Regulation of glucose metabolism by adenine nucleotides in round spermatids from rat testes

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Regulation of glucose metabolism in glycolysis by round spermatids was studied. Assay of activities of 11 glycolytic enzymes in cell-free spermatid extracts showed that hexokinase, phosphofructokinase, and glyceraldehyde-3-phosphate dehydrogenase had the lowest activities. When the cells were incubated with glucose (10 mM), the intracellular level of ATP fell rapidly and 5'-AMP increased. The ADP level remained unchanged. During incubation with glucose, fructose-1,6-bisphosphate, dihydroxyacetone phosphate, and glyceraldehyde-3-phosphate were accumulated without any change in a mass action ratio of fructose bisphosphate aldolase. Glyceraldehyde-3-phosphate dehydrogenase appeared to play a regulatory role in glycolysis. Glyceraldehyde-3-phosphate dehydrogenase was inhibited by the following compounds (K_i values in parentheses): adenosine (4.34 mM), 5'-AMP (3.50 mM), ADP (2.35 mM), ATP (5.34 mM), and 3',5'-cAMP (0.60 mM). In each case, the inhibition was competitive with NAD (K_i = 0.20 mM). The 2'-hydroxy group of the adenine-linked ribose moiety was essential for binding. The compounds adenosine, 2'-deoxyadenosine, 2'-AMP, 3'-AMP, CTP, UTP, and NAD showed little inhibition. These findings suggest that regulation of glycolysis in round spermatids by glyceraldehyde-3-phosphate dehydrogenase is most likely and that glyceraldehyde-3-phosphate dehydrogenase is inhibited by the adenine nucleotides, particularly by 5'-AMP and ADP as inhibitors competitive with NAD.

It has been known that glucose is a major substrate for testicular metabolism (1). Several reports indicate that glucose is mainly utilized by spermatids and spermatocysts (2–4). In fact, glucose was shown to stimulate protein synthesis and oxygen consumption of the isolated spermatids from rat testes (5, 6). However, recent studies from this (7–9) and other laboratories (10) have shown that lactate is a preferred substrate for spermatid metabolism. Although it is very likely that spermatids utilize exogenous lactate as a substrate for their metabolism, it was decided to examine glucose metabolism and its regulation in round spermatids from rat testes. We here report the regulation of glycolysis in spermatids by glyceraldehyde-3-phosphate dehydrogenase which is inhibited by adenine nucleotides that compete with NAD.

MATERIALS AND METHODS

Preparation of Spermatids—Round spermatids (steps 1–8) from 40-day-old rats (Sprague-Dawley strain) were prepared by the method of Nakamura et al. (6). The dissociated cells were separated by sedimentation velocity at unit gravity at 4 °C, using a 2-4% linear bovine serum albumin gradient in PBS (11). The spermatid fraction was greater than 80% pure and more than 97% of the cells prepared by this method exhibited trypan blue.

Determination of Glycolytic Intermediates and Adenine Nucleotides—Spermatids were incubated at 34 °C in 0.5 ml of PBS with glucose (10 mM). The incubation was stopped by injection of perchloric acid at a final concentration of 5%. The cells were homogenized with a Teflon-glass homogenizer (10 strokes) and centrifuged at 3,000 \( \times g \) for 10 min at 2 °C. The supernatant was neutralized to pH 6.8 by careful addition of cold saturated K_2CO_3. After keeping in ice for 30 min, the samples were centrifuged and the supernatant was used for the assay of glycolytic intermediates and adenine nucleotides. Determination of all the glycolytic intermediates was carried out spectrophotometrically by standard methods (12). Adenine nucleotides were determined spectrophotometrically by standard methods (13, 14). Estimation of 3',5'-cAMP was made by the method of Gilman (15). Orthophosphate was determined by the method of Fiske and SubbaRow (16).

