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Studies on the Metabolism of Semen

3. FRUCTOSE AS A NORMAL CONSTITUENT OF SEMINAL PLASMA. SITE OF FORMATION AND FUNCTION OF FRUCTOSE IN SEMEN

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Previous investigations (Mann, 1945*a*, *b*, *c*) have shown that the glycolytic pathways and enzymes involved in the carbohydrate metabolism of spermatozoa resemble those of other animal tissues and yeast. These studies have established the participation of hexokinase, zymohexase and of certain phosphopherases and dehydrogenases in sperm glycolysis and they disclosed that adenosinetriphosphate (ATP) forms a link between the activity of spermatozoa and glycolysis. In all these experiments washed spermatozoa were used. It remained to be seen what is the glycolytic mechanism which operates in whole semen, i.e. in semen which has not been divided artificially into its two natural components, spermatozoa and seminal plasma.

Since the early researches by Iwanow (1931) and Redenz (1933) it has been known that in one important aspect at least the washed spermatozoa show a strikingly different behaviour from whole semen. Whereas washed spermatozoa can survive anaerobically only in presence of glucose or some other glycolyzable carbohydrate, in whole semen the spermatozoa continue living without additional sugar, at the expense of a reducing and yeast-fermentable carbohydrate already present in the seminal plasma. In the extensive literature dealing with the subject of seminal sugar, this substance has hitherto been described either as glucose or simply as the reducing sugar of semen (Killian, 1933; Bernstein, 1933; Goldblatt, 1935; Shergin, 1937; McKenzie, Miller & Bauguess, 1938; Davis & Cole, 1939; Huggins, Scott, & Heinen, 1942; Moore & Mayer, 1941; MacLeod & Hotchkiss, 1942; Salisbury & Van Demark, 1945), and the only reference to the probable occurrence of fructose in semen is found in a paper by Yamada (1933) who surveyed human tissues and body fluids for the presence of fructose by means of a colour test with 'Cryogenine' (the antipyretic drug 'Cryogénine Lumière').

In view of the inadequate evidence as to the identity of the reducing carbohydrate present in semen, an investigation of its chemical nature was undertaken. The sugar has been purified and shown to be identical with *d*(−)-fructose (preliminary communication, Mann, 1946). In this study it will be demonstrated that fructose accounts for practically the whole of the yeast-fermentable carbohydrate present in the seminal plasma of several species, including bull, ram, rabbit, boar and man. Evidence will also be given that fructose originates in the accessory glands of reproduction and that spermatozoa come in contact with it during their passage through the male generative tract. Finally, it will be shown that fructose in whole semen is the natural substrate which provides life energy to the spermatozoa by its glycolytic breakdown to lactic acid.

EXPERIMENTAL

Methods

The experiments were carried out with ejaculated semen. The semen of animals was collected by Dr A. Walton at the Animal Research Station, Cambridge, by the method of Walton (1945). Several samples of bull semen were kindly supplied by Veterinary Officers in charge of Cattle Insemination Centres in England. The semen was used whole or it was separated by centrifugation into seminal plasma and spermatozoa. The spermatozoa were washed free of the seminal plasma by means of a Ca-free Ringer's solution composed as follows: 100 ml. 0·9% NaCl + 4 ml. 1·15% KCl + 1 ml. 2·11% KH_2PO_4 + 1 ml. 3·82% $MgSO_4.7H_2O$ + 2 ml. 1·3% $NaHCO_3$. In some experiments this solution was further supplemented by 0·5% fructose ('Ringer-fructose') or by 20 ml. 0·25 M-phosphate buffer, pH 7·4

('Ringer-phosphate'), or by both fructose and phosphate ('Ringer-fructose-phosphate').

Total reducing sugar was determined by the method of Hagedorn & Jensen (1923); with this method the reducing value of fructose is only about 2% lower than that of an equivalent amount of glucose (Baranowski, 1935). The value obtained by the reduction of ferricyanide in the Hagedorn-Jensen method will be referred to as the 'total reducing sugar'; it represents the sum of yeast-fermentable sugar and some reducing but non-fermentable substances.

Yeast-fermentable sugar. In order to distinguish between the yeast-fermentable and non-fermentable components of the seminal plasma the following technique was adopted. Fresh baker's yeast (2 g.) was suspended in 20 ml. tap water, left 1 hr. at room temperature and then centrifuged. The yeast was resuspended in 20 ml. of 0·05M-phosphate buffer (pH 6) or in 20 ml. water. Portions of 2 ml. of the washed yeast suspension were pipetted into two conical centrifuge tubes, *A* and *B*. *A* had no further additions, *B* received 1 ml. seminal plasma. Both tubes were incubated for 2 hr. at 30°. The incubation was terminated by centrifugation, and the centrifuged yeast washed with 2 ml. water. The supernatant solutions and wash-fluids were then transferred quantitatively into two 10 ml. volumetric flasks, and 1 ml. fresh seminal plasma was added to *A*. After diluting the content of each flask with water to the 10 ml. mark, suitable samples (1–5 ml.) were removed and examined for reducing sugar content. The difference in the reducing value between *A* and *B* represents the amount of 'yeast-fermentable sugar'.

The time required for 2 ml. yeast suspension to remove the whole yeast-fermentable sugar from 1 ml. seminal plasma was rather long (up to 2 hr.); much longer than the time needed for yeast to remove the sugar from 1 ml. blood plasma. This is only partly due to the fact that the seminal plasma contains several times more sugar than the blood plasma. The main reason is that the yeast-fermentable sugar present in the seminal plasma is not glucose but fructose. Baker's yeast available locally was found to be much less effective towards fructose than towards glucose.

