Glycolytic Enzymes in Mammalian Spermatozoa

ACTIVITIES AND STABILITIES OF HEXOKINASE AND PHOSPHOFRUCTOKINASE IN VARIOUS FRACTIONS FROM SPERM HOMOGENATES

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1. Methods of homogenizing suspensions of washed mammalian spermatozoa were studied. The most useful methods were those using sonication and those using a French press. 2. Hexokinase, phosphofructokinase, glucose phosphate isomerase and adenosine triphosphatase activities in ram, bull and boar spermatozoa were investigated by using these two homogenization methods. Glucose phosphate isomerase, representative of soluble cytoplasmic material, was very readily extracted and remained entirely in the supernatant after centrifugation at 145000g for 60min. In contrast, the other three activities were less easily extracted and were sedimented in various proportions under the described conditions of centrifugation. 3. Attempts to obtain subcellular fractions from sperm homogenates by 'classical' methods failed, owing apparently to the inhomogeneity of subcellular particles in the homogenates. It is concluded that, after removal of sperm heads, the only meaningful fractionation is a separation of spermatozoal material which sediments at 145000g during 60min from that which does not. 4. The stabilities of hexokinase and phosphofructokinase activities in bull, boar and ram sperm homogenates were investigated. Hexokinases showed very little dependence on the various environments tested, whereas the optimum conditions for phosphofructokinase stability were: a minimum of sonication, the presence of phosphate ions and of a thiol-group protectant, and a pH 7.5. Activities of hexokinase, phosphofructokinase and glucose phosphate isomerase per sperm cell were compared with published data on rates of fructolysis by spermatozoa; the potential catalytic activities were shown to be considerably in excess of these rates. However, phosphofructokinase may be the rate-limiting enzyme of glycolysis *in vivo* in bull and ram spermatozoa.

Glycolysis and respiration constitute the main sources of energy for motility in mammalian spermatozoa (Mann, 1964, pp. 265–307); but although much work has been done on the metabolism in spermatozoa of a great number of substrates, and particularly on those of the glycolytic pathway, little work has been done on the isolation and study of the enzymes involved in these metabolic events.

The present paper describes experiments on homogenization and subsequent fractionation of spermatozoa from bull, boar and ram, and also some studies on the stability of hexokinase (EC 2.7.1.1) and phosphofructokinase (EC 2.7.1.11) in these homogenates.

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MATERIALS AND METHODS

Semen. Ejaculated semen was obtained from bulls, boars and rams at the Unit by the usual methods (see Maule, 1962). Pooled ejaculates were used in the cases of bull and ram semen, and single ejaculates in the case of boar; several animals of each species were involved. The usual precautions were taken to avoid cold shock and the processing of semen was always begun within 1 h of collection.

Preparation of milk diluent. Fresh cow's milk was centrifuged at 4°C at 6000g for 45min, and the infranatant solution carefully decanted. This skimmed milk was heated at 90–95°C for 10min, cooled and dialysed thoroughly at 4°C against five 12h changes of deionized water (12.5 litres of water/pint of milk). The dialysed milk was used as solvent for salts, fructose and antibiotics to give the following diluent composition: 2% (w/v) of

dialysed skim-milk solids, 36g of fructose/l (200mM), 1.8g of NaCl/l (31mM), 3.0g of NaH_2PO_4/l (19mM), 5mM-KOH, 500i.u. of penicillin/ml and 750i.u. of streptomycin/ml, adjusted to pH7.4 with 1M-NaOH. It was centrifuged at 20000g for 20 min, and the partially clarified supernatant was removed without disturbing the loose pellet, and used for diluting semen.

Washing of spermatozoa. Boar semen, strained through muslin immediately after collection to remove the gel, was concentrated by centrifugation at 20°C in an MSE High Speed 18 centrifuge, using the 6×250ml angle head, at 360g for 10min (all centrifugal forces are expressed as g_{max}. values calculated to the bottoms of the tubes); most of the seminal plasma was removed and the loose sperm pellet was gently resuspended. Semen from bull and ram was used without prior concentration. The semen was diluted with the milk diluent, at 20–25°C to a final sperm density of 1×10^8–2×10^8 cells/ml, its container was surrounded by a water jacket at the same temperature, and it was left to cool overnight to 4°C in the cold-room. To obtain a sufficiently slow rate of cooling, the total volume of diluted semen plus water jacket was between 600 and 1000ml. All further operations were carried out at 4°C.

Next day the diluted semen was centrifuged at 300g for 12min in the 6×250ml angle head (High Speed 18); the supernatant was removed and discarded and the sperm pellet gently resuspended to the same volume in a medium containing 250mM-sucrose and 4mM-EDTA (sodium salt), adjusted to pH7.4 (at 20°C) with 1M-KOH. The sperm suspension was recentrifuged at 360g for 12min and the pellet resuspended and recentrifuged in the sucrose medium once more. This twice-washed sperm pellet was used as the starting material in all enzyme studies.

Homogenization. In preliminary experiments, various methods for homogenization of spermatozoa were compared. These methods and the results obtained are described briefly in the Results section.

Homogenization of some sperm suspensions was carried out by using a French press (Hughes & Cunningham, 1963) by courtesy of Dr D. Kerridge of the Department of Biochemistry, University of Cambridge. Samples (5ml) were forced through the apparatus by using a hand-operated hydraulic ram, either under low pressure (150–00kg/cm²) or under high pressure (1000–1200kg/cm²).

As a routine, however, homogenization was performed with a Kerry's Ultrasonic Cell Disruptor K100, at the medium power input (50W). The sample (approx. 5ml) was contained in a flat-bottomed 51mm$\times 19$mm glass specimen tube. It was kept cool in a stirred ice–water bath. The vibrating tungsten probe was immersed not more than 1cm into the sample.

In all manipulative procedures during and after homogenization the temperature of the preparations was maintained below 4°C unless otherwise stated.

