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Motile Life of Bovine Spermatozoa in Glycine and Yolk-Citrate Diluents at High and Low Temperatures. (19878)

ALBERT TYLER AND T. Y. TANABE.*

From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena.

In experiments with sea urchins and other invertebrates it has been found(1,2) that the addition of any one of a number of amino acids or peptides to the spermatozoa, diluted in sea water, extends very greatly their duration of motility and fertilizable life-span. The extension of functional life-span is accomplished(3) without metabolic utilization of the added amino acid. Experiments with fowl sperm(4) have also shown an extension of motile life-span as a result of the addition of an amino acid to the saline diluent. It was of interest, then, to learn whether or not such agents would be effective in extending the life-span of mammalian spermatozoa. The experiments with the invertebrates were performed at temperatures in the range of their usual environment. Life-span studies on spermatozoa of mammals have generally been carried out at low temperatures since the purpose evidently is to extend, as much as possible, the period during which the sperm may be stored before use in artificial insemination. It should be noted, however, that mammalian spermatozoa must survive at body temperature in the female genital tract for some time after insemination before the eggs are encountered. Their duration of survival at such temperature may, then, be an important factor in determining whether or not successful fertilization will occur.

In view of these considerations the present experiments with bovine spermatozoa were performed at both body and refrigerator temperatures. The effect of the amino acid solution was compared with that of isotonic NaCl, balanced salt solution and the yolk-citrate diluent(5) which, following the discovery(6) of the efficacy of hen's egg-yolk, is generally employed for storage of bovine spermatozoa at low temperatures.

Material and methods. Semen was obtained from Holstein and Guernsey stud bulls of the Roger Jessup Certified Farm† in Los Angeles, Calif. The samples were gradually cooled and kept at 2° to 4° until use at the laboratory, usually between 1¾ and 6¼ hours later. Sperm counts of the various samples ranged from 1 to 14 x 10^8 per ml. The semen was diluted 1/100 and 1/1000 in the various test solutions. Pyrex, glass-stoppered, 25 ml Erlenmeyer flasks were used with 4 ml of the diluted semen. In the experiments at 38.6°C, these were shaken at 80 c.p.m. and 5 cm excursion. In the experiments at 4°C, 50 ml flasks were used with 4 ml of diluted semen and these were not shaken. Estimates of the percentage of motile sperm were made hourly in the 38.6°C series and daily in the 4°C series, with 2 or 3 readings taken during the first period. Motility was scored as 0, 1, 5,

* Present address: Department of Dairy Husbandry, Pennsylvania State College, State College, Pa.,

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TABLE I. Duration of Motility of Bovine Spermatozoa at 38.6°C and 4°C in Glycine as Compared with Balanced Salt and Yolk-Citrate Diluents.

