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Studies on the Metabolism of Semen

2. GLYCOLYSIS IN SPERMATOZOA

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The presence of oxygen is not an essential condition for the survival of spermatozoa. Walton, Hammond & Asdell (1928) were the first to point out that rabbit semen can be successfully stored if placed in narrow test-tubes under a thick layer of paraffin oil which protects the semen from the access of air. Shortly afterwards, Iwanow (1931) showed that dog spermatozoa suspended in an isotonic solution of glucose and phosphate retain their motility when their respiratory activity has been abolished either by poisoning with cyanide or by replacing the air with hydrogen. Redenz (1933) found that the presence of glucose or some other glycolyzable substrate is indispensable if the spermatozoa are to remain alive under anaerobic conditions; without carbohydrate the anaerobic life of the spermatozoa is very short, and in this respect the anaerobiosis differs markedly from the aerobiosis. More recently, it has been conclusively demonstrated that the metabolism of spermatozoa is predominantly of a glycolytic character, and it has been established that the glycolytic activity can serve as a useful guide in assessing the potential fertility of the semen (Shergin, 1939; Comstock, 1939; MacLeod, 1939; Lardy & Phillips, 1941; Moore & Mayer, 1941; Ross, Miller & Kurzrock, 1941; Henle & Zittle, 1942). Up to the present,

studies on the glycolysis of spermatozoa were largely confined to investigations of optimal conditions, various substrates and inhibitors of the glycolysis, leaving open the question of the pathways and enzymes involved in the glycolytic processes. In this study an attempt is made to elucidate the intermediary phases in sperm glycolysis by examining the enzymes and coenzymes which control the glycolytic mechanism. It will be shown that adenosinetriphosphate (ATP) takes part in several intermediary reactions of sperm glycolysis. The evidence presented gives support to the view that ATP is the substance through which a link is established between the activity of spermatozoa on the one hand and the glycolysis on the other (Mann, 1945 a, b). However, further study is required to explore more fully the precise nature of this link.

EXPERIMENTAL

Methods

Ejaculated semen of the ram and bull was used. The spermatozoa were separated from the seminal plasma and washed with calcium-free Ringer-bicarbonate solution as previously described (Mann, 1945 b). The suspensions of washed spermatozoa had the same density as the whole semen (ram, 2.5–5 million cells/ μ l.; bull, 0.7–1 million cells/ μ l.).

The respiration was measured in Barcroft differential manometers, in air. The results are expressed in terms of $Z_{O_2} = \mu\text{l. O}_2$ taken up by 10^8 spermatozoa in 1 hr. at 37° (Redenz, 1933). The anaerobic glycolysis was studied by incubating the washed sperm in Thunberg tubes filled with pure N_2 or with 95% N_2 + 5% CO_2 . The methods used for the estimation of lactic acid and phosphate were the same as those previously described (Mann, 1945*b*). Pyruvic acid was estimated by the method of Clift & Cook (1932). The figures for lactic acid are presented in mg. From these figures the quotient Z_L was calculated on the basis $Z_L = \mu\text{l. CO}_2$ equivalent to lactic acid produced by 10^8 spermatozoa in 1 hr. at 37° .

Respiration and glycolysis of washed spermatozoa

Aerobically, ram spermatozoa which have been washed free of seminal plasma show a high respiratory activity. The average figure found was $Z_{O_2} = -20$ which agrees with the value reported by Lardy, Winchester & Phillips (1945). In order to convert the Z_{O_2} into Q_{O_2} , the Ringer solution was removed from the suspension of spermatozoa by washing the sperm with water and drying at 100° to constant weight. This procedure, however, is not satisfactory since it involves the use for washing of an unphysiological medium. On the whole, therefore, the use of Z_{O_2} is preferable. The average Q_{O_2} of the ram sperm based on the dry weight as determined after washing with water was -8 .

The respiration of washed spermatozoa, if examined in the complete absence of seminal plasma, was not accompanied by any appreciable formation of lactic acid. On the addition of glucose, however, a glycolysis developed which was well pronounced even in a continuously aerated sperm suspension. The rate of the aerobic glycolysis of ram spermatozoa was seldom less than half of the anaerobic glycolysis, but there was much seasonal and individual variation in both the aerobic and anaerobic glycolysis. Anaerobically the highest value observed was 6 mg. lactic acid produced by 1 ml. sperm suspension (3.5×10^9 cells) in 1 hr. at 37° , which corresponds to a Z_L of +42. The lowest value observed was one-sixth of the above figure.

