Biochem. J. (1970) **118**, 851–857
Printed in Great Britain

Acid-Soluble Phosphorus Compounds in Mammalian Semen

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(*Received* 16 *March* 1970)

1. A method is described for the extraction, purification and separation of acid-soluble phosphorus compounds from mammalian semen. [8-^{14}C]ATP and [8-^{14}C]-AMP were used as internal recovery standards to measure the breakdown and loss of these nucleotides in the procedure. 2. Bull, ram, boar and stallion semen was separated into seminal plasma and spermatozoa and the two fractions were examined separately. The overall composition of the mixture of the phosphorus compounds extracted from the two fractions was similar for the four species. 3. Glycerylphosphorylcholine and glycerylphosphorylinositol were the two phosphorus compounds identified in extracts of seminal plasma. ATP, ADP, AMP, GTP, GDP, NAD, fructose 1,6-diphosphate and glucose 6-phosphate were identified in extracts prepared from spermatozoa.

The chemical nature of acid-soluble phosphorus compounds in mammalian semen, and their distribution between spermatozoa ('sperm') and seminal plasma, has been the subject of relatively few studies in the past. One of the well-established constituents of this nature is ATP, which was first isolated from ram sperm by Mann (1945). Other nucleotides that have been reported to occur in bull sperm are ATP, ADP, AMP and IMP (Newton & Rothschild, 1961). The occurrence of guanine and cytosine nucleotides has been inferred from their presence in acid hydrolysates from bull and buffalo sperm (Abraham & Bhargava, 1962). Values for the nicotinamide coenzymes extracted from bull and rabbit sperm have been published by Bistocchi, D'Alessio & Leone (1968). One of the main difficulties in studies of this kind is that sperm are by no means easy to disrupt. Additional difficulty arises from the presence of powerful phosphatases and nucleotidases in the seminal plasma, capable of rapidly metabolizing organic phosphorus compounds (Mann, 1964). The present study had a twofold aim: first, to evolve an efficient extraction procedure and secondly, to identify and to determine quantitatively the various acid-soluble phosphorus compounds extracted from mammalian spermatozoa and seminal plasma.

MATERIALS AND METHODS

Collection and handling of semen. Mature Friesian bulls, Suffolk rams, a Landrace boar and a Welsh Mountain pony were used. Semen was collected by means of an artificial vagina and samples were pooled, if necessary. In rams, for example, as many as 20 ejaculates were pooled before extraction to supply sufficient material. The semen was either frozen as a whole, as soon as possible after collection, by dripping it into liquid N_2, or it was first separated into sperm and seminal plasma by centrifugation. Boar ejaculates were strained through muslin cloth to remove gelatinous material and then centrifuged for 12min at 250*g* to sediment the sperm. Bull, ram and stallion sperm were sedimented from seminal plasma by centrifuging in 10ml tapered tubes for 15min at 700*g*. The seminal plasma was siphoned off with a Pasteur pipette and frozen in liquid N_2; the sperm were then resuspended in a small volume of 0.9% NaCl and the suspension was frozen by dripping it into liquid N_2. When it was necessary to store material before extraction, the samples were kept frozen in solid CO_2. The sperm concentration of bull semen was determined by the method of Bishop, Campbell, Hancock & Walton (1954) with a Spekker absorptiometer (Hilger and Watts Ltd., London N.W.1, U.K.). Sperm concentrations of ram, boar and stallion semen were measured in a haemocytometer by counting the spermatozoa in semen diluted with 0.9% NaCl containing 0.2% (v/v) of formaldehyde.

