Carbon Dioxide Fixation by Bovine Semen, Washed Spermatozoa, and Seminal Plasma 1

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Abstract
Radioactive carbon dioxide, released by acidifying NaH\textsuperscript{14}CO\textsubscript{3} in a side arm of a Warburg flask, was incorporated by whole semen, washed spermatozoa, and seminal plasma. When the incubation mixture was fractionated into supernatant, total lipids, nucleic acids, and protein, radioactivity was recovered in both the supernatant and total lipid fractions of whole semen but only in the supernatant of washed spermatozoa. Fixation of \textsuperscript{14}CO\textsubscript{2} by washed spermatozoa was significantly less in Krebs-Ringer-phosphate buffer, pH 7.4, than in Krebs-Ringer buffer, pH 7.4. When pyruvate was not added, there was little or no CO\textsubscript{2} fixation by washed spermatozoa; however, substantial \textsuperscript{14}CO\textsubscript{2} incorporation occurred in the presence of 15mM pyruvate. Increasing pyruvate above 15mM resulted in no significant additional increase in CO\textsubscript{2} fixation. Malic enzyme activity was found in acetone powder extracts of washed bovine spermatozoa, and NADP\textsuperscript{+} and MnCl\textsubscript{2} were required for maximal activity.

Introduction
Incorporation of carbon dioxide into organic compounds by animal tissues has been established (6, 13, 15). Carbon dioxide plays an important role in the metabolism of spermatozoa; in the presence of CO\textsubscript{2} there was a marked stimulation of respiration (4), aerobic glycolysis (12), and fructolysis (7) by bull spermatozoa. However, the mechanism of action of CO\textsubscript{2} on the metabolism of bovine spermatozoa is obscure.

The objectives of this investigation were to measure carbon dioxide incorporation by bovine semen, washed spermatozoa, and seminal plasma, to study the effects of pyruvate and phosphate on CO\textsubscript{2} fixation by washed spermatozoa, and to study possible mechanisms for CO\textsubscript{2} incorporation by washed spermatozoa.

Experimental Procedure
Semen was collected from six bulls on a routine schedule by an artificial vagina. Following semen collection, volume, percent motility, and sperm cell concentration were determined. An aliquot of pooled first ejaculates from three bulls was centrifuged and the seminal plasma was removed. Spermatozoa were washed twice and resuspended in, depending on the experiment, Krebs-Ringer [(KR), 2mM phosphate] or Krebs-Ringer-phosphate [(KR-P), 20mM phosphate] buffer, pH 7.4 (14). Warburg flasks had one single side arm and one double side arm (14). Buffer, sodium pyruvate (20 \textmu moles), except in the pyruvate concentration study, and either whole semen (10\textsuperscript{9} cells per 0.5 ml), washed spermatozoa (10\textsuperscript{9} cells per 0.5 ml), or seminal plasma (0.5 ml) were placed in the main chamber of the flask, final volume, 1.0 ml. The double side arm was prepared by adding 0.6 ml of 2 N HCl to the smaller sac and 20 \textmu Ci (5.75 \textmu moles) of NaH\textsuperscript{14}CO\textsubscript{3} to the larger sac and 0.1 ml of 6 N HCl was added to the single side arm. After 5 min of equilibration in a 37 C water bath, the \textsuperscript{14}CO\textsubscript{2} was released into the gas phase of the flask by tipping 2 N HCl into the radioactive bicarbonate. Incubation at 37 C was continued for 60 min with constant agitation (84 cycles per min); then the reaction was stopped by tipping 6 N HCl into the incubation mixture. Potassium hydroxide (20% w/v) was added with a hypodermic needle through the self-sealing membrane of a rubber stopper into the center well and an additional 60 min at 37 C was allowed for absorption of CO\textsubscript{2}.

The incubation mixture was transferred to a centrifuge tube, centrifuged at 3,000 \times g for 10 min, and the supernatant removed. Aliquots were then dried and assayed for radioactivity with a thin-window gas flow counter (counting efficiency, 25%). The residue was further fractionated into the total lipids, nucleic acids, and protein according to the procedure described by Cohen (1). Boiled controls (95 C for 5 min) were included in all experi-

1 Authorized for publication as Paper 3851 in the Journal Series of the Pennsylvania Agricultural Experiment Station. Supported in part by Public Health Service Grant HD 00039-06.
TABLE 1. Total incorporation of $^{14}$CO$_2$ by whole semen, washed spermatozoa, and seminal plasma.$^a$

<table>
<thead>
<tr>
<th>Preparation tested</th>
<th>Control$^b$</th>
<th>Active</th>
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<tbody>
<tr>
<td>Whole semen</td>
<td>638 ± 150</td>
<td>13,227 ± 3,533</td>
</tr>
<tr>
<td>Washed spermatozoa</td>
<td>464 ± 119</td>
<td>4,188 ± 1,080</td>
</tr>
<tr>
<td>Seminal plasma</td>
<td>170 ± 47</td>
<td>1,566 ± 457</td>
</tr>
</tbody>
</table>

$^a$ Mean and standard deviation for six determinations.