Fructose. The identification of fructose as the sugar present in the seminal plasma was carried out by the purification of the sugar and by the preparation of the methylphenyl-fructosazone which has been shown by Neuberg (Neuberg, 1902, 1904; Neuberg & Strauss, 1902; Langstein & Neuberg, 1907) to be one of the few chemical derivatives by means of which fructose can be identified and distinguished from glucose and from other sugars. According to Neuberg, pure methylphenyl-fructosazone has m.p. 153°.

For rapid quantitative determinations of fructose in small samples of semen (0·05–0·5 ml.) two colorimetric methods were adapted, those of Roe (1934) and of Stewart, Scarborough & Davidson (1938). Both gave satisfactory results provided that the proportions of the solutions used for deproteinizing, 10% (w/v) $ZnSO_4.7H_2O$ and 0·5N-NaOH, were carefully adjusted so as to yield mixtures of pH 7·4–7·5. The routine procedure was similar to that used for blood (Somogyi, 1930): The semen was diluted with water to 8 ml., 1 ml. $ZnSO_4$ and 1 ml. NaOH added, the mixture heated for 1 min. in boiling water and filtered; 2 ml. of the clear filtrate were used for fructose estimation. Roe's method could be used also for trichloroacetic acid filtrates.

Glucose. When fructose was established as the principal yeast-fermentable sugar of semen, it became necessary to decide whether fructose occurs in the semen alone or with other yeast-fermentable sugars, particularly glucose, thus requiring a method for the accurate determination of small quantities of glucose in presence of excess of fructose. This was accomplished enzymically by glucose oxidase, an enzyme extracted from moulds, which rapidly oxidises glucose but scarcely attacks fructose. Two sources of the enzyme were used, one a purified preparation of 'Notatin' kindly supplied to Prof. D. Keilin by Prof. Raistrick and Dr Birkinshaw (Keilin & Hartree, 1945); the other a crude but very potent preparation ($Q_{O_2}=1000$) obtained from *Aspergillus niger* in the following manner. The fungus was grown as described by Mann (1944). After 2 days of growth at 30°, 500 ml. filtered culture medium, pH about 4·5–5, were concentrated *in vacuo* to 50 ml., dialyzed overnight, and the enzyme precipitated with 5 vol. of cold acetone. The acetone-dried preparation was stable at 0°. It was dissolved before use in 0·2M-phosphate buffer, pH 6, so as to give a 0·5% solution. For the estimation of glucose, two incubation flasks were used. Both flasks received 2 ml. seminal plasma + 1 ml. 0·2M-phosphate buffer, pH 6, and in addition 0·1 ml. glucose oxidase and 1 drop of a catalase preparation were added to one flask. The flasks were then shaken in air at 20° for 2 hr. The samples were then deproteinized and the reducing sugar was determined. The difference in the reducing value between the oxidase-treated and untreated sample gives directly the glucose content in 2 ml. seminal plasma.

Other methods. The methods employed for the estimation of lactic acid and of acid-soluble phosphate were the same as previously described (Mann, 1945*b*, *c*). In a few experiments the 'total carbohydrate' was estimated by the orcinol method of Tillmans & Philippi (1929) and glycogen by the method of Good, Kramer & Somogyi (1933). However, a full interpretation of the data obtained by means of the last two methods will be possible only when the seminal 'glycogen' has been purified and its chemical nature properly investigated.

RESULTS

Purification and identification of fructose in semen

For the purification of fructose bull seminal plasma was chosen because it has an even higher fructose content than the seminal plasma of other mammals. Altogether 120 ml. bull seminal plasma were used, containing 780 mg. fructose. This amount represented a mixture of seven samples, each originating from three to four bulls. The quantitative analysis of the seven samples gave the following results:

Reducing sugar (mg./100 ml.)	785	635	498	447	1090	531	1098
Fructose (colorim.) (mg./100 ml.)	779	592	480	376	1040	435	1062

The seminal plasma was made just acid to methyl red with 0·1N-HCl and precipitated with 5 vol. of ethanol. The clear filtrate was concentrated by

distillation *in vacuo* at 40° and the thick syrup extracted with several successive portions of boiling ethanol till the residue gave only a very weak Seliwanoff reaction. The combined ethanolic extracts, 250 ml. in all, were concentrated *in vacuo*, and the extraction of the syrup was repeated with boiling ethanol. This extract, on concentration, yielded a syrup which when dissolved in water and filtered through a thin layer of kieselguhr gave 15 ml. of a perfectly clear and almost colourless solution containing 1030 mg. dry matter. Of this material 630 mg., or 65 %, was fructose as assessed by reduction of ferricyanide and by colorimetric determination. This concentrated solution of fructose obtained from seminal plasma was used for the following tests.

Optical activity. A portion was diluted with water to a concentration of 0·848 % reducing sugar, and the solution, examined in the polarimeter in a 2 dm. tube, gave $\alpha = -1{\cdot}56°$, $[\alpha]_D^{20°} = -92{\cdot}2°$. Pure fructose in aqueous solution had $[\alpha]_D^{20°} = -92{\cdot}3°$.

Yeast-fermentation test. A 1 ml. sample (42 mg. seminal fructose) suitably diluted with phosphate buffer, pH 6, was divided into two portions of which one was incubated for 2 hr. with yeast, the other serving as control. In both samples the reducing sugar and fructose were determined. The yeast-incubated sample no longer contained any fructose or reducing sugar.

Absence of glucose. 1 ml. suitably diluted was examined for the presence of glucose by means of glucose oxidase but there was no change in either reducing value or fructose content as the result of this treatment.

Phenylosazone. 1 ml. was used for the preparation of phenylosazone. Two recrystallizations from ethanol gave 19 mg. of osazone; m.p. 205°.

