HEXOSE OXIDATION BY AN ENZYME SYSTEM OF MALLEOMYCES PSEUDOMALLEI

J. H. DOWLING AND H. B. LEVINE

Naval Biological Laboratory, Department of Bacteriology, University of California, Berkeley 4, California

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The widespread occurrence of non-glycolytic mechanisms of carbohydrate metabolism in mammalian tissues, plant tissues and microorganisms has recently been extensively investigated (Racker, 1954; Gunsalus et al., 1955; DeLey, 1955). These studies have indicated that the oxidation of glucose by way of gluconic acid or 6-phosphogluconate is common in Escherichia coli, Pseudomonas species, Azotobacter vinelandii and other bacterial genera. The reaction, in many instances, proceeds via phosphorylated glucose and is linked to adenosine triphosphate (ATP) which supplies phosphate to carbon 6 of the hexose. Triphosphopyridine or diphosphopyridine nucleotides (TPN, DPN) have been shown to act as hydrogen acceptors in the oxidation of glucose-6-phosphate to 6-phosphogluconate by yeasts, bacteria and animal tissues.

Similarly, the oxidation of galactose by way of galactonic acid has been demonstrated in Aerobacter cloacae (Vermeulen and DeLey, 1953), Pseudomonas species (Assi et al., 1952; DeLey, 1955) and in the alga Iridophycus flaccidum (Bean and Hassid, 1955).

The paucity of comparable metabolic data on pathogens and the observations that glucose, galactose, gluconate or galactonate served well as the sole energy sources for growth of Malleomyces pseudomallei prompted an investigation on the hexose oxidases of this species. The present report describes an enzyme system derived from M. pseudomallei which catalyzes the oxidation of both glucose and galactose to their corresponding hexonic acids. The system was not stimulated by added ATP, DPN, TPN or Mg++, and, in the case of galactose, did not involve a demonstrable "galactowaldenase" reaction.

METHODS

Bacteriological. Strain 103 (Levine et al., 1954) of M. pseudomallei was grown in a chemically-defined medium of the following composition: d-glucose, 0.08 M; KH2PO4, 0.1 M; Citric acid, 0.01 M; MgSO4, 0.003 M; FeSO4, 0.00001 M; NH4OH, to pH 7.2. One liter of the medium yielded approximately 12 g of cells (wet weight) after 48 hr of aerated incubation at 35 to 37 C. The cells were harvested at 12 C by centrifugation at 1300 X G (International Refrigerated Centrifuge, Model PR-2), washed twice with 0.85 per cent NaCl and resuspended in 30 ml saline.

After thorough mixing of the suspension with 60 g of levigated alumina (Norton Company, Worcester, Mass.), the mixture was transferred to a monel metal semi-micro jar accessory for the Waring blender. The jar was fitted with a welded stainless steel jacket which was filled with crushed ice.

The suspension was then ground in the blender for a total of 150 sec in 10-sec intervals, allowing a cooling period of 20 sec between each 10-sec grinding period. The number of viable cells was determined by surface plate count (Miles et al., 1938) before and after the grinding procedure. Subsequently, 25 ml of chilled saline was added to the mixture which was then centrifuged at 0 to 4 C for 2 hr at 1700 X G. The resulting opalescent amber colored supernatant fluid was decanted and this extract was employed for further enzymatic studies. This crude extract was partially purified by treatment with MnCl2 (Scott and Cohen, 1953) or precipitation with (NH4)2SO4 (table 1) and the oxidation of glucose and galactose to their corresponding hexonic acids was followed manometrically and chemically.

