the rabbit, to investigate this point, was negative. That porphobilinogen has here been demonstrated to be present in the liver in acute porphyria and that uroporphyrin has been obtained from liver and from faeces (Grinstein, Schwartz & Watson, 1945; Prunty, 1945), is of considerable significance. These facts do not accord with the view put forward by Waldenström & Vahlquist (1944) that the porphyrin formation in this condition is purely a secondary event, taking place after the formation of the urine.

SUMMARY

1. The preparation of purified solutions of porphobilinogen from the urine and from the liver of a patient suffering from acute porphyria is described.

2. The reaction of these solutions with *p*-dimethylaminobenzaldehyde is studied. Urea and ascorbic acid inhibit the reaction. Creatine and creatinine are without effect.

3. The formation of porphyrin from porphobilinogen heated in acid solution is confirmed. Quantitative data afford no evidence for formation of porphyrin from porphobilinogen injected into the rabbit.

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Studies on the Metabolism of Semen

1. GENERAL ASPECTS. OCCURRENCE AND DISTRIBUTION OF CYTOCHROME, CERTAIN ENZYMES AND COENZYMES

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Semen as ejaculated is composed of two parts, the spermatozoa or 'sperm' and the seminal fluid or 'plasma'. There are numerous analytical data on the chemical composition and the metabolic activities of semen, but for the most part they refer to whole semen without drawing a clear distinction between the sperm and the plasma (for references see Anderson, 1945). Yet, from many points of view, the two components of the semen must be considered separately, in the same sense as, for instance, the blood cells and blood plasma.

The spermatozoa exhibit two outstanding biological properties, fertility and motility, and they possess a characteristic metabolism of their own, maintained by a number of intracellular catalysts. In the presence of oxygen they show a considerable respiratory activity which is correlated significantly with both the number of cells and the intensity of movement; at the same time, the rate of respiration gives a quantitative measure of the viability of spermatozoa and is therefore used by some investigators as an indicator in assessing the potential fertilizing capacity of the semen (Walton, 1939). In the absence of oxygen, the activity of spermatozoa is maintained by glycolytic processes. Recent research indicates that the glycolysis, like the respiration, is correlated with the motility and fertility of spermatozoa. There seems to be little doubt that in whole semen the spermatozoa utilize glucose as the substrate for glycolysis (cf. MacLeod, 1943*a*). This is supplied by the plasma which in itself is devoid of a glycolyzing system. It is frequently said that the seminal plasma acts towards the spermatozoa both as a fluid vehicle for their transport and as a

nutrient medium. However, the significance of the seminal plasma goes far beyond that description. It is not a homogeneous fluid but a complex mixture secreted by several important glands. It carries a large number of highly active enzymes and it has a metabolism of its own.

This paper deals with the presence and distribution in the semen of several constituents. First, it will be shown that the spermatozoa contain a complete cytochrome system. Next, evidence will be given for the existence in the spermatozoa of adenosinetriphosphate (ATP). It will be shown that during the metabolic activity of spermatozoa, ATP undergoes characteristic changes which suggest that the function of this coenzyme is in some way connected with the motility of the cells. A study is also included of seminal deaminases and phosphatases with special reference to those enzymes which act on ATP and on other adenyl derivatives (preliminary communication, Mann, 1945*a*).

