METABOLISM OF ALLOXANIC ACID IN A SOIL MICROORGANISM

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Alloxan is a polyoxy-pyrimidine of unusual biological interest, being the agent of choice for producing experimental diabetes (Shaw-Dunn, Sheehan, and McLetchie, 1943). Despite the fact that it has been used extensively for many years, very little is known of its ultimate fate in mammals or other forms of life. It is exceedingly unstable at physiological pH values and is spontaneously converted to alloxanic acid within a few minutes in blood (Leech and Bailey, 1945; Seligson and Seligson, 1951), or other milieus (Richardson and Cannan, 1929; Archibald, 1945; Patterson, Lazaro, and Levey, 1949). Although mammals receiving alloxan excrete small quantities of murexide (Cerecedo, 1931; Lee and Stettin, 1952) and a compound which yields mesoxalic acid on hydrolysis (Seligson et al., 1951), the major decomposition products would appear to be unidentified substituted urea compounds which arise from alloxanic acid metabolism (Lee and Stettin, 1952).

A study of the metabolism of alloxanic acid in a bacterium obtained by soil enrichment was undertaken to establish a route for alloxan catabolism. The method of simultaneous adaptation was used as this technique has been successful in studying the breakdown of related pyrimidines (Wang and Lampen, 1952; Hayaishi and Kornberg, 1952; Lara, 1952).

An additional reason for studying alloxanic acid catabolism was the hope that the knowledge gained might suggest means for evaluating the hypothesis that certain cases of diabetes may arise from the endogenous formation of alloxan from various natural precursors. According to this hypothesis (Lazaro, 1946) normal products of metabolism such as uric acid or substances in vegetable foodstuffs may give rise to alloxan under certain conditions. The fugitive nature of alloxan makes it difficult to test this hypothesis by direct means. The detection of specific decomposition products of alloxan, which might be expected to accumulate in large quantities, would seem to offer more promise. In instances where bacteria might be suspect in the conversion of precursors to alloxan, the presence of a specific alloxanic acid decarboxylase, as in the bacterium studied, may be taken as presumptive evidence that alloxan or a precursor was present to form the specific inducer, alloxanic acid. Should alloxanic acid accumulate, purified alloxanic decarboxylase might be used to assay this compound.

MATERIALS AND METHODS

Isolation of the microorganism. Standard soil enrichment technique was used with 0.4% alloxan (carefully neutralized to avoid murexide formation) as a sole source of carbon, nitrogen, and energy. The basal medium (double strength) contained: NaCl, 2.0 g; MgSO4·7H2O, 0.8 g; KH2PO4, 24.0 g; K2HPO4, 8.0 g; water, 1 liter. The pH was adjusted to 6.2. For use equal volumes of this medium and an aqueous solution of the compound under study were mixed. The solid plating medium contained in addition 2% agar. Cultures were incubated at room temperature. The primary enrichment cultures were streaked on the solid medium, and the resulting colonies were picked and replated until a pure culture was obtained. The gram-negative aerobe selected for study was a highly pleomorphic, unpigmented rod, of undetermined species. Inasmuch as alloxan is unstable at physiological pH values, the microorganism was actually selected for growth on alloxanic acid. Attempts to isolate a microorganism which would grow on alloxan, that is to say at a pH below 4.0, were not successful.

Determination of growth response. Growth in shaken liquid cultures was determined by measuring the optical density at regular intervals in a model B Beckman spectrophotometer at

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590 m\(\mu\) until a plateau was reached. Growth was considered negative when there was no increase in optical density within 120 hr. The final concentration of the test compound was 0.4\% when it served as a sole source of carbon and nitrogen. When it was used as a source of carbon only, the concentration was lowered to 0.2\% and nitrogen was supplied by 0.2\% ammonium sulfate or urea. Compounds of questionable stability were sterilized by passing them in solution through Millipore filters.

**Cell extracts.** (1) Crude extracts:—The bacteria were cultured aerobically at 30 C using 2,000-ml Erlenmeyer flasks containing 1,000 ml of medium and employing continuous shaking. The cells were washed twice in 0.02 \(M\) phosphate buffer (pH 7.4) and one volume of cell paste was suspended in four volumes of 0.05 \(M\) tri(hydroxymethyl)aminomethane buffer (pH 8.0). The suspension was subjected to sonic oscillation in a Raytheon 10 kc magnetoeonstrictive oscillator at 4 C for 12 min. The disrupted cell suspensions were clarified by centrifugation at 20,000 \(\times\) \(g\) for 30 min in a Servall model SS-1 centrifuge. On occasion the extracts so obtained were dialyzed against cold distilled water for 4 to 18 hr.

