde (10 mM) | 64 ± 0 |
| | | D-glucose | 65 ± 6 | NADPH (2 mM) | 85 ± 0 |
| | | | | NADH (2 mM) | 66 ± 4 |
| | | | | D-glucose (80 mM) | 65 ± 6 |
| NEM (30 µM) | 5 | D-glucose | 42 ± 2.4 | NADPH (2 mM) | 42 ± 4 |
| | | | | D-glucose (80 mM) | 75 ± 0 |
| p-CMB (2 µM) | 1½ | D-glucose | 29 ± 0 | NADPH (2 mM) | 53 ± 8 |
| | | | | D-glucose (80 mM) | 59 ± 0 |

Residual activity values are expressed with standard deviation.

Table 3.10.2 Protection by NADP and its Analogs Against Phenylglyoxal and and 2,3-Butaneidione Inactivation of Ketoacyl Reductase, Enoyl Reductase, and Overall Activity of FAS

| Protecting agent | Percentage protection | | | | | |
|---|---|---|---|---|---|---|
| | Fatty acid synthetase | | Ketoacyl reductase | | Enoyl reductase | |
| | Phenyl-glyoxal (2 mM) | 2,3-Butane-dione (20 mM) | Phenyl-glyoxal (2 mM) | 2,3-Butane-dione (20 mM) | Phenyl-glyoxal (2 mM) | 2,3-Butane-dione (5 mM) |
| NADP | 29 | 32 | 100 | 79 | 58 | 29 |
| NAD(H) | 2 | 2 | 4 | 23 | 0 | 2 |
| 2'-AMP | 27 | 23 | 96 | 56 | 48 | 44 |
| 2',5'-ADP | 23 | ND | ND | ND | ND | ND |
| 3'-AMP,5'-AMP or 3',5'-ADP | <1 | <1 | <1 | <8 | <21 | <2 |

ND = Not determined.

Table taken from Poulose and Kolattukudy (1980)

## 3.11 Kinetic Parameters for Various Substrates of Aldehyde Reductase B

$K_m$, $V_{max}$, $K_{cat}$ and $K_{cat}/K_m$ for these substrates are listed in Table 3.11.1. Of these substrates p-nitrobenzaldehyde, DL-glyceraldehyde, DL-glyceraldehyde-3-phosphate, malondialdehyde showed substrate inhibition at above 2 mM, 20 mM, 1 mM and 800 μM concentrations respectively. $K_m$ is the substrate concentration giving half-maximal velocity, $K_{cat}$ or the turnover number of the enzyme represents the maximum number of molecules of substrates converted into products per active site per unit time. The ratio of $K_{cat}/K_m$ signifies the substrate specificity regardless of concentration. It also represents second order rate constant for association of enzyme and substrate and in a diffusion-controlled association the ratio $\frac{Kcat}{Km}$ should be $10^9 s^{-1} M^{-1}$. All the $\frac{Kcat}{Km}$ values for substrates of aldehyde reductase B studied, are $10^{+3}$ order which is much too low compared with diffusion-controlled rate constant. However, the $\frac{Kcat}{Km}$ values listed in Table 3.11.1 suggest that the best substrate is p-nitrobenzaldehyde followed by DL-glyceraldehyde. $\frac{Kcat}{Km}$ values are nearly the same for glyceraldehyde-3-phosphate and malondialdehyde. $\frac{Kcat}{Km}$ ratio for fructose is nearly 2.5 times higher than that for glucose but $V_{max}$ for glucose is 25 times higher than that for fructose. Succinic-semi-aldehyde and L-glucose are not acted on by aldehyde reductase.

Table 3.11.1 Kinetic Parameters for Bovine Lens Aldehyde Reductase

| Substrate | $K_m$ (mM) | $V_{max}$ (µmoles/min/ mg enzyme) | $K_{cat}$ ($s^{-1}$) | $\frac{K_{cat}}{K_m} \times 10^3$ ($M^{-1}s^{-1}$) |
|---|---|---|---|---|
| p-nitrobenzaldehyde | 0.03 | 0.54 | 0.27 | 9 |
| DL-glyceraldehyde | 0.123 (0.05*) | 1.27 | 0.77 | 6.41 |
| D-glucose | 120.00 (47.0*) | 0.5 | 0.3 | 0.0025 |
| D-glucuronate | 4.3 | 0.42 | 0.25 | 0.058 |
| 3-O-methylglucose | 150 | 0.23 | 0.06 | 0.0004 |
| Fructose | 23 | 0.02 | 0.015 | 0.0065 |
| Glyceraldehyde-3-phosphate | 0.2 | 0.014 | 0.08 | 0.4 |
| Malondialdehyde | 0.25 | 0.19 | 0.13 | 0.52 |
| Succinic semi-aldehyde | ND | ND | | |
| L-glucose | ND | ND | | |
| NADPH | 0.009 | 0.49 | 0.28 | 31.0 |
| NADH | 1.64* | 0.0003* | | |

ND = Not Detectable.

* Values determined with crude enzyme.

$K_m$ values with asterisks indicate the values obtained with the crude enzyme with DL-glyceraldehyde and D-glucose as substrates. It is interesting to note that $K_m$ values for pure enzyme are nearly 2.5 times higher than those obtained with crude enzyme. The result is discussed in Chapter 4.

## 3.12 Eadie-Hofstee Plots for DL-glyceraldehyde, D-glucose and NADPH

Lineweaver-Burke plots, Eadie-Hofstee plots and Hanes plots are the linearizing plots for initial velocity against substrate concentration data. As Lineweaver-Burke plot can misinterpret data, Eadie-Hofstee plot was chosen to see if aldehyde reductase obeys Michaelis-Menten equation. Figs.3.12.1 and 3.12.2 are the best fit Eadie-Hofstee plots for observed data of D-glucose and NADPH respectively which are linear over a 100-fold concentration range. The linearity was checked by feeding the initial velocity data against substrate concentrations for both glucose and NADPH to a non-linear regression programme, based on the equation $v = \frac{VS}{K_m+S}$ as described in Chapter 2 (Computing, Section 2.2.16). The predicted plots were superimposable with the observed ones for both glucose and NADPH. Eadie-Hofstee plot of DL-glyceraldehyde was found to be non-linear. The initial velocity data against substrate concentration were fed into BMDP3R

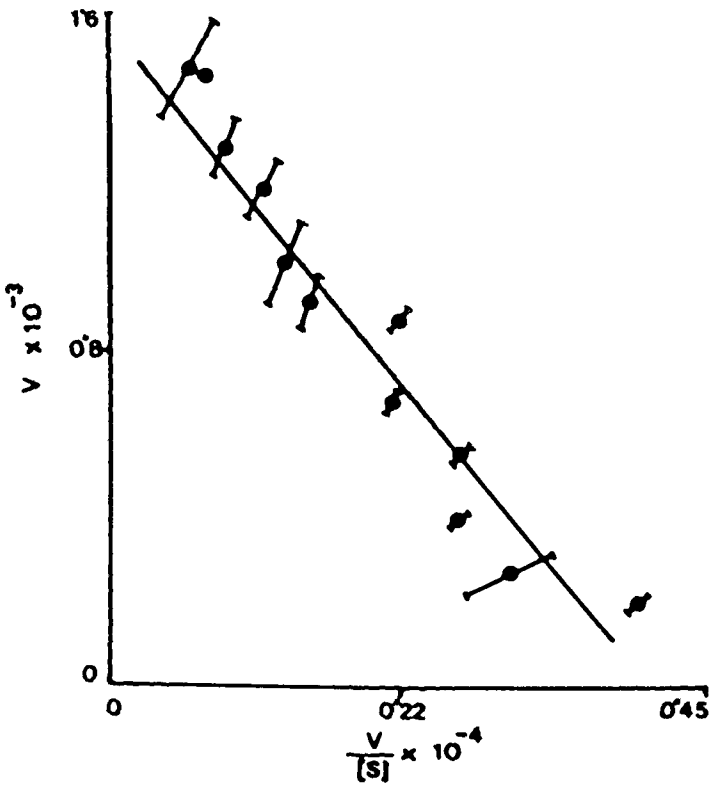



Fig. 3.12.1 Eadie-Hofstee plot with D-glucose (see Section 3.12 for details).

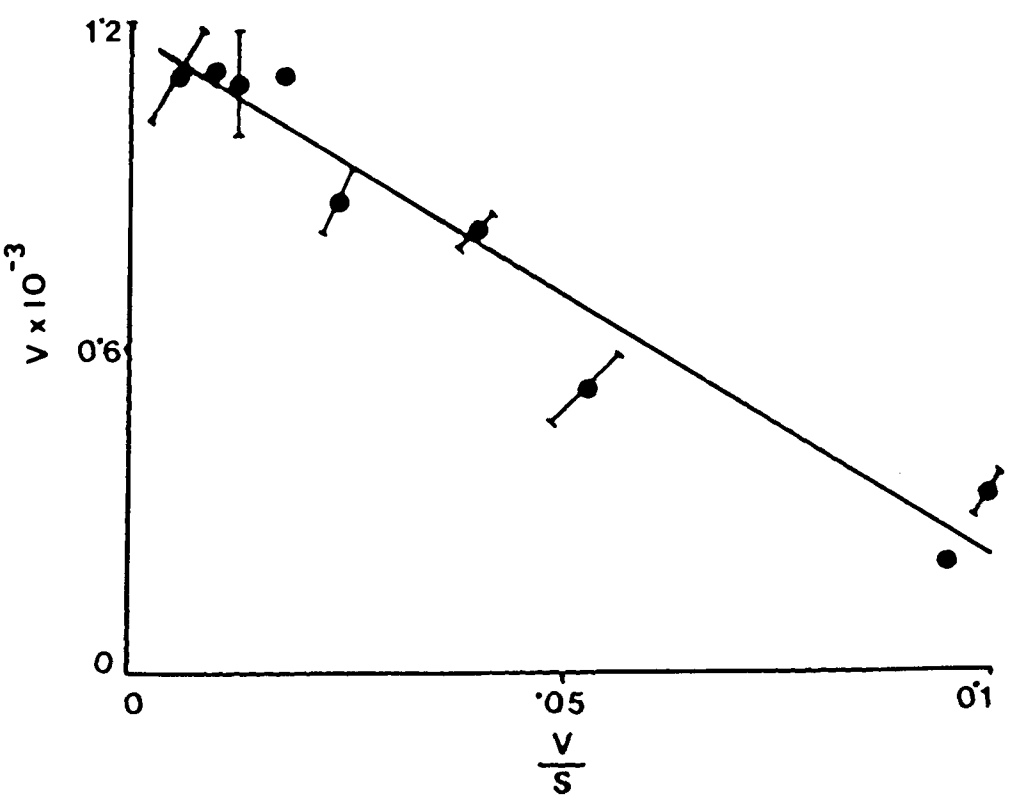



Fig. 3.12.2 Eadie-Hofstee plot with NADPH (see Section 3.12 for details).

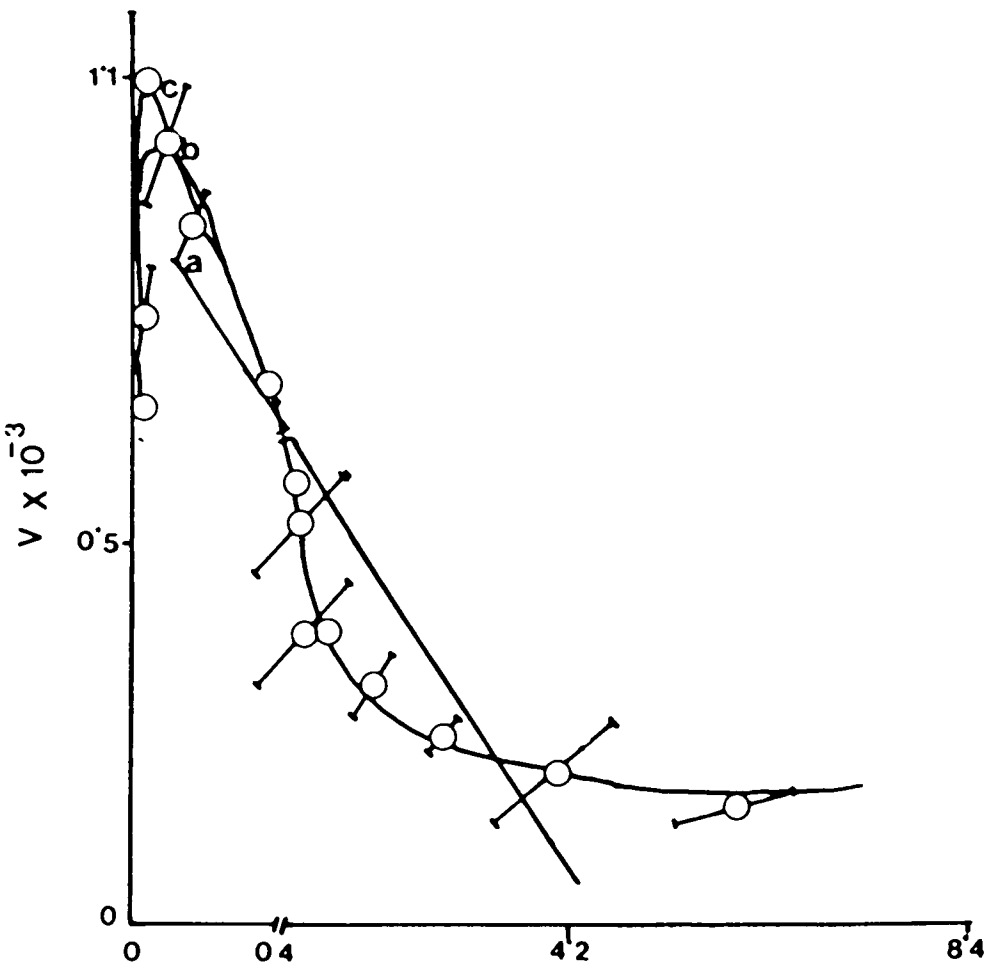



Fig. 3.12.3 Eadie-Hofstee plot with DL-glyceraldehyde. a, b and c represent the computer predicted graph plots for 1:1, 2:2 and 3:3 functions. O represents the observed data (see Section 2.2.16 for details).

non-linear regression programme (see Section 2.2.16) to find out the minimum degree of rate equation. The data were fitted to 1:1, 2:2 and 3:3 function and then an Eadie-Hofstee plot was constructed with the predicted velocities for all these functions. The observed data was found to match with 2:2 function but fitted better with 3:3 function. Hence the minimum degree of rate equation found with DL-glyceraldehyde is 2:2. This study of kinetics was perfomed with both crude and pure enzyme in both Tris-HCl pH 7.3 and phosphate 6.2 buffer. No change was observed due to buffer or nature of enzyme, crude or pure.

## 3.13 Study of Mechanism

The mechanism of enzyme action was studied with D-glucose as the aldehyde substrate, as glucose showed linear kinetics and glucose is more important a substrate to study with diabetic cataract in mind.

The steady-state initial velocity study with the two substrates in absence of product plotted in Lineweaver-Burke space, i.e. $\frac{1}{v}$ vs. $\frac{1}{s}$ (v = initial velocity; s = substrate concentration) show that they are linear (Fig. 3.13.1a and c) and converge on the second quadrant. Initial velocity data when fitted to $v = \frac{Vs}{K_m+s}$ equation and predicted result plotted in the similar manner, their linear and converging pattern were

confirmed (Fig. 3.13.1b and d). The observed secondary plots of primary slopes and intercepts against $\frac{1}{S}$ were also linear (Fig. 3.13.2a,b) as the computer predicted plots were.

