EFFECT OF PHOSPHATE AND CHLORIDE IONS ON AEROBIC METABOLISM OF BOVINE SPERMATOZOA

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INTRODUCTION

The oxygen consumption of mammalian spermatozoa is influenced by the type of diluent used. Both the respiratory rate and the motility of spermatozoa are greater when the semen is diluted with isotonic sodium chloride than with phosphate buffer. Although the pH of the unbuffered saline falls to pH 5-62, no deleterious effects are observed.

The present report extends the observations of Bishop & Salisbury (1955b). The data indicate that the aerobic metabolic activity of bull spermatozoa varies in isosmotic solutions of sodium salts according to the presence of chloride or phosphate ions.

MATERIALS AND METHODS

The collection of semen, estimation of cell concentration and semen pH have been previously described (Bishop & Salisbury, 1955a). A total of sixty-eight individual semen samples was collected, of which fifty three were used in the direct comparison of two diluents. Twenty-four of these semen samples were collected from 17 May to 21 July 1954, six from 16 October to 4 November 1954, and twenty-three samples from 21 January to 16 February 1955. The diluents used were isotonic (0.154M) sodium chloride and an isosmotic phosphate buffer at pH 7.0 (701.3 ml. of 0.13M-Na₂HPO₄.12H₂O and 280.0 ml. of 0.17M-NaH₂PO₄.H₂O). The osmotic pressure of both diluents, determined by freezing-point estimations in a Fiske osmometer, was 288 ± 5 milliosmols.

In most tests the metabolism of the spermatozoa was measured in diluted whole semen, but in some cases the cells were washed with isotonic sodium chloride or phosphate buffer. Whole semen was centrifuged for 10 min. at 1500 r.p.m. and the supernatant seminal plasma removed. Sodium chloride solution or phosphate buffer was added to restore the original volume and the spermatozoa were gently resuspended by drawing up and down in a large bore Pasteur pipette. The resuspended cells were again centrifuged, the supernatant fluid was removed and replaced with fresh diluent. The process was repeated once more to obtain twice-washed cells suspended in a medium free from seminal plasma.(56,529),(929,953)
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One part of semen or washed cells was diluted with 4 parts of diluent in the Warburg flasks. In order to measure immediate or transitory effects on respiration, 0.8 ml. of diluent was placed in the flask, and after a 10 min. equilibration period 0.2 ml. of semen or cells was added from the side arm.

Aliquots were taken at the beginning and end of each experiment to determine the fructose and lactic acid content of the suspensions. Fructose was determined by the method of Roe (1934) as modified by Mann (1948), and lactic acid by the procedure of Barker & Summerson (1941).

In an attempt to demonstrate the hexosemonophosphate pathway, a cell-free extract of sperm cells was prepared by disrupting the washed cells suspended in saline, in a 10 kV. Raytheon sonic oscillator at 9000 c./s. for 10 min. with glass beads. Glucose-6-phosphate (G-6-P) dehydrogenase or 'Zwischenferment', prepared by the method of Kornberg (1950), served as the standard of comparison. The optical density change of triphosphopyridine nucleotide (TPN) reduction was observed at 340 m\(\mu\) with the Beckman Model DU Spectrophotometer.

RESULTS

(1) Effect of phosphate and isotonic sodium chloride diluents on semen

The effect of sodium chloride and phosphate diluents on the average rate of \(O_2\) uptake, fructose utilization and the accumulation of lactic acid is summarized in Table 1.

Table 1. Effect of diluent on the metabolism of bovine semen

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Final motility</th>
<th>Aerobic activity</th>
<th>Lactic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>Rate</td>
<td>(-Z_{O_2}) ((\mu)g.)</td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>26 ±15</td>
<td>1 ±1</td>
<td>5 ±2</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>44 ±13</td>
<td>2 ±1</td>
<td>10 ±3</td>
</tr>
</tbody>
</table>

\(* Z = \text{activity/}10^8 \text{spermatozoa/hr. Redenz (1933).} \)

Mean of fifty-three semen samples from twelve bulls. Initial motility 51 ± 9%. Initial rate of motility 3 ± 0. Mean cell count 2.77 \(\times\) 10^7 ± 0.73 cells/ml. of diluted semen.

