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Glycerol Metabolism by Spermatozoa

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Although the inclusion of glycerol in media for freezing and long-term storage of bull semen is now a generally established practice, very little has been known hitherto about the mechanism underlying the uptake and metabolism of glycerol by spermatozoa. Recently, however, it has been shown by White, Blackshaw & Emmens (1954) that although glycerol does not affect the motility and respiration of spermatozoa in a fructose-containing medium, it increases markedly the oxygen uptake of washed ram- and bull-sperm suspensions, giving rise under certain conditions to an accumulation of lactic acid. Evidence was also brought forward recently by O'Dell, Flipse & Almquist (1956) that [^{14}C]glycerol is taken up readily and utilized by bovine spermatozoa, with the production of $^{14}\text{CO}_2$, and that the rate of glycerol uptake is higher in the absence than in the presence of seminal plasma. Further experiments on glycerol metabolism by ram spermatozoa are described in the present study, together with new observations on the metabolic behaviour of certain related substrates, and, particularly, phosphoglycerol, dihydroxyacetone and sorbitol (preliminary communication, Mann & White, 1956). These findings are discussed in the light of another recently completed study (Dawson, Mann & White, 1957), which describes the occurrence in ram seminal plasma of large quantities of bound glycerol in the form of glycerylphosphorylcholine.

MATERIAL AND METHODS

Semen. This was collected from ten rams by means of an artificial vagina and pooled for each experiment. Washed sperm suspensions were made by diluting 1.0–1.5 ml. of semen to 10 ml., in a tapered centrifuge tube, with one of the diluents listed below, centrifuging at about 1000 g, and washing the spermatozoa twice with the same amount of fresh diluent. The diluents were (i) the 'phosphate diluent' of White *et al.* (1954), containing 0.032 M- $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$,

0.048 M- $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.040 M- NaCl and 0.004 M- KCl , and (ii) the calcium-free 'Ringer solution for spermatozoa' (Mann, 1946); in some experiments phosphate was left out from the Ringer solution ('P-free Ringer solution'), and in some other experiments each 100 ml. of the Ringer solution was supplemented further with 20 ml. of 0.25 M phosphate buffer, pH 7.4, prepared by mixing 190 ml. of an aq. soln. of 7.1 g. of Na_2HPO_4 (or 17.9 g. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) with 10 ml. of N-HCl ('Ringer-phosphate solution').

Determinations. Glycerol was determined in protein-free filtrates from samples deproteinized with $\text{Ba}(\text{OH})_2$ and ZnSO_4 , by the method of Lambert & Neish (1950) as modified by Ryley (1955), and in some cases the identification of glycerol was carried out also by chromatography by the method of Trevelyan, Procter & Harrison (1950). The methods used for the determination of fructose, lactic acid, phosphorus, sperm respiration (in air, at 37°) and fructolysis (in N_2 , at 37°) were as described by Mann (1946). The chromatographic identification of fructose was carried out by the method of Jermyn & Isherwood (1949).

RESULTS

Aerobic formation of lactic acid from glycerol by spermatozoa

The rate at which glycerol (0.01 M) is utilized by washed ram spermatozoa at 37° was determined in nine experiments, a fresh sperm suspension in 'phosphate diluent' being used each time, with about 3×10^8 cells/ml. The disappearance of glycerol under these conditions was nearly linear over the 3 hr. experimental period. The rate of disappearance was 0.5–1 mg. of glycerol/ 10^9 sperm/hr., with an average of 0.8 mg. Throughout the period of 3 hr., the utilization of glycerol was accompanied by an increase in O_2 uptake of the order of 0.1–0.25 c.c. of O_2 / 10^9 sperm/hr. over that of the glycerol-free control sample. The disappearance of glycerol was accompanied by the accumulation of lactic acid, at a fairly linear rate of 0.2–0.6 mg./ 10^9 sperm/hr. Assuming that one atom of oxygen was required for

the conversion of each molecule of glycerol into lactic acid ($\text{C}_3\text{H}_8\text{O}_3 + \text{O} \rightarrow \text{C}_3\text{H}_6\text{O}_3 + \text{H}_2\text{O}$), and assuming further that the excess of observed O_2 uptake was due to the oxidation of lactic acid ($\text{C}_3\text{H}_6\text{O}_3 + 3\text{O}_2 \rightarrow 3\text{CO}_2 + 3\text{H}_2\text{O}$), it can be calculated that between one-half and three-quarters of metabolized glycerol is accounted for by the oxidative breakdown.

