Mitochondrial Metabolism of Pyruvate in Bovine Spermatozoa*

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Treatment with the polyene antibiotic, filipin, renders the spermatozoan cell membrane permeable to small molecules, but not to the intracellular enzymes aldolase and lactate dehydrogenase. Pyruvate (10 mM) as the sole substrate was metabolized very slowly. L-Carnitine increased pyruvate metabolism 3- to 4-fold and allowed limited rates of oxidative phosphorylation. When spermatozoa treated with filipin were supplemented with malate, there was a rapid, almost linear rate of pyruvate metabolism which was slightly increased by L-carnitine. In the absence of malate, 20 to 30% of the pyruvate used was reduced to lactate; this increased to 57% in the presence of malate. Without malate, about 90% of the pyruvate metabolized was converted to lactate and acetate or L-acetylcarnitine. Rutamycin or rotenone increased both the rate of pyruvate use and the lactate/acetate ratio. Under all treatments, L-carnitine consistently reduced the percentage of pyruvate converted to lactate by about 10%; part of the pyruvate was preferentially shunted into L-acetylcarnitine rather than lactate. The mitochondrial inhibitors, rotenone or rutamycin, did not change the amount of pyruvate that was converted to metabolites other than lactate, or L-acetylcarnitine, or both.

Pyruvate-supported State 3 respiration was linear only if L-carnitine, or malate, or both, were added to the incubation medium. Added malate was necessary to produce a rapid State 3 respiratory rate and was also required for significant respiratory activity in the presence of rotenone or rutamycin.

From cells metabolizing [2-14C]pyruvate (1.4 mM), 14C-labeled acid-extractable metabolites were separated by ion exchange column chromatography. All of the [2-14C]pyruvate (±5%) used was recovered in 14C-labeled metabolites and 14CO2. In the presence of malate, citrate accumulation was significant, and was always large in comparison to flux through the citric acid cycle. Glutamate, β-hydroxybutyrate, acetoacetate, fumarate, aspartate, and α-ketoglutarate did not accumulate in significant amounts. Some 14C-labeled succinate was produced but only in the presence of malate. Alkaline hydrolysis of a fraction containing carnitine esters yielded acetate and a compound tentatively identified as β-hydroxybutyrate or lactate.

As in intact cells, intramitochondrial lactate dehydrogenase competes successfully with the electron transport system for the NADH generated by pyruvate metabolism. The role of lactate and L-carnitine, and conclusions suggested by the accumulation of certain metabolites are discussed in relation to control of citric acid cycle activity.

The natural substrates available to spermatozoa include endogenous lipids (1), fructose which is in the seminal plasma (2), and glucose and lactate which are in the fluids of the female reproductive tract. However, pyruvate, a product of aerobic glycolysis, is also found in bull semen at 4.7 mg/100 ml (2). Seminal plasma from vasectomized bulls contains 17 to 47 mg of pyruvate/100 ml, indicating that pyruvate rapidly disappears in the presence of viable spermatozoa (3). More recently, it has been shown that pyruvate is necessary for in vitro capacitation of guinea pig sperm (4) and for maintenance of mouse gametes (5).

In ejaculated porcine spermatozoa and bovine epididymal spermatozoa, the stoichiometry of pyruvate metabolism does not fit that of a simple dismutation (6-8). In intact bovine spermatozoa, the intramitochondrial location of lactate dehydrogenase allows pyruvate to oxidize the NADH produced from its own oxidation (7, 8). It also allows pyruvate to serve as an energy source in the presence of rotenone, an inhibitor of NADH oxidation, and of rutamycin, an inhibitor of oxidative phosphorylation. Of several substrates tested, only pyruvate or a glycolyzable sugar were able to restore motility in the presence of these inhibitors.

Spermatozoa contain a high concentration of L-carnitine and high carnitine acetyltransferase activity (9, 10). Milkowski et al. (11) and Van Dop et al. (7, 8) have shown that added pyruvate rapidly acetylates the free carnitine pool, even under anaerobic conditions (8). The effects of L-carnitine on pyruvate metabolism in heart and blowfly flight muscle mitochondria have been well documented (12-15).

