The Metabolism of D-Glyceraldehyde by the Lens

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1. The metabolism of D-glyceraldehyde by the lens was examined.
2. When low concentrations of D-[U-¹⁴C]glyceraldehyde were incubated with lens extracts there was no incorporation of the label into protein; more than two-thirds of the labelled metabolites consisted of glyceric acid and glycerol, their relative proportions depending on the species. Lactic acid, a phosphate, glutathione-glyceraldehyde compounds and a neutral compound were also formed.
3. When high concentrations of D-[U-¹⁴C]glyceraldehyde were incubated with lens, extensive incorporation of the label into protein occurred and the protein became yellow-brown. This coloured protein did not exhibit the fluorescent properties shown by the brown proteins of human cataractous senile lens, or of naphthaquinone-treated lens.
4. Evidence that D-glyceraldehyde is formed by the lens was sought but not found.

In the 1930s it was considered likely that glycolysis might occur by way of non-phosphorylated intermediates. Most of the evidence in support of non-phosphorylating glycolysis rested on conclusions drawn from the use of glyceraldehyde as an inhibitor. Dorfman (1943) reviewed the literature and concluded that there was no positive evidence for the existence of such a pathway. Since then much has been found out about the mechanism by which glyceraldehyde (D- and L-) inhibits glycolysis (Lardy, Wiebelhaus & Mann, 1950; Needham, Siminovitch & Rapkine, 1951; Riddick & Bressler, 1967), but there has been no evidence that glyceraldehyde is concerned in non-phosphorylating glycolysis, or that such a process occurs.

However, it has been suggested (Moonsammy & Stewart, 1967) that glyceraldehyde might be the 'natural' substrate of aldose reductase (EC 1.1.1.21). The K_m of this enzyme isolated from the lens (Hayman & Kinoshita, 1965) for DL-glyceraldehyde ($3 \times 10^{-5} M$) is 0.0005 of the K_m for glucose ($7 \times 10^{-2} M$). Glucose is the only substrate of aldose reductase known to be present in the lens. Because of the high K_m for glucose, aldose reductase in the lens forms sorbitol only to a slight extent under normal conditions but to a greater extent when the glucose concentration is raised, as in diabetes (van Heyningen, 1959). Sorbitol is converted into fructose by a second enzyme, ketose reductase (EC 1.1.1.14). The occurrence of this non-phosphorylating sequence of reactions, and also the oxidation of glucose with formation of gluconic acid under similar conditions (van Heyningen, 1964), prompted the question whether other non-phos-

phorylating pathways might occur in the lens, possibly with the production of glyceraldehyde as the substrate of aldose reductase.

The metabolism of D-GA* by the lens was therefore studied with this idea in mind. Possible pathways of synthesis of D-GA by the lens were also examined.

The fact that the effect of glyceraldehyde, usually DL, has been studied on a variety of systems, and as an inhibitor of tumour growth (e.g. Warburg, Gawehn, Geissler & Lorenz, 1963; Bennett & Cannon, 1966), makes it of value to have more knowledge of the metabolism of the compound itself. Landau & Merlevede (1963) examined the initial reactions involved in the metabolism of D-GA and L-glyceraldehyde by rat liver. Only D-GA was used in the present investigation.

A further reason for the study of the effect of D-GA on the lens was the fact that the lens, intact or disrupted, becomes yellow-brown in colour when incubated in a medium containing D-GA (Süllmann, 1938; Nordmann, Mandel & Achard, 1954). Most of this colour is associated with the non-diffusible fraction, probably protein. Human lens is yellow and becomes a deeper colour with age. It is possible that the colour could be due to the cross-linking of lens proteins caused by glyceraldehyde.

There are at least four known enzymic reactions whereby D-GA can be formed in animal tissues:

* Abbreviation: D-GA, D-glyceraldehyde.

Enzymic reactions in which D-GA may be metabolized in animal tissues include reactions (1), (2) and (3) above, in the reverse direction, and:

Among possible non-enzymic reactions of D-GA in animal tissues are reactions with the thiol groups of cysteine, GSH and protein with the formation of hemimercaptals, mercaptals and (with cysteine) substituted thiazolidinecarboxylic acid (Schubert, 1936; Guidotti, Loreti & Ciaranfi, 1965), and reactions with the amino groups of amino acids and proteins to form Schiff bases of hydroxymethyl derivatives (see Milch, 1963). Reactions with thiol and amino groups are unlikely to be simple and would be expected to lead to intramolecular cyclization and cross-linking (see Harding, 1965).

MATERIALS

Chemicals. D-[U-¹⁴C]GA (lot no. 292-49) was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. It was supplied in ethanolic solution (0.52 mg.; 0.05 mc; 0.5 ml. of 90% ethanol). Examination by paper electrophoresis, chromatography and radioautography (van Heyningen, 1965) showed that contaminants, chiefly glycolic acid, accounted for less than 5% of the radioactivity. D-[U-¹⁴C]Glucose and D-[U-¹⁴C]fructose were obtained from The Radiochemical Centre, Amersham, Bucks., and aqueous solutions of each were made containing 3.3 μC/mg. of the sugar (van Heyningen, 1965). Contaminants were found to account for less than 1% of the total

radioactivity in each case. All labelled compounds were stored at -20°.

D-GA was obtained from Sigma (London) Chemical Co., London S.W.6. It was stored at -20°. Enzymes and coenzymes were supplied by Boehringer Corp. (London) Ltd., London W.5.

