GLYCEROKINASE AND ITS POSSIBLE ROLE IN GLYCEROL METABOLISM OF BULL SPERMATOOZOA*

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Summary. Glycerokinase occurs in ejaculated bull spermatozoa. Characteristics of the spermatozoan glycerokinase are quite similar to those of rat liver glycerokinase, except that the enzyme is firmly bound to the midpieces (mitochondria). The addition of L-glycerol-3-phosphate, as well as of free glycerol, to the washed bull spermatozoa causes both an increase in oxygen uptake and an accumulation of lactic acid. Since bull spermatozoa possess neither NAD⁺- nor NADP⁺-linked glycerol dehydrogenase, L-glycerol-3-phosphate would be formed by the action of glycerokinase as the initial step of glycerol metabolism in bull spermatozoa, followed by its oxidation to dihydroxyacetonephosphate by the mitochondrial dehydrogenase, via the glycolytic pathway. Among other animals, fowl and ram spermatozoa show high specific activity of glycerokinase. The enzyme activity is low in boar spermatozoa, and is not detectable in rabbit, human, rainbow trout, starfish and sea-urchin spermatozoa.

INTRODUCTION

It is now well established that glycerol exerts beneficial effects on spermatozoa during long-term storage at -79°C or below. Since the observation by White, Blackshaw & Emmens (1954) that glycerol markedly increases the oxygen uptake of washed ram and bull spermatozoa, evidence has been accumulated to indicate that these spermatozoa metabolize glycerol under aerobic conditions (O'Dell, Flipse & Almquist, 1956; Mann & White, 1957; White, 1957), although this does not seem to be responsible for the protecting action of glycerol in deep freezing. From the results that washed ram spermatozoa oxidize dihydroxyacetone as readily as glycerol, and dephosphorylate α - and β -phosphoglycerol which also stimulate the oxygen uptake, it has been suggested that the initial dehydrogenation of glycerol to dihydroxyacetone is involved in glycerol metabolism (Mann & White, 1957). On the other hand, studies on the isolated midpieces, consisting of mitochondrial complex, from bull spermatozoa revealed that L-glycerol-3-phosphate (α -phosphoglycerol) is oxidized at the highest rate among several substrates tested, while no oxidation of free

* This paper is dedicated to Professor Juro Ishida on the occasion of his sixtieth birthday.

glycerol takes place in the isolated midpieces (Mohri, Mohri & Ernster, 1965). Furthermore, the presence of glycerokinase, an enzyme catalysing the formation of L-glycerol-3-phosphate from glycerol and adenosinetriphosphate (ATP), was demonstrated in bull spermatozoa. These results raised the possibility that glycerol is phosphorylated as the first step of its utilization and then oxidized by mitochondrial enzyme to dihydroxyacetonephosphate, which in turn enters the glycolytic machinery.

The present investigation was undertaken to examine the properties and the localization of bull sperm glycerokinase. The distribution of spermatozoan glycerokinase among several species was also examined. The results will be discussed in relation to glycerol metabolism in bull and other spermatozoa.

MATERIALS AND METHODS

Bull semen was collected with an artificial vagina. Spermatozoa were separated from seminal plasma by centrifugation, and washed once with Ca^{2+} -free Ringer solution for spermatozoa (Mann, 1946). The washed spermatozoa were diluted with 0.25 M sucrose up to the original volume and sonicated in cold for 2 min in a Kubota KMS-100 sonic disintegrator at a frequency of 10 kilocycles. The sonicate thus obtained was then fractionated into head, mid-piece, tail and soluble fractions as described by Mohri *et al.* (1965). To determine the enzyme activity of whole semen, either the semen was directly sonicated, or the separated seminal plasma and the sperm sonicate were combined in the original proportion. The same result was obtained in both cases. In some cases, semen and spermatozoa were homogenized, but incomplete disruption of the spermatozoa resulted.