In view of the possible importance of pyruvate in intact spermatozoan metabolism, we have investigated mitochondrial pyruvate metabolism using the polyene antibiotic, filipin, to render the spermatozoan cell membrane permeable to small molecules (16). The results confirm the important role of intramitochondrial lactate dehydrogenase postulated for intact cells (8, 17). In addition, L-carnitine was found to influence pyruvate metabolism via pyruvate dehydrogenase and lactate dehydrogenase. The distribution of 14C label in acid-
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Preparation and Treatment of Bovine Epididymal Spermatozoa — Bovine caudal epididymal spermatozoa were prepared as described previously (8). The collecting medium contained 250 mM mannitol, and 15 mM Tris/Cl, pH 7.4. Sperm were sedimented at 600 x g for 5 min, washed twice in collecting medium, and suspended at a concentration of about 1 x 10⁹ cells/ml. Filipin treatments were washed twice and finally resuspended in the standard incubation medium. The standard incubation medium (similar to the one used by Morton and Lardy (18)) contained 15 mM phosphate, 10 mM MgSO₄, 0.5 mM ADP, 35 mM 2-deoxyglucose, 0.1 mg/mℓ of hexokinase (dialyzed into 15 mM phosphate, pH 7.4, immediately before use), and 250 mM mannitol, 15 mM Tris/Cl, pH 7.4. All acidic compounds were neutralized with Tris. In later experiments, and when 50 mM carnitine (pH 7.4) was added, the sperm were resuspended at a concentration of 1.5 to 2 x 10⁹ in the mannitol/Tris medium and diluted into the incubation medium. The final salt concentrations were identical as those stated above. After 5 min at room temperature, the filipin-treated cells were washed twice and finally resuspended in the standard incubation medium. The standard incubation medium (similar to the one used by Morton and Lardy (18)) contained 15 mM phosphate, 10 mM MgSO₄, 0.5 mM ADP, 35 mM 2-deoxyglucose, 0.1 mg/mℓ of hexokinase (dialyzed into 15 mM phosphate, pH 7.4, immediately before use), and 250 mM mannitol, 15 mM Tris/Cl, pH 7.4. All acidic compounds were neutralized with Tris. In later experiments, and when 50 mM carnitine (pH 7.4) was added, the sperm were resuspended at a concentration of 1.5 to 2 x 10⁹ in the mannitol/Tris medium and diluted into the incubation medium. The final salt concentrations were identical as those stated above. When L-carnitine was added to the sperm suspensions, the total osmolarity was kept below 390 to 400 mosM by deleting Tris/mannitol. Below 400 mosM, osmotic (81, but lactate production was much lower than in intact cells (93%). Small amounts of L-carnitine and L-acetylcarnitine were lost during the washing procedure. The small amount of L-carnitine remaining (approximately 10%) may be intramitochondrial. In addition, nucleotides and citric acid cycle intermediates were low or undetectable after treatment with filipin and washing.

It is not surprising that State 3 pyruvate metabolism (ADP present) in these cells was limited. Fig. 1A shows plots of pyruvate deacooxaration, and lactate and acetate accumulation. In the absence of 4-carbon dicarboxylic acids that permit more active metabolism, at least 70 to 80% of the pyruvate used was converted to lactate and lactate. Significant amounts of acetate were also produced in intact cells when electron transport was blocked with inhibitors or by anaerobiosis (8), but lactate production was much lower than in intact cells. This agrees with the data of Van Dop et al. (7, 8) which show that lactate production is an outlet for the mitochondrial NADH produced during pyruvate metabolism.

