BACTERIAL FERMENTATION OF CREATININE

I. ISOLATION OF N-METHYL-HYDANTOIN

JEKISIEL SZULMAJSTER1

Section on Enzymes of the Laboratory of Cellular Physiology, National Heart Institute, National Institutes of Health, Bethesda, Maryland

Received for publication December 4, 1957

Although creatinine is, other than urea, the most important nitrogenous end product excreted by many animals, very little is known about its metabolic pathway in soil bacteria. Some incomplete studies have been made by early workers (Ackerman, 1913; Linneweber, 1930), mostly with putrefactive bacteria, and they claimed to have identified, besides ammonia, methyl-hydantoin as an end product.

Dubos and Miller (1937), in a deliberate attempt to find a microorganism which could be used for a bio-assay of creatinine in the blood of higher animals, were the first to isolate from soil two aerobic strains (NC and HR), capable of growing specifically on creatinine. No attempt was made by these authors to study the complete metabolism of creatinine. However, they did observe that the oxidation of creatinine involves the formation of an adaptive enzyme and that urea and ammonia are the end products.

Kreb and Egleston (1939) have identified one of the strains of Dubos and Miller as a coryne-bacterium and have shown that a number of other compounds can be oxidized by this microorganism. The reversible transformation of creatinine to creatine and the oxidative breakdown of the latter by different strains of pseudomonads has been the subject of more recent papers (Kopper and Beard, 1947; Kopper and Robin, 1950; Roche et al., 1950; Akamatsu and Kanai, 1951; Akamatsu and Miyashita, 1952; Nimmo-Smith and Appleyard, 1956; Appleyard and Woods, 1956). However, the method of fermentation of creatinine by anaerobic bacteria still remained unknown. In view of these facts it was of considerable interest to investigate the complete pathway of the bacterial fermentation of creatinine by a pure culture of a soil bacterium.

The present paper deals with the first steps in the anaerobic degradation process of creatinine by a clostridium isolated by the enrichment culture technique.

MATERIAL AND METHODS

Culture. The enrichment culture technique was employed for the isolation from sewage sludge of a pure culture capable of decomposing creatinine under anaerobic conditions. After numerous successive transfers in a liquid medium and of single colonies in agar shake tubes, a pure culture was obtained.

Morphology. The microorganism was found to be a gram-positive, slender, motile rod, 3 to 15 μ in length containing terminal oval spores that bulge the sporangium. Chain formation was rarely observed. Deep agar colonies were lens shaped to flat, 2 to 4 mm in diameter, becoming lobate.

Growth medium. The bacteria were grown anaerobically in a medium containing the following ingredients in g per cent: creatinine, 0.5; yeast extract, 0.1 to 0.2; malt extract, 0.025 to 0.05; NH₄Cl, 0.05; MgSO₄·7H₂O, 0.02; FeSO₄·7H₂O, 0.001; CaCl₂, 0.001. An addition of 500 μg each of MnSO₄·4H₂O and Na₂MoO₄ was made together with potassium phosphate buffer (0.1 M, pH 7.4) plus 0.05 ml of 0.5 per cent methylene blue (as an indicator for anaerobiosis). Since Clostridium strain Bs is an obligate anaerobe it was necessary to add to the medium a reducing agent such as hydrogen sulfide: after autoclaving, 0.3 ml of 10 per cent sterile solution of Na₂S·9H₂O was added to 100 ml of the medium

1 Creatinine medium described above containing 1.5 per cent agar.

2 For the purposes of this paper the strain will be referred to as Clostridium Bs (Bethesda Strain). Since this paper was submitted for publication, Dr. L. S. McClung has tentatively identified Clostridium Bs as Clostridium paraputrificum. The complete description of this organism will be given in a separate communication.
prior to inoculation. A pyrogallol-carbonate seal was used. Cultures were incubated at 31 C. The stock cultures were maintained in the above medium supplemented with 0.5 per cent agar and stored at 3 to 4 C. The pH range for growth was from 6.5 to 7.8 with an optimum at about 7.4.

