(0.1 M phosphate buffer), either in modified Thunberg tubes or, more conveniently, by bubbling the gases through the solutions in test tubes provided with two-holed rubber stoppers bearing inlet and outlet tubes. Amounts of the enzyme preparation were used that corresponded to 4 ml of the original phosphate suspension. The gases used were deoxygenated by passing over hot reduced copper filings.

In the presence of active preparations the dye was reduced under an atmosphere of hydrogen but not under nitrogen. Heating for twenty minutes at 100°C destroyed the activity of the enzyme.

Summary. Stable dry powders and cell-free solutions that possess hydrogenase activity have been prepared from cultures of *B. coli communior.*

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Effect of Gramicidin on Metabolism of Bovine Spermatozoa.*

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Gramicidin,†,‡ a bactericidal agent for gram-positive organisms,[1] seems to have a wider range of activity than the name indicates. It acts as a hemolytic agent in low concentrations,[2,§] and it is highly

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† The preparation of crude and purified gramicidin, and purified tyrocidine, used in these studies were obtained through the kindness of Dr. R. J. Dubos, who independently suggested this investigation. We are indebted also to Dr. Dubos for reading this paper before publication.

‡ Crude gramicidin has recently been renamed tyrothricin by Hotchkiss and Dubos[4] and the name gramicidin retained for one of the purified components. The other principal component has been purified also and named tyrocidine; it exhibits bactericidal activity against both Gram positive and Gram negative organisms.

1 Dubos, R. J., *J. Exp. Med.*, 1939, **70**, 1, 11; Dubos, R. J., and Cattaneo, C., *ibid.*, 249; Dubos, R. J., *Ann. Int. Med.*, 1940, **13**, 2025.

2 Heilman, D., and Herrell, W. E., PROC. SOC. EXP. BIOL. AND MED., **1941, 46**, 182.

§ Hotchkiss and Dubos[5] recently have stated that tyrocidine is hemolytic but that purified gramicidin is not hemolytic. Further studies[6] have confirmed the hemolytic effect of gramicidin in saline or buffer solutions[2] but have shown that hemolysis is completely inhibited by small amounts of glucose.

toxic if injected into animals by the intravenous route.[3] The effect of gramicidin on mammalian cells other than erythrocytes has not been investigated to our knowledge.

Bovine spermatozoa, which are being studied extensively in this laboratory, appeared to be suitable material for such an investigation, since they can readily be obtained in pure suspension, and, being a type of mammalian cell, might be expected to throw some light on the observed toxicity of this substance in animals. In the following we shall describe the action of gramicidin on the metabolism and the motility of bovine spermatozoa. Some data are available for the normal respiration and glycolysis of these cells;[7-10] further information is being collected in this laboratory at present.

Experimental. Spermatozoal suspensions were prepared from the bovine epididymis as described elsewhere.[11] Ringer solution containing a final concentration of 0.2% glucose with or without the addition of M/15 phosphate buffers (added in ratio of 1 to 4) of pH 5.3 to 8.0 served as medium for the determination of the oxygen consumption, while Ringer glucose with sodium bicarbonate (0.025 M) was used for the measurement of the glycolysis. Alcoholic solutions of the gramicidin preparations were added to Ringer solution and appropriate volumes of the resulting suspension used.

Oxygen consumption and anaerobic glycolysis were measured by the direct method of Warburg[12] under air and under nitrogen with 5% CO_2, respectively. In a number of experiments the respiratory quotient (RQ) and at the same time the aerobic glycolysis were determined in Dixon-Keilin flasks[12] attached to Warburg manometers filled with Clerici solution. The results were calculated according to the second method of Dickens and Simer.[12] In these experiments Ringer bicarbonate solution was used as medium, the suspensions being in equilibrium with a mixture of oxygen and 5% CO_2. Two ml of suspensions containing 150 to 700 million spermatozoa per ml were used per vessel. All of the experiments were performed at 37°C.

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3 McLeod, C. A., Mirick, G. S., and Curnen, E. C., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 461.