Ram, boar and rabbit semen were also obtained by using artificial vaginæ. In the case of boar, a sperm-rich fraction of semen was used. Cock semen was obtained by the massaging method. Human semen was supplied by the Department of Obstetrics and Gynecology, School of Medicine, Keio University, and rainbow trout semen by the Irikawa Hatchery. Sea-urchin semen was obtained by injecting a few drops of 0.5 M KCl into the body cavity of male animals. Starfish semen was shed following injection of radial nerve extract (Kanatani, 1964). Semen or spermatozoa of these materials were also treated as described above.

Glycerokinase activity was assayed according to the method of Bublitz & Kennedy (1954), the L-glycerol-3-phosphate formed being measured enzymatically by following the optical change at 340 m μ of NAD^+ (Hohorst, 1963a). In some cases, glycerokinase activity was directly observed by recording the change at 340 m μ in the following medium: 0.2 M hydrazine-0.5 M glycine buffer pH 9.5; 10^{-3} M MgCl_2 ; 6×10^{-3} M ATP; 2×10^{-2} M cysteine; 2.5×10^{-2} M KF; 10^{-3} M NAD^+ ; 10^{-2} M glycerol; 10 μ l Baranowski enzyme (crystalline NAD^+ -linked L-glycerol-3-phosphate dehydrogenase from rabbit muscle) and enzyme sample, in a total volume of 3 ml. The amount of enzyme catalysing the formation of 0.1 μ moles of L-glycerol-3-phosphate/hr is expressed as one unit.

NAD^+ - and NADP^+ -linked glycerol dehydrogenase activity were measured

according to the method of Burton (1955), by following the increase in optical density at 340 $m\mu$ at an alkaline pH, using glycerol as substrate. The reverse reactions, i.e. the oxidation of NADH or NADPH coupled with the reduction of dihydroxyacetone or glyceraldehyde to glycerol, were also examined as described by Chino (1960). Phosphatase assay was made in a system consisting of 0.025 M Tris-HCl buffer, pH 7.4; 10^{-3} M $MgCl_2$; 2.5×10^{-2} M KF; 3×10^{-3} M DL-glycerol-3-phosphate and enzyme preparation, in a total volume of 1 ml, at 37° C. Liberated inorganic phosphate was determined according to Fiske & Subbarow (1925).

Lactate (Hohorst, 1963b), dihydroxyacetonephosphate (Bücher & Hohorst, 1963) and glycerol (Wieland, 1963) were determined by enzymatic methods. Glycerol was also determined by the method of Ryley (1955), as modified by White (1959).

The oxygen uptake was measured by the direct Warburg method, at 37° C. Once-washed spermatozoa were suspended in the Ca^{2+} -free Ringer solution with care to avoid injuring the sperm cells.

TABLE 1
GLYCEROKINASE ACTIVITY OF SEMEN, SEMINAL PLASMA AND
SPERMATOZOA

	No. of exps.	Activity (unit/mg protein)		
		Semen	Seminal plasma	Spermatozoa
Bull				
Homogenate	4	0.04 ± 0.01	0.00	0.46 ± 0.08
Sonicate	6	0.03 ± 0.02	0.00	0.81 ± 0.20
Ram				
Homogenate	1	0.07	0.00	0.32
Sonicate	5	0.17 ± 0.04	0.00	0.95 ± 0.17

One unit corresponds to 0.1 μ moles of L-glycerol-3-phosphate formed/hr.

The total nitrogen content was estimated by direct Nesslerization after the combustion of samples, and multiplied by 6.25 in order to calculate the amount of protein. Sperm counts were made with a haemocytometer.

All chemicals and enzymes used were commercially available products. De-ionized water was used in the preparation of all solutions.

RESULTS

Glycerokinase activity in bull and ram semen

As shown in Table 1, homogenates or sonicates of both bull and ram spermatozoa washed free of seminal plasma exhibited relatively high glycerokinase activities, while no activities were detectable in the seminal plasma of either material. Although the whole semen also showed some glycerokinase activity, such activity was very low as compared with that of the washed spermatozoa, even if the presence of the seminal plasma in the whole semen is taken into consideration. This could be interpreted as indicating that the potent seminal

phosphatase immediately hydrolysed the L-glycerol-3-phosphate formed by the action of the spermatozoan glycerokinase, resulting in an apparently low glycerokinase activity in homogenates or sonicates of whole semen. In fact, the seminal plasma showed sufficiently high phosphatase activity toward DL-glycerol-3-phosphate when glycerokinase assays were performed under similar conditions (Table 2).