Effects of L-Carnitine and Malate on Pyruvate Metabolism — L-Carnitine increases decarboxylation of [1-14C]pyruvate in rat heart mitochondria (13). In filipin-treated bovine epididymal spermatozoa, L-carnitine addition enhanced the rate of pyruvate metabolism nearly 3-fold (Fig. 1B). There was a large accumulation of L-acetylcarnitine, but no measurable acetate. A concentration of 50 mM L-carnitine was used, because it approximates the intracellular carnitine concentration (about 90 mM). The intracellular carnitine concentration was estimated using the specific gravity of the droplet, mid-piece, and tail portions, the weight of a spermatozoon (2) and the total cellular L-carnitine (6, 24). This open system can only approximate intracellular conditions, because metabolites like L-acetylcarnitine can freely diffuse out of the filipin-treated cell. Lactate production was low but the sum of the lactate and L-acetylcarnitine accounted for more than 80% of the total pyruvate metabolized.

Malate addition, which provides a ready source of oxalacetate for citrate synthetase, resulted in a rapid, almost linear rate of pyruvate disappearance (Fig. 1C). Malate also has been shown to stimulate pyruvate-supported respiration in hypotonia treated rabbit sperm (25). Pyruvate disappearance was rapid in the presence of L-carnitine plus malate, although...
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FIG. 1 (left). Effects of L-carnitine and malate on pyruvate metabolism. Twice washed, filipin-treated bovine epididymal spermatozoa were incubated in the standard incubation medium with pyruvate (10.0 mM) (see "Materials and Methods"). L-Carnitine was 50 mM (B and D) and malate was 0.77 mM (C and D). The sperm concentrations were 7 x 10⁸ (A), 8.3 x 10⁸ (B), and 8.1 x 10⁸ (C and D) cells/ml. The following symbols were used: pyruvate, (O); acetate, (A); lactate, (C); L-acetylcarnitine, (□); and malate, (▲). The numbers in parentheses designate the percentage of pyruvate used that was converted to the respective metabolite.

The effects of L-carnitine and malate on pyruvate conversion to L-acetylcarnitine were significantly decreased by malate (compare Fig. 1, B and D). Lactate production was dramatically increased in the presence of malate (Fig. 1, C and D).

The effects of L-carnitine and malate on 2-deoxyglucose 6-phosphate synthesis are shown in Fig. 2. In filipin-treated cells metabolizing pyruvate alone, little phosphate was incorporated into 2-deoxyglucose unless L-carnitine was also added (Fig. 2, A and B). However, there was a rapid linear rate of synthesis with cells metabolizing pyruvate-malate (Fig. 2C); this rate was not influenced by L-carnitine (data not shown).

Mitochondrial Inhibitors - Fig. 3 illustrates the effects of rutamycin and rotenone on State 3 pyruvate-malate metabolism and lactate accumulation. The rate of pyruvate use was significantly increased over the corresponding control shown in Fig. 1C. As in untreated bovine epididymal spermatozoa (7, 8), the presence of an inhibitor of oxidative phosphorylation (rutamycin) or a site I inhibitor of electron transport chain (rotenone) increased the percentage of lactate formed from pyruvate. Both rotenone and rutamycin greatly decreased ATP synthesis, although there was a small net accumulation of 2-deoxyglucose 6-phosphate (data not shown). In cells oxidizing 10 mM malate in the absence of pyruvate, either rutamycin or rotenone eliminated all 2-deoxyglucose 6-phosphate accumulation. Malate alone, at the concentrations (about 0.8 mM) used in the experiments with pyruvate, did not support the synthesis of significant amounts of 2-deoxyglucose 6-phosphate. L-Carnitine had no effect on malate oxidation or ATP synthesis (data not shown).

The results of the metabolic experiments are summarized in Table I where the pyruvate used and the lactate, acetate, and acetylcarnitine produced at 15 min are compared. The dramatic effect of carnitine on the rate of pyruvate use (p < 0.001) is evident. The pyruvate converted to metabolites other than lactate and acetylcarnitine or acetate is quite small. Carnitine appeared to increase pyruvate use even in the presence of malate (p < 0.05), and the difference largely was accounted for as L-acetylcarnitine.

