Aldose Reductase and Ketose Reductase in Male Accessory Organs of Reproduction

DISTRIBUTION AND RELATION TO SEMINAL FRUCTOSE

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Fructose, the sugar of mammalian semen, is produced by the male accessory organs of reproduction. In man, ram, bull, boar and certain other species, it is formed in the seminal vesicles or analogous organs; in the rat the main fructose-producing organs are the coagulating gland and the dorso-lateral prostate (Mann, 1946, 1949). Previous studies showed that the biosynthesis of the seminal sugar is testosterone-dependent, and involves the conversion of blood glucose into seminal fructose by the accessory organs, probably with glucose phosphate and fructose phosphate as intermediary metabolites (Mann & Lutwak-Mann, 1948, 1951*a, b*; Mann & Parsons, 1950). Hers (1957, 1960*a, b*) confirmed the existence in accessory-gland tissue of the metabolic mechanism whereby glucose is converted into fructose. This author, however, also found that, in the ram at any rate, there are two enzymes in seminal vesicles: (i) an aldose reductase (polyol-NADP oxidoreductase, EC 1.1.1.21) which reduces glucose to sorbitol, and depends on reduced nicotinamide-adenine dinucleotide phosphate (NADPH); (ii) a ketose reductase or so-called sorbitol dehydrogenase (α -iditol-NAD oxidoreductase, EC 1.1.1.14), shown by Williams-Ashman & Banks (1954) to be present in rat accessory organs, which oxidizes sorbitol to fructose, and depends on oxidized nicotinamide-adenine dinucleotide (NAD^+). Hers concluded that the transformation of glucose into seminal fructose involves sorbitol as an intermediary metabolite. A further development was the identification of sorbitol as a normal constituent of fresh ram semen (King & Mann, 1958; King, Isherwood & Mann, 1958). However, King & Mann (1959) showed that the sorbitol content of ram semen usually increases, rather than decreases, on incubation, and that ram spermatozoa possess a very active NAD-dependent sorbitol dehydrogenase which is responsible for the ability of the sperm cells to oxidize sorbitol aerobically to

fructose, and which also catalyses, under suitable circumstances, a rapid reduction of fructose to sorbitol at a rate considerably in excess of that observed for the reduction of glucose to sorbitol. This led King & Mann (1959) to suggest that, in semen, the steady state of the sorbitol-fructose interconversion depends on the ratio between the oxidized and reduced forms of NAD within the sperm cells. These authors considered, however, that the initial content of sorbitol in ram semen, about 60 mg./100 ml., is too high to be attributable solely to the action of spermatozoa on seminal fructose before ejaculation, and that accordingly sorbitol must largely be derived from the seminal vesicles, in agreement with Hers's views.

Assuming that the mechanism of fructose formation in the male accessory glands depends on the presence of aldose reductase and ketose reductase, one would expect some correlation between the activities of these two enzymes and the actual rate of fructose formation in the glands of different animals. An attempt was therefore made to compare the rates of fructose secretion in the accessory organs of different species with the respective activities of aldose reductase and ketose reductase.

EXPERIMENTAL

Tissue preparations. The following organs were examined: the seminal vesicles of the bull, ram, boar and stallion; and the coagulating gland, seminal vesicle, ventral prostate and dorso-lateral prostate of the rat. In the bull and ram the seminal-vesicle secretion contains up to 1% of fructose, in the boar usually less than 0.05%, and in the stallion almost none; in the rat, most fructose is produced in the coagulating gland, some in the dorso-lateral prostate, but none in the seminal vesicles and ventral prostate.

Bull, ram, boar, and stallion organs were collected at slaughter. Rat organs were obtained from adult males which had been pretreated for 1 week with testosterone propionate (1 mg. injected parenterally/day/rat). All organs were preserved in solid CO_2 until required for analysis.

The frozen tissues were ground in a mortar with 0.15M-KCl and then homogenized in ice-cold 0.15M-KCl in a

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TenBroeck (1931) homogenizer, the ratios of fluid volume to tissue weight being 3:1, 5:1 or 10:1 depending on the expected degree of enzymic activity. The homogenates were centrifuged for 10 min. at 15 000g in a refrigerated centrifuge. Two samples of the supernatant were set aside, one for fructose and citric acid analyses, and the other for the assay of aldose reductase and ketose reductase.