Lenses. Bovine lenses were obtained from a slaughterhouse and used at once. Rabbit and rat lenses were removed from the eye and used immediately after the animal had been killed. The animals (Dutch rabbits and albino rats) were bred in the laboratory.

Buffers. Tris and phosphate buffers were prepared by adjusting solutions of tris and Na₂HPO₄ of the required molarity to the required pH with 6M-HCl; 0.1M-triethanolamine buffer was prepared by adjusting a solution of 0.1M triethanolamine hydrochloride to the required pH by addition of M-NaOH.

Antibiotics. A solution of sodium benzylpenicillin and streptomycin sulphate (10mg. of each/ml.) was added in the proportion of 10 μl./ml. of incubation mixture

METHODS

Preparation of dialysed lens extracts for measurement of enzyme activity. Lenses were ground in a mortar with a little sand and three to ten times their weight of triethanolamine buffer, pH 7.4, containing EDTA (final concn., 1 mM). The dispersions were dialysed against this buffer overnight at 4° with stirring, and the insoluble part was then removed by centrifugation at 4° for 30 min. at 12000g.

Measurement of enzyme activity. Reduction of NAD⁺ and oxidation of NADPH by lens extracts in the presence of D-GA were measured in 1 cm. cells at 340 nm. at room temperature in a Unicam SP.500 spectrophotometer. The reaction mixture consisted of lens extract, containing the equivalent of 20–100 mg. of lens, 0.5 ml. of 0.1M-triethanolamine-EDTA buffer, 0.1 ml. of 10 mM-NAD⁺ or 0.03 ml. of 4 mM-NADPH and water to a final volume of 2.9 ml. The reference cell contained all reagents except the coenzyme. To start the reaction 0.1 ml. of 8.1 mM-D-GA was added. The reaction was followed for 10 min. except when the content of glycerol in the reaction mixture was to be measured (see below); in this case the reaction was allowed to continue for 30–60 min.

Assay of glycerol in reaction mixtures. After the extinction at 340 nm. had been read the reaction was stopped by the addition of 1 ml. of 0.5M-sodium acetate buffer. The tube containing the mixture was immersed in water at 100° for 5 min., cooled and centrifuged (Waley & van Heyningen, 1962). The clear supernatant was analysed for glycerol by the method of Wieland (1963).

Radioautography, paper electrophoresis and chromatography, and detection and identification of substances on paper. These were described by van Heyningen (1965). Incubation mixtures were examined without deproteinization. An additional buffer used for paper electrophoresis was barbitone acetate, pH 8.6, supplied by Oxoid Ltd., London S.E.1; this buffer was used to distinguish between glyceric acid and lactic acid, which are partially separated in this buffer after electrophoresis for 3 hr. at 10 v/cm. The radioactivity of a spot of glyceric acid plus lactic acid on a radioautogram was counted. The spot was then sewn into a sheet of paper and subjected to chromatography in butanol-1-ol-acetic acid-water (40:9:20, by vol.) and radio-

autography. The acids were well separated and, although the lactic acid partly evaporates with the acidic solvent, its radioactivity can be assessed from the difference between the value for the combined acids before and that for glyceric acid after chromatography. The results of the assays, done in duplicate, differed by less than 5%.

Assay of total thiol. This was carried out in incubation mixtures by the method of Ellman (1959). For GSH thiol the same method was used, after removal of protein by precipitation with trichloroacetic acid (final concn. 6%, w/v), and the value for protein thiol was obtained by difference.

Incubation of D-[¹⁴C]GA with lens extracts. Whole lenses were ground with washed sand and an equal weight of 0.05 M-phosphate buffer, pH 7.4. The dispersion was centrifuged at 12000g at 4° for 30 min. To the clear extract (0.2 ml.) was added 5 μ l. of 10% (w/v) glucose and 5 μ l. of 11.56 mM-D-GA. The final concn. of D-[¹⁴C]GA was 0.27 mM and that of glucose 13.2 mM. The mixture was contained in a small tube (8 mm. $\times$ 3 mm.), well stoppered and placed upright in a stoppered bottle completely immersed in a water bath at 37°. After incubation for the required time, samples of the mixture (5 μ l. or 10 μ l.) were submitted to paper electrophoresis followed by chromatography and radioautography.

Incubation of D-[¹⁴C]fructose and D-[¹⁴C]glucose with extracts of lens and dispersions of kidney. Extracts of whole lens were made as described above except that 0.1 M-phosphate buffer was used. To two 0.2 ml. samples of the extract was added 5 μ l. of D-[¹⁴C]fructose (containing 0.3 mg. and 1 μ C), and to another two 0.2 ml. samples, 5 μ l. of D-[¹⁴C]glucose (containing 0.3 mg. and 1 μ C). Then 0.01 ml. of freshly neutralized 80 mM-cysteine was added to one tube containing D-[¹⁴C]glucose and one containing D-[¹⁴C]fructose, and 0.01 ml. of freshly neutralized 80 mM-GSH was added to the other two tubes, and all four were incubated at 37° for 1 hr. as described above.

The same procedure was applied to a fine dispersion of rat kidney, prepared by chopping the tissue with scissors and grinding it with an equal volume of 0.1 M-phosphate buffer, pH 7.4, without the aid of sand. After incubation, the products were examined by paper electrophoresis and chromatography followed by radioautography.