Fractionation of homogenates. Attempts to fractionate sperm homogenates into heads, mitochondria, microsomes and cytoplasm were unsuccessful (see the Results section).
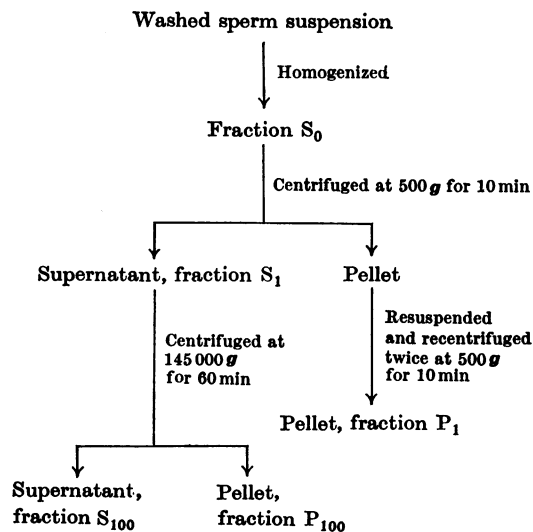
Subsequently, unless otherwise stated, the sperm homogenates (fraction S_0) were fractionated as shown in Scheme 1, to yield fractions P_1, S_1, P_{100} and S_{100}. Fraction P_1 contained heads and large midpiece and tail fragments, which could be identified by phase-contrast microscopy; fraction P_{100} contained microscopic and sub-microscopic particles (assumed to be mitochondria, fragments of mitochondria, membrane fragments and tail fragments, etc.); fraction S_{100} contained soluble material and very fine particles, probably mainly cytoplasmic membrane fragments.

During the fractionation procedures the pellets were resuspended in the homogenization medium relevant to the particular experiment.

Enzyme assays. Assays were based on well-established methods, and were carried out with a Unicam SP.800 recording spectrophotometer, fitted with an automatic cell changer and a constant-temperature cell housing. The pre-warmed assay mixture was pipetted into a semi-micro silica cuvette, maintained at the assay temperature. A sample of the experimental material was then added, the contents of the cuvette mixed thoroughly and rapidly, and the extinction at 340nm measured and recorded automatically. After 5min it was remeasured, and any change in extinction was assumed to be linear with time (an assumption that was proved to be valid in preliminary experiments).

As a routine all assays were performed at 37°C at pH8.2 in a total volume of 1ml, and the reactions initiated by the addition of experimental material. One unit of enzyme activity converts 1μmol of substrate/min under the specified conditions of assay. Blank rates (i.e. without substrate) were negligible in all cases, and the rates with substrate were proportional both to time and to the amount of enzyme added. The absence of 6-phosphogluconate dehydrogenase activity in sperm homogenates (R. A. P. Harrison, unpublished work) precluded the need for any correction for proportionality of reduced

Washed sperm suspension

| Homogenized
↓

Fraction S_0

| Centrifuged at 500g for 10min
↓

Supernatant, fraction S_1 Pellet

Centrifuged at 145000g for 60min   Resuspended and recentrifuged twice at 500g for 10min

Pellet, fraction P_1

Supernatant, fraction S_{100} Pellet, fraction P_{100}

Scheme 1. Fractionation scheme for sperm homogenates. Low-speed centrifugation was carried out on an MSE High Speed 18 centrifuge, using the 8×50ml angle head; high-speed centrifugation was carried out on a Spinco model L ultracentrifuge (50 Ti head). Centrifugal forces are expressed as g_{max}. values calculated to the bottoms of the tubes.

NADP$^+$ to oxidized glucose 6-phosphate in the hexokinase and glucose phosphate isomerase assays.

Assay systems. (*a*) Hexokinase. Final concentrations of constituents in the system were: 100 mM-tris–HCl, 1 mM-EDTA, 1 mM-2-mercaptoethanol, 10 mM-MgCl$_2$, 1 mM-glucose, 5 mM-ATP (neutralized), 0.5 mM-NADP$^+$, and 2 μg of crystalline glucose 6-phosphate dehydrogenase (0.56 unit)/ml.

(*b*) Phosphofructokinase. Final concentrations of constituents in the system were: 100 mM-tris–HCl, 1 mM-EDTA, 1 mM-2-mercaptoethanol, 2 mM-MgCl$_2$, 1 mM-fructose 6-phosphate, 0.01% bovine plasma albumin, 1 mM-ATP (neutralized), 0.2 mM-NADH, 50 μg of crystalline aldolase (0.9 unit)/ml, 4 μg of crystalline triose phosphate isomerase (19 units)/ml and 10 μg of crystalline α-glycerophosphate dehydrogenase (0.8 unit)/ml.

(*c*) Glucose phosphate isomerase (EC 5.3.1.9). Final concentrations of constituents in the system were: 100 mM-tris–HCl, 1 mM-EDTA, 1 mM-2-mercaptoethanol, 10 mM-MgCl$_2$, 1 mM-fructose 6-phosphate, 0.5 mM-NADP$^+$ and 2 μg of crystalline glucose 6-phosphate dehydrogenase (0.56 unit)/ml.

(*d*) ATPase* (EC 3.6.1.4). Final concentrations of constituents in the system were: 100 mM-tris–HCl, 1 mM-EDTA, 1 mM-2-mercaptoethanol, 10 mM-MgCl$_2$, 5 mM-ATP (neutralized), 60 mM-KCl, 1 mM-phosphoenolpyruvate, 0.2 mM-NADH, 2 μg of crystalline pyruvate kinase (0.6 unit)/ml and 2.5 μg of crystalline lactate dehydrogenase (1.8 units)/ml.

Protein determinations. These were performed by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine plasma albumin as a standard. Samples and standards were preincubated with 1 M-NaOH for 30 min to dissolve insoluble protein as described in the original method.