The initial stage of sperm glycolysis

When glucose is added to washed spermatozoa, lactic acid is formed in large amounts. Glycogen, on the other hand, yields hardly any lactic acid (Table 1). The ability of spermatozoa to utilize glucose but not glycogen resembles that of yeast where the initial step in the metabolism of glucose involves a reaction between adenosinetriphosphate (ATP) and glucose (Lutwak-Mann & Mann, 1935). This reaction is catalyzed by hexokinase and yields adenosinediphosphate (ADP) and 6-phosphoglucose (Meyerhof, 1935; Colowick & Kalckar, 1943). It can be conveniently studied in presence of fluoride

Table 1. *Formation of lactic acid from glycogen, glucose, pyruvic acid and Harden & Young ester*

(Washed spermatozoa, 10^9 cells/sample. Glycogen, 0.4%. Other substrates, 0.05M. Total volume of samples, 1–2 ml.)

Exp. 1. Ram spermatozoa suspended in Ringer-bicarbonate buffered with 0.1M-phosphate, pH 7.3. Incubated 1 hr., 37° , in Thunberg tubes filled with 95% N_2 and 5% CO_2 .

	Lactic acid (mg.)
No substrate	0.150
Glycogen	0.165
Glucose	1.940
Pyruvate	1.435
Harden & Young ester	0.330

Exp. 2. Ram spermatozoa suspended in Ringer solution buffered with 0.1M-phosphate, pH 7.3. To each sample added 0.2 mg. cozymase (coz.). Incubated 1 hr., 37° , in open test-tubes.

	Lactic acid (mg.)
No substrate	0.036
Glycogen	0.045
Glucose	0.289
Pyruvate	0.540
Pyruvate + glucose	1.210
Harden & Young ester	0.089
Harden & Young ester + pyruvate	1.623

Exp. 3. Conditions as in Exp. 2.

	Lactic acid (mg.)
No substrate	0.004
Glycogen	0.017
Glycogen + coz.	0.020
Pyruvate	0.690
Pyruvate + coz.	0.512
Glycogen + pyruvate	0.515
Glycogen + pyruvate + coz.	0.555

Exp. 4. Bull spermatozoa. Otherwise conditions the same as in Exp. 2.

	Lactic acid (mg.)
Glycogen	0.080
Glucose	1.580
Pyruvate	0.620
Harden & Young ester	0.335
Harden & Young ester + pyruvate	2.010

which hardly affects hexokinase but helps to preserve ATP by inhibiting the adenosinetriphosphatase.

In spermatozoa, as in other animal tissues and yeast, ATP is readily dephosphorylated unless NaF is added to check the adenosinetriphosphatase activity. However, in addition to the phosphatase which acts on ATP, the semen also contains the powerful adenyolphosphatase or '5-nucleotidase', so that any adenylic acid formed from ATP is rapidly broken down to adenosine. It should be pointed out, however, that the rate at which adenylic acid is dephosphorylated is smaller in the washed spermatozoa than in the whole semen; the bulk of the

5-nucleotidase is supplied by the seminal plasma and is effectively removed by washing. That the metabolism of glucose in the washed spermatozoa is in fact initiated by the shift of a labile phosphate group from ATP to the sugar is shown by the experiments recorded in Table 2. Even in absence of NaF, a slight esterification between ATP and glucose can be observed provided the time of incubation is brief and the temperature low. However, much more definite results are obtained when the reaction between ATP and glucose is examined in the presence of NaF. Thus, in Exps. 1 and 2 of

Table 2, 1 ml. ram sperm, to which glucose, ATP and 0.04N-NaF were added, brings about the disappearance of almost half of the readily hydrolyzable phosphorus of ATP within $\frac{1}{2}$ –1 hr. at 37°. Simultaneously, a difficultly hydrolyzable P compound is formed which behaves like 6-phosphohexose in that it can be precipitated quantitatively as Ba salt at pH 8 in the presence of 75% ethanol and which yields, after hydrolysis with N-HCl, inorganic phosphate at approximately the same rate as pure Embden ester. However, the primary products of the hexokinase reaction, namely 6-phosphohexose