(2) Ammonium sulfate fractionation:—The crude undialyzed extract was treated with sufficient solid ammonium sulfate to give 0.30 saturation and then centrifuged at 20,000 \(\times\) \(g\) for 20 min. The precipitate was discarded and the supernatant fluid brought to 0.60 saturation with a further quantity of ammonium sulfate. The resulting precipitate was collected, dissolved in distilled water, and dialyzed with stirring against cold distilled water for 4 hr.

Protein was determined turbidimetrically by the method of Stadtman, Novelli, and Lipmann (1951), or by ultraviolet extinction at 280 and 260 m\(\mu\).

**Chemicals and reagents.** Most of the compounds were obtained from commercial sources and were tested for purity by chromatography and melting point. Dialiric acid was kindly supplied by C. E. Carter, Department of Pharmacology, Yale Medical School. Allooxid acid and 3-hydroxyhydantoin were synthesized by the methods of Bitt, Heyn, and Berghaus (1916) and Biltz and Kobel (1921). Allantoic acid was prepared from allantoin (Young and Conway, 1942).

**Analytical procedures.** The following procedures were used to detect and quantitate the various enzymes or their reaction products:

1. Manometric determinations:—Oxygen consumption and carbon dioxide evolution were measured in a conventional Warburg apparatus at 30 C.

2. Urea and urease:—Urea was determined manometrically (Krebs and Henseleit, 1932) or in Conway diffusion cells (Conway, 1950) using crude or crystalline jack bean urease. Urease was determined by the same technique in a reciprocal manner.

3. Identification of glyoxylate:—Glyoxylic acid was clearly distinguished from glycolic acid and oxalic acid in deproteinized reaction mixtures by ascending paper chromatography in an n-butanol-water-formic acid solvent (77:10:13) and spraying the dried paper with neutral alcoholic bromocresol green. The respective \(R_p\) values were 0.77, 0.60, and 0.04. Glyoxylate was also identified by the migration of its 2,4-dinitrophenylhydrazones using descending paper chromatography in a solvent composed of n-butanol, ethanol, and 0.5 \(N\) ammonia (70:21:10). It was later found that glyoxylate could also be determined by adding thiamine pyrophosphate to the 0.30 to 0.60 ammonium sulfate enzyme fraction and observing the evolution of 1 \(\mu\) mole of CO\(_2\) for every 2 \(\mu\) moles of glyoxylate present; a reaction first described by Krakow and Barkulis (1956).

4. Identification of other compounds:—Alloxic acid, parabanic acid, and oxaluric acid were detected on paper using the n-butanol-water-formic acid solvent mentioned above and observing ultraviolet absorbing areas and acidic areas. As work progressed it was possible to distinguish alloxic acid from other compounds by the use of the specific alloxic acid decarboxylase described in the text.

**RESULTS**

**Growth.** The results of growth studies are indicated in Table 1. It should be pointed out that the duration of the lag phase and the total crop achieved on certain substrates varies with the source of inoculum. Thus, cells taken from an alloxic or glyoxylate medium grew poorly when transferred to an acetate medium and the reverse was also true. The cells grew well on conventional carbon compounds such as glucose,
glycerol, pyruvate, and inositol, and conventional members of the tricarboxylic acid cycle such as succinate and malate. The compounds which were of more direct concern, neutral alloxan and alloxanic acid, always supported moderate growth. Of the compounds related to alloxanic acid by structure, 5-hydroxyhydrantoine supported growth, whereas oxaluric acid or parabanic acid were inactive. Hydantoin and hydantoic acid also failed to support growth. Uric acid and the compounds usually associated with its enzymatic breakdown, allantoin and allantoic acid, supported good growth. It was interesting to observe that conventional pyrimidines and double pyrimidines were inactive.

Behavior of resting cell suspensions. Results obtained with resting cell suspensions were difficult to interpret because of questions of permeability. However, two findings were of interest. Neutral alloxan and alloxanic acid were oxidized without lag only by cells grown on either of these substrates. This interchangeability was expected because both compounds exist as alloxanate in solutions which are neutral or approaching neutrality. One characteristic of alloxanic acid oxidation worthy of mention is that it was always preceded by a rapid evolution of approximately 1 μmole of carbon dioxide per μmole of neutral alloxan or alloxanic acid present.

Uric acid was oxidized without lag only when the cells were grown in its presence.

