A Study of Three Enzymes Acting on Glucose in the Lens of Different Species

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1. The activities of three enzymes which act on glucose, namely hexokinase, aldose reductase and glucose dehydrogenase, were measured in extracts of eye lens from cow, calf, rabbit, rat and guinea pig, and in human cataractous lenses. 2. The K_m (glucose) of these three enzymes in extracts of cow lens was found to be 0·12mM, 28mM and 690mM respectively. 3. The physiological importance of hexokinase, aldose reductase and glucose dehydrogenase in the lens of normal and diabetic animals is discussed.

Three enzymes which act on glucose have been reported to be present in eye lens: hexokinase (Green & Solomon, 1959; Nordmann & Mandel, 1955; van Heyningen & Pirie, 1957); aldose reductase (van Heyningen, 1959*a*; Hayman & Kinoshita, 1965); glucose dehydrogenase (van Heyningen, 1964). The reactions catalysed by these enzymes are shown below:

(1) Hexokinase (ATP–D-hexose 6-phosphotransferase, EC 2.7.1.1)

$$\text{Glucose} + \text{ATP} \rightarrow \text{glucose 6-phosphate} + \text{ADP}$$

(2) Aldose reductase (polyol–NADP oxidoreductase, EC 1.1.1.21)

$$\text{Glucose} + \text{NADPH}_2 \rightleftharpoons \text{sorbitol} + \text{NADP}$$

(3) Glucose dehydrogenase [β-glucose–NAD(P) oxidoreductase, EC 1.1.1.47]

$$\begin{array}{lll} \text{Glucose} & \rightleftharpoons \text{glucose } \delta\text{-lactone} & \rightarrow \text{gluconic acid} \\ +\text{NAD(P)} & +\text{NAD(P)H}_2 & \end{array}$$

Phosphorylation of glucose by hexokinase is the initial step in glucose utilization by the Embden–Meyerhof pathway and the hexose monophosphate shunt. Both pathways are active in the lens (Green, Bocher & Leopold, 1955; Kinoshita & Wachtl, 1958). Aldose reductase together with polyol dehydrogenase (L-iditol–NAD oxidoreductase, EC 1.1.1.14) forms the 'sorbitol pathway' (van Heyningen, 1962*a*), for the interconversion of glucose and fructose by lens and other mammalian tissues (van Heyningen, 1959*b*; Kinoshita, Futterman, Satoh & Merola, 1963; Hers, 1957, 1960). Aldose reductase may have an important role in the production of sugar cataracts (van Heyningen, 1959*a*; Kuck, 1961; Kinoshita, Merola & Dikmak, 1962*a*,*b*). It would appear from the findings of these workers that a high concentration of the aldoses, glucose, xylose or galactose in the blood and aqueous humour of animals results in increased formation of the corresponding polyols in the lens. Lens membranes appear to be impermeable to polyols and these accumulate in lens producing a hypertonic condition, which results in movement of water into the lens fibres; these fibres swell and eventually burst with resulting opacity.

The physiological importance of glucose dehydrogenase is still uncertain. The low turnover number and high K_m for glucose of this enzyme and its inhibition by low concentrations of glucose 6-phosphate led Brink (1953) to conclude that liver glucose dehydrogenase has no practical importance in the metabolism of glucose by this tissue. However, since free gluconic acid is present in human lens (van Heyningen, 1964) it seems possible that in this tissue glucose dehydrogenase may have some activity on glucose under physiological conditions. The concentration of glucose in human lens is elevated in diabetes and the concentrations of gluconic acid and sorbitol are also increased (van Heyningen, 1964; Pirie & van Heyningen, 1964).

The present paper reports the activities of hexokinase, aldose reductase and glucose dehydrogenase found in cow, calf, rat, rabbit and guinea-pig lenses and in human cataractous lenses. The K_m for glucose of these enzymes in extracts of bovine lens was also measured in an attempt to make some assessment of the relative contribution of the three enzymes to glucose utilization at abnormal glucose concentrations such as may be found in diabetes.

