Enzymes of Intermediary Carbohydrate Metabolism in
Ureaplasma urealyticum and Mycoplasma mycoides subsp. mycoides

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Cell extracts of Ureaplasma urealyticum and Mycoplasma mycoides were examined for enzymes of intermediary carbohydrate metabolism using a sensitive radiochemical assay procedure. For M. mycoides, the enzyme activities detected were supporting evidence for the existence of a glycolytic pathway giving lactate anaerobically and acetate aerobically. U. urealyticum also had activities of many glycolytic enzymes. Enzymes of the pentose phosphate pathway occurred in both M. mycoides and U. urealyticum. Their presence allowed the proposal of a sequence for the synthesis from glycolytic pathway intermediates of ribose 5-phosphate, and hence phospho-ribosyl diphosphate, for the synthesis of nucleotides. Pathways for the further metabolism of deoxyribose 1-phosphate and ribose 1-phosphate produced from nucleoside phosphorylase reactions operated in extracts from both organisms.

INTRODUCTION

Recent studies (Neale et al., 1983a) on Mycoplasma mycoides subsp. mycoides have suggested that the organism may metabolize deoxyribose 1-phosphate via glyceraldehyde 3-phosphate and reactions of the pentose phosphate pathway to ribose 5-phosphate, a precursor of 5-phosphoribosyl 1-diphosphate used in the salvage synthesis of nucleotides. Preliminary investigations had indicated that extracts of another mollicute, Ureaplasma urealyticum, also had activities for the degradation of nucleotides and nucleosides, and for the further metabolism of the ribose and deoxyribose phosphates produced. M. mycoides is a fermentative mycoplasma, able to produce acid from glucose and other hexoses effective as energy sources. Studying cell suspensions, Rodwell (1969) showed that M. mycoides can metabolize glucose, mannose or fructose to acetate and CO₂ aerobically, and to acetate, lactate and CO₂ anaerobically, probably via the Embden–Meyerhof pathway together with pyruvate dehydrogenase, phosphate acetyltransferase and acetate kinase. U. urealyticum is non-fermentative and no energy sources for its growth have been defined with the possible exception of the hydrolysis of urea (Romano et al., 1980; Shepard & Masover, 1979).

As reviewed by Shepard & Masover (1979), extracts of U. urealyticum have the following enzyme activities: urease, histidase, esterases, malate dehydrogenase, glycerol-3-phosphate dehydrogenase, ATPase, acid and alkaline phosphatases, and also ribonuclease and deoxyribonuclease (Romano & La Licata, 1978). We have examined the extent to which extracts of U. urealyticum can utilize carbohydrates resulting from nucleotide and nucleoside degradation as possible energy sources. In this report, we present data on the activities of enzymes related to the Embden–Meyerhof and pentose phosphate pathways in cell-free extracts of U. urealyticum and M. mycoides. The use of sensitive radiochemical assays has helped to overcome the limitation imposed by the low yield of cells obtainable for U. urealyticum.

Abbreviations: Ap₃A, P₃,P₅-di(adenosine-5') pentaphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; PGK, phosphoglycerate kinase; TPP, thiamin pyrophosphate; PEP, phosphoenolpyruvate.
METHODS

Organisms and culture media. M. mycoides subsp. mycoides, goat strain Y, supplied by Dr A. Rodwell, CSIRO Animal Health Laboratory, Parkville, Victoria, Australia was grown in PPLO broth (Rodwell et al., 1975) as described by Mitchell et al. (1978). U. urealyticum (Belacosa H) was provided by Dr D. Birch, Nephrology Unit, Royal Melbourne Hospital, Melbourne, Australia. Growth medium for U. urealyticum contained per litre: 15 g Spirolate broth (BBL microbiological culture medium), 5 g NaCl, 4 g MES, 0.2 g KH₂PO₄, 0.5 g urea, 40 mg phenol red, 40 ml normal horse serum (not inactivated), 50 mg methicillin, 40 mg ampicillin and 20 μg glycyl-L-histidyl-L-lysine acetate. The pH of the medium was adjusted to 6-0.