Estimations of sperm concentration. The concentration of sperm-cell suspensions was estimated by an absorptiometric method by using a Hilger–Spekker absorptiometer with a red filter and relating the readings to a calibration curve based on haemocytometer counts (Dott, 1960).

Reagents. Crystalline enzymes and biochemicals were obtained from Sigma (London) Chemical Co. Ltd. (London S.W.6, U.K.) and from Boehringer Corp. (London W.5, U.K.). Bovine plasma albumin was obtained from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex, U.K.). All chemicals were of A.R. grade where possible and double-distilled water or high-purity deionized water was used to make up solutions.

RESULTS

Observations on hexokinase and phosphofructokinase assays using sperm extracts

Sperm hexokinase had a broad pH optimum between 7.6 and 8.6 in all three species of spermatozoa, and no sign of a high K_m glucokinase could be observed nor any inhibitory effect of glucose at high concentration (cf. Grossbard & Schimke, 1966).

* Abbreviations: ATPase, adenosine triphosphatase (EC 3.6.1.4); HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.

The optimum concentration of ATP for hexokinase may be greater than 5 mM, but a lag period in the rate of NADP$^+$ reduction appeared at higher ATP concentrations, probably because of the inhibitory action of ATP on glucose 6-phosphate dehydrogenase at low glucose 6-phosphate concentration (Avigad, 1966). Increasing the amount of auxiliary enzyme led to undesirable increases in the amount of contaminating hexokinase activity in the blank.

Sperm phosphofructokinase had a pH optimum at pH 8.2 in all species. The optimum Mg^{2+} concentration was 2 mM, above which inhibition occurred whatever the ATP concentration, and neither K$^+$ nor ADP or AMP had any effect in the assay (cf. Underwood & Newsholme, 1965). The activity of ram sperm phosphofructokinase decreased on standing at 4°C, after which incubation with ATP and/or fructose 6-phosphate was required to restore full activity (cf. Mansour, 1965). The enzyme in bull or boar spermatozoa did not show this effect, but pre-incubation was desirable, because intermediates which interfered with the assay were then used up. Thus it was routine to incubate the sample in the assay mixture for 3 min before commencing readings, allowing the mixture to warm up to 37°C during this time. Ram sperm phosphofructokinase also showed dependence on the order in which the substrates were added to it: fructose 6-phosphate added before ATP gave maximum activity, although the enzyme from the other species did not show this effect. There was no effect if the substrates were added simultaneously, full activity being maintained under these conditions (cf. Mansour & Ahlfors, 1968).

Comparison of methods for disruption of spermatozoa and extraction of enzymes

General. In preliminary experiments, the efficacy of methods of homogenizing spermatozoa in causing cell disruption were compared, the extent of disruption being estimated with a phase-contrast microscope. The results were not noticeably dependent on the suspension media or on cell concentration, provided that these did not markedly increase the viscosity of the suspensions. Thus, for example, high concentrations of sucrose and densities of more than 2×10^9 cells/ml did decrease disruption.

The conclusions from these experiments are set out in Table 1. Apart from efficiency in disruption, some other criteria have also been compared: these were of importance when a final choice was made.

Table 1 shows that the two clearly useful methods were those using either sonication or a French press. These methods were studied to assess how well they extracted enzymes.

Table 1. *Comparison of methods for homogenization of spermatozoa*

Suspensions of washed ram spermatozoa in 0.154 M-NaCl at a cell density of 0.5×10^9–1.5×10^9 cells/ml were subjected to the stated treatments; samples of the homogenates were examined under high-power magnification with a phase-contrast microscope. For references to methods, see the text. Disruption was assessed at 5 min or after a single treatment. For explanation of the criteria assessed see the Discussion section.

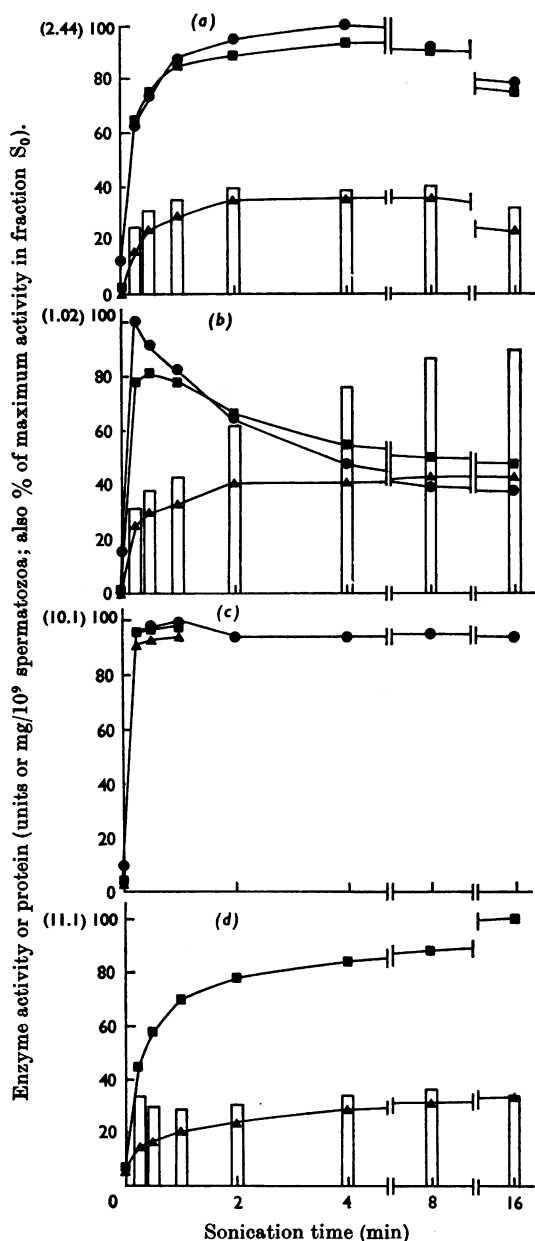| Methods	Disruption	Completeness of sample treatment	Controllability	Recovery of material	Minimum vol. treatable (ml)	Disadvantages
1. Grinding: (a) alone	Poor: most heads detached from midpieces, some tail breakage	Incomplete	Poor	Quite good	2–3	
(b) with abrasives	Better: heads detached, some midpieces fragmented, most tails fragmented	Incomplete	Poor	Poor	4	
(c) while freezing and thawing	As (b)	Incomplete	Poor	Quite good	2–3	Needs to be frozen
2. Mechanically operated Potter homogenizer	Very poor: hardly any visible damage	Complete	Good	Good	1–2	
3. Waring Blendor	Rather poor: all heads detached from midpieces only after 15 min at full power; tails disrupted	Complete	Good	Fair	30	Heating occurs
4. Shaking with glass beads: (a) low speed—Mickle	Rather poor and slow: after 15 min, all heads detached, many midpieces and tails fragmented	Complete	Poor	Poor	5	
(b) high speed—Nossal	Quite good: heads detached, all midpieces and tails fragmented	Complete	Poor	Poor	5	Heating occurs
5. Colloid mill: (a) alone	Poor	Incomplete	Poor	Fair	5	Frothing occurs
(b) with glass beads	Quite good	Incomplete	Poor	Poor	5	Frothing occurs
6. Hughes press	Very good: all heads detached, very few midpieces visible, tails completely disrupted	Complete	Poor	Good	2	Needs to be frozen
7. French press	Very good	Complete	Good	Good	2	
8. Sonication	Very good	Complete	Good	Good	1–2	Heating occurs