In the absence of L-carnitine, rotenone or rutamycin increased the rate of pyruvate use (p < 0.05). The percentage of pyruvate converted to lactate in the presence of inhibitors approached that seen in untreated cells (8). The lactate data support our hypothesis that pyruvate metabolism is intramitochondrial, with the intramitochondrial location of LDH-X' (21-23) allowing pyruvate to oxidize the NADH produced during its metabolism via the citric acid cycle.

In the intact cell, the intracellular carnitine is acetylated within the first 5 min after pyruvate addition (8). In the filipin-treated cells, the rate of acetylation of added free carnitine is linear throughout the 20-min incubation period. In cells oxidizing pyruvate, L-carnitine consistently reduced the percentage of pyruvate converted to lactate by about 10%. It appears that part of the pyruvate was preferentially shunted into L-acetylcarnitine, rather than to lactate. The data also indicate that during State 3, pyruvate dehydrogenase is probably not operating maximally in the absence of L-carnitine. Similarly, in the absence of malate, L-carnitine can activate pyruvate decarboxylation, possibly by freeing coenzyme A (15). This increased flux through pyruvate dehydrogenase produces NADH, which can be used to reduce pyruvate to lactate.

The abbreviation used was: LDH-X, lactate dehydrogenase-X.
or oxidized via the electron transport chain to produce ATP (see Fig. 2B). Without L-carnitine or malate, disposal of acetyl units produced by pyruvate dehydrogenase is limited and free acetate accumulates.

**Effects on Respiration** — Data obtained on the oxygraph support the data from the metabolic studies (see Fig. 4). In these experiments, the pyruvate concentration was lowered to 1.4 mM; however, the State 3 respiratory rate (ADP present) was the same as with 10 mM pyruvate. State 3 respiration supported by pyruvate was slow and nonlinear without L-carnitine (Fig. 4A). With L-carnitine, the respiratory rate increased 2- to 3-fold and remained linear until anaerobiosis. This agrees with earlier work by Bremer (12). Malate further increased the respiratory rate (3-fold) and this enhanced rate was also linear until anaerobiosis (Fig. 4B); this rate was not affected by L-carnitine. As expected, rotenone or rutamycin inhibited State 3 (Fig. 4C). Among the combinations tested, only antimycin A plus rotenone blocked respiration completely. Pyruvate (Fig. 4C) but not malate (Fig. 4D) supported respiration in the presence of rotenone. At 0.8 mM malate, respiration was almost undetectable.

Although the respiratory rate was low with rutamycin or rotenone, respiration decreased even further within 6 to 10 min. A second addition of pyruvate reinitiated respiration. This is predictable based on the rapid rate of pyruvate disappearance in the presence of rutamycin or rotenone (see Table I), but the oxygen consumed accounts for only a small portion of the pyruvate used (Fig. 4). It is also somewhat surprising that the addition of rotenone or rutamycin did not influence the amount of pyruvate that was converted to metabolites other than lactate, or acetylcarnitine, or both (Table I).

**Metabolism of [2-14C]Pyruvate Separation of 14C-Labeled Metabolites** — In order to determine the fate of pyruvate in these experiments, the chromatographic procedure of LaNoue et al. (19) was used to separate and quantitate 14C-labeled citric acid cycle intermediates. A typical elution pattern for cells metabolizing malate and [2-14C]pyruvate is shown in Fig. 5. While the order of elution resembled that of LaNoue et al. (19), the metabolite pattern was quite different from that of rat heart mitochondria. In addition to acetylcarnitine, Peak I contained a minor component (less than 1% of the counts added) that resisted alkaline hydrolysis and rechromatographed on Dowex 1 as Peak I. It ranged in size from 0.4 to 1.8 nmol/10^8 cells (calculation based on the specific activity of the added pyruvate). It was most likely alanine (19) and was not characterized further. The major product was usually acetate, derived from L-acetylcarnitine. A third product of alkaline hydrolysis of Peak I co-chromatographed with lactate; it usually ranged from 1 to 2 nmol and occasionally reached concentrations as high as 7 nmol/10^8 cells (3.5% of added disintegrations per min; Table III). This product appeared to be unaffected by borohydride reduction, which ruled out acetoacetate or keto acids. The chromatographic pattern suggested it was lactate or possibly β-hydroxybutyrate, although L-carnitine esters of either of these compounds have not been reported previously in biological systems. Peak II was identified as containing mainly acetate, some glutamate and small amounts of aspartate. Acetate was identified by co-chromatography with [14C]acetate standard and volatilization. Acetate