	No. of exp.	Mean half-life (hr)	Mean increase over corresponding control: Hr	Mean increase over corresponding control: Fraction	Mean 90%-life (hr)	Mean increase over corresponding control: Hr	Mean increase over corresponding control: Fraction
38.6°C; 1/100 semen							
Balanced salt	15	5.9			11.2		
.13 M glycine	14	.6	−5.7 ± .9†	−13.9 ± 3.7†	1	−10.3 ± 1.1†	−24.4 ±10 *
.075	15	4.7	−1.2 ± 1.2	−1.9 ± 1.9	12.9	1.7 ± .9	.2 ± .1
.038	15	7.2	1.3 ± .9	1.5 ± 1	12.8	1.6 ± 1	.2 ± .1
.019	15	7.2	1.3 ± .7	.4 ± .3	12.5	1.3 ± .9	.2 ± .1
.009	14	7	.8 ± .7	.1 ± .2	12.2	1 ± .6	.1 ± .1
Yolk-citrate	14	8	2.3 ± .8*	.5 ± .2	10.6	−.7 ± 1.1	.2 ± .3
38.6°C; 1/1000 semen							
Balanced salt	15	3.2			4.6		
.13 M glycine	14	.5	−2.9 ± .5†	−10 ± 5.1	.7	−4 ± .7†	−14.7 ± 6.4*
.075	15	3.8	.6 ± .9	.2 ± 1	7.9	3.3 ± .9†	1 ± .4*
.038	15	6.9	3.7 ± .5†	5.3 ± .3†	9.2	4.6 ± .5†	1.2 ± .2†
.019	15	6.6	3.4 ± .5†	6.5 ± .4†	8.7	4.1 ± .4†	1.3 ± .4†
.009	14	5.7	2.3 ± .5†	3.4 ± .3†	7.7	3.1 ± .6†	.8 ± .2†
Yolk-citrate	14	2.3	−.8 ± .7	−4.9 ± .5†	6.6	2.1 ± 1.2	.5 ± 1.2
4°C; 1/100 semen							
Balanced salt	7	14			33		
.13 M glycine	7	4	−10 ± 2†	−3.9 ± 1.2*	11	−22 ± 10	−9.3 ± 2.1†
.075	7	14	0 ± 4	−.1 ± .4	34	1 ± 7	.1 ± .3
.038	7	15	1 ± 2	.2 ± .2	31	−2 ± 5	0 ± .2
.019	7	15	1 ± 1	.2 ± .2	33	0 ± 4	.1 ± .1
.009	7	14	0 ± 1	0 ± .1	33	0 ± 4	.1 ± .1
Yolk-citrate	7	185	171 ±18†	13.2 ± 2 †	300	267 ± 26†	9.1 ± 1.3†
4°C; 1/1000 semen							
Balanced salt	7	12			23		
.13 M glycine	7	3	−9 ± 1†	−3.5 ± .6†	9	−14 ± 4*	−5.4 ± 1.7*
.075	7	11	−1 ± 2	−.2 ± .3	25	2 ± 2	.1 ± .1
.038	7	14	2 ± 1	.2 ± .1	24	1 ± 1	.1 ± .1
.019	7	13	1 ± 1	.1 ± .1	24	1 ± 1	.1 ± .1
.009	7	14	2 ± 1	.2 ± .1	24	1 ± 1	.1 ± .1
Yolk-citrate	7	47	35 ± 6†	2.9 ± .4†	97	74 ± 17†	3.2 ± .8†

* Exceeds 5% level of significance. † Exceeds 1% level of significance.
See text for further explanation of table.

10, 25, 50, 75 and 100%. In each set of experiments aliquots of a single semen sample were diluted in the various glycine solutions, a balanced salt solution and yolk-citrate. The balanced salt solution was of the following composition: 1000 ml of 0.15 M NaCl, 22 ml 0.15 M KCl, 195 ml 0.10 M $MgCl_2 \cdot 6H_2O$, 103 ml 0.10 M Na_2SO_4, 35 ml 0.10 M $CaCl_2$, 5.9 ml 0.15 M $NaHCO_3$. The pH was adjusted when necessary to 7.3-7.4. The glycine solutions were prepared by dilution of an isotonic stock solution in the balanced salt solution. The yolk-citrate consisted of 1 volume of hen's egg yolk plus 1 volume of 3.6% $Na_3C_6H_5O_7 \cdot 2H_2O$ and contained 0.3% sulfanilamide, 1000 units per ml potassium penicillin G, and 1000 units per ml of dihydro streptomycin sulphate. In certain experiments, yolk-citrate without the antibacterial agents was also employed. Tests were also made of glycine and balanced salt solutions containing the antibacterial agents. No marked effect of the presence or absence of these agents on the duration of motility was observed and the data are not included in the present report(7-11).

Results. Summarized data for 15 sets of experiments at 38.6°C and 7 sets of experiments at 4°C are presented in Table I. Two end points of motile life-span are employed for presentation of the data. These are termed half-life and 90%-life, and represent the time at which half and 90%, respectively, of the initially motile sperm had become immotile. The values in most cases were obtained by interpolation of consecutive readings of percentage motility. Two methods of ex-

pressing the effect of treatment on the duration of motility are employed in the table. One consists simply in the means of the difference, in life-span, between a particular test solution (glycine or yolk-citrate) and the corresponding (*i.e.*, same semen sample) balanced salt solution control. Negative values, of course, signify shorter life-span than in the control. The standard error of the means is given and significance, as determined by the t-value(12,13), is indicated by a single asterisk or a double asterisk where it exceeds the 5% or 1% levels respectively. The other method, listed under the heading "fraction" in the table, gives the means of the ratio of the difference in life-span between test solution and corresponding control to life-span in the control or the test solution, depending upon which is the lower value. Thus, these fractions have negative values when motility in the control solution lasts longer than in the test solution.

