Metabolism of Xylose by the Lens

CALF LENS *IN VITRO*

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When a 21-day-old rat is fed with a diet containing more than 20% of D-xylose, complete opacity of the lens (cataract) results within about 3 weeks (Mitchell, Merriam & Cook, 1937; Darby & Day, 1939, 1940; Sterling & Day, 1951; Booth, Wilson & DeEds, 1953). Loss of weight, diarrhoea and abdominal distension also result from feeding with this diet. If the diet is not begun until the rat is 40 days old, no cataract occurs (Booth *et al.* 1953).

The mechanism whereby xylose brings about cataractous changes in the lens is unknown. The feeding of galactose (Mitchell & Dodge, 1935) or the raising of blood glucose in experimental diabetes (Chaikoff & Lachman, 1933; Patterson, 1953) also causes cataract in young rats. It has been believed that all three sugars act by means of a similar mechanism directly on the lens, by virtue of their elevated levels in the aqueous humour, but the evidence is conflicting (Patterson, 1955).

It was decided therefore to investigate the effect of one of the sugars, xylose, on the lens, first *in vitro* and later *in vivo*, in the hope of elucidating the metabolic changes within the lens. Experiments on galactose cataract in young rats have shown the accumulation of galactose 1-phosphate in the epithelial cells just beneath the lens capsule (Schwarz & Golberg, 1955).

D-Xylose appears to be only slightly metabolized by rats (Blatherwick *et al.* 1936; Miller & Lewis, 1932). Evidence that xylose can be used to some extent in the formation of glycogen in the liver of rats has recently been obtained (Hiatt, 1957). The pathways of xylose metabolism in the animal body are not known, and its phosphorylation by animal tissues has not been demonstrated. Xylulose has been shown to be phosphorylated in the liver to form xylulose 5-phosphate (Hickmann & Ashwell, 1956), and thence it can be metabolized by way of the pentose phosphate cycle. In some bacteria xylose has been shown not to be phosphorylated directly, but converted first into xylulose (Hochster, 1955; Stumpf & Horecker, 1956; Kline & Baron, 1957) and thence into xylulose 5-phosphate. In *Pseudomonas* xylose is rapidly converted into xylonic acid (Lockwood & Nelson, 1946), but the mechanism was not examined.

Only the very young rat is susceptible to xylose cataract; its lens is small (20 mg.) and therefore yields very little experimental material, and it also is difficult to remove without damage. However, the lens of the calf is relatively easy to remove from the eye without perceptible damage and is available in sufficient quantity. It has the further advantage that a considerable amount of information about its acid-soluble phosphates is available (Klethi & Mandel, 1957; van Heyningen & Pirie, 1958). It was decided therefore to make preliminary investigations on the calf lens *in vitro* before investigating the metabolism of the rat lens. The calves were usually about a week old when killed.

In this work evidence of the formation of xylulose and of phosphorylated intermediates of xylose metabolism was sought but not found. Instead, it has been shown that xylose is oxidized to xylonic acid by the intact calf lens *in vitro* by means of a dehydrogenase reducing diphosphopyridine nucleotide (DPN).

EXPERIMENTAL

Methods

Lens dispersions. Calf lens was ground in a mortar until a rather thick smooth gruel was obtained.

Samples (1–2 g.) of this dispersion were poured into weighed centrifuge tubes and the weights of the samples determined by reweighing. Small volumes of concentrated solutions of the substances with which the lens was to be incubated were added to the dispersions to give the desired final concentration and the mixtures stirred with a glass rod, which was then left in the tube. Thus the lens dispersion was scarcely diluted. When glucose was to be present in all the experimental tubes at the same concentration it was added to the dispersion in the mortar. The open tubes were incubated in a water bath at 37°. To stop the reaction ice-cold trichloroacetic acid was added, usually 5 vol. of 6% (w/v), and after a thorough stirring the rod was removed and the mixture centrifuged at 5000 g for 10 min. The extracts were poured off, and analysed at once or stored frozen. When phosphorus compounds were to be analysed, precautions were taken to keep the mixtures as cold as possible after the addition of trichloroacetic acid and centrifuging was at 0° at 10 000 g for 15 min.

Lens extracts for measurement of enzyme activity in a Beckman spectrophotometer. Calf lens was ground in a mortar with twice its weight of water and the dispersion

was dialysed overnight at 4°, against deionized water or 0.9% NaCl. The insoluble part was removed by centrifuging.

Incubation of intact lens. Calf eyes were removed as soon as possible after death and brought to the laboratory at the ambient temperature. With as little handling as possible, and without touching the front surface (on which the epithelium is located), the lens was removed from the eye and placed, front surface upwards, on a watch glass. It was then at once gently slid into an open vessel containing Krebs-Ringer phosphate solution (Umbreit, Burris & Stauffer, 1949), with suitable additions, at 37°, so that it rested on its back surface and was completely covered with fluid. The open vessels were incubated in a water bath at 37° and occasionally gently moved. When lactic acid formation, penetration of xylose or glucose or use of xylose was to be measured, each lens was incubated in a separate vessel (usually with 10 ml. of fluid) and the two lenses from one animal were compared. For the chromatographic analysis of extracts after incubation several lenses (five to ten) were incubated together in a beaker of diameter 7.5 cm. and height 7.0 cm., in a xylose-containing medium, and the same number in a medium without xylose. All the lenses lay on the bottom of the beaker and there was plenty of room for free circulation of the fluid. Usually lenses were incubated in Ringer solution containing 1 mg. of D-glucose/ml., with or without the addition of 4 mg. of D-xylose/ml. On one occasion (see Table 4) 5 mg. of D-xylose/ml. was added. The volume of fluid was about 10 ml./lens.