Chemicals. All chemicals were of the highest grade available and all solvents were redistilled before use. Propan-1-ol was purified by the procedure of Isherwood & Barrett (1967). 1,4-Dioxan was freed from peroxides as described by Eastoe (1955) and redistilled in the presence of sodium. Dowex resins were purchased from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Dowex AG1 (X2) resin was obtained in the Cl^- form and was converted into the formate form by washing it successively with 6M-formic acid, water, 3M-ammonium formate (until the eluate was free from Cl^-) and water. Dowex AG50W (X8) was cycled with M-NH_3, water, 2M-HCl and water. [8-^{14}C]AMP (53mCi/mmol) and [8-^{14}C]ATP (38mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The various phosphate esters were supplied by Sigma (London) Chemical Co., London

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S.W.6, U.K., and the preparations of enzymes and co-enzymes were obtained from the Boehringer Corp. (London) Ltd., London W.5, U.K. Glycerylphosphorylcholine and a phospholipid composed principally of phosphatidylserine and phosphatidylinositol were kindly given by Dr E. F. Hartree; this phospholipid was subjected to alkaline hydrolysis by Method 1 of Dawson (1960), and the glycerylphosphorylinositol set free by hydrolysis was purified from the aqueous fraction by chromatography on Dowex resin as described below for phosphorus compounds.

Extraction procedure. The extraction procedure was a modification of that used by Isherwood & Barrett (1967) for plant material. The entire procedure (except freeze-drying) was conducted in a cold-room at 2°C. The frozen material (usually representing about 60×10^9 sperm) was first ground to a fine powder in a porcelain mortar containing liquid N_2. Usually 0.025 μCi of radioactive tracer (recovery standard) in buffer (0.005M-sodium cacodylate adjusted to pH 7.0 with 0.01M-HCl) was added and ground with the material. Then an extraction was performed in a small Waring Blendor holding a volume of extracting solution [aq. 20% (w/v) trichloroacetic acid containing 0.15% of 8-hydroxyquinoline] equal to the volume of the sample. Excess of liquid N_2 was allowed to evaporate from the powder, which was slowly added into the vortex of the acid contained in the Blendor. Homogenization was continued for 10min after all the powder had been added and the mixture was then centrifuged at −2°C for 10min at 35000*g* in a Spinco model L ultracentrifuge. The centrifuged precipitate was extracted twice more by rehomogenization in 20ml of aq. 10% (w/v) trichloroacetic acid containing 0.15% of 8-hydroxyquinoline. The supernatant fractions were next shaken six times with equal volumes of ether to remove trichloroacetic acid. They were then passed through a column (0.6cm × 10cm) of Dowex AG50W (X8; H^+ form; 100–200 mesh) and the column was washed with 30ml of water. The eluate was shaken with ether six more times and neutralized to pH 7.0 with dilute NH_3. The Dowex column was eluted with a further 750ml of water to ensure good recovery of AMP, since this was found to be considerably retarded on the column. High-molecular-weight material was removed from the eluates by dialysis, which was performed in a pressure dialysis cell (model 80-0011; National Instrument Laboratories Inc., Rockville, Md., U.S.A.) under 8.5atm N_2; the last traces of the eluent were flushed from the cell by filling it with 140ml of water and continuing the dialysis until the cell was empty. The diffusate was freeze-dried, dissolved in 0.5ml of water and mixed with 1g of cellulose powder (Whatman Chromedia CF1; W. and R. Balston Ltd., Maidstone, Kent, U.K.). Then 5ml of propan-1-ol was mixed with the powder and the resulting slurry was stirred into the solvent present in the upper part of a column (1cm diam.) containing 4g of cellulose powder pre-equilibrated with propan-1-ol–water–aq. NH_3 (sp.gr. 0.88)–0.2M-EDTA (90.4:9.5:0.1:0.1, by vol.; sp.gr. 0.83 at 15°C). After the cellulose had settled, the column was eluted with 1 litre of solvent; this quantity was found to be sufficient to remove Cl^-, sugars, amino acids and glycerylphosphorylcholine without elution of other phosphorus compounds, except for some glycerylphosphorylinositol. The phosphorus compounds were eluted with 400ml of solvent containing more water (60:39.8:0.1:0.1, by vol.; sp.gr. 0.894 at 15°C). The eluate was freeze-dried and this material was later used for chromatography without any further purification.