Assay of Glycolytic Enzymes—The isolated spermatids were homogenized in 0.5 ml of a cold hypotonic buffer (15 mM KCl, 10 mM Tris-HCl, 1.5 mM Mg acetate, 6 mM mercaptoethanol at pH 7.4 (17). The homogenate was then centrifuged at 105,000 \( \times g \) for 90 min at 2 °C and the resultant supernatant was used for an assay of 11 glycolytic enzymes. Activities of enzymes in cell-free spermatid extracts were estimated, in a final volume of 2 ml, in 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM MgCl_2 and 40 \( \mu \)M NADP, or NADH at 30 ± 0.1 °C spectrophotometrically by recording the change in the absorbance at 340 nm due to reduction of NADP, or oxidation of NADH (18). The assay used for glyceraldehyde-3-phosphate dehydrogenase activity contained, in a final volume of 2 ml, 20 mM KPO_4, 0.2 mM EDTA, 20 mM NaF, 3.3 mM cysteine, 0.5 mM NAD, 0.5 mM glyceraldehyde-3-P in 133 mM Tris-HCl buffer (pH 7.4) (19). All enzymes, coenzymes, glycolytic intermediates, ATP, ADP, and AMP except glyceraldehyde-3-P were obtained from Boehringer Mannheim Co. Glyceraldehyde-3-P was purchased from Sigma. The kit for 3',5'-cAMP was obtained from Hoechst Japan (Tokyo).

RESULTS

Activities of Glycolytic Enzymes—As shown in Fig. 1, activities of 11 glycolytic enzymes were determined. Hexokinase, phosphofructokinase, and glyceraldehyde-3-phosphate dehydrogenase had the lowest activities, whereas lactate dehydrogenase had the highest activity. The rest were intermediate. The low activities of hexokinase and phosphofructokinase

The abbreviations used are: PBS, phosphate-buffered saline; Glc-6-P, glucose 6-phosphate; Fru-6-P, fructose 6-phosphate; Fru-P, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; glyceraldehyde-3-P, glyceraldehyde 3-phosphate.

This work was supported in part by grants from the Japan Ministry of Education, Science, and Culture and the Morinaga Co.

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1 The abbreviations used are: PBS, phosphate-buffered saline; Glc-6-P, glucose 6-phosphate; Fru-6-P, fructose 6-phosphate; Fru-P, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; glyceraldehyde-3-P, glyceraldehyde 3-phosphate.
were not due to the activity of phosphatase, since the phosphatase activity in the assay system used in this experiment was very low when p-nitrophenyl phosphate was used as a substrate (data not shown).

Glycolytic Intermediates in Spermatids—Glycolytic intermediates, determined when the cells were incubated with glucose (10 mM), are presented in Table I. During incubation with glucose (10 mM), there were a significant accumulation of Fru-P2, DHAP, and glyceraldehyde-3-P and a considerable production of lactate (Fig. 2 and Table I). Without glucose in the medium, the levels of Fru-P2, DHAP and glyceraldehyde-3-P dropped rapidly (Fig. 2). However, this drop was accounted for by the amount of produced lactate. It was also found that there was a small accumulation of glucose in spermatids as compared with lactate production when 10 mM glucose was present in the incubation medium. This result indicates that the glucose which was taken up by spermatids was rapidly transformed into lactate, but a part of glucose was accumulated as Fru-P2, DHAP and glyceraldehyde-3-P.

Next, the levels of the adenine nucleotides were determined. As can be seen in Fig. 3, the level of ATP fell rapidly in the presence and absence of glucose, while the AMP level increased. However, the level of ADP remained unchanged. In addition, intracellular 3',5'-cAMP was determined after incubation of the cells at 34 °C for 60 min with or without glucose (10 mM). Determination of the concentration of glycolytic intermediates, orthophosphates, and adenine nucleotides were carried out as described under "Materials and Methods." All values are expressed as nanomoles/106 cells ± S.E. from triplicate determinations except the values from the glucose-treated cells. In the same experiments, spermatids were incubated in quadruplicate when glucose was added. After incubation, 5 ml of ice-cold PBS were introduced into two out of four flasks and the samples were centrifuged at 150 × g for 5 min at 2 °C. The cells were washed five times with cold PBS by centrifugation at 150 × g for 5 min at 2 °C, homogenized in 0.5 ml of 0.5% perchloric acid with a Teflon-glass homogenizer, and centrifuged at 3000 × g for 10 min at 3 °C. The resulting supernatant was adjusted to pH 6.8 with careful addition of cold K2CO3. The neutralized samples were used for determination of intracellular levels of glucose (the value with an asterisk), glycolytic intermediates except lactate, and adenine nucleotides. On the other hand, perchloric acid at the final concentration of 5% was added to half of four samples at the end of incubation in order to stop the reaction. Then, the samples were homogenized and prepared for determination of glycolytic intermediates including lactate (the value with two asterisks) and adenine nucleotides as mentioned above. There was no difference in the values of the concentration of glycolytic intermediates and adenine nucleotides between cells washed with PBS and unwashed cells. When orthophosphate was determined, the cells were washed with 9% NaCl containing 0.9 mM CaCl2, 0.5 mM MgCl2 five times after incubation at 34 °C for 60 min in 0.5 ml of PBS.