The major portion was subjected to fractional precipitation with neutral ammonium sulphate. The first fraction was that precipitated at 50% saturation, the second that precipitated between 50 and 70% saturation, and the third that remaining in solution at 70% saturation with ammonium sulphate. The sample of the original supernatant and the three fractions were then dialysed against 0.15M-KCl at 0° for 16 hr. with three changes of fluid, at least 150 times the volume of the four fractions being used each time. After dialysis, each fraction was again centrifuged to remove insoluble material and the clear supernatants were assayed immediately or stored at the temperature of solid CO₂ until used.

Fructose and citric acid determinations. These were carried out by the methods of Roe (1934) and Speck, Moulder & Evans (1946) respectively.

Aldose-reductase assay. The basis for the determination of aldose-reductase activity was the measurement, in the Unicam SP. 500 spectrophotometer, of the oxidation of NADPH [triphosphopyridine nucleotide (reduced), Sigma Chemical Co., U.S.A., and C. F. Boehringer und Soehne, Germany] in the presence of glucose (British Drug Houses Ltd., AnalaR); the decrease in the u.v. absorption was measured at 340 m μ . As a preliminary to the actual determinations, a study was made of the dependence of the activity of aldose reductase on pH and substrate concentration. As expected in view of the participation of H⁺ ions in the reduction of glucose, the rate of reaction increased with decreasing pH over the range 8.0-6.2 (Fig. 1). The rate of destruction of the nucleotide increased markedly on the acid side of neutrality, however, and pH 7.0 was adopted for the routine assay. The relationship between enzymic activity and substrate concentration is illustrated in Fig. 2. To ensure saturation of the system, a glucose concentration of 67 μ moles/ml. was used in all measurements. For routine assay of aldose reductase, each cuvette contained 1.0 ml. of 0.15M-potassium phosphate buffer, pH 7.0, 0.5 ml. of 0.40M-glucose, 0.1 ml. of 0.05-0.1 mM-NADPH and enzyme extract plus water to make 3.0 ml. Incubation was at 25° in the jacketed carrier of the Unicam spectrophotometer. The decrease in absorption at 340 m μ over the first 10 min. was taken as the measure of NADPH oxidized, the molar extinction coefficient, ϵ , being taken as 6.22×10^3 . Under these conditions the initial rate of reaction was proportional to enzyme concentration up to at least 20 units. A unit of activity is that amount of enzyme which will oxidize 1 μ m-mole of NADPH/min.

Ketose-reductase assay. The reduction of NAD⁺ (diphosphopyridine nucleotide, Sigma Chemical Co., U.S.A.) in the presence of sorbitol (Thomas Kerfoot Ltd., Vale of Bardsley, Lancs.) was used for the ketose-reductase assay. As Williams-Ashman & Banks (1954) had found that pH 9 was optimum for the reaction, a pyrophosphate buffer of pH 9.0-9.1 was used for the assay. As the result of a study in which the activity of ketose reductase was determined in the presence of various substrate concentrations but at a constant concentration of 0.33 μ mole of NAD⁺/ml. (Fig. 3),

Fig. 1. Variation in aldose-reductase activity with pH over the range 6.2-8.0. Conditions of assay other than pH were as given in text. The enzyme concentration was the same in all cuvettes.

Fig. 2. Variation in aldose-reductase activity with glucose concentration. All other conditions of assay were as given in text. The enzyme concentration was the same in all cuvettes.

Fig. 3. Variation in ketose-reductase activity with sorbitol concentration. All other conditions of assay were as given in text. The enzyme concentration was the same in all cuvettes.

a concentration of 25 μ moles of substrate/ml. was adopted for the routine assay. In the standard ketose-reductase assay each cuvette contained 1.0 ml. of 0.15M-sodium pyrophosphate buffer, pH 9.0-9.1, 0.5 ml. of 0.15M-sorbitol, 0.2 ml. of 6 mM-NAD⁺ and enzyme solution plus water to make 3 ml. Incubation was at 25° and the increase in absorption at 340 m μ was read over the first 10 min. The NAD⁺ reduced was then calculated, ϵ being taken as 6.22×10^3 . Under such conditions a linear relationship between the enzyme concentration and the rate of NAD⁺ reduction was obtained. A unit of enzyme activity is defined as the enzyme required to reduce 1 μ m-mole of NAD⁺/min.