The product inhibition study was undertaken to get a clear picture regarding the mechanism of action. Both $NADP^+$ and sorbitol inhibited the enzyme. All the double reciprocal plots with products as inhibitors were linear. $NADP^+$ showed competitive inhibition against NADPH as variable substrate (Fig. 3.13.3a) and showed non-competitive inhibition with glucose as variable substrate (Fig. 3.13.3b). Sorbitol showed non-competitive inhibition with both glucose (Fig. 3.13.4a) and $NADP^+$ (Fig. 3.13.4b). The insets show the replots of primary slopes or intercepts against inhibitor concentrations. The $K_{ii}$ and $K_{is}$ values obtained from these replots are given in Table 3.13.2.

The initial velocity study with two substrates and the product inhibition study were compatible with a mechanism of

$$E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \rightleftharpoons EPQ \longrightarrow EP \longrightarrow E$$

compulsory-order-ternary-complex formation (Cleland, 1963) and the rate equation considering the initial velocity in absence of product for such mechanism is

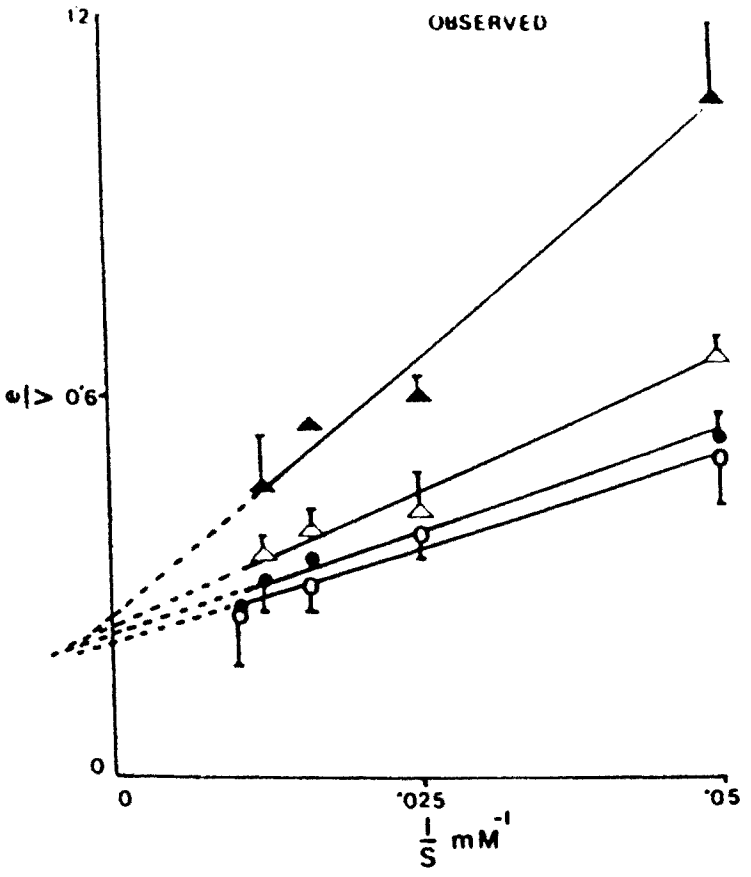



Fig. 3.13.1a Double reciprocal plot of initial velocity (observed) against glucose at (o) 10, (●) 20 μM, (Δ) 40 μM, (▲) 80 μM NADPH, respectively. v is the initial velocity in μmoles/min. e is the amount of enzyme in μmoles.

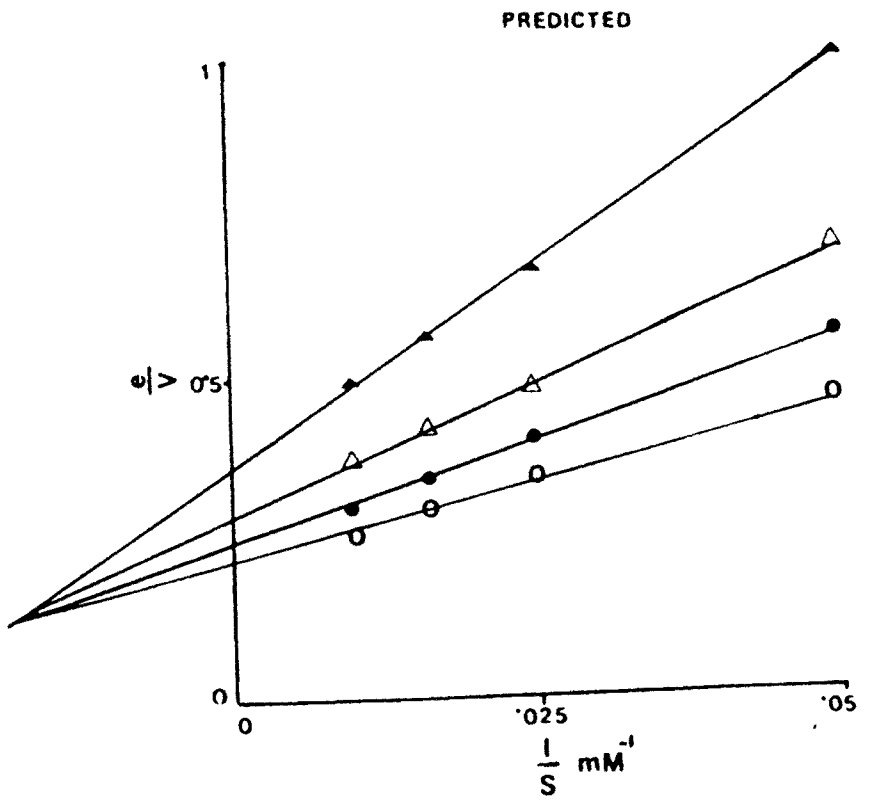



Fig. 3.13.1b Double reciprocal plot of computer calculated initial velocity against glucose at NADPH concentrations mentioned in Fig. 3.13.1a.

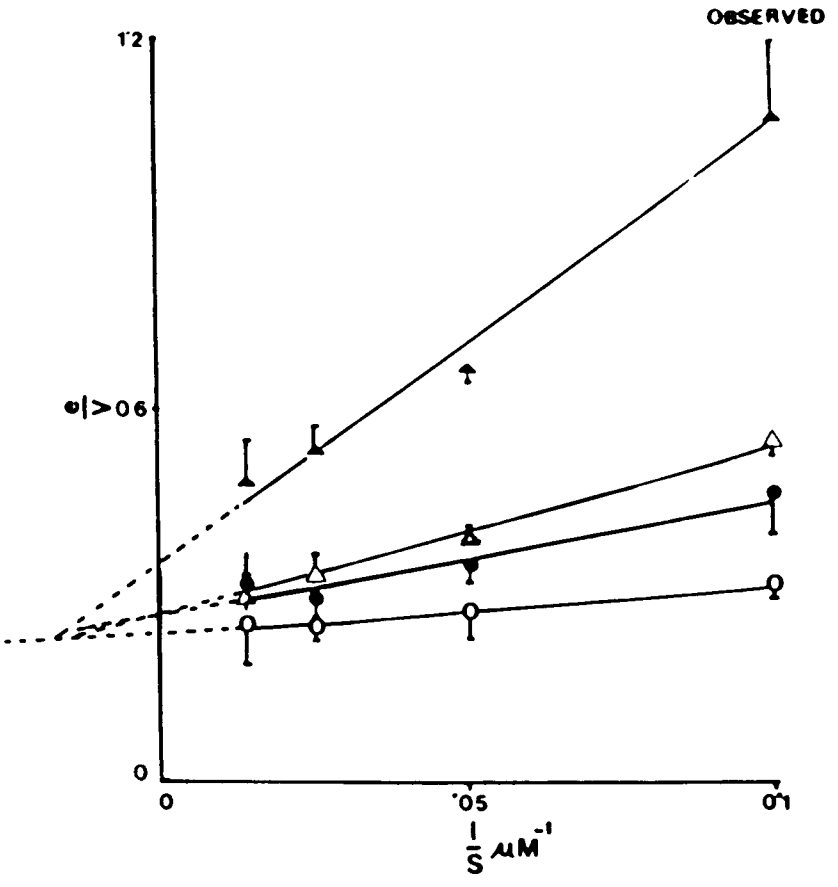



Fig. 3.13.1c Double reciprocal plot of initial velocity (observed) against NADPH at (o) 20 mM, (●) 40 mM, (Δ) 80 mM, (▲) 100 mM glucose concentration. v is the initial velocity expressed in μmoles/min. e is the amount of enzyme in μmoles.

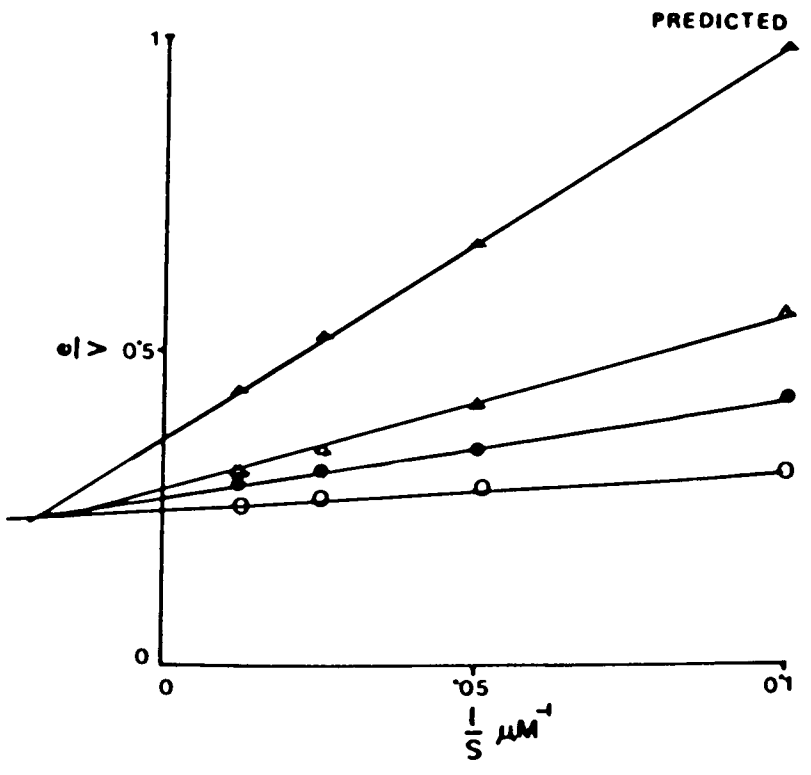



Fig. 3.13.1d Double reciprocal plot of computer calculated initial velocity against NADPH at glucose concentrationsmentioned in Fig. 3.13.1c.

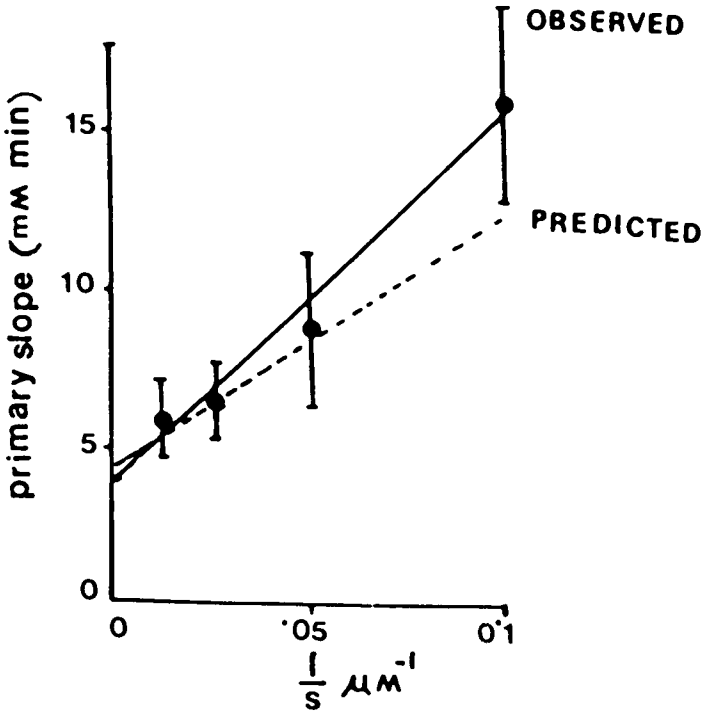



Fig. 3.13.2a Secondary plot of primary slope against reciprocal NADPH concentration. The dotted line is the secondary plot with the computer calculated data, whereas the solid line represents that with the observed data.

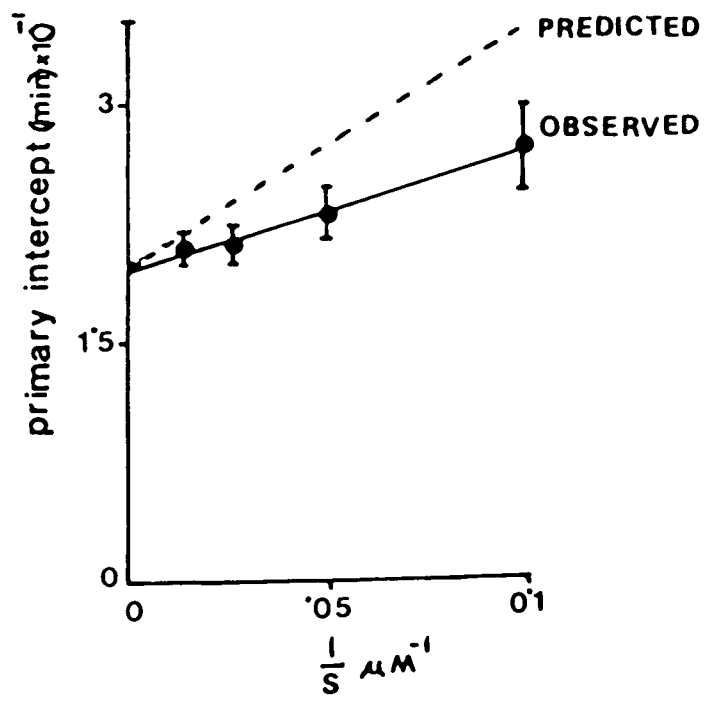



Fig. 3.13.2b Secondary plot of primary intercept against reciprocal of NADPH concentration. The dotted line is the secondary plot with the computer calculated data and the solid line represents that with the observed data.

Table 3.13.1 Kinetic Parameters Obtained from Secondary Plots

| | Predicted | Observed |
|---|---|---|
| $K_m$ glucose (mM) | 21.8 ± 3.9 | 18.6 ± 3.45 |
| $K_m$ NADPH (μM) | 8.2 ± 1.5 | 3.2 ± 0.7 |
| $K_s$ NADPH (μM) | 5.41 ±0.59 | 4.6 ±0.64 |
| $V_{max}$ (μmoles/min/mg) | 0.01 ± 0 | 0.01 ± 0 |

The steady-state initial velocity study in absence of product was carried out in phosphate buffer pH 6.2. The predicted velocity data were obtained by feeding the data to a non-linear regression programme as described in Section 2.2.16. Values are expressed with standard deviation.