EXPERIMENTAL

Methods

The ejaculated semen of the ram, bull and boar was used. The semen of rams, being composed of nearly equal parts of sperm and plasma, has a higher concentration of spermatozoa (2·5–5 million/μl. semen) than that of other mammals and is therefore particularly suitable for metabolic studies on spermatozoa. Usually a mixed sample of semen was used, collected from seven rams, the volume varying from 5 to 18 ml. according to the season. Bull semen (0·8–1·1 million cells/μl. semen) lends itself to studies on spermatozoa as well as on the seminal plasma; single ejaculates were used varying in volume from 5 to 6·5 ml. Boar semen, although very voluminous (250 ml. in a single ejaculate), has but a small proportion of spermatozoa. Ram and bull semen was collected by Dr A. Walton, and that of boar by Miss C. Williams-Ellis, at the Animal Research Station, Cambridge. The method of collection was that described by Walton (1945). It was brought to the laboratory within 1 hr. after ejaculation. The sperm was separated from the seminal plasma by centrifugation. In order to remove the plasma from the spermatozoa the samples were suspended in several volumes of a calcium-free Ringer solution and centrifuged. Finally, the washed cells were resuspended in fresh Ringer solution up to the original volume of the whole semen, thus yielding a suspension of spermatozoa equal in concentration to that of the original whole semen. In a few experiments, e.g. in spectroscopic experiments on bull spermatozoa, the volume of suspensions was kept smaller than that of the original semen. The suspensions of washed spermatozoa are referred to as the 'washed sperm', and the results of chemical estimations carried out with the washed sperm are expressed in mg./100 ml. washed sperm, equivalent to 100 ml. semen.

The washing of the spermatozoa usually causes only little damage to the cells provided that certain precautions are observed. Considerable damage can easily result, for instance, from vigorous shaking of the sperm suspension. To avoid this type of inactivation it is convenient to add the Ringer solution gradually to the centrifuged sperm in the centrifuge tube and to stir it up gently with the bottom-end of a loosely fitting test-tube till a homogeneous suspension is obtained. As a rule, the washing of spermatozoa should not be repeated more than once. Exhaustive washing, particularly if carried out with a large volume of Ringer solution, may cause a great deal of damage. Only in exceptional cases such as, for instance, in studies on phosphatases and diastases, is it necessary to wash the spermatozoa more than once so as to achieve a complete separation of the enzymes present in the spermatozoa from those carried by the seminal plasma. Another factor which may influence the experimental results concerns the time and speed of centrifugation; these should be kept as low as possible. In this respect, ram spermatozoa seem to be much more resistant and therefore much more suited for metabolic studies than bull or boar spermatozoa. Less time is required to achieve a separation by centrifugation of the sperm from the plasma in the case of ram than that of other animals.

The observations on cytochrome were made by means of a microspectroscope ocular attached to a microscope; the tube containing the sperm was illuminated by a strong Pointolite lamp. Cu was determined in the semen as diethyl-dithiocarbamate, Zn with dithizone and Fe with $\alpha\alpha'$-dipyridyl. The production of lactic acid was followed by the method of Friedemann, Cotonio & Shaffer (1929), using the apparatus of Lieb & Zacherl (1932); before the estimation of lactic acid, protein was removed with trichloroacetic acid and the extracts treated with copper-lime. Ammonia was estimated according to Parnas & Heller (1924). The acid-soluble phosphate was estimated in trichloroacetic acid extracts by the method of Lohmann & Jendrassik (1926). The following fractions were determined: (1) $P_{inorg.}$, the true inorganic phosphate determined as $MgNH_4PO_4$; (2) P_0, phosphorus determined as phosphate which reacts directly with ammonium molybdate; (3) P_7, P_{30} and P_{180}, the phosphorus which appears as orthophosphate after 7, 30 and 180 min. hydrolysis at 100° with N-HCl; (4) $P_{tot.}$, phosphorus as the total acid-soluble phosphate after incineration. The alkali-hydrolyzable phosphate of phosphotrioses was determined by the method of Meyerhof & Lohmann (1934).

Cytochrome

For the spectroscopic examination, the washed sperm of ram and bull was used. 2 ml. of a thick suspension of spermatozoa (3×10^9 cells/ml.) were placed in a Thunberg tube and examined under the microspectroscope, the thickness of the layer being increased gradually from about 0·2 to 1 cm. In a freshly prepared suspension the cytochrome is only partially reduced and the absorption bands are therefore weak. On standing, however, or better still on adding a little glucose and replacing the air by nitrogen, the cytochrome becomes fully reduced and the suspension then shows distinctly the spectrum of all three reduced cytochromes, *a*, *b* and *c*. The absorption band of the cytochrome *a* is particularly strong and, on passing CO through the sperm, the formation of the CO-compound can be clearly observed. This compound is identical with the CO-compound of cytochrome oxidase or cytochrome a_3