Table 2. *The phosphorylation of glucose by adenosinetriphosphate*

(Washed spermatozoa. Total volume of samples 2–5 ml. Samples incubated in Thunberg tubes filled with 95% N₂ and 5% CO₂, pH 7.3, 37°. Labile P=pyro-P=that part of ATP phosphorus which appears as inorganic phosphate after 7 min. hydrolysis in N-HCl at 100°.)

Exp. 1. Ram spermatozoa. 10⁹ cells/sample. Incubated with ATP (0.422 mg. labile P).

Additions	Incubation (min.)	P ₀ (mg.)	P ₇ (mg.)	P ₃₀ (mg.)	Labile P of ATP esterified with hexose	
					(mg.)	(%)
0	0	0.198	0.620	0.626	0	0
0.05 M-glucose	30	0.528	0.578	0.581	0.042	10
0.05 M-glucose + 0.04 N-NaF	30	0.251	0.438	0.521	0.182	43
0.05 M-glucose + 0.04 N-NaF	120	0.420	0.446	0.500	0.174	41

Exp. 2. Ram spermatozoa. 10⁹ cells/sample. Incubated with ATP (0.725 mg. labile P).

Additions	Incubation (min.)	Acid-soluble P (mg.)	
		(P ₇ -P ₀)	(P _{tot.} -P ₇)
0	0	0.725	0.370
0	30	0.540	0.290
0.05 M-glucose	30	0.160	0.430
0.05 M-glucose + 0.03 N-NaF	30	0.530	0.520

Exp. 3. Ram spermatozoa. 2.5 × 10⁹ cells/sample. Incubated with ATP (0.123 mg. NH₄-N, corresponding to 0.544 mg. labile P). In the trichloroacetic acid extract ATP + ADP were precipitated with Ba acetate at pH 8 and the precipitate analyzed for the content of NH₄-N; the supernatant solution treated with 3 vol. ethanol and the precipitate (free from pyro-P) was analyzed for content of difficultly hydrolyzable phosphate.

Additions	Incubation (min.)	Ba precipitate at pH 8		Ba ethanol precipitate: P-fractions determined	
		Estimated NH ₄ -N (mg.)	Calculated labile P (mg.)	(P ₁₈₀ -P ₀) (mg.)	(P _{tot.} -P ₁₈₀) (mg.)
0.03 M-glucose	0	0.123	0.544	0.005	0.030
0.03 M-glucose	10	0.029	0.122	0.082	0.102
0.03 M-glucose + 0.025 N-NaF	60	0.011	0.046	0.085	0.219

Exp. 4. Bull spermatozoa, 0.7 × 10⁹ cells/sample. Incubated with ATP (0.7 mg. labile P) + 0.001 N-MgCl₂ + 0.013 N-NaF.

Additions	Incubation (min.)	P ₀ (mg.)	P ₇ (mg.)	Labile P of ATP esterified with hexose	
				(mg.)	(%)
0.01 M-glucose	0	0.146	0.850	0	0
0.01 M-glucose	15	0.212	0.785	0.065	9
0	15	0.244	0.820	0.030	4
0.01 M-glucose	60	0.264	0.730	0.120	17
0	60	0.354	0.830	0	0

(P_{inorg.}=true inorganic phosphate determined as MgNH₄PO₄; P₀=phosphorus determined as phosphate which reacts directly with ammonium molybdate; P₇, P₃₀ and P₁₈₀=phosphorus which appears as orthophosphate after 7, 30 and 180 min. hydrolysis with N-HCl; P_{tot.}=phosphorus as the total phosphate after incineration of the trichloroacetic acid extract.)

and ADP, do not accumulate in the spermatozoa but are further metabolized. Thus in Exp. 1, together with a sharp rise in the difficultly hydrolyzable fraction ($P_{\text{tot.}}-P_{30}$), there is also an increase in the more readily hydrolyzable fraction ($P_{30}-P_7$); this, as we shall see later, is due to the formation of diphosphohexose from monophosphohexose. Similarly, ADP is not stable in the sperm but undergoes further dephosphorylation as shown by Exp. 3 of Table 2. Here, the sperm was incubated for 1 hr. at 37°, with ATP, glucose and fluoride. After deproteinization, the sample was precipitated with Ba acetate at pH 8, and the Ba precipitate was analyzed for its content of $\text{NH}_2\text{-N}$ by enzymic deamination; it was found that the $\text{NH}_2\text{-N}$ content which represents the sum of ATP and ADP has decreased as a result of the incubation by about 90 %.

