THE EFFECT OF SOME SEMINAL CONSTITUENTS AND RELATED SUBSTANCES ON DILUTED MAMMALIAN SPERMATOZOA

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Summary

The effect has been studied of a number of seminal constituents and related substances on the motility of diluted ram, bull, rabbit, and human spermatozoa. All tests were made with a fructose phosphate diluent over a 4-hr period at room temperature.

Trace concentrations ($< 0{\cdot}20$ mg/100 ml) of copper, cobalt, manganese, iron, and zinc had little effect on ram, bull, or rabbit spermatozoa, except that copper depressed the motility of ram spermatozoa.

Biotin improved the viability of bull spermatozoa but had no effect on ram or rabbit spermatozoa. Thiamine, riboflavin, niacin, inositol, *p*-aminobenzoic acid, pantothenic acid, and folic acid were inactive in all species.

None of 21 amino acids had any significant beneficial action on ram, bull, or rabbit spermatozoa, and some were toxic.

Of several miscellaneous seminal constituents, cytochrome *c* and spermine phosphate improved the motility of rabbit spermatozoa but had no effect on ram or bull spermatozoa. Citric acid, choline chloride, CoI, and adenosine triphosphate were inactive and ascorbic acid toxic to the spermatozoa of all three species.

Human spermatozoa were found to be particularly susceptible to dilution and no protective action was shown by substances that were beneficial to other mammalian spermatozoa.

I. Introduction

The harmful effect of dilution on the motility of spermatozoa was first reported by Milovanov (1934*a*, 1934*b*) who found that spermatozoa could be immobilized if sufficient of 1 per cent. sodium chloride was added to semen. This was the basis for his test of the resistance of ejaculates; the index of resistance, *R*, being the volume of diluent required, divided by the volume of semen. Milovanov's observation has been confirmed for rabbit, human, bull, and ram spermatozoa and it would appear that the dilution phenomenon is of general occurrence with mammalian spermatozoa (Chang 1942; Kennedy 1947; Rao and Hart 1948; Blackshaw 1953).

The effect is not, as Milovanov supposed, due to the toxicity of sodium chloride, since it occurs with chloride-free sulphate, tartrate, and citrate diluents (Salisbury *et al.* 1943; Emmens and Swyer 1948; Cheng, Casida, and Barrett 1949; Blackshaw 1953). Tonicity changes and the dilution of seminal substrate are not important factors either, since motility is still depressed in isotonic diluents containing glucose or fructose (Emmens and Swyer 1948; Blackshaw 1953; White 1953*a*).

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Emmens and Swyer (1948) found that accessory secretion protected rabbit spermatozoa to some extent from dilution, but it was not as effective as supernatants from spermatozoa left overnight. The depression of motility at low cell concentrations would seem, therefore, to be due to both dilution of plasma material and loss of substances from the spermatozoa. Miscellaneous high-molecular-weight substances (e.g. proteins and starch) that have a protective action on diluted spermatozoa presumably act by preventing the latter (Emmens and Swyer 1948; Blackshaw 1953; White 1953*a*).

White (1953*a*, 1953*b*, 1953*c*) found that potassium increases the motility and glycolysis of repeatedly washed or moderately diluted (20×10^6/ml) ram and bull spermatozoa. This suggests that potassium is lost from the cells under these conditions. At very low spermatozoa concentrations ($< 2 \times 10^6$/ml), however, substances other than potassium must be limiting since motility in potassium-containing diluents is little or no better than in potassium-free diluent (White 1953*a*).

The effect has now been studied of a number of seminal constituents and related substances on the motility of diluted ram, bull, rabbit, and human spermatozoa and the results are presented in this paper.

II. Materials and Methods

(*a*) *Semen*

Ram semen was obtained by electrical ejaculation (Gunn 1936); bull and rabbit semen was collected in an artificial vagina, and human semen was obtained from a sterility clinic. Only normal ejaculates of good initial motility were employed, and were used singly. Experiments with ram and rabbit semen were started immediately after collection and those with bull semen within 2 hr of collection; the bull semen was slowly cooled to about 10°C and kept at this temperature during transport to the laboratory. The human semen was between 4 and 6 hr old before use.

