Relation between the Oxidation State of Nicotinamide–Adenine Dinucleotide and the Metabolism of Spermatozoa

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1. The existing procedures for extraction of oxidized and reduced nicotinamide coenzymes were adapted to spermatozoa to overcome the coenzyme-degrading activity of seminal plasma. 2. The content of total NAD^+ and NADH was determined in the spermatozoa of ram, bull, boar, stallion and cock. NADP^+ and NADPH were not detected in ram spermatozoa. 3. The oxidation state of sperm NAD depended on the seminal plasma, the removal of which produced a change in the percentage oxidation state of the coenzyme, $100 \times [\text{NAD}^+ / (\text{NAD}^+ + \text{NADH})]$, without altering the total content of $\text{NAD}^+ + \text{NADH}$. 4. In suspensions of washed ram spermatozoa, incubated anaerobically at 25°C , the percentage oxidation state of NAD declined with increasing spermatozoa concentration. 5. When ram or boar spermatozoa that had been previously washed and resuspended in Ringer phosphate medium, were incubated anaerobically at 25°C with various substances, pronounced effects on the percentage oxidation state of NAD could be observed with L-lactate, pyruvate, oxaloacetate, dihydroxyacetone, formaldehyde and glyceraldehyde; sorbitol and acetoacetate acted only on ram spermatozoa; fructose, glucose, mannose and acetaldehyde acted predominantly on boar spermatozoa. Formaldehyde lowered the $(\text{NAD}^+ + \text{NADH})$ content of ram spermatozoa, but none of the other substances had a comparable effect. 6. The percentage oxidation state of sperm NAD was not influenced by exogenous cysteine, cystine, ergothioneine or ascorbate. 7. A highly active sorbitol dehydrogenase could be prepared from ram, but not from boar, spermatozoa. 8. Sorbitol, acetoacetate and 3-hydroxybutyrate effectively supported the respiration of ram, but not boar, spermatozoa. 9. 'Cold shock', resulting from sudden cooling of spermatozoa, abolished motility completely and irreversibly but produced only a slow and partial decrease in the total NAD content. Slight over-heating, sufficient to produce loss of motility, had no adverse effect on the total NAD content. 10. Storage of ram sperm at 14°C produced only a small decrease of NAD after 2 days, but subsequently the loss became greater.

Spermatozoa represent an excellent experimental material for studies on the relationship between extracellular metabolites and intracellular coenzymes in living cells of higher vertebrates, for several reasons. Spermatozoa do not either grow or multiply, yet they are intensely motile, very active metabolically, and capable of prolonged survival both within and outside the body. As mammalian spermatozoa possess no intracellular glycogen, they depend on extracellular carbohydrate, mainly fructose and its metabolites, for the supply of energy-yielding glycolysable substrates; fructolysis, moreover, proceeds at a rate that is quantitatively related to the degree of sperm motility (Mann, 1946, 1964). Thus, any changes in the state of intracellular coenzymes observed during

fructolysis are attributable directly to the metabolism of fructose and its breakdown products.

The present study was undertaken to obtain information about the regulatory influence which energy-yielding processes in semen exert on the ratio between total NAD^+ and total NADH within the motile spermatozoa. By determining the content of the two coenzyme forms we were able to deduce which extracellular substances are capable of undergoing oxidoreductive metabolism by spermatozoa. At the same time, we could define to what extent factors such as removal of seminal plasma, storage *in vitro*, cold shock, and dilution influence the relationship between extracellular and intracellular processes in semen. Predictably, our study also revealed certain marked species differences in sperm metabolism, in relation to extracellular substrates such as fructose, glucose, mannose, sorbitol, acetaldehyde, glyceraldehyde and acetoacetate (Brooks & Mann, 1971).

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Materials and Methods

Chemicals and standardization of stock solutions of coenzymes

NAD⁺, NADH, NADP⁺, NADPH, yeast alcohol dehydrogenase (EC 1.1.1.1), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), lactate dehydrogenase (EC 1.1.1.27), isocitrate dehydrogenase (EC 1.1.1.42) and glutamate dehydrogenase (EC 1.4.1.2), were obtained from Boehringer Corporation (London) Ltd., London W.5, U.K. Most of the substrates and other chemicals were obtained from Sigma (London) Chemical Co., London S.W.6, U.K., and BDH Chemicals Ltd., Poole, Dorset, U.K.; all acids were used either as sodium salts or neutralized to pH 7.4 with NaOH; acetoacetate, however, was used as the lithium salt, as obtained from Sigma. Testosterone and androstenedione were from Organon Laboratories Ltd., London W.C.2, U.K.; benzylpenicillin (1670 units/mg) and streptomycin sulphate (745 units/mg) were from Glaxo Laboratories Ltd., Greenford, Middx., U.K. Acetaldehyde was prepared by distillation of paraldehyde from 0.5 M-H₂SO₄. Stock solutions of the coenzymes (about 1 mM) were standardized by spectrophotometric procedures (Klingenberg, 1963), the final concentrations of substances in the cuvettes being as follows. NAD⁺ was standardized by adding alcohol dehydrogenase to 75 mM-sodium pyrophosphate – 75 mM-semicarbazide – HCl – 1% (v/v) ethanol, adjusted to pH 8.7 with 1 M-NaOH; NADH, by adding lactate dehydrogenase to 100 mM-triethanolamine – HCl, adjusted to pH 7.6 with 1 M-NaOH, in 3 mM-sodium pyruvate; NADP⁺, by adding isocitrate dehydrogenase to 100 mM-triethanolamine-HCl, adjusted to pH 7.6 with 1 M-NaOH, in 3 mM-MgCl₂–3 mM-DL-sodium isocitrate; NADPH, by adding glutamate dehydrogenase to 100 mM-triethanolamine – HCl, adjusted to pH 7.6 with 1 M-NaOH, in 15 mM-NH₄Cl–7 mM-2-oxoglutaric acid. The solutions were suitably diluted for use as recovery standards, and NAD⁺ and NADP⁺ were used to calibrate the assay procedure as recommended by Greenbaum *et al.* (1965).