After incubation the lenses were quickly washed in several changes of deionized water and 5 vol. of ice-cold 6% (w/v) trichloroacetic acid was added. The lenses were disintegrated with a glass rod, and the mixture was chilled for 30 min. at 4° and then centrifuged for 15 min., 12 000 *g* at 0°. The extracts were poured off and analysed at once or stored frozen.

For the measurement of lactic acid, which diffuses into the medium during incubation, the lens plus medium was precipitated with 10 ml. of 10% (w/v) trichloroacetic acid, or else the fluid was poured off and analysed separately.

For the measurement of xylose or glucose, precipitation with Ba(OH)₂ and ZnSO₄ was used (Somogyi, 1945) to remove phosphorylated pentoses and most reducing substances. Usually the lens was ground with 10 ml. of water and 1 ml. of 0.3 N-Ba(OH)₂ solution; 1 ml. of 5% (w/v) ZnSO₄·7H₂O solution was added and after thorough shaking the mixture was filtered through hardened filter paper (Whatman no. 42).

Preparation of lens extracts for paper chromatography and electrophoresis. Trichloroacetic acid extracts were shaken three times with 3 vol. of ether to remove most of the acid. They were then treated in one of several ways. (i) Extracts were neutralized with dil. aq. NH₃ soln. and washed through a column of Amberlite IR-120 (H), 1 cm. in diam. and of length dependent on the volume of extract, neutralized with dil. aq. NH₃ soln., concentrated *in vacuo*, evaporated to dryness in a desiccator and made up to a suitable volume with water. (ii) Extracts were brought to pH 8.2 with dil. aq. NH₃ soln. and fractionated according to LePage (1949) into 'barium salts insoluble at pH 8.2', 'barium salts soluble at pH 8.2 but insoluble in 80% (v/v) ethanol' and 'barium salts soluble at pH 8.2 and in 80% (v/v) ethanol'. Each fraction was treated with Amberlite IR-120 (H). The

fraction containing insoluble barium salts was shaken with several lots of the resin in a small volume of water until no more would dissolve. The combined acid extract and washings were neutralized and concentrated as above. The fraction containing barium salts insoluble in 80% (v/v) ethanol was dissolved in water and passed through a column of the resin as above. For investigation of sugars a neutral solution of the fraction containing barium salts soluble in 80% (v/v) ethanol was passed through a mixed column of Amberlite IR-120 (H) and IR-4B (OH). The neutral effluent was concentrated as above. (iii) The unneutralized trichloroacetic acid extract was washed through a column of Amberlite IR-4B (OH) and the effluent concentrated as above. The effluent contains phosphorylcholine (PC), phosphorylethanolamine (PE), glycerophosphorylcholine (GPC) and glycerophosphorylethanolamine (GPE) (van Heyningen & Pirie, 1958).

Preparation of extracts for estimation of periodate-labile phosphate of α -glycerophosphate. A water solution of the fraction containing 'barium salts soluble at pH 8.2 but insoluble in 80% (v/v) ethanol' from about 3 g. of lens (see above) was made 0.05 N with respect to HCl. It was shaken with successive amounts of Amberlite IR-4B (OH) until the pH was about 6. The α -glycerophosphate (α GP) is absorbed on the resin and GPC and GPE remain in solution. The resin was washed with water and the total α GP was then eluted with three successive lots of 5 ml. of aq. 3 N-NH₃ soln. Periodate-labile P was estimated in the eluate by the method of Olley & Blewett (1950). α GP, GPC and GPE are the only periodate-labile phosphate compounds present in calf lens in appreciable quantities (van Heyningen & Pirie, 1958).

Paper chromatography of lens extracts. Descending chromatography on Whatman no. 1 paper was used. The paper was washed in 2 N-acetic acid, followed by six successive changes of water. The following solvents were used: Phenol-water (100:28, w/v). Butanol-propionic acid-water [prepared fresh by mixing equal volumes of A and B (A, 1246 ml. of butan-1-ol, 84 ml. of water; B, 620 ml. of propionic acid, 790 ml. of water); Benson *et al.* 1950]. Benzene-butan-1-ol-pyridine-water (1:5:3:3, upper phase; Hough, 1950). *iso*Butyric acid-aq. N-NH₃ soln. (100:60; Krebs & Hems, 1953). Propan-1-ol-aq. NH₃ soln. (sp.gr. 0.880)-water (60:30:10; Hanes & Isherwood 1949).

Paper electrophoresis. Horizontal strips of Whatman no. 3MM paper between sheets of polythene and glass were used (Kunkel & Tiselius, 1951). The potential difference along the paper was about 10 v/cm. Pyridine-acetic acid buffer, pH 4, was used (50 ml. of acetic acid, 15 ml. of pyridine and 2.5 l. of water; Grassmann, Hannig & Plöckl, 1955).

Detection and determination of substances after chromatography and electrophoresis. Phosphates were visualized by dipping the papers in the molybdate reagent of Burrows, Grylls & Harrison (1952) and, after drying, exposing the papers to sunlight or to ultraviolet light. P was estimated quantitatively by ashing the paper according to the method of Hanes & Isherwood (1949) and determining the resulting orthophosphate (P_i) by the method of Berenblum & Chain (1938). Nucleotides were located on the chromatograms by their ultraviolet absorption with a Hanovia Chromatolite (Hanovia, Ltd., London) and their positions ringed in pencil.

The AgNO_3 -NaOH method of Trevelyan, Procter & Harrison (1950) was used to detect carbohydrates which react under these conditions. Aniline phthalate (Partridge, 1949) was used to detect aldoses, orcinol-trichloroacetic acid (Klevstrand & Nordal, 1950) to detect heptuloses, and a combination of the two sprays to detect xylulose (Hochster, 1955). Lactones were detected by the hydroxylamine method of Abdel-Akher & Smith (1951).