When these experiments were repeated with, instead of the 'phosphate diluent', either a Ringer solution, which had a much lower P content, or a 'P-free Ringer solution', the ratio between glycerol disappearance on one side, and the O_2 uptake and lactic acid formation on the other, was found to be entirely different. In the P-poor or P-free medium,

Table 1. *Effect of phosphate on lactic acid accumulation and oxygen uptake of spermatozoa respiring in the presence of glycerol*

Each manometer flask contained 3.6 ml. of sperm suspension, 3×10^8 cells/ml.; the contents of each flask were analysed after 3 hr. incubation in air, at 37° .

Medium	Lactic acid (mg./3 hr.)	O_2 uptake ($\mu\text{l.}/3$ hr.)
Phosphate diluent, no glycerol	0	178
Phosphate diluent + 0.01 M glycerol	1.05	459
P-free Ringer soln., no glycerol	0	224
P-free Ringer soln., + 0.01 M glycerol	0.15	705

Fig. 1. Effect of phosphate on the oxidation of sodium lactate (0.01 M) by washed ram-sperm suspensions, 10^8 cells/3.3 ml., in air, at 37° . ●, With P-free Ringer as suspending medium for spermatozoa; ○, with the 'phosphate diluent' (0.08 M phosphate) as the medium.

the rate of O_2 uptake was much higher, whereas that of lactic acid accumulation was found to be lower (Table 1).

These observations suggested that the high rate of lactic acid accumulation in the presence of phosphate may be due not to a higher rate of glycerol conversion into lactic acid but to a depressing effect of phosphate on the oxidative disposal of lactic acid by spermatozoa. This assumption was borne out fully by experiments in which the rate of lactate oxidation in the presence of phosphate was compared with that in a phosphate-free medium. As shown in Fig. 1, washed ram spermatozoa to which lactate has been added consumed O_2 at a lower rate in the presence than in the absence of phosphate. The depression in O_2 uptake brought about by the presence of phosphate corresponded also to a decreased disappearance of lactic acid, as determined chemically.

The metabolism of glycerol depends strictly on the presence of motile, i.e. fully active, spermatozoa. It can be abolished completely by heating the sperm suspensions at 70° for 2 min., and it can also be arrested promptly by the addition of spermicidal agents such as cetyltrimethylammonium bromide (Table 2). In this respect the process of glycerol oxidation resembles that of fructolysis, which is also extremely sensitive to spermicidal agents, particularly those which belong to the group of surface-active substances (Mann, 1954). Like the breakdown of fructose, that of glycerol appeared to be unaffected by the presence of antibiotics such as penicillin; no adverse effect was observed even after the addition of 1000 penicillin units/ml. of sperm suspension. Whereas, however, fructolysis can proceed in spermatozoa both anaerobically and aerobically, the process of glycerol utilization

Table 2. *Effect of cetyltrimethylammonium bromide (CTAB) on the utilization of glycerol by washed ram spermatozoa*

Glycerol, 0.01 M; incubation for 3 hr., in air, at 37° , with 2.2 ml. of a suspension of 'normal sperm' and of 'CTAB-treated sperm'. 'Normal sperm' suspension was prepared by mixing 1.5 ml. of ram semen with 1.5 ml. of phosphate diluent, leaving the mixture standing at 37° for 10 min. before centrifuging, and then washing it twice with 10 ml. of fresh phosphate diluent. 'CTAB-treated sperm' suspension was prepared by mixing 1.5 ml. of ram semen with 1.5 ml. of 0.005 M cetyltrimethylammonium bromide (CTAB), leaving the mixture standing at 37° for 10 min. before centrifuging, and then washing it twice with 10 ml. of fresh phosphate diluent.

	Glycerol utilized (mg./3 hr.)	O_2 uptake ($\mu\text{l.}/3$ hr.)
Normal sperm + glycerol	1.83	381
CTAB-treated sperm + glycerol	0	16

depends strictly on the presence of O_2 and does not proceed in washed sperm suspensions incubated in the presence of N_2 instead of air. The essentially aerobic character of glycerol metabolism by spermatozoa is further reflected in the observation that in the presence of both fructose and glycerol a definite 'sparing effect' was exerted by glycerol on the rate of fructolysis, provided, however, that O_2 was also present; the rate of anaerobic fructolysis, unlike that of the aerobic fructolysis, remained unaffected in the presence of glycerol (Table 3). Although purely aerobic, the process of glycerol oxidation by living spermatozoa did not lead to the formation of hydrogen peroxide. All attempts to detect hydrogen peroxide by chemical means gave a consistently negative result, and the ratio between O_2 consumption and glycerol disappearance was found to be unaffected by the addition of catalase.