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MATERIALS

Lenses. Cow and calf eyes were obtained from the local slaughterhouse and the lenses removed as soon as possible (1–2hr. after killing). Rats (albino) and guinea pigs from laboratory suppliers were killed with ether; rabbits from the same source were killed by Nembutal injection. Lenses were removed immediately after death. Human cataractous lenses were removed at surgery and either used immediately or stored frozen for 1–4 days before use.

Chemicals. All enzymes and coenzymes were purchased from Boehringer Corporation (London) Ltd., and were of the highest grade of purity available.

Buffers. For the phosphate buffer, a solution of K_2HPO_4 of the required molarity was titrated with NaH_2PO_4 of the same molarity to the pH value required by using a glass electrode. For the glycine–NaOH buffer, a solution of glycine of twice the required molarity was adjusted to the required pH with 5N-NaOH; the solution was then diluted to a volume twice that of the original glycine solution. For the tris–HCl buffer, tris solution of twice the required molarity was adjusted to the required pH value with 10N-HCl and diluted to twice the original volume of the tris solution.

METHODS

Preparation of lens extract. The lenses were weighed and ground with sand by mortar and pestle with 10mM-phosphate buffer, pH7·2 (2ml./g. of lens). The lens mush was centrifuged at 10000*g* for 30min. at 1–2°. The supernatant fluid was then dialysed with stirring against 6l. of 5mM-phosphate buffer, pH7·2, for 15–20hr. at 2° to remove glucose 6-phosphate, sorbitol and lactic acid, known to be present in lens tissue (van Heyningen, 1962*b*); these compounds would interfere with the enzyme assay procedures (see reactions 1–3). The dialysis of lens extracts decreased high blank rates but did not significantly affect activity of the three enzymes even for dialysis periods of up to 28hr.

Measurement of enzyme activities. All enzyme activities were measured by following the change in extinction at 340mμ measured in a Unicam SP.500 spectrophotometer fitted with a water-jacketed cell compartment maintained at 37°. Two control cuvettes (no substrate added and no enzyme added) were included for each determination of enzyme activity. For all three assay procedures the rate of change of extinction was proportional to the volume of the lens extract added up to a rate of change in extinction of 0·070/min.

Hexokinase. Glucose 6-phosphate formation from ATP and glucose by hexokinase was coupled to reduction of NADP in the presence of added glucose 6-phosphate dehydrogenase (EC 1.1.1.49). Each cuvette contained 300μmoles of tris–HCl buffer, pH7·4, 30μmoles of $MgCl_2$, 30μmoles of glucose, glucose 6-phosphate dehydrogenase (3 international units), 3μmoles of NADP and lens extract. The reaction was started by addition of 12μmoles of ATP (a saturating concentration under all conditions employed), giving a total volume of 3·0ml.

For determination of the pH optimum of this enzyme in cow lens extracts, the activity was measured by the usual assay procedure in 0·1M-phosphate buffer over the pH range 6·0–7·0 and in 0·1M-tris–HCl buffer over the pH range 7·0–8·5.

Aldose reductase. The activity of aldose reductase was determined by a procedure similar to that of Kinoshita *et al.* (1963), involving measurement of the decrease in extinction at 340mμ due to oxidation of $NADPH_2$. Each cuvette contained 300μmoles of phosphate buffer, pH7·2, 300 μmoles of glucose and lens extract. The reaction was started by the addition of 0·6μmole of $NADPH_2$ (a saturating concentration under all conditions employed) to give a final volume of 3·0ml. The non-enzymic decay of $NADPH_2$ was automatically subtracted by using a blank cuvette containing the same incubation mixture as for the test (including $NADPH_2$) but without the addition of lens extract.

The pH optimum of this enzyme in cow lens extracts was determined by measurement of the activity in 0·1M-phosphate buffer, pH5·7–8·0.

Glucose dehydrogenase. The oxidation of glucose catalysed by glucose dehydrogenase was determined by a method similar to that of Metzger, Wilcox & Wick (1964), involving measurement of the increase in extinction at 340mμ due to reduction of NAD. Each cuvette contained 500μmoles of phosphate buffer, pH7·4, 3m-moles of glucose and dialysed lens extract. The reaction was started by addition of 6μmoles of NAD (a saturating concentration under all conditions employed) in a final volume of 3·0ml.