Preparation of the thiazolidinecarboxylic acid formed between D-[¹⁴C]GA and cysteine. A 10 μ l. portion of 80 mM-cysteine hydrochloride, 5 μ l. of 11.56 mM-D-[¹⁴C]GA and 0.2 ml. of 0.1 M-phosphate buffer, pH 7, were incubated for 1 hr. at 37°. Separation by paper electrophoresis and chromatography followed by radioautography showed that all the radioactivity was in a charged compound that ran close to, but separate from, spot *e* (Fig. 3). This preparation was stored frozen and used as a reference compound for radioautography.

Attempt to assay triokinase in the lens. (1) The method described by Hers (1962), based on measurement of disappearance of D-GA in the presence of ATP and Mg²⁺, was followed closely, with a dialysed cow lens dispersion (2.0 g. of cow lens ground with 2.0 g. of buffer and dialysed against the same buffer overnight at 4°); 0.5 M-tris buffer, pH 7.2, was used in place of imidazole-HCl buffer. (2) The method of Sherman (1963) was used. It is based on the conversion of a labelled uncharged substrate (D-GA) into a labelled anionic product (D-glyceraldehyde phosphate), which is retained on an anion-exchange paper (DEAE-

cellulose). The course of the reaction is followed by applying portions of the reaction mixture to the paper, removing the uncharged substrate by elution with water and counting the radioactivity retained on the paper. The reaction mixture consisted of 0.13 ml. of dialysed extract of bovine lens (Pottinger, 1967), 45 μ l. of 3% (w/v) ATP (sodium salt), 5 μ l. of 0.1 M-MgCl₂, 5 μ l. of 11.56 mM-D-[¹⁴C]GA and 15 μ l. of 12.5 mM-D-GA. The total volume was 0.2 ml., containing approx. 0.2 μ mole of D-GA. Samples (10 μ l.) were removed for assay at 30 min. intervals.

Preparation of lens proteins coloured yellow by reaction with D-GA. Bovine lens (10 g.) was ground in a mortar, with a little sand and 5 ml. of water, 0.5 ml. of 0.5 M-D-GA, 0.5 ml. of M-phosphate buffer, pH 7, and 0.1 ml. of antibiotic solution. This was incubated for 12 hr. at 37° in a stoppered tube. After incubation the dispersion, now yellow-brown in colour, was dialysed with stirring for 72 hr. at 4° against several changes of water (1 l. each time). The protein was then freeze-dried.

Action of triose phosphate isomerase on D-GA. A 50 μ l. portion of 0.5 M-D-GA was added to 0.95 ml. of 0.05 M-triethanolamine or 0.05 M-phosphate buffer, pH 8.6. Antibiotic solution was added and the buffered D-GA (25 mM) was incubated with or without triose phosphate isomerase (20 μ g. in 2 μ l.) in small stoppered tubes at 37°. After 1 hr. or 24 hr., samples (10 μ l.) were submitted to paper chromatography in ethyl acetate-pyridine-water (12:5:4, by vol.) for 5 hr. with marker spots of D-GA and dihydroxyacetone. Dihydroxyacetone ran faster than D-GA, and was well separated from it. The compounds were detected by the AgNO₃-NaOH method of Trevelyan, Procter & Harrison (1950). A 10 μ l. spot of 0.1 mM-dihydroxyacetone is easily detected by this method, equivalent to a 0.5% conversion of D-GA into dihydroxyacetone.

RESULTS

Structure of D-GA in aqueous solution. The study of glyceraldehyde is complicated by lack of knowledge of the molecular forms in which this compound occurs in solution. Apart from the simple monomeric form, dimeric, monomeric oxycyclo, dioxalane and hydrated forms are possibilities. I used a water solution of syrupy D-GA that had been kept for 2 or more days; the D-[U-¹⁴C]GA was in ethanol and was stored at -20°. Baer & Fischer (1939) recommended heating the dried compound at 55° for 2 hr. *in vacuo*. J. M. Charlton & R. van Heyningen (unpublished work) have not found that this or other treatment either affected its overall metabolism appreciably, or improved its ability to be a substrate for liver triokinase.

Reaction of D-GA with the thiol groups of GSH and protein. GSH (6.7 mM) was incubated with D-GA (33 mM). The mixture was buffered at pH 7.2 with phosphate and tris to a final concentration of 167 mM. Fig. 1 shows that the removal of thiol by D-GA was very slow in the presence of tris and absence of phosphate (line *D*, 0.08 μ mole/hr.). In the presence of 33 mM-phosphate this rate increased to 0.6 μ mole/hr. (line *C*); lines *B* and *A* show that

Fig. 1. Reaction of D-GA with GSH thiol. Incubations were in stoppered tubes at 37°. All tubes contained 0.25 ml. of 0.1 M-D-GA, 0.25 ml. of 0.02 M-GSH and 0.25 ml. of 0.5 M-buffer, pH 7.2, in a total volume of 0.75 ml.; both buffers contained EDTA (1 mM). (A) 0.15 ml. of phosphate + 0.10 ml. of tris buffer; (B) 0.10 ml. of phosphate + 0.15 ml. of tris buffer; (C) 0.05 ml. of phosphate + 0.20 ml. of tris buffer; (D) no phosphate, 0.25 ml. of tris buffer. Two 0.05 ml. samples were taken at intervals and assayed for thiol.