The average fructose utilization during aerobic incubation was about 20% higher in the phosphate than in the chloride diluent, and more lactic acid was accumulated in the phosphate diluent. The increase of lactic acid determined at the end of the incubation period averaged 65% of the fructose utilized in the phosphate and 26% in the saline diluent. However, the variability observed, as indicated by the standard deviations shown in Table 1, was large. One hundred per cent or more of the fructose utilized was found as lactic acid in fifteen of the fifty-three semen samples diluted in phosphate and in only nine diluted in saline.

When these semen samples were grouped according to periods of collection the following differences were noted. The average lactic acid determined for the fructose utilized was 59.1% for twenty-four phosphate-diluted semen samples collected in May, June and July. On the other hand, the accumulation of lactic
acid averaged 4.3% in chloride-diluted samples. In the October-November collections the average accumulation was 100% for the six semen samples diluted in each diluent. For the January-February collections, the average was 62% in the phosphate and 33% in the chloride solution. The reasons for these differences in the metabolic behaviour of sperm cells with the seasons of the year are not known.

The fifty-three semen samples collected from twelve bulls were not distributed equally in the different seasons of the year. Thus, a random selection of the data was made to obtain an unbiased estimate of the effect of the diluents and season.

![Figure 1](image)

**Fig. 1.** Effect of diluent on semen respiration. Mean oxygen uptake of fifty-three semen samples diluted 1:4 with 0.9% saline and isosmotic sodium phosphate at pH 7.0.

Thirty samples were proportionally distributed among the bulls for the analysis of variance of these data. It was found that the time of the year did not affect the oxygen uptake but that the differences in fructose utilization and lactic acid formation from one season to another were significant ($P<0.01$ and $<0.05$ respectively). Between the two diluents, chloride and phosphate, the differences of $O_2$ uptake and lactic acid formation were both highly significant ($P<0.01$), but for fructose utilization no significant difference ($P>0.05$) was present.

Characteristically, the $Z_{O_2}$ values in the phosphate diluent averaged about one-half as much as those in the chloride diluent. The initial rate of oxygen consumption (Fig. 1) was maintained without appreciable change throughout the 4 hr. period in the chloride diluent; while that in the phosphate diluent was less and declined somewhat more rapidly. Motility of the cells was also maintained better in samples diluted with chloride than in those diluted with phosphate.
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(2) The effect of washing spermatozoa with sodium phosphate or isotonic sodium chloride and subsequent dilution

During the January–February collection period, when the total volume of the individual ejaculates permitted, one-third of each sample was used for the experiments reported above. The other two-thirds were washed twice, one-half with phosphate diluent and the other half with chloride diluent. Each of these was subsequently resuspended in the respective diluent to give approximately the original cell concentration. These were then split into two parts and diluted 1:4, one with the diluent used for washing plus 2.0 mg. of fructose per ml., and the other with seminal plasma. The seminal plasma, which had been withdrawn after the first centrifugation, was diluted in the proportion of 0.2 ml. plasma to 0.6 ml. of the diluent and added to the cells.

The process of washing reduced the percentage of motile cells by about 5% in chloride diluent and slightly more in the phosphate. The results of the metabolic measurements, the final percentage of motile cells, and the rate of motility after 4 hr. are given in Table 2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Diluent</th>
<th>Final Motility</th>
<th>Aerobic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>None</td>
<td>HPO₄⁻</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>None</td>
<td>Cl⁻</td>
<td></td>
<td>41</td>
</tr>
<tr>
<td>HPO₄⁻ washed</td>
<td>HPO₄⁻ + fruct.</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>HPO₄⁻ washed</td>
<td>HPO₄⁻ + s.p.*</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Cl⁻ washed</td>
<td>Cl⁻ + fruct.</td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Cl⁻ washed</td>
<td>Cl⁻ + s.p.*</td>
<td></td>
<td>39</td>
</tr>
</tbody>
</table>

* s.p. = seminal plasma. Mean of twenty-three semen samples.
Initial motility: unwashed, 51% + 3; Cl⁻ washed, 46% + 3; HPO₄⁻ washed, 44% + 3.
Mean cell count 2.55 x 10⁹ cells/ml. of diluted semen.