TABLE 2
PHOSPHATASE ACTIVITY OF SEMINAL PLASMA

<i>Animals</i>	<i>Activity (unit/mg protein)</i>
Bull	1.33
Ram	2.43

One unit corresponds to 0.1 μ moles of inorganic phosphate liberated from DL-glycerol-3-phosphate/hr.

Glycerokinase activities obtained with the sonicates were much higher than those of the homogenates. This might be due to the fact that sonication brought about more complete disruption of sperm cells, permitting the substrate to enter more readily. This interpretation is also supported by the fact that the enzyme activity increased somewhat after repeated freezing and thawing of the enzyme preparations.

Localization of bull sperm glycerokinase

Glycerokinase is known as one of the soluble enzymes, although the phosphorylation of glycerol catalysed by this enzyme is considered as an essential step in the synthesis of neutral fats or phospholipids, which takes place in mitochondria. The data presented in Table 3 indicate that the spermatozoan

TABLE 3
DISTRIBUTION OF GLYCEROKINASE ACTIVITY IN SPERM FRACTIONS

<i>Bull</i>	<i>Activity (unit/mg protein)</i>				
	<i>Sonicate</i>	<i>Head</i>	<i>Mid-piece</i>	<i>Tail</i>	<i>Soluble</i>
I	1.01	0.15	4.12	1.47	—
II	0.87	0.22	4.26	1.87	—
III	0.95	0.10	4.08	0.68	0.08

glycerokinase is highly concentrated in the isolated midpiece fraction. The head fraction possesses practically no enzyme activity and the activity of the tail fraction is much lower than that of the midpiece fraction. Little activity remained in the soluble fraction obtained after centrifugation of the tail fraction, i.e. the supernatant after removal of heads and midpieces, at 100,000 *g* for 60 min.

Furthermore, attempts to dissolve the glycerokinase from the midpiece fraction, including extraction with acetate buffer at pH 5.2 (which is effective in obtaining glycerokinase from rat liver or other sources in a soluble form), prolonged sonication, digitonin treatment and n-butanol extraction, have so far been unsuccessful, although a preparation with very weak activity was obtained after extraction of acetone-dried powder of the sonicated midpieces. These results seem to indicate that the spermatozoan glycerokinase is rather firmly bound to the midpieces, possibly to the mitochondria.

Properties of bull sperm glycerokinase

Since it was found difficult to dissolve the spermatozoan glycerokinase, some properties of this enzyme were examined with the isolated mid-piece fraction. Text-fig. 1(a) and (b) indicate that the amount of L-glycerol-3-phosphate

TEXT-FIG. 1. (a) Effect of enzyme concentration on the reaction velocity of glycerokinase from bull spermatozoa. (b) Effect of incubation time on the spermatozoan glycerokinase.

formed is proportional, in the ranges shown, to both enzyme concentration and time. As can be seen from Text-fig. 2, the maximal rate of the reaction was obtained at about pH 10.0. The pH-activity curve is similar to that of rat liver glycerokinase (Bublitz & Kennedy, 1954). Text-fig. 3 is a Lineweaver-Burk plot of the reciprocals of rate of enzyme action versus the reciprocals of substrate concentration. The Michaelis constant obtained from this figure was about 4×10^{-4} M. At higher concentrations of substrate, the reaction rate was depressed.

Both glycerol and dihydroxyacetone have been reported to be substrates for rat liver glycerokinase, the latter being phosphorylated more rapidly by the enzyme action (Bublitz & Kennedy, 1954). As indicated in Table 4, the spermatozoan glycerokinase also phosphorylated these two substrates. The rate, however, was higher with glycerol than with dihydroxyacetone under the present experimental conditions.