Formation of diphosphohexose

In the normal course of tissue glycolysis, 6-monophosphohexose is phosphorylated by ATP and forms the Harden & Young ester or 1:6 diphosphofructose (Ostern, Guthke & Terszakowec, 1936).

To study the action of the phosphopherase which effects the transfer of labile phosphate groups from ATP to monophosphohexose, use was made of the Embden ester. This ester represents an equilibrated mixture of two compounds, 6-phosphoglucose and 6-phosphofructose. When added to the sperm alone, the Embden ester was hardly metabolized at all; out of 2 mg. difficultly hydrolyzable P added as Embden ester to 1 ml. ram sperm, 0.03 mg. P appeared as inorganic phosphate after 30 min. incubation at 37°, the rest remained unchanged. However, if the same quantity of Embden ester was added to the sperm simultaneously with ATP (1 mg. pyro-P) and the sample incubated 30 min., then 26 % of the Embden ester was converted into a compound which, judging by its behaviour on acid hydrolysis, was diphosphohexose or the Harden & Young ester. The amounts of lactic acid formed after 30 min. incubation, using 1 ml. ram sperm, were as follows: with Embden ester alone 0.037 mg., with ATP alone 0.057 mg., but with Embden ester + ATP 0.558 mg. lactic acid.

In view of the successful demonstration of the phosphopherase activity in spermatozoa, a study was made of the behaviour of the Cori ester or 1-phosphoglucose in spermatozoa. However, when added to the sperm, the Cori ester was hardly metabolized at all; very little or no lactic acid was produced; it was not converted to any appreciable extent either to glucose + inorganic phosphate or to Embden ester.

The conversion of diphosphohexose to phosphotriose

When 2:6-diphosphofructose is added to washed spermatozoa, it is converted to phosphotriose

(Table 3) and finally to lactic acid (Table 1). A slight formation of phosphotriose from diphosphofructose is also noticeable in the seminal plasma, this in spite of the fact that the seminal plasma itself is devoid of glycolytic activity and is unable to produce lactic

Table 3. *Zymohexase activity of semen*

Exp. 1. Bull semen, diluted with 1 vol. Ringer solution, centrifuged, the dilute seminal plasma separated from the sperm, which, after one more washing, was brought with Ringer solution to the original volume of the whole semen. 1 ml. dilute seminal plasma corresponds to 0.5 ml. semen. 0.5 ml. 'washed sperm' corresponds to 0.5 ml. semen, and represents 0.3×10^9 cells. All samples contained 0.2 ml. neutral solution of Harden-Young ester (0.3 mg. P hydrolyzable in 30 min., in N-HCl). They were incubated at 37°.

Incubation (min.)	Alkali-labile P formed in presence of	
	0.5 ml. washed sperm (mg. P)	1 ml. dilute seminal plasma (mg. P)
0	0.0	0.0
5	0.02	0.0
15	0.10	0.04
60	0.15	0.16

Exp. 2. Washed spermatozoa, 0.8×10^9 cells/ml., incubated at 30° with Harden-Young ester (0.3 mg. P hydrolyzable in 30 min., in N-HCl).

Sperm (ml.)	Incubation (min.)	Alkali-labile P (mg.)
0.2	0	0.0
0.2	15	0.08
0.5	0	0.0
0.5	15	0.16

acid from either glucose, fructose or diphosphofructose. It appears that zymohexase, the phosphotriose-forming enzyme, although mainly concentrated in the spermatozoa, is not altogether absent from the seminal plasma. In this respect, the semen somewhat resembles the blood, where the essential glycolytic activity is restricted to the corpuscles, but the blood plasma also contains some intermediary enzymes, among them zymohexase. The rate of formation of phosphotriose in the spermatozoa and in the seminal plasma is represented in Table 3; phosphotriose has been assessed as the alkali-labile P, i.e. as inorganic phosphate which appears after exposure to 0.1 N-NaOH for 20 min. at room temperature.