Fig. 2. Reaction of D-GA with protein thiol in the presence and absence of GSH. A 0.1 ml. portion of cow lens extract dialysed against water and containing 21 mg. of protein/ml., 0.1 ml. of 0.2 M-D-GA and 1.9 ml. of 0.1 M-phosphate buffer, pH 7.4, was incubated at 37° in a stoppered tube with or without 0.05 ml. of 0.02 M-GSH. Samples (0.25 ml.) were removed at intervals for assay of GSH and protein thiol. ●, Protein thiol in the absence of added GSH; ■, protein thiol (—) and GSH thiol (----) in the presence of added GSH.

the rates were 0.7 and 0.8 μ mole/hr. with phosphate concentrations of 67 and 100 mM. In another experiment it was shown that in the presence of 167 mM-veronal buffer, pH 7.2, the removal of GSH thiol was at the rate of 0.09 μ mole/hr. The incubated solution became yellow in colour when phosphate, but not tris or veronal, was used.

When an extract of dialysed lens was incubated with D-GA in phosphate buffer there was some removal of protein thiol, but if GSH was added there was no such reaction. The D-GA reacted preferentially with the thiol of GSH (Fig. 2).

Use of D-GA by the whole lens. A number of preliminary experiments were carried out with high concentrations of D-GA (10 or 20 mM) and several hours incubation with intact or ground bovine lens; in these experiments there was yellowing of the lens proteins and, when D-[14 C]GA was used, substantial incorporation of the label into protein. The low-molecular-weight products were qualitatively the same whether intact or dispersed lens was used, and also the same as those formed when much lower concentrations of D-GA were used. Also cow, rat and rabbit lens gave the same products, although there were quantitative differences.

In most experiments a low concentration of D-[14 C]GA was used (0.27 mM) and incubation with lens dispersions was for 15 min. at 35°. Glucose (5 mM) and phosphate buffer were always added.

Fig. 3 is a tracing of a radioautogram, showing the compounds that became labelled after 15 min. incubation with D-GA. There was no incorporation of label into protein in these circumstances, and no unused D-GA remained. Table 1 shows how the radioactivity was distributed among the seven labelled spots, after incubation of cow, rat and rabbit lens. The spots were identified by paper electrophoresis and chromatography, as described by van Heyningen (1965).

(a) Glycerol. Presumably this is derived from the reduction of glyceraldehyde with aldose reductase or alcohol dehydrogenases, and not, as with lens incubated with [U- 14 C]glucose, from the action of alkaline phosphatase on L-3-glycerophosphate. It is not further metabolized by the lens (van Heyningen, 1965).

(b) Glyceric acid. This is presumably formed by the action of aldehyde dehydrogenase and, like glycerol, appears to be an end product.

(c) A phosphate. The mobility of this compound at pH 8.6 and pH 4 and its R_F in various solvents were the same as those of glyceraldehyde 3-phosphate and different from those of phosphoglyceric acid and L-3-glycerophosphate.

(d) Lactic acid. This is presumably L-lactic acid derived from glycolysis of D-glyceraldehyde 3-phosphate. Although trikinase activity in the lens is low (see below) the activities of glycerol kinase (EC 2.7.1.30) and of glycerate kinase (EC 2.7.1.31) are even lower. Methylglyoxal can be formed non-enzymically from D-GA in phosphate buffer (Riddle & Lorenz, 1968), and this would be converted into D-lactic acid by the glyoxalase system; however, the time of incubation was not long enough for this to occur to an appreciable extent,

Fig. 3. Tracing of a radioautogram of the mixture obtained after incubation of a lens extract with D-[U-¹⁴C]GA for 15 min. at 35°. A sample was subjected to paper electrophoresis at pH 4 for 3 hr. in direction 1, followed by chromatography in butan-1-ol-acetic acid-water (40:9:20, by vol.) for 12 hr. in direction 2. Table 1 gives the identity of the labelled spots.

Table 1. Radioactivity in labelled products of metabolism of D-[U-¹⁴C]GA in the lens

A lens extract was incubated with D-[U-¹⁴C]GA (see the Methods section) and a 10 μ l. sample was subjected to two-dimensional paper electrophoresis and chromatography, followed by radioautography. Radioactive spots were cut from the paper and counted on both sides. The two values for each spot were averaged and the background was subtracted.

Spot (see Fig. 3)	Compound	Percentage of total radioactivity		
		Cow	Rat	Rabbit
a	Glycerol	7	44	27
b	Glyceric acid	78	20	44
c	A phosphate	2	2	3
d	Lactic acid	2	18	15
e	D-GA-GSH compounds	2	2	11
f	Uncharged compound(s)	9	14	Trace
g	Glycollic acid	Trace	Trace	Trace
	Glyceric acid Glycerol ratio	11.2	0.45	1.6

especially since the D-GA was rapidly removed by other routes.

(e) Compounds between D-GA and GSH. There

were sometimes several contiguous spots in this region of the paper, all apparently compounds formed with GSH. They were absent if a dialysed extract of lens was incubated with D-GA, but present if GSH (but not GSSG) was added to the dialysed extract. They moved towards the negative pole in electrophoresis at pH 1.6, at similar rates to that of GSH.

(f) A neutral compound. This spot was uncharged at pH 1.6, 4 and 8.6. By chromatography in various solvents (van Heyningen, 1965) it was shown to be composed of three compounds, one of which may be fructose.