RESULTS

When assays of aldose reductase were carried out on crude, unfractionated tissue extracts, it proved difficult to determine the initial rate of oxidation of NADPH, even in the presence of 0.04M-nicotinamide, presumably because of excessive enzymic breakdown of NADP [described by Leone & Bonaduce (1959)]. This difficulty was partly overcome by ammonium sulphate fractionation. The distribution of the two reductases among the various fractions thus obtained is illustrated in Table 1. Ketose-reductase activity was highest in the material precipitated at 50% saturation of ammonium sulphate, but a significant amount was also present in the 50-70% fraction. Use of 55%, instead of 50%, saturation led to a better separation, but caused precipitation of a considerable amount of aldose reductase. Therefore the 50% saturation step for the first precipitation was used for routine fractionations, but all 50-70% fractions were also assayed for ketose reductase. No activity was found in the fraction soluble at 70% saturation.

Aldose-reductase activity was largely limited to the fraction that is precipitated between 50 and 70% saturation. Since, however, in two instances slight activity was present also in the fraction precipitated at 50% saturation, the two fractions precipitated at 50 and 70% saturation respectively were always assayed. There was no significant activity in the fraction soluble in 70% saturated ammonium sulphate.

Table 1 also shows that occasionally the 'total activity' of enzyme, i.e. the sum of the activities present in the various fractions, exceeded the activity present in the crude, unfractionated extract. This was presumably due to the higher rate of coenzyme destruction in the crude extract.

The aldose reductase was found to be quite stable during storage at the temperature of solid carbon dioxide, but the ketose-reductase activity decreased rapidly (Table 2). Both enzymes were adversely affected by repeated freezing and thawing. Ketose reductase was always determined within 3 days after preparation of the extracts.

Table 3 records the results of enzyme assays carried out on the four principal accessory organs of the rat, namely the seminal vesicles, coagulating glands, ventral prostate, and dorso-lateral prostate. The results of fructose and citric acid analyses in the four organs are also given. The rat seminal vesicles, which produce citric acid but no fructose, had a remarkably high ketose-reductase activity; this agrees with an earlier finding by Williams-Ashman & Banks (1954). On the other hand, they were poor in aldose-reductase activity, an observation which explains their inability to produce fructose. In the coagulating glands, which produce

Table 1. *Distribution of aldose-reductase and ketose-reductase activities among fractions obtained by ammonium sulphate precipitation from seminal-vesicle extracts of bull, boar and stallion*

Enzyme activities were assayed as described in the text.					
	Enzyme activity (units/g. of tissue)				
	Fractions pptd. by (NH ₄) ₂ SO ₄				
	Crude extract	0-50 % saturation	50-70 % saturation	70-100 % saturation	Total
Aldose reductase					
Bull no. 1	43	4.9	43	9.3	57.2
no. 2	56	—	52	0.0	52.0
no. 3	66	0.0	58	0.0	58.0
no. 4	—	0.0	42	0.0	42.0
Boar	—	Trace	10.8	0.0	10.8
Stallion	—	0.8	2.4	0.3	3.5
Ketose reductase					
Bull no. 1	143	120	16	—	136.0
no. 2	24.3	24.8	2.8	—	27.6
no. 2	24.3	21.0	6.1	—	27.1
no. 3	55	58	3.4	—	61.4
no. 4	85	94	5.6	—	99.6
Boar	—	0.5	0.0	—	0.5
Stallion	—	2.5	3.0	—	5.5

fructose but no citric acid, ketose-reductase activity was low, whereas that of aldose reductase was high. The ventral prostate, which like the seminal vesicles produces citric acid but not fructose, was almost devoid of either ketose reductase or aldose reductase. The dorso-lateral prostate, on the other hand, which produces both citric acid and fructose, contained both enzymes.