### Table I

**Glycolytic intermediates in round spermatids**

Spermatids were incubated at 34 °C for 60 min with or without glucose (10 mM). Determination of the concentration of glycolytic intermediates, orthophosphates, and adenine nucleotides were carried out as described under "Materials and Methods." All values are expressed as nanomoles/106 cells ± S.E. from triplicate determinations except the values from the glucose-treated cells. In the same experiments, spermatids were incubated in quadruplicate when glucose was added. After incubation, 5 ml of ice-cold PBS were introduced into two out of four flasks and the samples were centrifuged at 150 × g for 5 min at 2 °C. The cells were washed five times with cold PBS by centrifugation at 150 × g for 5 min at 2 °C, homogenized in 0.5 ml of 0.5% perchloric acid with a Teflon-glass homogenizer, and centrifuged at 3000 × g for 10 min at 3 °C. The resulting supernatant was adjusted to pH 6.8 with careful addition of cold K2CO3. The neutralized samples were used for determination of intracellular levels of glucose (the value with an asterisk), glycolytic intermediates except lactate, and adenine nucleotides. On the other hand, perchloric acid at the final concentration of 5% was added to half of four samples at the end of incubation in order to stop the reaction. Then, the samples were homogenized and prepared for determination of glycolytic intermediates including lactate (the value with two asterisks) and adenine nucleotides as mentioned above. There was no difference in the values of the concentration of glycolytic intermediates and adenine nucleotides between cells washed with PBS and unwashed cells. When orthophosphate was determined, the cells were washed with 9% NaCl containing 0.9 mM CaCl2, 0.5 mM MgCl2 five times after incubation at 34 °C for 60 min in 0.5 ml of PBS.

<table>
<thead>
<tr>
<th>Intermediates</th>
<th>Concentration</th>
<th>0 min control</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td></td>
<td>0.07 ± 0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Glc-6-P</td>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fru-6-P</td>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fru-P2</td>
<td>1.25 ± 0.19</td>
<td>0.20 ± 0.03</td>
<td>2.80 ± 0.16</td>
</tr>
<tr>
<td>DHAP</td>
<td>0.35 ± 0.02</td>
<td>0.15 ± 0.01</td>
<td>0.65 ± 0.02</td>
</tr>
<tr>
<td>Glyceraldehyde-3-P</td>
<td>0.21 ± 0.03</td>
<td>0.10 ± 0.01</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>3-Phosphoglycerate</td>
<td>0.18 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>2-Phosphoglycerate</td>
<td>0.07 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>0.09 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.11 ± 0.02</td>
<td>0.10 ± 0.00</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>Lactate</td>
<td>13.86 ± 2.25</td>
<td>16.43 ± 2.40</td>
<td>36.03 ± 1.97 **</td>
</tr>
<tr>
<td>P0</td>
<td>11.70 ± 0.20</td>
<td>19.50 ± 0.30</td>
<td>13.00 ± 0.20</td>
</tr>
</tbody>
</table>

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**Mass Action Ratios of Glycolytic Reaction in Spermatids**

As the levels of glycolytic intermediates and adenine nucleotides show in Table I and Fig. 3, the mass action ratios of each enzyme step are calculated as if spermatids have had a level of 3',5'-cAMP (12). The ratio of NAD/NADH was calculated from the pyruvate/lactate ratio given in Table I, although the levels of NAD and NADH were not estimated. Mass action ratios of three enzymes (hexokinase, glucose phosphate isomerase, and phosphofructokinase) could not be calculated, because Glc-6-P and Fru-6-P levels were too low to be detectable. The reactions catalyzed by glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase were far from the equilibrium state (Table II). This result suggest that the steps of glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase may be rate limiting in glycolysis of spermatids, since the other steps seem to be close to the equilibrium state. However, glyceraldehyde-3-phosphate dehydrogenase activity was found to be much lower than that of pyruvate kinase (Fig. 1), and Fru-P2 and triose phosphates were accumulated during incubation with glucose (10 mM) (Table I). Therefore, it seems very likely that glucose metabolism in glycolysis of spermatids is more limited by glyceraldehyde-3-phosphate dehydrogenase than by pyruvate kinase.