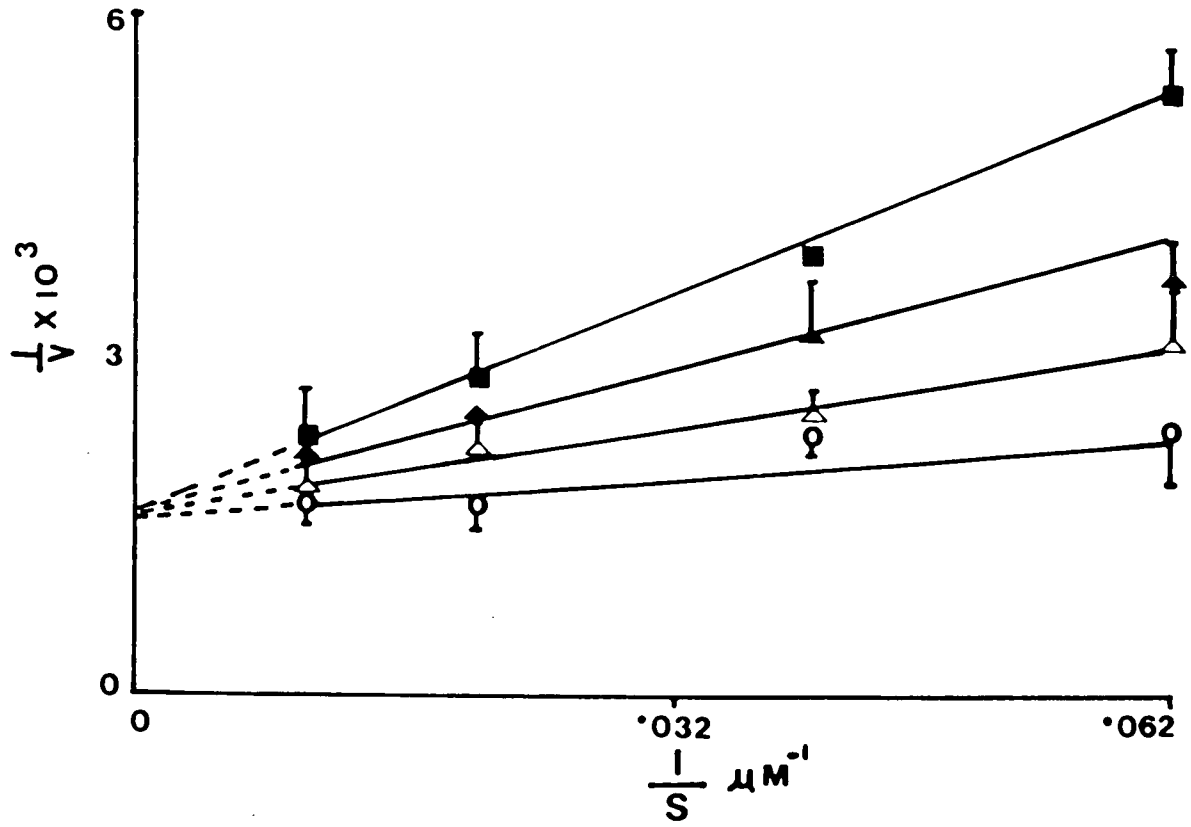
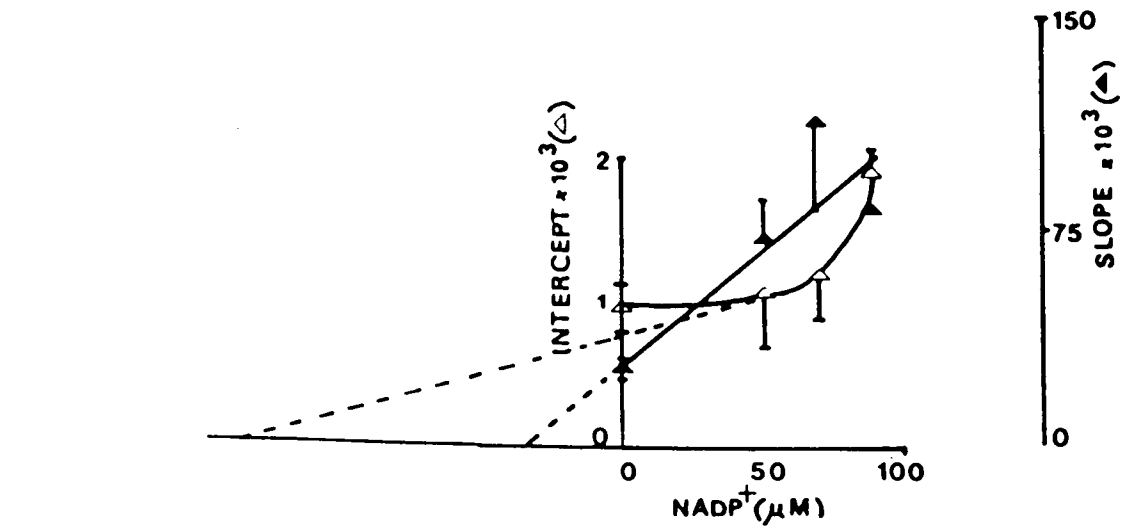



Fig. 3.13.3a A double reciprocal plot of initial velocity (v) against NADPH as variable substrate with $NADP^+$ as product inhibitor. The concentration of D-glucose was held constant at 80 mM. o 0, Δ 30 μM, ▲ 50 μM, ■ 70 μM $NADP^+$, respectively. The inset shows the replot of the primary slope and intercept against $NADP^+$ concentration.



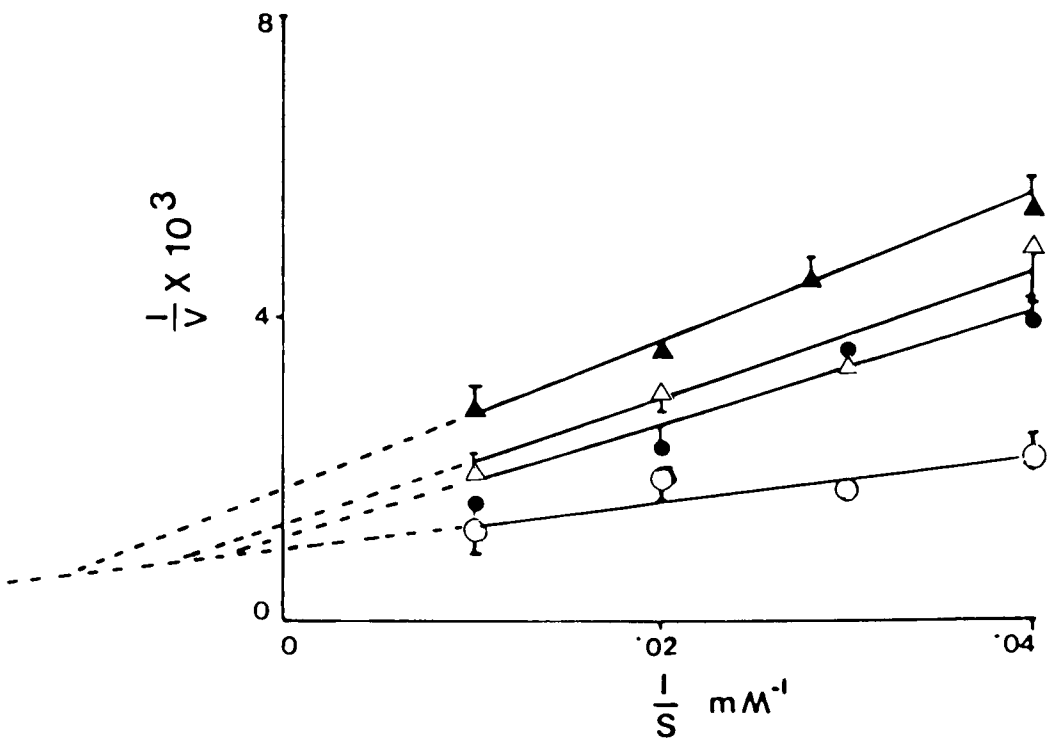
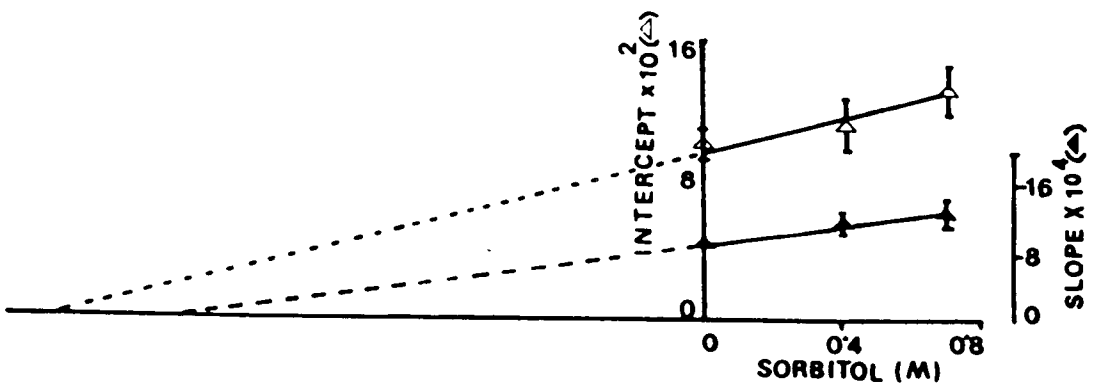




Fig. 3.13.3b Double reciprocal plot of initial velocity (v) against glucose as variable substrate with $NADP^+$ as product inhibitor. The concentration of NADPH was kept constant at 50 μM. o 0, • 50 μM, Δ 70 μM, ▲ 90 μM $NADP^+$ concentrations respectively. The inset shows the replot of primary slope and intercept against $NADP^+$ concentration.



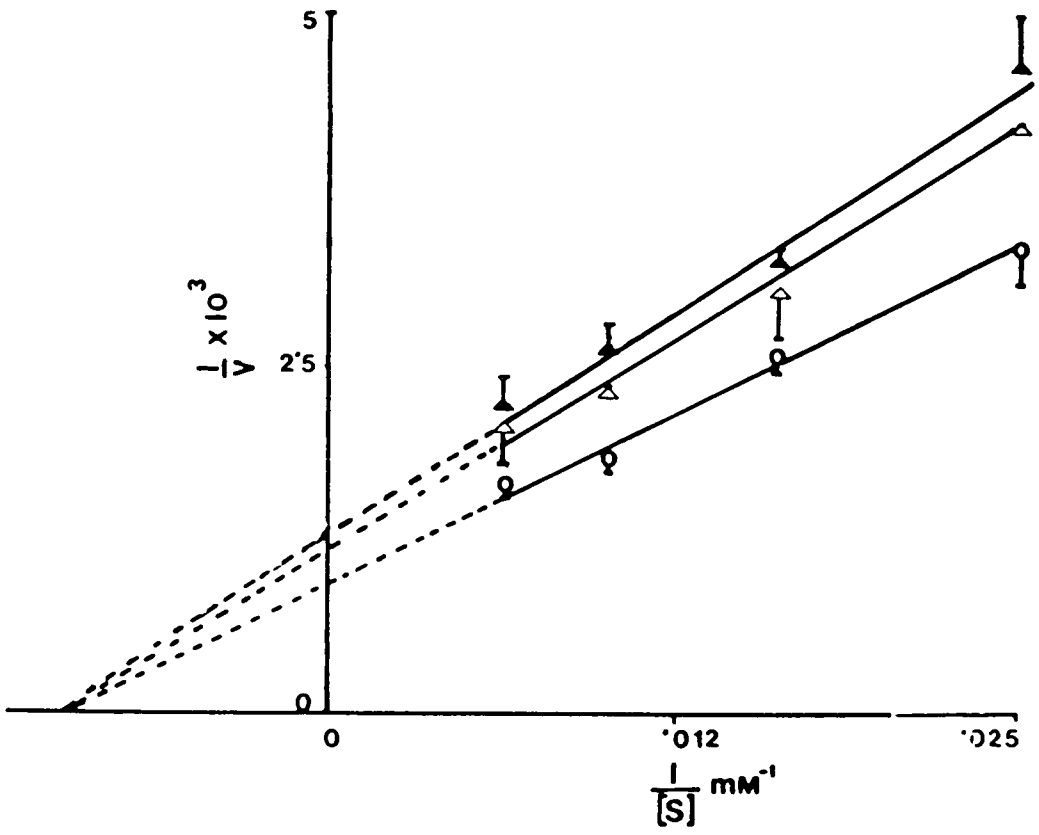




Fig. 3.13.4a Double reciprocal plot of initial velocity (v) against glucose as variable substrate and sorbitol as inhibitor. The concentration of NADPH was held constant at 50 μM. o 0, Δ 0.4 M and ▲ 0.7 M sorbitol, respectively. The inset is the replot of primary intercept and slope against sorbitol concentration.

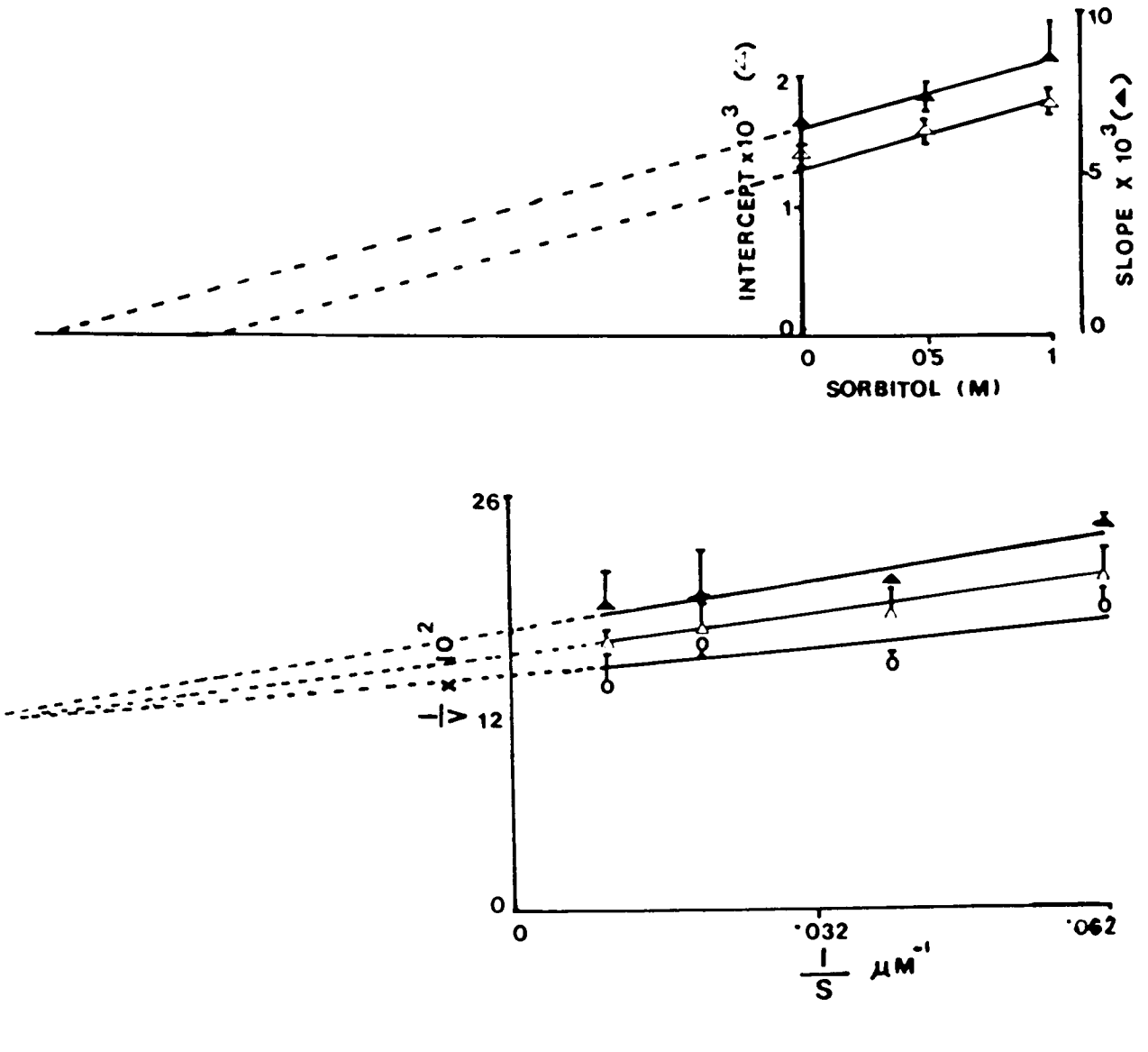



Fig. 3.13.4b Double reciprocal plot of initial velocity (v) against NADPH as variable substrate and sorbitol as inhibitor. Glucose concentration was held constant at 80 mM, o 0, Δ 0.5 M, 1 M ▲ sorbitol, respectively. The inset shows the replot of primary slope and intercept against sorbitol concentration.