as first described in heart-muscle preparations by Keilin & Hartree (1939). If a suspension of spermatozoa is vigorously shaken in air, the cytochrome is oxidized and the bands disappear. In the presence of cyanide, however, the oxidation is inhibited and cytochrome remains in the reduced form. No appreciable change in the spectrum of cytochrome was observed in ram and bull spermatozoa after they had been preserved for 1 week at 10°, under anaerobic conditions. The complete spectrum of cytochrome was also observed in whole semen of ram and bull. However, in the case of bull semen it was necessary to carry out the examination of a layer about 1 cm. thick, which is quite understandable in view of the comparatively low density of spermatozoa. No cytochrome was found in the seminal fluid from which spermatozoa had been removed.

Fe, Zn *and* Cu

One large sample (15 ml.) of mixed ram semen was examined for its content of Fe, Zn and Cu. The three elements were determined separately in the washed spermatozoa and in the seminal plasma. Table 1 shows that the concentration of all three elements is higher in the spermatozoa than in the plasma, and this in spite of the fact that the sperm had been repeatedly washed with Ringer solution. The form in which the three elements occur remains to be investigated.

Table 1. *Distribution of* Fe, Zn *and* Cu *in ram semen*

	100 ml. semen contain	
	In sperm	In plasma
Fe (mg.)	0·68	0·16
Zn (mg.)	0·70	0·28
Cu (mg.)	0·12	0·05

Acid-soluble phosphorus

The difference in the distribution of elements between the sperm and the plasma is also noticeable with P. In the ram semen, the readily hydrolyzable P compounds are mainly concentrated in the spermatozoa. Table 2 shows the distribution of the acid-soluble P in freshly washed sperm. On standing, the inorganic phosphate increases, mainly although not exclusively at the expense of the readily hydrolyzable P fraction.

Table 2. *Acid-soluble phosphorus in ram spermatozoa*

(4×10^9 cells/ml.)

($P_{inorg.}$ = true inorganic phosphate determined as $MgNH_4PO_4$; 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 at 100° with N-HCl; $P_{tot.}$ = phosphorus as the total phosphate after incineration of the trichloroacetic acid extract.)

$P_{inorg.}$	P_0	P_7	P_{30}	$P_{tot.}$
5·1	5·9	9·8	10·5	39·0 mg. P/100 ml.

Adenosinetriphosphate

As shown in Table 2, a large proportion of the acid-soluble P present in the ram sperm yields orthophosphate after 7 min. hydrolysis with N-HCl, and thus behaves like a typical readily hydrolyzable P compound. Lardy, Hansen & Phillips (1945) observed the occurrence of a similar P fraction in bull sperm and suggested that it might be adenosinetriphosphate. However, the possibility had to be considered that the substance might be one of the many adenosine polyphosphates other than ATP or one of the inorganic polyphosphates such as occur, for example, in moulds and yeast (Mann, 1944). In order to obtain more definite information with regard to the chemical nature of the readily hydrolyzable P compound, an attempt was made to isolate it from the spermatozoa. The procedure was based on the method recommended by Parnas & Lutwak-Mann (1935) for the quantitative estimation of adenosinetriphosphate in skeletal muscle.

10 ml. fresh ram semen were used, containing 3×10^9 cells/ml. It was diluted with 15 ml. Ringer solution, centrifuged, and the two parts, the sperm and the dilute plasma, were brought with Ringer solution to 20 ml., cooled in ice and twice extracted with 10 ml. 10% cold trichloroacetic acid, centrifuging each time. Tests carried out on small samples of the extracts have shown that most of the acid-soluble P passed into the first extract. The combined extracts were precipitated with Ba acetate at pH 8 and centrifuged 30 min. later. The supernatant solutions gave no further precipitate with Ba acetate and were discarded; they contained no readily hydrolyzable P but showed the presence of difficultly hydrolyzable P compounds.