Methylphenyl-fructosazone. The remaining solution of seminal fructose was concentrated *in vacuo* over P_2O_5 to 3·3 ml. Insoluble material was centrifuged off, and 3 ml. of the clear solution (450 mg. reducing sugar) was treated in a small tube with 1 g. α-methylphenylhydrazine and then ethanol added drop by drop till a clear solution was obtained. After addition of 1 ml. 50 % acetic acid the tube was immersed in boiling water for 5 min. and then cooled in ice. Within 30 min. there appeared a mass of yellow needle-shaped crystals. In addition, a brown oily material was deposited in the bottom of the tube. After 2 hr. the crystalline material was separated from the oil, and washed on a sintered glass filter with ice-cold water. It was recrystallized from 15 % (v/v) ethanol giving 200 mg. of the recrystallized osazone; m.p. 151°. Methylphenylosazone prepared from pure fructose had exactly the same m.p. (151°). An attempt to obtain in this manner methylphenylosazone from glucose was negative.

A further batch of methylphenylosazone was obtained from the oily residue. This was washed several times with cold water (by decanting), dried over H_2SO_4 and then dissolved in absolute ethanol. The solution (3 ml.) was filtered into a test-tube and immersed in a freezing mixture of acetone and solid carbon dioxide. The crystallization began almost at once and after 14 hr. at 0° the crystals were separated. A little more crystalline osazone was obtained from the filtrate by gradual addition of water. However, the product obtained in this manner had m.p. 148°, even after one recrystallization. The total yield of methylphenyl-fructosazone from the seminal sugar was 56 %. The yield obtained by Neuberg & Strauss (1902) from ascites fluid was 66 %; they reported even higher values in some of their other preparations.

Search for other sugars in seminal plasma

Phosphofructose. Bull seminal plasma contains more fructose than the plasma of other animals. It is also distinguished by a remarkably high though variable content of organic acid-hydrolyzable phosphorus compounds (130–200 mg. P/100 ml.). The possibility was envisaged that some of this organic phosphorus may occur in the form of phosphofructose. However, the presence of more than a negligible quantity of fructose in the phosphorylated form may be discounted in view of the following facts. When the seminal plasma is treated with yeast (2 hr., 30°) only 2–25 mg./100 ml. fructose remain out of several hundred mg./100 ml. fructose originally present, yet the disappearance of fructose is not accompanied by any appreciable diminution in the above-mentioned organic P-fraction. Moreover, if the seminal plasma is precipitated with Ba acetate in presence of ethanol, the precipitate contains very little fructose, practically all of which remains in the supernatant solution. Under these conditions monophospho- and diphospho-fructose would both be precipitated as Ba-salts.

The possibility was also considered that apart from fructose the seminal plasma may contain some ketotrioses, either phosphorylated or phosphate-free, which give the same colour reactions as fructose and its derivatives. However, this appeared unlikely in view of the very low content of bisulphite-binding substances. Trichloroacetic acid extracts from bull and ram seminal plasma treated with $NaHSO_3$ according to Clift & Cook (1932) used up less than 0·2 ml. 0·01 N-I_2/ml. semen.

Glucose. Yet another question to be considered was that the seminal plasma might contain some glucose in addition to the large amount of fructose. Table 1 shows that this can be ruled out. When the seminal plasma is shaken aerobically with glucose oxidase and the reducing value of such a sample is compared with that of fresh seminal plasma, the

difference found is very small indeed. Glucose added to seminal plasma disappears after treatment with glucose oxidase.

Table 1. *The effect of glucose oxidase on total reducing sugar and fructose content of seminal plasma*

(Total reducing sugar estimated by reduction of ferricyanide; fructose determined colorimetrically. Results expressed in terms of mg. fructose/100 ml. seminal plasma.)

	Before incubation with glucose oxidase		After incubation with glucose oxidase	
	Reducing sugar	Fructose	Reducing sugar	Fructose
Bull:				
Sem. plasma	559	513	546	505
Sem. plasma +0·8% glucose	1359	539	546	535
Ram:				
Sem. plasma	682	565	682	565
Sem. plasma	231	191	220	190
Rabbit:				
Sem. plasma	618	515	610	515
Blood serum	142	—	15	—
Blood serum +1% glucose	1142	—	16	—
Man:				
Sem. plasma	265	100	257	100
Boar:				
Sem. plasma	31	9	30	9

Concentration of fructose in seminal plasma of various animals

The quantitative assay of fructose was carried out in samples of semen from various animals, ranging from 0·05 ml. in bull to 1 ml. in boar. In view of the identification of fructose by the isolation of methylphenylosazone and by other means as described in the preceding parts, it was safe to regard the values obtainable by colorimetric methods as representing fructose. Whenever practicable, the determination of reducing sugar was carried out simultaneously with the colorimetric estimation of fructose; in all such experiments the same standard solution of pure fructose was used for both methods.

Comparative figures for total reducing sugar, yeast-fermentable sugar and fructose in the seminal plasma of several species in Table 2 reveal the strikingly high level of fructose, which in bull, ram, rabbit and man exceeds several times that of glucose in blood. In boar, on the other hand, only 9 mg./100 ml. fructose was found, or about 1/100 of the fructose concentration in bull. However, it must be remembered that the volume of a single ejaculate of boar (250–500 ml.) is almost 100 times larger than that of bull so that in both animals the quantity of fructose contained in a single ejaculate is about the same: 20–50 mg.

Table 2. *Total reducing sugar, yeast-fermentable sugar and fructose content of seminal plasma*

(Results expressed as mg. fructose/100 ml. seminal plasma.)