Collection and handling of semen

Semen was collected by artificial vagina. Ram semen was used as pooled ejaculates from 20 Suffolk rams. Boar semen was collected as the so-called 'sperm-rich fraction' from Large White and Landrace boars. Stallion semen was obtained from a Welsh mountain pony. The gel portions were removed from boar and stallion semen ejaculates by straining them through two layers of muslin.

Epididymal spermatozoa were flushed out from freshly excised ram epididymides; from boar epididymides they were obtained by method II of Lasley & Bogart (1944). Testicular spermatozoa were col-

lected by Dr. B. P. Setchell at the Agricultural Research Council Institute of Animal Physiology, Babraham, from a cannulated conscious ram, under sterile conditions, by using the technique described by Voglmayr *et al.* (1967); the collection was carried out at 15°C for 24 h and the testicular semen was concentrated by centrifugation before extraction of the coenzymes. Cock semen was collected by Miss D. M. Cooper at the Houghton Poultry Research Unit, Huntingdon.

To remove seminal plasma from spermatozoa, semen samples were suitably diluted with Ringer phosphate medium, pH 7.4 (119.3 mM-NaCl, 4.8 mM-KCl, 1.2 mM-KH₂PO₄, 1.2 mM-MgSO₄, 2.4 mM-NaHCO₃, 41.7 mM-NaH₂PO₄, adjusted to pH 7.4 with 1 M-NaOH), centrifuged, and the spermatozoa were washed twice more by resuspending and centrifuging in Ringer phosphate medium. The concentration of bull, ram and boar spermatozoa was determined as described by Bishop *et al.* (1954) by using a Spekker absorptiometer (Hilger and Watts Ltd., London N.W.1, U.K.). Stallion and cock spermatozoa concentration was measured in a haemocytometer after diluting the semen with 0.9% NaCl containing 0.2% (v/v) formaldehyde.

'Cold shock' treatment of semen and washed-sperm suspensions was carried out as recommended by Mann & Lutwak-Mann (1955); when warmed up to 37°C, the cold-shocked spermatozoa remained immotile.

Incubation procedures

Incubations were carried out in a water bath at either 25° or 37°C. For anaerobic incubation, the samples were pipetted into Thunberg tubes, and the tubes were four times evacuated and filled with N₂ which had been passed through copper filings at 400–450°C to remove traces of oxygen.

To test the effect of extracellular substrates on the sperm coenzymes under anaerobic conditions, 4.5 ml samples of sperm suspension were placed in the main compartment, and 0.5 ml of 1% (w/v) substrate (in Ringer phosphate medium) was in the side arm. Testosterone and androstenedione were placed in the side arms as solutions in ethanol, the final concentration of ethanol after mixing with the sperm suspension being 2% (v/v). The incubation was initiated by mixing the contents of both compartments. Aerobic samples were shaken in an atmosphere of air.

Sampling and extraction procedures

Samples (1 ml) of sperm suspension were taken for extraction in acid and alkali, to obtain the oxidized and reduced forms of NAD respectively. Pipetting was unsatisfactory for sperm sampling, owing to the large and variable proportion of sperm suspension

retained inside the pipette; disposable polystyrene syringes (1 ml) were used instead. The time required to open a Thunberg tube, and to take up in the syringe and deliver the sperm sample into the extracting medium was less than 10 s. The tube containing the extracting medium was held on a Rotamixer [Jencons (Scientific) Ltd., Hemel Hempstead, Herts., U.K.] to ensure instantaneous and complete mixing during the addition of the sample.

For reasons described in the Results section, the routine extraction procedure for oxidized coenzyme had to be modified: hot HCl was replaced by trichloroacetic acid. Semen (1 ml) was added to 1 ml of ice-cold 20% (w/v) trichloroacetic acid with instantaneous mixing. After a few minutes 1 ml of water was added and the sample centrifuged in the cold. The supernatant was shaken six times with 3 ml of ether and the volume made up with water to 4 ml. For reduced coenzyme the alkaline extraction procedure was satisfactory when applied to semen. Semen (1 ml) was rapidly mixed with 1 ml of 0.2M-NaOH containing 2mM-nicotinamide at 100°C. The mixture was heated for 2 min at 100°C, cooled in ice, neutralized with 1 ml of 0.2M-HCl containing 2mM-nicotinamide, made up with water to 4 ml, and centrifuged.

Assay of coenzymes

For measuring the coenzymes, the procedure of Greenbaum *et al.* (1965) was used, with an oxygen electrode assembly from Rank Bros, Bottisham, Cambs., U.K., connected to a Servoscribe recorder (Smiths Industries Ltd., Wembley, Middx., U.K.). To eliminate a blank reaction, which occurred in the assay of NAD⁺ and NADH at the higher concentrations of phenazine methosulphate, the glycylglycine buffer was replaced by a tris-HCl buffer. The volume of the final assay mixture was 2.1 ml; it was made up by 1 ml of 0.25M-tris containing 1mM-EDTA and 6% (v/v) ethanol adjusted to pH 9.0 at 25°C with 1M-HCl, the sample, water, and 0.1 ml of phenazine methosulphate (4mg/ml).