The Ba precipitates were washed with dilute Ba acetate and dissolved in 5 ml. 0·05N-HCl. Ba was then removed by adding Na_2SO_4, the $BaSO_4$ precipitate spun down and washed twice with 4 ml. 0·05N-HCl. The washings were added to the main solution, neutralized, with bromo-thymol blue as indicator, and made up to 15 ml. This solution was divided into three equal parts, *A*, *B* and *C*.

A was incubated for 2 hr. at 37° with 1 ml. frog muscle pulp, which serves as a source of adenyl deaminase. The incubation was terminated by the addition of 10 ml. saturated borax, and NH_3 was estimated in the Parnas and Heller apparatus.

B was treated with 10 ml. saturated borax; to this was added 1 ml. muscle pulp which had been incubated for 2 hr. at 37° and NH_3 was estimated. The difference between the NH_3 content in fractions *A* and *B* represents the NH_3 liberated by the enzymic deamination and corresponds to the NH_2 of ATP.

C was used for the determination of P_0 and P_7. The results of analysis were as follows:

Spermatozoa. *A*: 0·029 mg. NH_3-N. *B*: 0·006 mg. NH_3-N. Deaminated enzymically 0·023 mg. NH_2-N or 1·64 μmol.

C: 0·112 mg. (P_7–P_0) or 3·60 μmol. P; molecular ratio NH_2-N : P = 1 : 2·2; ATP content in spermatozoa from 10 ml. semen (3×10^9 cells/ml. semen): 0·069 mg. NH_2-N and 0·336 mg. readily hydrolyzable P.

Seminal plasma. *A*: 0·009 mg. NH_3-N; *B*: 0·006 mg. NH_3-N; deaminated enzymically 0·003 mg. NH_2-N; *C*: 0 mg. (P_7–P_0).

The analytical data show that the P compound present in spermatozoa has the properties of adenosinetriphosphate; it forms the Ba salt under identical conditions, and the molecular ratio between NH_3 liberated by the muscle deaminase and P liberated by the acid hydrolysis agrees satisfactorily with the theoretical ratio 1 : 2 required for ATP. Calculated from these figures, the amount of ATP contained in the spermatozoa of 100 ml. ram semen (3×10^9 cells/ml. semen) is 0·69 mg. ATP-amino-N and 3·36 mg. ATP-readily hydrolyzable P. It appears that in the semen all the ATP is confined to the cells.

The content of ATP was determined five times, using mixed samples of semen from the same seven rams, and once in bull semen (6·5 ml.). In each case the compound was first separated in the form of its Ba salt and then NH_2-N determined as NH_3. The estimation of the readily hydrolyzable P was sometimes omitted, since it was found to be less accurate; moreover, the P analysis does not always provide a reliable criterion for assessing the ATP content, especially under conditions where the occurrence of other readily hydrolyzable P compounds cannot be excluded with certainty. In the case of ram semen, the lowest content of ATP-amino-N recorded was 0·5 mg./100 ml., and the highest 1·5 mg./100 ml. The highest content of ATP in spermatozoa was observed in December, coinciding with the peak of the breeding season, and the lowest in April, at the end of the season. However, as this observation was made during one season only, it requires repetition for full confirmation. The ATP-amino-N of bull semen was 0·41 mg./100 ml., a rather high value considering the relatively low concentration of spermatozoa in the bull semen.

Behaviour of ATP in spermatozoa. For these experiments the ram sperm was used after the removal of the plasma by washing.

In one experiment two equal samples were incubated for 2 hr. at 37°, one aerobically, the other anaerobically; the contents of ATP-amino-N were 0·208 and 0·056 mg./100 ml. respectively, showing that the level of ATP in washed spermatozoa is maintained better in air than in nitrogen. In another experiment a sperm suspension was buffered by the addition of 0·05 M-phosphate, pH 7·3, and divided into two equal parts; 0·03 M-glucose was added to one part only, and both were incubated in N_2 for 2 hr. at 37°. Afterwards, the samples were deproteinized with trichloroacetic acid and in the filtrates ATP and lactic acid were determined. The results were 0·55 mg. ATP-amino-N/100 ml. and 605 mg. lactic acid/100 ml. in the sperm incubated with glucose, and 0·15 mg. ATP-amino-N/100 ml. and 35 mg. lactic acid/100 ml. in the sperm incubated without glucose. These results indicate that under anaerobic conditions the ATP content is preserved better in the presence than in the absence of glucose.