Other estimations. Lactic acid was measured by the method of Barker & Summerson (1941), glutathione by the method of Grunert & Phillips (1951) and pentose by that of Barrenscheen & Peham (1941). Ascorbic acid was estimated by titration with 2:6-dichlorophenol-indophenol.

Glucose (or reducing sugar) was measured by the method of Nelson (1944). To measure glucose in the presence of xylose, the $\text{Ba}(\text{OH})_2$ - ZnSO_4 extracts were estimated by this method before and after removal of the glucose by yeast fermentation (King & Wootton, 1956). Standards of glucose and xylose were included. The values for xylose were checked by the method of Barrenscheen & Peham (1941).

Phosphate was estimated by the methods of Fiske & Subbarow (1925) and Berenblum & Chain (1938).

Materials

D-Xylose and D-glucose were obtained from British Drug Houses Ltd., and triose phosphate acid esters (dihydroxyacetone phosphate and glyceraldehyde phosphate sodium salts, each approx. 0.02M) from C. F. Böhringer und Söhne G.m.b.H., Mannheim-Waldhof, Germany. D-Xylulose was kindly given by Dr J. S. Fewster; D-xylonic acid was made from D-xylose by way of the cadmium bromide double salt of cadmium xylonate (Elsner, 1935).

RESULTS

Effect of xylose on the formation of lactic acid by lens dispersions

About 70% of the glucose used by rabbit lens maintained in tissue culture is converted into lactic acid (Merriam & Kinsey, 1950; Wachtl & Kinsey, 1957). Measurement of lactic acid formed by the lens under various circumstances may therefore give a guide to the normality of its metabolism. Table 1 shows the formation of lactic acid by dispersions of calf lenses, in the presence or absence of glucose and xylose; xylose is not converted into lactic acid by lens dispersions, but does not prevent the formation of lactic acid from glucose. The small amount of lactic acid formed in the absence of added glucose comes from the glucose originally present in the lens. Table 2 shows that, although no lactic acid is formed, xylose is metabolized by the lens dispersions (about 0.1 mg. used/hr./g. of lens at 37°). This disappearance of xylose is inhibited by 25 mM-iodosobenzoate and 0.1M-iodoacetate but unaffected by fluoride or anaerobiosis. Lower concentrations of the inhibitors were not tried, since the undiluted dispersion contains much protein and glutathione thiol, with which they will also react.

Table 1. *Lactic acid formation in lens dispersions in the presence and absence of glucose and xylose*

For preparation of dispersions see Methods. Incubations in air at 37°.

Length of incubation (hr.)	Additions (mg./g. of lens)		Lactic acid (mg./g. of lens)	
	Glucose	Xylose	Found	Increase on incubation
0	0	0	0.97	—
2.5	0	0	1.21	0.24
2.5	1.3	0	1.87	0.90
2.5	1.1	1.2	1.85	0.88
2.5	0.9	0.7	1.95	0.98
2.5	0	0.9	1.20	0.23
0	0	0	0.93	—
1.5	0	0	1.06	0.13
1.5	20	0	1.81	0.88
1.5	20	30	1.83	0.90
1.5	0	30	1.10	0.17

Table 2. *Use of xylose by dispersions of lens*

Xylose (10 μl .; 10%, w/v) was added/g. of dispersion (for preparation of dispersions, see Methods). Incubation in air (unless stated otherwise) for 3 hr. at 37°. A different lens dispersion was used in each experiment.

Inhibitors	Decrease in xylose (mg./g. of lens)
None	0.22
NaF (0.1M)	0.23
Sodium iodosobenzoate (25 mM)	0.11
None	0.33
None (in N_2)	0.38
None	0.39
Sodium iodosobenzoate (0.01M)	0
Sodium iodoacetate (0.1M)	0.22

Effect of xylose on the phosphorylation of glucose by lens dispersions

The phosphorylation of glucose by adenosine triphosphate (ATP) in lens dispersions, in the presence and absence of xylose, was determined by measuring decrease in pyrophosphate P of ATP (Table 3). The incubation mixture was based on that used by Long (1952). The table shows that xylose does not interfere with the phosphorylation of glucose, but is not itself phosphorylated to form a compound stable to hydrolysis for 10 min. in N-HCl at 100°.

Lactic acid formation by and penetration of sugars into intact calf lens in vitro

Table 4 shows that, with the intact lens, unlike the dispersion, the presence of xylose inhibits the formation of lactic acid from glucose. It also shows that xylose penetrates into the lens and that a relative decrease in the concentration of glucose within the lens is brought about by the presence of xylose in the incubating fluid.

Table 3. *Effect of xylose on the phosphorylation of glucose by lens dispersion*

Mixture A contained 2 g. of lens dispersion, KF (0.05M), $MgCl_2$ (0.005M), KCl (0.042M) and ATP (0.004M approx.), final vol. 6 ml. In each tube, 1 ml. of mixture A was incubated in a final vol. of 1.2 ml., with sugars added as shown below. Reaction was stopped by addition of 5 ml. of 5% (w/v) trichloroacetic acid. P_i was estimated before and after hydrolysis in $N-HCl$ for 10 min. at 100°; difference gives value of the pyrophosphate P of ATP.

Concn. of sugar in final incubation mixture (mg./ml.)	Incubation time (hr.)	Pyrophosphate P of ATP (μ g. of P)	Decrease on incubation
1. None	0	116	—
2. None	1.5	116.5	0
3. Glucose 0.4	1.5	89	27
4. Xylose 6.0	1.5	116	0
5. Glucose 0.4 + xylose 6.0	1.5	85	31

Table 4. *Penetration of xylose and glucose into the intact calf lens and formation of lactic acid in the presence and absence of xylose*

Pairs of calf lenses were incubated at 37°, each lens in 10 ml. of Krebs-Ringer phosphate solution containing 1 mg. of glucose/ml. To the incubating fluid of one lens of each pair 5 mg. of xylose/ml. was added.