Preparation and assay of sorbitol dehydrogenase

To obtain a soluble preparation of sorbitol dehydrogenase (EC 1.1.1.14), washed ram or boar spermatozoa were extracted with aluminium oxide powder as described by King & Mann (1959). The enzymic activity of the preparations was assayed at 25°C by following the oxidation of NADH or reduction of NAD⁺ at 340 nm. The assay system consisted of 1.8 ml of 0.1 M-triethanolamine adjusted to pH 7.6 with 1M-HCl, and 1 ml of water plus sperm extract. To reduce NAD⁺, 0.1 ml of 25mM-NAD⁺ was used, and the reaction was initiated by adding 0.1 ml of 4.5M-sorbitol; to oxidize NADH, 0.1 ml of 5mM-

NADH was used and the reaction was initiated by adding 0.1 ml of 4.5M-fructose.

Manometric experiments

Sperm respiration was measured at 37°C, by using Bärcroft's differential manometers (Mann, 1945). A portion (3 ml) of a sperm suspension, representing approx. 10⁹ spermatozoa, was placed in the main compartment of the manometer flask, and 0.2 ml of 1% substrate was tipped in from a dangling cup at the start of incubation. CO₂ was absorbed on filter paper soaked in 0.2 ml of 10% (w/v) KOH, placed in the centre well.

Results

Extraction of NAD⁺, NADP⁺, NADH and NADPH from spermatozoa

The usual method for extracting the oxidized and reduced forms of nicotinamide coenzymes involves homogenization of tissues in hot acid and alkali, respectively. However, since spermatozoa are evenly dispersed in semen, homogenization is unnecessary. However, it is necessary to take into account the fact that the fluid portion of semen, i.e. the seminal plasma, is extremely rich in powerful and stable nucleotide-degrading enzymes (Leone & Bonaduce, 1959; Abdel-Latif & Alivisatos, 1962; Mann, 1964; Yuan & Anderson, 1971); and similar enzymes may also be present in the so-called 'cytoplasmic droplets' (Dott & Dingle, 1968).

To adapt the extraction procedures for the oxidized and reduced form of nicotinamide coenzymes to spermatozoa, two tests were performed. One was based on quantitative recovery of the coenzymes added to various extraction media immediately before, or after, the addition of semen, and the other depended on checking the stability of coenzymes extractable from the spermatozoa over a period of 24 h. The alkaline extraction procedure used as a routine for reduced coenzymes proved satisfactory. The recovery of added NADH and NADPH was about 98% and the concentrations stayed constant for 24 h at 0°C. Added NAD⁺ and NADP⁺ were destroyed by the alkaline extraction. In contrast, the acid extraction procedure by means of HCl at 100°C for the determination of NAD⁺ and NADP⁺, was inadequate, as the coenzyme-destroying activity of seminal enzymes was not abolished. The use of an equal volume of cold 1.2M-HClO₄ or 0.2M-HClO₄ at 100°C, to which 2mM-nicotinamide had been added, proved equally ineffective. Finally, extraction with trichloroacetic acid was tried and found to be satisfactory. In five experiments the average recovery of added coenzymes was 98.7% (S.E.M. 2.8) for NAD⁺, and 92.7% (S.E.M. 1.6) for NADP⁺. No loss of either

Table 1. *Content and percentage oxidation state of total NAD in spermatozoa of ram, bull, boar, stallion and cock*

The number of samples examined is given in parentheses; where more than one sample was analysed, the mean $\pm$ S.E.M. is given. Each sample of ejaculated ram spermatozoa represented pooled semen from about 20 rams. The sample of cock spermatozoa represented pooled semen from 7 birds. Ejaculated boar spermatozoa were collected in the form of the sperm-rich fraction of semen. Testicular and epididymal spermatozoa were obtained as described in the Materials and Methods section. Extraction of coenzymes was carried out on semen samples as collected, except for testicular semen, which was concentrated by centrifugation before extraction. Sperm concentration is given as 10^9 spermatozoa/ml, and the contents of NAD^+ and NADH as nmol/ 10^9 spermatozoa.

Animal	Source of spermatozoa	Concentration of spermatozoa	Content				$\frac{\text{NAD}^+}{\text{NAD}^+ + \text{NADH}} \times 100$
			NAD^+	NADH	$\text{NAD}^+ + \text{NADH}$		
Ram	Ejaculated (7)	3.34 ± 0.31	19.1 ± 0.7	5.5 ± 0.3	24.6 ± 0.8		77.6 ± 1.8
	Epididymal (1)	8.75	14.8	17.1	31.9		46.4
	Testicular (1)	1.37	3.5	0	3.5		100
Bull	Ejaculated (9)	1.08 ± 0.12	10.6 ± 0.8	2.1 ± 0.3	12.7 ± 1.0		83.7 ± 1.6
Boar	Ejaculated (6)	1.07 ± 0.17	7.5 ± 1.0	1.4 ± 0.4	9.0 ± 0.9		83.5 ± 5.3
	Epididymal (1)	5.75	4.6	5.9	10.5		43.8
Stallion	Ejaculated (3)	0.10 ± 0.03	14.9 ± 1.1	1.9 ± 0.6	16.8 ± 1.0		88.8 ± 3.5
Cock	Ejaculated (1)	4.33	7.4	3.5	10.9		67.9

NAD^+ or NADP^+ occurred over a period of 24 h at 0°C .