Adenosine derivatives other than ATP

Spermatozoa contain some other adenosine derivatives apart from ATP. In order to determine the joint content of these compounds, a method has been worked out similar to that used by Ostern, Baranowski & Terszakoweć (1938) for the estimation of adenylic acid + adenosine-polyphosphoric acids in yeast.

Whole semen, washed spermatozoa and the seminal plasma were each extracted with trichloroacetic acid. The centrifuged and filtered extracts were freed from trichloroacetic acid by ether extraction, neutralized to phenolphthalein by the addition of Na_2CO_3, free NH_3 was removed in the Parnas-Heller apparatus, and the NH_3-free solution neutralized to pH 7. The neutral solutions were then incubated for 2 hr. at 37° with 2 ml. deaminase prepared by grinding 50 mg. rat heart muscle in 0·1 M-phosphate, pH 7. Under these conditions both adenosine and adenylic acid as well as adenosine-polyphosphates are deaminated. The total content was 3·9 mg. NH_2-N/100 ml. whole semen, of which 3·45 mg. were derived from the sperm and 0·45 mg. from the plasma.

Ammonia formation in semen

The total NH_2-N content of the semen as assessed by means of the heart muscle adenyl deaminase undergoes little change during the storage of the semen in spite of the fact that there is a decrease in ATP-amino-N. For example, at the end of 7 hr. incubation at 37° the NH_2-N content of the ram semen decreased only from 3·9 to 2·9 mg. N/100 ml. In the same semen, however, there was a large evolution of free NH_3, the content of which increased from 1·3 mg. N/100 ml. in the fresh semen to 9·7 mg. N/100 ml. after 7 hr. incubation. This remarkable formation of NH_3 in semen will be discussed separately. However, it should be pointed out at once that the NH_3 formed in the semen far exceeds the amount which could be derived from the adenosine derivatives. Furthermore, the NH_3 formation is not dependent on the presence of spermatozoa but occurs also in the seminal plasma which, as was shown above, has a very low content of adenosine derivatives. Moreover, the evolution of NH_3 in the semen is not influenced by such substances as fluoride or iodoacetate or by the presence of glucose. The stability of the adenyl-amino-N content indicated that the semen must be poor in enzymes capable of deaminating adenosine derivatives. That this is so was shown by experiments designed to test the ability of whole semen, washed sperm and seminal plasma to deaminate added adenosine derivatives. There was no appreciable NH_3 production when the following sub-

stances were used as substrates: ATP, adenosine, muscle adenylic acid (adenine ribose-5-phosphoric acid) and yeast adenine nucleotide (adenine ribose-3-phosphoric acid) (Table 3). It is of some interest

Table 3. *Deaminases in semen*

(*m*-Adenylate = Na salt of muscle adenylic acid or adenine ribose-5-phosphoric acid; *y*-adenine nucleotide = Na salt of adenine ribose-3-phosphoric acid.)

Source of enzyme	Substrate 100 μg. NH_2-N	pH	NH_3-N liberated after 1 hr. at 37° (μg.)
Ram, 0·1 ml. whole semen	Adenosine	7	2
	m-Adenylate	7	4
	y-Adenine nucleotide	7	4
	ATP	7	3
	Glucosamine	7	4
	Cadaverine	7	20
Ram, 0·35 ml. seminal plasma	Adenosine	6	10
	,,	7·5	4
	m-Adenylate	6	9
	,,	7·5	12
Ram, 0·35 ml. washed sperm	Adenosine	6	0
	,,	7·5	7
	m-Adenylate	6	0
	,,	7·5	7
Boar, 0·5 ml. seminal plasma	Adenosine	7	3
	,,	7·5	5
	m-Adenylate	7	3
	,,	7·5	3
Bull, 0·5 ml. whole semen	Adenosine	7·5	5
	m-Adenylate	6	4
	,,	7·5	5

to compare this poor deaminase activity of spermatozoa and of the seminal plasma with the powerful phosphatase activity exhibited by the semen towards adenyl compounds.