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**Table I**

**Effects of L-carnitine, malate, and mitochondrial inhibitors on State 3 pyruvate metabolism**

Conditions were the same as in Fig. 1. The sperm concentration ranged from 8 to 10 × 10^8 cells/ml. Pyruvate concentration was 10 mM. When added, malate was 0.8 mM, L-carnitine was 50 mM, rutamycin was 15 μM, and rotenone was 15 μM.

| Additions       | L-Carnitine | Pyruvate used | Lactate produced | L-Acetyl/carnitine produced | Pyruvate converted to lactate | Pyruvate converted to other metabolites |
|-----------------|-------------|---------------|------------------|----------------------------|-----------------------------|----------------------------------------|
|                 |             |               |                  |                            |                             |                                        |
| None            | –           | 171 ± 51 (3)  | 52 (2)           | 85* 30% (3)                | 10                          |                                        |
|                 | +           | 476 ± 40 (3)  | 126 ± 6          | 301 ± 30% (5)             | 20                          | 49                                    |
| Malate          | –           | 671 ± 61 (5)  | 383 ± 55         | ND 66% (6)                 | 57                          | 285                                   |
|                 | +           | 806 ± 78 (3)  | 406 ± 69         | 141 (2) 50% (4)           | 249                         |                                        |
| Malate + rutamycin | –         | 882 (2)       | 551 (2)          | ND 66% (5)                 | 272                         |                                        |
|                 | +           | 867           | 423              | 114 (2) 40% (5)           | 330                         |                                        |
| Malate + rotenone | –          | 870 (2)       | 586 (2)          | ND 69% (4)                 | 272                         |                                        |
|                 | +           | 845           | 527              | 92 (2) 62% (3)            | 227                         |                                        |

* Acetate produced; L-acetyl/carnitine was not measurable.

* ND, not detectable.
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Fig. 4. Effect of various treatments on State 3 respiration of filipin-treated spermatozoa. In A to C, the sperm concentration was 3.2 x 10^8 cells/ml, pyruvate was 1.5 mM, malate was 0.8 mM, and, when added, L-carnitine was 50 mM. In D, the sperm concentration was 1.9 x 10^8 cells/ml and malate was 8 mM. The numbers in parentheses are respiratory rates expressed as nanogram atoms of oxygen/4 x 10^8 cells/min. Inhibitors were added at the following concentrations: rotenone, 15 µM; rutamycin, 13 µM; antimycin A, 2 µM. In C, rotenone, rutamycin, or antimycin A was added immediately after the substrates.

Fig. 5. Separation of 14C-labeled, acid-soluble metabolites by anion exchange column chromatography from spermatozoa oxidizing malate and [2-14C]pyruvate. The incubation conditions are described under "Materials and Methods" and the chromatographic procedure in (19). The elution rate was 2.0 ml/min and the eluent was collected in 2.0 ml fractions. I, acetylcarnitine (see "Results"); II, acetate, glutamate, aspartate; III, lactate; IV, acetoacetate (see "Results"); V, succinate; VI, malate; VII, pyruvate; VIII, citrate, α-ketoglutarate, fumarate (see "Results").