The respiration rate and fructose utilization were lower in samples washed in phosphate, diluted in phosphate-fructose (or in seminal plasma plus phosphate) than in those in which the phosphate was replaced with chloride. However, the proportion of lactic acid level in relation to the fructose utilized was greater than could be accounted for by the loss of fructose. When carbohydrate was added to washed cells suspended in chloride, about one-sixth of the fructose utilized was detected as lactic acid. But in the presence of seminal plasma a much higher proportion of lactic acid accumulated.

(3) Effect of varying osmotic pressures with phosphate and chloride diluents

The oxygen consumption data for these experiments, conducted with semen collected in May and June and diluted 1:4 with either phosphate or chloride solutions of varying concentrations, are shown in Fig. 2. Eleven ejaculates provided
the data for each point of curve A. Four other individual semen samples, two of which were of sufficient volume for complete duplications, provided the data for curve B which illustrate the repressive effect of phosphate concentration on respiration.

![Graph](image)

**Fig. 2. Effect of osmotic pressure on semen respiration.**

The greatest $Z_{O_2}$ was at a concentration of chloride having an osmotic pressure (approximately 8 atmospheres) similar to that of semen. [The osmotic pressure of semen is 7.2–7.6 atmospheres at 37° C. (Salisbury, Knodt & Bratton 1948; Rothschild & Barnes, 1954).] Fructose utilization and lactic acid accumulation were also greatest at that osmotic pressure. In curve B the $O_2$ uptake parallels that of curve A up to a molar concentration of 0.05M (approximately 3.9 atmospheres); then an inhibitory effect supervenes. The fructose utilization and lactic acid accumulation were again greatest not at the point of greatest $O_2$ uptake but at the approximate osmotic pressure of semen.

(4) *Addition of catalase to whole semen*

In order to eliminate the possible oxidation of amino-acids as a source of $H_2O_2$ (Tosic & Walton, 1950), which depresses motility and oxygen consumption in the phosphate solution, a series of samples was split four ways with two portions of each suspended in chloride diluent and two in phosphate diluent. To one of the subsamples of each diluent crystalline catalase was added to a final concentration of 1:10,000. The added catalase depressed respiration slightly in the phosphate (by an average of 9%) and increased it slightly in the chloride (by an average of 5%). The essential differences between the two diluents in the metabolism of semen, however, were not altered by the addition of catalase. Thus the formation
of toxic amounts of $\text{H}_2\text{O}_2$ from oxidation of amino-acids or of other substrates does not seem to be the primary cause of the inhibition of respiration and motility observed in the phosphate diluent.

(5) Tests for the hexosemonophosphate pathway

Experiments were undertaken to demonstrate the presence of the hexose-monophosphate pathway. The oxidation of glucose-6-phosphate (G-6-P) with triphosphopyridine nucleotide (TPN) as the hydrogen acceptor yields 6-phosphogluconate (6-PG) and reduced triphosphopyridine nucleotide (TPN.H).

Observations indicated that TPN reduction either did not occur—or amounted to less than $0.005 \mu\text{M/10 min.}$—with the sonic extract of twice-washed spermatozoa ($29.2 \times 10^8$ cells/ml.). Diphosphopyridine nucleotide (DPN) also failed to serve as a hydrogen acceptor under similar conditions. TPN.H generated by yeast G-6-P dehydrogenase in the presence of a limiting amount of G-6-P was reoxidized in an aerated cuvette at a rate less than $0.004 \mu\text{M/10 min.}$, indicating that TPN.H dehydrogenase activity was not masking the G-6-P dehydrogenase of the sperm cells. In addition, the rate of G-6-P oxidation by yeast dehydrogenase was the same in the presence or absence of sperm extract. Thus an inhibitor of this reaction was not responsible for the lack of dehydrogenase activity.