Coenzyme concentrations in spermatozoa

We confirmed the reports of Bistocchi *et al.* (1968) and Brooks (1970), that NAD occurs in bull spermatozoa, and we have established the concentrations of both NAD^+ and NADH in ejaculated spermatozoa of the ram, boar, stallion and cock. The results are listed in Table 1. However, in spite of several attempts we failed to demonstrate NADP in ram spermatozoa. Efforts to determine NADP were hampered by the fact that the activity of glucose 6-phosphate dehydrogenase, a component of the assay system, was so much dependent on the ionic strength of the medium (Fig. 1) that any attempt to increase the volume of the sperm extract diminished the sensitivity of the whole assay procedure. This difficulty could be partly overcome by using highly concentrated suspensions of spermatozoa which were washed before extraction of coenzymes, in a medium of low ionic strength, composed of 0.276M-sucrose and 0.016M-citric acid, adjusted with 1M-NaOH to pH 6.8. However, even with these modifications it was not possible to demonstrate either NADP^+ or NADPH in ram spermatozoa, despite the excellent recovery of added standards. Our calculations lead us to believe that if NADP had been present in ram spermatozoa, we should have been able to detect it by our method, even at a concentration as low as 0.1 nmol/ 10^9 spermatozoa.

Different animal species have different total contents of NAD in ejaculated spermatozoa. The sum of ($\text{NAD}^+ + \text{NADH}$) was highest in the ram ($24.6 \text{ nmol}/10^9$ spermatozoa) and lowest in the boar ($9.0 \text{ nmol}/10^9$ spermatozoa). However, the oxidation state of sperm NAD, expressed as $100 \times [\text{NAD}^+ / (\text{NAD}^+ + \text{NADH})]$, was similar in all species (Table 1). Table 1 also shows that the epididymal spermatozoa of ram and boar contained slightly more NAD than did ejaculated spermatozoa, and that more of the coenzyme was in the reduced form. However, in the single sample of ram testicular sperm that we analysed, there was less NAD^+ than in ejaculated ram sperm, and no NADH was found; whether the low content of NAD is a general characteristic of testicular semen, or a peculiarity of the sample examined, is not known.

Effect of removal of seminal plasma and washing of spermatozoa on the content and oxidation state of NAD

To remove the seminal plasma, ejaculated semen was diluted with Ringer phosphate medium, centrifuged, and the spermatozoa were washed at least

Fig. 1. Effect of increasing ionic strength by means of added NaCl on the response of the assay system for NADP to 1 nmol of NADP⁺

The assay was done at 25°C, by using the method of Greenbaum *et al.* (1965), and consisted of 1 ml of 0.1 M-tris-HCl-1 mM-EDTA-2 mM-glucose 6-phosphate, pH 8.0, 1 ml of sample, and 0.1 ml of phenazine methosulphate (1 mg/ml). Oxygen uptake was initiated by the addition of 0.05 ml of glucose 6-phosphate dehydrogenase (0.1 mg/ml) in 0.1 M-tris-HCl-1 mM-EDTA, pH 8.0.

twice. The washed spermatozoa were made up with Ringer phosphate into a suspension of the same sperm concentration as the starting sample. Washing did not alter significantly the total sum of (NAD⁺ + NADH) in either ram or boar spermatozoa, but it affected markedly the oxidation state of the coenzyme. Before being washed, ram spermatozoa showed a mean value of 77.6 (S.E.M. 1.8, 7 samples) for the percentage oxidation state of NAD, but after washing this value declined to 61.2 (S.E.M. 1.3, 9 samples). An opposite effect was observed in boar spermatozoa, the mean value being 83.5 (S.E.M. 5.3, 6 samples) before, and 96.6 (S.E.M. 0.9, 6 samples) after washing. Attempts to restore the oxidation state of NAD in washed spermatozoa by adding fructose (the sugar normally present in ram and boar seminal plasma) brought back the oxidation state of NAD in boar spermatozoa to approximately the value for fresh

Fig. 2. Relation between the concentration of spermatozoa and the percentage oxidation state of total NAD

Suspensions of washed ram spermatozoa, used in different experiments, were incubated anaerobically at 25°C; the line represents a linear regression with a correlation coefficient of 0.93, fitted by the method of least squares.

semen. However, fructose had no such effect in washed ram spermatozoa.

Effect of sperm concentration on the oxidation state of NAD

We found that the percentage oxidation state of NAD in washed suspensions of ram spermatozoa was closely dependent on sperm concentration, under anaerobic conditions at 25°C. This clearly follows from Fig. 2, in which the values obtained from a number of different experiments are plotted and a linear regression fitted to them by means of the method of least squares. A similar situation was encountered in experiments carried out with the same sperm suspension, progressively diluted and incubated anaerobically for 30 min at 25°C, before determinations of NAD⁺ and NADH. Tenfold dilution of a thick suspension containing 3×10^9 ram spermatozoa/ml caused a rise in the percentage oxidation state of sperm NAD from about 64 to nearly 100; the oxidation state remained at the high value on further dilution. Similar observations with washed boar-sperm suspensions were too few to permit a conclusion that the dependence of the oxidation state of NAD on sperm concentration is as close in the boar as it is in the ram, but from the limited number of observations it appears that in boar spermatozoa the percentage oxidation state of NAD does not fall significantly below the value of approx. 100%, until the sperm concentration has become more than 1.7×10^9 /ml.