The oxido-reduction between phosphotriose and pyruvic acid

The rate of lactic acid formation from diphosphohexose in spermatozoa is considerably increased by the addition of pyruvate. The mechanism underlying this process appears to be the same as in the muscle and may be defined as an oxido-reduction between

phosphotriose (derived from the enzymic cleavage of diphosphohexose) and pyruvic acid. It involves the participation of cozymase. The progress of the

reaction was studied in the following experiments. When diphosphohexose and pyruvate are added to spermatozoa and the mixture incubated in the presence of 0.04 N-NaF, the amount of lactic acid formed is as high as in the absence of fluoride. In the NaF-treated sperm, however, in addition to lactic acid there is also an accumulation of phosphoglyceric acid as indicated by the appearance of a difficultly hydrolyzable P fraction; this must be attributed to the inhibitory action of fluoride on enolase. Iodoacetate, 0.002–0.001 N., which inhibits the oxido-reduction between phosphotriose and pyruvate in yeast as well as in muscle, has a similar effect in the sperm.

The addition of cozymase to the system composed of washed spermatozoa, diphosphohexose, pyruvate and NaF, has an accelerating effect on the rate of the reaction, but even without added cozymase the reaction proceeds rapidly suggesting that the content of cozymase in the washed spermatozoa is sufficiently high to catalyze the oxido-reduction. When whole semen was used, even in the absence of NaF, the dismutation was found to proceed quite satisfactorily without additional cozymase; this may be due to an optimal concentration of cozymase in the unwashed spermatozoa; in any case, added cozymase undergoes inactivation by the seminal plasma.

In muscle and yeast, the oxido-reduction between phosphotriose and pyruvic acid is coupled with an esterification of inorganic phosphate and the synthesis of ATP (Needham & Pillai, 1937; Meyerhof, 1937; Warburg & Christian, 1939). This reaction postulates that not more than 1 mol. of inorganic phosphate should be esterified with adenylic acid for each molecule of lactic acid formed. It can be seen from the data in Table 4 that in the sperm the molecular ratio $\frac{\text{Inorganic phosphate esterified}}{\text{Lactic acid formed}}$ does not exceed 1. These experiments provide an illustration of the continuous resynthesis of adenosine-

Table 4. *The oxido-reduction between diphosphohexose and pyruvic acid and the coupling of this reaction with esterification of phosphate*

Exps. 1 and 2. Ram; washed spermatozoa suspended in Ringer-bicarbonate, 10^8 cells/ml., incubated at 37° with an equal volume of medium of the following composition, per ml.: 0.2 ml. of 0.3 M-Na-pyruvate + 0.2 ml. of 0.15 M-Harden-Young ester + 0.05 ml. of 0.1 M-MgCl₂ + 0.05 ml. of 0.5% cozymase + 0.2 ml. phosphate buffer, pH 7.4, + 0.1 ml. of 0.16 M-Na adenylate + 0.1 ml. of 1 M-NaF + 0.1 ml. of 6% NaHCO₃.

Exps.	Incubation (min.)	P _{inorg.} (mg.)	Esterified P		Lactic acid		ATP		P ₁₈₀ (mg.)	P _{tot.} (mg.)
			(mg.)	(10 ⁻⁵ mol.)	(mg.)	(10 ⁻⁵ mol.)	NH ₃ -N (mg.)	Pyro-P (mg.)		
Exp. 1	0	1.120	0	0	0.096	0.11	—	—	—	—
	30	0.875	0.335	1.08	1.342	1.49	—	0.28	—	—
Exp. 2	0	5.225	0	0	0.135	—	0.012	—	8.875	9.450
	30	3.850	1.375	4.35	3.890	4.30	0.330	1.407	5.545	9.350

Exp. 3. Ram; washed spermatozoa incubated with medium of the same composition as in Exps. 1 and 2, but with the exclusion of cozymase (coz.).