With the 1/100 dilution of semen at 38.6°C the data show no significant effect of glycine, in the concentration range 0.009 to 0.075 molar. In 0.13 M glycine a considerable decrease in life-span is shown. In yolk-citrate there is a small increase in half-life span, with significance slightly above the 5% level, when expressed as the difference from the balanced salt control, but no significant effect when expressed as fractional increase. This increase is not obtained for the 90% end point by either method of expression.

With the 1/1000 dilution of semen at 38.6°C the data show highly significant increases in life-span (both the half-life and 90%-life end points) in 0.009, 0.019 and 0.038 molar glycine solutions. Thus, the mean percentage increases in half-life span in these solutions are 340, 650 and 530 respectively. In 0.13 molar glycine there is again a decrease in life-span. In yolk-citrate there is either no significant effect or a decrease, depending upon which end-point and which method of expression is used.

At 4°C, with both dilutions of semen, the yolk-citrate shows the well-established favorable effect on the life-span of the sperm. Glycine solutions of 0.009 to 0.075 molar have no significant effect, while 0.13 molar again gives a decrease which is somewhat less marked than in the 38.6°C series.

Discussion. The results show that the favorable effect of yolk-citrate in extending the life-span of bovine spermatozoa at low temperature in not manifested at 38.6°C. Glycine, which extends considerably the life-span of spermatozoa of invertebrates(1-3) and also of fowl(4), has, at concentrations of 0.009 to 0.038 M, a marked favorable effect on bovine sperm at 38.6°C and 1/1000 dilution of semen. At the 1/100 dilution and this temperature, the effect is not shown. This is consistent with the lesser effect obtained in the experiments with invertebrates and fowl, when higher concentrations of sperm are used. No significant effect of glycine on the life-span of the bovine sperm is found at the lower temperature, except for the injurious effect of a high concentration (0.13 M) shown at both temperatures.

One of the factors that may be involved in the results is the effect of aeration. MacLeod(14) suggested that H_2O_2 was formed by mammalian (human) sperm under aerobic conditions and this shortened their life-span as compared with anaerobic conditions. Addition of catalase and hemoglobin prolonged aerobic survival, presumably by destruction of the H_2O_2 whose formation was attributed(15) to dehydrogenation of succinic acid. Tosic and Walton(16,17) identified as H_2O_2 an inhibitor of respiration and motility formed by bovine spermatozoa incubated aerobically in yolk-phosphate. They found that catalase and peroxidase could reverse the inhibition. The H_2O_2 was shown to be formed in the presence of a dialyzable fraction of egg-yolk and by phenylalanine, tyrosine and tryptophane but not by any of ten other amino acids, including glycine, that were tested. Prince and Almquist(18) showed that agitation of the partially filled tubes in which bovine sperm are customarily stored decreased livability to a statistically significant degree. Ram semen on the other hand seem to require periodic aeration for maintenance of motility according to Gunn(19). Van Demark *et al.*(20) found that the presence of oxygen decreased somewhat the livability and motility of bovine sperm in yolk-citrate at 46.5° and 5°C and

that the deleterious effect could be largely eliminated by addition of catalase. However, in a later investigation(21) no improvement from added catalase was obtained in either livability or fertility. It is suggested(21) that the difference may be correlated with whether or not the semen was diluted before cooling.

From these and other investigations there appears to be some uncertainty as to the effect of aeration on the life-span of mammalian spermatozoa under various conditions. The present experiments at 38.6° were performed under aerobic conditions since *in vivo* the spermatozoa would be under such conditions in the female genital tract. (The O_2 tension within the uterus of the rabbit has been found (22) to be 20-45 mm Hg which is evidently ample for aerobic metabolism of the spermatozoa.) In the 4° C series the low fluid level (ca 2 mm) also insured essentially aerobic conditions even without shaking. As the results show the yolk-citrate at this temperature gave the usual great increase in life-span of the spermatozoa. That the yolk-citrate fails to show such effect, or may even be deleterious at the higher temperature, might possibly be due to more rapid formation of a toxic agent such as H_2O_2, but in the absence of additional information there are many other possible interpretations based on changes in metabolic patterns of cells at different temperature.