Table 3.13.2 Kinetic Parameters Obtained from Product Inhibition Study

| Inhibitors | Variable substrate | Fixed substrate | Type of inhibition | $K_{ii}$ | $K_{is}$ |
|---|---|---|---|---|---|
| Sorbitol | Glucose | NADPH (50 μM) | Non-competitive | 1.9 M | 1.7 M |
| | NADPH | Glucose (80 mM) | Non-competitive | 2.25 M | 2.8 M |
| NADP | Glucose | NADPH (50 μM) | Non-competitive | 135 μM* | 30 μM |
| | NADPH | Glucose (80 mM) | Competitive | - | 60 μM* |

$K_{ii}$ and $K_{is}$ were determined from the replots of intercepts and slopes against inhibitor concentration. The fixed substrates were used at non-saturating concentration in all cases. Phosphate buffer pH 6.2 was used as an assay buffer.

*These values were determined from the tangents to the hyperbolic curves.

$$v = \frac{V\ a\ b}{Ks_a\ K_m^{\ b} + K_m^{\ a}b + K_m^{\ b}a + ab}$$

a,b = concentrations of A & B respectively

$Ks_a$ = dissociation constant for EA

$K_m^{\ a}$ = $K_m$ for substrate A

$K_m^{\ b}$ = $K_m$ for substrate B

From the slope and intercept of the secondary plots (Fig. 3.13.2a and b) the kinetic parameters were calculated and given in Table 3.13.1.

## 3.14 Fluorescence Spectroscopy

The initial velocity study with the two substrates and the product inhibition study indicated that it is a case of compulsory order ternary complex formation mechanism. That NADPH is the first substrate to bind to the enzyme was shown by the fluorescence spectroscopy study (Fig. 3.14.1). The Figure shows that the NADPH binds to the enzyme causing a red shift of the NADPH emission spectrum from 466 nM to 452 nM and the binding is accompanied by fluorescence quenching.

Sorbitol was used at a concentration of 0.4 M - 1 M for the product inhibition study, a viscosity effect

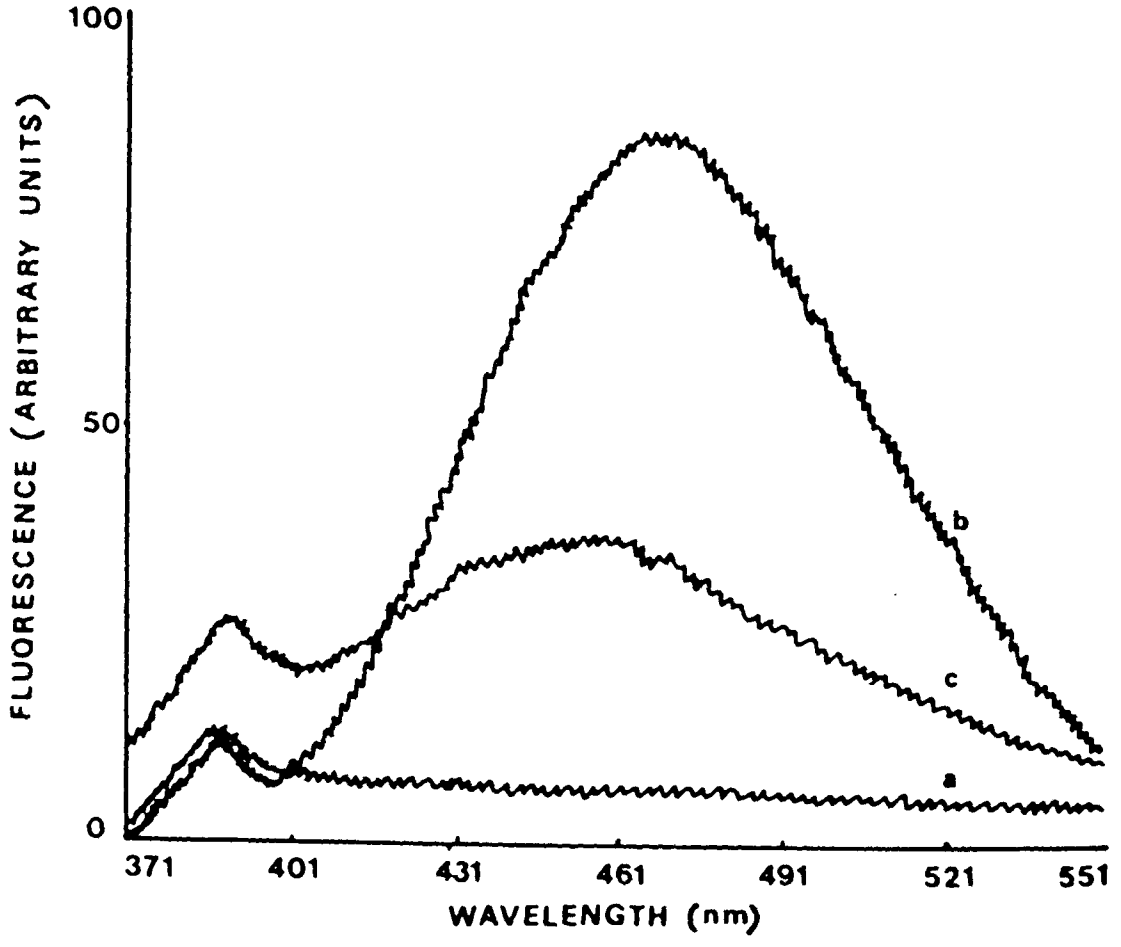



Fig. 3.14.1 Emission fluorescence spectrum of aldehyde reductase (0.2 mg/ml) and 1 μM NADPH (excitation wavelength = 340 nm) a = buffer alone, b = NADPH (1 μM) alone, c = enzyme (0.2 mg/ml) + NADPH (1 μM).

may be expected at these concentrations. Hence viscosities of assay mixturescontaining 0.5 M and 1 M sorbitol were measured by Ostwald's capillary viscometer and were found to be 1.37 cp and 1.44 cp respectively.

## 3.15 NADPH-oxidizing Activity in Human Blood

Blood samples from patients attending the Oxford Eye Hospital and John Radcliffe Hospital were collected and the samples were then prepared for the assay of NADPH-oxidizing activity, as described in Chapter 2 (Section 2.2.3.3). The data on haemoglobin $A_{1c}$, height and weight and other details of the patients were obtained from the Diabetic Research Laboratory (Oxford Eye Hospital). Patients were divided into 5 groups, normal control (group 1), diabetic controls (group 2), patients with senile cataract (group 3), patients with diabetic retinopathy but no cataract (group 4) and patients with diabetic retinopathy and cataract (group 5). Fig. 3.15.1 and Table 3.15.1 show that, compared with mean activity level in normal control group (group 1) and diabetic control (group 2), the mean glyceraldehyde reducing activity measured in terms of NADPH oxidation was significantly higher in diabetic patients with retinopathy and cataract (2.5-fold increase, $p<0.001$), diabetics with retinopathy but no cataract (2-fold increase, $p<0.01$) and non-diabetic subjects with senile cataract (1.5-fold increase, $p<0.05$). There was no significant

Table 3.15.1 Glyceraldehyde Reductase and Polyol Dehydrogenase Activities

| Group | Mean ± SD age (yr) | Mean ± SD enzyme activity ($U\ ml^{-1} \times 10$) | |
|---|---|---|---|
| | | Glyceraldehyde reductase | Polyol dehydrogenase |
| (1) Normal subjects without diabetes or cataracts (n = 18) | 69 ± 2.88 | 0.67 ± 0.126 | 0.18 ± 0.085 |
| (2) Diabetic subjects without retinopathy or cataract (n = 15) | 44 ± 5.9 | 0.64 ± 0.17 ($p<0.9$) | 0.011 ± 0.009* ($p<0.1$) |
| (3) Non-diabetics with senile cataract (n = 22) | 69 ± 2.1 | 1.01 ± 0.107 ($p<0.05$) | 0.067 ± 0.035 ($p<0.2$) |
| (4) Diabetics with retino-pathy but no cataract (n = 31) | 42 ± 2.1 | 1.31 ± 0.126 ($p<0.01$) | 0.23 ± 0.055† ($p<0.06$) |
| (5) Diabetics with retinopathy and cataract (n = 18) | 50 ± 3.8 | 1.61 ± 0.183 ($p<0.001$) | 0.11 ± 0.06 ($p<0.6$) |

*Tested in 14 subjects.

†Tested in 28 subjects.

p = significance of difference from group (1).

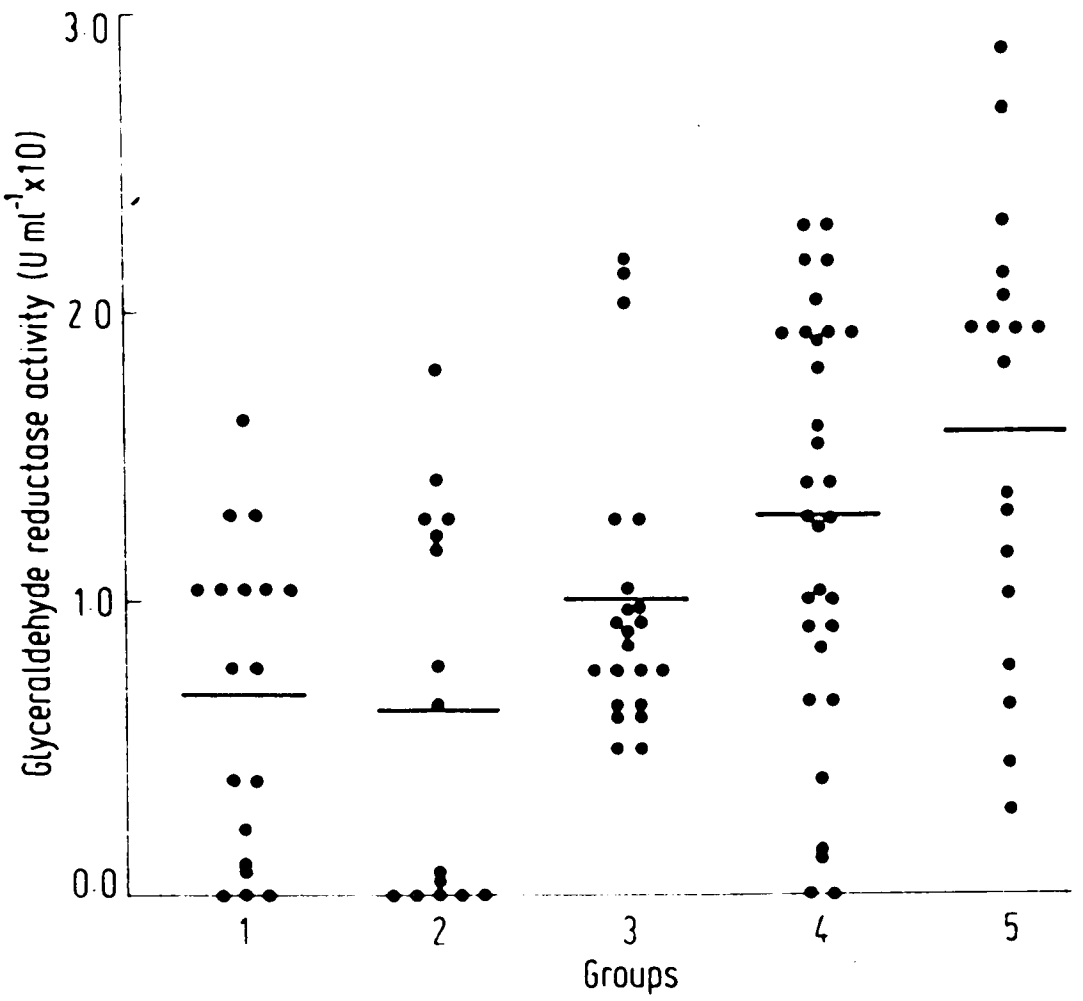



Fig. 3.15.1 **Individual levels of erythrocyte glyceraldehyde reductase activity in groups 1-5.**

Horizontal bars indicate the mean levels of enzyme activity for each group.

difference ($p>0.9$) between glyceraldehyde reducing activity levels in the normal controls (group 1) and the diabetic controls (group 2). There was little difference in the mean glyceraldehyde reducing activity levels between group 3 and 4 ($p<0.2$) and 4 and 5 ($p<0.2$) but groups 2 and 4 were significantly different ($p<0.01$). Although there was some overlap in the rate of glyceraldehyde reducing level between each group, all normal and diabetic control subjects had this activity level lower than $1.8 \times 10^{-1}$ U/ml, whereas 55% of the diabetics with retinopathy and cataract had the values above $1.8 \times 10^{-1}$ U/ml and all the patients in senile cataract group had activity level above $0.45 \times 10^{-1}$ U/ml., whereas 44% of normal controls had activity levels below this value. Juvenile diabetics had a significantly higher mean value ($1.5 \times 10^{-1}$ U/ml $p<0.05$) than maturity onset diabetics ($0.96 \times 10^{-1}$ U/ml). There was no significant correlation between this glyceraldehyde reducing activity and sex or age in any group, r being $<0.4$ in all cases, no correlation was found with glycosylated haemoglobin ($HbA_{1c}$) levels either in diabetics with retinopathy *and* cataract ($r = 0.53$) or with retinopathy ($r = 0.41$). There was no significant change in polyol dehydrogenase activity in diabetics with retinopathy or cataract or non-diabetics with senile cataract within each group, there was no correlation between glyceraldehyde reducing activity and duration of

diabetes. There was no correlation between glyceraldehyde reducing activity and raised body-weight in any of the diabetic groups or the normal control groups. There was also no significant difference in the mean body-weight between any of the groups. The glyceraldehyde reducing activity could be inhibited to 50% of the control value by quercitrin at $10^{-4}$ M (present work), a flavonoid found to delay cataract formation in rodents (Varma et al., 1977).