Oxidation of dihydroxyacetone

In the normal course of glycerol oxidation by washed spermatozoa, suspended either in a P-free Ringer solution or in the phosphate diluent, little or no accumulation of bisulphite-binding substances was observed. However, when such sperm suspensions were incubated in the presence of 0.02M-NaF, particularly if glycerol was used in high concentrations (0.1M), there was a marked increase in bisulphite-binding material, which accumulated after 3 hr. in amounts equivalent to 50–250 μ g. of dihydroxyacetone. At the same time, fluoride had a strong inhibitory effect on the rate of glycerol

utilization, O_2 uptake and lactic acid formation, as can be seen from Table 4, which also shows, for comparison, the effect of fluoride on fructose metabolism.

Over 50% of the bisulphite-binding material which accumulated in the aerobic incubation of spermatozoa with glycerol and fluoride was stable towards treatment with hot alkali, which suggests that at least some of it may have been pyruvic acid (Clift & Cook, 1932). No attempt has been made so far to identify the bisulphite-binding substances. It was found, however, that dihydroxyacetone added to washed sperm suspensions was oxidized as readily as glycerol, giving rise to an increased O_2 uptake and also to the formation of lactic acid in quantities as large as those which were produced from glycerol; the comparative effect of glycerol and dihydroxyacetone on the O_2 uptake of ram spermatozoa is shown in Fig. 2.

Metabolism of phosphoglycerol

Phosphoglycerol (both α - and β -) was observed to increase considerably the O_2 uptake of washed spermatozoa, though seldom to the same extent as

Table 3. *Effect of glycerol on aerobic and anaerobic sperm fructolysis*

Washed suspensions of 6×10^8 ram spermatozoa in 2 ml. of phosphate diluent were incubated for 3 hr. at 37° , in air and in nitrogen.

Additions to sperm	Fructose utilized (mg./3 hr.)	
	Aerobically	Anaerobically
Fructose (0.005M)	1.26	1.49
Fructose (0.005M) + glycerol (0.010M)	0.86	1.49

Fig. 2. Effect of glycerol (0.01M) and dihydroxyacetone (0.01M) on the oxygen uptake of sperm suspensions, 10^8 cells/3.3 ml. of phosphate diluent; in air, at 37° . ●, Glycerol; ○, dihydroxyacetone; ▽, no additions.

Table 4. *Effect of fluoride on the aerobic metabolism of glycerol and fructose by ram spermatozoa*

Washed sperm suspensions in 2.4 ml. of phosphate diluent were incubated for 3 hr. at 37° , in air.

Additions to the sperm suspension	Glycerol utilized (mg.)	Fructose utilized (mg.)	O_2 uptake (μ l.)	Lactic acid formed (μ g.)
None	—	—	124	0
Fluoride (0.02M)	—	—	89	0
Glycerol (0.01M)	1.75	—	415	990
Glycerol (0.01M) + fluoride (0.02M)	0.38	—	165	0
Fructose (0.005M)	—	1.75	324	738
Fructose (0.005M) + fluoride (0.02M)	—	0.24	133	0

free glycerol. On closer analysis of this phenomenon, however, the conclusion was reached that the increased O_2 uptake was due, not to a direct oxidation of phosphoglycerol as such, but to the oxidation of free glycerol which had been set free from phosphoglycerol by dephosphorylation. It was found that phosphoglycerol was readily dephosphorylated by spermatozoa not only aerobically but anaerobically as well (Table 5); this dephosphorylation was accompanied by the liberation of glycerol. While, however, anaerobically, glycerol accumulated, aerobically it was oxidized, thus giving rise to an increase in the consumption of O_2 . The effect of α - and β -phosphoglycerol, and that of free glycerol, on the O_2 uptake of spermatozoa is shown in Fig. 3.