Time of incubation (hr.)	Xylose (mg./g. of lens)	Glucose (mg./g. of lens)	Lactic acid in lens plus incubating fluid (mg./g. of lens)	Change due to presence of xylose in medium (mg./g. of lens)	
				Glucose in lens	Lactic acid formation
0	0	0.31	1.02	—	—
	0	0.33	0.97		
1.0	0	0.50	1.70	-0.07	-0.10
	0.87	0.43	1.60		
3.75	0	0.53	1.96	-0.12	-0.31
	1.37	0.41	1.65		
4.5	0	0.42	2.29	-0.08	-0.45
	1.36	0.34	1.84		
5.25	0	0.40	2.36	-0.23	-0.55
	1.23	0.17	1.81		
6	0	0.36	2.63	-0.21	-0.60
	1.48	0.15	2.03		

Table 5. *Lactic acid of intact calf lens incubated in the presence and absence of xylose*

One lens of a pair was incubated in 10 ml. of medium containing no glucose (see Methods) and the other in 10 ml. of the same medium containing 4 mg. of xylose/ml. Incubations were for 2 hr. at 37°. The initial level of lactic acid in calf lens is about 0.93-1.03 mg./g. of lens.

	Lactic acid (mg./g.)
No additions	1.10
With xylose	1.01
No additions	1.15
With xylose	1.15

The fact that no lactic acid is formed from xylose is shown by the finding that in the absence of glucose in the medium the amount of lactic acid formed is approximately the same whether xylose is present or not (Table 5). Presumably, in the

absence of added glucose, the small amount of lactic acid comes from the glucose initially present in the lens.

The intact lens, like the lens dispersions, was shown to metabolize xylose. When a lens was incubated in 5 ml. of the incubating fluid containing 0.5 mg. of xylose for 5 hr. at 37°, analysis showed that 0.135 mg. of xylose had disappeared. The concentration of xylose used in this experiment (0.1 mg./ml.) was much lower than in the standard experimental conditions (4 or 5 mg./ml.), in order that a decrease could be detected.

The above experiments showed that xylose was metabolized by the lens. The finding that the presence of xylose inhibited the penetration of glucose into the intact lens and caused a diminution of production of lactic acid led to a decision to investigate more fully the carbohydrate and phosphate metabolism of the intact lens in the presence and absence of xylose.

Table 6. *Analysis of phosphates in calf lens after incubation of intact lens in a medium containing glucose, or glucose plus xylose*

Five to ten lenses were incubated at 37° for 4-5 hr. in medium containing glucose (1 mg./ml.) and the same number in medium containing glucose (1 mg./ml.) plus xylose (4 mg./ml.) (see Methods). Trichloroacetic acid extracts of the lens were examined for total P, P_i , P contained in compounds which are hydrolysed in 10 min. at 100° in N-HCl to form P_i and P contained in compounds not hydrolysed under these conditions.

Presence of xylose in medium	Total P (mg./g. of lens)	Difference due to xylose	P_i (mg./g. of lens)	Difference due to xylose	P hydrolysed by HCl (mg./g. of lens)	Difference due to xylose	P not hydrolysed by HCl (mg./g. of lens)	Difference due to xylose
-	0.66	-0.01	0.10	0	0.22	-0.04	0.34	+0.03
+	0.65		0.10		0.18		0.37	
-	0.68	-0.09	0.16	-0.01	0.34	-0.18	0.18	+0.10
+	0.59		0.15		0.16		0.28	
-	0.71	0	0.12	0	0.18	-0.09	0.41	+0.09
+	0.71		0.12		0.09		0.50	

Table 7. *Fractionation of calf lenses after incubation in the presence or absence of xylose*

Two lenses were incubated in medium containing glucose (1 mg./ml.) and two in the same medium containing glucose (1 mg./ml.) plus xylose (4 mg./ml.) (see Methods). Trichloroacetic acid extracts of the lenses were partitioned into three fractions with barium and ethanol (see Methods).

Incubated in	Barium salts insoluble at pH 8.2 (mg. of P)	Barium salts soluble at pH 8.2 but insoluble in 80% (v/v) ethanol (mg. of P)	Barium salts soluble at pH 8.2 and soluble in 80% (v/v) ethanol (mg. of P)
Glucose	0.72	0.39	0.16
Glucose + xylose	0.42	0.66	0.14
Difference due to presence of xylose	-0.30	+0.27	-0.02

Phosphates in lens incubated in glucose plus xylose

Table 6 shows the results of several analyses of trichloroacetic acid extracts of lens, after incubation of whole lenses in a medium containing glucose, or glucose plus xylose. It shows that the presence of xylose does not affect the amount of P_i in the lens, but brings about a decrease in the amount of P in compounds which are hydrolysed in N-HCl for 10 min. at 100° to form P_i , and a corresponding increase in phosphate compounds not liberating P_i under these conditions. The former consists almost entirely of the labile phosphate groups of the di- and tri-phosphates of nucleotides, chiefly adenosine and uridine tri- and di-phosphates (ATP, UTP, ADP, UDP) (van Heyningen & Pirie, 1958).

Analysis of lenses fractionated with barium and ethanol (Table 7) (see Methods) showed that the presence of xylose caused a relative decrease in the fraction containing barium salts insoluble at pH 8.2, thus confirming the loss of nucleotide di- and tri-phosphates, since these, together with P_i , constitute almost the whole of this fraction (van Heyningen & Pirie, 1958). The table also shows an increase in the fraction containing barium salts insoluble in 80% (v/v) ethanol, and it is in this

fraction therefore that the 'difficultly hydrolysable' compound or compounds formed as the result of incubation in xylose is to be found. The monophosphates of pentoses, hexoses and sedoheptuloses would occur in this fraction if present as a result of the phosphorylation of xylose (Hochster, 1955). It has already been shown that normal calf lens contains only trace amounts of these substances (van Heyningen & Pirie, 1958).