Preparation of cell-free extracts. The preparation of extracts of M. mycoides was as described by Mitchell et al. (1978). U. urealyticum was grown at 37°C without shaking for 20 to 30 h. Cells were harvested by centrifuging at 25000 g for 15 min at 4°C when the cultures had reached late exponential phase, as indicated by a pH of 7. After washing twice with 0.25 M NaCl, the cells from 500 ml medium were resuspended in 0.5 ml 200 mM Tris/HCl (pH 7-8 at 37°C) containing 1 mg BSA ml⁻¹ and 1 mM-DTT.

The cells, cooled on ice, were disrupted by sonication in an MSE Soniprep 150 ultrasonic disintegrator using a cycle of 20 s sonication followed by 20 s cooling to give a total sonication time of 8 min. The suspension was centrifuged at 16000 g for 10 min in a Beckman air-driven ultracentrifuge. The supernatant was stored as small samples at -70°C. The protein content of cell-free extracts was estimated by the method of Lowry.

Chemicals and radiochemicals. Horse serum for growth media was from the Commonwealth Serum Laboratories, Parkville, Victoria 3052, Australia, and Spirolate broth was from Becton Dickenson, Cockeysville, MD 21030, USA. Glycyl-L-histidyl-L-lysine acetate and antibiotics were from Sigma.

Enzyme assays by radiochemical procedures. All radiochemical assays involved the production of [¹⁴C]glucose 6-phosphate, except for that of uridine phosphorylase which was assayed as described previously (Mitchell & Finch 1979). The reaction under investigation was coupled by addition of the appropriate enzymes and intermediates to the production of ATP which was then used by hexokinase to convert [¹⁴C]glucose to [¹⁴C]glucose 6-phosphate. Assay volumes were 50 μl unless otherwise indicated and incubations were at 37°C. Reactions were commenced by the addition of substrate, and sampled at various times by applying 5 μl reaction mixture to EDTA-treated DEAE-cellulose. For each assay there were two controls: one without extract and one without substrate.

(i) Isolation of [¹⁴C]glucose 6-phosphate on DEAE-cellulose paper. Sheets of DEAE-cellulose paper (Whatman DE81) were ruled into squares (15 × 15 mm) and 10 μl 5 mM-EDTA, pH 7.0, was applied to each. The dried paper was stored at -15°C. As required, pieces containing the appropriate number of squares were cut from a sheet and a sample was applied centrally to a numbered square. After the collection of all samples, the paper was dried, then washed twice by immersion in distilled water for 10 min without intermediate drying. After a final drying, the squares were cut out and placed in vials with 6 ml scintillation solution [toluene containing 0.5% (w/v) diphenyloxazole] for counting. This procedure removed [¹⁴C]glucose 6-phosphate bound.

(ii) Incubation components. The sequence of reactions required to achieve ATP generation was similar for many of the reactions assayed, allowing the use of the two standard assay mixtures described below.

Assay mixture A contained 1 mM-[U-¹⁴C]glucose (283 mCi mmol⁻¹ (10-47 GBq mmol⁻¹)) and [2-¹⁴C]uridine (57 mCi mmol⁻¹ (2.11 GBq mmol⁻¹)) were from Amersham. NADH, NAD⁺, NADPH and NADP⁺ were from Boehringer-Mannheim, and BSA and pyruvate kinase were from Calbiochem-Behring. All other enzymes, nucleotides, cofactors and intermediates were from Sigma.

(iii) Details of individual assays. Hexokinase (EC 2.7.1.1): 1 mM-[U-¹⁴C]glucose, 5 mM-MgCl₂, 5 mM-DTT, 40 mM-Tris/HCl (pH 7.8 at 37°C), 0.2-0.8 U hexokinase, 3-20 μg extracted protein and 100 μM-ZnCl₂, 0.8 mg BSA ml⁻¹ and 3 mM-fructose 1,6-bisphosphate.

Aldolase (EC 4.1.2.13): assay mix B with 1 mM-ZnCl₂, 0.8 mg BSA ml⁻¹ and 3 mM-fructose 1,6-bisphosphate. Triosephosphate isomerase (EC 5.3.1.1): assay mix B with 3 mM-dihydroxyacetone.