(g) Glycollic acid. This was a radioactive contaminant of the D-[¹⁴C]GA preparation; it accounted for about 2% of the total radioactivity. It was not metabolized by the lens, but was found to be slowly formed non-enzymically from D-GA during incubation at 37°.

Oxidation and reduction of D-GA by dialysed lens extracts and NAD(P). The formation of glycerol and glyceric acid, which together account for more than two-thirds of the products of D-[¹⁴C]GA metabolism (Table 1), was examined in dialysed lens extracts.

Aldose reductase is responsible for the reduction of D-GA to glycerol [reaction (6)], the rate with NADPH being 7–10 times as fast as that with NADH (Hayman & Kinoshita, 1965). Alcohol dehydrogenases [reaction (7)] will also bring about this reaction and probably contribute to the reduction of D-GA, but these enzymes have not been investigated in the lens.

Aldehyde dehydrogenase is the enzyme in the lens responsible for oxidation of D-GA to glyceric acid in the presence of NAD⁺, but not NADP⁺ [reaction (5)] (van Heyningen, Pirie & Boag, 1954).

Thus, in the presence of NADP, D-GA is reduced by dialysed lens extracts to give glycerol, and no oxidation takes place; in the presence of NAD, oxidation and reduction take place simultaneously. At pH 7.4 the formation of glyceric acid is more rapid than that of glycerol. Eqn. (10) illustrates how the rates of the two concurrent reactions were assessed:

where x , y and z are the numbers of $m\mu$ moles of NADH, glyceric acid and glycerol formed after a given interval of time.

Increase in extinction at 340 nm. was recorded for 30 or 60 min. From the final reading, x was calculated. The value for z was obtained by analysing the reaction mixture for glycerol, and y was calculated by substitution in eqn. (10). Thus the relative ratio of glyceric acid to glycerol (y/z) formed in the presence of NAD⁺ was obtained.

Table 2. *Formation of glycerol and glyceric acid from D-GA by dialysed lens extracts*

Lenses from adult animals were used. The D-GA was at a final concentration of 0.27 mM, pH 7.4. For other details see the Methods section. Average results of two experiments are given.

Species	Coenzyme ...	Glyceric acid (nmoles/g. of tissue/min.)		Glycerol (nmoles/g. of tissue/min.)			Glyceric acid Glycerol ratio
		NAD ⁺	NADPH	NADH	NADPH	Sum	
Cow		119		45	9	54	2.2
Rabbit		175		175	107	282	0.6
Rat		68		66	103	169	0.4

Since the rate of reduction of NAD⁺ was constant and more rapid during the first few minutes, values for the initial rate of formation of these two products were obtained by substituting the initial rate for x in eqn. (10) and using the value y/z obtained after the longer incubation. This procedure assumes that y/z remains constant during the reaction time.

Table 2 gives the results of these calculations and also the formation of glycerol by lens extracts with NADPH as coenzyme. The ratio of glyceric acid to glycerol formed by the cow lens is higher than that for the rabbit and rat lens, indicating species differences in the relative proportions of enzymes that oxidize and those that reduce D-GA. Table 1 confirms this; when whole lens dispersions are incubated with D-[¹⁴C]GA, the ratio of glyceric acid to glycerol is again higher in incubated dispersions of cow lens than in those of rabbit lens. In both dialysed extracts and dispersions, the initial concentration of D-GA was 0.27 mM and the pH 7.4. Differences in the concentrations of reduced and oxidized coenzymes, and other competing reactions, presumably account for the fact that a higher ratio of glyceric acid to glycerol is found in dispersions of cow and rabbit lens than in dialysed extracts of the lens of the same species with added coenzymes. It is fortuitous that the ratio of glyceric acid to glycerol found with the rat lens was almost the same in the experiments of Tables 1 and 2.

Triokinase in the lens. Triokinase phosphorylates D-GA and dihydroxyacetone [reaction (8)] (Hers & Kusaka, 1953; Heinz & Lamprecht, 1961). An attempt was made to assay the enzyme in the cow lens by the methods of Hers (1962), but the activity was too low to be measured by this method. An experiment based on that of Sherman (1963), in which a dialysed lens extract was incubated with D-[¹⁴C]GA and the acid products were separated by adsorption on DEAE-cellulose paper, showed a fairly rapid formation of acid. However, analysis of the acid products by paper electrophoresis and chromatography, followed by radioautography, revealed that nearly all the radioactivity was

associated with the protein (which streaked in paper electrophoresis at pH 1.6, and stayed at the origin in chromatography in butanol-acetic acid-water). About 5% of the radioactivity appeared in a spot corresponding in position to a phosphate, presumably D-glyceraldehyde 3-phosphate. These results, and also the labelling pattern, show that, compared with aldehyde dehydrogenase and aldose reductase, triokinase is sluggish in the lens.