	Total reducing sugar	Fructose	Yeast-fermentable sugar	Non-fermentable residue
Bull	785	779	779	16
	1090	1040	1055	35
Ram	458	340	350	108
	315	268	269	46
	472	285	394	78
	525	466	466	59
Rabbit	438	318	300	138
	618	515	521	97
Man	260	100	100	160
	262	120	128	134
	362	204	208	154
Boar	31	9	10	21

There is on the whole a close agreement between the values for fructose and for yeast-fermentable sugar. This furnishes an additional proof that glucose, if at all present, occurs in the seminal plasma only as a slight admixture with fructose. However, occasionally a discrepancy has been observed between the content of yeast-fermentable sugar and that of fructose, indicating the presence of a small amount of yet another yeast-fermentable component. On the other hand, there is in almost every instance a distinct difference between the total reducing sugar and the yeast-fermentable sugar; this points to the existence in the seminal plasma of some reducing but non-fermentable substances. The chemical nature of these substances remains to be investigated.

Another characteristic feature of the seminal sugar is the variability of the fructose content in the semen of the same species. Considerable variations were observed in the semen from the same animal collected on different occasions, in spite of the fact that all estimations of fructose were carried out as soon after ejaculation as possible. These findings agree with statements by earlier investigators who recorded similar variations in reducing sugar in semen (Goldblatt, 1935; Shergin, 1937; McKenzie *et al.* 1938; Davis & Cole, 1939; Ross, Miller & Kurzrok, 1941; Moore & Mayer, 1941). The variations in the content of seminal fructose are difficult to explain. If they were solely due to fructolysis taking place in semen already before ejaculation, i.e. inside the male body, one would expect that samples of semen comparatively poor in fructose would have at the same time a rather high content of lactic acid. This, however, is not so (Table 3). It seems, therefore, that the level of fructose in the semen is not controlled by that mechanism which so strictly regulates the level of glucose, for instance, in blood and some other body fluids.

Table 3. *Fructose and lactic acid content of whole semen (ram)*

(Semen deproteinized within 10 sec. after ejaculation.)

Ram no.	Fructose (mg./100 ml.)	Lactic acid (mg./100 ml.)
1	188	43
	274	25
2	280	43
	428	56
3	238	42
4	300	37
5	176	22
6	340	55
	500	62

Fructose in accessory organs of reproduction

As shown above, freshly ejaculated semen of ram or bull contains several hundred mg./100 ml. fructose. If this high content of fructose is connected with the presence of spermatozoa as such, one would expect in the testes and in the epididymis an even higher concentration of fructose. Yet both these organs were found to contain only 1–4 mg./100 ml. fructose. Moreover, the epididymal semen, i.e. the thick suspension of spermatozoa which can be obtained directly from the ram or bovine epididymis, gives a barely perceptible Seliwanoff reaction. This shows that the site of fructose formation in the male body is situated along the generative passage between the epididymis and the urethral outlet.

Huggins & Johnson (1933), to whom we owe an extensive study of the secretions produced by the prostate gland and the seminal vesicles in man, have noted that the high concentration of reducing sugar in the semen is due principally to the seminal vesicles. Similar observations were made on the bull (Bernstein, 1937), boar (McKenzie *et al.* 1938) and ram (Moore & Mayer, 1941). In view of these observations it seemed desirable to determine the fructose content in the various accessory glands of reproduction. Table 4 shows that in the bull and ram the seminal vesicles contain a high concentration of fructose. On the other hand, in rabbit, which does not possess seminal vesicles, the prostate (Gl. vesicularis) is rich in fructose. The form in which fructose occurs in the accessory glands is under investigation.

Table 4. *Distribution of fructose in organs of reproduction*

	Fructose (mg./100 ml.)				
	Bull	Ram	Rat	Rabbit	Cat
Testes	3	1	1	1	3
Epididymis	3	3	1	1	4
Prostate gland	5	8	10	230	35
Seminal vesicles	480	160	10	—	—
Fluid from seminal vesicles	840	570	—	—	—
Ampullae	—	—	—	12	—
Uterus masculinus	—	—	—	0	—
Cowper's gland	—	—	—	Trace	—

Function of fructose in semen

The main function of fructose is to supply the life energy to the spermatozoa in the form of an easily glycolyzable material. The incubation of a freshly ejaculated whole semen is followed by a progressive fall in fructose, and lactic acid accumulates as the result of fructolysis (Table 5). The rate of fructolysis is greater in N_2 than in air, and the ratio between lactic acid produced and fructose used up is appreciably higher in N_2 than in air (Table 5, Exp. 1). Aerobically, fructolysis is not the only source of energy for the spermatozoa which, even if deprived of fructose, can survive in O_2 owing to utilization of other substances mainly of undetermined nature. Anaerobically, however, the spermatozoa very largely depend on fructose and the cessation of fructolysis invariably terminates their activity. In order to deplete ram semen of most of its fructose it is usually enough to incubate the semen in N_2 for a few hours at 30–37°. If the anaerobic fructolysis is allowed to proceed almost to depletion of fructose and if at this point fresh 'Ringer-fructose-phosphate' is added, this enables the spermatozoa to retain their anaerobic activity and to continue to produce lactic acid with unabated vigour (Table 5, Exp. 2). Reinforcement of the fructose content by extra fructose added at the start of the incubation period has a similar effect (Table 5, Exp. 3). It must be stressed, however, that the survival under anaerobic conditions in semen can only be achieved if the pH of semen is not allowed to fall too low; for this reason sugar was added to semen in the form of 'Ringer-fructose-phosphate' solution which has a high buffering capacity.

Since there is such a close connexion between the disappearance of fructose and formation of lactic acid one might expect perhaps that the addition of excess carbohydrate to whole semen would not only prolong the duration of fructolysis but would at the same time enhance the rate of lactic acid formation. However, that is not so, at least not in semen which already has a high concentration of fructose. It can be seen from Exp. 3, Table 5, that during the first hour of anaerobic incubation, the yield of lactic acid formed is not markedly affected by the presence of additional sugar. Only later, when the semen has depleted its own sugar reserve, the beneficial effect of extraneous sugar becomes more apparent. In Table 6 fructose disappearance in whole semen is compared with lactic acid formation both in absence and in presence of various additional sugars. It can be seen that in presence of

Table 5. *Fructolysis in whole semen*

Exp. 1. Ram semen incubated at 30°; anaerobically in Thunberg tubes filled with N_2; aerobically by shaking in Barcroft manometers filled with air.