Phosphoglycerate kinase (EC 2.7.2.3): assay mix B with 1 mM-NAD⁺, 5 mM-sodium phosphate, 0.8 μg BSA ml⁻¹, 4 mM-pyruvate, 0.15 U LDH, 0.05 U G6PDH and 3 mM-glyceraldehyde 3-phosphate.

Phosphoglyceromutase (EC 2.7.5.3): assay mix A with 0.1 U pyruvate kinase, 0.2 U enolase, 1 mM-3-phosphoglycerate and 0.5 mM-2,3-bisphosphoglycerate.

Enolase (EC 4.2.1.11): assay mix B with 40 mM-sodium phosphate buffer pH 7.0 replacing Tris, 0.1 U pyruvate kinase and 1 mM-2-phosphoglycerate.

Pyruvate kinase (EC 2.7.1.40): assay mix A with 40 mM-sodium phosphate buffer pH 7.0 replacing Tris, and 2 mM-PEP.

Phosphatase acetyltransferase (EC 2.3.1.8): assay mix A with 40 mM-sodium phosphate buffer pH 7.0 replacing Tris, 3 mM-
acetyl CoA and 0.06 U acetate kinase. *Acetate kinase* (EC 2.7.2.1): assay mix A with 2 mM-acetyl phosphate. *Transketolase* (EC 2.2.1.1) *(U. urealyticum)*: assay mix B with 0.5 mM-thiamin pyrophosphate (TPP), 3 mM-erythrose 4-phosphate and 3 mM-xylose 5-phosphate. *Transketolase* (EC 2.2.1.1) *(M. mycoides)*: assay mix A with 1 mM-NAD*, 2.5 mM-sodium phosphate, 4 mM-pyruvate, 0.5 mM-TPP, 3 mM-erythrose 4-phosphate and 3 mM-xylose 5-phosphate. *Transaldolase* (EC 2.2.1.2) *(U. urealyticum)*: assay mix A with 1 mM-NAD*, 5 mM-sodium phosphate, 4 mM-pyruvate, 0.05 U G6PDH, 0.15 U LDH, 0.3 U PGK, 0.8 mg BSA ml⁻¹, 3 mM-erythrose 4-phosphate and 3 mM-fructose 6-phosphate. *Transaldolase* (EC 2.2.1.2) *(M. mycoides)*: assay mix A with 1 mM-NAD*, 5 mM-sodium phosphate, 4 mM-pyruvate, 3 mM-erythrose 4-phosphate and 3 mM-fructose 6-phosphate. *Ribosephosphate isomerase* (EC 5.3.1.6): assay mix B with 0.5 U transketolase, 0.5 mM-TPP, 0.5 U ribulose-5-phosphate epimerase, 3 mM-erythrose 4-phosphate and 3 mM-ribose 5-phosphate. *Ribosephosphate epimerase* *(M. mycoides)* (EC 5.3.1.1): assay mix B with 0.5 mM-TPP, 3 mM-erythrose 4-phosphate and 3 mM-ribulose 5-phosphate. *Phosphopentomutase* *(ribose 1-phosphate)* *(Hammer-Jespersen & Munch-Petersen, 1970)*: assay mix B with 0.5 mM-TPP, 0.5 U transketolase, 0.5 U ribose isomerase, 0.5 U ribulose-5-phosphate epimerase, 3 mM-erythrose 4-phosphate and 3 mM-ribose 1-phosphate. *Phosphopentomutase* *(Hammer-Jespersen & Munch-Petersen, 1970)*: assay mix A with 1 mM-NAD*, 2.5 mM-sodium phosphate, 4 mM-pyruvate, 0.05 U G6PDH, 0.15 U LDH, 0.3 U PGK, 4 mM-sodium citrate, 0.2 mM-glucose 1,6-bisphosphate and 2 mM-deoxyribose 1-phosphate. *Deoxyribose-5-phosphate aldolase* *(EC 4.1.2.4)*: assay mix A with 1 mM-NAD*, 2.5 mM-sodium phosphate, 4 mM-pyruvate, 0.05 U G6PDH, 0.15 U LDH, 0.3 U PGK, 4 mM-sodium citrate and 2 mM-deoxyribose 5-phosphate. *Adenylate kinase* *(EC 2.7.4.3)*: assay mix A with ApA omitted and 1 mM-ADP instead of 0.2 mM-ADP. *PEP:glucose phosphotransferase system*: 3 mM-PEP, 1 mM-[U-14C]glucose, 5 mM-MgCl₂, 5 mM-DTT, 40 mM-Tris/Cl⁻ (pH 7.8 at 37 °C), 0.8 mg BSA ml⁻¹ and 10⁻⁸⁰ µg cell protein. *Uridine phosphorylase* *(EC 2.4.2.3)* *(U. urealyticum)*: assayed as described previously by Mitchell & Finch (1979) with 0.5 mM-[2⁻¹⁴C]uridine [9⁻5 mCi mmol⁻¹ (351.5 MBq mmol⁻¹)], 5 mM-sodium phosphate, 5 mM-MgCl₂, 0.2 µg cell protein, 40 mM-Tris/Cl⁻ (pH 7.8 at 37 °C) in a total volume of 25 µl.