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TABLE 1

Purification of an enzyme system derived from Malleomyces pseudomallei

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Activity Units*</th>
<th>Specific Activity Units per mg Protein</th>
<th>No. of Viable Cells per ml Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>1. Crude extract†</td>
<td>236.5</td>
<td>301.8</td>
<td>349.8</td>
</tr>
<tr>
<td>2. MnCl₂ supernatant‡</td>
<td>—</td>
<td>—</td>
<td>365.3</td>
</tr>
<tr>
<td>3. 1st 0.5 sat. (NH₄)₂SO₄ ppt.</td>
<td>449.1</td>
<td>273.0</td>
<td>1.95</td>
</tr>
<tr>
<td>4. 2nd 0.5 sat. (NH₄)₂SO₄ ppt.</td>
<td>290.2</td>
<td>164.4</td>
<td>—</td>
</tr>
<tr>
<td>5. Centrifuge after dialysis</td>
<td>—</td>
<td>—</td>
<td>178.6</td>
</tr>
<tr>
<td>a. Precipitate</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>b. Supernatant</td>
<td>—</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

* Activity units = μL O₂ uptake per 10 min per ml extract.
† All extracts were dialyzed after steps 1 through 4 against a total of 240 × their volume of distilled water at 0 to 4 C.
‡ Extract was treated with 0.1 volume of 0.25 M MnCl₂.

The formation of gluconate and galactonate was demonstrated also in growth media inoculated with whole cells. In later studies the preparation was rendered cell-free by treatment with protamine sulfate (4 to 5 mg per 20 ml of crude extract), centrifugation (15000 × G, 20 min), and filtration of the supernatant through a Millipore Filter (Lovell Chemical Co., Watertown, Mass.). These additional steps did not alter the stoichiometry of the reactions described below.

Analytical. The identification of gluconate and galactonate was accomplished by lactonizing the acids and comparing the Rf values obtained after ascending paper chromatography with known samples of both lactones. Two solvent systems which provided clear separation of D-galactono-γ-lactone and D-glucono-δ-lactone were employed. These were the butanol layer of a n-butanol-ethanol-water (5:1:4) system (Abdel-Akher and Smith, 1951) and 2-butanol saturated with 1 N HCl.

After conversion of the lactones to their hydroxamic acids (Abdel-Akher and Smith, 1951), color as well as Rf value aided the identification. The spot corresponding to galactono-lactone was red-brown while gluconolactone reacted with the formation of a lighter brown color. Small quantities of the isomeric glucono-γ-lactone were found in known samples of glucono-δ-lactone but these were of sufficiently different Rf value to cause no interference. In all experiments, reaction products, knowns and mixtures of the knowns were co-chromatographed and compared directly. The lactones in the biological samples were chromatographically pure and their concentration was determined by a modification of the method of Brodie and Lipmann (1955).

Hexose utilization was followed by the procedure described by Morris (1948). Respiratory data were obtained by Warburg manometry (Umberg et al., 1949). The pH was measured potentiometrically. Protein concentration was determined according to Lowry et al. (1951).

RESULTS

Properties of cell extract. The partial purification of the enzyme system derived from M. pseudomallei was accomplished by procedures summarized in table 1. Washed cell suspensions contained approximately 2 × 10⁸ viable cells per ml. Subsequent to the grinding procedure viability declined by 83 per cent. An additional 100-fold reduction in the viable cell concentration was obtained by centrifugation of the crude extract. The first (NH₄)₂SO₄ fractionation increased the total activity in two of the three experiments. The second precipitation with this salt was discontinued in routine preparations since it was attended by a loss in both total and specific activity.

Treatment of the extract with MnCl₂ precipitated undetermined quantities of nucleic acid and also lowered the viable cell concentration. Previous assays demonstrated that measurable
O₂ uptake activity occurred in Warburg vessels containing approximately 3 × 10⁸ viable cells. However, fractionation of the extract with either (NH₄)₂SO₄ or MnCl₂ or both was sufficient to reduce the viable cell population 10 to 10,000 fold below this level.

The partially purified extract could be preserved as the (NH₄)₂SO₄-precipated fraction at 0 to 3 C for at least 36 days without loss of activity. After dialysis of either the precipitated fraction or untreated extracts against distilled water (0 to 3 C), a precipitate was recovered from the dialysis bag. Activity was contained in this fraction and not in the supernatant fluid.

The crude ground extract had a high rate of endogenous O₂ uptake which was lowered by 10 to 20 per cent by dialysis. Following either (NH₄)₂SO₄ precipitation or MnCl₂ treatment, the endogenous O₂ rate was essentially zero (figure 1).