	Incubation (min.)	Fructose (mg./100 ml.)	Lactic acid (mg./100 ml.)	Fructose loss accounted for as lactic acid (%)
	0	490	112	—
Anaerobically	150	312	290	100
	300	160	396	86
	450	41	500	86
Aerobically	150	336	220	70
	300	215	292	65
	450	94	336	42

Exp. 2. Ram semen; anaerobic incubation at 37°.

	Additions to semen	Incubation (min.)	Fructose (mg./100 ml.) Content	Fructose (mg./100 ml.) Decrease	Lactic acid (mg./100 ml.) Content	Lactic acid (mg./100 ml.) Increase
Fresh semen	—	0	465	—	118	—
	—	120	35	430	394	276
Same semen after 120 min. incubation	1 vol. Ringer-phosphate	0	35	—	394	—
		90	9	26	407	17
	1 vol. Ringer-fructose-phosphate	0	535	—	394	—
		90	287	248	593	199

Exp. 3. 1 ml. samples of semen diluted with 1 ml. Ringer-phosphate (R. phosph.), Ringer-fructose-phosphate (R. fruct. phosph.) and Ringer-phosphate containing 0·5% glucose instead of fructose (R. gluc. phosph.), respectively. Otherwise conditions as in Exp. 2.

Additions to semen	Incubation (min.)	Fructose (mg./100 ml.) Content	Fructose (mg./100 ml.) Decrease	Lactic acid (mg./100 ml.) Content	Lactic acid (mg./100 ml.) Increase
—	0	393	—	127	—
R. phosph.	60	57	336	284	157
	120	42	351	291	164
	180	9	384	289	162
R. fruct. phosph.	60	582	311	161	161
	120	450	443	240	240
	180	389	504	297	297
R. gluc. phosph.	60	318	75	164	164
	120	265	128	236	236
	180	187	206	315	315

Table 6. *Effect of additional carbohydrate on the rate of glycolysis and on the fructose content of whole semen*

(1% carbohydrate added to whole semen and mixture incubated at 30°.)

Carbohydrate added to whole semen	Incubation (hr.)	Fructose (mg./100 ml.)	Lactic acid (mg./100 ml.)	Decrease n fructose (%)	Ratio: Lactic acid formed / Fructose broken down
None	0	331	201	0	—
None	3	41	397	88	0·67
Glucose	3	274	390	18	3·30
Fructose	3	—	432	—	—
Mannose	3	277	394	17	3·60
Glycogen	3	43	405	87	0·72
Galactose	3	59	369	82	0·61
Pyruvate	3	28	560	92	1·18
Arabinose	3	31	351	91	0·50
Maltose	3	128	401	62	0·99
Lactose	3	131	393	61	0·96
Sucrose	3	—	390	—	—
Raffinose	3	—	371	—	—

added glucose or mannose the rate of fructose disappearance from semen is considerably diminished, but the rate of lactic acid formation remains unimpaired. In other words, the addition of glucose or mannose to whole semen has a suppressing influence on the lactic acid formation from fructose, but this decrease is made good by lactic acid produced from glucose or mannose respectively. The 'preserving effect' on the seminal fructose is evident only with glycolyzable sugars such as glucose and mannose. Glycogen and other non-glycolyzable sugars have no effect.

The rate of fructolysis in semen is highly dependent on temperature (Table 7). At 5–10°, which is the usual temperature for storing semen, an appreciable proportion of seminal fructose still remains after 2 days' incubation. At 30–37°, on the other hand, the disappearance of fructose is only a matter of a few hours.

Table 7. *Effect of temperature on the rate of fructolysis in whole semen*

(Ram semen stored in narrow test-tubes at constant temperature.)

Exp. 1

Incubation Temp.	Hr.	Fructose (mg./100 ml.)	Lactic acid (mg./100 ml.)	Decrease in fructose (%)	Fructose loss accounted for by lactic acid (%)
—	—	414	204	0	—
5°	48	335	242	18	48
10°	48	113	278	77	25
24°	4	125	364	70	55
30°	3	83	421	80	69
37°	2	50	423	87	60

Exp. 2

Incubation Temp.	Hr.	Fructose (mg./100 ml.)	Decrease in fructose (%)
—	0	265	0
5°	1	259	4
10°	1	254	8
15°	1	222	17
24°	1	40	65
28°	1	11	95
30°	1	10	96

Apart from fructose, the semen contains some reducing substances which are not fermented by yeast as well as a certain amount of carbohydrate material which resembles glycogen. So far, however, no evidence could be produced that any of these compounds can be utilized by the spermatozoa as an additional source of glycolysis (Table 8).

The seminal 'glycogen', i.e. the alkali-resistant polysaccharide which behaves on ethanol-precipitation and hydrolysis like the glycogen of other animal tissues, seldom exceeds 0·1 % in ram semen; of seven samples of ram semen, in five the glycogen content ranged from 0·019 to 0·078 %, the remaining two had 0·118 and 0·190 % respectively. Even on prolonged incubation at 37°, under conditions where fructose is completely exhausted, the level of glycogen in semen underwent little change. The chemical properties and function of seminal glycogen remain to be investigated.