Oxidation of sorbitol

Apart from glycerol, the effect of various other alcohols and related substances on the sperm respiration was tested, including ethanol, *n*-

propanol, isopropanol, *n*-butanol, isobutanol, ethylene glycol, propylene glycol, inositol, mannitol, dulcitol, erythritol, glycidol (1:3-epoxypropan-1-ol), DL-glyceraldehyde and sorbitol. Of these substances, two, namely glycidol and DL-glyceraldehyde, had some depressing effect on the rate of O_2 uptake by washed spermatozoa, and sorbitol produced a marked increase in the O_2 consumption. It can be seen from Fig. 4 that the O_2 uptake of washed ram spermatozoa was increased by 0.1M sorbitol to the same extent as by 0.01M glycerol. The oxidation of sorbitol, like that of glycerol, was found to be accompanied by the formation of lactic acid and also, at the same time, by the appearance of a reducing sugar which gave the same ketose reactions as fructose and was yeast-fermentable; chromatographic analysis confirmed the identity of this sugar with fructose. The amounts of ketose sugar accumulating depended on the concentration in which sorbitol was used, and they were particularly large when sorbitol was added to spermatozoa simultaneously with glycerol. Thus in four experiments, the amounts of colorimetrically determined and yeast-fermentable ketose which accumulated at the end of a 3 hr. incubation period, in the presence of 10^9 sperm cells/3 ml. of phosphate diluent, were (in terms of fructose): 0–30 μ g. from 0.01M glycerol; 0–87 μ g. from 0.1M glycerol; 65–136 μ g. from 0.01M sorbitol; 430–970 μ g. from 0.1M sorbitol; and 570–1030 μ g. from 0.1M sorbitol + 0.01M glycerol.

Table 5. *Dephosphorylation of phosphoglycerol by washed ram spermatozoa*

Incubation for 3 hr. at 37°; 3.4 ml. of sperm suspension (10^9 cells) in P-free Ringer solution. Each value represents an average from two experiments.

	Increase in inorganic phosphate (μ g. of P)		Increase in the content of glycerol (μ g.)
	Anaerobic	Aerobic	Anaerobic
α -Phosphoglycerol (0.01M)	107	177	281
β -Phosphoglycerol (0.01M)	63	150	240

Fig. 3. Effect of α -phosphoglycerol, β -phosphoglycerol and glycerol on sperm respiration; 3.3 ml. of washed sperm suspension (10^9 cells) in phosphate diluent, incubated in air, at 37°. ●, Glycerol (0.01M); ○, α -phosphoglycerol (0.01M); ▽, β -phosphoglycerol (0.01M); ▼, no additions.

Fig. 4. Effect of sorbitol on sperm respiration; 3 ml. of suspension of washed ram spermatozoa (10^9 cells) in phosphate diluent, incubated in air, at 37°. ▽, No additions; ●, sorbitol (0.01M); ○, glycerol (0.01M).

DISCUSSION

In several mammalian species, including the ram, the seminal plasma is distinguished by a high content of glycerylphosphorylcholine; the concentration of glycerol bound in that form is of the order of 1 g./100 ml. of ram seminal plasma (Dawson *et al.* 1957). The evidence so far obtained, however, does not warrant the conclusion that this large reserve of bound glycerol can act either as a source of free glycerol or provide the sperm cells with a substrate for respiration. It is only after choline has been removed from glycerylphosphorylcholine by acid hydrolysis that the resulting product, phosphoglycerol, can be utilized by spermatozoa. It is shown in the present study that when phosphoglycerol is brought into contact with ram spermatozoa it is first dephosphorylated and then glycerol arising from this process is oxidized to lactic acid, probably via dihydroxyacetone. The exact mechanism underlying the oxidative dissimilation of glycerol by spermatozoa still remains to be explored in the light of the numerous recent studies on the various pathways of glycerol oxidation in other animal tissues and micro-organisms (Baranowski, 1949; Gunsalus & Umbreit, 1953; King & Cheldelin, 1952*a, b*; Asnis & Brodie, 1953; Magasanik, Brooke & Karibian, 1953; Wiame, Bourgeois & Lambion, 1954; Hauge, King & Cheldelin, 1955*a, b*). A possibility which merits particular attention is that the oxidation involves an initial dehydrogenation of glycerol to dihydroxyacetone, followed by a phosphorylative, glycolytic breakdown to lactic acid via phosphodihydroxyacetone. Another possibility is that the oxidation of dihydroxyacetone involves the 'phosphohexose shunt' and 'pentose cycle' as postulated recently by Hauge *et al.* (1955*b*) for *Acetobacter suboxydans*. The existence of a link between the oxidation of glycerol and that of sorbitol is another subject for future investigations. The oxidation of sorbitol to fructose by spermatozoa is probably due to sorbitol dehydrogenase, an enzyme analogous to that which occurs in the liver (Blakley, 1951; McCorkindale & Edson, 1954) and in male accessory organs (Williams-Ashman & Banks, 1954; Hers, 1955); fructose formed in that way would readily be broken down to lactic acid by the normal process of aerobic fructolysis. Such a mechanism would explain why glycerol, which itself is a lactic acid-producing substrate, should be able to exert a 'sparing effect' on the rate of aerobic fructolysis, thus facilitating the accumulation of fructose from sorbitol.