The action of glycine was of special interest in this work in view of the beneficial effects of various amino acids shown in the earlier experiments with invertebrate sperm. At relatively low concentrations this amino acid also extends the life-span of the bovine spermatozoa. The effect is manifested only at the higher temperature and with the more dilute semen. At high concentration (0.13 M) the glycine is injurious. According to Tosic and Walton(17) glycine is among the ten amino acids that do not cause H_2O_2 formation. This agent is, then, presumably not responsible for the injurious effect of high concentrations of glycine and, of course, not for the favorable effect of lower concentrations. Possibly the extension of life-span obtained with glycine in bovine spermatozoa involves the same sort of action as in the case of the invertebrate and fowl sperm. In these the experiments(1-4) suggest that the amino acids act by binding certain heavy metal ions normally present in trace amounts in the dilution medium. Further unpublished experiments on invertebrates with other kinds of chelating agents support this view. However, preliminary tests on sperm with the metal-chelating agents diethyldithiocarbamate and ethylenediaminetetraacetate have given no very marked favorable effect. The present evidence does not, then, permit a decision to be made on the extent to which the presence of trace metals in the dilution medium may be involved in the life-span of bovine sperm, nor on the manner in which glycine is able to extend the life of these cells.

Summary. The addition of glycine, at concentrations of 0.009 to 0.038 molar, to a balanced salt diluent, increases considerably the motile life-span of bovine sperm at 38.6°C. The effect is manifest in 1/1000, but not in 1/100, dilutions of semen. It is not exhibited at 4°C. In yolk-citrate the usual extension of life-span is obtained at 4°C, but at 38.6°C there is either no significant effect or a decrease as compared with the balanced salt solution controls. The results are discussed in relation to experiments on sperm of invertebrates and fowl in which the life-span extending action of amino acids is attributable to the binding of trace metals present in the dilution medium.

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Thermal Inactivation of a New Vibrio Phage.*† (19879)

LUCILE SCHULTZ SMITH AND ALBERT PAUL KRUEGER.
From Department of Bacteriology, University of California, Berkeley.

Susceptibility of bacteriophages to inactivation by heat has been observed by several workers to be a general phenomenon(1-4a). Nanavutty(5) studied rates of inactivation of a coli-dysentery phage exposed to various temperatures. He plotted the logarithms of residual active phage against time and obtained concave curves. He also observed a difference in heat susceptibility of the phage when different suspending fluids were used. Application of an accurate analytical method for phage assay to experiments on the thermolability of staphylococcal phage demonstrated that: 1) heat inactivation at various temperatures follows the course of a monomolecular reaction; and 2) the temperature coefficient is of a magnitude characteristic of protein denaturation(6). Other races of bacteriophage have since been shown to behave similarly during heat inactivation(7,8).

Recently we have been making a survey of the properties of a new Vibrio-phage system. The present papers deal with some interesting aspects of the heat inactivation of this particular phage.

Materials and methods. The virus-host system under study was recently isolated from San Francisco Bay mud. The bacterium is of the genus Vibrio, and will not grow in ordinary media without the addition of NaCl. A concentration of 3% sodium chloride supports maximum growth of the organism and optimal phage production. The phage lysate was prepared in yeast extract mineral 3% salt medium‡ and was passed through a Seitz filter (Type ST) to secure a bacteria-free suspension of phage at pH 7.9. When it was desired to compare the susceptibility of the phage suspended in various fluids, the lysate was ultracentrifuged, the supernatant fluid decanted, and the phage resuspended in the medium indicated, *i.e.*, M/15 phosphate buffer pH 7.9, M/15 phosphate buffer pH 7.9 plus 3% NaCl, 1% aqueous yeast extract plus NaCl in concentrations of 5, 4, 3, 2, 1, 0.5, 0.25, and 0.125%, respectively and no NaCl. The phage was exposed to a given temperature by dispensing 0.5 ml aliquots into prewarmed test-tubes and immediately placing these in the water bath at the desired temperature. At the end of each time interval a sample was removed from the water bath and immediately

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‡ The composition of Yeast Extract Mineral 3% salt medium is:

Yeast Extract (Difco)	%	Bacto dehydrated	1%
			%
$MgSO_4$	.05	NaCl	3.0
K_2HPO_4	.1	Tap water	10
NH_4NO_3	.1	Dist. water q.s.	90