TEXT-FIG. 2. pH-activity curve of glycerokinase from bull spermatozoa. 0.025 M histidine buffer, adjusted with HCl or NaOH to desired pH.

TEXT-FIG. 3. Lineweaver-Burk plot of glycerokinase from bull spermatozoa. Ordinate: reciprocal of rate of phosphorylation of glycerol ($1/v = 1/10$ unit = $1/\text{m}\mu\text{mole/hr/mg protein}$); abscissa: reciprocal of substrate concentration ($1/s = 1/\text{mM}$).

TABLE 4
PHOSPHORYLATION OF GLYCEROL AND
DIHYDROXYACETONE BY BULL SPERM
GLYCEROKINASE

Exp. no.	Activity (unit/mg protein)	
	Glycerol	Dihydroxyacetone
1	6.11	3.31
2	5.73	3.36

One unit corresponds to 0.1 μ moles of L-glycerol-3-phosphate or dihydroxyacetonephosphate formed/hr.

Distribution of spermatozoan glycerokinase

Among several animal species examined, cock spermatozoa exhibited the highest activity of glycerokinase (about three times as high as that of bull spermatozoa) followed by ram and bull spermatozoa (Table 5). Boar spermatozoa showed a relatively low glycerokinase activity, while no appreciable activity was detectable in human, rabbit, rainbow trout, two species of sea-urchin or starfish spermatozoa. The enzyme activity of seminal plasma was negligible in all of these species.

TABLE 5
DISTRIBUTION OF SPERMATOZOAN GLYCEROKINASE

Animals	No. of exps.	Activity (unit/mg protein)		
		Semen	Seminal plasma	Spermatozoa
Bull	6	0.03 $\pm$ 0.02	0.00	0.81 $\pm$ 0.20
Ram	5	0.17 $\pm$ 0.04	0.00	0.95 $\pm$ 0.17
Boar	2	—	0.09 $\pm$ 0.06	0.25 $\pm$ 0.08
Rabbit	2	—	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01
Human	2	0.00	0.00	0.00
Cock	2	—	0.02 $\pm$ 0.02	2.44 $\pm$ 0.42
Rainbow trout	1	—	0.02	0.01
Sea-urchin				
<i>Pseudocentrotus depressus</i>	1	0.00	—	—
<i>Hemicentrotus pulcherrimus</i>	1	0.00	—	—
Starfish (<i>Asterias amurensis</i>)	1	0.00	—	—

Such differences in glycerokinase activity of spermatozoa from species to species seem to be correlated with the difference in their pattern of glycerol metabolism, as will be discussed later.

Glycerol dehydrogenase

Two pathways at least of glycerol metabolism are known as follows:

In determining which pathway is operating in the spermatozoa under investigation, it is desirable to test for the presence or absence of glycerol dehydrogenase. Two types of glycerol dehydrogenase have been reported, namely NAD^+ -linked (Burton, 1955) and NADP^+ -linked glycerol dehydrogenase (Faulkner, 1958). By the action of the former, glycerol would be converted to dihydroxyacetone, provided that a high concentration of glycerol is added or the product is continuously removed; the latter catalyses the inter-conversion between glycerol and dihydroxyacetone or glyceraldehyde.

Text-fig. 4 indicates that bull spermatozoa show no appreciable activity of either type of glycerol dehydrogenase as compared with the high activity of glycerokinase in the same sample, when glycerol was used as substrate.

TEXT-FIG. 4. Glycerokinase and glycerol dehydrogenase of bull spermatozoa. ●, Glycerokinase; 0.2 M hydrazine-0.5 M glycine buffer, pH 9.5, 0.001 M MgCl_2 , 0.02 M cysteine, 0.025 M KF, 0.006 M ATP, 0.01 M glycerol, 0.001 M NAD^+ and 10 μl Baranowski enzyme. ○, Glycerol dehydrogenase; 0.033 M sodium pyrophosphate, 0.17 M glycerol, 0.001 M NAD^+ or NADP^+ , 0.05 ml bull sperm sonicate. Total volume 3 ml.