Seminal phosphatases

Hitherto, work concerning the phosphatases of semen has been carried out mainly with human semen or, strictly speaking, with the human seminal plasma. Kutscher & Wolbergs (1935) discovered the high phosphatase activity of the human semen and were the first to point out that the seminal phosphatase originates chiefly in the prostate gland, from whence it passes into the semen together with the other components of the prostatic secretion. The range of activity of the prostatic phosphatase was defined by the discoverers as that of an enzyme which acts optimally at a slightly acid pH, splitting equally well α- and β-phosphoglycerol but which has only a slight effect on diphosphohexose and on pyrophosphate. Reis (1937, 1938), who tested a number of other substrates, found that a dominant characteristic of the phosphatase present in the human seminal plasma is its high activity towards certain nucleotides. He found that inosinic acid, adenylic acid and yeast adenine nucleotide are dephosphorylated several hundred times more rapidly than phosphoglycerol, and arrived at the conclusion that the so-called seminal phosphatase is in fact a complex mixture of dephosphorylating enzymes originating partly in the prostate and partly in the testicles. Reis pointed out that among the seminal phosphatases there is one which is specifically concerned with the dephosphorylation of adenylic acid. This enzyme, the '5-nucleotidase' as he called it, was shown to be highly active against adenylic acid but completely inactive towards ATP and yeast adenine nucleotide.

Using bull semen, it was possible to confirm the existence of a highly active 5-nucleotidase. In the bull seminal plasma the activity found can be expressed by the ratio

$$\frac{\text{P split from adenylic acid}}{\text{P split from } \beta\text{-phosphoglycerol}} = \frac{300}{1}.$$

The potency of the seminal plasma can be judged by the fact that from 160 μg. P added as Na adenylate to 0·001 ml. seminal plasma, 140 μg. P were found in the form of inorganic phosphate after 1 hr. incu-

Table 4. *Phosphatases in bull semen*

Source of enzyme	pH	μg. P liberated as orthophosphate in 1 hr. at 37° from 160 μg. P added as: β-Phosphoglycerol	ATP	*m*-Adenylate	*y*-Adenine nucleotide
0·1 ml. whole semen	6	25	160	160	40
	7·3	23	160	160	37
0·01 ml. whole semen	6	8	30	160	8
	7·3	8	50	160	8
0·01 ml. seminal plasma	6	7	35	160	8
	7·3	7	30	160	8
0·001 ml. seminal plasma	7·3	0·5	9	140	0
Washed sperm corresponding to 0·4 ml. semen	6	7	120	120	9
	7·3	7	140	130	9
Washed sperm corresponding to 0·04 ml. semen	6	0	35	20	1
	7·3	0	35	35	1

bation at 37°. Expressed in phosphatase units, the nucleotidase activity in the bull seminal plasma is 3000–5000 as compared with 30 in brain, 2 in skeletal muscle and 0·05 in normal serum. Yeast adenine nucleotide is dephosphorylated by bull seminal plasma much more slowly than muscle adenylic acid. ATP, on the other hand, occupies an intermediary position between the two nucleotides. Table 4 shows the phosphatase activity of the bull seminal plasma towards the various substances and the comparison between the seminal plasma and the spermatozoa. The bull spermatozoa, in spite of the fact that they have been repeatedly washed with Ringer solution, still exhibit a remarkably high phosphatase activity which is particularly noticeable with ATP. The 5-nucleotidase, on the other hand, is much weaker in the spermatozoa than in the seminal plasma. Altogether it appears that the bull semen contains at least two enzymes concerned with the breakdown of ATP. One of these enzymes converts ATP into adenylic acid, the other carries the degradation one step further and produces adenosine from adenylic acid. Analogous enzymes were also found in the semen of boar and ram. The activity here, however, is not nearly as strong as in the bull semen. The boar in particular has a low phosphatase activity which may be due to the fact that a large proportion of the voluminous semen ejaculate is contributed by the seminal vesicles. With adenylic acid as substrate, 0·01–0·03 ml. ram seminal plasma was required to bring about the same effect as 0·001 ml. bull seminal plasma. The activity of ram spermatozoa towards ATP was of the same order as that of the equivalent number of bull spermatozoa.