**RESULTS**

**Enzyme assays**

In developing the coupled assays, various background sources of ADP phosphorylation, which would lead to estimation of falsely elevated production of [¹⁴C]glucose 6-phosphate, had to be considered. Continued phosphorylation by hexokinase after sampling was stopped by the pretreatment of the DEAE-cellulose with EDTA. Extracts from both organisms contained adenylate kinase activity which formed ATP via the reaction 2 ADP → ATP + AMP. To lessen this effect, the ADP concentration was kept low (0-0.2 mM) compared to the [¹⁴C]glucose concentration (1 mM), and the specific adenylate kinase inhibitor, ApA, was included in reaction mixtures. At 0-1 mM, ApA inhibited the activity from *U. urealyticum* by 98% and that from *M. mycoides* by 77%. Contaminating activities in some of the commercial enzyme preparations were another source of spurious phosphorylation and necessitated use of the lowest effective amounts of the contaminated enzymes. Controls without extract were used to correct for residual activity for both the assays coupled to ADP phosphorylation and those in which activity was measured spectrophotometrically. For spectrophotometric assays of extracts of *M. mycoides*, exogenous NADH oxidase activity was inhibited by flushing the assay mixtures with N₂.
Table 1. Enzyme activities in extracts of *U. urealyticum* and *M. mycoides*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction catalysed*</th>
<th>Specific activity [μmol min⁻¹ (g cell protein)⁻¹]</th>
<th><em>U. urealyticum</em></th>
<th><em>M. mycoides</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>PEP:glucose phosphotransferase</td>
<td>1</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexokinase</td>
<td>2</td>
<td>115</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphate isomerase</td>
<td>3</td>
<td>0</td>
<td>2.1 × 10³</td>
<td></td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>4</td>
<td>72</td>
<td>2.26 × 10³</td>
<td></td>
</tr>
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<td>Aldolase</td>
<td>5</td>
<td>1.7</td>
<td>368</td>
<td></td>
</tr>
<tr>
<td>Triose-phosphate isomerase</td>
<td>6</td>
<td>24</td>
<td>240</td>
<td></td>
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<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>7</td>
<td>260</td>
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<td></td>
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<td>Phosphoglycerate kinase</td>
<td>8</td>
<td>-†</td>
<td>251</td>
<td></td>
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<tr>
<td>Phosphoglyceromutase</td>
<td>9</td>
<td>-†</td>
<td>82</td>
<td></td>
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<tr>
<td>Enolase</td>
<td>10</td>
<td>29</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>11</td>
<td>7.0</td>
<td>56</td>
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<tr>
<td>Lactate dehydrogenase</td>
<td>12</td>
<td>0</td>
<td>82.4 × 10³</td>
<td></td>
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<tr>
<td>Phosphate acetyltransferase</td>
<td>13</td>
<td>-†</td>
<td>233</td>
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<tr>
<td>Acetate kinase</td>
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<td>-†</td>
<td>733</td>
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<tr>
<td>NADH oxidase</td>
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<td>6.8 × 10³</td>
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<tr>
<td>NADPH oxidase</td>
<td>-‡</td>
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<td></td>
</tr>
<tr>
<td>Glycerol-1-phosphate dehydrogenase</td>
<td>16</td>
<td>260</td>
<td>0</td>
<td></td>
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<tr>
<td>Transketolase</td>
<td>17</td>
<td>183</td>
<td>168</td>
<td></td>
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<tr>
<td>Transaldolase</td>
<td>18</td>
<td>-‡</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Ribulose-5-phosphate epimerase</td>
<td>19</td>
<td>127</td>
<td>102</td>
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<tr>
<td>Ribosephosphate isomerase</td>
<td>20</td>
<td>145</td>
<td>173</td>
<td></td>
</tr>
<tr>
<td>Phosphopentomutase (ribose 1-phosphate)</td>
<td>21</td>
<td>99</td>
<td>28</td>
<td></td>
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<tr>
<td>Phosphopentomutase (deoxyribose 1-phosphate)</td>
<td>21</td>
<td>3.5</td>
<td>22</td>
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<tr>
<td>Deoxyribose-5-phosphate aldolase</td>
<td>22</td>
<td>12</td>
<td>89</td>
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<tr>
<td>Uridine phosphorylase</td>
<td>23</td>
<td>84</td>
<td>860</td>
<td></td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>-§</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>366</td>
<td>3.5 × 10³</td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>