With regard to the enzymic mechanism in semen which regulates the fructose metabolism, this is similar in many ways to the mechanism of glucose metabolism previously described in studies on washed spermatozoa (Mann, 1945*c*). There it was shown that glucose is first phosphorylated by adenosinetriphosphate and that the monophosphohexose thus formed is further metabolized through diphosphofructose, phosphotriose, phosphoglyceric acid and pyruvic acid to lactic acid. Table 9 shows that glucose, fructose and mannose are metabolized by washed spermatozoa at a rate which is of an equally high order for all three sugars; galactose and glycogen are glycolyzed only very slowly. Also, the hexokinase reaction can be demonstrated for glucose, fructose and mannose alike when these sugars are incubated with washed spermatozoa in presence of fluoride and adenosine triphosphate (ATP). Table 10 shows that almost half of the readily hydrolyzable phosphorus of ATP is esterified within ½ hr. at 30°, and to the same extent with glucose, fructose and mannose. Among the various other sugars examined, only galactose seems to undergo esterification with ATP. However, a small degree of esterification with ATP can be observed even without additional sugar (Exp. 2, Table 10). This is probably due to small amounts of carbohydrate present in the spermatozoa despite the washing.

The hexokinase reaction also takes place in whole semen (Table 11), the chief reactants being fructose contained in the seminal plasma and preformed ATP in the spermatozoa. As a result of this reaction phosphohexose accumulates and can be precipitated as Ba-salt at pH 8 in the presence of 80 % ethanol. Emphasis must be laid upon the peculiar dependence which was formerly shown to exist between the level of ATP and the glycolysis in washed spermatozoa. This dependence is an equally outstanding feature in whole semen; here, too, the maintenance of the ATP level is intimately linked, at any rate anaerobically, with the unhampered progress of glycolysis (Table 12).

DISCUSSION

Generally, the evidence for the occurrence of free fructose in the animal body has been rather scanty. The presence of fructose in the embryo is one of the few instances where the nature of this sugar was adequately proved. A laevorotatory constituent in foetal fluids was first noticed by Claude Bernard (1855) but its identity with fructose was recognized

Table 8. *Changes in various carbohydrate fractions brought about by anaerobic glycolysis in whole semen*

Exp. 1. 10 ml. ram semen divided into 2 equal parts, A and B. A centrifuged immediately; B centrifuged after 3 hr. incubation at 37° in N_2. Centrifuged seminal plasma subdivided into 2 equal parts, one examined without further treatment, and the other after 2 hr. treatment with washed yeast. Results expressed in mg./100 ml. seminal plasma.

	A Seminal plasma from fresh semen			B Seminal plasma from semen allowed to glycolyze for 3 hr., 37°, in N_2		
	Yeast-fermentable	Non-fermentable	Together	Yeast-fermentable	Non-fermentable	Together
Total carbohydrate in non-deproteinized seminal plasma	508	370	878	51	368	419
Total carbohydrate in protein-free filtrates	491	170	661	49	167	216
Total reducing sugar in protein-free filtrates	457	78	535	19	97	116
Fructose	447	19	466	21	11	32

Exp. 2. 20 ml. ram semen divided into 2 equal parts, A and B. A used fresh; B after 3 hr. incubation at 37°, in N_2. Glycogen estimated in 5 ml. samples of whole semen. Free fructose and 'Embden ester' estimated in filtrates after treatment of whole semen with trichloroacetic acid. The 'Embden ester' was separated as follows. Ba acetate added at pH 8, precipitate discarded, supernatant solution treated with 5 vol. ethanol, left overnight, and centrifuged. The precipitate dissolved in dilute HCl, Ba removed with Na_2SO_4, the Ba-free extracts analyzed. Results in mg./100 ml. whole semen.

	A Fresh semen	B Semen allowed to glycolyze for 3 hr. at 37°
Free fructose	330	23
Glycogen:		
Reducing sugar	118	101
'Embden ester':		
Reducing sugar	21	36
Fructose	3	6
P_0	0	0
P_{30}	1	2
$P_{tot.}$	7	12

(P_0 = phosphorus determined as phosphate which reacts directly with ammonium molybdate; P_7 and P_{30} = phosphorus which appears as orthophosphate after 7 and 30 min. hydrolysis with N-HCl; $P_{tot.}$ = phosphorus as total phosphate after incineration of trichloroacetic acid extract.)

only some time later; fructose was definitely shown to be a normal constituent of both the allantoic and amniotic fluid and in urine of newly-born animals (Majewski, 1858; Grüber & Grünbaum, 1904; Paton, Watson & Kerr, 1907; Langstein & Neuberg, 1907); it was also stated to be present in foetal blood (Orr, 1924). Fructose has also been demonstrated in certain pathological dysfunctions like fructosuria and in transudates (Rosin & Laband, 1902; Neuberg & Strauss, 1902; Moraczewski, 1907; Adler, 1911; Barrenscheen, 1922). In most, if not in all of these instances, the concentration of fructose was sufficiently high to make practicable a proper identification of the sugar by such means as purification, optical activity measurements and the preparation of specific derivatives. Occasionally, however, claims were made of the alleged presence of fructose in animal tissues and body fluids where the chemical criteria applied were less satisfactory. Not infrequently statements were based merely on positive reactions with certain colour-producing substances such as resorcinol (Seliwanoff, 1887), diphenylamine (Ihl, 1885) and cryogenine (Yamada, 1933). Unfortunately, these colour reactions, unless supplemented by more direct and specific methods, seldom disclose the true chemical nature of the sugar studied, since they give positive results not only with fructose but also with a variety of other substances such as the Neuberg ester (6-phosphofructose), Harden-Young ester (2:6-diphosphofructose), sucrose, methylglyoxal, dihydroxyacetone, and numerous ketoses and ketose derivatives.

Among the body fluids, semen is distinguished by a very high content of reducing sugar which the present study proves to be *d*(−)-fructose. The purified seminal sugar shows the same reducing power and the same specific optical activity as pure fructose, and it yields the crystalline methylphenylfructosazone typical of fructose. It also gives the phenylosazone and it is yeast-fermentable but,

Table 9. *Glycolysis in washed spermatozoa*

(Spermatozoa washed free from seminal plasma and made up to the original vol. of whole semen with Ringer-phosphate.)