(*b*) *Technique*

Spermatozoal counts were made in duplicate on the neat semen using a haemocytometer; the semen was then diluted with an isotonic diluent of pH 7·0 having the following composition:

0·032M $NaH_2PO_4.H_2O$, 0·048M $Na_2HPO_4.12H_2O$, 0·036M NaCl, 0·004M KCl, 0·004M $MgCl_2$, 0·022M fructose.

With the exception of 500 mg/100 ml glutamic acid, which brought the pH of the diluent down to 6·0, the other substances added had no appreciable effect. Tests were made on ram and bull spermatozoa at a cell concentration of 2×10^6/ml and on rabbit and human spermatozoa at $0{\cdot}4 \times 10^6$/ml. All experiments were done with open tubes at room temperature.

Motility was scored at hourly intervals over a 4-hr period by the system of Emmens (1947). Full motility was rated as 4 and complete immotility as

zero, but in presenting results the actual scores have been multiplied by 4 since quarter grades were frequently used.

(*c*) *Statistical Treatment*

The results have been subjected to standard analyses of variance (cf. Snedecor 1946) with isolation of sums of squares attributable to differences between ejaculates and treatments. The total motility score ($\times$ 4) has been used as unit observation and the interaction mean square as error term. Differences between ejaculates are often very significant, so that the accuracy of comparisons is much improved by analysis in this way.

F values have been given in the text. When, as is sometimes the case, motility falls rapidly, treatment variances are probably not completely independent of the level of response. Since, however, an effect has only been judged significant when the probability of its being due to chance is less than 1 in 100 this is of little consequence.

(*d*) *Chemicals*

AR sulphate salts of the minerals were used. The L or DL amino acids were B.D.H. laboratory reagents; except that ornithine, arginine, leucine, isoleucine, valine, cystine, and di-iodotyrosine were supplied by Light & Co. Ltd. Glycine was supplied by May and Baker Ltd.

The vitamins and other substances were obtained from the following sources:

Nicotinamide and folic acid—Roche Products Ltd.

Para-aminobenzoic acid, riboflavin, and pyridoxide—Andrews Laboratories.

Calcium pantothenate, thiamine hydrochloride, and vitamin B_{12} (saline solution)—General Biochemicals.

Inositol—Difco Laboratories.

Biotin (in phosphate buffer)—British Drug Houses Ltd.

Ascorbic acid—British Drug Houses Ltd. and Colonial Sugar Refinery Co.

Adenosine triphosphate (free acid; total P = 14·6%, 64·5% hydrolysable; N = 11·0%; N/P = 0·75); and CoI (61·4% DPN)—Schwarz Laboratories.

Cytochrome *c* (0·34% Fe)—National Biochemical Corp.

Spermine phosphate and choline chloride—Light & Co. Ltd.

Crystalline bovine albumin—Armour Laboratories.

III. Results

(*a*) *Ram, Bull, and Rabbit Spermatozoa*

The nature of the dilution effect is illustrated by the results of preliminary tests (Table 1) in which motility was scored at high and low spermatozoa concentration for each species. Not only was the motility depressed initially by dilution but it fell off more rapidly at the lower cell concentrations. The *F* values for the analyses of variance are 70·0, 30·5, and 84·8 for the ram, bull, and rabbit respectively and $P < 0{\cdot}01$ in each case.

In subsequent tests the semen was diluted for convenience in two stages. Motility in the neat semen and at the intermediate cell concentration has not been recorded but was invariably better than at the final dilution to which the figures in subsequent tables refer. It may be noted that the susceptibility to dilution of ejaculates from the same species often varied considerably although initial motility in the neat semen was good in each.