Separation of phosphorus compounds by chromatography on Dowex resin. The freeze-dried eluate was dissolved in a small quantity of water and applied to a column (0.6cm × 20cm) of Dowex AG1 (X2) resin (formate form 200–400 mesh), which was developed at 20ml/h with the aqueous ammonium formate system of Hurlbert, Schmitz, Brumm & Potter (1954). This system, however, was altered in one respect: elution was continued until the chambers of the gradient apparatus were almost empty, then the mixing chamber was filled with the solution that previously filled the reservoir and the reservoir was simultaneously filled with an equal volume of the next eluting solution (the mixing chamber contained 100ml for the first phase of elution and 50ml for the second phase). The elution of the nucleotides was followed by measurement of E_{260}, and 0.1ml portions were taken from the fractions (2.75ml) for determinations of phosphorus and radioactivity. Fractions representing separate peaks were pooled, freeze-dried and freed from ammonium formate as described by Selvendran & Isherwood (1967). Material from the isolated peaks was rechromatographed on columns (0.6cm × 10cm) of Dowex AG1 (X2) resin at 20ml/h with the formic acid system of Hurlbert *et al.* (1954) modified as described above (volume of mixing chamber 25ml). The fractions (2.75ml) were treated in the same manner as for the first column.

Radioactivity measurements. Samples were introduced into 5ml of dioxan-based scintillation fluid (Bray, 1960). Radioactivity was assayed in a Packard liquid-scintillation spectrometer. Some of the earlier work was performed in a model 3003 instrument where c.p.m. was corrected to d.p.m. by internal standardization; later measurements were made with a model 3380 instrument fitted with an Absolute Activity Analyser that was calibrated to record d.p.m. directly.

Chromatography and electrophoresis. T.l.c. of nucleotides was carried out on layers prepared from cellulose powder (MN Cellulose powder 300; Mackerey, Nagel and Co., Düren, Germany) impregnated with polyethyleneimine hydrochloride as described by Randerath & Randerath (1964). The chromatograms were developed with one of the systems at pH 7, 3.4 or 2 as recommended by these authors.

Paper chromatography and electrophoresis were carried out on Whatman no. 1 paper, washed with M-formic acid and water. Electrophoresis was performed under white spirit (BDH Chemicals Ltd., Poole, Dorset, U.K.) with the voltage applied at 50V/cm; 0.05M-ammonium formate, pH 3.5, containing 0.05% of EDTA was used as the buffer system for the separation of nucleotides. Compounds suspected to be identical with glycerylphosphorylcholine and glycerylphosphorylinositol were separated with the systems described by Dawson, Hemington & Davenport (1962), namely by electrophoresis in the volatile buffer pyridine–acetic acid–water (1:10:89, by vol.), pH 3.6, and by descending chromatography with phenol (saturated with water)–acetic acid–ethanol (50:5:6, by vol.).

The chromatograms and electrophoretograms were examined under u.v. light and in addition treated by one of the following methods. For the detection of phos-

phorus they were treated with the acid molybdate dip of Harrap (1960); for the detection of sugars with the ammoniacal $AgNO_3$ reagent described by Block, Durrum & Zweig (1958); and for the detection of amino groups by spraying with a solution of 0.5% ninhydrin in butan-1-ol and heating in an oven at 80°C for 10min.

Other analytical procedures. Total phosphorus was determined by a micro-procedure based on the method of Chen, Toribara & Warner (1956). Samples were dried in 10ml tapered tubes in an oven at 130°C; an overnight incubation was sufficient to drive off ammonium formate contained in samples from column fractions without causing any loss of phosphorus. After the addition of 0.1ml of 60% (w/w) $HClO_4$, incubation was continued at 130°C for 8h. When samples had cooled, 0.4ml of water and 0.5ml of a freshly prepared mixture of 5% (w/v) ammonium molybdate–water–10% ascorbic acid (1:7:2, by vol.) were added. The tubes were then incubated at 37°C for 2h and E_{820} values were measured. For the determination of P_i in the presence of organic phosphorus, the micro-procedure of Berenblum & Chain (1938) was carried out in 10ml tapered centrifuge tubes; emulsification of the 2-methylpropan-1-ol and aqueous layers was achieved with a Rotamixer [Jencons (Scientific) Ltd., Hemel Hempstead, Herts., U.K.].