The oxygen consumption of the whole semen in chloride diluent, before sonic oscillation, was $8.7 \mu\text{l./hr./10}^8 \text{ cells at 37}^\circ \text{C. measured in air.}$ On the assumptions that (1) all of the oxidation was mediated through the hexosemonophosphate pathway, and (2) all of the dehydrogenase activity was present in the extract, the theoretical rate of reduction should have been $0.112 \mu\text{M/10 min.}$ It was concluded that G-6-P dehydrogenase, if present in the cells in such concentration, could have been easily detected in the sonic extracts. However, the reaction was at best $26.8\%$ of the theoretical value.

DISCUSSION

In most cases with the phosphate-diluted semen lactic acid appeared to have been oxidized or not to have been formed since the accumulated lactic acid was less than the fructose utilized. In most of the washed cells diluted in phosphate-fructose diluent the accumulation of lactic acid was greater than the loss of fructose. This would suggest that an endogenous substrate was utilized for the formation of the excess lactid acid.

The lactic acid accumulation of semen and of washed cells, both in chloride diluent, was, however, much lower than when phosphate was used as the diluent. The oxygen consumption in chloride diluent was approximately twice as great as in phosphate, but was insufficient to account for the loss of lactic acid on a quantitative basis, except in one case. Since the addition of catalase to whole semen diluted in chloride or phosphate did not essentially alter the metabolic response, it appeared that the depression of respiration of whole semen diluted in phosphate was not due to $\text{H}_2\text{O}_2$. The motility of washed spermatozoa was maintained best in cells washed and resuspended in chloride diluent plus seminal plasma.
Further, a higher percentage of lactic acid accumulated in plasma-containing diluents than in diluted whole semen.

Oxygen may be partly involved in the initiation and maintenance of motility of bovine spermatozoa as in sea-urchin spermatozoa (Rothschild, 1948). Thus in the process of washing, inhibitory substances or waste products are removed and oxygen is more easily available to the organism. However, seminal plasma may contain substances which inhibit oxidative processes; hence the larger accumulation of lactic acid.

Phosphate ions in levels greater than 0.05 M (3.9 atmospheres) have an inhibitory effect on respiration; however, fructolysis appears to be influenced by the tonicity of the diluent. On the other hand, the tonicity of the chloride diluent influenced both respiration and fructolysis. Sperm cells washed and resuspended in chloride diluent utilized more fructose, but accumulated less lactic acid than those in phosphate diluent. Chloride ions apparently favoured the oxidation of lactic acid (or inhibited the formation of lactic acid), whereas phosphate ions, in the concentration used, inhibited the oxidation of this substance.

Though G-6-P and 6-PG dehydrogenase were not demonstrated, Mann (1951) and van Tienhoven, Salisbury, VanDemark & Hansen (1952) reported that intact spermatozoa utilized glucose in the presence of fructose. This would suggest that the hexosemonophosphate pathway may be operative in spermatozoa.

Significant differences were observed in the metabolic pattern of bovine spermatozoa collected during the different seasons of the year. Work is in progress at present to confirm these observations.

SUMMARY

Under the aerobic conditions of these experiments, dilution of bovine semen with phosphate ion depressed oxygen consumption and motility of sperm cells. However, fructose utilization and lactic acid accumulation were increased in the presence of phosphate ion. When semen was diluted with isotonic sodium chloride oxygen uptake was greater, motility was maintained better and fructose utilization and lactic acid accumulation were lower than in the phosphate-diluted semen.

Twice-washed cells in a diluent containing seminal plasma utilized more fructose and accumulated more lactic acid than those in plasma-free diluent. The depressive effect of phosphate on respiration was not due to $H_2O_2$ formation nor to changes of osmotic pressure.

The season of the year in which semen was collected influenced its metabolic response in each of the diluents studied. The presence of glucose-6-phosphate dehydrogenase activity was not conclusively demonstrated in cell-free extracts of bovine spermatozoa prepared by sonic oscillation.

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REFERENCES