**Inhibition of Glyceraldehyde-3-phosphate Dehydrogenase by Adenine-containing Compounds**—To elucidate the mechanism by which glyceraldehyde-3-phosphate dehydrogenase is regulated, the inhibition of glyceraldehyde-3-phosphate dehydrogenase by adenine-containing compounds was studied. As shown in Table III, all the adenine-containing compounds inhibited glyceraldehyde-3-phosphate dehydrogenase. 3',5'-cAMP was the strongest inhibitor among various compounds as shown in Table III. The inhibition by adenosine, 5'-AMP,
ADP, and ATP was moderate. However, other purine and pyrimidine compounds as listed in Table III inhibited only weakly.

 Kinetics of Inhibition of Glyceraldehyde-3-phosphate Dehydrogenase by Adenosine, 5′-AMP, ADP, and ATP—The reaction in which glyceraldehyde-3-phosphate dehydrogenase converts glyceraldehyde-3-P to 1,3-bisphosphoglycerate requires NAD as a cofactor. Since the inhibitors listed in Table III have a common structural feature to NAD, it was decided to examine whether adenine-containing inhibitors bound to glyceraldehyde-3-phosphate dehydrogenase at the same site as NAD. The Lineweaver-Burk plots are shown in Fig. 4. The inhibition of glyceraldehyde-3-phosphate dehydrogenase by adenosine appeared to be competitive with NAD (Fig. 4a). The apparent inhibition constant (K_i) for adenosine was calculated to be 4.34 mM. The K_i value for NAD was calculated to be 0.2 mM from the same figure. Fig. 4, b–d shows the inhibition of glyceraldehyde-3-phosphate dehydrogenase by 5′-AMP, ADP, and ATP. All of the three inhibitors were competitive with NAD as well as adenosine. The values of K_i for 5′-AMP, ADP, and ATP respectively. The similarity of the K_i values for adenosine to those for 5′-AMP, ADP, and ATP suggests that the mono-, di-, or triphosphate at position 5 of the adenine-linked ribose moiety is essential for the inhibition of the enzyme by these compounds. The inhibition of the enzyme by other purine and pyrimidine compounds as listed in Table III was rather weak. The inhibition of the enzyme by other purine and pyrimidine compounds as listed in Table III was rather weak.

### Table II

*Comparison of mass action ratios with apparent equilibrium constants (K_{eq}) for enzymic steps in glycolysis of spermatids*

Mass action ratios were calculated from the data in Table I. The NAD/NADH ratio was calculated from the glucose/lactate ratio. Apparent K_{eq} values were obtained from the report of Yasumasu et al. (12).

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Apparent K_{eq}</th>
<th>Mass action ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 min control</td>
</tr>
<tr>
<td><strong>Hexokinase</strong></td>
<td>6.2 x 10^5</td>
<td>10</td>
</tr>
<tr>
<td><strong>Glucose phosphate isomerase</strong></td>
<td>0.43</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>Phosphofructokinase</strong></td>
<td>1.2 x 10^5</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Aldolase</strong></td>
<td>9.1 x 10^{-5}</td>
<td>7.4 x 10^{-5}</td>
</tr>
<tr>
<td><strong>Triose phosphate isomerase</strong></td>
<td>4.6 x 10^{-2}</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>Glyceraldehyde-3-phosphate dehydrogenase + 3-phosphoglycerate kinase</strong></td>
<td>2.4 x 10^2</td>
<td>1.7 x 10^{-1}</td>
</tr>
<tr>
<td><strong>Phosphoglyceromutase</strong></td>
<td>0.17</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>Enolase</strong></td>
<td>2.9</td>
<td>1.3</td>
</tr>
<tr>
<td><strong>Pyruvate kinase</strong></td>
<td>2.9 x 10^{-1}</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*Data from Yasumasu et al. (12).*

### Table III

*Inhibition of spermatid glyceraldehyde-3-phosphate dehydrogenase by various compounds*