In this particular experiment, glycerokinase activity was measured under the conditions where pH was 9.5 instead of 7.4 used in other experiments, and dihydroxyacetonephosphate formed by the combined action of glycerokinase and crystalline NAD^+ -linked L-glycerol-3-phosphate dehydrogenase was caught up by hydrazine. The activity thus measured was much higher than that obtained with the routine method. The experiment was then repeated in order to observe glycerol dehydrogenase activity under the same experimental conditions as those for glycerokinase activity, i.e. with hydrazine-glycine buffer, pH 9.5. There was no increase in the optical density at 340 mμ. Even if the reverse reactions, i.e. the oxidation of NADH or NADPH with glyceraldehyde or dihydroxyacetone as substrates, were followed, no decrease

was observed in the optical density at 340 $m\mu$ by the addition of either bull sperm sonicate or seminal plasma.

Effect of glycerol and L-glycerol-3-phosphate on oxygen uptake and lactate formation

As reported by other investigators (White *et al.*, 1954; Mann & White, 1957; White, 1957), glycerol stimulated the oxygen uptake of washed bull

TEXT-FIG. 5. Effect of glycerol and DL-glycerol-3-phosphate on oxygen uptake of washed bull and boar spermatozoa. (a) Bull, $8.5 \times 10^8/2$ ml; (b) boar, $4 \times 10^8/2$ ml. ∇ , No addition; $\circ$, DL-glycerol-3-phosphate (0.02 M); $\bullet$, glycerol (0.01 M).

TABLE 6

METABOLISM OF GLYCEROL AND DL-GLYCEROL-3-PHOSPHATE BY WASHED BULL AND BOAR SPERMATOZOA

Animal	Additions	Increase or decrease (μ moles/3 hr)			
		Glycerol	L-Glycerol-3-phosphate	Lactate	Dihydroxy-acetone-phosphate
Bull (7×10^8 sperm.)	Glycerol (20 μ moles)	-11.23	+1.47	+0.76	± 0.00
	DL-Glycerol-3-phosphate (40 μ moles)	+1.35	-9.10	+1.98	+0.20
Boar (4×10^8 sperm.)	Glycerol (20 μ moles)	-0.98	± 0.00	+0.05	-
	DL-Glycerol-3-phosphate (40 μ moles)	+29.50	-18.91	+0.19	-

spermatozoa and led to the formation of lactic acid (Text-fig. 5a and Table 6). Since the present experiments were performed with a phosphate-poor medium, the amount of lactic acid formed was much less than that reported with a phosphate-rich medium, probably due to the fact that phosphate inhibits the lactate oxidation (cf. Mann & White, 1957). The disappearance of added

glycerol was also followed by an accumulation of L-glycerol-3-phosphate, suggesting the possibility that this compound is formed as an intermediate of glycerol utilization.

In Text-fig. 5 is also shown the effect of DL-glycerol-3-phosphate on the oxygen uptake of washed bull spermatozoa. The addition of DL-glycerol-3-phosphate also caused an increased oxygen uptake, nearly up to the rate obtained with glycerol. In some cases the rate of oxygen uptake with DL-glycerol-3-phosphate exceeded that with glycerol. At the same time a marked disappearance of added L-glycerol-3-phosphate was observed (Table 6). Living organisms utilize only L-glycerol-3-phosphate, but not its D-isomer, and this is also true of bull sperm mitochondria (Mohri *et al.*, 1965). The disappearance of L-glycerol-3-phosphate was accompanied by an accumulation of lactic acid and a small amount of dihydroxyacetonephosphate, a logical intermediate of the metabolism of L-glycerol-3-phosphate. The observed formation of free glycerol from the added DL-glycerol-3-phosphate would be a result of the action of the phosphatase contained in the spermatozoa. In contrast to the high activity of phosphatase in the seminal plasma, however, neither washed bull spermatozoa nor any of their parts obtained after fractionation showed appreciable phosphatase activity, at least at neutral pH.