Fig. 1. Extraction of enzymes and protein after sonication of washed ram sperm suspensions for various periods of time. Washed ram spermatozoa were resuspended in ice-cold 20 mm-HEPES buffer, pH 7.2 (at 20°C), containing 250 mm-sucrose, 1 mm-EDTA and 5 mm-2-mercaptoethanol. They were sonicated for various periods of time, and the homogenates then fractionated and assayed for hexokinase, phosphofructokinase, glucose phosphate isomerase and protein amounts. Values are all normalized to a percentage of the maximum value obtained in fraction S_0 for each parameter, but the absolute maximum value in each case is also given in parentheses, as units of enzyme activity or mg of protein/10^9 spermatozoa. ●, Fraction S_0; ■, fraction S_1; ▲, fraction S_{100}; ⊓, % non-sedimentable (100 × activity in fraction S_{100}/activity in fraction S_1). (a) Hexokinase; (b) phosphofructokinase; (c) glucose phosphate isomerase (in all cases after zero-time >95% of the activity was non-sedimentable); (d) protein.

Duration of sonication. Washed spermatozoa were resuspended in ice-cold 20 mm-HEPES buffer (pH 7.2 at 20°C), containing 1 mm-EDTA and 5 mm-2-mercaptoethanol, and sonicated for various lengths of time. The homogenates were fractionated and the fractions assayed for hexokinase, phosphofructokinase and glucose phosphate isomerase activities. The results with ram spermatozoa are shown in Fig. 1; similar patterns of results were obtained with bull and boar spermatozoa, although different absolute amounts were found. The morphological appearances of bull sperm suspensions after various periods of sonication are shown in Plate 1.

In general the soluble enzymes, represented by glucose phosphate isomerase (de Duve, Wattiaux & Baudhuin, 1962; Hernandez & Crane, 1966), were extracted very rapidly whereas the particulate enzymes were extracted more slowly (although their activities rapidly became detectable in the S_0 fraction); this suggests that the particles to which the bound enzymes were attached were gradually decreased in size, since the specific activities of the non-sedimentable fractions remained relatively constant for these enzymes. In HEPES buffer, the phosphofructokinase from spermatozoa of bull and ram was unstable, losing its activity rapidly during sonication (see below). The proportion of extracted activity which was non-sedimentable is also shown in Fig. 1. A clear difference is seen between the behaviour of hexokinase, where the proportion remained relatively constant even after prolonged sonication, and phosphofructokinase, where the proportion increased continuously.

Comparison of the effect of the ultrasonic disruptor with that of the French press. Washed spermatozoa were resuspended in ice-cold 20 mm-phosphate buffer (pH 7.6 at 20°C), containing 1 mm-EDTA and 5 mm-2-mercaptoethanol, and disrupted either by sonication or in a French press. Various periods of sonication or pressures were employed. The homogenates were fractionated and the fractions assayed for hexokinase, phosphofructokinase and ATPase activity. The results for ram spermatozoa are shown in Fig. 2; similar patterns of results were obtained for bull and boar spermatozoa. The morphological appearances of different homogenates of bull spermatozoa are shown in Plate 1.
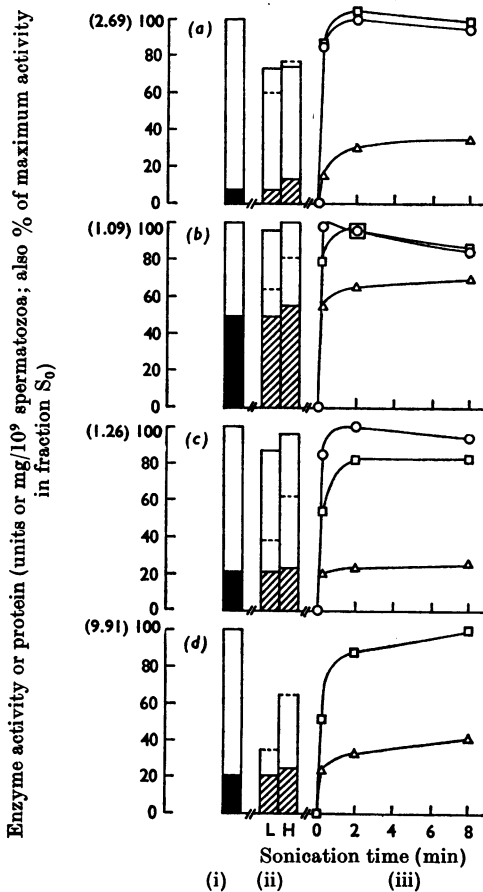
Fig. 2. Extraction of enzymes and protein from disrupted ram sperm suspensions: comparison of sonication with use of a French press. Suspensions of washed ram spermatozoa in ice-cold 20mM-phosphate, 1mM-EDTA and 5mM-2-mercaptoethanol, pH7.6, were sonicated for 15s, 2min or 8min, or treated in the French press at low or high pressure. The homogenates were then fractionated, and assayed for (a) hexokinase, (b) phosphofructokinase, (c) ATPase and (d) protein amounts. All values are normalized to a percentage of the maximum value obtained in fraction S_0 for each parameter, but the absolute maximum value is also given in parentheses, as units of enzyme activity or mg of protein/10^9 spermatozoa. (i) $\sqcap$, Total and $\blacksquare$, 'true' non-sedimentable activity as percentage comparison (see the text). (ii) French press, low (L) and high (H) pressures; $\sqcap$, fraction S_0; $\sqcap$, fraction S_1; $\blacksquare$, fraction S_{100}. (iii) Sonication; $\bigcirc$, fraction S_0; $\square$, fraction S_1; $\triangle$, fraction S_{100}.