Search for reactions forming D-GA in the lens. The possibility that fructose might be metabolized in the lens by a non-phosphorylating route by way of D-GA was tested in two ways. The first method was based on the finding that *N*-acetylglucosamine is an inhibitor of the phosphorylation of fructose by hexokinase (EC 2.7.1.1) (Kerly & Leaback, 1957*a,b*). It was thought possible that, if the phosphorylation of fructose was inhibited, the activity of other metabolic routes might be revealed (provided that they were not also inhibited). Cow and rabbit lens dispersions were accordingly incubated with [U-¹⁴C]fructose in the presence or absence of 5 mM-*N*-acetylglucosamine for 20 and 150 min. at 37°. The products were examined by paper electrophoresis, chromatography and radioautography in the usual way. The finding that little lactic acid was formed in the presence of *N*-acetylglucosamine, whereas in its absence nearly all the fructose was broken down to lactic acid in 150 min., shows that phosphorylation of fructose by hexokinase was greatly inhibited. However, there was no evidence of any formation of D-GA; in particular there was no formation of glyceric acid, glycerol or glyceraldehyde-GSH compounds. Sorbitol was formed in both the presence and the absence of the inhibitor.

The second way in which it was hoped to examine the possible formation of D-GA from fructose was based on the fact that D-GA reacts rapidly with cysteine to form a thiazolidinecarboxylic acid derivative. The reaction between GSH and D-GA is, by contrast, slow. However, the product of incubation of a dispersion of rat kidney with [U-¹⁴C]fructose and [U-¹⁴C]glucose in the presence of cysteine (4 mM) or GSH (4 mM) was in all cases only lactic acid. Although this tissue, unlike the

Table 3. *Action of triose phosphate isomerase on D-GA*

All reactions were carried out at pH 7.6 at 37°.

Buffer* (mm)		Other additions	Time of incubation (hr.)	Extent of reaction†	
Triethanolamine	Phosphate			No triose phosphate isomerase	Plus triose phosphate isomerase
50	—	—	1	0	0
50	—	—	24	0	+
50	—	(NH ₄) ₂ SO ₄ (final concn. 100 mm)	24	0	+
45	5	—	24	0	+
—	50	—	24	++	++

* Buffer (0.1 ml.) contained D-[U-¹⁴C]GA (25 mm), with or without triose phosphate isomerase [20 µg. in 2 µl. of 2.8 mm-(NH₄)₂SO₄].

† Conversion of D-GA into dihydroxyacetone: 0, none; +, about 10%; ++, about 30%.

lens, is known to phosphorylate fructose to form fructose 1-phosphate, which is split by ketose 1-phosphate aldolase to give D-GA and dihydroxyacetone phosphate [reaction (1)], no thiazolidinecarboxylic acid was detected in the incubation mixtures containing fructose and cysteine. Since this method did not trap D-GA even when it is known to be formed, the failure to detect formation of a thiazolidinecarboxylic acid when a similar experiment was performed with the lens was to be expected.

Lens colour. A yellow protein was prepared by the reaction of D-GA with an extract of bovine lens. The fluorescence of a solution in water (2 mg./ml.) at pH 10 was measured. With excitation at 298 nm. there was a fluorescence peak at 354 nm.; this is characteristic of proteins in general. With excitation at 350 nm. there was no fluorescence at 450 nm. such as occurs with the brown proteins prepared from senile cataractous human lens (Pirie, 1968), and also with naphthaquinone-treated lens protein reduced with sodium borohydride (van Heyningen & Pirie, 1967).

Action of triose phosphate isomerase (EC 5.3.1.1) on D-GA. Triose phosphate isomerase catalyses the interconversion of D-glyceraldehyde phosphate and dihydroxyacetone phosphate, the equilibrium being in favour of the formation of dihydroxyacetone phosphate. It was found that triose phosphate isomerase also catalyses the conversion of D-GA into dihydroxyacetone, but the rate is extremely low. Table 3 shows that, in triethanolamine buffer and with a high concentration of enzyme, no reaction could be demonstrated after 1 hr. at 37°. After 24 hr. about 10% of the D-GA had been converted into dihydroxyacetone. This is a rate of conversion about 10⁻⁷ of the rate of conversion of glyceraldehyde phosphate (Czok & Bücher, 1960; Burton & Waley, 1968). That the reaction was caused by the triose phosphate isomerase and not the ammonium sulphate solution

in which it is contained was shown by the fact that no reaction occurred when 5 µl. of 2M-ammonium sulphate was added in place of triose phosphate isomerase.

Phosphate, which inhibits the conversion of D-glyceraldehyde phosphate into dihydroxyacetone phosphate, did not affect that of D-GA into dihydroxyacetone at a concentration of 5 mm. However, in the presence of 50 mm-phosphate, D-GA was converted into dihydroxyacetone irrespective of the presence of triose phosphate isomerase, and faster than in the presence of the enzyme in triethanolamine buffer. This is in accord with the findings of Riddle & Lorenz (1968), who showed that a solution of DL-glyceraldehyde in phosphate or bicarbonate, when spotted on to paper for chromatography, was converted into dihydroxyacetone. In their experiments the conversion was quantitative.

DISCUSSION

Use of D-GA by the lens. Most of the D-GA added to a lens, intact or dispersed, is converted into glycerol or glyceric acid (Table 1). The lens does not appear to possess kinases that phosphorylate these products, and the rate of phosphorylation of D-GA itself is low. This situation is different from that in the liver and kidney; these tissues contain kinases that phosphorylate D-GA, glycerol and glyceric acid (see Heinz & Lamprecht, 1967a,b).

Glyceric acid is formed in the lens from D-GA by the action of aldehyde dehydrogenase, with NAD⁺ as coenzyme. In general, aldehyde dehydrogenases of mammalian tissues use only NAD (Jacoby, 1963). It is not known if aldehyde oxidase [reaction (9)] occurs in the lens, but in any case its contribution to the formation of glyceric acid from D-GA is negligible.