Assuming that L-glycerol-3-phosphate is first oxidized to dihydroxyacetonephosphate by the mitochondrial oxidase system and then to $\text{CO}_2 + \text{H}_2\text{O}$ via lactic acid (more correctly via pyruvic acid) through the tricarboxylic acid cycle, the amount of glycerol or L-glycerol-3-phosphate removed during the incubation of sperm suspensions, corrected for the accumulation of intermediates, well accounts for the increment in the oxygen uptake of the sperm suspensions. In washed bull spermatozoa, the enhancement of oxygen uptake by glycerol or L-glycerol-3-phosphate did not lead to any remarkable change in sperm motility.

In Text-fig. 5(b) and Table 6 are also shown the results obtained with washed boar spermatozoa, where the activity of glycerokinase was found to be relatively low. The endogenous oxygen uptake of washed boar spermatozoa was much lower than that of washed bull spermatozoa, and the addition of glycerol did not improve markedly this low oxygen uptake. Analyses of glycerol and possible products before and after the incubation of sperm suspension also indicated that washed boar spermatozoa could not utilize the added glycerol effectively.

On the other hand, a considerable increase in the oxygen uptake was caused by adding DL-glycerol-3-phosphate, up to a rate almost comparable to that obtained with this compound in washed bull spermatozoa. Although most of the added L-glycerol-3-phosphate (about 50% of DL-glycerol-3-phosphate) disappeared after 3 hr incubation of the sperm suspension, there was an accumulation of free glycerol over the amount of L-glycerol-3-phosphate removed, probably due to the action of spermatozoan phosphatase. Half or more of the formed glycerol, however, would originate from D-glycerol-3-phosphate, which is not utilized directly by living organisms. Thus, at least the difference between the L-glycerol-3-phosphate removed and half of the free glycerol accumulated would be the amount of L-glycerol-3-phosphate directly utilized by washed boar spermatozoa. This amount is still quite sufficient to account

for the observed increase in the oxygen uptake, if calculation is made according to the above-mentioned assumption.

Furthermore, in washed boar spermatozoa, the addition of DL-glycerol-3-phosphate also brought about an improvement of motility, while this was not the case with free glycerol.

Effect of fructose on glycerokinase activity

It has been reported that in the presence of both fructose and glycerol a definite 'sparing effect' was exerted by glycerol on the rate of aerobic fructolysis in washed ram spermatozoa (Mann & White, 1957). Since the first step of fructose utilization is either phosphorylation to fructose-6-phosphate by the action of hexokinase or a conversion to fructose-1-phosphate by the action of fructokinase, both of which require ATP, there is a possibility that the 'sparing effect' is a result of competition between hexokinase or fructokinase and glycerokinase for available ATP molecules.

As can be seen from Table 7, the glycerokinase activity of the mid-piece

TABLE 7
EFFECT OF FRUCTOSE ON GLYCEROKINASE
ACTIVITY

	Activity (unit/mg protein)	
	-Fructose	+Fructose
Bull		
Sperm sonicate	1.83	1.22
Mid-piece fraction	5.65	5.71

fraction of bull spermatozoa, which contains little activity of the enzymes involved in fructolysis, was not affected by the presence or absence of fructose. In agreement with the above postulation, however, the formation of L-glycerol-3-phosphate from glycerol in the sperm sonicate containing the whole fructolysis system was somewhat depressed in the presence of fructose.

DISCUSSION

The properties of glycerokinase obtained from bull spermatozoa, i.e. pH-activity curve, Michaelis constant, etc., are quite similar to those of rat liver glycerokinase reported by Bublitz & Kennedy (1954). However, in contrast to glycerokinase from other animal sources, known to be a soluble enzyme, the spermatozoan glycerokinase appears to be firmly bound to mitochondria, since it is resistant to the usual extraction procedures. This characteristic may have some relation to the dehydration process which takes place during spermiogenesis, or to the limited space in mature spermatozoa. In this respect, it is interesting that glycerokinase found in peanut seedlings has been also reported to be bound to mitochondria (Stumpf, 1955). It is likely that glycerokinases from other sources are loosely bound to mitochondria and thus easily obtain-

able in a soluble form, because the reaction catalysed by glycerokinase is one of the initial steps in fat synthesis, which proceeds in mitochondria.