# CHAPTER 4

## DISCUSSION

### 4.1 Activities of Polyol Pathway Enzymes in Rat, Rabbit, Bovine, Mouse and Human Lens

The pattern of activities of polyol pathway enzymes as emerged from the data presented in Section 3.1 can be summarized as follows. The order of specific activity of aldehyde reductase with DL-glyceraldehyde as aldehyde substrate in adult rat, rabbit, bovine, human and mouse lenses is rat > rabbit > human > bovine >> mouse. The order of total activity of aldehyde reductase expressed as total I.U/lens is rabbit > bovine > rat = human >> mouse. With D-glucose as aldehyde substrate the order of specific activity of aldehyde reductase is bovine > human > rabbit > rat >> mouse. Polyol dehydrogenase activity in these lenses gave a completely different picture, the order of specific activity of polyol dehydrogenase is human >> bovine and that in rat, rabbit and mouse is zero. The relative age difference of these species (Section 3.1) may be responsible for giving such pattern but it seems that it is the species rather than the age that is responsible for this. All the studies on aldehyde reductase, made so far, dealt mostly with animal lens. The present work and the work done by Jedziniak et al. (1981) have clearly shown that cataract

formation in human probably occurs through a mechanism which is different from that in animal lens. More work is needed to evaluate the role of aldehyde reductase in the formation of cataract in human. However, sorbitol was found in human diabetic lens (Pirie and van Heyningen, 1964; Varma et al., 1979). Polyol dehydrogenase actually catalyzes a reversible reaction sorbitol $\rightleftharpoons$ fructose. $V_{max}$ for the reverse reaction, i.e. fructose $\longrightarrow$ sorbitol is 10 times higher than that of the forward reaction (Jedziniak et al., 1981). Hence, Jedziniak et al. (1981) suggested that it is polyol dehydrogenase which has to be investigated to understand development of cataract formation in human. Aldehyde reductase too can use fructose as its substrate but as $V_{max}$ and $K_{cat}$ are 0.02 µmoles/min/mg and 0.015 $sec^{-1}$ respectively reduction of fructose will be very slow (Table 3.11.1). Polyol dehydrogenase needs to be studied in both human and animal lens in order to understand the contribution of these two polyol pathway enzymes to cataract formation in man. However, recently Inagaki et al. (1982) reported that bovine lens aldehyde reductase acts only on the open chain form or aldehyde form of glucose which is present in the solutions in a very small amount when compared with the cyclized form. On the basis of free aldehyde-glucose form, $K_m$ of aldehyde reductase will be 0.66 µM. In other words, glucose is a physiological substrate when only the free aldehyde form is considered.

## 4.2 Properties of Aldehyde Reductase B and Polyol Dehydrogenase

Some properties of crude polyol dehydrogenase have been studied and compared with those of aldehyde reductase in order to evaluate them as enzymes of the same pathway. Aldehyde reductase was assayed with both DL-glyceraldehyde and D-glucose, except where stated otherwise. This was done to see if a multi-substrate enzyme acts on its substrates in an exactly similar manner. Most of the work on lens aldehyde reductase used DL-glyceraldehyde as the main substrate. D-glucose was used besides DL-glyceraldehyde because glucose is more important from the viewpoint of diabetic cataract.

The pH optimum for glyceraldehyde reducing activity was found to be 6.3-6.7 (Fig. 3.7.1.1) and with glucose the pH optimum was 6.4. There is a hint of inflexion at pH 7.4 for both glyceraldehyde and glucose but this could not be confirmed as no single buffer was used in the range pH 7.0-8.4. Variation of $K_{cat}/K_m$ against pH may be done to find out the pka value of the enzyme from which the nature of amino-acid residue responsible for pka value can be deduced. The effect of variation of pH on polyol dehydrogenase activity (Fig. 3.7.1.2) shows that the pH optimum is at 8.9 and a secondary pH optimum is observed at around pH 9.6. Since no buffer was used in the range 8.8-9.5, it is difficult to confirm this hump but

it can be said that the optimum pH of aldehyde reductase is different from that of polyol dehydrogenase. The pH optimum for human lens polyol dehydrogenase, however, has been reported to be broad 8-9.5 for the forward reactions (Jedziniak et al., 1981). The effect of ions on the activity of aldehyde reductase and polyol dehydrogenase enzymes is given in Fig. 3.7.2.1 and 3.7.2.2 respectively and the result is discussed in Chapter 3. Both $(NH_4)_2SO_4$ and $Li_2SO_4$ activated aldehyde reductase approximately 1.8-fold at 0.2 M concentration, whereas $NH_4Cl$ inhibited the enzyme particularly at above I = 0.2 M. So it seems that it is $SO_4^{--}$ ion that is responsible for this activation. There could be a positively charged site on the enzyme at which $SO_4^{--}$ binds and hastens the catalytic rate. Above 0.2 M concentration the decline in activation may indicate the binding of $SO_4^{--}$ ion elsewhere on the enzyme or it could be $NH_4^+$ ion which opposes the activation effect because no such effect is seen with $Li_2SO_4$. As both $NH_4Cl$ and KCl were tried on aldehyde reductase and with KCl, a little activation but no inhibition was observed, it can be said that $NH_4^+$ is inhibitory to aldehyde reductase activity. The effects of $(NH_4)_2SO_4$ and KCl on polyol dehydrogenase are clearly opposite to that on aldehyde reductase. $(NH_4)_2SO_4$ is inhibitory to polyol dehydrogenase, particularly at above I = 0.8 M, whereas KCl activated the enzyme even at as low a concentration as 0.2 M. No other compounds were

tried on polyol dehydrogenase so a conclusion regarding the nature of ion causing this activation or inhibition cannot be made. To get a clear picture of the activities of these enzymes a more detailed and comparative study is needed. The data presented here suggest that when forward reactions of these two enzymes are considered they do not seem to work in unison with each other. However, the pH optimum of human lens polyol dehydrogenase for the reverse reaction, i.e. sorbitol ← fructose, has been found to be 6.8 which is close to that for the forward reaction of aldehyde reductase. $K_m$ of crude bovine lens polyol dehydrogenase for D-sorbitol and $NAD^+$ are 4.81 mM and 40 μM respectively (present work) which are very close to the values obtained for human lens polyol dehydrogenase (Jedziniak et al., 1981). The physiological concentrations of sorbitol and $NAD^+$ in the normal lens are (0.3-2.4)mM and 570 μM respectively (Jedziniak et al., 1981) which indicate that they can be physiological substrates of polyol dehydrogenase.

## 4.3 Purification of Aldehyde Reductase B

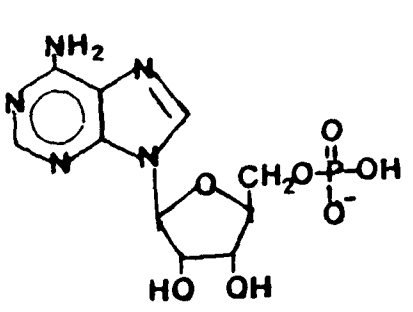
Aldehyde reductase has been purified to different extent by using a combination of gel-filtration and ion-exchange columns (see 2.2.6)as well as by using affinity columns. The 60-85% ammonium sulphate dialysate (see 2.2.6)was used as the starting material for further purification using the columns mentioned above. The 60-85%

ammonium sulphate fraction was found to contain the highest aldehyde reductase activity with both glyceraldehyde and glucose (Fig. 3.3.1.1). Though extensive dialysis was performed to remove ammonium sulphate and a simple conductometer was used to check the conductivity of the dialysate, the possibility of having ammonium sulphate in this fraction cannot be ruled out. $SO_4^{--}$ has been shown to increase the activity of aldehyde reductase by 1.8-fold (Fig. 3.7.2.1) at 0.2 M but above 0.2 M concentration the activity was found to decrease and reach the original value. The concentration of ammonium sulphate is 2 M at 60-85% saturation, so it seems the activity of the enzyme seen in 60-85% ammonium sulphate fraction is not due to ammonium sulphate activation. In the purification of aldehyde reductase using a combination of gel-filtration and ion-exchange columns in a 6-step procedure, a purification of 3,951-fold was obtained with DL-glyceraldehyde as substrate (Table 3.3.1). Using a similar procedure which included an isoelectric focussing column and involved 8 steps, Scheaff and Doughty (1976) reported a 9,250-fold purification. The method of purification reported here is simpler and easier. The SDS polyacrylamide gel electrophoresis photograph (Fig. 3.3.1.7a) obtained with 50 µg enzyme protein is shown which is a single major band. The yield (= 1.2%) by this method, however, was very poor compared to the yield obtained by Scheaff and Doughty

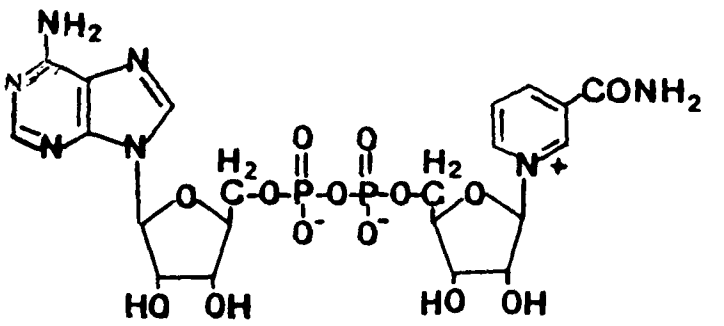
(1976). An interesting finding of the present work is that different extent of purification was obtained when the enzyme was assayed with DL-glyceraldehyde and D-glucose (Table 3.3.1). The work with other aldehyde reductases carried out so far did not simultaneously deal with all or some of its substrates while purifying the enzyme. It seems, although the enzyme showed similar behaviour with glyceraldehyde and glucose in the crude state, some properties are certainly different and the difference becomes obvious as the enzyme becomes more pure. From the present data it appears that the glucose and glyceraldehyde binding sites are probably two close subsites and the glucose binding site is more vulnerable to the damage by purification which could be due to (i) oxidation of some essential amino-acid, i.e. sulphydryl group which has been shown to be involved in glucose binding (Section 3.10.3); (ii) loss of other lens proteins which could have a stabilizing effect on the enzyme especially on the glucose binding site. This reflects on the $K_m$ values of the enzyme for glucose and glyceraldehyde as $K_m$ values are lower for impure enzyme than for the pure enzyme (Table 3.11.1). Both glyceraldehyde and glucose reducing activities were found in the same fraction all throughout the purification procedure mentioned before. The elution from Sephadex-G-200 in gel-filtration experiment with protein markers showed that the enzyme activity resides in the 32 000 molecular

weight fraction and also on SDS gel with the pure enzyme a major band at 40 000 was obtained. Though this evidence can be taken to indicate that this is a single species of enzyme protein, one cannot rule out completely the possibility that they are two very closely related enzymes because if they have the same molecular weight and if there is no molecular association between these forms, both activities will elute at 32 000 molecular weight fraction and SDS gel would not show it. Scheaff and Doughty (1976) used iso-electric focussing column to purify the enzyme but they did not check glucose reducing activity.

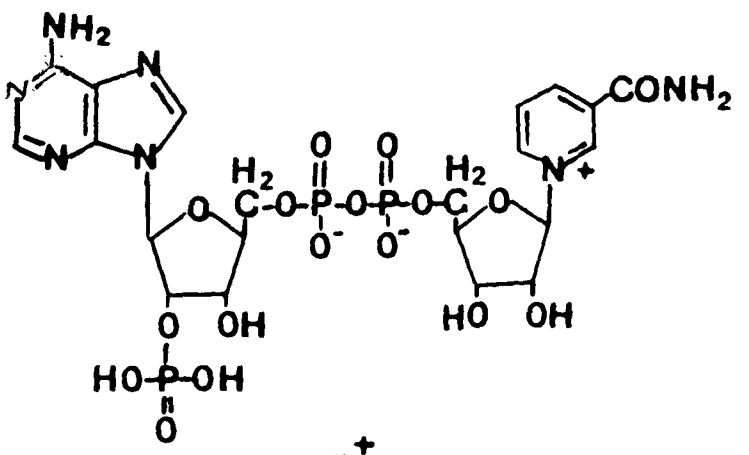
To reduce the number of purification steps and to make the purification a quick and easy method, affinity columns 5'AMP Sepharose, Reactive Red Agarose and NADP Sepharose were tried. The structures of 5'AMP, $NAD^+$ and $NADP^+$ are shown as follows:



5'AMP



$NAD^+$



$NADP^+$

Because of the structural similarities between $NAD^+$/NADH and 5'AMP, $NAD^+$/NADH dependent dehydrogenase or ATP dependent kinases bind to 5'AMP Sepharose. NADH is also a co-substrate for the bovine lens aldehyde reductase, though $K_m$ for NADH (= 1.64 mM) is much higher than that for NADPH ($K_m$ = 9 μM). Hence the enzyme bound to the 5'AMP Sepharose column.

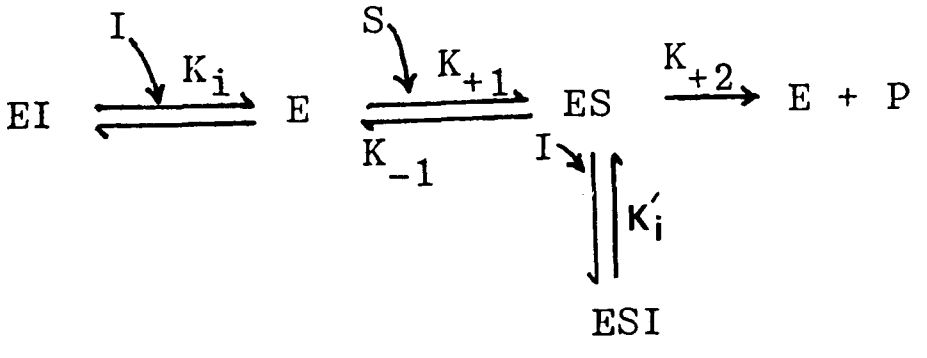
Salt elution from Reactive Red Agarose gave 670-fold purification. When NADPH was used as an eluent with Reactive Red Agarose column 2,461-fold purification (Table 3.3.2.1) was obtained. NADP-Sepharose in just one step gave 1,333-fold purification and 50% yield with NADPH as eluent (Table 3.3.2.2) when compared to 60.-85% $(NH_4)_2SO_4$ fractionation step. Both these columns gave apparently homogenous bands (Fig. 3.3.2.6 and 3.3.2.8a). The rat liver aldehyde reductase was purified using a Procion Red HE3B Sepharose column and a Blue Dextran Sepharose column (Turner and Hryszko, 1980). Porcine brain aldehyde reductase was purified using Blue Sepharose column (Boghosian and McGuinness, 1981). The triazine dyes, i.e. Cibacron Blue F3GA and Procion Red, interact with a specific class of proteins known to possess the dinucleotide fold (Thompson et al., 1975; Watson et al., 1978). Procion Red also showed competitive inhibition with bovine lens aldehyde reductase when NADPH was used as a variable substrate (Fig. 3.9.2.1). Hence

it is likely that the enzyme possesses dinucleotide fold. The purification of lens aldehyde reductase using affinity columns, NADP-Sepharose and Reactive Red Agarose served the purpose of reducing purification steps but there was a common problem associated with this. The enzyme lost its activity very rapidly after elution with NADPH. This could be due to the reasons (i) both Procion Red and $NADP^+$ are inhibitors of aldehyde reductase; (ii) breakdown of NADPH eluent to $NADP^+$ which then inhibited the enzyme. The enzyme eluted from Reactive Red column lost its activity completely within 12 hrs of elution. Extensive dialysis for 20 hrs could recover 36% of its original activity. Enzyme eluted from NADP-Sepharose showed the same phenomenon. For this reason, the enzyme purified by the alternative method using gel-filtration and ion-exchange column chromatography was used for most of the experiments. The SDS polyacrylamide gel electrophoresis (Fig. 3.4.1) and gel-filtration (Fig. 3.4.2) experiments showed that the enzyme is a monomer with a relative molecular weight between 32 000 - 40 000. The amino-acid composition of the enzyme purified using gel-filtration and ion-exchange columns (Fig. 3.3.1.7) showed the predominance of acidic amino-acids, glutamic and aspartic acid in it (Table 3.6.1).

The activity band on polyacrylamide gel with bovine lens aldehyde reductase has been reported (Scheaff and Doughty, 1976) but no activity band was obtained with bovine lens aldehyde reductase following the method of Scheaff and Doughty (1976). The rate of reverse reaction was checked and found to be very slow. This could be the reason why no activity band was obtained as the method uses the reverse reaction (see Section 3.5).