In all cases the French press was less efficient at extracting hexokinase and ATPase activities into fraction S_1 although similar extraction of phosphofructokinase was equally efficient by either method in spite of the presence of considerable particulate

activity; also there was no evidence of instability of the latter enzyme during disruption in the French press. Changing the buffer from HEPES to phosphate and raising the pH slightly seemed to protect phosphofructokinase against inactivation during sonication, but dithiothreitol (results not given) gave no further protection of enzyme activity in sperm homogenates from any species.

There were large differences between hexokinase and phosphofructokinase in their extractability by the French press into fraction S_{100}. This is shown in Fig. 2, for ram spermatozoa, by the relative heights of the hatched bars. This variation in extractability by the French press together with the observed ease of extraction of glucose phosphate isomerase during sonication suggested that an estimation of the proportion of enzyme activity that is soluble or only loosely bound in the living sperm cell could be made by comparing the proportion of activity extracted into fraction S_{100} by the French press at low pressure with the maximum assayable activity in the total homogenate (fraction S_0). When the French press was used at low pressure sufficient damage was done to rupture the cell membranes and to extract the soluble cytoplasmic constituents, whereas the membranes themselves stayed relatively unfragmented, with the membrane-bound enzymes still attached.

A comparison of the behaviour of the enzymes assayed in ram spermatozoa in these experiments is shown in Fig. 2 by the dark and light bars (i), and reveals clear differences in the distribution of the enzymes. About 49% of the phosphofructokinase in ram spermatozoa appeared to be soluble, whereas only about 20% of the ATPase and 7% of the hexokinase appeared to be soluble; in bull spermatozoa only 20% of the phosphofructokinase, 14% of the hexokinase and 12% of the ATPase was soluble, and in boar spermatozoa about 74, 15 and 10% respectively was soluble.

Fractionation of sperm homogenates

The fractionation procedure originally adopted was one based on the classical nuclei–mitochondria–microsomes–cell sap separation procedures described by Hogeboom (1955). Thus spermatozoa, homogenized either in hypo-osmotic phosphate or in iso-osmotic potassium chloride, were fractionated successively at 500g for 10min, 10000g for 30min, and 145000g for 60min.