comprised 50 to 60% of this peak and in the absence of malate or L-carnitine was nearly the sole component. β-Hydroxybutyrate which should have chromatographed in this region was not detected under any treatments tested, including prolonged incubation. Glutamate (II), aspartate (II), lactate (III), malate (VI), pyruvate (VII), citrate (VIII), and α-ketoglutarate (VIII) were measured enzymatically. One hundred per cent of Peak V was identified as succinic acid by recrystallization of the pooled material with carrier succinic acid. Occasionally, a small peak (0.2% of added disintegrations per min) was found near Fraction 40 (Peak IV). It was probably acetoacetate (19) but in such small amounts that it was not detectable by enzymatic analysis of the perchloric acid extracts of any treatment group. Citrate comprised the major portion of Peak VIII. Fumarate was too low to be measured enzymatically in the pooled peak. Similarly, α-ketoglutarate never exceeded 1 to 2 nmo/10^8 cells, the limit of detectability. Marker α-ketoglutarate did chromatograph in this region, immediately after the citrate peak. Isocitrate was not measured.

14C-labeling of Lactate, Acetate, and L-Acetylcarnitine—The effects of various mitochondrial inhibitors on 14C-labeling of citric acid cycle intermediates are shown in Table II. Essentially all of the initial [2-14C]pyruvate (±5%) used was recovered in 14C-labeled metabolites and 14CO2. The labeling pattern for the major metabolites fits that seen with 10 mM pyruvate. In the absence of malate, the rate of pyruvate metabolism was low and only about 30% of the added pyruvate was metabolized. In the short period of 4 min, and with the lower pyruvate concentration (Table II), the effect of L-carnitine on pyruvate use was not obvious. Fig. 1A and oxygraph data (Fig. 4) showed that the enhancement by carnitine of pyruvate disappearance and oxidation was most apparent after the first 5 min. Again, lactate production was low while acetate and acetylcarnitine have the reciprocal relationship seen in Fig. 1, A and B.

In agreement with the data of Table I (10 mM pyruvate), rutamycin or rotenone enhanced the rate of pyruvate disappearance. As in intact cells (8), neither antimycin A nor antimycin A plus rotenone blocked pyruvate use.

L-Carnitine did not influence pyruvate disappearance in the presence of inhibitors, but did increase the counts recovered in Peak I (see Table II), partly at the expense of lactate. At least 10 to 20% of the pyruvate used was recovered as L-acetylcarnitine. The results of typical alkaline-hydrolysis patterns (Peak I) for rutamycin or rotenone treatments are shown in Table III. The small amounts of endogenous free L-carnitine present at the time of substrate addition were acetylated within 4 min. Incorporation of 14C-label into L-acetylcarnitine was significant in the presence of L-carnitine; the unknown (lactate?) fraction was highest in the rotenone-treated samples.

Citrate Production—Citrate accumulation was significant in the presence of malate. In a nonradioactive experiment, the average amount of citrate that accumulated in 4 min (44 nmo/l/10^8 cells) closely approximated malate used (58 nmo/l/10^8 cells). In the presence of inhibitors, citrate accumulation was 36 nmo/l versus 42 nmo/l of malate used. In all cases, citrate accumulation was large in comparison to flux through the citric acid cycle as indicated by the negligible release of 14CO2 (Table II).
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TABLE II
Effects of mitochondrial inhibitors on 14C-labeling of metabolites in cells metabolizing [2-14C]pyruvate

Incubation conditions are described under "Materials and Methods." The sperm concentration ranged from 6 to 8 x 10^8 cells/ml. Pyruvate was 1.4 mM and malate, when added, was 0.8 mM. L-Carnitine was added at 50 mM, rotenone at 15 μM, rutamycin at 13 μM, and antimycin A at 1 μM. Roman numerals heading five of the columns designate the chromatographic fraction and the major components of the fractions are designated.