Exp. 1. Bull spermatozoa, incubated 30 min. at 30° with 0·02 M-sugar.

	Lactic acid (mg./100 ml.)
No substrate	1·5
Anaerobically:	
Glucose	30·5
Fructose	42·5
Mannose	30·5
Aerobically:	
Glucose	24·5
Fructose	24·5

Exp. 2. Ram spermatozoa, incubated anaerobically for 2 hr. at 37° with 0·1 M-sugar.

	Lactic acid (mg./100 ml.)
No substrate	7·0
Glucose	447·0
Fructose	417·0
Mannose	405·0
Galactose	103·0
Glycogen	32·0

unlike glucose, it is not acted upon by glucose oxidase. In addition, it reacts in a characteristic manner with the Seliwanoff reagent and with other ketose-reagents.

At the site of their origin, in the testes and in the epididymis, the spermatozoa, still immotile, have hardly any fructose at their disposal. However, when they traverse the male generative passages from the testes onwards they receive the complementary secretions of several accessory organs of reproduction—seminal ducts, seminal vesicles, prostate gland, Cowper's gland and other urethral glands. These secretions, which together constitute the seminal plasma, form the natural environment and nutrient medium for the reproductive cells. Fructose is one of the numerous constitutents of the seminal plasma. Its concentration varies greatly but in some species it may amount to as much as 1 g./100 ml. plasma. The seminal vesicles are the main contributors of fructose and the final concentration of fructose in the whole semen as ejaculated largely depends on the contribution made by these organs. In some animals, which do not possess seminal vesicles, e.g. rabbit, part of the prostate provides the source of fructose. In the semen itself fructose occurs in a free, non-esterified form. This, however, does not exclude the possibility that fructose may be the end-product of enzymic reactions involving more complex compounds.

Once in contact with spermatozoa, fructose diffuses readily into the cells and enters the characteristic chain of reactions initiated by the hexokinase-catalyzed reaction with adenosinetriphosphate, leading finally to lactic acid. However, even under purely anaerobic conditions the production of lactic acid does not account for the total fructose metabolized. In this respect semen behaves like other animal tissues, except that whereas the majority of tissues have glucose or glycogen at their disposal, semen uses fructose which is the naturally available sugar. It should be pointed out, however, that although normally spermatozoa utilize fructose they are well able to glycolyze other sugars as well. If glucose or mannose, for instance, is added to the semen, this offers the spermatozoa a choice of several glycolyzable substrates and they actually make use of all of them so that whereas in untreated semen the entire lactic acid would have been the outcome of fructolysis, in presence of additional sugar only a certain proportion of lactic acid is derived from the seminal fructose and the rest is the result of glycolysis of the extraneous sugar. This

Table 10. *Phosphorylation of carbohydrate by adenosinetriphosphate in washed sperm*

Exp. 1. 10^9 ram spermatozoa + 0·422 mg. pyro-P of ATP + 0·05 M-sugar + 0·04 N-NaF; anaerobic incubation 30 min. at 30°, in 95% N_2 + 5% CO_2.

	Incubation (min.)	P_0 (mg.)	P_7 (mg.)	P_{30} (mg.)	Pyro-P esterified with sugar (mg.)	(%)
No sugar	0	0·198	0·620	0·626	0	0
Glucose	30	0·251	0·428	0·521	0·182	43
Fructose	30	0·251	0·418	0·500	0·202	48
Mannose	30	0·261	0·475	0·525	0·145	35

Exp. 2. 10^9 ram spermatozoa + 0·283 mg. labile P of ATP + 0·03 M-sugar + 0·05 N-NaF; incubation in test-tubes open to air, 30 min. at 30°.

	Incubation (min.)	P_0 (mg.)	P_7 (mg.)	Pyro-P esterified with sugar (mg.)	(%)
No sugar	0	0·222	0·505	0	0
No sugar	30	0·272	0·495	0·011	4
Glucose	30	0·243	0·381	0·124	44
Fructose	30	0·222	0·376	0·129	45

Exp. 3. 10^9 ram spermatozoa + 0·251 mg. pyro-P of ATP + 0·03 M-sugar + 0·04 N-NaF; incubation in test-tubes open to air, 30 min. at 30°.

	Incubation (min.)	P_0 (mg.)	P_7 (mg.)	Pyro-P esterified with sugar (mg.)	(%)
No sugar	0	0·244	0·495	0	0
No sugar	30	0·282	0·447	0·048	19
Glucose	30	0·232	0·383	0·112	44
Fructose	30	0·225	0·369	0·156	62
Mannose	30	0·251	0·388	0·107	42
Galactose	30	0·252	0·398	0·097	39
Arabinose	30	0·294	0·466	0·029	11
Sucrose	30	0·293	0·473	0·022	9
Maltose	30	0·294	0·478	0·017	6
Raffinose	30	0·294	0·448	0·047	19
Lactose	30	0·318	0·478	0·017	6

Table 11. *Phosphorylations in whole semen*

Exp. 1. Ram semen diluted with equal volume of 'Ringer-fructose-phosphate' and incubated at 30°, then centrifuged and the sperm extracted with trichloroacetic acid. Results are expressed in mg. P/100 ml. non-diluted semen; NaF, 0·02N; iodoacetate (IAA), 0·001N.