Chromatography of fraction containing barium salts insoluble in 80% (v/v) ethanol

α -Glycerophosphate. Amounts of extract containing about 10 μ g. of P were chromatographed in phenol-water and the paper was developed for the detection of P-containing compounds. Preparations from lens incubated with glucose or glucose plus xylose showed only two spots, P_i (R_F about 0.18) and a spot of R_F about 0.36. In unincubated lens this spot has been identified with α GP, which accounts for about 5.5% of the acid-soluble P in calf lens (van Heyningen & Pirie, 1958). When spots of authentic α GP were superimposed on spots of the lens extracts, the compound in the lens and the α GP did not separate after paper electrophoresis and chromatography in several solvents (phenol-water; butan-1-ol-propionic acid-water; propanol-aq. NH_3 soln.-water).

On one occasion P was estimated in the spots corresponding to α GP after paper electrophoresis at pH 4 and values of 80 μ g. of P/g. of lens incubated in glucose alone, and 160 μ g. of P/g. of lens incubated in glucose plus xylose, were obtained. Estimation of periodate-labile P of α GP (see Methods), in another experiment, gave values of 48 μ g. of P/g. and 93 μ g. of P/g. respectively for lenses incubated in the absence and presence of xylose.

Other phosphates. If more extract (20–30 μ g. of P) were chromatographed in phenol–water, a spot of R_F 0.9 (PC and GPC) was seen in extracts from both types of incubation, and also a spot corresponding in position to adenylic acid (AMP). Estimation of P showed that concentrations of PC and GPC in the lenses was approximately the same whether xylose was present or not.

Spraying of trichloroacetic acid–orcinol on chromatograms of these extracts in phenol–water or in butanol–propionic acid–water showed areas in the position expected of sedoheptulose monophosphate (Benson *et al.* 1950). The spots were faint, possibly stronger in the extracts from xylose than in those from glucose incubation, and their P content was not measured as they overlapped in position with other P-containing compounds. Over-spraying with aniline phthalate did not show up the presence of any xylulose phosphate or ribulose phosphate and, on heating the papers, no evidence of the presence of aldose phosphates appeared.

Capsule plus epithelium. Schwarz & Golberg (1955) in experiments on galactose cataract in the rat were able to detect the formation of galactose 1-phosphate by examination of the capsule plus epithelium. Therefore, in a further search for phosphorylated derivatives of xylose the capsules and epithelia were removed from lenses after incubation at 38° for 5 hr. in medium containing glucose and glucose plus xylose. Ten lenses were used in each group. The fraction containing ‘barium salts insoluble in 80% (v/v) ethanol’ was prepared and examined by chromatography, followed by treatment with the appropriate sprays. There was no trace of phosphorylated aldoses, ketopentoses or sedoheptuloses.

Non-phosphorylated compounds. Treatment of the chromatograms with alkaline silver nitrate showed

two faint areas common to both extracts, glucose-incubated and (xylose plus glucose)-incubated. One of these spots had R_F 0.15 in phenol–water and has not been identified; the other, R_F 0.22 in phenol–water, corresponds to inositol, and was of equal strength in the two extracts. A third spot, very large and dark and coming up slowly after treatment with NaOH, appeared only in the extracts prepared from lenses incubated in the xylose-containing medium. An intense spot due to the presence of this compound was found in extracts of capsule plus epithelium from lenses incubated in the xylose-containing medium. This compound was found to be D-xylonic acid.

Identification of xylonic acid

This compound was detected by means of its positive reaction with AgNO_3 –NaOH (Trevelyan *et al.* 1950). Its presence was shown either in trichloroacetic acid extracts after extraction with ether, or in the fraction containing barium salts insoluble in 80% (v/v) ethanol. Paper electrophoresis of these fractions at pH 4 showed the compound to be an acid, with a mobility of 0.62 relative to P_i (1.00). α GP had a relative mobility of 0.79.

The compound had the same R_F as synthetic xylonic acid in several solvents (Table 8), and the same electrophoretic mobility at pH 4.

The rate of formation of D-xylono- γ -lactone from synthetic D-xylonic acid was measured. The acid was treated with N-HCl at 100° and, at 15 min. intervals, samples (5 μ l.) were removed for chromatography in phenol–water. The relative concentration of acid and lactone was judged by the intensity of the spots developed in AgNO_3 –NaOH. It was found that about 50% of the acid was converted into lactone in 2 hr. Confirmation of the formation of lactone was given by means of the hydroxylamine– FeCl_3 spray for lactones and esters (Abdel-Akher & Smith, 1951). The compound formed from xylose by calf lens behaved in the same way as the synthetic acid, about 50% being converted in 2 hr. at 100° in N-HCl into a compound having the same R_F as the lactone formed from the synthetic acid. Table 8 gives the R_F of D-xylose, D-xylonic acid and D-xylono- γ -lactone in several solvents.

Table 8. R_F values for D-xylose, D-xylonic acid and D-xylono- γ -lactone in various solvents

Descending chromatography on acid-washed Whatman no. 1 paper was used (see Methods for details about solvents).

Solvent	D-Xylose	D-Xylonic acid	D-Xylono- γ -lactone
Phenol–water	0.46	0.29	0.62
Butan-1-ol–propionic acid–water	0.33	0.33	0.48
Benzene–butanol–pyridine–water	0.42	0.10	0.73
isoButyric acid–aq. NH_3 soln.–water	0.50	0.40	0.59

Oxidation of xylose by dialysed lens extracts

Extracts of mammalian liver have been shown to oxidize xylose (Wainio, 1947) and to reduce di-phosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) (Strecker & Korkes, 1952; Brink, 1953). Glucose was oxidized by the same systems at a faster rate; the optimum pH for the reaction was about 8. Very high concentrations of glucose are needed for the oxidation to proceed at its maximum rate, the Michaelis constant being about 37 mM at pH 7.8. The primary product of the reaction, with glucose as substrate, is D-glucono- δ -lactone, which, in alkaline solution, is converted into D-gluconic acid. The appearance of xylonic acid in lenses incubated in a xylose-containing medium suggested that a similar enzyme occurs in the calf lens.