| Additions               | L-Carnitine | Pyruvate used | I Acetyl-carnitine | II Acetaldehyde + glutamate | III Lactate | VI Malate | VIII Citrate | 14CO2 |
|-------------------------|-------------|---------------|-------------------|-----------------------------|-------------|-----------|-------------|-------|
| None                    | –           | 28*           | 11                | 25                          | 15          | 3         | <1          | 3     |
| Malate                  | +           | 32            | 36                | 10                          | 14          | <1        | 4           | 1     |
| Malate + rutamycin      | –           | 70            | 9                 | 4                           | 74          | 8         | 25          | 7     |
| Malate + rotenone       | +           | 42            | 14                | 6                           | 34          | 4         | 29          | 1     |
| Malate + antimycin A    | –           | 92            | 7                 | 6                           | 86          | 4         | 30          | 3     |
| Malate + rotenone       | +           | 94            | 31                | 6                           | 78          | 4         | 22          | 2     |
| Malate + antimycin A    | +           | 89            | 12                | 9                           | 124         | 5         | 15          | 5.5   |
| Malate + antimycin A + rotenone | –           | 81            | 32                | 7                           | 101         | 6         | 11          | 2.5   |
| Malate + antimycin A + rotenone | +           | 62            | 18                | 6                           | 59          | 2         | 27          | <1    |
| Malate + antimycin A + rotenone | –           | 44            | 4                 | 8                           | 54          | <1        | 21          | <1    |
| Malate + antimycin A + rotenone | +           | 40            | 13                | 8                           | 49          | <1        | 19          | <1    |

* Calculation based on the specific activity of the [2,14C]pyruvate.
Per cent of initial pyruvate used.

Also, the specific activity of citrate formed at 4 min was essentially the same as that of the added pyruvate. Therefore, a continuous flux of pyruvate through the citric acid cycle did not occur at a significant rate. Citrate accumulation was not influenced by any mitochondrial inhibitors, except possibly rotenone (Table II).

14C-Labeling of Malate Pool – The data in Table II suggest that limited 14C-label is entering the malate pool. This was measured quantitatively in several experiments. For example, after 4 min of incubation, the specific activity of the remaining malate was 7 to 9% of that of the added pyruvate. Even after 20 min, the specific activity of the malate was only one-third of the pyruvate. Malate use was similar under all conditions tested.

14C-Labeling of Minor Metabolites – Accumulation of 14C-labeled succinate was variable. The largest labeling of succinate from [2,14C]pyruvate occurred with malate in the absence of inhibitors (5 nmol/10^8 cells in 4 min); no 14C-labeled succinate was found in the absence of malate. Enzymatically assayed glutamate and fumarate varied from about 4 to 12 nmol/10^8 cells in 4 min, and were not influenced by the various inhibitors. At zero time neither glutamate, fumarate, nor malate were detectable and aspartate was less than 5 nmol/10^8 cells. Aspartate never increased above 6 nmol/10^8 cells during incubation with pyruvate-malate.

DISCUSSION

The importance of carnitine in fatty acid oxidation has been recognized since the report of Fritz (26). Later, carnitine was shown to have a significant function in carbohydrate use, specifically pyruvate metabolism, in blowfly flight muscle (15). During flight, pyruvate is the end product of glycolysis, either α-glycerophosphate nor lactate accumulate, but a large share of the pyruvate is transaminated to alanine (27). Reports from Casillas (10, 24) and this laboratory (7, 8, 15) suggest that carnitine and acetylcarnitine are important in bovine spermatozoa carbohydrate and fatty acid metabolism. Pyruvate metabolism in filipin-treated bovine epididymal spermatozoa is quite different from that in blowfly flight muscle (15) or heart mitochondria (18, 28) because of the intramitochondrial location of LDH-X.

The observations made on intact spermatozoa oxidizing pyruvate have been confirmed with filipin-treated cells in which the mitochondria are readily accessible to added metabolites and co-factors. The data indicate that intramitochondrial lactate dehydrogenase competes successfully with the electron transport system for the NADH generated by pyruvate metabolism. Malate addition increased the percentage of pyruvate converted to lactate as a result of increased NADH production by malate dehydrogenase and some increase in pyruvate flux through the citric acid cycle. Increased lactate production was also observed in ejaculated human spermatozoa oxidizing pyruvate plus fumarate or succinate (29). The percentage of pyruvate converted to lactate was greater under aerobic than anaerobic conditions in ejaculated porcine spermatozoa (5).