The supernatants and precipitates were examined morphologically and assayed for hexokinase and for cytochrome oxidase (by the method of Smith, 1955). Some results obtained by using iso-osmotic potassium chloride are shown in Table 2. Use of hypo-osmotic media led to essentially similar results.
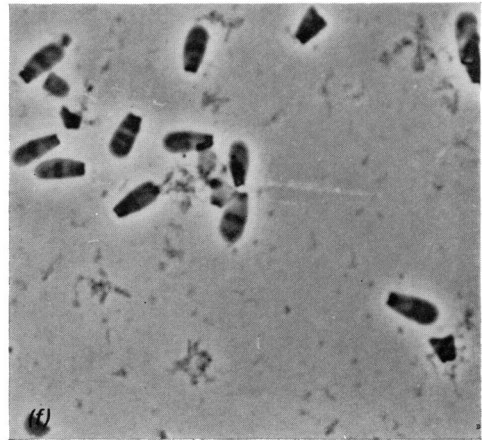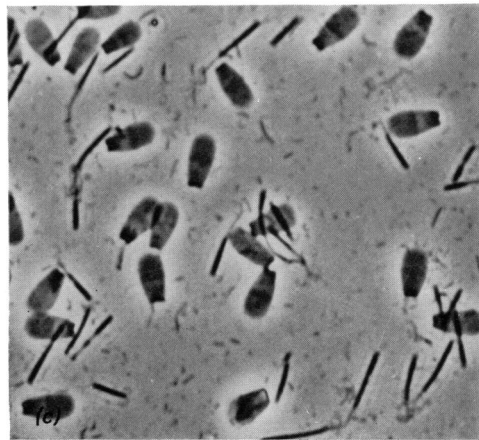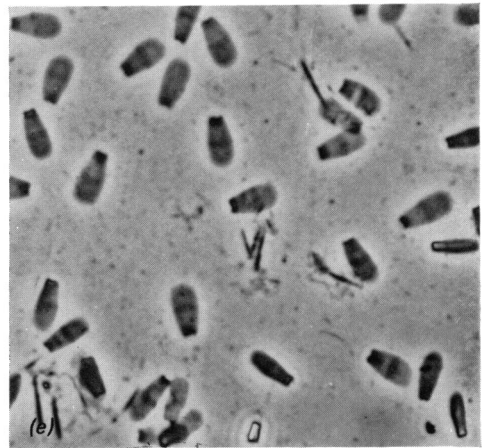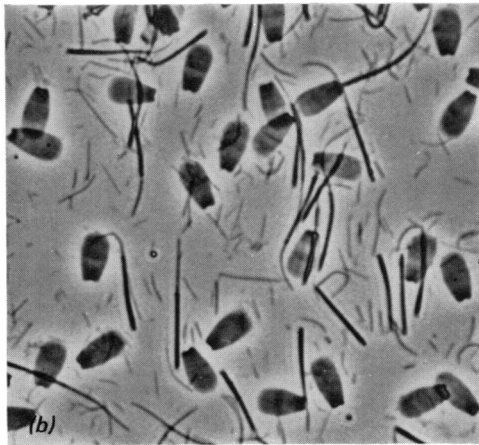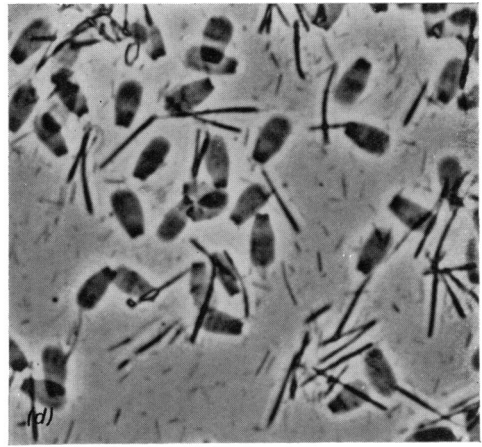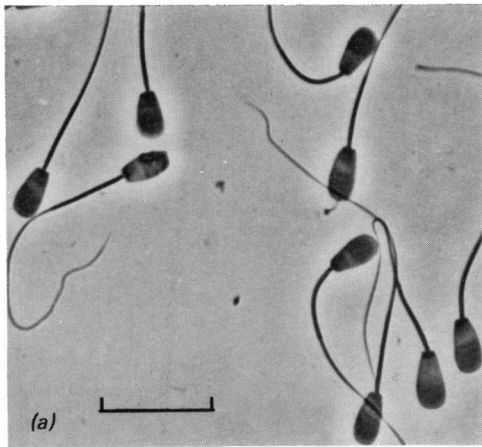
EXPLANATION OF PLATE I

Morphological appearance of homogenates of washed bull spermatozoa. (Phase-contrast magnification ×800.) (*a*) Untreated. (*b*) French press, low pressure. (*c*) French press, high pressure. (*d*) Sonication, 15s. (*e*) Sonication, 2min. (*f*) Sonication, 8min. The bar represents $20\,\mu$m.

Table 2. *Fractionation of hexokinase and cytochrome oxidase activities in ram sperm homogenates*

Washed ram spermatozoa were suspended in ice-cold 150mm-KCl, containing 5mm-MgSO$_4$, 5mm-EDTA and 10mm-2-mercaptoethanol, buffered to pH7.4 (at 20°C) with KHCO$_3$. The suspension was sonicated for 2min, the homogenate fractionated as described in the text, and the supernatant fractions assayed for hexokinase and cytochrome oxidase activities. The sedimentable activities were calculated by subtraction; they have been expressed as a percentage of the activity not sedimented at 500g for 10min.

	Hexokinase	Cytochrome oxidase
Sedimented between 500g for 10min and 10000g for 30min	6	40
Sedimented between 10000g for 30min and 145000g for 60min	62	54
Not sedimented at 145000g for 60min	32	7

Since cytochrome oxidase is known to occur only in mitochondria (de Duve *et al.* 1962), fragmentation of mitochondria must have occurred during sonication, since intact mitochondria should sediment during centrifugation at 10000g for 30min. Similar findings were reported by Nelson (1955), who used a freezing–thawing–grinding method of homogenization.

In another experiment attempts were made to sediment fractions at successively higher centrifugal forces. Considerable hexokinase activity was detected in all fractions, suggesting that the enzyme was attached to particles with a wide variation of size. No attempt was made to fractionate sperm homogenates further than is described in the Materials and Methods section.

Stability of enzymes

The stability of hexokinase and phosphofructokinase, the two enzymes under main study, was investigated with respect to the buffer, pH and storage conditions. In one experiment, washed spermatozoa were resuspended in various ice-cold media and sonicated. Various fractions of the homogenates were stored at 2°C or frozen at −20°C, and all samples were assayed at intervals for hexokinase and phosphofructokinase activities. It was found that whereas hexokinase activity was essentially independent of the medium used, and was stable whether stored at 2°C or in the frozen state, phosphofructokinase activity was always highest in buffered media containing phosphate (phosphate probably protected against inactivation

during sonication). Phosphofructokinase was unstable, particularly during storage in an iso-osmotic potassium chloride medium, and F$^-$ showed a protective action only on the boar sperm enzyme (cf. Ling, Marcus & Lardy, 1965; Layzer, Rowland & Bank, 1969). Freezing was always the better method of storage.

In another experiment, phosphate and HEPES buffers, each 20mm and containing 1mm-EDTA and 5mm-2-mercaptoethanol, were prepared at pH6.4 (phosphate only), 6.8, 7.2, 7.6 and 8.0 at 20°C. Washed sperm samples were resuspended in each of these buffers at 0°C, sonicated for 15s, and centrifuged at 145000g for 60min. The whole homogenates (fraction S$_0$) and the centrifuged supernatants (fraction S$_{100}$) were assayed for hexokinase and phosphofructokinase activities. The results are shown in Fig. 3. Hexokinase stability varied little with the buffer or the pH of the homogenizing medium, although the proportion of non-sedimentable activity decreased with decreasing pH. In contrast, the stability of boar and bull sperm phosphofructokinase depended markedly on the pH of the homogenizing medium, and was best stabilized by phosphate buffer above pH7.5; over a narrow range of pH the differences between phosphate and HEPES were considerable; the proportion of non-sedimentable activity (similar in phosphate and HEPES buffers) decreased only below pH6.5.