Effects of various compounds on the enzyme activity of glyceraldehyde-3-phosphate dehydrogenase were assayed in a final volume of 2 ml as described under “Materials and Methods.” The assay system contained 5 μl of the 105,000 × g supernatant of spermatid extract (approximately 4.5 μg of protein).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>K_i (mM)</th>
<th>Inhibition (%)</th>
<th>1 mM</th>
<th>5 mM</th>
<th>10 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3',5'-cAMP</td>
<td>0.69</td>
<td>32</td>
<td>64</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Adenosine</td>
<td>4.34</td>
<td>17</td>
<td>22</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>5′-AMP</td>
<td>3.50</td>
<td>17</td>
<td>34</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>2.35</td>
<td>27</td>
<td>39</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>5.34</td>
<td>12</td>
<td>26</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Adenine</td>
<td>0</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2′-Deoxyadenosine</td>
<td>0</td>
<td>10</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2′-AMP</td>
<td>0</td>
<td>19</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3′-AMP</td>
<td>7</td>
<td>17</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTP</td>
<td>5</td>
<td>11</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>3</td>
<td>12</td>
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<tr>
<td>UTP</td>
<td>5</td>
<td>10</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADP</td>
<td>0</td>
<td>20</td>
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<td></td>
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</tr>
</tbody>
</table>
Phosphofructokinase was shown to be the key enzyme in regulating glycolysis of human sperm because of its low concentration. However, it was found that the ATP/ADP ratio was low in spermatids (Fig. 3) and that Fru-P₂ and triose phosphates were accumulated during incubation with glucose (Fig. 2). In addition, the Glc-6-P level was too low to be detected (Table I). These results suggest that the reaction catalyzed by phosphofructokinase is probably not the main rate-limiting step in glycolysis of spermatids. A similar conclusion was made in glycolysis of buffalo spermatozoa by Gandhi and Anand (21). They also showed a possible regulation of glycolysis by glyceraldehyde-3-phosphate dehydrogenase in those cells (21). Furthermore, Peterson and Freund (23) suggested that a possible limitation of glycolysis in human spermatozoa was at the step of glyceraldehyde-3-phosphate dehydrogenase.

**DISCUSSION**

The result of experiments described here clearly showed several features of spermatid metabolism. First, most of glucose which was taken up by the cells was rapidly transformed into lactate, although the products of the aldolase step accumulated and a small amount of glucose remained without being converted to Glc-6-P (Table I). The accumulation of Fru-P₂, DHAP, glyceraldehyde-3-P, glucose, and lactate in spermatids as shown in Table I is able to account for the utilization of exogenous glucose (17.9 ± 1.0 nmol/h/10⁶ cells).

Second, the ATP level fell rapidly in contrast to the ADP level which increased, but the ADP level remained unchanged (Fig. 1). This pattern was similar to that found in buffalo spermatozoa (21). In view of these findings, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase were far from the equilibrium state (Table II). Fourth, activities of hexokinase, phosphofructokinase, and glyceraldehyde-3-phosphate dehydrogenase were markedly lower than those of other glycolytic enzymes in spermatid extracts (Fig. 1). This pattern was similar to that found in buffalo spermatozoa (21). In view of these findings, glyceraldehyde-3-phosphate dehydrogenase and phosphofructokinase seem to be rate limiting in glycolysis of spermatids. Hexokinase and phosphofructokinase require ATP as a cofactor and both enzymes are very sensitive to ATP. However, hexokinase is probably not involved much in the control of glycolysis in spermatids. This conclusion was drawn from the following findings. The ratio of ATP/ADP was low and the glucose level in cells was relatively low when compared to that of lactate (Fig. 2).

Moreover, Glc-6-P and Fru-6-P were undetectable (Table I), although utilization of exogenous glucose was at a high rate (17.9 ± 1.0 nmol/h/10⁶ cells).