TABLE 1

EFFECT OF DILUTION ON THE HOURLY MOTILITY SCORE (× 4) OF RAM, BULL, AND RABBIT SPERMATOZOA. EACH VALUE IS THE MEAN OF SIX EJACULATES

Species	Sperm Concentration ($\times 10^{-6}$/ml)	Hours					Total
		0	1	2	3	4	
Ram	200·0	15	15	15	15	15	75
	2·0	10	6	6	3	1	26*
Bull	200·0	14	13	12	11	8	57
	2·0	8	6	5	4	3	26*
Rabbit	20·0	14	13	12	12	11	62
	0·4	8	6	4	3	2	23*

*Significantly<score at higher cell concentration, $P<0·01$.

(i) *Trace Elements.*—Mammalian semen is known to contain small amounts of copper, iron, and zinc (Zittle and Zitin 1942; Mann 1945), and the report of Lardy, Phillips, and Rupel (1942) suggests that manganese might be important for the maintenance of the motility of bull spermatozoa. The addition to the diluent of these elements in physiological concentrations (copper, cobalt, manganese = 0·05 mg/100 ml; iron, zinc = 0·20 mg/100 ml) did not, however,

TABLE 2

TOTAL MOTILITY SCORE (× 4) OF DILUTED RAM, BULL, AND RABBIT SPERMATOZOA IN THE PRESENCE OF HEAVY METAL IONS. EACH VALUE IS THE MEAN OF FOUR EJACULATES

Species	Control	Copper	Cobalt	Iron	Manganese	Zinc
Ram	38	27*	41	43	43	42
Bull	19	20	23	18	23	20
Rabbit	9	7	8	8	8	7

*Significantly toxic, $P<0·01$.

significantly improve the motility of diluted ram, bull, or rabbit spermatozoa. The results of tests on four ejaculates from each species are given in Table 2. Copper, it may be noted, significantly depressed the motility of diluted ram spermatozoa ($F = 12·0$, $P < 0·01$).

TABLE 3

TOTAL MOTILITY SCORE (× 4) OF DILUTED RAM, BULL, AND RABBIT SPERMATOZOA IN THE PRESENCE OF THE B VITAMINS. EACH VALUE IS THE MEAN OF FOUR EJACULATES

Species	Control	Thiamine (0·5 mg/ 100 ml)	Riboflavin (0·5 mg/ 100 ml)	Niacin (0·5 mg/ 100 ml)	Inositol (0·5 mg/ 100 ml)	PAB (0·5 mg/ 100 ml)	Pantothenic Acid (0·5 mg/ 100 ml)	Pyridoxine (0·5 mg/ 100 ml)	Folic Acid (0·5 mg/ 100 ml)	Biotin (25 μg/ 100 ml)	Vitamin B_{12} (40 μg/ 100 ml)
Ram	38	40	43	34	41	41	43	44	39	37	42
Bull	10	12	10	11	11	10	11	8	9	36†	11
Rabbit	34	36	37	34	35	35	29	27	34	36	34

† Significantly beneficial, $P<0·01$.

(ii) *Vitamin B Complex.*—Thiamine, riboflavin, niacin, and pantothenic acids are present in bull semen (Lardy and Phillips 1941; VanDenmark and Salisbury 1944), and inositol has recently been found to be a major constituent of boar semen (Mann 1951*a*). It was of interest, therefore, to see if these or other members of the vitamin B complex were involved in the dilution effect, particularly as VanDenmark and Salisbury (1944) claimed that the thiamine, riboflavin, and niacin levels of bull semen are correlated with the initial motility of the spermatozoa. Thiamine, riboflavin, niacin, inositol, *p*-aminobenzoic acid, pantothenic acid, pyridoxine, and folic acid were each tried at a concentration of 0·5 mg/100 ml. Biotin and vitamin B_{12} had to be used in lower concentrations (25 and 40 μg/100 ml respectively) because they were supplied as solutions in ampoules.

The results for four ejaculates from each species are set out in Table 3. Biotin increased the viability of bull spermatozoa ($F = 108·4$) but did not significantly influence the motility of ram or rabbit spermatozoa. The other members of the vitamin B complex had little or no effect on the spermatozoa of any of the three species.