For large scale experiments the CO_2 added with the seal was insufficient for growth. Therefore, 2 ml of a 10 per cent solution of potassium carbonate (neutralized to pH 7 with sterile HCl in the presence of phenol red) were added to the medium after sterilization. In experiments in which CO_2 was to be analyzed, the pyrogallol-carbonate seal was replaced by “Oxsorbent” (Burrell Corporation, Pittsburgh).

With the above conditions 100 to 150 mg of lyophilized cells per L of 50 to 60 hr culture were obtained. The yield could be increased 4 to 5 times by growing the cells in the presence of 0.5 per cent glucose in addition to the creatinine. Sufficient carbonate (0.3 to 0.4 per cent) in sterile solution was added during growth to maintain the medium approximately at pH 7.

Quantitative determinations. For quantitative growth measurements, the experiments were carried out in colorimeter tubes (final volume 7 ml) and growth estimated by optical density measurements with a no. 660 filter in the Klett colorimeter.

Creatinine was estimated by the Jaffe reaction. Creatine was determined by the method of Eggleston et al. (1943) as modified by Ennor and Stocken (1948). The total volatile acids were determined by titration following steam distillation in the Markham (1942) apparatus. Identification of the volatile acids was carried out by the paper chromatographic method of Kennedy and Barker (1951). When only acetic and formic acids were present, the latter was destroyed by heating with HgO in H_2SO_4 and the acetic acid was then distilled and titrated. The difference between total steam volatile acids and acetic acid corresponds to formic acid. Urea was determined by the urease method of Krebs and Henseleit (1932), and ammonia by Nesslerization or by titration after distillation from alkali.

1-C^14-Creatinine was prepared from 1-C^14-creatinine by autoclaving 1 hr at 121 C in 1 N acid solution. Total conversion was obtained under these conditions.

RESULTS

Nutrition of Clostridium Bs. No growth of Clostridium Bs was observed in a medium containing mineral salts, phosphate buffer, and creatinine, unless yeast extract (0.1 to 0.2 per cent) was added. The requirement for yeast extract was absolute. Attempts to replace the yeast extract by casein hydrolyzate, peptone, tryptone, or a mixture of known vitamins, in the presence or in the absence of a purine-pyrimidine mixture, were unsuccessful. Growth on creatinine plus yeast extract was further improved by the addition of 0.025 to 0.05 per cent malt extract.

The effect of creatinine on growth in the presence of yeast and malt extracts is shown in figure 1 and the effect of creatinine concentration in figure 2.

CO_2 requirement. Clostridium Bs, like certain other clostridia (Tomlinson and Barker, 1954), required carbon dioxide for growth. Figure 3 shows the effect of CO_2 concentration on growth in the complete medium.

Tracer experiments utilizing K_2C^14O_3 have

Figure 1. Growth of Clostridium Bs on creatinine. 1, Yeast extract (0.1 per cent); malt extract (0.025 per cent) and creatinine (0.5 per cent). 2, Yeast extract (0.1 per cent), malt extract (0.025 per cent) and no creatinine. 3, Disappearance of creatinine.
shown that CO₂ is incorporated into cell material and volatile acids. Table 1 summarizes the analysis of the cells and culture filtrate from a typical experiment with labeled carbonate. The protein fraction insoluble in trichloroacetic acid at 100°C contains 65 per cent of the total radioactivity incorporated into the cells. Acid hydrolysis of this fraction has shown that serine, glycine, and glutamic acid contain most of the isotope.

Specificity. Among the guanido and other compounds tried (creatine, guanidine, methyl-guanidine, guanido-acetic acid, arginine, hydantoin, 5-methyl-hydantoin, N-methyl-hydantoin (isolated and purified from culture filtrates), methylhydantoinic acid, sarcosine, urea, sarcosine plus urea, glycine, methyl-urea and methyl-urea plus glycine) only creatine and, to a slight extent, guanido-acetic acid could partially replace creatinine for growth.

All of the carbohydrates tested (glucose, ribose, and xylose) produced heavy growth. With these carbohydrates as substrates, yeast extract was still required for growth.