Fig. 1 shows that at concentrations of 0.44 M the rate of reduction of DPN by dialysed lens extract (see Methods) was nearly three times faster with xylose as substrate than with glucose. When the concentration of the sugars was reduced to 0.02 M, the rate of reduction of DPN with xylose as substrate was not much decreased, but the reaction with glucose was greatly reduced, to about one-tenth of the rate with xylose. Xylose (20 mM, i.e. 3 mg./ml.) corresponds approximately to the level of sugars found in the blood of rats fed with a diet

Fig. 1. Xylose dehydrogenase in calf lens; xylose and glucose as substrates. Beckman cells contained 1 ml. of dialysed lens extract, 2 ml. of phosphate buffer (M, pH 8) containing xylose or glucose (1320 or 60 μ moles), and DPN (approx. 0.2 μ mole). Sugars were omitted from blank.

of 35 % xylose (Sterling & Day, 1951). In another experiment separate extracts of the cortex and the nucleus of calf lens were made, and these were shown to contain almost the same concentration of the enzyme. An attempt was made to measure the activity of the epithelium plus capsule of lens, but material from three lenses did not contain measurable activity, thus showing that the enzyme is not greatly concentrated in the epithelium. The enzyme did not reduce TPN in the presence of either xylose or glucose.

It appears from Fig. 2 that a single enzyme is responsible for the oxidation of both glucose and xylose, since the rate of reduction of DPN in the presence of glucose plus xylose is no greater than that in the presence of xylose alone. With the lens extract used in this experiment, the difference between glucose and xylose as substrates was not so great as with the extracts used in Fig. 1.

The oxidation of xylose by DPN was carried out on a larger scale in order to compare the product of oxidation with the xylonic acid formed on incubation of whole lenses in a xylose-containing medium. A dialysed lens dispersion was brought to pH 8.2 by careful addition of 0.1 N-KOH. A volume (1 ml.) of this dispersion, DPN (1 μ mole) and xylose (67 μ moles), in a final volume of 3 ml., were incubated for 4 hr. at 37°. A blank was run without DPN. The reaction was stopped by adding 6 ml. of 7.5 % (w/v) trichloroacetic acid to each tube, and the mixtures were centrifuged. The extracts were shaken three times with ether, neutralized, concentrated and taken up in 0.3 ml. of water. Chromatography

Fig. 2. Xylose dehydrogenase in calf lens; glucose, xylose and glucose plus xylose as substrates. Contents of Beckman cells were as described in Fig. 1, but with 660 μ moles of xylose or 660 μ moles of glucose, or 660 μ moles of each.

in phenol-water showed a large spot in the position of xylonic acid which appeared only in the extract from the incubation containing DPN.

Preparation of cadmium bromide-cadmium xylonate double salt from xylonic acid made from xylose by calf lens

The identity of xylonic acid formed from xylose by calf lens was confirmed by converting it into cadmium bromide-cadmium xylonate double salt, $(\text{CdC}_5\text{H}_9\text{O}_6)_2 \cdot \text{CdBr}_2 \cdot 2\text{H}_2\text{O}$, which forms characteristic whetstone-shaped crystals (Elsner, 1935). It was not found possible to precipitate the crystals from xylonic acid in extracts of whole lens incubated in xylose-containing media, probably because of interfering compounds. The following method was therefore used.

Calf lens was ground with its own weight of water and dialysed against distilled water overnight at 4°. This dispersion (15 ml.) was cautiously brought to pH 8.2 with 0.1N-KOH and incubated for 6 hr. with xylose (1000 μ -moles), sodium pyruvate (500 μ -moles) and DPN (about 10 μ -moles). The presence of pyruvate ensured the continual regeneration of the oxidized form of DPN, through lactic dehydrogenase. After 6 hr. the incubation mixture was diluted with an equal volume of water, brought to pH 5.5 with dil. acetic acid, placed in a boiling-water bath for 3 min. to precipitate protein, cooled and centrifuged. A chromatogram of 10 μ l. of the clear extract in phenol-water showed that about 10% of the xylose had been oxidized to the acid. The rest of the extract (containing very approx. 100 μ -moles of xylonic acid) was evaporated to dryness *in vacuo*. The residue was taken up in 0.5 ml. of water and centrifuged to remove remaining protein; 0.1 g. of $\text{CdBr}_2 \cdot 4\text{H}_2\text{O}$ and 0.1 g. of CdCO_3 were then added to the extract, which after 30 min. at room temperature was evaporated almost to dryness on a water bath. Water (2 ml.) was added and the mixture stirred and centrifuged. The supernatant was reduced to 0.1 ml. on a water bath, cooled and 0.15 ml. of absolute ethanol added. After some days there was a heavy deposit of crystals in the extract, a large proportion of them being identical in appearance with those formed by the oxidation of xylose by bromine in the presence of cadmium carbonate (Elsner, 1935).

Chromatography of the fraction containing barium salts soluble in 80% (v/v) ethanol

This fraction was examined for the presence of xylulose, by chromatography and a combination of the trichloroacetic acid-orceinol and aniline phthalate sprays. Extracts of lens incubated in medium containing glucose plus xylose gave a small spot in the position of glucose and a large spot in the position of xylose, but nothing else. Cochromatography of these extracts together with authentic xylulose in a mixed spot showed that if 0.05% of the xylose present in the lens had been converted into xylulose its presence would have been detectable. The medium in which the lenses had been incubated was also examined for xylulose, but none

was found. Comparisons of extracts from lenses incubated in medium with and without xylose confirmed the finding that less glucose is present in the lenses incubated with xylose in the medium although both media contained the same concentration of glucose.