## 4.4 Some Structural Aspects of Aldehyde Reductase B

More information regarding the structure of the enzyme was obtained from the study of kinetics with the inhibitors, sulindac and FPL 58665. Sulindac inhibited the lens aldehyde reductase in in vitro studies (Sharma and Cotlier, 1982) and FPL 58665 has been tried with the rat lens aldehyde reductase (Kador - unpublished work). Both these compounds showed linear non-competitive inhibition with all the three substrates of aldehyde reductase, DL-glyceraldehyde, D-glucose and NADPH (Fig. 3.9.1.1.a,b,c and 3.9.1.2a,b,c). This means that these inhibitors (I) can bind to the free enzyme (E) as well as to the enzyme substrate (ES) complex and this can be represented as follows:




$K_i$ and $K'_i$ = dissociation constants of EI and ESI respectively

$K_{+1}$, $K_{-1}$ and $K_{+2}$ = rate constants

The replots of intercept against inhibitor concentration were all linear. Non-linear slope effect was observed with FPL 58665 when NADPH was used as variable substrates. Sulindac showed non-linear slope effect with NADPH and DL-glyceraldehyde only. Non-linear slope and linear intercept or vice versa or both intercept and slope being non-linear are observed in the case where more than one molecule of inhibitor binds to the enzyme (Cleland, 1963). In other words, the enzyme has more than one site at which the inhibitor can bind. As both sulindac and FPL 58665 showed non-linear slope effect with NADPH, a chemical interaction between these inhibitors and NADPH cannot be ruled out, especially when this effect is very prominent at high inhibitor concentration. Nicotinamide, 2'5' ADP showed competitive inhibition with NADPH as variable substrates which is expected as they are structural analogues of $NADP^+$. Procion Red competes with NADPH for the same site on the enzyme. As Kis value (20 μM) for 2'5' ADP was much lower than that for nicotinamide (Kis = 10 mM) it may be assumed that phosphate groups at 2' and 5' are more essential for binding than nicotinamide moiety of NADPH. The nature of the substrate binding to the enzyme is known from the study of kinetics of initial velocity against variable substrate concentrations. Eadie-Hofstee plots were then drawn to see if there was any deviation from the linearity or, in other words, if this enzyme showed non-Michaelis-Menten

behaviour. Eadie-Hofstee plots with both D-glucose (3.12.1) and NADPH (3.12.2) were linear over 100-fold of concentration range which means that one molecule of glucose or NADPH binds to one molecule of enzyme. In other words, the degree of rate equation is 1:1 for NADPH and D-glucose. But DL-glyceraldehyde showed non-linear kinetics (see Fig. 3.12.3). The data for DL-glyceraldehyde could not be treated in the same manner as the data for glucose and NADPH. Treatment of data on glyceraldehyde required the use of a polynomial function shown below.

The initial velocity (v) of an enzyme catalyzed reaction can be expressed algebraically in the form of a poly-nomial function:

$$V = \frac{\sum_{i=1}^{n} \alpha_i S^i}{1 + \sum_{j=1}^{m} \beta_j S^j} \qquad (1)$$

where S = substrate, $\alpha_i$, $\beta_j$ = kinetic constants

and $\alpha_i$ and $\beta_j \geqslant 0$

Equation (1) can be expanded as follows:

$$V = \frac{\alpha_i S + \alpha_2 S^2 + .... + \alpha_n S^n}{1 + \beta_1 S + .... \beta_m S^m} \qquad (2)$$

where $n \leqslant m$ and the equation (2) is referred to as n:m function.

By feeding the initial velocity data against variable substrate concentrations to the equation (2) the degree of rate equation can be deduced. Computing on initial velocity data against substrate concentrations(with 95% confidence limit) was done using a non-linear BMDP 3R programme (see Section 2.2.16) and an Eadie-Hofstee plot was obtained with predicted velocities. Fig. 3.12.3 shows that the observed data can match with the plot predicted for 2:2 degree of rate equation but fits better with the 3:3 degree so we can say that the minimum degree of rate equation is 2:2. D- and L- glyceraldehyde were not tried separately. More than 1:1 degree of rate equation could be due to the presence of both D- and L-form of glyceraldehyde. A substrate binding experiment with radioactive glyceraldehyde is suggested here. The purity of DL-glyceraldehyde which was used for this work was checked by infrared spectroscopy and found to contain all the usual absorption peaks and no extra peak was observed.

## 4.5 Nature of Amino Acids Involved in Catalysis

To ascertain the nature of the amino acid residues involved in catalysis, chemical modification of aldehyde reductase was carried out with diethylpyrocarbonate, butanedione, phenylglyoxal, potassium cyanate, pyridoxal-5'-phosphate and tetranitromethane.

Time dependent inhibition of enzyme by modifying reagents involves a rapid pre-equilibration of enzyme (E) and inhibitor (I) to give a complex which is irreversibly converted into a modified enzyme (E*) according to $E + I \rightleftharpoons EI \longrightarrow E^*$ - (1) (Kitz and Wilson, 1962). The process has been shown to be pseudo-first order and gives linear semi-logarithmic plots. However, the above process could yield non-linear semi-logarithmic plots that are concave up in practice and usually reach a horizontal asymptote after sufficient time and in that case the process should be described as

$$E_1 \underset{K_{-1}}{\overset{K_{+1}I}{\rightleftharpoons}} E_2 \underset{K_{-2}}{\overset{K_{+2}I}{\rightleftharpoons}} E_3 \cdots E_{n-1} \underset{K_{-(n-1)}}{\overset{K_{+(n-1)}I}{\rightleftharpoons}} E_n$$

where n = number of enzyme species involved.

Time dependent inhibition of rabbit muscle pyruvate kinase by pyridoxal-5'-phosphate (Johnson and Deal, 1970), the inhibition of diamine oxidase by various carbonyl group reagents (Crabbe et al., 1975) were non-linear and showed asymptotic effect. Time dependent inhibition by modifying reagents and protection by substrates have been taken as evidence of a particular residue to which the modifying reagent selectively binds, being present at the substrate binding site.

Diethylpyrocarbonate inhibited the enzyme with both glyceraldehyde and glucose as aldehyde substrates in a time-dependent fashion (Fig. 3.10.1a). Formation of

carboethoxy derivative of histidine is indicated by the increase of absorbance at 250 nm (Fig. 3.10.1b). Both NADPH and NADH offered protection against this inhibition (Table 3.10.1) and the protection is comparable to that obtained by Poulose and Kolattukudy (1980) which is given in Table 3.10.2. The data, therefore, suggest that histidine is probably present at or near the binding site of NADPH which is similar to the finding in pig kidney aldehyde reductase (Flynn et al., 1981).

Phenylglyoxal and butanedione, the two widely used arginine specific reagents, were used in this study. Inhibition of the enzyme with butanedione was time-dependent up to 8 min but with longer incubation time a reactivation phenomenon was observed. However, modification with butanedione can be reversible in borate buffer depending upon the concentration of borate used (Daemn and Riordan, 1974). The pattern of inactivation of human liver alcohol dehydrogenase by butanedione has been reported to be confusing (Lange et al., 1974). Inactivation of bovine lens aldehyde reductase B by phenylglyoxal was very rapid with DL-glyceraldehyde but the time dependent inactivation pattern with D-glucose was found to be a little different (Fig. 3.10.2.1). Inactivation of the enzyme with DL-glyceraldehyde as substrate by both diethylpyrocarbonate and phenylglyoxal was clearly

pseudo-first order which indicates that the inactivation process obeyed scheme (1). Phenylglyoxal, however, was found to be a good substrate of the enzyme with $K_m$ = 7 μM and $V_{max}$ = 0.61 μmoles/min/mg of enzyme. It might be argued that the inhibition effect is nothing but a competition phenomenon between two aldehyde substrates. But because the reagent showed time dependent inhibition and protection against this inhibition was achieved with both NADPH and NADH, phenylglyoxal seems to act as an active site-directed reagent rather than a competitive substrate. Binding studies have shown that horse liver alcohol dehydrogenase modified with phenylglyoxal no longer binds NADH suggesting that arginine is directly involved in binding the cofactor (Lange et al., 1975). But butanedione modified aldehyde reductase binds NADPH (Davidson and Flynn, 1979c), so it seems that probably arginine plays a role in orientating the co-enzyme in proper position for catalysis to occur. Whether phenylglyoxal modified bovine lens aldehyde reductase still binds NADPH is not known because the binding study was not performed. But phenylglyoxal was not found to be competitive with NADPH (present work). It is probable, therefore, that arginine does not directly bind NADPH but it is needed for orientation of the coenzyme. Though the result with butanedione is somewhat confusing, time dependent inhibition with phenylglyoxal and protection with NADH and NADPH strongly suggests that arginine is present near the NADPH binding site and

helps orientate the co-factor in the proper position for catalysis to occur. Arginine may interact with the pyrophosphate moiety of NADPH as suggested for other pyridine nucleotide requiring enzymes (Lange et al., 1974; Poulose and Kolattukudy, 1980). DL-glyceraldehyde when used as a protecting substrate augmented the inhibition by modifying reagents (Table 3.10.1). Glyceraldehyde probably binding to the inhibitor site (degree of binding of glyceraldehyde to the enzyme is 2:2 - Fig. 3.12.3) shows this inhibitory effect.

Both N-ethylmaleimide and p-chloromercuribenzoate inhibited only glucose reducing activity. Glyceraldehyde reducing activity remained unaffected even at 1 mM NEM concentration. This is in contrast to that reported by Jedziniak and Kinoshita (1971) who obtained inhibition of glyceraldehyde reducing activity by NEM (1 mM or above). Glucose reducing activity was inhibited by low (μM) concentration of NEM (Fig. 3.10.3.1). NEM showed time-dependent inhibition up to nearly 5 min, after which an inexplicable reactivation phenomenon was observed which is unexpected of an irreversible covalent modification. Inhibition of glucose reducing activity by phenylglyoxal (Fig. 3.10.2.1) was non-linear unlike glyceraldehyde reducing activity, again with a hint of reactivation. Perhaps a glucose-induced conformational change occurred in the enzyme which resulted in such reactivation. However, dependence of glucose reducing activity on sulphydryl group was shown by the loss of 70% activity after passage through a Sephadex-G-25 column to remove mercaptoethanol and addition of mercaptoethanol

restored the activity (see Section 3.10.3). Glucose reducing activity was also inhibited by p-CMB at the concentrations at which it is specific for sulphydryl group (3.10.3.2). Both NEM and p-CMB inhibition could be prevented by the pre-incubation of the enzyme with glucose alone or glucose and NADPH together (see Section 3.10.3, Table 3.10.1). All this evidence indicates that glucose probably binds to the enzyme via thiol group forming a hemithioacetal linkage.

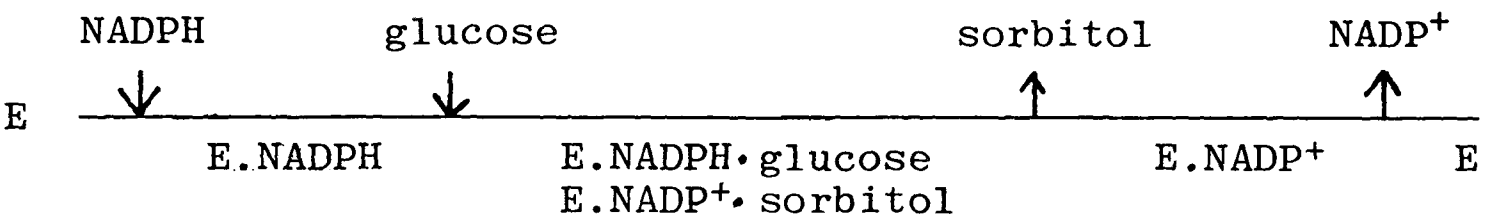
The notion of involvement of tyrosine or tryptophan in the catalytic reaction was rejected on the basis of the result obtained with 0.2% tetranitromethane (see Section 3.10.5). Unlike pig kidney aldehyde reductase (Flynn et al., 1981) the involvement of amino group (lysine) was not found by the use of pyridoxal-5'-phosphate, a fairly specific, widely-used amino group reagent, and KNCO which under the experimental conditions used would have reacted with amino groups.

So, in conclusion, we can say that (1) histidine and arginine are present at or near the NADPH binding site, (2) glucose and glyceraldehyde probably do not bind at the same site, and (3) glucose binding may occur via thiol group. However, the assignment of functional roles to amino acids based solely on chemical modification reactions can be questioned. To confirm this finding, further experiments are to be done on the modification of 100% pure enzyme followed by peptide mapping.

## 4.6 Study of Enzyme Mechanism

The mechanism of enzyme action came from the study of steady-state initial velocity in absence of product as well as from product inhibition study. Both the computed and observed data of initial velocity in absence of product when plotted in double reciprocal space (Fig. 3.13.1a,b,c and d) show that they are all linear and they converge on the second quadrant. The secondary replots (primary slopes or intercepts against $\frac{1}{S}$) were also linear. The computer calculated kinetic parameters and those calculated with the observed data obtained from the slopes and intercepts of the secondary plots were comparable (see Table 3.13.1). The linear and converging nature of double reciprocal plots with initial velocity in absence of product excludes the possibility of both a substituted-enzyme mechanism (ping-pong) and a random-order mechanism. The observed pattern is suggestive of either a Theorell-Chance mechanism or a ternary complex formation mechanism. The product inhibition study shows that $NADP^+$ is a competitive inhibitor with NADPH as variable substrate and a non-competitive one with glucose as variable substrate, and sorbitol is a non-competitive inhibitor with both glucose and NADPH as variable substrates. All the double-reciprocal plots obtained in the product inhibition study with both sorbitol and $NADP^+$ were linear. The type of product inhibition obtained and the initial velocity study in absence of product reject Theorell-Chance mechanism

and a rapid-equilibrium-random order mechanism. The data obtained are compatible with a compulsory order ternary complex formation mechanism in which NADPH binds first to the enzyme. More confirmatory evidence for the latter came from fluorescence study when binding of NADPH to the enzyme was shown by a red shift in the emission spectrum of NADPH and fluorescence quenching (Fig. 3.14.1). However, the product inhibition study was carried out at non-saturating level of the fixed substrate. Further work with fixed substrates at saturating level and study with alternate substrate or product could strengthen the observed data. The mechanism thus seems to tally with that proposed for other dehydrogenases and can be represented as:




The concentrations of sorbitol used for product inhibition were very high because sorbitol did not show measurable product inhibition below 0.4 M. At such high concentrations (0.4 M - 1 M) of sorbitol the viscosity could have interfered with the reaction velocity. At 0.5 M and 1 M sorbitol the viscosity values of the assay mixture were 1.37 and 1.44 cp respectively, which were 25% and 32% higher than that without sorbitol. However, the product inhibition pattern with sorbitol was linear and all the

replots of slopes and intercepts against sorbitol concentration were also linear (Fig. 3.13.4a and b). The double reciprocal plots with $NADP^+$ as inhibitor were all linear (3.13.3.a and b) but the replots of primary intercepts and slopes against $NADP^+$ concentration showed interesting features. With NADPH, $NADP^+$ showed hyperbolic slope effect and the slope effect was very prominent at high $NADP^+$ concentration (= 70 μM). The Kis obtained from the extrapolation of the tangent drawn to the hyperbola is 60 μM. At 50 μM, however, the value reduces to approximately 5 μM which is the dissociation constant for NADPH (see Table 3.13.1). Hence, a random order mechanism of product release at high $NADP^+$ concentration may be operative. However, NADPH induced isomerization of enzyme cannot be ruled out, in which case $NADP^+$ can bind to both forms and show hyperbolic effect. However, at near saturating concentration of NADPH, $NADP^+$ did not show hyperbolic slope effect with glucose but showed hyperbolic intercept effect, which is again more prominent at high $NADP^+$ concentration (= 90 μM) and the tangent drawn to the curve when extrapolated to zero ordinate gave a Kii of 135 μM but at 70 μM it reduces to about 5 μM, which is similar to that observed with NADPH. However, $NADP^+$ used was 98-100% pure (Sigma specification) hence the non-linearity at high $NADP^+$ concentration could be due to a contaminant. It is also worth remembering here that the enzyme has a separate

inhibitor site as evidenced from the study with FPL 58665 and sulindac. Hence, $NADP^+$ could also bind to the inhibitor site and show hyperbolic effect.