From the above analyses it is clear that the ability of the different rat accessory organs to produce fructose coincides with high aldose-

Table 2. *Stability of aldose reductase and ketose reductase; activity after various periods of storage in solid carbon dioxide*

Extract was prepared from the seminal vesicles of a 6-year-old Friesian bull. For the preparation of aldose reductase, the fraction precipitated by ammonium sulphate in the range 50–70% saturation was dissolved in 0.15M-potassium chloride (5 ml./g. of tissue) and dialysed against 0.15M-potassium chloride. For the preparation of ketose reductase, the fraction precipitated by ammonium sulphate in the range 0–50% saturation was dissolved in 0.15M-potassium chloride and dialysed. The dialysed solutions were divided, each into 12 equal samples, and stored at the temperature of solid carbon dioxide. A fresh sample was thawed for each test except that marked *. Under the heading 'Time' are recorded the days between preparing the extract and testing the samples. Enzyme activities were assayed as described in the text.

Aldose reductase		Ketose reductase	
Time (days)	Activity (units/g. of tissue)	Time (days)	Activity (units/g. of tissue)
1	113	3	60
8	88	4	66
25	89	35	37
29	91	40	17
43	105		
44	88		
98	93		
98*	27		
104	59		
114	24		

* The same sample which had been thawed and assayed was refrozen in a solid CO₂-ethanol bath and held in solid CO₂ for 2 hr. It was then re-thawed and assayed again.

reductase activity, but bears no direct relation to that of ketose reductase. This was also borne out by the results obtained with the accessory organs of the bull, ram, boar and stallion. Variations in aldose-reductase and ketose-reductase activities in the seminal vesicles of all these animals are presented in Table 4. The bull and ram seminal vesicles, which are capable of producing large amounts of fructose, showed by far the highest aldose-reductase activity, and also the highest ketose-reductase activity, which indicates that these glands possess equally high capacities to reduce glucose to sorbitol and to oxidize sorbitol to fructose. The seminal vesicles of the boar, which produce much less fructose than those of the bull or the ram, also had a much lower, though still distinguishable, aldose-reductase activity. However, they lacked ketose reductase and therefore would not be expected to be able to convert sorbitol into fructose efficiently. This is in accordance with the observation made by King & Mann (1959) that the boar seminal plasma contains more sorbitol than fructose. Stallion seminal vesicles contained little of either enzyme; stallion seminal plasma was shown by King & Mann (1959) to contain some sorbitol but only a trace of fructose.

Since the process of fructose secretion in male accessory organs is closely linked with the activity of male sex hormone, an experiment was done to determine the effect of testosterone administration on the aldose-reductase and ketose-reductase activity in bull seminal vesicles. A pair of Hereford twins was used for this purpose. They were castrated at the age of 6½ months; a month later, one was given 20 mg. of testosterone in 1 ml. of arachis oil by injection every other day for 4 weeks, the control twin being untreated. The seminal vesicles of both animals were then analysed for fructose, citric acid, aldose reductase and ketose reductase. The results (Table 5) show that testosterone treatment produced more than a sixfold increase in the concentration of fructose and citric acid and an approximately similar increase in the activities of both aldose reductase and ketose reductase in the seminal vesicles.

Table 3. *Activities of aldose reductase and ketose reductase, and concentrations of fructose and citric acid, in accessory organs of the male rat*

All determinations were carried out as described in the text.

	Activity (units/g. of tissue)		Concentration (mg./100 g. of tissue)	
	Aldose reductase	Ketose reductase	Fructose	Citric acid
Seminal vesicles	2.7	39.5	0	43
Coagulating glands	15.2	27.0	121	0
Ventral prostate	2.8	1.6	0	78
Dorso-lateral prostate	10.4	20.4	47	56

Table 4. *Activities of aldose reductase and ketose reductase, and concentrations of fructose and citric acid, in the seminal vesicles of sexually mature rat, bull, ram, boar, and stallion*

All determinations were carried out as described in the text.