Influence of exogenous substrates on the percentage oxidation state of NAD in washed spermatozoa under anaerobic conditions

The experiments concerned with the effects of fructose and various other added substances on NAD in washed spermatozoa were done mainly under anaerobic conditions, to exclude the involvement of oxygen and of the cytochrome system in oxidoreductive processes. The anaerobic incubations were carried out at 25°C, and in the experiments recorded in Table 2 samples were taken for analysis of NAD⁺ and NADH at 0, 5 and 20 min. A two-way analysis of variance and *t* test for a significant difference between samples with and without added substance were applied to determine whether the effect of any given substance was significant. The variance due to interaction between substances and times was used as the error term for experiments in which the treatments were not done in duplicate; when

duplicates were used, the variance due to replicates served as the error term. The extent to which some of the exogenous substances affected the oxidation state of NAD, is indicated in Fig. 3 (*a*, *b* and *c*). As shown in Table 2 and Fig. 3, ram and boar spermatozoa differed markedly in their response to several of the tested substrates.

Glycolysable sugars produced no significant change in the percentage oxidation state of NAD in ram spermatozoa, but reduced some of the NAD⁺ to NADH in boar spermatozoa; this species difference shows clearly when Figs. 3 (*a*) and 3(*c*) are compared with Fig. 3(*b*).

Sorbitol had a strongly reducing influence on NAD⁺ in ram spermatozoa (Fig. 3*a*), but its slight effect on boar spermatozoa was not significant (Fig. 3*b*) and may have been due to a trace amount of reducing sugar in the sorbitol. The reducing effect of sorbitol on NAD⁺ in ram spermatozoa was both

Table 2. *Exogenous substrates examined for their ability to exert a significant effect on the percentage oxidation state of intracellular NAD in spermatozoa*

Sperm suspensions, prepared from ram and boar semen by washing them with Ringer phosphate medium (pH 7.4), were incubated anaerobically at 25°C, in the presence of various substances added at a final concentration of 0.1% (unless stated otherwise). Samples were taken for analyses of total NAD⁺ and NADH at 0, 5 and 20 min. An increase in the ratio $100 \times [\text{NAD}^+]/([\text{NAD}^+ + \text{NADH}])$ is indicated by +, a decrease by –, no change by 0; a blank space indicates that the substance listed was not tested. After 20 min of incubation spermatozoa were motile in all samples except those containing formaldehyde, acetaldehyde and DL-glyceraldehyde. The sum of (NAD⁺ + NADH) was not significantly affected by 20 min of incubation except in the presence of formaldehyde which decreased it to 76% of the control value.

Compound tested	Ram sperm	Boar sperm
Fructose	0	—
Glucose	0	—
Mannose	0	—
Sorbitol	—	0
Inositol	0	
Galactose		0
L-Lactate	—	—
D-Lactate	0	
Pyruvate	+	+
Acetoacetate	+	0
DL-3-Hydroxybutyrate	0	0

Compound tested	Ram sperm	Boar sperm
Oxaloacetate	+	+
2-Oxoglutarate	0	0
DL-Malate	0	
Succinate	0	
Fumarate	0	
Citrate	0	
DL-Isocitrate	0	
Formate		0
Acetate	0	
Propionate	0	
Butyrate	0	
L-Alanine	0	0
L-Glutamate	0	
L-Cysteine		0
L-Cystine		0
Dihydroxyacetone	+	+
Glycerol	0	
Propane-1,2-diol	0	
DL-Glycerol 3-phosphate	0	
DL-Glyceraldehyde 3-phosphate	0	
Formaldehyde	+	+
Acetaldehyde	0	—
DL-Glyceraldehyde	+	—
Ethanol	0	
Testosterone (0.002%)	0	
Androstenedione (0.002%)	0	
Ascorbate		0
Ergothioneine		0
Choline		0
Nicotinamide	0	0

* Effect only evident after 5 but not 20 min.

Fig. 3. Changes in the percentage oxidation state of total NAD in spermatozoa

Washed sperm suspensions were incubated as follows: C, control (without substrate); and with D, dihydroxyacetone; E, ergothioneine; F, fructose; G, glucose; L, lactate; O, oxaloacetate; P, pyruvate; or S, sorbitol. The incubations were carried out at 25°C, with (a), ram sperm anaerobically, (b), boar sperm anaerobically, (c), ram sperm anaerobically, and (d), boar sperm aerobically.

marked and rapid: after 1 min of anaerobic incubation it was as great as after 5 or 20 min. The observation that sorbitol reduces NAD⁺ effectively in ram, but hardly at all in boar, spermatozoa, suggested the possibility that boar spermatozoa, in contrast with ram spermatozoa, lack sorbitol dehydrogenase. By applying the method of King & Mann (1959), it was possible to obtain a highly active preparation of sorbitol dehydrogenase from ram spermatozoa, but not from boar spermatozoa (Fig. 4). Further, manometric measurements of oxygen consumption in washed

sperm suspensions showed that sorbitol maintained the respiration of ram, but not of boar, spermatozoa, in agreement with reports by King & Mann (1959) and Aalbers *et al.* (1961).

Acetoacetate also did not alter the oxidation state of NAD in boar spermatozoa but produced a marked effect in ram spermatozoa. The effect of acetoacetate on ram sperm was, however, the reverse of that recorded for sorbitol; acetoacetate increased the oxidation state of NAD, indicating thereby that ram spermatozoa, in contrast to those of the boar, possess

Fig. 4. *Sorbitol dehydrogenase activity in extracts from ram and boar spermatozoa*

Extraction and determination were done by the method of King & Mann (1959). (a) Reduction of NAD (due to oxidation of sorbitol) in the presence of A, 1 ml of ram sperm extract; B, 0.2 ml of ram sperm extract; and C, 1 ml of boar sperm extract. (b) Oxidation of NADH (due to reduction of fructose) in the presence of 1 ml of boar sperm extract, supplemented later, at the time indicated by the arrow, with 0.05 ml of ram sperm extract.

an active 3-hydroxybutyrate dehydrogenase. DL-3-Hydroxybutyrate produced only a slight reduction of NAD^+ in ram spermatozoa, but none at all in boar spermatozoa. Subsequent manometric experiments showed that acetoacetate as well as 3-hydroxybutyrate effectively maintained the rate of oxygen consumption of ram, but not boar, spermatozoa (Fig. 5).