The finding of glycerokinase in bull and ram spermatozoa also threw light on the hitherto unexplained mechanism of the transformation of glycerol in these spermatozoa. From the facts that glycerol increases the oxygen uptake of washed bull and ram spermatozoa, resulting in an accumulation of lactic acid, Mann & White (1957) and White (1957) postulated the conversion of glycerol to dihydroxyacetone as the initial step of glycerol utilization, followed by a glycolytic breakdown to lactic acid via dihydroxyacetonephosphate. As the present experiments failed to detect the activity of glycerol dehydrogenases, both NAD^+ - and NADP^+ -linked, which are indispensable for the oxidation of glycerol to dihydroxyacetone, the above process would not be the main pathway for glycerol utilization. The fact that dihydroxyacetone gives rise to an increased oxygen uptake, and also to the formation of lactic acid to the extent obtained with glycerol (Mann & White, 1957; White, 1957), could be explained by the present result that this compound is readily phosphorylated to dihydroxyacetonephosphate by the spermatozoan glycerokinase, although at a somewhat slower rate than the phosphorylation of glycerol.

The following evidence, on the other hand, favours the view that glycerol is first phosphorylated to L-glycerol-3-phosphate, which, in turn, is oxidized to dihydroxyacetonephosphate and then enters into the glycolytic system. (1) Bull and ram spermatozoa possess glycerokinase of relatively high activity. (2) L-Glycerol-3-phosphate added to washed full spermatozoa was oxidized as readily as glycerol and also produced lactic acid. (3) L-Glycerol-3-phosphate accumulated after the aerobic incubation of sperm suspensions with glycerol. (4) Dihydroxyacetonephosphate was detected after the aerobic incubation of sperm suspensions with DL-glycerol-3-phosphate, although in a small amount. (5) The process of glycerol utilization depends strictly on the presence of oxygen (Mann & White, 1957). This coincides with the fact that L-glycerol-3-phosphate is stoichiometrically oxidized to dihydroxyacetonephosphate by mitochondrial L-glycerol-3-phosphate dehydrogenase in bull spermatozoa (Mohri *et al.*, 1965). Soluble NAD^+ -linked L-glycerol-3-phosphate dehydrogenase is lacking in these spermatozoa. Under anaerobic conditions, the glycerol utilization would stop at this stage, giving rise to no accumulation of lactic acid. (6) Although some free glycerol was formed after the incubation of washed bull spermatozoa with DL-glycerol-3-phosphate (cf. also Mann & White, 1957), the amount of L-glycerol-3-phosphate utilized after subtracting the amount of accumulated glycerol and other intermediates was quite enough to account for the increase actually observed in oxygen uptake, as shown even in an extreme case of washed boar spermatozoa. (7) In washed boar spermatozoa, which show a relatively low activity of glycerokinase, only L-glycerol-3-phosphate was actively metabolized, causing the increased oxygen uptake. Free glycerol, on the other hand, exerted no appreciable effects. In sea-urchin spermatozoa, which have L-glycerol-3-phosphate dehydrogenase bound to a particulate component (Mohri, 1957), but not glycerokinase, glycerol also failed to increase their oxygen uptake.