Chromatography of fraction containing insoluble barium salts

The concentrated extracts were chromatographed in propan-1-ol-aq. NH_3 soln.-water, and the dried papers examined for nucleotides in ultraviolet light. Although the size of this fraction was much smaller in the lenses incubated in medium containing xylose (Table 7), the relative proportions of the four main nucleotides, ATP, ADP, UTP, UDP, appeared to be the same in the extracts from incubations in the presence or absence of xylose. This fraction was not examined further.

Effect of incubation with xylose on phosphorylcholine, glycerophosphorylcholine, phosphorylethanolamine and glycerophosphorylethanolamine in intact lenses

Chromatography of the fraction containing barium salts insoluble in 80% (v/v) ethanol (see earlier) showed no change in PC and GPC in lenses incubated with or without xylose. Precipitation of these compounds may not be quantitative under these conditions and a comparison was therefore made of extracts of type (iii) (see Methods), from lens incubated in media with or without xylose. These contain PC, GPC, PE and GPE (van Heyningen & Pirie, 1958). Five lenses were used in each group; the extract from the lenses incubated in glucose alone contained 513 μ g. of P, and that from lenses incubated in glucose plus xylose 456 μ g. of P. The difference is unlikely to be significant, considering the number of experimental manipulations involved.

Glutathione and ascorbic acid in the lens after incubation

Glutathione and ascorbic acid were measured in three pairs of lenses after incubation with and without xylose. The presence of xylose in the medium caused no change in the concentration of either of the substances.

DISCUSSION

The addition of xylose to the glucose-containing medium in which intact calf lenses were incubated brought about the following metabolic changes: (1) depression of formation of lactic acid (Table 4); (2) decrease in nucleotide di- and tri-phosphates (see Table 7); (3) increase in α GP; (4) formation of xylonic acid. These findings all fit in with the assumption that DPN, normally available for the

oxidation of 3-glyceraldehyde phosphate (GAP) to 1:3-diphosphoglyceric acid in the glycolytic pathway, is preferentially reduced by xylose dehydrogenase in the presence of xylose. The Michaelis constant for the use of DPN by glucose dehydrogenase in liver is $4.3 \mu\text{M}$ at pH 7 (Brink, 1953), whereas that for DPN in GAP dehydrogenase in muscle is $39 \mu\text{M}$ (Cori, Slein & Cori, 1948). Thus if the enzymes in lens are the same as these, the xylose dehydrogenase would be expected to have a greater affinity for DPN than that of the GAP dehydrogenase.

Reoxidation of reduced DPN, normally occurring through the action of lactic dehydrogenase on pyruvate, would in the presence of xylose be brought about instead by the action of αGP dehydrogenase on dihydroxyacetone phosphate. Owing to the blockage of GAP dehydrogenase the concentration of GAP, and of its isomeric form dihydroxyacetone phosphate, would be expected to be at an increased level. It is assumed that the rate of isomerization is not limiting.

Thus the glycolytic cycle is interrupted, production of lactic acid is prevented, and ATP converted into ADP in the earlier unobstructed reactions of the glycolytic cycle is not able to be regenerated.

However, analysis of insoluble barium salts, containing nucleotide di- and tri-phosphates, did not show a higher proportion of ADP in lenses incubated in a xylose medium, although the whole fraction was considerably smaller (Table 7). Presumably other unidentified reactions are also involved.

The presence of an active glycerophosphate dehydrogenase in the calf lens is shown by the finding that DPN, reduced by a dialysed lens extract in the presence of xylose, is rapidly reoxidized on the addition of triose phosphate (Fig. 3).

Xylose interferes with the production of lactic acid from glucose by an intact lens (Table 4), but not by a lens dispersion (Table 1), although xylic acid and glycerophosphate are formed in both cases. Presumably when the lens fibres are disrupted sufficient DPN is available both for reactions (1) and (2) and for the reactions in the glycolytic cycle. Even in the intact lens, the glycolytic cycle continues to operate to some extent, as shown by the formation of lactic acid, which is decreased but not abolished.

The xylose dehydrogenase in lens has not been examined in detail but it does not appear to be

identical with the glucose dehydrogenase of liver (Strecker & Korkes, 1952; Brink, 1953). TPN, as well as DPN, is a coenzyme of the liver enzyme, but not of the lens enzyme. In the liver xylose is oxidized at a slower rate than glucose, whereas in the lens it is oxidized faster, especially at low concentrations of sugar. The possibility that D-xylonolactone is an intermediate in the formation of xylic acid by the lens has not been investigated.

The findings presented in this paper accord with those of Wachtl & Kinsey (1957), who used rabbit lenses cultured *in vitro* under conditions which maintained a normal level of mitosis in the epithelial cells for 9 hr. They showed that substitution of glucose in the medium by xylose (both 1.25 mg./ml.) abolished the formation of lactic acid, although some xylose was consumed; lactic acid was formed when lenses were incubated with glucose and xylose together (each at a concentration of 0.625 mg./ml.), and some of each of these sugars was metabolized. Xylose alone did not support mitosis in the lens epithelium; when present together with glucose, the mitotic activity was about half that seen in the presence of glucose alone.