* Reactions corresponding to the numbers are shown in Fig. 1.
† Activity not significantly above high background.
‡ Activity possibly obscured by inhibitory factors in coupling enzyme preparations (see text).

The enriched medium used for the cultivation of the bacteria could also be a source of extraneous enzyme activity, particularly in the case of *U. urealyticum*, since the yield of cellular protein was relatively low compared with the initial protein content of the medium. Correction for this latter source of error was made by applying the extraction procedure to uninoculated media incubated under conditions of temperature and pH which simulated growth conditions of the cultures.

Enzymic activities observed in extracts of *M. mycoides* and *U. urealyticum*, after corrections for background activity as discussed above, are shown in Table 1; the results were for a single extract from each organism, but are representative of assays made with several different extracts.

Transaldolase activity was observed in extracts from *M. mycoides* only when using endogenous coupling enzymes. The addition of commercial preparations of coupling enzymes inhibited this reaction. With the extracts of *U. urealyticum*, the endogenous levels of the coupling enzymes GAPDH, PGK and LDH (nos 7, 8 and 12 in Table 1) were inadequate, so the presence of any transaldolase may have been masked by the inhibitory effect of added coupling enzymes.

As well as the enzymes listed in Table 1, extracts of both organisms were assayed for alcohol dehydrogenase, acetaldehyde dehydrogenase, malic enzyme, aspartate transaminase, alanine

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Fig. 1. Potential reactions of carbohydrate metabolism in *U. urealyticum* and *M. mycoides*. The numbers 1 to 23 against individual reactions are those assigned in Table 1 to the corresponding enzyme for catalysis of the reaction.
transaminase, glutamate dehydrogenase and alanine dehydrogenase, none of which was detected.

DISCUSSION

An outline of potential reactions of carbohydrate metabolism in *U. urealyticum* and *M. mycoides*, keyed to the numerical listing of enzymes in Table 1, is shown in Fig. 1.

For *M. mycoides*, the activities shown in Table 1 demonstrate the enzymic activities implied by the previous observations of Rodwell (1960, 1969) of a glycolytic pathway giving lactate anaerobically and acetate aerobically. Aerobically, NADH oxidase allows the regeneration of NAD+ from NADH coupled with the conversion of molecular O₂ to H₂O₂. An observed stimulation of growth by gentle aeration of *M. mycoides* cultures is consistent with the possibility that two additional molecules of ATP are produced per glucose molecule catabolized via the aerobic pathway (Rodwell, 1969).