It has been reported that both α - and β -phosphoglycerol were effective in

producing an increase in oxygen uptake of washed ram spermatozoa (Mann & White, 1957). These results appear somewhat in conflict with the above idea, because β -phosphoglycerol (glycerol-2-phosphate) is not oxidized by the mitochondrial oxidase system. There are two possibilities, however, which would explain such results. One possibility is that the sample of glycerol-2-phosphate used was contaminated with L-glycerol-3-phosphate. In fact, in a previous experiment using the mid-pieces isolated from bull spermatozoa, a commercially available sample of glycerol-2-phosphate was oxidized at exactly the same rate as L-glycerol-3-phosphate, and enzymatic analysis revealed that this product contained a certain amount of L-glycerol-3-phosphate (Mohri *et al.*, 1965). Another possibility is that glycerol-2-phosphate was converted into L-glycerol-3-phosphate via free glycerol, owing to the combined action of spermatozoan phosphatase and glycerokinase, and then oxidized by the mitochondrial oxidase system. On the other hand, White (1957) reported that phosphoglycerol neither produced any increase in the oxygen uptake nor formed appreciable lactic acid in washed bull spermatozoa. Although there is no further description of the phosphoglycerol used in his experiments, the compound should be glycerol-2-phosphate. Since phosphatase activity of bull spermatozoa was found to be quite low, there would be less possibility that glycerol once formed from glycerol-2-phosphate is again phosphorylated to L-glycerol-3-phosphate.

The accumulation of fructose during the course of aerobic glycerol metabolism reported in washed bull spermatozoa (White, 1957) can be explained consistently with the present view of glycerol metabolism. It has been reported that the addition of fructose resulted in less glycerol-1- ^{14}C utilization by twice-washed bull spermatozoa (O'Dell, Almquist & Flipse, 1959) and, conversely, that glycerol exerted a 'sparing effect' on fructolysis in washed bull spermatozoa (White, 1957). These phenomena could be understood as the result of competition for ATP between glycerokinase and hexokinase or fructokinase, both of which catalyse the initial reaction in glycerol and fructose utilization, respectively.

A question then arises as to why these spermatozoa contain glycerokinase, because glycerol as such is not present in semen except perhaps in traces. A most likely source of free glycerol would be diglycerides, which are suggested by Turner & Korsh (1962) to be an adequate substrate to support endogenous respiration of bull spermatozoa by the oxidation of the fatty acids. Phospholipids would be another possible source of glycerol. According to Hartree & Mann (1960), however, choline-plasmalogen, the main phospholipid of bull and ram spermatozoa, is hydrolysed only to the stage of lysoplasmalogen by phospholipase A. Even if glycerophosphorylesters were formed from lysophospholipids, no diesterase to hydrolyse glycerophosphorylesters has so far been detected in sperm cells, in spite of the fact that high concentrations of glycerophosphorylcholine are found in seminal plasma of many species. In this connection, it is interesting that a diesterase is found in the female genital tract of many animals (White, Wallace & Stone, 1963). The liberated L-glycerol-3-phosphate from glycerophosphorylcholine could be directly oxidized and utilized as an energy source by the spermatozoa in the female genital tract.

An entirely different possibility also exists concerning the formation of free glycerol (Hers, 1957). Namely, fructose, another main constituent of seminal plasma, is first phosphorylated to fructose-1-phosphate with the aid of fructokinase, and then split into glyceraldehyde and dihydroxyacetonephosphate by aldolase. Glyceraldehyde thus formed would be then converted into glycerol by either NADP^+ -linked glycerol dehydrogenase (aldose-reductase) or NAD^+ -linked alcohol dehydrogenase. The existence of such a pathway in spermatozoa, however, can be excluded by the fact that no oxidation of NADH or NADPH occurred in bull sperm sonicate or seminal plasma with glyceraldehyde as substrate, although the presence of aldose-reductase in ram seminal vesicle has been reported (Hers, 1957).

In summarizing the above facts, the following pathway can be drawn concerning the metabolism of glycerol and related compounds in bull (and possibly also ram, goat and cock) spermatozoa.

Finally, the present results raise another question as to whether phosphorylation of glycerol is an essential step in the transfer of glycerol into sperm cells across the membrane. It has been shown empirically that the deep freeze preservation of boar spermatozoa (possessing weak glycerokinase activity and utilizing only L-glycerol-3-phosphate) in the presence of glycerol is not so successful as that of bull spermatozoa (possessing high glycerokinase activity and utilizing both glycerol and L-glycerol-3-phosphate).

The uptake of glycerol into boar spermatozoa, which is necessary to protect the sperm cells from the adverse effects of freezing, might not occur so efficiently.

More damage might thus be caused than with bull spermatozoa. This interpretation must await further elucidation.

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