Glycerol is formed from D-GA by two types of

dehydrogenases, aldose reductase [reaction (6)] and alcohol dehydrogenase(s) [reaction (7)]. The former was purified from the lens by Hayman & Kinoshita (1965); it is more active with NADP than with NAD. Alcohol dehydrogenases have not been studied in the lens; the enzyme found in most animal tissues uses NAD as coenzyme, but an enzyme that reduces glyceraldehyde to glycerol with NADPH as coenzyme was found in rat liver (Moore, 1959) and skeletal muscle (Toews, 1966). Heinz & Lamprecht (1967*a,b*) consider that, since the enzyme using NAD (EC 1.1.1.1) has K_m for DL-glyceraldehyde $3 \times 10^{-2} \text{ M}$ whereas the form using NADP (EC 1.1.1.2) has K_m $6.2 \times 10^{-4} \text{ M}$, the former is unlikely to operate on this substrate *in vivo*. In the lens, the formation of glycerol from D-GA is probably partly due to enzymes of this type and partly to aldose reductase. The fact that the activity of aldose reductase is higher in the lens of the rat and rabbit than in the bovine lens (Kinoshita, Futterman, Satoh & Merola, 1963; Pottinger, 1967) contributes to the higher proportion of glycerol formed in these species.

The metabolism of D-GA by the lens resembles that of xylose and shares with it an interesting species difference, illustrated by a comparison between the bovine and rat lens. In the bovine lens both D-GA and xylose are oxidized to the corresponding acids, glyceric acid [reaction (5)] and xylonic acid, which is formed by the action of glucose dehydrogenase (EC 1.1.1.47) (van Heyningen, 1958). In the rat lens, however, the oxidizing capacity is much less. Glucose dehydrogenase is absent (Pottinger, 1967) and therefore no xylonic acid is formed from xylose (van Heyningen, 1958); aldehyde dehydrogenase is present but less active (Table 2). In the lens of both species D-GA and xylose are reduced to the corresponding polyhydric alcohols, glycerol and xylitol.

Xylose is not phosphorylated by the lens (or by any mammalian tissue), but the metabolism of glucose resembles that of D-GA in that three routes are possible: reduction, oxidation and phosphorylation. At physiological concentrations of glucose (about 5 mM) the last of these is quantitatively the most important.

Effect of D-GA on the metabolism of glucose by the lens. The effect of D-GA on other aspects of lens metabolism was beyond the scope of this work, but it was shown in a single experiment that its oxidation to glyceric acid by cow lens was accompanied by a shift in glucose metabolism from the formation of lactic acid to that of L-3-glycerophosphate (R. van Heyningen, unpublished work). The same shift occurs when the cow lens is incubated with glucose and a high concentration of xylose; xylonic acid and L-3-glycerophosphate accumulate (van Heyningen,

1958). In both cases, the reason is the increase in the NADH/NAD⁺ concentration ratio as a result of dehydrogenase action on the added substrate. Süllmann (1938) showed that glyceraldehyde decreased the formation by the lens of lactic acid from glucose.

Formation of D-GA by the lens. None of the four enzyme reactions [reactions (1)–(4)] whereby D-GA can be made in animal tissues is known to occur in the lens. Fructose is phosphorylated by lens hexokinase to give fructose 6-phosphate, not the 1-phosphate (R. van Heyningen, unpublished work), and the activity of aldolase on fructose 1-phosphate is very low in the lens (Schmack, Ong & Shah, 1963) [reaction (1)]. Formation of D-GA by transaldolase [reaction (2)] is a possibility, although Hollmann (1964) considers that this enzyme is more likely to be involved in the reverse direction, namely the utilization of D-GA. As xylulose is not known to be present in the lens, there is little likelihood that transketolase operates to yield D-GA [reaction (3)]; and as D-glyceraldehyde phosphate occurs in the lens at a low concentration (J. M. Charlton & R. van Heyningen, unpublished work) it is unlikely that appreciable amounts of D-GA are formed by phosphatase action [reaction (4)].

D-GA and dihydroxyacetone condense non-enzymically in dilute alkali to form D-fructose (and D-sorbose) (Fischer & Baer, 1936; Gutsche *et al.* 1967). Neither this reaction, nor the cleavage of the ketohexoses to yield trioses, has been shown to occur enzymically. The attempts described in the present paper to demonstrate the enzymic cleavage of free fructose, by inhibiting its phosphorylation in the lens, were unsuccessful. So also was an attempt to trap any D-GA formed in the lens and kidney, by the addition of a large excess of cysteine. In spite of the rapid reaction between D-GA and cysteine, no thiazolidinecarboxylic acid was formed, even in a homogenate of kidney, which is known to metabolize fructose by way of D-GA. Heinz & Lamprecht (1967*a,b*) consider that phosphorylation of D-GA itself, and formation of both glyceric acid and glycerol, followed by phosphorylation, may take place in the rat kidney. Presumably the rate of one or more of the enzymic reactions that metabolize D-GA in the kidney is much greater than the reaction between D-GA and cysteine. Ballard (1965) could not detect the presence of labelled D-GA after incubation of rat liver slices with D-[U-¹⁴C]fructose.