4 Hotchkiss, R. D., and Dubos, R. J., *J. Biol. Chem.*, 1940, **136**, 803.

5 Hotchkiss, R. D., and Dubos, R. J., *J. Biol. Chem.*, Proceedings, 1941, lxiii.

6 Dubos, R. J., and Hotchkiss, R. D., *J. Exp. Med.*, 1941, **73**, 629.

7 Redenz, E., *Biochem. Z.*, 1932-33, **257**, 234.

8 Iwanow, E. E., *Biochem. Z.*, 1935, **278**, 101.

9 Comstock, R. E., *J. Exp. Zool.*, 1940, **81**, 147.

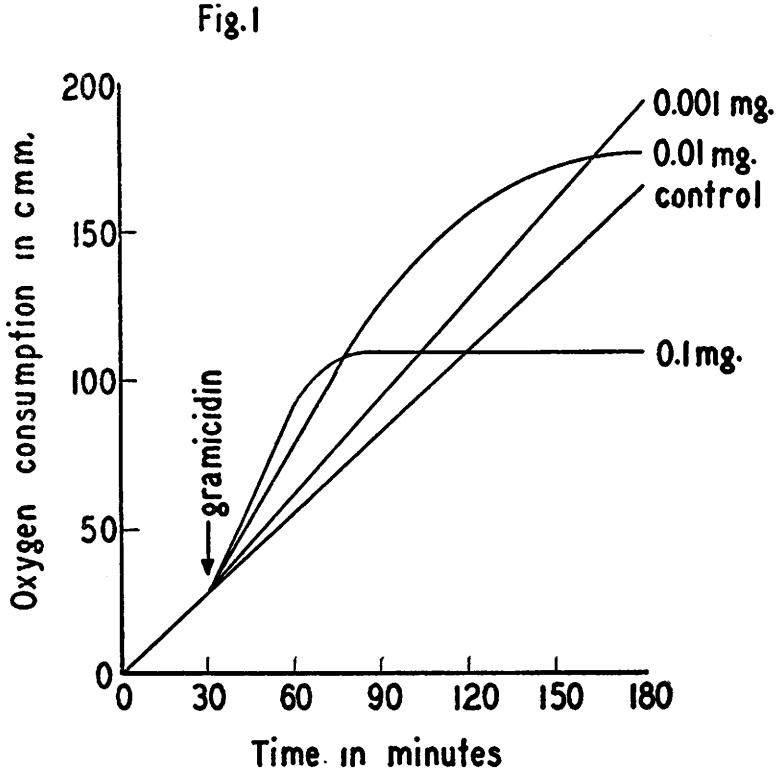
10 Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, 1941, **138**, 195.

11 Henle, W., *J. Immunol.*, 1938, **34**, 325.

12 Dixon, M., *Manometric Methods*, Cambridge, 1934.

It was found that small amounts of the crude preparation of gramicidin altered strikingly the oxygen consumption of bovine spermatozoa. Fig. 1 shows the effect of various concentrations of this agent on 400 million spermatozoa suspended in Ringer glucose (pH 6.0) over a period of 150 minutes. Concentrations of 0.1 mg and 0.01 mg of crude gramicidin per ml of suspension caused an initial stimulation of the oxygen uptake, but the respiration fell off rather abruptly after 30 and 90 minutes, respectively. The smallest amount of gramicidin used (0.001 mg per ml suspension) caused only a moderate stimulation of the oxygen consumption, but there was no decrease during the period of observation. As the suspensions of gramicidin were prepared from alcoholic solutions, control experiments were made with comparable amounts of alcohol. No influence on the oxygen uptake or on the motility of the spermatozoa was noticed.

In cases where the oxygen uptake had ceased, the spermatozoa were immobile whereas the cells in the control suspensions were



Influence of gramicidin on the oxygen consumption of bull spermatozoa suspended in Ringer glucose.

quite active. In the experiments where a stimulation of respiration was obtained by the addition of a small amount of gramicidin, the spermatozoa had about the same activity as the control suspensions.

This action of gramicidin on the respiration of spermatozoa suspended in Ringer glucose which had a pH of about 6.0 was also observed when the spermatozoa were suspended in a medium containing phosphate buffers of pH 5.3 and 6.6. When alkaline phosphate buffers (pH 7.9) were added to the Ringer solution, a medium in which the spermatozoa showed maximal respiration,[13] the addition of gramicidin caused a stimulation of oxygen consumption only, and there was no decrease in the uptake during the 4 hours of observation. In a few cases a decrease occurred but only when enough acid had been produced by the cells to lower the pH considerably.

For the simultaneous determination of the O_2 consumed and the CO_2 produced by the technic mentioned the spermatozoa were suspended in Ringer bicarbonate solution. Although the pH of this medium was approximately 7.5 the oxygen consumption of the spermatozoa in this case was reduced 70 to 80% by the addition of gramicidin (Table I), when measured at the end of one hour. Therefore, the stimulating action of gramicidin in alkaline phosphate buffer unaccompanied by final inhibition may not be due to the pH alone, for in bicarbonate at the same pH the depression in oxygen consumption is considerable. However, a direct comparison cannot be made, for in the former experiments air was present, whereas in the latter an atmosphere of 95% O_2 and 5% CO_2 was used. These differing conditions must also be kept in mind in considering the glycolysis experiments in which the inhibition of glycolysis by gramicidin is only partial.