Total reducing sugar was measured by the orcinol reaction (Vasseur, 1948). Glycerol was determined by the periodate method of Lambert & Neish (1950) as modified by Ryley (1955), but with all volumes reduced tenfold. Glycerylphosphorylcholine was hydrolysed in M-HCl for 20min at 100°C and the liberated choline was determined as the reineckate by the method of Glick (1944); the extinction of the acetone solution was read at 345nm. Inositol was measured by the method of Lornitzo (1968) for use with samples containing no sugar, amino sugar or sugar alcohol. Guanidine phosphates were measured after hydrolysis in 0.1M-HCl for 9min at 65°C by the method of Rosenberg, Ennor & Morrison (1956) with all volumes reduced tenfold.

Spectra of nucleotides were recorded at pH2, pH7 and pH12 in a Unicam SP800 recording spectrophotometer. The E_{250}/E_{260}, E_{280}/E_{260} and E_{290}/E_{260} ratios were calculated and compared with the same ratios for standard nucleotides.

Enzymic assays for phosphorus compounds were carried out at 37°C. The components of the assay system were prepared as described in the information leaflets supplied by the Boehringer Corp. (London) Ltd., but with the total assay volume decreased to 1ml. ADP, AMP and phosphoenolpyruvate were determined with the pyruvate kinase (EC 2.7.1.40)–lactate dehydrogenase (EC 1.1.1.27)–myokinase (EC 2.7.4.3) system. Fructose 1,6-diphosphate was determined with the aldolase (EC 4.1.2.13)–triosephosphate isomerase (EC 5.3.1.1)–α-glycerophosphate dehydrogenase (EC 1.2.1.12) system. ATP and 3-phosphoglycerate were measured by the 3-phosphoglycerate kinase (EC 2.7.2.3)–glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) system. Glucose 6-phosphate, glucose 1-phosphate and fructose 6-phosphate were measured by using glucose 6-phosphate dehydrogenase (EC 1.1.1.49) coupled with phosphoglucomutase (EC 2.7.5.1) and phosphoglucose isomerase (EC 5.3.1.9) respectively. NAD^+ and NADP were determined by using alcohol dehydrogenase (EC 1.1.1.1) and isocitrate dehydrogenase (EC 1.1.1.42) respectively. Creatine phosphate was determined with the creatine kinase (EC 2.7.3.2)–hexokinase (EC 2.7.1.1)–glucose 6-phosphate dehydrogenase (EC 1.1.1.49) system.

RESULTS

Extraction of acid-soluble phosphorus compounds. Means of using various acids for enzyme denaturation and for extraction of phosphorus compounds were studied in detail; other methods reported by Isherwood & Barrett (1967) to suffer from the disadvantages of low recovery (aqueous ethanol) and brief stimulation of enzyme activity (boiling water) were not investigated. The most suitable acid was regarded as one that satisfies best the following five criteria: (*a*) prevents enzyme activity, (*b*) effectively precipitates protein and other high-molecular-weight material, (*c*) does not cause degradation of phosphate esters, (*d*) achieves maximum recovery of phosphate esters, and (*e*) can be easily removed from the extract. Organic acids have been found to satisfy these criteria when used for the extraction of phosphorus compounds from other tissues, and therefore the use of these acids was examined in the present work.

To determine the recovery and the degree of possible degradation of phosphorus compounds, recovery standards were added to the frozen material before it was ground and added to the acid. However, since the tracer was added to the extracellular medium, it cannot be regarded as an ideal representative of intracellular phosphorus compounds, i.e. those residing within the spermatozoa. To overcome this problem, at least to some extent, the spermatozoa were subjected to partial homogenization. The best results were obtained by grinding the material thoroughly under liquid N_2 in a porcelain mortar. Microscopic examination of the ground material revealed that extensive fragmentation of sperm heads, middle pieces and tails had occurred. On the addition of the frozen and powdered material to the acid in the Blendor, the acid is more likely to penetrate into the broken sperm and make contact with the enzymes before complete thawing of the aqueous phase occurs, a mechanism that has been postulated to occur under similar extraction conditions with other tissue (Minard & Davis, 1962).