Addition of rutamycin or rotenone to intact bovine epididymal spermatozoa resulted in a Δlactate/Δpyruvate ratio of 0.8 which indicates that the pyruvate not reduced to lactate was completely oxidized via the citric acid cycle and that all of the NADH produced during pyruvate oxidation was used to reduce pyruvate via lactate dehydrogenase (8). In filipin-treated cells, with rotenone present to block NADH oxidation at Site I, the Δlactate/Δpyruvate ratio would be 0.66 if all the pyruvate used which is not reduced to lactate is converted exclusively to lactate. This is close to the observed value of 0.69. If one NADH is allowed for each L-acetylcarnitine produced, this calculation also holds when L-carnitine is present. This calculation also holds when L-carnitine is present. This calculation also holds when L-carnitine is present.
tion (about 1.4 mM) a calculation of NADH produced, based on the metabolite pattern and $^{14}$CO$_2$ data, indicates that when NADH oxidation was blocked with rotenone, there was a good correlation between potential NADH and lactate accumulated.

L-Carnitine stimulates the oxidative decarboxylation of pyruvate in mitochondria from several tissues (13-15). L-Carnitine competes successfully with oxalacetate for acetyl-CoA at low concentrations of citric acid cycle intermediates (15) or high pyruvate concentration (12). Carnitine also enhances pyruvate oxidation in filipin-treated bovine epididymal spermatozoa under similar conditions. L-Carnitine consistently reduced the percentage of pyruvate converted to lactate but flux of pyruvate into the citric acid cycle was not significantly altered. L-Acetylcarnitine can thus serve as a reserve of "active" acetate. L-Carnitine may reduce $^{14}$CO$_2$ release from [2-$^{14}$C]pyruvate, but flux through the citric acid cycle (at 4 min) is so low as to make the data inconclusive.

The fact that L-carnitine did not change the amount of pyruvate that was metabolized via the citric acid cycle suggests that pyruvate dehydrogenase activity does not appear to be rate-limiting during State 3 pyruvate-malate oxidation. The factor(s) that control sperm pyruvate dehydrogenase activity are still unknown. Although citrate is an inhibitor of rat skeletal muscle pyruvate dehydrogenase (30), there is no clear evidence for regulation by citrate in filipin-treated bovine epididymal spermatozoan mitochondria. The assay conditions used in these experiments - high ADP (31) and high Mg$^{2+}$ and phosphate (32) - would favor maximal dehydrogenase activity. Although citrate is an inhibitor of rat pyruvate dehydrogenase activity (31, 36), control by citrate in filipin-treated bovine epididymal spermatozoan mitochondria is not certain whether aconitase maintains equilibrium under all the conditions tested. Data from heart (34) and blowfly (35) suggest there may be a block at aconitase or isocitrate dehydrogenase. Because neither isocitrate nor the intra- versus extramitochondrial oxalacetate concentrations. It is clear that the limited accumulation of a-ketoglutarate and succinate preclude any significant exchange of these two acids with malate.

It has been suggested that a high ratio of NADH to NAD inhibits flux through the citric acid cycle (34, 36). Control exerted by high concentrations of NADH in sperm mitochondria seems unlikely for pyruvate maintains spermatozoan NAD greater than 90% oxidized (37).

Finally, the necessity for a number of substrate-linked cycles (for a review, see Ref. 38) to transfer reducing equivalents from the cytosol to the mitochondria is limited because lactate produced during glycolysis can be oxidized directly by the mitochondria. Several recent reports have postulated a shuttle system involving mitochondrial lactate dehydrogenase-X (17, 39). In addition, a citrate-malate exchange could be used to shuttle reducing equivalents from cytosol to mitochondria during aerobic glycolysis when pyruvate is produced (40). Therefore, use of filipin-treated bovine epididymal spermatozoa can provide a unique system for studying control of citric acid cycle activity.

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