DISCUSSION

Homogenization and fractionation of spermatozoa. In the past various methods have been used to disrupt spermatozoa: shaking with glass beads (Morton & Lardy, 1967), grinding with abrasives (King & Mann, 1959), freezing (Nelson, 1955), Waring Blendor (Dallam & Thomas, 1953), Potter homogenizer (Clausen & Øvlisen, 1965), colloid mill (Morton, 1968), Ribi cell fractionator (Bell, 1969), and sonication (Mohri, Mohri & Ernster, 1965). However, apart from Morton's (1968) work, little critical appraisal of these methods has been published. A discussion of some general principles of cell disruption together with more detailed studies of sonication processes has been published by Hughes & Cunningham (1963); other aspects of enzyme extraction are reviewed by Morton (1955).

The main considerations for a choice of homogenization method are (*a*) efficacy of homogenization, (*b*) completeness of sample treatment (i.e. whether or not all the sample has been treated), (*c*) controllability, (*d*) recovery of material and (*e*) convenience. As described above, the only methods that proved successful by most criteria were those that used either the French press or sonication.
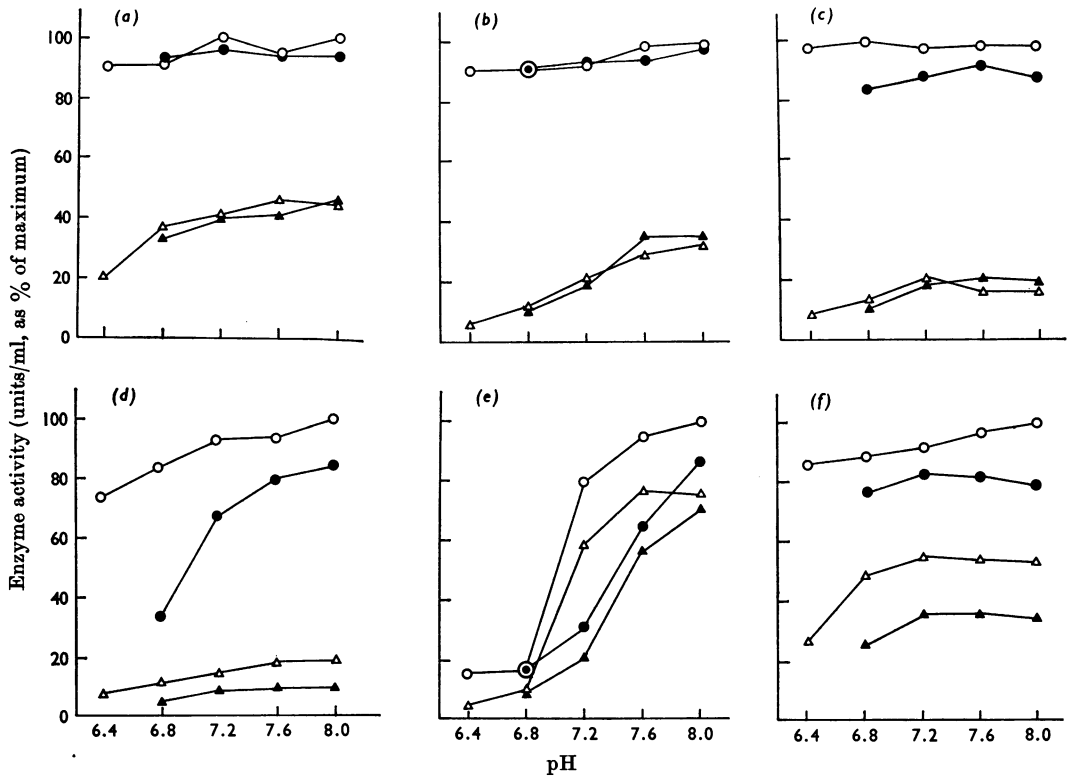
Fig. 3. Stability and distribution of hexokinase and phosphofructokinase in phosphate and HEPES buffers at various pH values. Washed spermatozoa were resuspended at various pH values in ice-cold 20 mM concentrations of either phosphate or HEPES (which also contained 1 mM-EDTA and 5 mM-2-mercaptoethanol), and were sonicated for 15 s. The whole homogenate (fraction S_0) and the non-sedimentable reaction (fraction S_{100}) of each sample were assayed for hexokinase and phosphofructokinase activity. All values have been normalized to a percentage of the maximum total activity in fraction S_0 obtained/10^9 spermatozoa. (a) Bull, (b) boar and (c) ram hexokinase activities. (d) Bull, (e) boar and (f) ram phosphofructokinase activities. $\bigcirc$, Fraction S_0 in phosphate buffers; $\bullet$, fraction S_0 in HEPES buffers; $\triangle$, fraction S_{100} in phosphate buffers; $\blacktriangle$, fraction S_{100} in HEPES buffers.

Compared with other animal cells, mammalian spermatozoa are small, very asymmetric, contain very little cytoplasm and have a tough fibrillar structure (Mann, 1964, pp. 19–29). It is thus much more difficult to prepare from them organelles and subcellular fractions in a relatively pure or undamaged state. Inevitably a range of fragmentation occurs and careful differential centrifugation will not prevent cross-contamination of the fractions. The French press system seems very suitable for preparing subcellular sperm fractions; an advantage of this system is that breakage is a 'single event', occurring only during the very brief period when the cell passes through the needle valve; therefore less damage to organelles may be expected. Sonication is too powerful, or needs to be applied for too long to achieve reasonably complete cell breakage: since it is a continuous process, it fragments released mitochondria at the same time as it disrupts intact midpieces. Extraction of total sperm enzyme activities, however, requires extensive homogenization, particularly if the activity is held within organelles or bound within membrane vesicles: in this case, sonication is usually the most suitable method. A check of individual enzyme activities during extraction processes should always be made to ensure that maximal extraction has been obtained: the effect of sonication on hexokinase, phosphofructokinase and glucose phosphate isomerase activities in spermatozoa illustrates this point.