In view of the observation made by Winberg (1941) that spermatozoa, but not the seminal fluid, contain cozymase, the possibility has been envisaged that the bull seminal plasma, which has such a high phosphatase activity towards several adenyl derivatives, may also act on cozymase. As a matter of fact, cozymase was found to be slowly dephosphorylated both by the seminal plasma and by the washed spermatozoa of bull. Of 0·2 mg. P added as cozymase to 0·1 ml. seminal plasma and 0·4 ml. washed sperm, respectively, only 0·02 mg. P appeared as inorganic phosphate after 1 hr. incubation at 37°; 0·7 whole semen was required to set free 0·08 mg. or 40 % P after 1 hr. at 37°.

DISCUSSION

As might be expected, the two chief components of the semen, the spermatozoa and the seminal plasma, differ biochemically from each other in many respects. In conformity with their function, the spermatozoa carry several important intracellular catalysts required to maintain the respiratory activity as well as the glycolytic activity of the cells. On the high metabolic activity ultimately depends the motility of the spermatozoon and, according to some investigators, the male fertility. However, it may be pointed out here that the exact correlation between motility and fertility is still uncertain and it appears that motile spermatozoa are not always fertile (Hammond, 1930).

The respiratory mechanism of the spermatozoa has been the subject of a long dispute which centred chiefly around the question of the presence of cytochrome in the spermatozoa. Keilin (1925) has shown that among all the organs of a perfused frog two are distinguished by the highest concentration of cytochrome: the heart and the testes. Iwanow (1931) has shown that the respiration of spermatozoa is cyanide-sensitive, suggesting the probable presence of the cytochrome system, and Ball & Meyerhof (1940) detected spectroscopically the bands of cytochrome in the sperm of *Arbacia punctata*. However, so far attempts to prove spectroscopically the presence of cytochrome in mammalian spermatozoa have failed repeatedly, and the only facts from which one could deduce the functioning of the cytochrome system were experiments showing the succinic dehydrogenase and cytochrome oxidase activity of spermatozoa (Lardy & Phillips, 1941; Zittle & Zitin, 1942; MacLeod, 1943*b*). The successful demonstration in the present study of the existence of cytochromes *a*, *b* and *c* in the spermatozoa was achieved by using fairly thick suspensions of the sperm and examining them by means of the microspectroscope, an instrument particularly suited to this kind of investigation because of its small dispersion and strong illumination. There can now be little doubt that the respiratory activity of the mammalian spermatozoa is in fact maintained by the cytochrome system, and thus there is no further need to uphold the old views whereby the respiratory mechanism of spermatozoa was said to differ markedly from that of other respiring cells. There are, however, certain aspects of the semen respiration which still remain to be clarified. One of them concerns the alleged differences between the 'immature' epididymal spermatozoa and the 'mature' ejaculated spermatozoa. Then there is the problem concerning the chemical nature of the substrate for the 'endogenous' respiration of washed spermatozoa. According to Lardy *et al.* (1945), the substrate utilized by the sperm aerobically is not carbohydrate but a phospholipid; they are of the opinion that the motility of the epididymal spermatozoa is initiated and maintained by the oxidation of the phospholipid stores, which results in an uptake of inorganic phosphate and the formation of an easily hydrolyzable ester, possibly identical with ATP. Finally, it remains to be seen in what way and to what extent the seminal plasma can contribute towards the O_2

uptake of the whole semen. It is stated that in some, but not in all, mammals the sperm-free plasma can utilize O_2 (Zeller, 1941; MacLeod, 1943*a*). In view of the absence of cytochrome from the seminal plasma this O_2 uptake must be the outcome of processes entirely different from those on which depends the O_2 uptake of spermatozoa.