The recoveries of [8-^{14}C]ATP added to frozen semen before the grinding were determined and compared, by using bull semen extracted with an equal volume of 2M-formic acid, aq. 10% (w/v) trichloroacetic acid or 2M-perchloric acid. With formic acid, the recovery of radioactivity was only 23%, whereas the recoveries with trichloroacetic acid and perchloric acid were at least 98%. It was also found that trichloroacetic acid is far more

effective as a protein precipitant than perchloric acid; when the concentration of trichloroacetic acid was increased to 20% (w/v), the supernatant after centrifugation was completely clear. After further purification of the extract obtained with 20% (w/v) trichloroacetic acid and chromatography on Dowex resin, the final recovery of radioactivity was at least 92%. Conversion of [8-^{14}C]ATP into ADP and AMP was less than 2%.

It was noticed that the recovery of added [8-^{14}C]-AMP was poor unless large quantities of water were used for elution of the column of Dowex 50. However, when this column was eluted with an additional 750ml of water and the procedure then continued in the usual manner, the final recoveries were at least 92%.

Separation and identification of phosphorus compounds. Semen from the bull, ram, boar and stallion was separated into seminal plasma and sperm, and the two fractions were then extracted, purified and chromatographed separately. Qualitatively the content of phosphorus compounds in the seminal plasma and sperm fractions was identical for the four species. The three main constituents identified in seminal plasma were glycerylphosphorylcholine, glycerylphosphorylinositol and P_i. The pattern obtained by chromatography of an extract from the sperm fraction (still contaminated with some residual seminal plasma) is shown in Fig. 1. Six main peaks were isolated apart from a small amount of glycerylphosphorylcholine, which was eluted at the void volume. These six peaks were subsequently chromatographed with the formic acid system; the compounds identified are shown in Table 1.

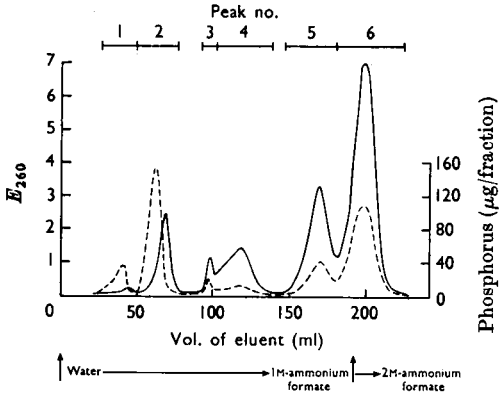

Fig. 1. Elution sequence of a purified trichloroacetic acid extract from 68×10^9 boar sperm on a Dowex 1 (formate form) column. The column (0.6cm×20cm) was developed with the ammonium formate system and 2.75ml fractions were collected; the volume of the mixing chamber was 100ml for the first phase and 50ml for the second phase of elution. ——, E_{260}; – – – –, total phosphorus. The material under the numbered intervals was pooled for subsequent chromatography (see Table 1).

Evidence for the identity of the various compounds was gained by the following means: (*a*) Dowex chromatography of standard compounds by using the two chromatographic systems; (*b*) t.l.c. and paper chromatography and electrophoresis with reference compounds; (*c*) characteristics of the u.v. spectrum of nucleotides; (*d*) correlation of extinction coefficients and phosphorus content for nucleotides; (*e*) correlation of glycerol, phosphorus, choline and inositol content for glycerylphosphorylcholine and glycerylphosphorylinositol; (*f*) enzymic assay for adenine nucleotides, NAD, fructose 1,6-diphosphate and glucose 6-phosphate. NADP, phosphoenolpyruvate, fructose 6-phosphate, glucose 1-phosphate and 3-phosphoglycerate could not be demonstrated by enzymic methods.