$^b$ Preparation tested was boiled at 95°C for 5 min before incubation.

As shown in Table 3, a significant incorporation of radioactive CO$_2$ occurred in washed bovine spermatozoa suspended in either KR or KR-P buffer at pH 7.4. However, total fixation of $^{14}$CO$_2$ by washed spermatozoa was significantly less in KR-P than in KR.

The effect of various concentrations (0, 15, 30, 60 mM) of pyruvate on $^{14}$CO$_2$ incorporation was measured in washed spermatozoa (KR, pH 7.4). With no pyruvate added to the system, little $^{14}$CO$_2$ incorporation took place, 842 ± 191 cpm per incubation flask. Concentrations of 15, 30, and 60 mM pyruvate all significantly increased $^{14}$CO$_2$ incorporation, 4,037 ± 510, 4,941 ± 471, and 4,033 ± 490 cpm per incubation flask. But above 15 mM there was little evidence of additional response.

Malic enzyme activity was measured by the procedure of Ochoa (10). Malic enzyme activity, 33.7 μmoles NADPH formed per milligram of protein, was determined in acetone powder extracts (40 mg per ml) of washed bovine spermatozoa. In the absence of NADP$^+$ and MnCl$_2$, malic enzyme activity was reduced by 86 and 100%, respectively.

Discussion

These investigations have demonstrated that CO$_2$ fixation occurs in bovine spermatozoa. The results are consistent with the theory that bicarbonate, which is the sole sperm-stimulating component in oviduct fluid, may be responsible for the increase in respiration of capacitated spermatozoa compared to ejaculated spermatozoa (3) by providing an anaplerotic means for the replenishment of oxaloacetate (via malate) that is required for the operation of the citric acid cycle. Therefore, the ability of spermatozoa to fix CO$_2$ may be important in the capacitation process.

In agreement with O'Shea and Wales (11) we found more carbon dioxide fixation occurring...
TABLE 3. Effect of added phosphate on total incorporation of $^{14}$CO$_2$ by washed bovine spermatozoa.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>KR buffer</th>
<th>KR-P buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control$^b$</td>
<td>714 ± 217</td>
<td>324 ± 191</td>
</tr>
<tr>
<td>Active</td>
<td>4396 ± 968</td>
<td>2253 ± 160</td>
</tr>
</tbody>
</table>

$^a$ Mean and standard deviation of four determinations.

$^b$ Washed spermatozoa were boiled at 95°C for 5 min before incubation.

$^c$ Significantly different from each other and from the respective control (P < 0.05).

in semen than in washed spermatozoa and seminal plasma. They concluded that this was presumably due to endogenous substrates in whole semen acting as metabolic traps.

Most of the radioactivity was incorporated into the supernatant fraction of both whole semen and washed spermatozoa. This finding suggests that the majority of CO$_2$ fixation reactions in spermatozoa are involved with connecting the glycolytic pathway to the oxidative system. Mounib and Eisan (9) reported that almost all of the $^{14}$CO$_2$ incorporation by washed cod spermatozoa occurred in the supernatant. Of the three preparations only whole semen incorporated a significant amount of $^{14}$CO$_2$ into total lipids. These data indicate that seminal plasma may contribute substrates or cofactors for lipid synthesis or both.

Several factors including pyruvate concentration (2) apparently influence the rate of carbon dioxide fixation in some animal tissues. In our study carbon dioxide fixation by washed spermatozoa was depressed in the absence of pyruvate. Kraft and Lodge (5) reported that pyruvate was required for anaerobic carbon dioxide uptake by bovine spermatozoa. The rate of $^{14}$CO$_2$ fixation by washed spermatozoa was reduced with a 10-fold increase in phosphate concentration (2 to 20mM) of the diluent. Mann noted (8) that phosphate concentrations in the range of 15 to 80mM depress metabolism.

Malic enzyme activity was demonstrated in acetone powder extracts of bovine spermatozoa. Mounib and Eisan (9) reported similar results in cod sperm. One possible mechanism for fixation of CO$_2$ with pyruvate by bovine spermatozoa is by way of the malic enzyme pathway which could provide a mechanism for the replenishment of oxaloacetate for the operation of citric acid cycle and for the synthesis of amino acids. However, the possibility cannot be excluded that other pathways such as pyruvate carboxylase may be involved in CO$_2$ fixation by bovine spermatozoa.

References