The presence of D-GA in the lens was sought, but the values obtained were too low for any reliance to be placed on them, although D-GA added to the lens immediately before precipitation of the proteins with perchloric acid was largely recovered (J. M. Charlton & R. van Heyningen, unpublished work).

Much of the D-GA metabolized by a lens dispersion is oxidized to glyceric acid or reduced to glycerol, which are not metabolized further. These compounds, if present in the lens *in vivo*, could therefore be derived from D-GA, and constitute evidence of its formation. However, glycerol, found in the lens at a concentration of $0.1\text{--}1.0\text{ }\mu\text{mole/g.}$, is probably all formed by the action of a phosphatase on L-3-glycerophosphate (van Heyningen, 1965). Glyceric acid has not been found in the lens; in the rat liver it has been detected at a concentration of $0.02\text{ }\mu\text{mole/g.}$; this value increases to $0.19\text{ }\mu\text{mole/g.}$ after a large intraperitoneal dose of fructose (Kattermann, Dold & Holzer, 1961).

Reaction of D-GA with GSH. The reaction between D-GA and GSH is more complex than that between glyceraldehyde and cysteine, which rapidly condense to form a thiazolidinecarboxylic acid (Schubert, 1936; Ratner & Clarke, 1937; Guidotti *et al.* 1965). When GSH is incubated at 37° at pH 7.2 with a fivefold excess of D-GA, the thiol group is removed, presumably by the formation of a mercaptal or hemimercaptal. The reaction is slow in the presence of tris or veronal buffer, but more rapid in the presence of phosphate; the rate of removal increases with increasing concentration of phosphate buffer (Fig. 1). The reason for this difference in reactivity, according to the molarity and nature of the buffer, may be that the reactive aldehyde is not glyceraldehyde itself, but pyruvic aldehyde (methylglyoxal). Riddle & Lorenz (1968) have shown that DL-glyceraldehyde and dihydroxyacetone are converted non-enzymically into methylglyoxal in the presence of certain anions; phosphate was one of the most effective, and the reaction increased with increasing concentration. Tris buffer also catalysed the conversion, but at a much lower rate. Methylglyoxal is known to be an intermediate in the non-enzymic isomerization of DL-glyceraldehyde and dihydroxyacetone (Speck, 1958).

The rates of removal of GSH thiol by D-GA in the presence of phosphate and tris (Fig. 1) are similar to those given by Riddle & Lorenz (1968) for the conversion of DL-glyceraldehyde into methylglyoxal. The rates of both processes increase with increasing concentration of phosphate, and also with increasing pH (R. van Heyningen, unpublished work; Riddle & Lorenz, 1968).

In the experiments discussed above (Fig. 1) there was fivefold excess of D-GA over GSH. In the experiments in which labelled D-GA was incubated with undialysed lens extracts (Table 1 and Fig. 3), there was an excess of endogenous GSH, and both D-GA and GSH were at lower concentrations (0.27 mM and about 4 mM). In these circumstances three labelled compounds were formed, which partly separated on paper electrophoresis at

pH 8.6 for 3 hr. at 10 V/cm. and had closely similar R_F values in butanol-acetic acid-water (Fig. 3, spot e). The finding that the compounds were formed if lens extract was replaced by 0.05 M -phosphate or 0.05 M -triethanolamine buffer, pH 7.4, containing GSH (4 mM) showed that the reaction was non-enzymic. There was no reaction when GSH was replaced by GSSG.

In the presence of a dialysed lens extract, phosphate buffer and added GSH, some lactic acid was formed from D-GA. This could not have been derived from phosphorylation followed by glycolysis, and was probably D-lactic acid, formed by the action of the glyoxalase system (known to occur in lens) on the hemimercaptal formed from methylglyoxal and GSH (the probable substrate of the glyoxalase system; Cliffe & Waley, 1961). In the absence of GSH no lactic acid was formed by dialysed lens extracts.

The nature of the three D-GA-GSH compounds, formed in the presence or absence of lens, was not examined, except to show that none of them was itself a substrate of the glyoxalase system. The aldehyde group of glyceraldehyde itself probably reacts with the thiol in the presence of excess of GSH, followed by the formation of Schiff bases or hydroxymethyl derivatives with the *N*-terminal amino groups of GSH and possible intramolecular cyclization. The finding that GSSG did not react with D-GA implies that combination with thiol is the first reaction in the formation of these compounds.

Lens colour. Incubation with a high concentration of D-GA is needed to colour the lens protein, and the resulting yellow-brown protein differs from the brown protein of the cataractous senile human lens in that it is partly water-soluble, and does not show the characteristic fluorescence. This finding, together with the evidence that brown proteins prepared by reaction with various quinones closely resemble those from the human lens in solubility and fluorescence spectra (Pirie, 1968), disposes of the theory that reaction with D-GA could be responsible for lens colour.

The coloured protein was prepared by incubating a lens dispersion with D-GA in phosphate buffer (see the Methods section). The condensation product of methylglyoxal and cysteine is dark brown (Schubert, 1935) and it seems likely that the yellowing of the lens protein was in part due to the combination of methylglyoxal with lens protein thiol. Removal of GSH by reaction with excess of glyceraldehyde or methylglyoxal would inhibit glyoxalase activity and favour the accumulation of methylglyoxal. The earlier experiments of Süllmann (1938) and Nordmann *et al.* (1954) were also done under conditions in which some glyceraldehyde would be converted into methylglyoxal.

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