	No. of animals	Activity (units/g. of tissue)		Concentration (mg./100 g. of tissue)	
		Aldose reductase	Ketose reductase	Fructose	Citric acid
Rat	5	0-2.7	19.4-39.5	0-2	35-80
Bull	4	66-165	59.0-136.0	197-970	178-925
Ram	2	140, 191	385, 542	265, 293	45, 72
Boar	1	11	0.5	35	110
Stallion	1	3.5	5.5	1	163

Table 5. *Effect of testosterone on bull seminal vesicles*

A pair of castrated Hereford bull twins was used. One twin was given 20 mg. of testosterone by injection every other day for 4 weeks; the control twin was untreated. The seminal vesicles of both animals were then examined for fructose, citric acid, aldose reductase and ketose reductase by the methods described in the text.

	Castrated, untreated control twin	Castrated, testosterone- treated twin
Weight (g.)	5.9	15.0
Fructose (mg./100 g. of tissue)	19.8	124.0
(mg./pair of glands)	1.17	18.6
Citric acid (mg./100 g. of tissue)	9.8	70.0
(mg./pair of glands)	0.58	10.5
Aldose reductase (units/g. of tissue)	6.1	33.5
Ketose reductase (units/g. of tissue)	4.2	21.5

These findings suggested that testosterone has about the same stimulating effect on fructose formation as on the activity of both aldose reductase and ketose reductase in the bull seminal vesicles. To explore these relationships further, another experiment was carried out in which analyses were performed on seminal vesicles removed from normal, non-castrated bulls at different stages of sexual maturation. An opportunity to do this experiment arose in conjunction with an investigation by Lindner & Mann (1960) of the relationship between the content of testosterone and androstenedione in bull testes and the concentration of fructose and citric acid in the seminal vesicles; samples of vesicular tissues from several bulls became available for assay of aldose reductase and ketose reductase, the youngest animal being 70 days old, and the oldest 17½ years. The results are given in Table 6. There was no direct proportionality between the values for fructose and the activity of either aldose reductase or ketose reductase in the seminal vesicles of six bulls. However, in the youngest and immature animal of the

series, not only the fructose concentration but also the activity of both aldose reductase and ketose reductase in the seminal vesicles was lowest; further, in the oldest bull, in which (presumably owing to advanced age) the rate of fructose production had already declined, the activities of aldose reductase and ketose reductase in the seminal vesicles were also low.

Finally, the possibility was considered that testosterone might influence the activity of either aldose reductase or ketose reductase under the conditions used *in vitro*. However, the addition of 10^{-7} – 10^{-14} M-testosterone to enzyme preparations *in vitro* produced no noticeable effect.

DISCUSSION

In bull, ram and boar, fructose is produced by the same organ as is citric acid, namely the seminal vesicle. In these species, however, the seminal vesicles have a rather complex cellular structure, and in the bull, for example, the vesicular epithelium contains three distinct types of cells which probably have different secretory functions (Mann, Davies & Humphrey, 1949). In the rat, on the other hand, the secretion of fructose and citric acid proceeds in separate organs, making it possible to study the two processes independently (Humphrey & Mann, 1949).

Studies by Williams-Ashman, Banks & Wolfson (1957) and Hers (1957, 1960*a, b*) have shown that, apart from the phosphorylative pathway of carbohydrate metabolism demonstrated by Mann & Lutwak-Mann (1951*a, b*), the male accessory organs possess a non-phosphorylative mechanism for fructose formation, which involves as a final stage the oxidation of sorbitol by ketose reductase (sorbitol dehydrogenase). Hers has also shown that in the ram seminal vesicles yet another enzyme is present, aldose reductase, which converts glucose into sorbitol. We were able to confirm Hers's observation, and extend it to other species. The male rat was shown to contain ketose reductase not only in the coagulating gland and dorso-lateral

Table 6. *Activities of aldose reductase and ketose reductase, and fructose concentration, in seminal vesicles of bulls varying in age from 70 days to 17½ years*

All determinations were carried out as described in the text.

Bull (Age and breed)	Weight	Activity (units/g. of tissue)		Concn. of fructose (mg./100 g. of tissue)
		Aldose reductase	Ketose reductase	
70 days, Friesian	4.5	24.4	5.3	15
167 days, Friesian	16.0	63	40	91
169 days, Friesian	17.6	57	27.5	196
21 months, Friesian	98.5	66	100	420
7 years, Lincoln Red	171.0	128	136	321
17½ years, Shorthorn	164.5	52	61	126

prostate (where fructose is normally formed) but also in the seminal vesicle (which normally produces only citric acid). In the rat, therefore, there is no direct relationship between the occurrence of ketose reductase and the ability of accessory-gland tissues to produce fructose. On the other hand, our experiments have shown that in the rat, as well as in the bull and boar, those glands which produce fructose are always distinguished by the presence of aldose reductase. We regard this finding as evidence in support of Hers's views.