To determine the pH optimum of this enzyme in cow lens extracts, the activity was measured in 0·1M-phosphate buffer, pH5·5–8·0, and in 50mM-glycine–NaOH buffer, pH8·0–10·5.

RESULTS

Hexokinase. Maximum activity of hexokinase in cow lens extracts was observed at pH7·4 (Fig. 1), but activity was not much lower even at pH6·5 and at pH8·0 (65% and 70% of maximum activity respectively). Green & Solomon (1959) reported that hexokinase in homogenates of lenses from young and old rabbits had maximum activity at pH6·5–7·7 and 7·3–8·0 respectively.

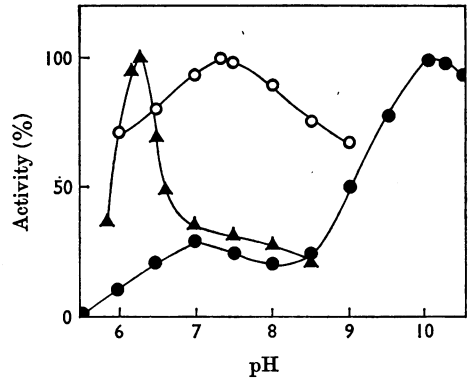

Fig. 1. Effect of pH on the activity of hexokinase (○), aldose reductase (▲) and glucose dehydrogenase (●). The assay conditions are described in the Methods section. Activity is expressed as a percentage of the maximum activity observed for each enzyme.

Lens hexokinase thus appears similar to hexokinase from other animal tissues that have pH optima in the range 6–8 with near maximal activity over a broad pH range (Crane, 1961; Crane & Sols 1955).

The K_m for glucose of hexokinase of cow lens was estimated from the Lineweaver & Burk (1934) plot to be 0·12mM (Fig. 2). Green & Solomon (1959) reported K_m (glucose) values for hexokinase in extracts of young and old rabbit lenses of 0·39mM and 0·29mM respectively. Hexokinases from animal tissues have been found to have K_m (glucose) values in the range 0·1–0·001mM, e.g. calf brain hexokinase (Sols & Crane, 1954), 0·006mM, and human leucocyte hexokinase (Beck, 1958), 0·25mM.

Aldose reductase. Maximum activity of this enzyme in cow lens extract was found at pH 6·3 (Fig. 1). Kinoshita *et al.* (1963) reported that aldose reductase of crude lens extracts had a pH optimum near 6·5 and Hayman & Kinoshita (1965) obtained maximum activity with purified calf lens aldose reductase at pH 5·5 with DL-glyceraldehyde as substrate.

The K_m for glucose of aldose reductase in cow lens extract was estimated from Lineweaver & Burk (1934) plots to be 28·5mM (Fig. 3). This value is in close agreement with that of 33mM for aldose reductase of crude rabbit lens extracts (Kinoshita *et al.* 1963), 70mM for the purified aldose reductase from calf lens (Hayman & Kinoshita, 1965) and 20mM for aldose reductase from the seminal vesicles of sheep (Hers, 1957).

The enzyme was found to have tenfold greater activity with $NADPH_2$ as cofactor than with $NADH_2$, a finding in agreement with that of Hayman & Kinoshita (1965).

Glucose dehydrogenase. The glucose dehydrogenase activity of cow lens extracts increased steadily from pH 5·5 to 7·0, remained fairly constant over the pH range 7·0–8·5 and increased sharply from pH 8·5 to 10·0, exhibiting a maximum in the range pH 10·0–10·5 (Fig. 1). It seems unlikely that the apparent activity at pH 10·0–10·5 is due to a non-enzymic artifact as two blanks, similar to the test except that for one no enzyme was added, and for the second no glucose was added, were included for measurement at each pH value. However, the physiological significance of glucose dehydrogenase activity at this pH seems doubtful and activity was normally measured at pH 7·4. The pH optimum of ox liver glucose dehydrogenase has been reported to be 7·5 (Nakamura, 1954), 8·0 (Brink, 1953), 8·5 (Metzger, Wilcox & Wick, 1965) and 9·8 (Strecker & Korkes, 1952). In a survey of the glucose dehydrogenase of liver of vertebrates, Metzger *et al.* (1965) reported pH optima in the range pH 8·5–8·9 with the exception of the porcine enzyme, which had maximum activity at pH 9·5.