According to the report of Peterson and Freund (22), phosphofructokinase was shown to be the key enzyme in regulating glycolysis of human sperm because of its low concentration. However, it was found that the ATP/ADP ratio was low in spermatids (Fig. 3) and that Fru-P₂ and triose phosphates were accumulated during incubation with glucose (Fig. 2). In addition, the Glc-6-P level was too low to be detected (Table I). These results suggest that the reaction catalyzed by phosphofructokinase is probably not the main rate-limiting step in glycolysis of spermatids. A similar conclusion was made in glycolysis of buffalo spermatozoa by Gandhi and Anand (21). They also showed a possible regulation of glycolysis by glyceraldehyde-3-phosphate dehydrogenase in those cells (21). Furthermore, Peterson and Freund (23) suggested that a possible limitation of glycolysis in human spermatozoa was at the step of glyceraldehyde-3-phosphate dehydrogenase. Therefore, it does not seem to be an unusual feature that glyceraldehyde-3-phosphate dehydrogenase plays a regulatory role in glycolysis of spermatids rather than the so-called rate-limiting enzymes, hexokinase and phosphofructokinase. This is supported by the following findings. During 1-h incubation of cells with 10 mM glucose, the glucose equivalent to about 14 nmol/h/10⁶ cells was utilized as shown in Table I. The activities of hexokinase and phosphofructokinase appeared to be low (Fig. 1), but seem to be high enough to phosphorylate this amount of glucose to Glc-6-P and then to Fru-P₂. The activities of hexokinase and phosphofructokinase were shown to be 0.49 ± 0.02 nmol/min/10⁶ cells and 0.42 ± 0.03 nmol/min/10⁶ cells, respectively. Therefore, glucose would be phosphorylated in spermatids by hexokinase at an average rate of 29.4 nmol/h/10⁶ cells which is about twice as much as the utilized amount of glucose (approximately 14 nmol/h/10⁶ cells). The step of phosphorylation of hexose by hexokinase and phosphofructokinase is probably not the main step in regulating glycolysis in spermatids. However, intracellular levels of glucose, Glc-6-P and Fru-6-P were found to be

![Fig. 4. Lineweaver-Burk plots of the inhibition of spermatid glyceraldehyde-3-phosphate dehydrogenase by adenosine (a), 5'-AMP (b), ADP (c), ATP (d), and 3',5'-cAMP (e) at concentrations shown at 30 °C and pH 7.4. Glyceraldehyde-3-phosphate dehydrogenase activity was assayed as described under "Materials and Methods."](image-url)
very low (Table I), which suggests that the concentration of these substrates in intact cells might be lower than the $K_m$ value in the respective reaction in glycolysis. The role of steps of phosphorylation by hexokinase and phosphofructokinase would be more easily explained if hexokinase and phosphofructokinase are activated by some factor. Recently, Pilkis and co-workers (24, 25) have shown that phosphofructokinase in rat liver was activated by fructose 2,6-bisphosphate. Phosphofructokinase in spermatids might be activated by this bisphosphorylated sugar as well as in rat liver.

Regarding pyruvate kinase, it is possible that it could be one of the key enzymes in the control of glycolysis of spermatids, judging from the calculated mass action ratio as shown in Table II. However, during incubation of cells with glucose (19 mM), Fru-P₄, and triose phosphates were accumulated. The levels of 3-phosphoglycerate, phosphoenolpyruvate, and pyruvate remained constant (Table I) and pyruvate kinase activity in spermatid extracts was relatively high (Fig. 1). Pyruvate kinase may not be, therefore, at the main step of a regulation of glycolysis of spermatids either.

Glyceraldehyde-3-phosphate dehydrogenase was demonstrated to be inhibited by adenine-containing compounds which were competitive with NAD. The ribose moiety of adenosine was primarily involved in binding, since adenosine, 5'-AMP, ADP, and ATP, but not adenine were inhibitory. The 5'-diphosphate and triphosphate group on the adenine-linked ribose did not have a pronounced effect on binding, because the $K_v$ values of 5'-AMP, ADP, and ATP did not differ from one another (Table III). However, the 5'-phosphate group on the ribose moiety of adenosine enhanced the binding when linked to the 3'-hydroxy group on the ribose moiety as in 3',5'-cAMP or to a free phosphate group as in NAD. By contrast, the 2'- and 3'-phosphate group was not essential for binding. In particular, it is evident that the binding of adenine-linked ribose heavily relies on the 2'-hydroxy group for binding to glyceraldehyde-3-phosphate dehydrogenase, since 2'-deoxadenosine, 2'-AMP, and NADP were not inhibitory. These features of glyceraldehyde-3-phosphate dehydrogenase inhibition by adenine nucleotides which were seen in spermatids, were also observed in yeast glyceraldehyde-3-phosphate dehydrogenase (26).