The system catalyzed the oxidation of glucose between pH 4.0 and pH 7.0 with a maximum rate at pH 5.5 (figure 2). Typical oxidative activity by the enzyme system (1.8 × 10⁸ viable cells per ml) on glucose and galactose is shown in figure 1. Both hexoses were oxidized with the uptake of 0.5 μ moles O₂ per μ mole of substrate. The rate of the reaction was not accelerated by the addition of TPN, DPN, ATP, Mg²⁺ individually or in combination. No CO₂ evolution was observed. The 1 and 6 phosphate esters of glucose were oxidized, but there was no measurable oxidation of the following hexoses and related metabolites: L-allose, L-altrose, d-arabinose, d-fructose, d-fructose-6-phosphate, d-fructose-1,6-diphosphate, d-gulose, d-mannose, d-ribose, d-talose, d-xyllose, d-gluconic acid, calcium-bi-malate, fumaric acid, glycerol, α-ketoglutaric acid, lithium lactate, oxalacetic acid, sodium acetate, sodium citrate, sodium pyruvate, and succinic acid.

The effect of glucose concentration upon
the reaction velocity is shown in figure 3. The oxidative rate increased linearly over a concentration of 1 to 10 μmoles per ml in preparations containing 3 mg of protein per ml. The Michaelis constant derived by the method of Lineweaver and Burk (1934) was $K_m = 3.5 \times 10^{-3}$ M.

Reaction products. The oxidation of glucose and galactose to their corresponding hexonic acids was demonstrated by paper chromatography of the reaction mixtures containing either cell extracts or whole cells. Typical data are shown in table 2. The extracts were clarified by centrifugation and filtration prior to analyses. Since the acids existed largely in the free form, they were converted to lactones by heating at pH 1. In growth media 58 per cent of the glucose and 25 per cent of the galactose were recovered as the corresponding lactones after 72 hr of incubation. In no instance were we able to detect glucose or gluconate formation from galactose by the enzyme preparation or whole cells. Similarly, the only acid detected during the oxidation of glucose was gluconic acid.

The stoichiometry of the reaction over a period of 160 min is presented in table 3. A final conversion of 78 per cent of glucose to gluconolactone and 103 per cent conversion of galactose to galactonolactone was observed. Oxygen uptake was 97 per cent of theoretical for the oxidation of both substances to hexonic acid according to the reaction:

$$C_6H_{11}O_5\cdot CHO + 0.5 \text{ O}_2 \rightarrow C_6H_7O_5\cdot COOH$$

![Figure 3](image)

* Chromatograms of endogenous extracts were clear.

### TABLE 2

Chromatographic analyses of endproducts of glucose and galactose oxidation by cell extracts and intact cells of Malleomyces pseudomallei

<table>
<thead>
<tr>
<th>Experiment*</th>
<th>Solvent System</th>
<th>$R_f$ Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Product</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gluconolactone</td>
</tr>
<tr>
<td>1. Extract + glucose</td>
<td>BuOH—EtOH—H$_2$O</td>
<td>0.34</td>
</tr>
<tr>
<td>Extract + galactose</td>
<td>BuOH—EtOH—H$_2$O</td>
<td>0.28</td>
</tr>
<tr>
<td>2. Extract + glucose</td>
<td>BuOH—EtOH—H$_2$O</td>
<td>0.29</td>
</tr>
<tr>
<td>Extract + galactose</td>
<td>BuOH—EtOH—H$_2$O</td>
<td>0.22</td>
</tr>
<tr>
<td>Extract + glucose</td>
<td>Butanone—HCl</td>
<td>0.33</td>
</tr>
<tr>
<td>Extract + galactose</td>
<td>Butanone—HCl</td>
<td>0.18</td>
</tr>
<tr>
<td>3. Whole cells + glucose</td>
<td>BuOH—EtOH—H$_2$O</td>
<td>0.34</td>
</tr>
<tr>
<td>Whole cells + galactose</td>
<td>BuOH—EtOH—H$_2$O</td>
<td>0.29</td>
</tr>
</tbody>
</table>