Analysis of culture filtrate. The results of the chemical analysis of culture filtrates from creatinine-grown cells are summarized in Table 2. These analyses were carried out on samples removed during growth after 2, 4, and 7 days of incubation. The concentrations of creatinine, creatine, ammonia, the total volatile acids, and the pH were determined. The results were corrected for uninoculated controls from parallel samples. It can be seen that all of the creatinine disappeared during growth. The amount of ammonia formed was equivalent to the amount of creatinine which disappeared and which was not recovered as creatine. Only 3 to 4 per cent of the creatinine could be accounted for as volatile acids.

Paper chromatographic studies using n-butanol:pyridine:water (2:2:1) as the solvent system and alkaline ferricyanide-nitroprusside reagent as the spray (Berry et al., 1951), revealed

4 In the preliminary experiments (Szulmajster and Stadtman, 1957), ammonia was determined by direct Nesslerization to avoid heating or prolonged contact with alkali. There was no difference in ammonia measured in this manner between the inoculated and the uninoculated media. Subsequent experimentation has shown the surprising fact that creatinine itself will give a positive Nessler's reaction.

![Figure 2](http://jb.asm.org/) Effect of creatinine concentration on growth of Clostridium Bs. Cells were grown 52 hr in the usual medium containing 0.1 per cent yeast extract and 0.025 per cent malt extract.

![Figure 3](http://jb.asm.org/) Effect of CO₂ on growth of Clostridium Bs. Cells were grown in Klett test tubes for 34 hr in the usual medium containing 0.1 per cent yeast extract and 0.025 per cent malt extract. Sterile potassium carbonate (neutralized to pH 7 in the presence of phenol red) was added to the medium after sterilization.
TABLE 1
Recovery in culture filtrate and cell material of C14O2 incorporated during growth of Clostridium Bs

<table>
<thead>
<tr>
<th></th>
<th>C14</th>
<th>Total Radioactivity Incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>%</td>
</tr>
<tr>
<td>A. Culture filtrate*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) Volatile acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formic</td>
<td>16,500</td>
<td>52.5</td>
</tr>
<tr>
<td>Acetic</td>
<td>10,480</td>
<td>51</td>
</tr>
<tr>
<td>(2) Volatile base</td>
<td>6,020</td>
<td>21</td>
</tr>
<tr>
<td>B. Cell material†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) Proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold TCA soluble</td>
<td>7,18</td>
<td>65</td>
</tr>
<tr>
<td>TCA insoluble at 100 C</td>
<td>2,120</td>
<td>18.5</td>
</tr>
<tr>
<td>Alcohol soluble at 45 C</td>
<td>1,122</td>
<td>10</td>
</tr>
</tbody>
</table>

* The culture filtrate was freed of C14O2 by flushing with N2 until no more counts were in the medium. The analysis was carried out as given in Methods.
† Cells harvested from 100 ml culture, washed until no more counts were in the washings. The fractionation of the cell material was carried out by the procedure of Roberts et al. (1955).

Initial C14O2 added to 100 ml culture: 472 μmoles containing 490 X 10^2 total cpm.

TABLE 2
Analysis of culture filtrates

<table>
<thead>
<tr>
<th>Age of Culture</th>
<th>Final pH</th>
<th>Creatinine Disappeared</th>
<th>Creatine Formed</th>
<th>Ammonia Formed</th>
<th>NH3 Formed/Creatinine-creatinine disappeared</th>
<th>Volatile Acid Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7.6</td>
<td>90</td>
<td>0</td>
<td>93</td>
<td>1.03</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>8.7</td>
<td>340</td>
<td>70</td>
<td>292</td>
<td>0.81</td>
<td>13.5</td>
</tr>
<tr>
<td>7</td>
<td>8.7</td>
<td>340</td>
<td>70</td>
<td>307</td>
<td>0.85</td>
<td>15.0</td>
</tr>
</tbody>
</table>


that the disappearance of creatinine was associated with the accumulation of a new compound in the medium. The Rf of the new compound in this solvent system was 0.65 (creatinine, 0.38 and creatine, 0.1). The unknown compound was also distinct chromatographically from other guanido compounds such as guanidine, methyl guanidine, guanido-acetic acid, etc. (The same results were observed when the initial pH of the media was maintained by the introduction of sterile HCl during growth.)