There are no figures in the literature for phosphorus in the lens in xylose cataract. In galactose cataract in rats, and also in diabetic cataract induced with alloxan, it has been shown that, when the lenses are partially opaque, compounds containing acid-labile P are decreased in concentration and P_i is unchanged (Nordmann & Mandel, 1953). Müller *et al.* (1955) used paper electrophoresis to

Fig. 3. Reduction of DPN by xylose dehydrogenase and reoxidation by glycerophosphate dehydrogenase. Beckman cell contained 1.5 ml. of dialysed lens extract, 1.4 ml. of phosphate buffer (M, pH 8) containing xylose ($67 \mu\text{moles}$) and DPN (approx. $0.02 \mu\text{mole}$). Triose phosphate ($1 \mu\text{mole}$ of dihydroxyacetone phosphate and $1 \mu\text{mole}$ of glyceraldehyde phosphate) was added where indicated by the arrow.

compare the lenses of rabbits made diabetic with alloxan with normal rabbit lenses after incubation in a medium containing ^{32}P . They found that the lenses from the diabetic rabbits incorporated a smaller proportion of ^{32}P into ATP, and a correspondingly larger proportion into a fraction consisting chiefly of phosphorus compounds not hydrolysed in 7 min. in N-HCl at 100° .

SUMMARY

1. Xylose is not phosphorylated by the calf lens; it does not interfere with the phosphorylation of glucose by lens dispersions.

2. The formation of lactic acid from glucose is inhibited by xylose in the intact lens but not in lens dispersions. Penetration of glucose into the intact lens is inhibited by xylose.

3. Xylose is oxidized by the calf lens (intact or dispersed) to xylonic acid by means of a dehydrogenase using diphosphopyridine nucleotide (DPN). Reduction of DPN by dialysed lens extract at pH 8 is faster with xylose (0.02M) as substrate than with glucose at the same concentration.

4. Comparison between intact lenses incubated in a medium containing glucose, without or with the addition of xylose, shows that the formation of xylonic acid from xylose is accompanied by an increase in the amount of α -glycerophosphate (αGP) in the lens and a corresponding decrease in the acid-labile phosphate contained in the nucleotide di- and tri-phosphates. There is no change in orthophosphate or in the sum of phosphorylcholine, phosphorylethanolamine, glycerophosphorylcholine and glycerophosphorylethanolamine.

5. It is suggested that, in the presence of xylose, DPN normally available for oxidation of 3-glyceraldehyde phosphate in the glycolytic cycle is preferentially reduced by xylose dehydrogenase to xylonic acid. The observed increase in αGP is explained on the assumption that the reduced DPN thus formed is reoxidized, not as in the normal lens through pyruvic acid and lactic dehydrogenase, but by the action of αGP dehydrogenase on dihydroxyacetone phosphate.

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The Heterogeneity of β_2 -Lactoglobulin

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The discovery by Aschaffenburg & Drewry (1955, 1957) that individual cows differed in the β -lactoglobulins that they produce (they have either β_1 - or β_2 -lactoglobulin or a mixture of the two), although going far to explain the heterogeneity of β -lactoglobulin prepared indiscriminately from mixed milk, does not completely account for all the observed indications of heterogeneity.

Thus Ogston & Tombs (1957) found that both β_1 - and β_2 -lactoglobulin gave complex boundaries on electrophoresis, and although most of this heterogeneity could be explained as due to polymerization in solution (Ogston & Tilley, 1955) there remained a possibility that some of the electrophoretic heterogeneity was due to the presence of distinct minor components.

This possibility was investigated for β_1 -lactoglobulin mainly by use of solubility tests (Tombs, 1957), and the presence of about 10% of a minor component was detected. It could be removed by salt fractionation; the purified β_1 -lactoglobulin had a constant-solvent solubility curve, and a variable-solvent solubility curve characteristic of a very nearly homogeneous substance. It still had a complex boundary in electrophoresis, but this could be completely explained as due to polymerization in solution. Some results obtained with β_2 -lactoglobulin by using an approach similar to that already described for β_1 -lactoglobulin are reported here.

EXPERIMENTAL

Experimental details of the preparation of β -lactoglobulins, electrophoresis, determination of concentration and measurements of ultraviolet absorption were the same as in

Ogston & Tombs (1957). Solubility tests were substantially the same as were used for β_1 -lactoglobulin (Tombs, 1957).

Variable-solvent solubility curves

The protein was dissolved in 1.6M-sodium chloride and dialysed against it for at least 24 hr. This solution was then mixed with an equal volume of acetate buffer (pH 4.5; 1.6M-sodium acetate, 2.9M-acetic acid) and samples (1 ml.) were placed in centrifuge tubes. Then 3 ml. of an appropriate ammonium sulphate solution was run in to give final concentrations of 0.2M-sodium chloride, 0.2M-sodium acetate and ammonium sulphate in the range 1.9–2.4M.

After initial experiments in which equilibration took place at 25° (Fig. 2) it was found that cooling was necessary in order to obtain amorphous precipitates; subsequently therefore all solutions were precooled to 4° before mixing, and the tubes were kept in a water bath at 4° during equilibration. The tubes were sealed with silicone-treated rubber bungs. After centrifuging at 4° (10 000g for 20 min.), 2 ml. of the supernatant was removed from each tube and diluted to 3 ml. with water. β_2 -Lactoglobulin has a negative temperature coefficient of solubility in these solvents and dilution was necessary to prevent precipitation occurring when the tubes warmed up to room temperature. Protein concentration in the diluted supernatants was estimated directly by the absorption at 278 m μ in 1 cm. cells.

Constant-solvent solubility curves

β_2 -Lactoglobulin was dissolved in solvent containing 0.8M-sodium chloride, 0.8M-sodium acetate, 1.35M-acetic acid (pH 4.5), and dialysed against the same solvent. Then volumes of the β_2 -lactoglobulin solution not exceeding 1 ml. were carefully measured into centrifuge tubes with an Agla micrometer syringe. Then each sample was made up to 1 ml. with diffusate, and 3 ml. of ammonium sulphate solution was added to give the desired final concentration. The volume of protein solution added to each tube gave a measure of the total amount of protein in the system, and the amount in solution was found by measurement of the absorption of the supernatants at 278 m μ . Tubes were equilibrated at 4°, and supernatants diluted after centrifuging in exactly the same way as for the variable-solvent test.

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