Additions	Incubation (min.)	P ₀ (mg.)	Lactic acid (mg.)	ATP-amino-N (mg.)	Adenylate converted to ATP (%)
0	0	3.55	0.18	0.003	0
0	30	3.45	1.60	0.060	11.7
0.2 ml. 1% coz.	30	3.26	2.25	0.192	37.6
0.2 ml. coz. + 0.001 N-iodoacetate	30	4.00	0.65	0.018	3.5

Exp. 4. Ram; 0.5 ml. whole semen incubated in N₂, at 37° , diphosphohexose = diphhex., pyruvate = pyruv.

	Incubation (min.)	Lactic acid (mg.)	P ₀ (mg.)
Diphhex. + pyruv.	0	0.66	0.375
Diphhex. + pyruv.	60	2.13	0.505
Diphhex. + pyruv. + 0.1 ml. 1% coz.	60	2.28	0.555
No additions	60	1.32	0.120
0.1 ml. 1% coz.	60	1.26	0.175

triphosphate in the course of sperm glycolysis. The 'sparing effect' of the glycolysis on the content of ATP can be also demonstrated in the following way. If ATP is added to the sperm alone it disappears much more rapidly than if added together with diphosphohexose and pyruvate (Table 5). If, however, ATP and the two substrates are added together in the presence of iodoacetate, then ATP again disappears very quickly.

small amounts of pyruvic acid and lactic acid can be observed. However, when extra adenylic acid is added, then phosphoglyceric acid is rapidly converted to pyruvic, and subsequently to lactic acid. The phosphate group of phosphoglyceric acid is taken up by adenylic acid and ATP is formed. When 1 ml. washed sperm of ram was incubated anaerobically for 30 min. at 37° with Na phosphoglycerate (3 mg. P), the result was 0.13 mg. pyruvic

Table 5. *The maintenance of the ATP-level in glycolyzing spermatozoa, non-glycolyzing spermatozoa and in seminal plasma*

(Ram semen centrifuged. Seminal plasma decanted. Spermatozoa washed with Ringer solution. Both seminal plasma and spermatozoa diluted with Ringer solution up to the original volume of semen, and 1 ml. used. Pyruv. = pyruvate 0.03 M, diphhex. = diphosphohexose 0.01 M, coz. = 0.1 ml. 1% cozymase, ATP = 0.216 mg. ATP-amino-N corresponds to 0.96 mg. pyro-P and 1.44 mg. P_{tot.}. Incubation at 37°, in N₂.)

mg. pyro-P and 1.44 mg. P _{tot.} . Incubation at 37°, in N ₂ .)					
Sample	Incubation (min.)	Lactic acid (mg.)	ATP-amino-N		Inorganic phosphate set free from ATP (mg. P)
			(mg.)	(% decom- position)	
Spermatozoa					
ATP	0	0.00	0.216	0	—
ATP	60	0.06	0.079	64	0.92
ATP + pyruv. + diphhex. + coz.	60	2.75	0.189	15	—
Seminal plasma					
(a) ATP	0	1.50	0.216	0	—
(b) ATP	60	1.39	0.022	90	1.30
(c) ATP + pyruv. + diphhex. + coz.	60	1.59	0.026	88	—

Table 6. *The metabolism of phosphoglyceric acid*

(Ram; washed sperm, 3×10^9 cells/ml.; Pglyc. = 3 mg. P as Na phosphoglycerate; MA = Na adenylate (from muscle; 0.62 mg. NH₃-N and 1.37 mg. P); to all samples 0.1 ml. of 0.1 N-MgCl₂ added. Incubation at 37°, in Thunberg tubes filled with 95% N₂ + 5% CO₂.)