Stability of enzymes. Instability of hexokinases in homogenates has not so far been reported to be a problem and stabilization of hexokinases generally

has been effected with glucose, phosphate, EDTA and thiol-group protectants (Berger, Slein, Colowick & Cori, 1946; Sols & Crane, 1954; Uyeda & Racker, 1965; Grossbard & Schimke, 1966).

Little inactivation of sperm hexokinase occurred during sonication and thus excellent extraction of the enzyme with high recoveries was obtained. No intrinsic differences between the effects of buffers at the same pH could be detected, either during homogenization or during prolonged storage, and no stabilizing action of glucose was found.

The instability of phosphofructokinase in homogenates and during purification processes has been widely discussed (e.g. Ling et al. 1965; Wakid & Mansour, 1965; Mansour & Ahlfors, 1968; Brock, 1969; Hoskins & Stephens, 1969; Layzer et al. 1969). The findings of the present study agree in general with those of studies performed on other tissues; i.e. the requirements for optimum stability of this enzyme in homogenates are: a final pH above 7.5, the presence of phosphate ions, and probably of a thiol-group protectant; too high a salt concentration is detrimental and freezing is the best method of storage.

The instability of phosphofructokinase from ram and bull spermatozoa during sonication seems to be connected with the actual process of sonication, because loss of activity does not occur when the French press is used for homogenization. The enzyme is known to be particularly sensitive to oxidation (Kemp & Forest, 1968; Ahlfors & Mansour, 1969) and free radicals, which may attack its sensitive groups, are produced during sonication processes (Hughes & Cunningham, 1963).

Amount and distribution of enzyme activities found in sperm cells. The results of the experiments described above allow assessments of the probable amount and distribution of some enzymes in the sperm cells (Table 3). These values are the first published results on hexokinase and phosphofructokinase activities and distributions in spermatozoa of these species, expressed on the basis of cell numbers. Rates of fructolysis under optimum conditions are also given in Table 3 (from Mann, 1964, pp. 267 and 297).

Both hexokinase and phosphofructokinase are considered as possible rate-controlling enzymes in glycolysis (e.g. reviews by Passonneau & Lowry, 1964; Walker, 1966), and although the potential catalytic activities of these two enzymes are high compared with the maximum fructolytic rate in the spermatozoa, the intracellular amounts of the metabolites may so decrease turnover rates that these enzymes become rate-limiting *in vivo* (Hoskins & Stephens, 1969). Moreover it is possible that the fructolysis rates quoted are lower than the potential maximum saturated fructolysis activities of the enzymes in intact spermatozoa. For example, Rikmenspoel & Caputo (1966) have found the maximum saturated rate of fructolysis in bull spermatozoa to be $0.29\,\mu\text{mol/min}$ per 10^9 spermatozoa at 35°C (using a Michaelis–Menten plot of the fructolytic rates); fructolysis rates in boar spermatozoa have rarely been reliably measured and appear to decrease considerably after the spermatozoa have been ejaculated (C. Polge, personal communication). In bull and ram spermatozoa particularly, therefore, the relative amounts of hexokinase and phosphofructokinase activities do not preclude these steps from being rate-limiting to glycolysis *in vivo*.

In most tissues that have been studied, hexokinase has been found either in the cytoplasm or associated reversibly with mitochondria or other organelles (Hernandez & Crane, 1966; Rose & Warms, 1967; Spydevold & Borrebaek, 1968; Thompson & Bachelard, 1970). From the results of the present study, sperm hexokinases appear to be largely, if not entirely, membrane- or particle-bound. Thus during sonication of spermatozoa, hexokinase is extracted relatively slowly into the non-sedimentable fraction, as compared with the rapid extraction of a soluble enzyme, glucose phosphate isomerase; after homogenization when using the French press at low pressures, very little hexokinase is extracted, compared with the complete extraction of soluble enzymes. Morton & Lardy (1967) and Morton (1968) have also suggested that bull sperm hexokinase is membrane-bound, from circumstantial evidence and from the results

Table 3. *Some glycolytic enzyme activities and their distributions in bull, boar and ram spermatozoa*

Washed sperm suspensions in ice-cold 20 mM-phosphate buffer, pH 7.6, containing 1 mM-EDTA and 5 mM-2-mercaptoethanol, were sonicated for the optimum time-periods and fractionated as described in the text. Enzyme activities are expressed as units/10^9 spermatozoa, and distribution as % of non-sedimentable activity ($100 \times$ activity in fraction S_{100}/activity in fraction S_0); each value is the average of three observations. The fructolysis index is μmol of fructose utilized/min per 10^9 spermatozoa, at 37°C (Mann, 1964, pp. 267 and 297).

	Bull	Boar	Ram
Hexokinase	3.0 (25%)	1.3 (26%)	2.5 (13%)
Phosphofructokinase	0.85 (17%)	0.48 (76%)	1.0 (25%)
Glucose phosphate isomerase	21.2 (100%)	2.4 (100%)	9.6 (100%)
Fructolysis index	0.16	0.032	0.16

of fractionation of homogenates of bull epididymal spermatozoa, and Linford (1968) found a species of the enzyme of very high apparent molecular weight in boar sperm extracts, together with a species of lower molecular weight.

I am most grateful to Professor T. R. R. Mann and Dr E. F. Hartree for their valuable criticism, advice and encouragement. I also thank Dr H. M. Dott and Mr I. Wilmut for collecting semen samples, and Professor D. E. Hughes of the Department of Microbiology, University College of South Wales and Monmouthshire, for helpful advice and discussion.

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