L-Lactate, pyruvate and dihydroxyacetone strongly influenced the oxidation state of NAD in the spermatozoa of both species, but whereas L-lactate reduced NAD^+ to NADH anaerobically, pyruvate and dihydroxyacetone had the opposite effect, i.e. they increased the percentage oxidation state of NAD. D-Lactate had no effect on the state of NAD in ram spermatozoa.

L-Lactate, pyruvate and dihydroxyacetone are well known to be capable of increasing, or at any rate maintaining, the rate of respiration of washed ram spermatozoa (Mann & Lutwak-Mann, 1948; Mann & White, 1957). We found that these three substances also enhanced the respiration of boar spermatozoa, dihydroxyacetone being the least effective. The stimulation of respiration of washed boar spermatozoa by dihydroxyacetone was comparable with that of succinate. We found that the μl of O_2 consumed by 10^8 spermatozoa in 1 h during the first hour of aerobic incubation was 2.5 in the absence of substrate, 3.6 with succinate and 4.0 with dihydroxyacetone; during the third hour the rates declined to 1.3, 2.5 and 2.7 respectively.

Oxaloacetate increased the percentage oxidation state of NAD, but the effect, unlike with pyruvate or dihydroxyacetone, was transient. It was evident in ram and boar sperm suspensions incubated anaerobically at 25°C for 5 min, but disappeared on longer incubation.

Most substances that affected the oxidation state of NAD either stimulated or had no visible effect on sperm motility. However, DL-glyceraldehyde, acetaldehyde and formaldehyde, although influencing significantly the oxidation state of NAD, inhibited the motility of spermatozoa. Formaldehyde was unique in that as well as altering the oxidation state of NAD, it also decreased the total content of $\text{NAD}^+ + \text{NADH}$ in spermatozoa. Presumably, this effect was due to increased cell permeability.

Malate and alanine, like several other substances listed in Table 2, produced no significant change in the oxidation state of sperm NAD anaerobically, and were without effect on the rate of respiration in either ram or boar spermatozoa aerobically.

Influence of oxygen and temperature on the oxidation state of NAD in spermatozoa

A small series of experiments was done to compare the behaviour of sperm NAD under anaerobic and aerobic conditions. In both the ram and boar, the relative content of the oxidized form of coenzyme in washed sperm suspensions incubated at 25°C was

Fig. 5. Oxygen consumption by washed suspensions of motile spermatozoa

The oxygen uptake was measured in Barcroft manometers at 37°C with 3 ml of washed sperm suspension + 0.2 ml of 1% substrate in Ringer phosphate medium. (a) 1.23×10^9 ram spermatozoa per flask; (b) 1.1×10^9 boar spermatozoa per flask. The substrates added were as follows: A, acetate; AA, acetoacetate; Ala, alanine; C, control (Ringer phosphate); H, 3-hydroxybutyrate; L, lactate; M, malate; P, pyruvate; S, sorbitol.

distinctly higher aerobically than anaerobically. In boar spermatozoa kept aerobically without added substrate, NAD occurred almost exclusively in the oxidized form, even at a sperm concentration as high as 3×10^9 /ml. Anaerobically, as pointed out above, a substantial proportion of NAD in such a thick sperm suspension would have been present in the reduced form. In a washed suspension of ram spermatozoa containing 2×10^9 spermatozoa/ml and incubated aerobically, the percentage oxidation state of NAD was 87, compared with 59 in the absence of oxygen (see regression line in Fig. 2).

The aerobic response of sperm NAD to exogenous substances was qualitatively similar to that recorded under anaerobic conditions, except that the changes were much less pronounced. This can be seen, for example, by comparing the anaerobic and aerobic effects of fructose in Figs. 3(b) and 3(d).

As a routine the incubation experiments were done at 25°C, but in a few experiments lower and higher temperatures were also examined. In washed boar spermatozoa incubated anaerobically without added substrate, the mean percentage oxidation state of NAD, calculated by averaging the values determined after 30 and 60 min, was 92.8 (S.E.M. 1.4) at 21.5°C, and 79.8 (S.E.M. 3.4) at 37°C. In the presence of added substrates, the difference was less. Thus, for example, after the addition of fructose to an anaerobically incubated suspension of boar spermatozoa, the average percentage oxidation state of NAD (mean of four determinations covering an incubation period of 1 h) was 68.5 (S.E.M. 0.9) at 25°C, and 72.6 (S.E.M. 0.7) at 37°C. However, when ram spermatozoa

were incubated anaerobically with fructose, the oxidation state of NAD after 5 min was similar at 21.5° and 37°C, but after 15 min the value at 37°C became distinctly lower.

During routine collections of semen for artificial insemination, care must always be taken to maintain correct temperature in the artificial vagina and the receiving vessel, since a temperature even slightly above that of the animal's body can immobilize the spermatozoa. But we have found that ram spermatozoa immobilized by such slightly elevated temperatures still retain their characteristic content of NAD.