Although glycerol is metabolized aerobically by spermatozoa it seems unlikely that the preserving effect of glycerol on frozen sperm, described by Polge, Smith & Parkes (1949), is dependent on the metabolic activity of spermatozoa. The concentra-

tions of glycerol which must be added to semen for freezing purposes are many times higher than those required for the respiratory activity of spermatozoa. Moreover, although glycerol is most effective in protecting spermatozoa from the harmful effects of freezing in certain animals such as cock, bull and herring, it appears to be much less effective in other species, including ram (White *et al.* 1954). A much more likely explanation is that glycerol exerts its action through a protective influence on sperm colloids, possibly owing to its high electrolyte- and water-binding capacity. As pointed out by Lovelock & Polge (1954), the damage suffered by spermatozoa during freezing and thawing could be explained by the exposure to excessive concentrations of salt after the removal of water as ice. The protective action of glycerol could thus be ascribed to its ability to prevent the salt concentration rising above a critical level. Another possibility which must be borne in mind is that glycerol may act as a 'stabilizing' agent on sperm proteins and enzymes; glycerol, as it is well known, combines the properties of a protein solvent with the ability to protect proteins from denaturation.

SUMMARY

1. Ram spermatozoa, separated from seminal plasma by centrifuging and washing, metabolize glycerol aerobically to lactic acid. The yield of lactic acid is higher in the presence than in the absence of phosphate owing to an inhibitory effect of phosphate on the oxidation of lactic acid by spermatozoa.

2. Dihydroxyacetone is oxidized by spermatozoa as rapidly as glycerol, giving rise to lactic acid.

3. Phosphoglycerol (both α - and β -) increases the respiration of spermatozoa. This is due to the oxidation of glycerol set free from phosphoglycerol by dephosphorylation. Glycerylphosphorylcholine, which is a natural constituent of ram semen, is neither dephosphorylated nor oxidized by spermatozoa.

4. Apart from glycerol a number of alcohols and related substances have been tested with respect to their effect on respiration. One of them, sorbitol, is shown to be oxidized rapidly by spermatozoa to fructose, which is then broken down to lactic acid.

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A Method for the Dermination of Aldosterone, Cortisol and Corticosterone in Biological Extracts, Particularly Applied to Human Urine

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Increased amounts of aldosterone have been found in the urine of patients with certain pathological conditions (Gaunt, Renzi & Chart, 1955), and its analysis in human urine and body fluids is therefore of some interest. Methods previously used for the estimation of this compound in biological extracts have been based on these procedures: (a) bioassay of crude (Liddle, Cornfield, Casper & Bartter, 1955; Liddle, Bartter, Duncan, Barber & Delea, 1955; Mader & Iseri, 1955; Gordon, 1955) or purified (Luetscher & Axelrad, 1954; Venning *et al.* 1955; Cope & Llauro, 1954; Genest, 1955; Simpson & Tait, 1955a) extracts, partition-column chromatography being used for the separation of the steroids by Simpson & Tait and paper chromatography by the other workers; (b) separation in the propylene glycol-toluene system (Zaffaroni & Burton, 1951), followed by chromatography in the Bush C system (Bush, 1952), the resulting purified aldosterone being estimated by visual examination of the soda-fluorescent spot (Neher & Wettstein, 1955).

Both methods have serious disadvantages. The bioassay results can be influenced by the effects of administered impurities or, in some cases, by other

corticoids (Luetscher & Curtis, 1955). The double-chromatography method, first used for the analysis of adrenal gland extracts (Simpson & Tait, 1953), has not yet been shown to be either specific or quantitative when relatively large amounts of other corticoids are present. Moreover, the procedure described by Neher & Wettstein (1955) gives low values for aldosterone in normal urine (see below).

The method described here depends on the extensive purification of aldosterone diacetate followed finally by an objective measurement of the fluorescence on a paper chromatogram. Specific measurements of cortisol and, if necessary, of corticosterone, can be made at the same time. It seems possible, by this method, to measure aldosterone in one-third of a 24 hr. specimen of normal urine with a precision and accuracy of 10–15%. It has been applied to the analysis of adrenal homogenate, human urine and peripheral blood. From the stage immediately preceding the silica-gel column (Fig. 1), the method is basically the same for the analysis of urinary, blood or adrenal gland extracts. This paper is concerned only with its use with human urine. A preliminary account of some of this work has already been published (Ayres, Simpson & Tait, 1956).

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