Among several kinds of adenine nucleotides, 3',5'-cAMP was found to be a potent inhibitor. However, 3',5'-cAMP does not seem to control the glycolysis of spermatids. This conclusion was reached from the following findings. First, the intracellular level of 3',5'-cAMP did not change significantly when the cells were incubated with or without glucose (see "Results"). Second, the 5',5'-cAMP level in spermatids was not high enough to inhibit glyceraldehyde-3-phosphate dehydrogenase, judging from the $K_v$ value for 5',5'-cAMP (Table III). On the contrary, the 5'-AMP, ADP, and ATP levels in spermatids appeared to be high enough to inhibit glyceraldehyde-3-phosphate dehydrogenase. During incubation of cells with glucose, the 5'-AMP level increased from 0.9 to 1.5 nmol/10⁶ cells in contrast to the ATP level which decreased from 1.1 to 0.4 nmol/10⁶ cells (Fig. 3). If the average volume of spermatids is 524 µm³ (20), the 5'-AMP and ATP levels are calculated to increase from 1.8 to 3.0 mm and to decrease from 2.2 to 0.8 mm, respectively. Considering the $K_v$ values for 5'-AMP and ATP as shown in Table III, it is quite likely that the increased level of 5'-AMP inhibits glyceraldehyde-3-phosphate dehydrogenase strongly, while glyceraldehyde-3-phosphate dehydrogenase inhibition by ATP is somewhat less. The steady concentration of ADP (1.8 mM) is sufficient to inhibit glyceraldehyde-3-phosphate dehydrogenase significantly, because the $K_v$ for ADP was found to be 2.35 mm.

In view of these findings, the following metabolic control by adenosine nucleotides is postulated. Without administration of glucose to spermatids, the 5'-AMP, ADP, and ATP levels are relatively high (1.8, 1.9, and 2.2 nm, respectively). A glycolytic flow is regulated at the step of glyceraldehyde-3-phosphate dehydrogenase due to the inhibition by adenine nucleotides, particularly by ADP. Since ATP inhibits phosphofructokinase and pyruvate kinase, the steps of these enzymes may participate in the control of glycolysis when glucose is not added. However, assume that the levels of 5'-AMP and ATP change during incubation of cells with glucose, namely an increase in the 5'-AMP level and a decrease in the ATP level. The inhibition of glyceraldehyde-3-phosphate dehydrogenase is more pronounced because of the increased level of 5'-AMP, even though glyceraldehyde-3-phosphate dehydrogenase inhibition by ATP is diminished. Inhibition of glyceraldehyde-3-phosphate dehydrogenase by ADP would be unaltered, since the ADP level remains unchanged. In the meantime, the inhibition of phosphofructokinase and pyruvate kinase by ATP is released due to the decrease in the ATP level. Therefore, if glucose taken up by spermatids seems to be rapidly converted to Fru-P₄ without a regulation of phosphofructokinase which is activated by the decreased level of ATP. However, since a glycolytic flow is more limited at the step of glyceraldehyde-3-phosphate dehydrogenase when glucose is present in the outer medium, the build-up of Fru-P₄ and triose phosphates occurs. An accumulation of the products of aldolase indicates that the inflow rate of Fru-P₄ to the step of aldolase is a little greater than the outflow rate of glyceraldehyde-3-P. Then, once glyceraldehyde-3-P is converted to 1,3-bisphosphoglycerate by glyceraldehyde-3-phosphate dehydrogenase, the conversion of 1,3-bisphosphoglycerate through phosphoenolpyruvate may rapidly take place by the relatively higher active enzymes (3-phosphoglycerate kinase, phosphoglyceromutase, and enolase) and then, phosphoenolpyruvate is converted to pyruvate by pyruvate kinase. Finally, pyruvate is transformed to lactate by highly active lactate dehydrogenase.

However, there is a question about the concentration of NAD in spermatids. Glyceraldehyde-3-phosphate dehydrogenase inhibition by adenine nucleotides will be less if NAD concentration is extremely high. In the several reports, NAD concentration was estimated to be 0.6 mm in rat liver (27), nearly 1 mm in bovine spermatozoa (28), and 1-3 mm in yeast (29). Although the concentration of free NAD in the cytoplasm has not been determined since it is known that an estimation of free NAD is very difficult (30), the levels of 5'-AMP, ADP, and ATP in spermatids seem to be high enough to compete with NAD.

Together with these findings, it is quite probable that glycolysis in spermatids may be largely regulated at the step of glyceraldehyde-3-phosphate dehydrogenase with competitive inhibitors with NAD such as 5'-AMP, ADP, and ATP rather than by the so-called rate-limiting enzymes (hexokinase, phosphofructokinase and pyruvate kinase). In particular, the inhibitory effect of 5'-AMP and ADP is pronounced when glucose is present.

REFERENCES

Regulation of Glucose Metabolism in Spermatids