'Cold shock' of ram semen, that is, a sudden lowering of temperature from that of the body to 5°C or less, increases the permeability of spermatozoa, and produces at the same time a strong detrimental effect on sperm motility and glycolytic ability (Mann & Lutwak-Mann, 1955). One might expect that such a complete and irreversible loss of motility might be also reflected in a much lowered NAD content. We found, however, that in a cold-shocked suspension of washed ram spermatozoa incubated at 25°C, the sum of NAD⁺ + NADH was after 30 min 62%, and after 60 min 53%, of the control value. In another experiment, the cold-shocked sample of ram spermatozoa had after 30 min 78%, and 5 h later still 18%, of the NAD value in the non-shocked control sample. The total NAD content of boar spermatozoa responded to cold shock in a similar way. However, the oxidation state of NAD was profoundly affected by the cold-shock treatment in both boar and ram spermatozoa: cold shock increased markedly the percentage oxidation state of the coenzyme, and this was shown to be

due mainly to a loss of NADH, and not to an increase in the NAD^+ content.

Effect of prolonged storage of spermatozoa at 14°C on motility and the content and oxidation state of NAD

For purposes of artificial insemination, semen is often stored for a day or more, at 5–15°C. We examined the effect of 3 days' storage at 14°C on the concentration and the oxidation state of NAD in ram spermatozoa. A sample of freshly collected ram semen was divided into two portions: (A) was left untreated; in (B) the spermatozoa were separated by washing them twice and adjusting the suspension to the original volume of semen. Samples of portions A and B were diluted with 2 vol. of Ringer phosphate medium (pH 7.4) containing 3 mg of fructose/ml and 750 units each of penicillin and streptomycin/ml, and incubated anaerobically at 14°C. The initial concentration of fructose was higher in A than in B, since some fructose present in A was derived from the seminal plasma. After 24 h of incubation, fructose was almost completely exhausted in both A and B; the pH was 5.5 in A and 6.5 in B; motility was practically nil in A and low in B. During the further 48 h period, the pH remained constant in both A and B; in B motility declined further. Coenzyme determinations showed that the sum of NAD^+ + NADH remained practically unchanged in both A and B during the first 2 days of storage; it was 90% of the initial value at the end of that period. But after 3 days, the total coenzyme content in both samples had decreased to about 50% of the initial value. The percentage oxidation state of NAD remained remarkably constant in both samples; the mean of four determinations over the incubation period was 88.7% (S.E.M. 2.4) in A, and 58.3% (S.E.M. 3.7) in B.

Discussion

Much information is available about the redox state of nicotinamide nucleotide coenzymes in the cytosolic and mitochondrial compartment of animal cells, and the role of both NAD and NADP in the integration of metabolic pathways within animal cells (Chance, 1954; Chance & Jöbsis, 1959; Krebs & Veech, 1969; Bücher, 1970; Greenbaum *et al.*, 1971; Gumaa *et al.*, 1971; Stubbs *et al.*, 1972). But less is known about the events associated with the action of extracellular nutrients on the oxidation state of coenzymes in intact animal cells. We are equally ignorant about the correlation between changes induced in coenzyme concentrations by the exogenous factors and physiological parameters such as cell viability, motility, growth, and in the case of gametes, their fertilizing ability. We have therefore chosen for our study the spermatozoon, a functionally well defined and metabolically intensely active cell.

First, we have confirmed and extended the observation that although freshly ejaculated and highly motile ram spermatozoa contain NAD, they have little, if any, NADP (Brooks, 1970). This peculiar situation may be attributable to the special character of mammalian semen, in which the spermatozoa have practically no glycogen and hardly any synthetic ability, but depend largely on catabolic processes, such as the breakdown of fructose, for the supply of metabolic energy (Mann, 1964). As to NAD in the sperm cell, its total content in ram, bull, boar, stallion and cock semen was remarkably constant within each investigated species, but showed characteristic interspecies variations. In the bull, for example, the total content of NAD was 12.7 amol/spermatozoon; and as the osmotically active cell volume of a bull spermatozoon is estimated at 13.8 al (Drevius, 1971), it follows that the actual concentration of total NAD in bovine spermatozoa must be nearly 1 mM. This value may be compared with about 1 mM in liver (Greenbaum *et al.*, 1965) and 1–3 mM in yeast (Polakis & Bartley, 1966).

We regard as particularly significant the finding that the oxidation state of NAD within motile spermatozoa, expressed as $100 \times [\text{NAD}^+ / (\text{NAD}^+ + \text{NADH})]$, can be rapidly and markedly modified by the addition of various substances to the extracellular sperm medium, without interfering with the total coenzyme content, i.e. the sum of ($\text{NAD}^+ + \text{NADH}$). In several instances, determinations of both the NAD^+ and NADH contents, done before and after the addition of a substance, made it possible to predict whether or not the spermatozoa possess a NAD-linked dehydrogenase capable of metabolizing that particular substance. Sorbitol provides a good example in this respect. We observed that the addition of sorbitol produces within 1 min (at 25°C) a reduction of NAD^+ to NADH in ram, but not boar spermatozoa, and from this we deduced first and demonstrated later that unlike the spermatozoa of ram, those of the boar are deficient in sorbitol dehydrogenase. We made similar deductions about the presence or absence of 3-hydroxybutyrate dehydrogenase, in the case of acetoacetate, which alters the oxidation state of NAD in ram, but not boar, spermatozoa.