Experiments with cell suspensions. Washed cells of Clostridium Bs were able to ferment creatinine only if they were grown in its presence. Cell suspensions from cultures in which glucose was the only substrate were unable to metabolize creatinine. This showed that the fermentation involved an adaptive enzyme. Therefore cell suspensions were prepared from bacteria grown on glucose plus creatinine. After harvesting, the cells were washed twice with phosphate buffer (0.02 m containing 0.03 per cent Na2HPO4), resuspended in phosphate buffer, and incubated in Warburg vessels with creatinine in an atmosphere of N2 + 5 per cent CO2. A rapid CO2 uptake took place in the presence of creatinine. The disappearance of creatinine was determined at the end of incubation. About 0.5 to 0.7 mole of CO2 was consumed per mole of creatinine decomposed. The ratio of ammonia formed to creatinine utilized was 0.8. In similar experiments it was found that C14O2 was not incorporated into compounds stable to drying under slight acid conditions. Therefore it was concluded that with cell suspensions, carbon dioxide uptake may be due to a neutralization of the ammonia formed from creatinine.

Chromatographic analysis of the reaction mixtures from the Warburg experiments has shown that the same compound found in the culture filtrates was formed from creatinine by the cell suspensions.

In order to obtain quantitative data on the amounts of the unknown compound accumulated in culture media and cell suspensions, experiments were carried out using 1-C14-creatine. The balance studies of such experiments have shown that 75 to 80 per cent of the initial counts appeared in the unknown compound. Except for the unused creatinine no other labeled compound was found on the chromatogram. No urea nor sarcosine has been found in the culture filtrates or in the reaction mixtures from cell suspensions.

Isolation and identification of N-methyl-hydan- toin. The compound accumulating during the fermentation of creatinine was not retained on anion or cation exchange resins (Dowex-50-H+ or Dowex-1-HCOOH). Therefore the following
BACTERIAL FERMENTATION OF CREATININE

Figure 4. Infrared spectra of N-methyl hydantoin: 1. from culture medium and 2. synthetic compound.

method was used for the isolation of this substance from culture medium.

The culture medium (1700 ml) was passed through a Dowex-1-formate column (12 by 3.5 cm diam). The effluent was acidified to pH 3.5 with formic acid and passed through a Dowex-50-H+ column of the same size. The resulting effluent was concentrated under vacuum. The yellow crystalline residue was dissolved in a minimal amount of hot ethanol. Charcoal (0.5 g Norit) was added, the mixture was heated until boiling and filtered rapidly while hot. Crystallization in the filtrate took place immediately. After three recrystallizations from ethanol a white crystalline material was obtained (2.5 g).

The compound has been identified as N-methyl-hydantoin on the basis of the following information: (1) It has a sharp melting point at 157 C ±0.5 (156 C for synthetic N-methyl-hydantoin (Salkowski, 1874), which is not depressed when it is mixed with the synthetic compound). (2) From elemental analysis by Dr. Alford at the National Institutes of Health the compound was found to contain: C, 42.5 per cent; N, 24.4; H, 5.32; O, 28.8. The calculated values for N-methyl-hydantoin (C16H12N2O2) are C, 42.1; N, 24.55; H, 5.03; O, 28.3. (3) The infrared spectrum (figure 4) was identical with that of synthetic N-methyl-hydantoin. (4) In the ultraviolet region N-methyl-hydantoin and the fer-

A sample of synthetic N-methyl-hydantoin was prepared by a modification of the method of Gaebler (1926). Creatinine was hydrolyzed by boiling in the presence of barium hydroxide. The major part of methyl-hydantoic acid was separated by crystallization after acidification with H2SO4. The purification was carried out by the procedure employed for the fermentation product. The small amounts of methyl-hydantoic acid still present, were removed by paper chromatography and N-methyl-hydantoin was isolated by elution with water and recrystallization from ethanol.
mentation product both have identical spectra with an absorption maximum at 217 m\(\mu\). (5) Upon paper chromatography in two solvent systems (pyridine: n-butanol: water (2:2:1); tert-butanol: acetic acid: water (2:1:1)), the fermentation product and the synthetic compound moved as a single spot. They were further characterized by the fact that they gave a weak Jaffe reaction after 10 to 12 hr, under conditions in which the color produced by creatinine was apparent immediately. Hydrolysis of the synthetic and the enzymatic compounds in 1.0 N HCl at 120 C lead to the formation of sarcosine. The latter compound was identified by co-chromatography with synthetic sarcosine in tert-butanol:acetic acid:water (2:1:1) solvent system, by nitroprusside-acetaldehyde spray which gave a blue spot, specific for secondary amines, and by the ninhydrin reaction.