Confirmatory tests on six other bull ejaculates (Table 4) showed that biotin significantly improved motility at 5 μg/100 ml ($F = 18·8$) and 10 μg/100 ml ($F = 54·5$).

TABLE 4

EFFECT OF BIOTIN ON THE TOTAL MOTILITY SCORE (× 4) OF DILUTED BULL SPERMATOZOA

Ejaculate	Biotin (μg/100 ml)		
	0	5	10
1	12	46	62
2	13	36	63
3	7	55	72
4	26	38	76
5	28	51	53
6	33	47	55
Mean	20	46*	64*

*Significantly beneficial, $P<0·01$.

(iii) *Amino Acids.*—Recent reports indicate that free amino acids are present in bull semen and accessory fluid (Gassner and Hopwood 1952; Larson and Salisbury 1953). A number have therefore been tested on diluted bull, ram, and rabbit semen. Twenty-one amino acids were each tried at two concentrations (500 and 50 mg/100 ml). The tests were done on four ejaculates from each species at both concentrations.

Table 5 shows that none of the amino acids at either concentration improved the motility of diluted ram, bull, or rabbit spermatozoa to any great extent.

On the other hand, some of the amino acids significantly depressed motility. Di-iodotyrosine at 500 mg/100 ml rapidly immobilized ram ($F = 13{\cdot}5$), bull ($F = 69{\cdot}4$), and rabbit spermatozoa ($F = 29{\cdot}9$) and also depressed the motility of ram ($F = 21{\cdot}8$) and rabbit spermatozoa ($F = 31{\cdot}5$) at 50 mg/100 ml. Tyrosine was toxic to ram spermatozoa at 500 ($F = 13{\cdot}9$) and 50 mg/100 ml ($F = 12{\cdot}1$) and to rabbit spermatozoa at the higher concentration ($F = 9{\cdot}9$).

TABLE 5

TOTAL MOTILITY SCORES (× 4) OF DILUTED RAM, BULL, AND RABBIT SPERMATOZOA IN THE PRESENCE OF AMINO ACIDS AND GLUTATHIONE. EACH VALUE IS THE MEAN OF FOUR EJACULATES

Amino Acids	Ram		Bull		Rabbit	
	Amino Acid Concentration		Amino Acid Concentration		Amino Acid Concentration	
	500 mg/ 100 ml	50 mg/ 100 ml	500 mg/ 100 ml	50 mg/ 100 ml	500 mg/ 100 ml	50 mg/ 100 ml
Nil	22	42	31	23	25	33
Glycine	25	40	34	25	29	36
Alanine	18	42	36	25	31	31
Valine	25	36	37	25	24	29
Leucine	24	39	36	24	29	29
Isoleucine	24	38	35	23	29	29
Serine	23	36	37	25	25	34
Glutamic Acid	15	38	32	36	8*	34
Tryptophane	21	38	28	22	28	24
Proline	25	38	38	24	29	26
Histidine	23	35	36	27	20	29
Ornithine	21	39	36	19	26	30
Citrulline	29	—	36	—	29	—
Arginine	19	40	35	26	31	24
Lysine	23	38	35	21	27	30
Cysteine	3*	14*	13*	18	9*	16*
Cystine	28	37	29	24	20	26
Methionine	27	36	37	25	29	32
Glutathione	6*	32*	19*	25	11*	22*
Phenylalanine	15	34	28	23	24	34
Tyrosine	4*	31*	27	19	11*	37
Di-iodotyrosine	4*	27*	6*	20	1*	12*

*Significantly toxic, $P < 0{\cdot}01$.