Concerning enzyme-mediated interactions between extracellular substrates and intracellular NAD, one must remember that a metabolite, rather than the added substrate itself, may contribute to the effect on the oxidation state of the coenzyme. Thus, for instance, the increase of the percentage oxidation state of sperm NAD by dihydroxyacetone can be interpreted as the result of either a direct reduction of that substance to glycerol, or else the formation of triose phosphate, before the reduction. Dihydroxyacetone was shown to support the respiration of ram spermatozoa (Mann & White, 1957), and this has now been confirmed for boar spermatozoa. Also, free

glycerol, as well as glycerol 3-phosphate, exerts an enhancing effect on the rate of oxygen uptake by spermatozoa (Mann & White, 1957; Mohri & Masaki, 1967). We have demonstrated in the present study that dihydroxyacetone increases anaerobically the oxidation state of NAD within the spermatozoa, but we could not demonstrate any decrease in the oxidation state of NAD when incubating spermatozoa anaerobically with either glycerol, glycerol 3-phosphate or with propane-1,2-diol (another substrate for glycerol dehydrogenase). Without assuming the presence of a glycerol dehydrogenase in spermatozoa, the oxidizing effect of dihydroxyacetone on NADH is difficult to explain; the mechanism by which dihydroxyacetone and glycerol influence sperm respiration will have to be explored further. Fructose is another substrate that can influence NAD indirectly, that is, via its metabolites. On addition of fructose to a washed-ram-sperm suspension, practically no change is immediately detectable in the oxidation state of NAD. In boar spermatozoa, however, the response of NAD to fructose is different. The addition of a glycolysable sugar to washed boar spermatozoa always produces a rapid decrease in the oxidation state of NAD. In this species the reduction of pyruvate to lactate, with concomitant NADH oxidation to NAD^+ , may proceed at a distinctly lower rate than the reaction mediated by glyceraldehyde 3-phosphate dehydrogenase, in which NAD^+ is reduced to NADH. This would inevitably cause accumulation of NADH. Equally, the species difference in the behaviour of sperm NAD relative to glycolysable substrates could be explained, for fructose at any rate, by the fact that ram, but not boar, spermatozoa possess an active sorbitol dehydrogenase, so that only the ram spermatozoa are capable of reducing fructose directly, utilizing at the same time some of the NADH which otherwise would have accumulated.

Lack of change in the oxidation state of NAD on addition of such strongly reducing or oxidizing agents as ascorbic acid, ergothioneine, cysteine and cystine (Table 2) confirms our opinion that intracellular co-enzymes are highly resistant to the action of extracellular agents that are unable either to enter the sperm cell or to be metabolized by it. Ethanol, so far as it is known, is not metabolized by spermatozoa; *in vitro*, the motility and fertilizing ability of spermatozoa do not seem to be affected by ethanol at all, even at concentrations as high as 20% (Mann, 1968). The two steroids that we examined were testosterone and androstenedione, both well-known constituents of mammalian testis. There have been some claims in the past that testosterone can influence sperm metabolism *in vitro*, but the present evidence is by no means conclusive (see Murdoch *et al.*, 1970; Voglmayr *et al.*, 1970), and in our study we failed to produce any significant change in the oxidation state of NAD in ram spermatozoa with either testosterone or andro-

stenedione. Inositol and galactose were given special attention, since both occur normally in seminal plasma but are not metabolized by spermatozoa either anaerobically or aerobically (Mann, 1954, 1964; Baronos, 1971).

Volatile fatty acids had no effect on the oxidation state of sperm NAD under anaerobic conditions, nor is there any indication at present that they can be metabolized anaerobically by spermatozoa. In the presence of oxygen, however, some volatile fatty acids are known to be capable of supporting sperm respiration, and we confirmed this with boar spermatozoa in respect of acetate. Lack of change in NAD on addition of a tricarboxylic acid or dicarboxylic acid is presumably due to the inability of these acids to penetrate the cell membrane or mitochondrial sheath of spermatozoa; this would be in line with the behaviour of such compounds towards mitochondria from other tissues (Klingenberg, 1970). It is known (Koefoed-Johnsen & Mann, 1954) that whereas motile ram spermatozoa do not respond aerobically to added succinate with an increased O_2 uptake, as soon as they have been rendered immotile by detergent treatment or cold shock, they begin to show a marked response to succinate because of greater cell permeability and therefore accessibility of succinate dehydrogenase.

Cold shock, though it induces a rapid and complete loss of motility, does not deplete spermatozoa equally rapidly or completely of their NAD. In general, from this and several other instances cited in the present study, it can be concluded that the total content of NAD, that is, the sum of ($\text{NAD}^+ + \text{NADH}$), remains remarkably constant under a variety of conditions, irrespective of the percentage oxidation state of the coenzyme.

One other phenomenon that merits comment is the peculiar inverse relationship encountered in washed-sperm suspensions incubated anaerobically without substrate, between the percentage oxidation state of NAD and the concentration of spermatozoa. The reason for this is not clear, but it may possibly arise from the so-called contact inhibition or allelostatic effect, that is, diminished motility in dense sperm suspensions owing to mechanical overcrowding, the cells having a mutually restraining effect on their movements and thereby on their own metabolic activity. It remains to be shown whether the difference in behaviour between dense and dilute sperm suspensions is also reflected in differing concentrations of intracellular nucleotides other than NAD, such as ATP, ADP or adenosine 3':5'-cyclic monophosphate (cyclic AMP), all of which represent normal sperm constituents. As regards cyclic AMP, there is already some evidence that it performs the role of a regulator of cell motility by mediating the contact inhibition of cell movements (Johnson *et al.*, 1972). It could be, therefore, that the difference in behaviour between dense and dilute sperm suspensions is due to a higher

concentration of the diffusable nucleotide in the external milieu of the more highly concentrated sperm suspensions.

Finally, it should be stressed that all our observations about the content and oxidation state of NAD in spermatozoa, are based on determinations of total, not free, NAD⁺ and NADH. We have not measured the ratio between free NAD⁺ and free NADH, and therefore cannot state what the enzyme equilibria within the spermatozoa are. Nor do the results give us definite information about the rate of coenzyme turnover inside the sperm cells.

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