It is difficult at present to assess the relative importance of the two pathways of fructose formation, one involving sorbitol and the other phosphorylated sugar esters, for the normal secretory function of the male reproductive tract *in vivo*. The occurrence of fructose in the placenta and foetus, where there are two enzymic mechanisms, creates a similar problem (Neil, Walker & Warren, 1961).

There also remains the question as to why some male accessory glands secrete fructose and other closely adjacent glands accumulate citric acid. Talalay & Williams-Ashman (1958) suggested that 'citrate—and no other organic acid—probably accumulates in response to steroids because the unfavourable TPNH/TPN ratio acts to brake the isocitric dehydrogenase reaction and prevents the rate of oxidation of citrate from keeping pace with its synthesis'. Much can be said for this concept. Harding & Samuels (1961) showed that there is little, if any, mitochondrial NADPH-cytochrome *c* reductase in rat ventral prostate. If this, the major mechanism of oxidation in tissues like the liver, is relatively inactive in male secondary sex organs, other routes of oxidation of NADPH would become limiting. Our experiments have demonstrated not only that there exists a positive correlation between aldose-reductase activity and fructose-forming ability but that the absence of aldose reductase from a gland often coincides with a high rate of citric acid production. In the absence of aldose reductase there can be no reduction of glucose to sorbitol with the accompanying

oxidation of NADPH to NADP⁺, which the isocitrate dehydrogenase requires as its coenzyme. Consequently one might expect the rate of citrate oxidation to be small. In a fructose-producing gland, on the other hand, where aldose reductase is present, NADP⁺ would be generated from NADPH during the reduction of glucose to sorbitol, and that would facilitate the oxidative breakdown of citrate. Owing to the presence of ketose reductase in the fructose-producing glands, sorbitol is oxidized to fructose and NAD⁺ reduced to NADH. The NADH is probably oxidized by the NADH-cytochrome *c* reductase which occurs in certain accessory organs (Harding & Samuels, 1961). Thus the presence or absence of aldose reductase in a secretory tissue constitutes a factor in determining whether the cells produce fructose or citric acid. Fig. 4 shows how the two systems, one producing fructose and the other metabolizing citric acid, would have to be coupled to explain our observations on the inverse relationship between fructose and citrate secretion in the accessory-gland tissues.

The relationship between testosterone and the enzymic processes of fructose and citric acid formation does not appear to be a direct one. Testosterone has a powerful stimulating effect on fructose and citrate secretion by the male accessory organs *in vivo*, but it does not affect appreciably the ratio between the secretory rates of the two substances *in vivo*, and has no influence on the enzymic reactions *in vitro*. Moreover, attempts to demonstrate in the accessory glands a testosterone- or androstenedione-dependent transhydrogenase have so far been unsuccessful (Harding & Samuels, 1962). It is possible that testosterone stimulates protein synthesis in general by increasing the rate of transfer of amino acids from the soluble to the microsomal form of ribonucleic acid (Wilson, 1961). Thus the role of testosterone would be to provide a stimulus for the biosynthesis of enzyme proteins (amongst others aldose reductase and ketose reductase) without, however, a direct influence on the activity of any one enzyme.

Fig. 4. Possible influence of fructose synthesis on citrate oxidation in male secondary sex organs with low NADPH-cytochrome *c*-reductase activity.

SUMMARY

1. The activity of aldose reductase and ketose reductase (sorbitol dehydrogenase) has been determined quantitatively in the male accessory organs of rat, ram, bull, boar, and stallion.

2. The ability of an accessory-organ tissue to produce fructose has been shown to coincide with the presence of aldose reductase. Glands which lack this enzyme were devoid of the ability to form fructose, but formed citric acid instead.

3. Administration of testosterone to a castrated animal stimulated the production of fructose, and increased simultaneously the activity of both aldose reductase and ketose reductase.

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