**DISCUSSION**

The experiments described show that Clostridium Bs, isolated by enrichment culture technique was able under anaerobic conditions to convert creatinine almost quantitatively to N-methyl-hydantoin and ammonia:

\[
\begin{equation}
\text{HN} = \text{C}\bigg\langle \begin{array}{c}
\text{NH} \\
\text{CO}
\end{array}\bigg\rangle
\end{equation}
\]

\[
\text{HN} = \text{C}\bigg\langle \begin{array}{c}
\text{N} \\
\text{CH}_2
\end{array}\bigg\rangle
\]

\[
\text{CH}_3
\]

\[
\begin{equation}
\text{O} = \text{C}
\end{equation}
\]

\[
\begin{equation}
\text{NH} = \text{CO}
\end{equation}
\]

\[
\begin{equation}
\text{N} = \text{CH}_2
\end{equation}
\]

\[
\text{NH}_3
\]

The formation of N-methyl-hydantoin from creatinine may take place in different ways:

1. Creatinine \(\rightarrow\) methyl-hydantoin + NH\(_3\)
2. Creatinine \(\rightarrow\) creatine \(\rightarrow\) urea + sarcosine
3. Creatinine = NH \(\rightarrow\) methyl-hydantoin + X = NH

Reaction (2) is the least likely, since it has not been possible to show the formation of sarcosine, glycine, or urea from creatinine in growth media, nor the formation of methyl-hydantoin from sarcosine plus urea or from glycine plus methyl-urea.

In view of the fact that optimum growth of the organism depended on very high concentrations of creatinine it is rather tempting to speculate that the formation of methyl-hydantoin from creatinine is an energy-yielding process which provides energy for growth of this organism. If this is true, one would expect that the formation of methyl-hydantoin would involve an energy rich intermediate. To test this hypothesis studies are now in progress with cell-free extracts and purified enzyme preparations.

The formation of methyl-hydantoin and ammonia may take place by a mechanism shown in (3) in a very simplified form, namely, the guanido group of creatinine is transferred to an acceptor X with formation of X = NH which may be subsequently decomposed with the liberation of X plus ammonia.

Another possibility is that the energy for growth might be supplied through the complete degradation of some creatinine, since the recovery in the creatinine \(\rightarrow\) methyl-hydantoin reaction was only about 75 to 80 per cent. This would also suggest that some creatinine was assimilated by the cells. To determine whether creatinine is partly utilized for the synthesis of cell material, uniformly labeled creatinine would be required. Experiments with creatinine labeled in the 1-position have shown that little if any of the C-1 carbon was incorporated into cell material.

**ACKNOWLEDGMENTS**

I am grateful to Doctors T. C. Stadtman and E. R. Stadtman for the generous hospitality of their laboratory, for their helpful suggestions and constant interest during the course of this work.

**SUMMARY**

An organism belonging to the genus *Clostridium* was isolated from sewage sludge by the anaerobic enrichment culture technique. Growth on creatinine occurred only in the presence of yeast extract and carbon dioxide. The latter was incorporated into cell material as shown by experiments with C\(^{15}\)O\(_2\).

During growth, creatinine disappeared, a mole
of ammonia was formed per mole of creatinine decomposed, and a new compound accumulated in the medium. Only 3 to 4 per cent of the creatinine was converted to volatile acids. The new compound has been isolated from culture filtrates, crystallized, and identified as N-methyl-hydantoin.

Cell suspensions from creatinine-grown cells were able to carry out the same reaction. Possible mechanisms of the formation of methyl-hydantoin from creatinine are discussed.

REFERENCES

KREBS, H. A. AND EGGLESTON, L. V. 1939 Bacterial urea formation. Enzymologia, 1, 310–320.