The K_m for glucose of glucose dehydrogenase in extracts of cow lens estimated from Lineweaver & Burk (1934) plots was found to be 640mM, 690mM and 740mM at pH 7·2 in three separate experiments (Fig. 4). It has been reported that the K_m for ox liver glucose dehydrogenase varies with pH (Strecker & Korkes, 1952; Brink, 1953). However, Metzger, Wilcox & Wick (1964) found that the K_m of rat liver glucose dehydrogenase (300–700mM) was independent of pH in the range pH 7·6–9·8. The same workers (Metzger *et al.* 1965) later measured the K_m (glucose) of glucose dehydrogenase from the liver of 13 species of vertebrates including

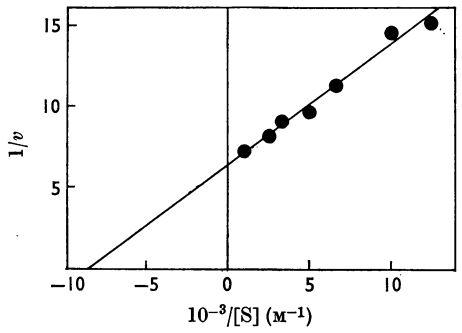

Fig. 2. Lineweaver–Burk plot of the effect of substrate concentration on hexokinase of lens extracts. The velocity units are arbitrary. Reaction rates were measured at pH 7·4 (as described in the Methods section) in the presence of 10mM-$MgCl_2$ and 4mM-ATP with glucose concentrations from 0·08 to 1·00mM. Under these conditions the rate of change of extinction varied from 0·010 to 0·030/min.

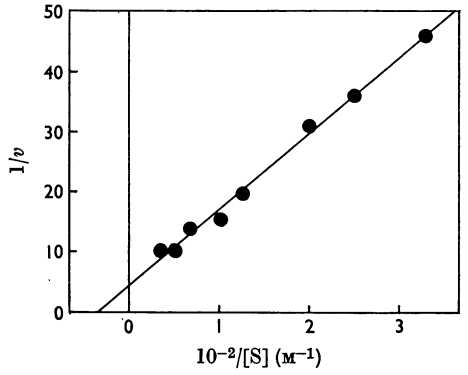

Fig. 3. Lineweaver–Burk plot of the effect of substrate concentration on aldose reductase of lens extracts. Velocity units are arbitrary. Reaction rates were measured (as described in the Methods section) at pH 7·2 with glucose concentrations from 3 to 30mM. Under these conditions the rate of change of extinction varied from 0·010 to 0·050/min.

cow (60mM), pig (60mM), human (160mM), rat (700mM) and duck (2·0M).

The glucose dehydrogenase activity of bovine lens extracts with NADP as hydrogen acceptor was less than 5% of the corresponding activity with NAD. Metzger *et al.* (1964) have reported that NAD was at least three times as effective as NADP as the coenzyme for glucose dehydrogenase from rat liver.

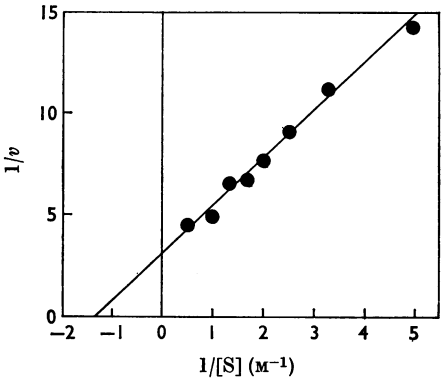

Fig. 4. Lineweaver–Burk plot of the effect of substrate concentration on glucose dehydrogenase of lens extracts. Velocity units are arbitrary. Reaction rates were measured (as described in the Methods section) at pH 7·4 with glucose concentrations from 0·2 to 2·0M. Under these conditions the rate of change of extinction varied from 0·015 to 0·060/min.