NAD contributed towards the E_{260} in peak 2, but the major proportion of the extinction was due to a non-phosphorylated compound that was eluted later than the phosphorus-containing peak when rechromatographed with the ammonium formate system. The E_{260} of peak 3 appeared to be due to some complex of EDTA. The concentrated material obtained from this peak had a light-blue colour and a u.v. spectrum corresponding to that of a sample of EDTA that had been complexed with Cu^{2+}.

It was noted that some interference with separation occurred in the region of peaks 3 and 4. This interference was caused by citrate (which occurs in large quantities in seminal plasma) and EDTA (derived from the solvent system used with the cellulose column in the purification procedure).

Table 1. *Compounds identified in trichloroacetic acid extracts of sperm sedimented from seminal plasma*

The six peaks isolated after chromatography with the ammonium formate system (Fig. 1) were rechromatographed on columns (0.6cm × 10cm) of Dowex 1 (formate form) with the formic acid system (volume of mixing chamber 25ml).

Peak no.	Compounds identified (in order of elution)
1	Glycerylphosphorylinositol
2	NAD, P_i+glucose 6-phosphate
3	AMP (trace), fructose 1,6-diphosphate
4	AMP, fructose 1,6-diphosphate (trace)
5	AMP (trace*), ADP, GDP, ATP (trace)
6	AMP (trace*), ADP, GDP, ATP, GTP

* The traces of AMP eluted from these columns are attributed to minor degradation of ADP and ATP.

Table 2. *Content of phosphorus compounds in extracts from sperm sedimented from seminal plasma*

Determinations were made on peaks separated by Dowex chromatography of purified trichloroacetic acid extracts as described in the Materials and Methods section. Values are expressed as nmol/10^9 sperm.

Phosphorus compound	Bull	Ram	Boar	Stallion
ATP	111	206	117	37
ADP	75	154	50	43
AMP	20	59	12	217
GTP	3.7	4.9	5.0	6.7
GDP	4.6	4.2	3.1	7.0
NAD	10.6	24.4	6.7	10.8
Fructose 1,6-diphosphate	6.0	8.7	5.2	27.3
Glucose 6-phosphate	5.2	5.3	0.7	0
Total nucleotides	225	443	194	322

Table 3. *Content of phosphorus compounds in extracts from seminal plasma after the removal of sperm*

Determinations were made on peaks separated by Dowex chromatography of purified trichloroacetic acid extracts as described in the Materials and Methods section. Values are expressed as nmol/ml of seminal plasma.

Phosphorus compound	Bull	Ram	Boar	Stallion
Glycerylphosphorylcholine	13500	52700	4230	2130
Glycerylphosphorylinositol	1400	1520	260	250
P_i	2800	2460	370	550

Although their effect was not sufficiently severe to warrant their removal, it is probable that a further purification step would be required if enzymic assays were to be carried out directly on the extract rather than chromatography on Dowex 1. Such an additional step to remove organic acids and P_i is described by Isherwood & Barrett (1967).

Concentrations of phosphorus compounds in sperm and seminal plasma. Quantitative measurements were made on the peaks separated by Dowex chromatography (Table 1) of extracts from sperm that had been separated from seminal plasma before extraction. The results obtained in four such extracts are shown in Table 2. The values represent averages based on extinction coefficients, phosphorus contents and enzymic assays. The values for total nucleotides were obtained by a summation of the values for the individual nucleotides. It is possible that the concentrations of the adenine nucleotides in the extract of stallion sperm are not representative of the situation *in vivo* but that considerable breakdown of ATP and ADP occurred during separation of the sperm from the seminal plasma. The seminal plasma that had been separated from the sperm was extracted and chromatographed in a manner identical with that used for the sperm. The concentrations of the three components identified are shown in Table 3; the values represent averages based on phosphorus, glycerol, choline and inositol content.