Exp. 1: in each sample 1 ml. sperm

Additions	Incubation (min.)	P ₀ (mg.)	Pyruvic acid (mg.)	Lactic acid (mg.)	ATP-amino-N (mg.)	Adenylate converted to ATP (%)
(a) MA	0	0.1	0.0	0.0	—	—
(b) MA	30	0.2	0.02	0.27	0.007	1
(c) Pglyc.	0	0.2	0.01	0.04	—	—
(d) Pglyc.	30	0.34	0.13	0.30	—	—
(e) Pglyc. + MA	30	1.35	3.45	1.62	0.166	30

Exp. 2

	Sperm (ml.)	Additions	ATP-amino-N (mg.)	Adenylate converted to ATP (%)
(a)	1	Pglyc. + MA	0.166	30
(b)	0.1	Pglyc. + MA	—	6
(c)	1	Pglyc. + adenosine	—	3

The transfer of phosphate from phosphoglyceric acid to adenylic acid

The accumulation in sperm of phosphoglyceric acid during the oxido-reductive breakdown of diphosphohexose, in the presence of NaF, indicates that phosphoglyceric acid is an intermediary product of the sperm glycolysis.

Phosphoglyceric acid alone added to the sperm is only slowly dephosphorylated and the formation of

acid, 0.3 mg. lactic acid and 0.34 mg. P_{inorg.}. However, in the presence of adenylate the same amount of phosphoglycerate yielded 3.45 mg. pyruvic acid, 1.62 mg. lactic acid, 1.3 mg. P_{inorg.}, and 30% of the added adenylate was converted into ATP. The experimental details are contained in Table 6. When adenosine was substituted for adenylic acid, only 3%, instead of 30%, was converted to ATP. It should be pointed out that the successful demonstration of the reaction between phosphoglyceric

and adenylic acid depends not only on the substrate concentration but also on the amount of sperm. It can be seen from Table 6 that if the sperm is reduced from 1 to 0.1 ml. (0.3×10^9 cells), the synthesis of ATP from adenylic acid falls considerably.

DISCUSSION

The question as to what constitutes the substrate for glycolysis in spermatozoa has been studied by several investigators; considerable differences have been observed especially between different species of animals. These discrepancies may be due to the fact that the metabolism of the whole semen is not merely the outcome of enzymic reactions which take place in the spermatozoa themselves, but to a large extent it represents also the processes which occur in the seminal plasma. In ram semen, for instance, of which only half is taken up by the seminal plasma, one cannot expect to find the same conditions as in human semen, which contains a much higher proportion of plasma. For similar reasons it is not surprising that washed spermatozoa behave differently from the whole semen. For example, washed spermatozoa are hardly able to produce lactic acid from added glycogen, and yet in the whole semen of certain species, notably in human semen, lactic acid is said to be formed from extracellular glycogen. Human seminal plasma, however, is known to be particularly rich in diastase (Goldblatt, 1935) and unless the spermatozoa are thoroughly washed and the seminal plasma completely removed, there exists the possibility that the observed formation of lactic acid from glycogen is the result of an amylolytic decomposition of glycogen to glucose and a subsequent fermentation of glucose to lactic acid. It appears highly probable that free glucose forms the substrate for the lactic acid formation under natural conditions, especially as it is a normal constituent of the semen (Goldblatt, 1935). It is interesting to note in this connexion that seminal plasma is several times richer in glucose than blood serum; the concentrations of glucose (mg./100 ml.) in semen are: man 200–300 (Goldblatt, 1935), ram 217–744 (Moore & Mayer, 1941), bull 281–495 (Shergin, 1939).

In the intermediary reactions of sperm glycolysis, several points deserve comment. The experiments above show that the glycolytic pathways and enzymes involved in the carbohydrate metabolism of spermatozoa resemble those of other animal tissues and yeast. The metabolism of glucose in the sperm is initiated by the shift of labile phosphate groups from ATP to glucose. The monophosphohexose thus formed is further phosphorylated with the intervention of ATP to diphosphohexose, which is then broken down to phosphotriose and oxidized to phosphoglyceric acid. From phosphoglyceric acid, pyruvic acid is formed which is then reduced

to lactic acid. The reconstitution of ATP broken up as the result of the initial reactions of glycolysis is accomplished by means of two reactions identical with those in muscle and yeast. The one occurs through the reaction between adenylic acid and the phosphate group set free during the conversion of phosphoglyceric acid to pyruvic acid; the other occurs between adenylic acid and inorganic phosphate and forms part of the phosphorylative oxidation-reduction. This involves the participation of cozymase which is first reduced in the course of the oxidation of phosphotriose to phosphoglyceric acid and then re-oxidized in the course of the reduction of pyruvic acid to lactic acid.