In these experiments it was found that gramicidin decreased the RQ (CO_2/O_2) from 1.0 to 0.8 in 2 cases. However, the reduction of respiration was such that in view of the small total volumes measured the differences may very well fall within the limits of accuracy of the method.[12]

As the crude gramicidin used in the original experiments was a mixture of gramicidin and tyrocidine, we studied the effect of both substances in purified form on the respiration of spermatozoa. The results described above were duplicated with the purified gramicidin, while tyrocidine showed a definite depression in oxygen consumption which, however, amounted to only about 10 to 20%, when 0.05 mg per ml was used.

[13] Henle, G., and Zittle, C. A., unpublished data.

TABLE I.

Influence of Gramicidin on Respiratory Quotient and Aerobic Glycolysis of Bull Spermatozoa.

Exp.	Millions of sperm per ml	Concen. gramicidin mg/ml suspension	RQ		Z_{O_2}			$Z_M^{O_2}$		
			Control	Gram-icidin	Control	Gram-icidin	% de-pression	Control	Gram-icidin	% de-pression
1	650	0.09 crude	1.02	0.80	27.1	6.5	76	36.6	22.8	38
2	335	0.10 "	1.01	0.85	27.9	7.0	75	30.0	25.5	15
3	200	0.05 "	0.98	1.10	16.2	5.1	69	42.7	23.4	45
4	180	0.02 "						48.0	31.0	35
5	155	0.05 purif.						41.6	17.4	58

$$Z_{O_2} = \frac{\text{mm}^3\ O_2}{100 \times 10^6 \text{ sperm/hour}}$$

$$Z_M^{O_2} = \frac{\text{mm}^3\ CO_2 \text{ (lactic acid)}}{100 \times 10^6 \text{ sperm/hour}}$$

Some of the experiments for the determination of glycolysis under aerobic conditions are cited in Table I. The values obtained with gramicidin showed a reduction in comparison with the controls, but the glycolysis continued in most cases over the period of observation. The inhibition of glycolysis caused by 0.05 mg of purified gramicidin per ml was on the average 42.5% (6 experiments). Purified tyrocidine in the same concentration caused only a depression of about 15% of the control values.

Similar experiments were conducted under anaerobic conditions. The inhibition of glycolysis amounted to about 45%, when the spermatozoa had been in contact with gramicidin for one hour, and in 2 to 3 hours the glycolysis was depressed up to 75% of the controls.

The motility of the spermatozoa in bicarbonate medium was greatly impaired by gramicidin under aerobic as well as under anaerobic conditions. The cells were found to be immobile in some cases, in others they no longer showed forward movements. The spermatozoa of the control suspensions were very actively motile in some experiments; in others, where the pH had dropped in a period of several hours the spermatozoa were less motile, but their activity could be restored by addition of a small amount of sodium bicarbonate to the suspension. Spermatozoa, whose motility had been impaired by gramicidin, could not be reactivated.

Discussion. The stimulation of the respiration of spermatozoa in moderately acid solution by small amounts of gramicidin, and the toxic action of larger amounts has been paralleled with other kinds of cells and reagents. The action of substituted phenols on the respiration of the fertilized eggs of *Arbacia punctulata*[14] is similar,

[14] Krahl, M. E., and Clowes, G. H. A., *J. Gen. Psysiol.*, 1940, **23**, 413.

and the action of detergents on the respiration of bacteria has in some cases shown this phenomenon also.[15] In the latter studies also a considerable effect of pH was observed; the cationic detergents were most active in the alkaline range, *i. e.*, when least ionized, and the anionic in the acid range. The degree of ionization can not explain the observed effect of pH on the activity of gramicidin, for gramicidin has recently[4] been described as a neutral polypeptide containing neither free amino nor carboxyl groups.

The inability of the purified gramicidin to hemolyze red blood cells in the presence of small amounts of glucose[6] eliminates this as an explanation of its toxic action in animals. The effect on spermatozoa we have observed suggests that perhaps its harmful action is exerted through the enzymes involved in respiration and glycolysis. Further studies will be necessary to determine the particular enzymes affected.

The action of gramicidin on spermatozoa seems to be of about the same magnitude as its action on pneumococci. Dubos has found 0.01 mg completely inhibits the growth of 10^9 pneumococci within 2 hours; an enormous stimulation of the oxygen consumption of Gram-positive organisms occurs.[16] No pH effect was observed.

Summary. Crude and purified gramicidin after initial stimulation inhibits the oxygen consumption of bovine spermatozoa completely in Ringer phosphate of acid pH and renders the cells immobile. By the use of sufficiently small amounts only stimulation is observed during the period of the experiment. In alkaline phosphate buffer only the increase of oxygen consumption is noted. However, in Ringer bicarbonate medium of the same pH, the respiration again is greatly inhibited; aerobic as well as anaerobic glycolysis are depressed on the average 40% and the motility of the spermatozoa is markedly impaired. Tyrocidine, in the concentrations studied, caused a small reduction in the oxygen uptake and the glycolysis.

[15] Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, 1941, **73**, 249.

[16] Dubos, R. J., personal communication.