HEXAMINE DEHYDROGENATION OF BACTERIA

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(Prof. U. Kurimoto)

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The discovery of this new chemical activity of bacteria is based on a suggestion of Prof. U. Kurimoto that much more new biological reactions should be presented for the classification of Enterobacteriaceae, in which nowadays serological methods are predominantly used. The study of antigen analysis of Enterobacteriaceae is so far advanced that several detailed tables of antigenic structures have been presented, which are, however, liable to be changed by the complicated problems of minor antigens and the variation of antigenic structures. On the other hand only a few biological characters available for the classification have recently been proposed. The Wood's test(1,2), being one of them, consists in the reduction of Trimethylamine-oxide by bacteria and proved very useful(3). Hexamine (Methenamine, Hexamethylenetramine, C₆H₁₂N₄) was chosen for its structural similarity with the former reagent. Investigations of the dehydrogenatic activities of strains in Enterobacteriaceae against Hexamine will be reported in this paper. This new reaction might have some significance in the classification of Enterobacteriaceae.

MATERIAL AND METHODS

Strains studied: All strains of Shigella, Salmonella, Paracolon and Alkalescens-Dispar were kindly supplied from the National Institute of Health of Japan, Escherichia from the Kitazato's Institute to Tokyo; others from the Prefectural Institutes of Health to Gifu and Aichi.

Reagent: Hexamine (Hex) was three times recrystallized from ethanol.

Technique: The technique measuring the time necessary for 90% decolorization of Methylenblue (Mb) in the Thunberg's tubes was used throughout the experiments.

Cell suspension: Bacteria were harvested from the surface of the plain nutrient agar of pH 7.4 after 18 hrs. incubation at 37°C, washed twice and suspended in saline containing usually one tenth of its volume of M/15 phosphate buffer at pH 8.0. The cell suspension containing nitrogen at 0.02 to 0.03 mg/ml proved most satisfactory in preliminary experiments causing no remarkable decolorization of Mb in the absence of substrate within 60 minutes, and standards of turbidity were used for the dilution of washed cells. The nitrogen contents of suspensions thus prepared, however, were determined in every experiments by the micro-Kjeldahl method.

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Constituents of reaction in a Thunberg's tube:

Lower part:
- Cell suspension ....................... 1.0 ml
- Buffer .................................. 1.0 ml

Upper part:
- Methylene blue solution (1:5000) ...... 0.5 ml
- Hexamine (M/2 M/10) .................... 0.5 ml

The tubes were evacuated by a vacuum pump for further 2 minutes after the manometer showed 10 mm Hg pressure (the final pressure arriving at around 2 to 5 mm Hg), then placed in a thermostat at 37°C for 10 minutes before mixing the upper and lower contents.

Velocity: The dehydrogenatic activities of bacteria have been expressed in several ways; e.g., Quastel and his co-workers\(^4\) as well as Nakagome\(^5\) proposed what they called “Reducing Coefficient” taking as standard substrates succinic acid or glucose respectively. In Toda's Textbook of Bacteriology\(^6\) one hundred times the reciprocal of the time in minutes necessary for Mb decolorization is adopted as “Reducing degree” which will be designated as “velocity” in this communication.

EXPERIMENTAL RESULTS

A. KINETICS OF THIS PRESUMABLY ENZYMIC REACTION

1. Influence of pH: As will be seen in Fig. 1, M/15 phosphate buffer (Sörensen) at pH 7.8-8.0 and veronal-Na buffer (Michaelis) at pH 8.0-8.4 were optimal but borate buffer (Sörensen) proved inhibitory to this reaction. In the following experiments M/15 phosphate buffer at pH 8.0 was principally used.

![Graph of pH Influence](image)

Fig. 1 Influence of pH

- M/15 Phosphate B
- Veronal B
- Borate B

2. Influence of the amount of cell suspension: As shown in Fig. 2 the velocity was usually directly proportional to the volumes of cell suspension, provided that the afore-said concentrations were used. However, with more con-
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Centered suspensions this was not true and also the endogenous reducing power without substrate became so marked that could not be neglected.

\[
\begin{align*}
\text{Fig. 2 Influence of Bacterial Concentration} & \quad \begin{cases} 
\text{Arizona D. C. 5} \\
\text{Hex. M/2, 0.5 ml} \\
\text{37° C}
\end{cases} \\
\text{Michaelis constant:} & \quad \text{Assuming the availability of the Michaelis-Menten's equation for this reaction, the Michaelis constant was evaluated graphically as 0.013 Mol (Fig. 3) since it is equal to that substrate concentration which produces one-half the maximum velocity of the reaction}^{(7,8)}. \\
\end{align*}
\]

\[
\begin{align*}
\text{Fig. 3 Michaelis constant} & \quad \begin{cases} 
\text{Arizona D. C. 5 (N=0.036 mg)} \\
37° C, \text{pH 8.0} \\
\text{Hex. 7.1–1/360 Mol.}
\end{cases}
\end{align*}
\]
4. Temperature Coefficient and Apparent Energy of Activation: As will be seen in Table 1, the temperature coefficient of this reaction was calculated at 1.84 comparing the velocities at 27°C and 36.8°C.