The overall rate equation based on the data presented here can be represented as:

$$V = \frac{V_{max}\,[glucose]\,[NADPH]}{K_S^{NADPH} K_m^{glucose} + K_m^{glucose}[NADPH] + K_m^{NADPH}[glucose] + [glucose] \times [NADPH]}$$

An ordered Bi-Bi or iso-Theorell-Chance mechanism has been suggested for pig brain aldose reductase (Boghosian and McGuinness, 1981) but no binding experiment was carried out to distinguish between the two mechanisms. Pig kidney aldehyde reductase (Davidson and Flynn, 1979a; 1979b) and rat brain aldehyde reductase (Rivett and Tipton, 1981) have been reported to follow an ordered Bi-Bi mechanism in which NADPH is the first substrate to bind. The ox brain aldehyde reductase showed a random Bi-Bi mechanism (Bronaugh and Erwin, 1972) and Towes (1967) suggested that rat skeletal muscle glycerol dehydrogenase follows ping-pong mechanism. Ox kidney aldehyde reductase has been reported to follow a di-iso-ordered Bi-Bi mechanism (Daly and Mantle, 1982). As no significant rate was obtained for reverse reaction with bovine lens aldehyde reductase, the initial rate study with two substrates in reverse directions could not be

performed. Hence, a coenzyme dependent isomerization of E-NADPH complex could not be completely ruled out. The fluorescence spectroscopy study showed an interesting quenching effect (Fig. 3.14.1) when NADPH bound to the enzyme. This is in contrast to other dehydrogenases which show fluorescence enhancement which accompanies $NAD^+$/$NADP^+$ binding to the enzyme. This observation was similar to that made by Velick (1958) and Davidson and Flynn (1979c). Velic first noticed a fluorescence quenching with rabbit muscle glyceraldehyde-3-phosphate dehydrogenase and NADH. He interpreted this as the orientation of pyridine ring was not very rigid, which is usually the case where fluorescence enhancement occurs as a result of enzyme-coenzyme interaction, and the binding forces are directed towards adenine and ribose phosphate moiety. No protection experiment, however, was carried out with 2'5' ADP-ribose, an NADPH analogue while performing chemical modification experiments so as to confirm the above suggestion made by Velick.

The data on chemical modification of aldehyde reductase and different extent of purification obtained when glucose and glyceraldehyde were used as aldehyde substrates may raise an interesting question - do glucose and glyceraldehyde bind to the same site? No competitive effect was observed when initial velocity of glyceral-dehyde reduction was studied in presence of glucose

at different concentrations . This can be considered as an additional evidence to the assumption that glucose and glyceraldehyde binding sites are probably different. These could be different but close sub-sites in a broad substrate binding site of the enzyme. However, further experiments using pure enzyme and radioactive substrates, glucose and glyceraldehyde could reveal this structural aspect of aldehyde reductase. However, based on the data presented here, the following structure of the enzyme is proposed:

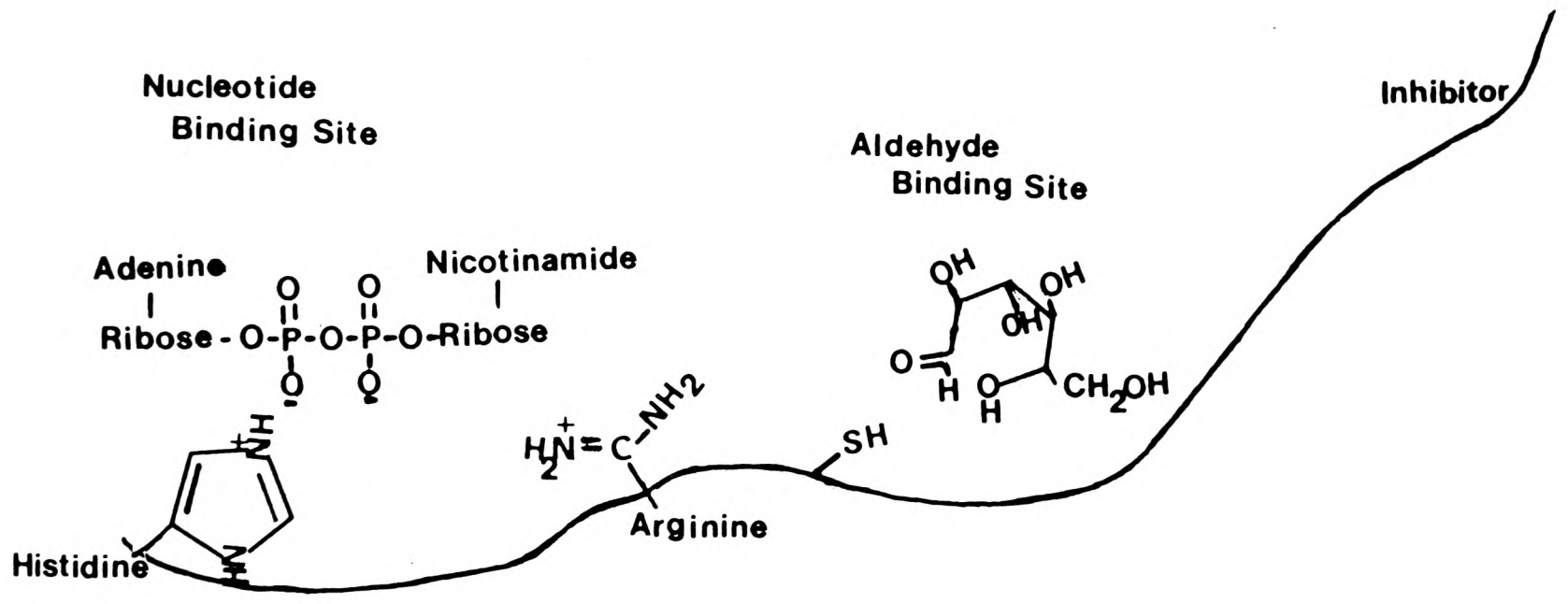



Fig. 4.1 A proposed structure for aldehyde reductase B. Glucose is shown at the aldehyde binding site.

## 4.7 Bovine Lens Aldehyde Reductase and Other Aldehyde Reductases

Bovine lens aldehyde reductase has been found to be similar to other aldehyde reductases. Sometimes different names were used for some of these enzymes but all of these

enzymes possess very similar properties (see Table 1.1). All are monomeric enzymes of 30 000 - 43 000 molecular weight, except calf liver aldehyde reductase (Attwood and Doughty, 1974) and aldehyde reductase from Rhodotorula (Sheys et al., 1971). The latter two have been shown to have a molecular weight of 61 000. Aldehyde reductases have similar amino acid composition, which is characterized by high acidic amino acid content, low proline content, low methionine and low cysteine content. Although many aldehyde reductases have not been studied in great detail, on the basis of the properties reported so far, these enzymes can be divided into two groups, aldehyde reductase A and aldehyde reductase B. DL-glyceraldehyde is a very good substrate of reductase B but not for reductase A. Reductase A is more sensitive to inhibition by sodium barbitone and valproates than reductase B. These two forms are also immunologically distinct protein molecules. Table 1.1 lists different aldehyde reductases from different species and tissues classified into two groups reductase A and reductase B. The nature of amino acid residues at the active centres has been investigated for some aldehyde reductases. For pig kidney aldehyde reductase arginine, histidine and lysine have been suggested to be involved in co-factor binding (Flynn et al., 1981). Two anion binding sites have been proposed for pig kidney aldehyde reductase to one of which binds the carbonyl group containing

substrates or inhibitors and arginine is present at one of these sites (Branlant et al., 1981). The result with bovine lens aldehyde reductase suggests that arginine and histidine are involved in cofactor binding. Glucose probably binds via thiol group. Most of the aldehyde reductases follow ordered Bi-Bi mechanism, except rabbit muscle glycerol dehydrogenase (Towes, 1967) and ox brain aldehyde reductase which follow a random Bi-Bi mechanism (Bronaugh and Erwin, 1972). Ox kidney aldehyde reductase obeys a di-iso ordered Bi-Bi mechanism (Daly and Mantle, 1982). Bovine lens aldehyde reductase, as the present data suggest, obeys an ordered Bi-Bi mechanism and bovine lens aldehyde reductase has been classified as aldehyde reductase B as its properties are similar to those belonging to reductase B group.

## 4.8 Role of Aldehyde Reductase in Diabetic Sequelae

Aldehyde reductase like activity measured in terms of NADPH oxidation with DL-glyceraldehyde or D-glucose as substrates has been reported in most of the tissues that are affected by diabetes. Presence of this activity was detected in the lens (van Heyningen, 1959a,b), in the nerve (Gabbay, 1973), in the retinal mural cells (Buzney et al., 1977). Corneal re-epithelialization in diabetic rats was improved by aldehyde reductase inhibitors (Fukushi et al., 1979) and the corneal endothelial cells were found to contain this activity (Friend et al., 1980).

Alrestatin (Dvornik et al., 1973), quercitrin (Varma and Kinoshita, 1976), sulindac (Sharma and Cotlier, 1982), sorbinil (Datiles et al., 1982) - all these drugs inhibited aldehyde reductase in in vitro studies and all others, except sulindac, delayed cataract formation in experimental animals; alrestatin improved nerve conduction in human patients (Fagius and Jameson, 1981). However, these compounds can also attack other cellular events, i.e. quercitrin can inhibit red cell Ca-pump (Wüthrich and Schatzmann, 1980), alrestatin decreased arginine induced glucagon levels and stimulated insulin release (Lippmann and Kobric, 1978).

Production of sorbitol which could give rise to osmotic pressure and delayed cataract formation by drugs that inhibited aldehyde reductase under in vitro condition havebeen taken as strong evidence that the enzyme plays an important role, if not the key one, in diabetic cataract formation in rats and degus. We now know from accumulating information that cataractogenesis is not a single step process; it is a combined effect of multiple injuries. Hence, in diabetic cataract or in other diabetic complications, apart from sorbitol production, glycosylation of protein (Cerami et al., 1979), NADPH depletion and a defective pentose phosphate pathway and probably defective fat metabolism resulting in membrane disruption too, all could contribute to the

formation of cataract or other diabetic sequelae. A scheme of diabetic cataract and diabetic retinopathy is shown below. Cataract in older diabetics is indistinguishable from those found in non-diabetics. Non-diabetic senile cataract patients were found to have high glucose tolerance curve (Dugmore and Tun, 1981). Hence, it may be assumed that cataract follows a common pathway at least in old age and in diabetes but, of course, cataract in old age may be partly due to other environmental insult like sunlight, etc.

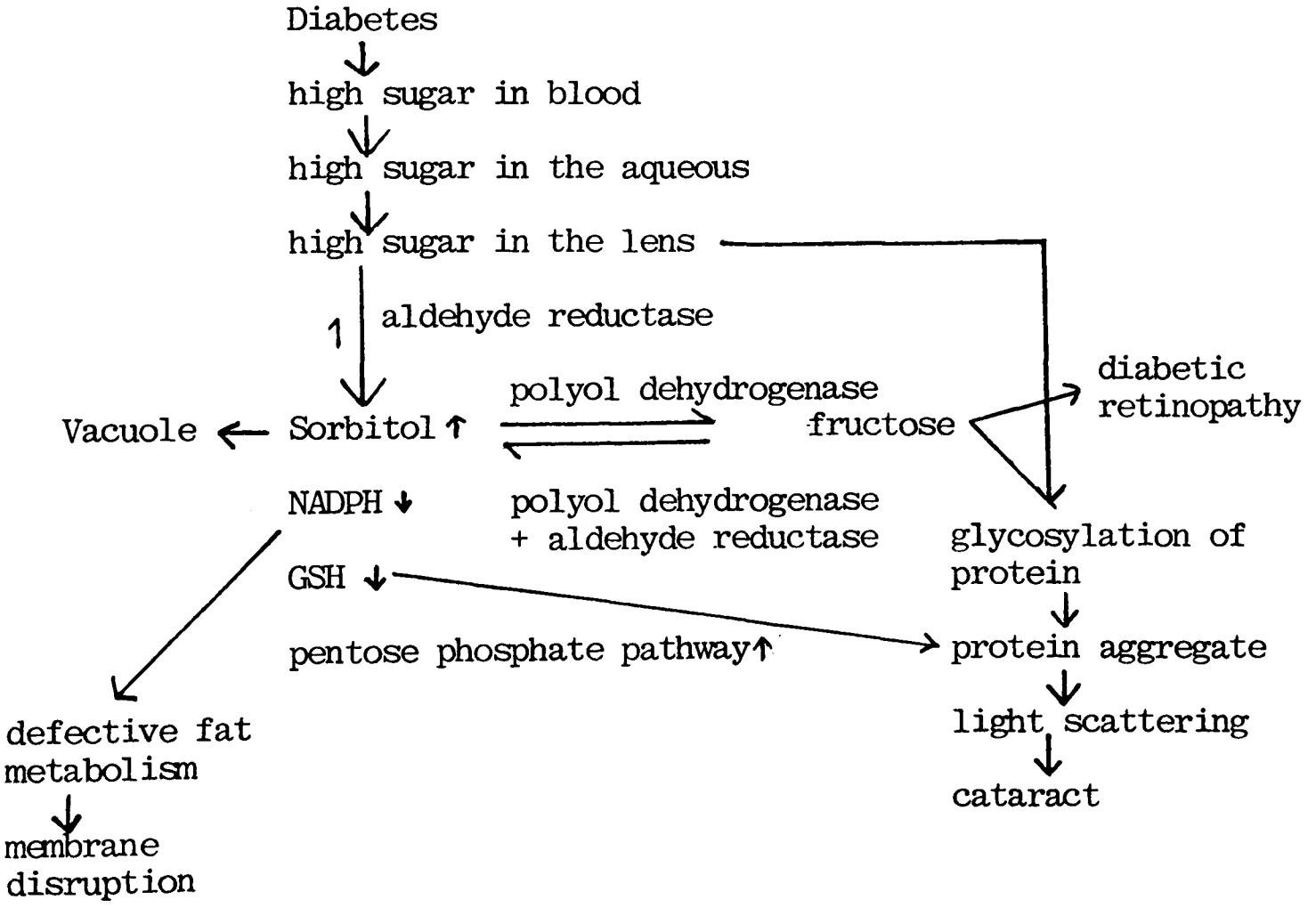



Fig.4.2 Scheme relating aldehyde reductase to diabetic cataract and retinopathy

Glycosylation of lens crystallins was found <u>in vitro</u> and in lenses from diabetic rats. Increased glycosylation

is reported in lenses from human diabetics (Pande et al., 1979) but no correlation was found between extent of glycosylation and the total disulphide content (Ansari et al., 1980). Free fatty acid levels increase in plasma in diabetics. An alteration in membrane phospholipid composition has been reported in cataract (Broekhuyse, 1969). This could be due to decreased level of NADPH which is needed for fat synthesis. High fat diets delayed cataract formation in animals (Patterson and Patterson, 1965; Hutton et al., 1976) even though sorbitol and fructose levels were high. High fat diets may help to maintain NADPH concentration and thus GSH concentration and thus protect the lens from having cataractous changes. Aldehyde reductase, however, can use glyceraldehyde-3-phosphate ($K_{cat}/K_m$ = 0.4 x $10^3$ $M^{-1}s^{-1}$) [see Table 3.11.1] to produce glycerol-3-phosphate which can be used to form phospholipid. Diabetic retinopathy is the most serious ocular problem of diabetic complication. Fructose has been reported to be a diabetic retinopathic agent in rat (Boot-Handford and Heath, 1980). Fructose is produced in polyol pathway and fructose may also be used as a substrate by aldehyde reductase ($K_{cat}/K_m$ = 0.0065 x $10^3$ $M^{-1}s^{-1}$) [see Table 3.11.1]. Retina also contains aldehyde reductase activity (Buzney et al., 1977; Merola et al., 1982).