The glycolytic mechanism of the spermatozoa will be discussed in the next paper (Mann, 1945*b*), where it will be shown that both ATP and cozymase act as coenzymes in several intermediary reactions in the sperm glycolysis.

Gray (1928), to whom we owe the first critical study of processes underlying the senescence and decay of spermatozoa, has pointed out that the progressive loss of activity of spermatozoa may be considered from two points of view: either as a process of spontaneous and irreversible depletion of available energy or as the result of auto-intoxication whereby the products of activity inhibit the essential reactions of life. In this connexion it is interesting to comment upon the function of ATP and its position in the sperm metabolism as it emerges from the experimental findings described above. In ejaculated spermatozoa the decrease in the content of ATP coincides with the impairment of activity. In the sperm which has been deprived by washing of all glycolyzable substrate the ATP is better preserved aerobically than in N_2. This behaviour is in agreement with the well-known phenomenon that in absence of glycolyzable substances the motility of spermatozoa can be maintained much longer in air than in N_2. No definite answer can be offered at present to the question as to why the level of ATP should depend on the presence of O_2. Analogy with the metabolism of other tissues seems to justify an assumption that aerobically, but not anaerobically, the breakdown of ATP is counterbalanced by a resynthesis at the expense of the non-glycolytic aerobic processes which form part of the 'endogenous' respiration of spermatozoa. If this is at all justified, one would expect that a resynthesis of ATP should be also possible under anaerobic conditions provided that a glycolyzable substrate is added. And, indeed, one finds that when spermatozoa are incubated anaerobically in the presence of glucose, the content of ATP is preserved much better than without it. Again, this behaviour is in conformity with the observation that in anaerobically stored spermatozoa motility is maintained more satisfactorily in the presence than in the absence of a glycolyzable substrate.

SUMMARY

1. A procedure is described for the preparation of suspensions of 'washed spermatozoa' with special reference to ram and bull semen.

2. The spermatozoa are shown to contain cytochromes *a*, *b* and *c*. It is possible to follow spectroscopically the reduction and oxidation of the cytochromes and their behaviour in the presence of respiratory inhibitors. The formation in spermatozoa of a compound between cytochrome *a* and carbon monoxide is described.

3. Iron, copper and zinc occur in a higher concentration in the spermatozoa than in the seminal plasma.

4. The distribution of acid-soluble-P compounds was studied. There are about 40 mg. acid-soluble P present in the spermatozoa in 100 ml. ram semen, of which 4 mg. are contributed by the readily hydrolyzable fraction. The rest is composed largely of difficulty hydrolyzable P compounds.

5. The readily hydrolyzable P fraction has been separated quantitatively as Ba salt and identified as adenosinetriphosphate (ATP). The content of ATP in ram spermatozoa is 0·6–1·5 mg. NH_2-N or 2·6–6·6 mg. labile P/100 ml. semen; the content in bull semen is 0·4 mg. NH_2-N or 1·7 mg. labile P/100 ml.

6. While the glycolytic activity of semen is restricted to the spermatozoa, enzymes which bring about the decomposition of ATP and cozymase are also found in the seminal plasma. Two enzymes at least are concerned with the breakdown of ATP. One converts ATP into adenylic acid, the other, the '5-nucleotidase', dephosphorylates adenylic acid to adenosine. Bull seminal plasma is particularly rich in '5-nucleotidase'.

7. ATP undergoes characteristic changes during the metabolic activity of ejaculated spermatozoa. Both under aerobic and anaerobic conditions, the decrease in the ATP content of the surviving sperm coincides with the loss of motility. It is suggested that the function of ATP is connected with the motility of spermatozoa.

8. In contrast to the powerful dephosphorylating enzymes, the semen lacks deaminases capable of deaminating adenyl derivatives. The considerable ammonia formation which takes place in the semen on storage depends on the presence of an enzyme system distinct from adenyl deaminases. The production of NH_3 also takes place in the sperm-free seminal plasma.

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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/μl.; bull, 0·7–1 million cells/μl.).