Cysteine depressed the motility of ram ($F = 15{\cdot}0$), bull ($F = 38{\cdot}1$), and rabbit spermatozoa ($F = 13{\cdot}3$) at 500 mg/100 ml and was also toxic to ram ($F = 75{\cdot}7$) and rabbit ($F = 21{\cdot}3$) spermatozoa at 50 mg/100 ml. Glutathione, which is a cysteine derivative, was similarly toxic to ram ($F = 10{\cdot}7$), bull ($F = 16{\cdot}7$), and rabbit spermatozoa ($F = 9{\cdot}6$) at the higher concentration and to ram ($F = 10{\cdot}0$) and rabbit spermatozoa ($F = 9{\cdot}0$) at 50 mg/100 ml.

Glutamic acid at 500 mg/100 ml also proved harmful to rabbit spermatozoa ($F = 14{\cdot}1$). This effect may, however, have been due to the fall in pH of the diluent caused by the glutamic acid. It should be noted that these effects may not always be seen if motility is too greatly depressed by dilution.

TABLE 6

TOTAL MOTILITY SCORE (× 4) OF DILUTED RAM, BULL, AND RABBIT SPERMATOZOA IN THE PRESENCE OF SOME MISCELLANEOUS SEMINAL CONSTITUENTS. EACH VALUE IS THE MEAN OF FOUR EJACULATES

Species	Experiment	Control	Cytochrome *C* (2·5 mg/100 ml)	Coenzyme I (2·5 mg/100 ml)	Adenosine Triphosphate (50 mg/100 ml)	Spermine Phosphate (50 mg/100 ml)	Citric Acid (25 mg/100 ml)	Choline Chloride (250 mg/100 ml)	Ascorbic Acid (10 mg/100 ml)
Ram	1	41	45	48	36	40	—	—	—
	2	44	—	—	—	—	40	40	—
	3	27	—	—	—	—	—	—	10*
Bull	1	18	20	19	23	17	19	21	—
	2	28	—	—	—	—	—	—	15
Rabbit	1	19	43†	29	24	31†	—	—	—
	2	27	—	—	—	—	26	29	9*

†Significantly beneficial, $P<0{\cdot}01$. *Significantly toxic, $P<0{\cdot}01$.

(iv) *Miscellaneous Seminal Constituents.*—The substances tested were citric acid (25 mg/100 ml), choline chloride (250 mg/100 ml), spermine phosphate (50 mg/100 ml), cytochrome *c* (2·5 mg/100 ml), CoI (2·5 mg/100 ml), adenosine triphosphate (50 mg/100 ml), and ascorbic acid (10 mg/100 ml). Each of these has been found in the semen of one or more mammalian species (see Mann 1949) and in choosing the concentrations, the amounts reported in neat semen have, where possible, been taken as a guide. The results for four ram, bull, and rabbit ejaculates are given in Table 6. None of the substances improved the motility of diluted ram or bull spermatozoa to any great extent. Cytochrome *c* ($F = 44{\cdot}2$) and spermine phosphate ($F = 11{\cdot}5$), however, were significantly beneficial to diluted rabbit spermatozoa.

Confirmatory tests on four rabbit ejaculates at three concentrations of cytochrome *c* (0·1, 1·0, and 10·0 mg/100 ml)and spermine phosphate (5·0, 50·0, and 500·0 mg/100 ml) are shown in Table 7. Cytochrome *c* was significantly beneficial at 10 ($F = 16{\cdot}6$) and 1·0 mg/100 ml ($F = 13{\cdot}7$) and spermine phosphate improved viability at 500 ($F = 11{\cdot}4$) and 50 mg/100 ml ($F = 8{\cdot}4$).

Ascorbic acid significantly depressed the motility of diluted ram ($F = 61{\cdot}2$) and rabbit spermatozoa ($F = 41{\cdot}6$) and had a similar effect on bull spermatozoa, although it is not quite significant at the 0·01 probability level.