The glycolytic metabolism of glucose by the Embden–Meyerhof pathway is initiated by its phosphorylation to glucose 6-phosphate. The results in Table 1 indicate that *M. mycoides* can effect this phosphorylation through both the phosphotransferase system with PEP as the phosphoryl donor as reported by Cirillo (1979), and by the action of hexokinase with ATP as phosphoryl donor. The use of glucose as a source of nucleotide pentoses (Rodwell & Mitchell, 1979) in *M. mycoides* could be achieved by the conversion of fructose 6-phosphate and glyceraldehyde 3-phosphate from the glycolytic pathway into ribose 5-phosphate by the action of the pentose phosphate pathway enzymes transketolase, transaldolase, ribulose-5-phosphate epimerase and ribose-5-phosphate isomerase (17, 18, 19 and 20 respectively in Table 1 and Fig. 1). Ribose 5-phosphate could then be used to synthesize 5-phosphoribosyl 1-diphosphate. While 5-phosphoribosyl-1-diphosphate synthetase has not been characterized in extracts of *M. mycoides*, the synthesis of its product has been observed in cell suspensions (Mitchell, 1976). The existence of phosphoribosyl transferases for adenine, guanine, hypoxanthine and uracil (Mitchell & Finch, 1979; Mitchell et al., 1978; Sin & Finch, 1972) completes the list of enzyme activities necessary for nucleotide synthesis from glucose and preformed bases.

*M. mycoides* catabolizes ribo- and deoxyribonucleosides by various nucleoside phosphorylases (Mitchell & Finch, 1977, 1979; Neale et al., 1983b). The observation of phosphoribomutase and deoxyribose-5-phosphate aldolase (21 and 22 in Table 1 and Fig. 1), together with the four pentose phosphate pathway enzymes, indicates a route which would enable the pentose 1-phosphate products to be converted to the glycolytic intermediates glyceraldehyde 3-phosphate and fructose 6-phosphate, thereby allowing the nucleoside sugars to serve as energy sources. The observation of Neale et al. (1983a) that carbon atoms from deoxyribonucleosides enter ribonucleotides could also be explained by this formation of glycolytic intermediates.

The activities observed for the glycolytic enzymes in extracts from *U. urealyticum* (Table 1) are considerably less than those from *M. mycoides*. This difference may be partly due to dilution of the small amount of *U. urealyticum* protein in the extracts by significant amounts of contaminating protein from the medium. However, the extent of the difference between the activities in the two extracts suggests a different role of the glycolytic pathway in the two organisms. A lack of activity in extracts of *U. urealyticum* for formation of glucose 6-phosphate from fructose 6-phosphate, is in keeping with the inability of *U. urealyticum* to ferment glucose. Many of the other enzymes of glycolysis appear to be present in *U. urealyticum*, although their low activity does not favour participation in a major energy generating pathway. Nevertheless, the presence of many glycolytic enzymes does suggest some role for them in carbohydrate metabolism in *U. urealyticum*. Demonstration of the presence of enzymes of pentose phosphate metabolism, except transaldolase (no. 18 in Fig. 1), which may have gone undetected because of an inhibitory action of the added commercial coupling enzymes, may indicate a capability for the formation of ribose 5-phosphate for nucleotide synthesis from glycolytic intermediates, or these enzymes could be part of a catabolic pathway from nucleoside to glycolytic intermediates, as discussed above for *M. mycoides*.

Glycerol-3-phosphate dehydrogenase (no. 16 in Fig. 1) and malate dehydrogenase activities, but not lactate dehydrogenase or NADH oxidase activities, were detected in extracts of *U.
urealyticum (Table 1), thus confirming the previous observations of Shepard & Masover (1979). Perhaps glycerol-3-phosphate dehydrogenase forms part of a pathway via glyceraldehyde 3-phosphate to allow a reversible interchange between lipid and nucleotide metabolites. This enzyme was absent from the M. mycoides extracts, in keeping with the requirement of that organism for glycerol (Rodwell, 1960).

An enzyme of comparatively high activity in the extracts of U. urealyticum is adenylyl kinase (Table 1). The significance of a capacity to rapidly interconvert AMP, ADP, and ATP in this organism remains unclear.

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REFERENCES