DISCUSSION

To obtain a true picture of the content of phosphorus compounds in spermatozoa, it is desirable to handle the semen in such a way as to prevent any breakdown of phosphorus constituents before extraction. Not only do phosphatases occur within the sperm, but large quantities of these enzymes also occur in the seminal plasma (Mann, 1964). Further, many substances are present in the seminal plasma that seriously interfere with chromatographic separations of extracts of whole semen. To remove the seminal plasma the sperm can be washed by centrifuging and resuspending the spermatozoa in fresh medium, the process usually being repeated several times. However, during the time taken for such manipulations metabolic activity could cause considerable alteration in the composition of intracellular phosphorus compounds, and, further, it has been noticed that a substantial leakage of phosphorus compounds from spermatozoa occurs during the washing process (D. E. Brooks, unpublished work). For these reasons it was decided to develop an extraction and purification procedure that would be applicable to whole semen. The procedure finally adopted enabled relatively pure samples of phosphorus compounds to be isolated with little loss or degradation.

All of the detected phosphorus-containing peaks separated by Dowex chromatography were identified. The presence of glycerylphosphorylcholine in mammalian semen was demonstrated by Dawson, Mann & White (1957), who found that this compound was derived from the epididymal secretion in the bull and boar. Further evidence that this compound is not of spermatozoal origin was indicated by its absence in washed ram sperm preparations and their inability to liberate this compound

Table 4. *Summary of data in the literature on nucleotide concentrations in acid extracts of mammalian sperm*

Results are for ejaculated sperm unless otherwise indicated. Values are expressed as nmol/10^9 sperm; numbers in parentheses indicate minimum and maximum experimental values.

Method of determination	Species	ATP content	Total nucleotide content	Report
Rate of hydrolysis in M-HCl	Bull (epididymal)	274		Lardy, Hansen & Phillips (1945)
Rate of hydrolysis in M-HCl and enzymic deamination	Bull	314		Mann (1945) as reported by Newton & Rothschild (1961)
U.v. extinction	Bull	129 (70–161)	384 (283–488)	Newton & Rothschild (1961)
U.v. extinction	Bull		393 (157–493)	Abraham & Bhargava (1962)
Rate of hydrolysis in M-HCl and enzymic deamination	Ram	164		Mann (1945)
U.v. extinction	Ram	225		Voglmayr, Scott, Setchell Waites (1967)
U.v. extinction	Ram (testicular)	300		Voglmayr *et al.* (1967)
U.v. extinction	Buffalo		392	Abraham & Bhargava (1962)
U.v. extinction	Goat		122	Abraham & Bhargava (1962)

during incubation. Glycerylphosphorylinositol has been reported to occur in bull and ram semen by Seamark, Tate & Smeaton (1968) and, like glycerylphosphorylcholine, appears to be derived from accessory sex organs as demonstrated by its presence in such organs in the rat and stallion. The present study shows that these two compounds together with P_i contribute the major proportion of acid-soluble phosphorus in semen; in bull semen, for example, which usually contains 10^9 sperm/ml, the ratio of acid-soluble phosphorus between sperm and seminal plasma is 1:35.

Abraham & Bhargava (1962) reported cytosine and guanine in hydrolysates of perchloric acid extracts of bull and buffalo sperm and inferred the presence of nucleotides of these bases. In the present study both GTP and GDP were identified in extracts, but no evidence was obtained for the presence of any cytosine derivatives. The absence of UDP-glucose and glucose 1-phosphate is not surprising, since mammalian spermatozoa are known to have only a negligible content of glycogen (Mann & Rottenberg, 1966). Contrary to the findings of Newton & Rothschild (1961), creatine phosphate was not identified in extracts, but it is of course possible that the acid treatment used in the extraction procedure was too prolonged for this highly labile compound to remain undegraded. NAD was identified in extracts in the present work, but the presence of NADP was not shown, probably because the methods of detection used were not sufficiently sensitive to detect the low concentrations (less than 1 nmol/10^9 sperm) reported by Bistocchi *et al.* (1968).

A definite correlation between ATP content and the proportion of liver sperm appears to exist for the bull (Newton & Rothschild, 1961), and it would seem probable that total nucleotide content would bear a similar relationship. Although the proportion of live sperm was not estimated in the present work, the concentrations of ATP and total nucleotides found are within the range previously reported (Table 4).