Additions to semen	Incubation min.	P_0	P_7	P_{30}	$P_{alk.}$	Pyro-P esterified with sugar	$P_{30}-P_7$	$P_{alk.}-P_0$
0	20	8·0	15·7	16·5	8·0	0	0·8	0·0
NaF	20	8·0	11·6	13·6	8·0	4·1	2·0	0·0
IAA	20	11·2	13·2	15·9	12·8	2·5	2·7	1·6

Exp. 2. Incubated 30 min. at 37°: (A) 5 ml. ram semen without any additions, (B) 5 ml. ram semen containing 0·02N-NaF. Incubation terminated by centrifugation. Centrifuged sperm extracted with trichloroacetic acid. Extracts precipitated with Ba acetate at pH 8 in presence of 10% ethanol, precipitates discarded. Supernatant solutions treated with 5 vol. of ethanol, the precipitate analyzed for P-fractions and fructose (from phosphofructose). Results in mg./100 ml. semen.

	P_0	P_7	P_{30}	$P_{tot.}$	$P_{tot.}-P_{30}$	Fructose
A	0	0	0·10	3·05	2·95	0·2
B	0	0·50	0·70	6·15	5·45	3·4

($P_{alk.}$ = alkali labile P = phosphorus which appears as orthophosphate after exposure to 0·1N-NaOH for 20 min. at 20°; P_0, P_7, P_{30} and $P_{tot.}$ = symbols explained in footnote to Table 8.)

Table 12. *Dependence of the ATP-level on the fructolysis in whole semen*

Exp. 1. 150 min. anaerobic incubation at 37°.

Inhibitor added to ram semen	Anaerobic incubation at 37° (min.)	Fructose (mg./ 100 ml.)	Lactic acid (mg./ 100 ml.)	ATP-amino-N (mg./ 100 ml.)
—	—	432	158	1·44
—	150	38	346	0·74
0·015N-NaF	150	168	158	0·28
0·001N-iodo-acetate	150	248	128	0·35

Exp. 2. 10 ml. ram semen allowed to glycolyze 2 hr. at 37°, i.e. till the fructose content was reduced to 2 mg. Then divided into two equal parts of which one (A) was diluted with 1 vol. Ringer-phosphate, and the other (B) with 1 vol. Ringer-phosphate containing 27·8 mg. fructose. Deproteinized with trichloroacetic acid after 2 hr. incubation at 37°. Results are expressed in mg./100 ml. semen.

	Fructose		Lactic acid	ATP-amino-N
	Before incubation	After incubation	After incubation	After incubation
A	2	0	10·2	0·31
B	29·8	16·7	22·6	0·92

explains why it has been possible in the past to employ glucose successfully as the nutrient component of artificial media for storing mammalian semen. The ability of spermatozoa to make equal use of fructose, glucose and mannose has its foundation in the fact that all three sugars enter the cycle of glycolysis by way of the same hexokinase-reaction with ATP, followed by the formation of monophosphohexose. From this point onwards the breakdown leads through diphosphofructose, phosphotriose, phosphoglyceric acid, pyruvic acid to lactic acid, and in this respect the process in whole semen is very much like that in washed spermatozoa to which sugar was added artificially (Mann, 1945*a*, *b*, *c*).

The progress of fructolysis in semen depends on several factors, such as the actual concentration of fructose in the seminal plasma, the density of spermatozoa, pH and temperature. In the field of artificial insemination it has been a practice of long standing to store the semen of farm animals at a comparatively low temperature of 5–10°, since at this temperature the spermatozoa were known to remain alive much longer than at body temperature. It is now probable that this is due to the slower rate of fructose utilization at lower temperature.

At present it is difficult to form an opinion as to why the animal body should choose fructose instead of glucose or glycogen as the natural sugar of semen. One may recall at this point another body fluid, milk, where the occurrence of lactose creates a similar problem (Kay, 1945). There may be, of course, several causes for the presence of fructose in semen but at least one already established fact must be taken into consideration. This relates to the rather specific behaviour of spermatozoa towards fructose as compared with that of most other animal tissues, few of which, according to Dickens & Greville (1932, 1933) who made a thorough comparative study of glucose and fructose metabolism in numerous animal organs, are capable of metabolizing fructose anaerobically to lactic acid. This should enable the spermatozoa to draw freely on the fructose reservoir as provided by the organs of reproduction without any serious competition from other animal tissues.

SUMMARY

1. Contrary to the views generally held, it was found that the reducing carbohydrate of the seminal plasma is not glucose but *d*(−)-fructose.

2. Fructose has been purified from seminal plasma and identified by its reducing value, optical activity, preparation of methylphenyl-fructosazone, yeast-fermentation test and by other means.

3. In the seminal plasma of several species, including bull, ram, rabbit, boar and man, fructose accounts for practically the whole of the yeast-fermentable reducing sugar. Little glucose, if any, is present in the seminal plasma; this was shown by applying a method which makes use of mould glucose oxidase which oxidizes glucose quantitatively but leaves fructose untouched.

4. The level of fructose in seminal plasma varies from one species to another and even within the same species there are individual differences. The highest values were observed in bull where the concentration sometimes exceeds 1 g. fructose/100 ml. seminal plasma.

5. Sperm obtained directly from the epididymis contains hardly any fructose. During the passage through the male generative tract the semen acquires fructose from the accessory glands of reproduction, of which the seminal vesicles are the chief contributors of fructose. In some animals which have no seminal vesicles proper, fructose is generated in the prostate.

6. The main function of fructose in semen is to supply the spermatozoa with readily glycolyzable material. On storage, the content of seminal fructose falls progressively and lactic acid accumulates.

7. Normally spermatozoa utilize fructose, as this is the chief sugar available in seminal plasma, but their enzymic equipment enables them to metabolize equally efficiently glucose and mannose. The glycolysis of these three sugars in sperm is initiated by a hexokinase interaction with adenosinetriphosphate. The monophosphohexose thus formed is further metabolized through diphosphohexose, phosphotriose, phosphoglyceric acid and pyruvic acid to lactic acid.

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