* Chromatograms of endogenous extracts were clear.
TABLE 3

The oxidation of glucose and galactose to hexonic acids by an enzyme preparation from Malleomyces pseudomallei

<table>
<thead>
<tr>
<th>Minutes</th>
<th>Micromoles per ml Reaction Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment A</td>
</tr>
<tr>
<td></td>
<td>O$_2$ Uptake</td>
</tr>
<tr>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>40</td>
<td>1.8</td>
</tr>
<tr>
<td>80</td>
<td>3.1</td>
</tr>
<tr>
<td>160</td>
<td>4.8</td>
</tr>
</tbody>
</table>

* Enzyme was prepared by following steps 1, 2, 3 (table 1). Vessels contained 2.5 ml extract (20 mg protein), 30 µmoles substrate, 33 µmoles phosphate, pH 5.5 and 0.2 ml 20 per cent KOH in center cup. Total volume was 3.2 ml.

Although total hexonic acid was measured after lactonization by heat, approximately 30 per cent of the end product was recovered as lactone at the termination of the oxidative reaction without heat treatment. However, known samples of gluconate were not lactonized enzymatically in detectable quantities. Thus, it appears that the acid end product is formed from intermediate lactone.

DISCUSSION

Similarities are evident in the enzyme system from M. pseudomallei described in this report and those derived from Pseudomonas fluorescens (Wood and Schwerdt, 1953) and Pseudomonas aeruginosa (Claridge and Werkman, 1953). In each case, activity was contained wholly within the fractions precipitated by (NH$_4$)$_2$SO$_4$ and added cofactor requirements were not demonstrated. However, the enzyme complex derived from the pseudomonads catalyzed the oxidation of glucose to 2-keto-6-phosphate via gluconate while only the first step of this reaction could be demonstrated in the extracts from M. pseudomallei which also oxidized galactose to galactonic acid.

Although the products of galactose oxidation were not reported in the abovementioned studies on the pseudomonads, others have demonstrated the intermediary role of galactonate in this genus as well as in A. cloacae and the alga I. flaccidum.

However, in other bacterial genera as well as in yeasts and animal tissues (Gunsalus et al., 1955; Hansen and Freedland, 1955) galactose was metabolized initially by interconversion with glucose by an enzyme system provisionally termed “galactowaldenase.” Hansen and Freedland (1955) demonstrated that this interconversion occurred in unfractionated extracts of Lactobacillus bulgaricus when uridine diphosphohexose (UDPH) or uridine triphosphate was supplied in the reaction. After (NH$_4$)$_2$SO$_4$ fractionation and dialysis, only UDPH served as a cofactor. In our study with M. pseudomallei, hexose interconversion was not detected in reactions employing either whole or disrupted cells, and only the corresponding hexonic acid was demonstrated after glucose or galactose was oxidized. Further, since added nucleic acid cofactors were not required for galactose metabolism by partially purified and dialyzed extracts, it is unlikely that galactowaldenase plays a significant role in this strain.

The procedure for disrupting cells in the Waring blender was suggested by the study of Lamanna and Malette (1954). However, it was necessary to substitute alumina for small glass beads and to incorporate the modifications described in order to obtain enzymatically active cell extracts. Previous attempts to obtain such preparations by manual grinding, lysis by glycine (Maculla and Cowles, 1948), lysozyme or toluene and alternate freezing and thawing were unsuccessful.

SUMMARY

The preparation and partial purification of an enzyme system from Malleomyces pseudomallei which catalyzed the oxidation of glucose to gluconic acid and galactose to galactonic acid is described. Intermediary lactone formation was shown and the reaction was demonstrated also in growth media. The complete system was precipitated by (NH$_4$)$_2$SO$_4$ and was not stimulated by added TPN, DPN, ATP or Mg$^{2+}$. Only the corresponding hexonic acids were formed by oxidation of glucose or galactose and a “galactowaldenase” rearrangement was not detected. A modified procedure is described for
disrupting cells by grinding in the Waring blender.

REFERENCES


DELEY, J. 1955 Galactose oxidation by Pseudomonas saccharophila. Meeting of the Northern California and Hawaii Branch of the S.A.B., Davis, California, November 17, 1955.