Yanagisawa, Hasegawa & Mohri (1968) found in sea-urchin sperm that the guanine nucleotides are bound to a protein (tubulin) constituting the microtubules of the flagellum. They obtained evidence for the presence of a nucleoside diphosphokinase that catalyses the transfer of the terminal phosphate from ATP to GDP, and they postulated that GTP functions in causing contraction of the tubulin molecule. The demonstration of guanine nucleotides from mammalian sperm extracted in the present study invites the same proposition.

I am grateful to Professor T. R. R. Mann, C.B.E., F.R.S. and Dr F. A. Isherwood for their interest and criticism. I also acknowledge financial assistance from the University of Adelaide in the form of the Barr-Smith Travelling Scholarship in Agriculture.

REFERENCES

Abraham, K. A. & Bhargava, P. M. (1962). *Experientia*, **18**, 575.

Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.

Bishop, M. W. H., Campbell, R. C., Hancock, J. L. & Walton, A. (1954). *J. agric. Sci., Camb.*, **44**, 227.
Bistocchi, M., D'Alessio, G. & Leone, E. (1968). *J. Reprod. Fert.* **16**, 223.
Block, R. J., Durrum, E. L. & Zweig, G. (1958). *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd ed., p. 178. New York: Academic Press Inc.
Bray, G. A. (1960). *Analyt. Biochem.* **1**, 279.
Chen, P. S., Toribara, T. Y. & Warner, H. (1956). *Analyt. Chem.* **28**, 1756.
Dawson, R. M. C. (1960). *Biochem. J.* **75**, 45.
Dawson, R. M. C., Hemington, N. & Davenport, J. B. (1962). *Biochem. J.* **84**, 497.
Dawson, R. M. C., Mann, T. & White, I. G. (1957). *Biochem. J.* **65**, 627.
Eastoe, J. E. (1955). *Biochem. J.* **61**, 601.
Glick, D. (1944). *J. biol. Chem.* **156**, 643.
Harrap, F. E. G. (1960). *Analyst, Lond.*, **85**, 452.
Hurlbert, R. B., Schmitz, H., Brumm, A. F. & Potter, V. R. (1954). *J. biol. Chem.* **209**, 23.
Isherwood, F. A. & Barrett, F. C. (1967). *Biochem. J.* **104**, 922.
Lambert, M. & Neish, A. C. (1950). *Can. J. Res.* B, **28**, 83.
Lardy, H. A., Hansen, R. G. & Phillips, P. H. (1945). *Archs Biochem.* **6**, 41.
Lornitzo, F. A. (1968). *Analyt. Biochem.* **25**, 396.
Mann, T. (1945). *Biochem. J.* **39**, 451.
Mann, T. (1964). *The Biochemistry of Semen and of the Male Reproductive Tract*, pp. 182–190. London: Methuen and Co. Ltd.
Mann, T. & Rottenberg, D. A. (1966). *J. Endocr.* **34**, 257.
Minard, F. N. & Davis, R. V. (1962). *J. biol. Chem.* **237**, 1283.
Newton, A. A. & Rothschild, Lord (1961). *Proc. R. Soc.* B, **155**, 183.
Randerath, K. & Randerath, E. (1964). *J. Chromat.* **16**, 111.
Rosenberg, H., Ennor, A. H. & Morrison, J. F. (1956). *Biochem. J.* **63**, 153.
Ryley, J. F. (1955). *Biochem. J.* **59**, 353.
Seamark, R. F., Tate, M. E. & Smeaton, T. C. (1968). *J. biol. Chem.* **243**, 2424.
Selvendran, R. R. & Isherwood, F. A. (1967). *Biochem. J.* **105**, 723.
Vasseur, E. (1948). *Acta chem. scand.* **2**, 693.
Voglmayr, J. K., Scott, T. W., Setchell, B. P. & Waites, G. M. H. (1967). *J. Reprod. Fert.* **14**, 87.
Yanagisawa, T., Hasegawa, S. & Mohri, H. (1968). *Expl Cell Res.* **52**, 86.