It was previously stated that, both under aerobic and anaerobic conditions, the decrease in the ATP content of surviving sperm coincides with the loss of motility (Mann, 1945 *a, b*). In this study it is shown how ATP is continually broken down and resynthesized in the course of sperm glycolysis. The content of ATP, and ultimately the anaerobic survival of the spermatozoa, both depend on the maintenance of the sperm glycolysis.

Apart from establishing the existence in spermatozoa of enzymes and coenzymes concerned with the phosphorylative breakdown of carbohydrate, the experiments described above show that the mode of action of the glycolyzing enzymes can be successfully explored in the intact cells without subjecting them to such treatments as acetone extraction, autolysis, etc. They provide welcome evidence in support of the concept that the carbohydrate metabolism in the living cells runs a course analogous to that demonstrable by means of cell-free enzyme systems.

SUMMARY

1. The course of the reactions which form part of the glycolytic mechanism in spermatozoa has been explored in the cells themselves without separating the enzymes from the cell structure.
2. The study of the intermediary enzymic reactions involved in the carbohydrate metabolism of spermatozoa has shown that these reactions closely resemble those of other animal tissues and of the earlier stages of yeast metabolism.
3. Washed spermatozoa use glucose as the substrate of glycolysis. The fermentative breakdown of glucose is initiated by a hexokinase type of reaction with adenosinetriphosphate (ATP). The monophosphohexose thus formed is further phosphorylated by ATP to diphosphohexose. The breakdown of diphosphohexose follows the course through phosphotriose, phosphoglyceric acid and pyruvic acid to lactic acid.
4. Two coenzymes participate in sperm glycolysis, adenosine triphosphate and cozymase. ATP, which yields its labile phosphate groups in

the introductory phase of glycolysis, is later reconstituted (a) through the transfer of the phosphate group from phosphoglyceric acid to adenylic acid and (b) by way of a reaction between inorganic phosphate and adenylic acid, which forms part of the coenzyme-catalyzed 'phosphorylative oxidation-reduction'.

5. Evidence is provided in support of the view that ATP is the substance through which a link is established between the activity of spermatozoa on the one hand and glycolysis on the other.

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Note added in proof: 27 November 1945. Since this paper was sent to press, the sugar present in seminal plasma has been purified and identified as *d*-fructose by means of optical rotation, reduction value and by the formation of crystalline methylphenyl-fructosazone. Washed spermatozoa can metabolize glucose and fructose alike. Yet in whole semen the natural substrate for the formation of lactic acid, as it is provided by the seminal plasma, is mainly fructose. (Mann (1945), *Nature, Lond.*, in the Press.)

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Euphorbain, a Protease Occurring in the Latex of the Weed *Euphorbia lathyris*

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Euphorbia lathyris, commonly known as Caper Spurge, is a biennial plant which grows to a height of about 3 ft. A survey of its distribution in Australia has shown that it is widespread in Victoria and New South Wales, but less plentiful in South Australia, and only rarely found in Queensland and Western Australia. When the stem or a branch of the living plant is broken near the tip, or when the stem is lanced, milky latex drips from the wound. Collection of the latex is best made in the spring, for then it flows freely.

The pH value of the latex samples examined ranges between 5.1 and 5.3, increasing by 0.5 pH units during dialysis. On addition of alkali either to the latex or acetone powder extracts (described later), a bluish purple colour appears at pH 6.5 reaching maximum intensity at pH 7.5. The colour

changes to deep red as the pH approaches 9.0 and yellow at pH 11.5.

Rod-shaped crystals are visible in the latex under the microscope but they disappear when the latex is dialyzed through cellophan. Both the undialyzed latex and the dialysate give reactions characteristic of an aromatic hydroxy acid.

As reported by Ellis & Lennox (1942), the latex possesses a highly active proteinase for which the name 'euphorbain I'* was suggested. Vines in 1903, however, referred to the work of Buscalioni & Fermi (1898), who demonstrated qualitatively the existence of a gelatinase in *E. lathyris* by placing portions

* 'Euphorbain I' is used to distinguish this protease from that of *Euphorbia cerifera* reported by Castañeda, Gavarron & Balcazar (1942).