Relative activities of the three enzymes in extracts of lens from various species. Table 1 shows the activity of the three systems in cow, calf, guinea-pig, rat and rabbit lens and in cataractous human lens. Hexokinase was detected in all these lenses. The animals used for this study were mature animals of approximately similar age within each group except for the cow and calf, where eyes were obtained from the local slaughterhouse and no information about age was available. The hexokinase activity/g. of calf lens was found to be much higher (about threefold) than the hexokinase activity of cow lens. This result is in agreement with the findings of Green & Solomon (1959) that the hexokinase activity of young rabbit lens/g. of lens was about four times that of old rabbit lens.

All the lenses examined were found to contain aldose reductase, highest activity being observed in guinea-pig and rat lens. Glucose dehydrogenase activity could not be detected in rat lens extracts and was low in extracts of rabbit lens. All other lenses examined contained glucose dehydrogenase, highest activity being found in calf lens.

Table 1. *Activity of hexokinase, aldose reductase and glucose dehydrogenase in extracts of lens from various animals*

The activity of these enzymes was determined by the methods described in the text and expressed as mμmoles of glucose utilized/min./g. of lens tissue. Glucose concentrations in the assay systems for hexokinase, aldose reductase and glucose dehydrogenase were 10mM, 100mM and 1·0M respectively.

	Activity			Activity proportions hexokinase:aldose reductase:glucose dehydrogenase (based on average values for each species)
	Hexokinase	Aldose reductase	Glucose dehydrogenase	
Cow (8 animals): Average	12	32	69	1:3:6
Range	(11–13)	(28–38)	(66–78)	
Calf	35	46	114	
	41	66	123	
	37	55	115	1:1·5:3
Guinea pig	36	134	52	
	63	144	50	
	54	140	50	1:3:1
Rat	25	125	0	
	41	116	1	
	55	118	1	1:3:0
Rabbit	26	85	4	
	34	128	7	
	41	77	12	1:3:0·2
Human (cataractous lens)	25	27	38	
	16	18	26	
	21	24	36	1:1:1·5

DISCUSSION

All lenses examined in this study were found to have aldose reductase and hexokinase activity (Table 1). However, glucose dehydrogenase activity could not be detected in rat lens and was low in rabbit lens, and highest activity of this enzyme was observed in calf lens extracts. van Heyningen (1958) found that calf lenses incubated in a medium containing xylose formed large amounts of xylonic acid and only a small amount of xylitol, whereas under the same conditions (van Heyningen, 1959*a*) rat lenses and rabbit lenses formed xylitol only and xylitol and a trace of xylonic acid respectively. These experiments with xylose (an alternative substrate for glucose dehydrogenase and aldose reductase) reflect the level of activity of glucose dehydrogenase and aldose reductase observed in this study, namely high glucose dehydrogenase and moderate aldose reductase in calf lens, low glucose dehydrogenase and high aldose reductase in rabbit lens, and no glucose dehydrogenase and high aldose reductase in rat lens. It seems possible therefore that the relative importance of these three enzymes in glucose utilization by the normal or diabetic lens may differ in different species.

The K_m values for glucose of hexokinase, aldose reductase and glucose dehydrogenase in bovine lens extract were found to be 0·1 mM, 28 mM and 690 mM respectively. The activities of these three enzymes at saturating concentrations of glucose for each were in the proportions 1:3:6 in bovine lens.

However, the glucose concentration of lens has been reported to be near 0·5 mM in the calf (Kinoshita *et al.* 1963) and in the rat (Kuck, 1963). At this glucose concentration (assuming an even distribution with the lens), hexokinase would be approximately saturated, but aldose reductase and glucose dehydrogenase would have less than 2% of maximal activity. Thus a more realistic comparison of activity of the three enzymes at a glucose concentration near that found in lens would give the proportions $100:3:<1$, assuming that other conditions were not rate-limiting.