In human lens, however, polyol dehydrogenase activity is much higher than aldehyde reductase (present work - see

3.1.1; Jedziniak et al., 1981). Polyol dehydrogenase. however, can produce sorbitol from fructose (Jedziniak et al., 1981) but so can bovine aldehyde reductase, although at a very slow rate (present work), and sorbitol was found in human diabetic lens (Pirie and van Heyningen, 1964; Varma et al., 1977). $K_m$ of lens aldehyde reductase for open chain glucose is 0.66 μM (Inagaki et al., 1982). Hence aldehyde reductase will produce sorbitol from glucose under physiological conditions. A glyceraldehyde reducing activity measured in terms of NADPH oxidation has been detected in human blood. Compared with the control groups this activity has been found to be significantly high in diabetics with retinopathy and cataract, diabetics with cataract and non-diabetics with cataract (Table 3.15.1). The activity in blood could be inhibited by quercitrin (present work). Aldehyde reductase has been isolated from red blood cells (Beutler and Guinto, 1974; Petrash et al., 1982). However, this NADPH oxidizing activity could be due to methaemoglobin (Wolff and Crabbe, unpublished work). NADPH oxidation with glyceraldehyde was also measured in human cataract lenses (n = 23) obtained from Edinburgh for a study in collaboration with Dr. R.M. Clayton and Dr. J. Cuthbert in Edinburgh. As no detailed description of the patients was obtained while the work was done, the result could not be produced here, but the preliminary analysis shows that in early cataract the glyceraldehyde reducing activity is higher than that in mature cataract.

Aldehyde reductase activity thus can be related to various events which occur in diabetic sequelae (Fig. 4.2) at least in rat but a look at the results presented in Table 3.11.1 makes it difficult to ascribe a constructive physiological role to lens aldehyde reductase. All the $K_{cat}/K_m$ values are of the order $10^{+3}$ which is much lower than the diffusion controlled rate constant (= $10^9$ $M^{-1}s^{-1}$). The activation energy for glyceraldehyde reduction is approximately 59 and that for glucose reduction 65 KJ respectively. Again, these values are too high for a diffusion-controlled reaction. Hence, it seems none of the substrates studied is the actual physiological substrate for this enzyme. Very recently, Inagaki et al. (1982) have suggested that the enzyme acts on free aldehyde form of glucose and based on the existence of actual amount of free aldehyde $K_m$ for glucose should be 0.66 μM. In that case, glucose will be a physiological substrate but the presence of an enzyme in a tissue for the sole purpose of damaging the tissue seems rather odd. The triose phosphate concentration in the human lens is nearly 1.1 mM (calculated from the value of Cheng, 1980); the $K_m$ of bovine lens enzyme for glyceraldehyde-3-phosphate is 0.2 mM. It suggests that glyceraldehyde-3-phosphate could be metabolized by the enzyme under physiological conditions, though $V_{max}$ and $K_{cat}$ values are very low being 0.014 μmoles/min/mg protein and

0.08 $sec^{-1}$ respectively. Malondialdehyde concentration in normal human lens is around 1 μM and 4-5 μM in cataract lens (calculated from the value given by Bhuyan et al., 1981). $K_m$ of bovine lens enzyme for malondialdehyde is 0.25 mM which discourages malondialdehyde to be a physiological substrate but it suggests that if the cellular concentration of malondialdehyde reaches a very high level by way of lipid peroxidation the enzyme can act on malondialdehyde, a toxic chemical, and thus could save the tissue from peroxidative damage. Malondialdehyde, however, shows substrate inhibition at above 1 mM concentration. This could be due to (1) cross-linking of enzyme molecule by this dialdehyde compound, (2) substrate depletion effect as malondialdehyde polymerizes rapidly.

It is only when a high amount of sorbitol is produced because of high glucose content that aldehyde reductase poses harm to the tissue. Then it is not only sorbitol, other events like glycosylation of protein, NADPH depletion, all together lead to cataract formation. Most of the so-called aldehyde reductase inhibitors inhibit other cellular events besides interfering with sorbitol production. Evaluation of a cataract drug on the basis of its ability to inhibit sorbitol production or inhibit aldehyde reductase under in vitro conditions will be wrong. It is also worth remembering that

sorbitol production by aldehyde reductase is necessary for glucose induced secretion of insulin in pancreas (Gabbay and Tze, 1972) so indiscriminate use of a drug solely for the inhibition of the enzyme could be more harmful than beneficial in the long run.

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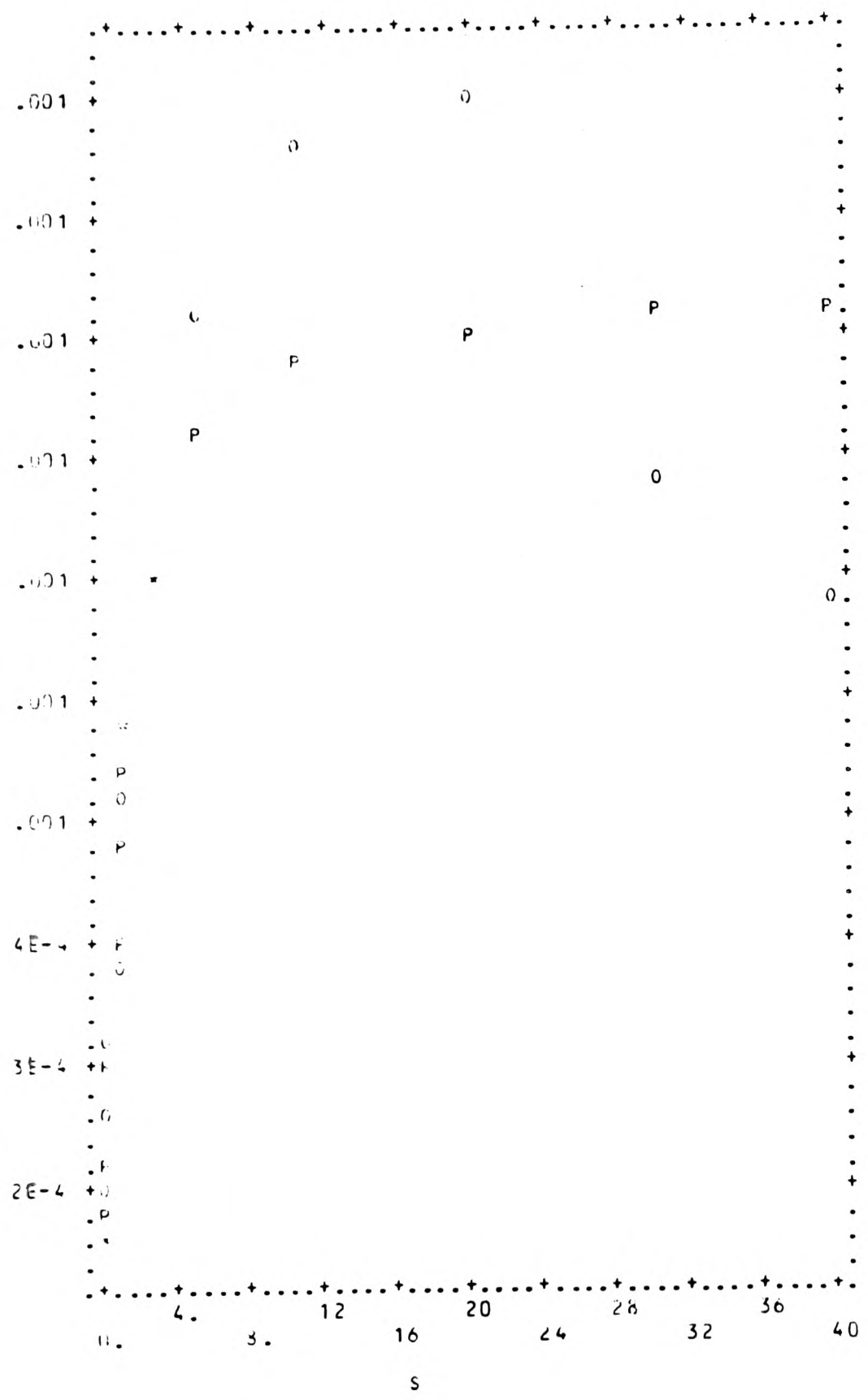
Appendix 1

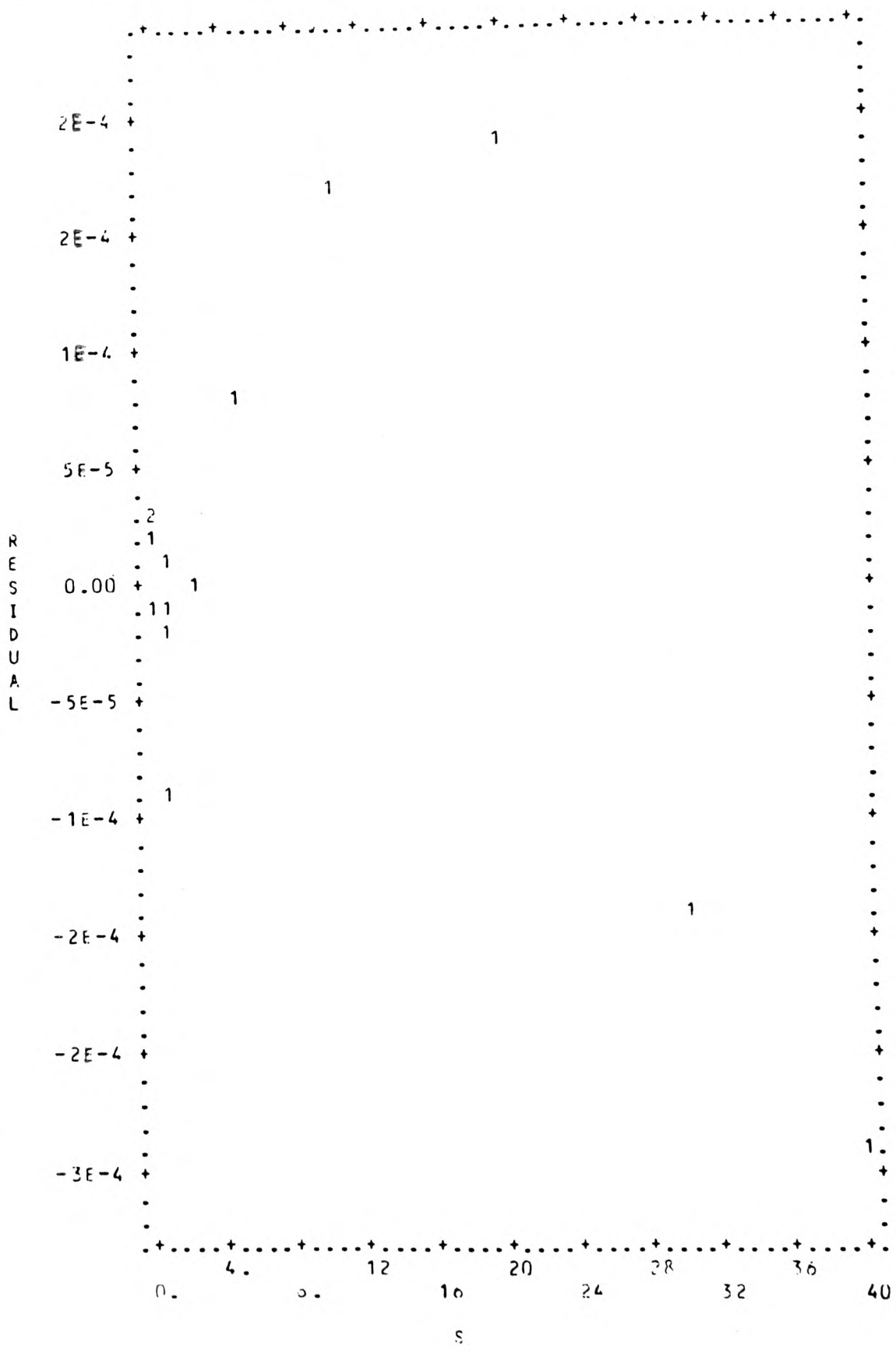
BMDP 3R Non-linear Regression Programme for 1:1 Function

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/PROBLEM      TITLE IS 'AR GLY'.
/COMMENT     'BOVINE LENS'.
/INPUT        VARIABLES ARE 2.
              CASES ARE 14.
              FORMAT IS '(2F9.5)'.
               UNIT IS 6.
/VARIABLE      NAMES ARE S,V.
/PLOT          VARIABLE IS S.
               PROBIT.
               RESIDUALS.
/REGRESSION    TITLE IS 'BEST FIT TO 1:1 FUNCTION'.
                NUMBER IS 4.
                 DEPENDENT IS V.
                 PARAMETERS ARE 3.
                 INITIAL ARE 1.,1.,1.0.
                  MAXIMUMS ARE 999.,999.,999.0.
                  MINIMUMS ARE 0.,0.,0.0.
                  NAMES ARE A,A1,B.
/END
```

| CASE NO. LABEL | PREDICTED V | STD DEV OF PRED VALUE | OBSERVED V | RESIDUAL | S |
|---|---|---|---|---|---|
| 1 | 0.000163 | 0.000075 | 0.000150 | -0.000013 | 0.025000 |
| 2 | 0.000185 | 0.000047 | 0.000200 | 0.000015 | 0.050000 |
| 3 | 0.000225 | 0.000056 | 0.000250 | 0.000025 | 0.100000 |
| 4 | 0.000294 | 0.000076 | 0.000320 | 0.000026 | 0.200000 |
| 5 | 0.000398 | 0.000049 | 0.000380 | -0.000018 | 0.400000 |
| 6 | 0.000473 | 0.000054 | 0.000380 | -0.000093 | 0.600000 |
| 7 | 0.000530 | 0.000056 | 0.000520 | -0.000010 | 0.800000 |
| 8 | 0.000575 | 0.000056 | 0.000580 | 0.000005 | 1.000000 |
| 9 | 0.000703 | 0.000050 | 0.000700 | -0.000003 | 2.000000 |
| 10 | 0.000825 | 0.000044 | 0.000910 | 0.000085 | 5.000000 |
| 11 | 0.000878 | 0.000050 | 0.001050 | 0.000172 | 10.000000 |
| 12 | 0.000908 | 0.000037 | 0.001100 | 0.000192 | 20.000000 |
| 13 | 0.000919 | 0.000040 | 0.000780 | -0.000139 | 30.000000 |
| 14 | 0.000924 | 0.000041 | 0.000680 | -0.000244 | 40.000000 |

PLOTS OF VARIABLE( 1) VERSUS PREDICTED AND OBSERVED VARIABLE( 2) AND VERSUS RESIDUALS.



Appendix 2

IR Spectroscopy of DL-glyceraldehyde (Aldrich, U.K.)