(*b*) *Human Spermatozoa*

Limited tests were made on human spermatozoa, using biotin, cytochrome *c*, spermine phosphate, CoI, glycine, choline chloride, and the high-molecular-

TABLE 7

EFFECT OF CYTOCHROME *c* AND SPERMINE PHOSPHATE ON THE TOTAL MOTILITY SCORE (× 4) OF DILUTED RABBIT SPERMATOZOA

Ejaculate	Control	Cytochrome *c* (mg/100 ml)			Spermine Phosphate (mg/100 ml)		
		0·1	1·0	10·0	5·0	50·0	500·0
1	3	10	37	24	19	21	32
2	23	22	29	47	41	45	46
3	4	13	38	37	22	20	13
4	9	8	14	18	8	15	20
Mean	10	13	28†	32†	23	25†	28†

†Significantly beneficial, $P<0·01$.

weight substances previously shown to improve the motility of diluted rabbit, bull, and ram spermatozoa (Emmens and Swyer 1948; White 1953*a*; Blackshaw 1953). Table 8 gives the results of four ejaculates. It is clear that human spermatozoa are particularly susceptible to dilution and that none of the compounds tried was of much value in maintaining motility.

TABLE 8

TOTAL MOTILITY SCORE (× 4) OF HUMAN SPERMATOZOA IN THE PRESENCE OF SEMINAL CONSTITUENTS AND MISCELLANEOUS SUBSTANCES

Treatment	Sperm Concentration (× 10^{-6}/ml)	Mean of Four Ejaculates
Control	20·0	54
Control	0·4	4
Cytochrome *c* (2·5 mg/100 ml)	0·4	4
Coenzyme I (2·5 mg/100 ml)	0·4	4
Spermine phosphate (50 mg/100 ml)	0·4	3
Choline chloride (250 mg/100 ml)	0·4	4
Biotin (10 μg/100 ml)	0·4	7
Glycine (500 mg/100 ml)	0·4	5
Plasma albumin (250 mg/100 ml)	0·4	6
Egg albumin (250 mg/100 ml)	0·4	4
Glycogen (250 mg/100 ml)	0·4	4
Starch (250 mg/100 ml)	0·4	5

IV. DISCUSSION

These tests (Tables 5 and 6) suggest that loss of biotin might be a factor in the harmful effect of dilution on bull spermatozoa. Egg yolk, which is widely used as an extender for bull semen, is a rich source of biotin and is

known to protect bull spermatozoa to some extent from dilution (Cheng, Casida, and Barrett 1949).

The two substances that improved the viability of rabbit spermatozoa in these tests were cytochrome *c* and spermine phosphate. Mann (1951*b*) has shown that cytochrome *c* is leached out of mammalian spermatozoa on storage and spermine phosphate is believed to be contributed to semen by the prostate (Rosenheim 1924; Harrison 1931, 1933; Bolliger 1935). These substances could therefore be the active compounds of the spermatozoa and accessory fluid, respectively, referred to in the introduction.

No clue, however, has been obtained as to the factors that might be involved in the deleterious effect of dilution on ram and human spermatozoa.

Knoop and Krauss (1944) claimed that 1 per cent. glycine or proline improved the efficacy of egg yolk as an extender for bull semen and more recently it has been reported that glycine increases the life span of fowl and bull spermatozoa diluted in saline and balanced salt solutions (Lorenz and Tyler 1951; Tyler and Tanabe 1952). The latter workers, however, believe that the beneficial effect of glycine in their experiments was due to the binding of toxic trace elements in the diluent. Glass-distilled water was used in the present experiments, which may account for the inactivity of glycine. Tosic and Walton (1950) reported that tyrosine, phenylalanine, and tryptophane are toxic to bull spermatozoa on shaking in air—an effect which they attributed to the formation of hydrogen peroxide. Macleod (1951) has also found that cysteine, but not glutathione, depresses the motility of human spermatozoa and suggested that its action is due to the oxidation of essential thiol groups in the spermatozoa.

The toxic action of ascorbic acid in these experiments is rather surprising and was apparently not due to an impurity in the chemical as similar results were obtained with two preparations from independent sources. However, the concentrations used were similar to those reported in neat semen (Phillips *et al.* 1940; Berg, Huggins, and Hodges 1941), and diluted spermatozoa would not normally be in contact with such amounts.

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