Any increase in glucose concentration above 0·5 mM will not greatly increase the activity of hexokinase, but will increase aldose reductase and glucose dehydrogenase activity. Pirie & van Heyningen (1964) found up to 5 μmoles of glucose/g. (approx. 5 mM) of lens in cataractous lenses from diabetic humans. At this glucose concentration aldose reductase is about half as active as hexokinase, assuming all other conditions are optimum.

The increase in activity of aldose reductase with increasing glucose concentration has been shown in experiments with whole rabbit lens incubated in a glucose medium (Kinoshita *et al* 1963). The accumulation of sorbitol was observed to increase with increasing glucose concentration in the medium. Sorbitol is found in small amounts in the lenses of normal animals but the concentration is much increased in lenses of alloxan-diabetic rats and in cataractous lenses from diabetic humans (van Heyningen, 1962*a*; Pirie & van Heyningen, 1964).

Since glucose dehydrogenase has a very high K_m for glucose, the activity of this enzyme in lens must be very low at normal glucose concentrations. van Heyningen (1964) reported that two diabetic human (post-mortem) lenses contained 0·4 and 2·5 μmoles of gluconic acid/g. of lens, whereas in non-diabetic lenses there was less than 0·1 μmole/g. of lens. Glucose dehydrogenase is also found in the liver of a wide variety of vertebrate animals (Ballard, 1965; Metzger *et al.* 1965), but as the K_m for glucose of the liver enzyme is also very high it seems unlikely that the enzyme has any significant activity in this tissue under physiological conditions.

In discussing the activity of these three enzymes in lens and the effect of glucose concentration on activity, it has been assumed that conditions are optimum for each enzyme and that the concentrations of the coenzymes ATP, NAD and $NADPH_2$ are not rate-limiting. The K_m values for the coenzymes of hexokinase, aldose reductase and glucose dehydrogenase were not measured in this study. The K_m for ATP of animal tissue hexokinase is about 0·1 mM (Crane, 1962). i.e. about one-twentieth of the ATP concentration reported for bovine lens (Klethi & Mandel, 1965). If lens hexokinase has a similar K_m value for ATP, then reported ATP concentrations would probably not be rate-limiting for this enzyme.

The K_m for NAD of glucose dehydrogenase from rat liver is reported to be 0·38 μM (Metzger *et al.* 1964) or about one-eightieth of the NAD concentrations reported for cow lens (Bullard, 1965; Klethi & Mandel, 1960). If glucose dehydrogenase of cow lens has a similar K_m (NAD), then the reported NAD concentrations would probably not be rate-limiting. The K_m for $NADPH_2$ of aldose reductase of rat lens was reported by Hayman & Kinoshita (1965) to be 6 μM with glyceraldehyde as substrate. However, no K_m ($NADPH_2$) value with glucose as substrate was given so it is not possible to speculate on the effect of the concentration of this coenzyme in lens on the activity of aldose reductase.

The concentration of coenzymes in the lens is known to vary with the age of the animal (Sippel, 1962) and in different areas of the lens (Mandel & Klethi, 1958). Also, it seems likely that coenzyme concentrations will vary with the glucose concentration in the lens. Sippel (1962) has shown that the concentration of the reduced form of nicotinamide nucleotides is higher in diabetic rat lens than in lenses from normal animals. Thus it is not possible at this stage to assess the effect of the concentra-

tions of coenzyme on the activity of hexokinase, aldose reductase and glucose dehydrogenase in lens.

Animal tissue hexokinases are known to be inhibited by the reaction products, ADP and glucose 6-phosphate (Crane, 1961). The concentrations of both ADP (0·3mM; Klethi & Mandel, 1965) and glucose 6-phosphate (0·12mM; van Heyningen, 1965) in lens are close to the literature K_i values for these compounds with hexokinase from other animal tissues. If the lens enzyme is similarly inhibited, measurement of the hexokinase activity in dialysed lens extracts (Table 1) is possibly an overestimation of the activity *in vivo*.

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