Kubo\(^9\) had showed in his study on the dehydrogenase of E. coli in Thunberg's tubes that the reciprocal of the time of decolorization could be substituted for the velocity constant in the Arrhenius's equation:

\[
\frac{d \ln K}{dT} = \frac{A}{RT}
\]

where

\(T\): absolute temperature

\(A\): energy of activation

\(R\): gas constant

\(K\): velocity constant

then he deduced the following equation:

\[
\log Z = \log C - \frac{A}{4.571} \cdot \frac{1}{T}
\]

where

\(Z\): time of decolorization

\(C\): constant

which made possible the graphical evaluation of \(A\) as the slope of \(\log Z:1/T\) line. As will be seen in Fig. 4 the apparent energy of activation of this reaction ranged from 10,000 to 11,000 Cal.

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**Fig. 4 Apparent Energy of Activation**

- Arizona D. C. 5 (N=0.06 mg)
- Hex. M/6, 0.5 ml
- pH 8.0

\[
\log Z = \log C - \frac{A}{4.571} \cdot \frac{1}{T}
\]

\[
\text{Slope} = \frac{0.26}{0.117} \times 4.571 \times 10^3 = 10200
\]
5. *Inactivation by heat:* Heating at 80°C for 20 minutes destroyed completely the activity of cell suspension.

6. *Inhibitors:* As shown in Fig. 5 monoiodoacetic acid, semicarbazide and urethane inhibited this reaction, provided that they were in contact with the cells for 30 minutes before the test.

### Table 1. Temperature and Velocity

<table>
<thead>
<tr>
<th>Temperature</th>
<th>100 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>27.0°C</td>
<td>8.84</td>
</tr>
<tr>
<td>30.0°C</td>
<td>11.42</td>
</tr>
<tr>
<td>33.3°C</td>
<td>13.5</td>
</tr>
<tr>
<td>36.8°C</td>
<td>16.3</td>
</tr>
<tr>
<td>40.0°C</td>
<td>17.4</td>
</tr>
</tbody>
</table>

Conditions equal to Fig. 4

![Fig. 5 Effect of Inhibitor](image)

- Arizona D. C. 5 (N=0.02 mg)
- Hex. M.4, 0.5 ml
- 37°C

(1) Monoiodoacetic acid 2%
(2) Semicarbazide M/11
(3) Urethane 10%
B. CLASSIFICAL MEANINGS OF THIS REACTION IN THE FAMILY OF ENTEROBACTERIACEAE.

About 160 strains of Enterobacteriaceae and a few strains of other families were examined as to their Hexamine dehydrogenatic activities. The results obtained are summarized as in Table 2 in which the "medium" degree was designated as about the same as the velocity of Paracolon Arizona D.C. 5; i.e., 0.02 to 0.03 mg nitrogen containing suspension decolorized 90% of Mb in 10 to 20 minutes; "strong" in much shorter time and "weak" in 30 to 90 minutes, "none" meant almost no difference between the test and the control test without the substrate in 60 minutes.

By the Thunberg's technique it was rather difficult, as was claimed by the previous investigators(5) to obtain just the same velocity in every experiments, even though the cultural conditions and other procedures were made fixed as similar as possible. Difficulties in comparing activities of many bacteria were increased by the endogenous reducing power of bacteria without the substrate which could not be neglected in some cases and necessitated repeat tests with more highly diluted suspension. All these reasons have made the activities of bacteria examined to be divided into 4 groups instead of numerical expression.

As will be seen in Table 2 this reaction might have some classifical meanings because the Genera Paracolon Arizona, Salmonella and Proteus including Proteus morganii were in general much more active than the Genera Shigella, Escherichia, Alkalescens-Dispar, Paracolon Bethesda and Paracolon Ballerup.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Number of strain tested</th>
<th>Hexamine dehydrogenatic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>none</td>
</tr>
<tr>
<td>Shigella</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>Escherichia</td>
<td>33</td>
<td>25</td>
</tr>
<tr>
<td>Alkalescens-Dispar</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Paracolon Bethesda</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>Paracolon Ballerup</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>Paracolon Arizona</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>Salmonella</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>Proteus</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Micrococcus</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Streptococcus</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
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SUMMARY

1. A new dehydrogenatic activity of bacteria against Hexamine (Methenamine, Hexamethylentetramine) was demonstrated.

2. Enzymic kinetics of this reaction were investigated at the level of cell suspension.

3. By the examination of about 160 strains this reaction proved to be significant to some extent for the classification of Enterobacteriaceae.

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REFERENCES