Cell-free extracts with alloxanic acid. The initial evolution of CO₂ from alloxanic acid, observed with whole cells taken from a medium containing this compound, was taken as a point of departure for studies with cell extracts. Table 2 shows

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**TABLE 1**

*Growth of soil isolate on various substrates in terms of days required to achieve maximal crop*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Optical Density</th>
<th>Days</th>
<th>Substrate</th>
<th>Optical Density</th>
<th>Days</th>
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</thead>
<tbody>
<tr>
<td>Neutral alloxan</td>
<td>0.85</td>
<td>3</td>
<td>Inositol</td>
<td>1.39</td>
<td>2</td>
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<tr>
<td>Alloxanic acid</td>
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<td>3</td>
<td>Glucose</td>
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<td>2</td>
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<tr>
<td>5-Hydroxyhydrantoine</td>
<td>0.48</td>
<td>3</td>
<td>Glycerol</td>
<td>1.62</td>
<td>2</td>
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<tr>
<td>Hydantoin</td>
<td>0.5</td>
<td>5</td>
<td>Glyceric acid</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Hydantoic acid</td>
<td>0.5</td>
<td>5</td>
<td>Pyruvic acid</td>
<td>1.30</td>
<td>2</td>
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<tr>
<td>Oxaluric acid</td>
<td>0.5</td>
<td>5</td>
<td>Acetic acid</td>
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<td>4</td>
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<tr>
<td>Parabanic acid</td>
<td>0.5</td>
<td>5</td>
<td>Citric acid</td>
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<tr>
<td>Uric acid</td>
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<td>Succinic acid</td>
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<td>2</td>
</tr>
<tr>
<td>Allantoin</td>
<td>0.80</td>
<td>2</td>
<td>Malic acid</td>
<td>1.16</td>
<td>2</td>
</tr>
<tr>
<td>Allantoic acid</td>
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<td>2</td>
<td>Malonic acid</td>
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<td>2</td>
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<td>Barbituric acid</td>
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<td>5</td>
<td>Methoxalic acid</td>
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<tr>
<td>Dialuric acid</td>
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<td>5</td>
<td>Mesoxylic acid</td>
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<td>Uramil</td>
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<td>5</td>
<td>Glycoaldehyde</td>
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<tr>
<td>Cytosine</td>
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<td>Glycolic acid</td>
<td>0.68</td>
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<td>Glyoxal</td>
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<td>Glyoxylic acid</td>
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<tr>
<td>Alloxantin</td>
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<td>5</td>
<td>Oxalic acid</td>
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<td>5</td>
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<tr>
<td>Murexide</td>
<td>0.5</td>
<td>5</td>
<td>Formic acid</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

* See text for details.

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**TABLE 2**

*Decarboxylation of alloxanic acid by cell-free extracts*

<table>
<thead>
<tr>
<th>Test Substrate</th>
<th>CO₂ Produced per μmole of Test Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alloxan (neutral) grown cells</td>
</tr>
<tr>
<td></td>
<td>μmole</td>
</tr>
<tr>
<td>Alloxan (neutral)</td>
<td>0.92</td>
</tr>
</tbody>
</table>

The reaction mixture contained 10 μmoles of neutral substrate, 2.5 mg of extract protein, and sufficient phosphate buffer (pH 7.2) to give a final molarity of 0.015 M. The total volume was 1.3 ml. Warburg vessels were shaken for 20 min in air at 30°C.
that similar results were obtained with extracts of cells grown on neutral alloxan. Succinate grown cells were inactive. As with whole cells, a slow O₂ consumption and CO₂ evolution followed the initial rapid release of CO₂. This rapid evolution of CO₂ from alloxanic acid also occurred anaerobically with whole cells or extracts. For purposes of future experimental design, these results were interpreted to mean that the removal of the free carboxyl group of alloxanic acid was the first point of attack. Such a reaction would give rise to a five membered compound containing urea and a two carbon fragment. On chemical grounds, the most likely product of a nonoxidative decarboxylation of alloxanic acid is 5-hydroxyhydantoin (Biltz and Kobel, 1921). This compound was found to support growth of the organism. Cell-free extracts of alloxanate grown cells oxidized alloxanic acid, 5-hydroxyhydantoin, and glyoxylic acid. Finally, after incubation of these extracts with alloxanic acid or 5-hydroxyhydantoin, the reaction mixtures were chromatographed directly and after treatment with dinitrophenylhydrazine, and glyoxylate or its phenylhydrazones were detected, although in less than theoretical amounts, in all instances.

Other five membered compounds containing urea and a two carbon fragment and worthy of consideration as the first breakdown product of alloxanic acid are: hydantoin, hydantoic acid, parabanic acid, and oxaluric acid. None of these compounds will alone support growth, but parabanic acid and oxaluric acid will serve as an excellent nitrogen source when a suitable carbon source is supplied. Hydantoin and hydantoic acid contain an incipient glycolic acid residue, whereas parabanic acid and oxaluric acid contain an oxalic acid residue. Oxalic acid does not provide a suitable carbon source for growth of the organism. Crude cell-free extracts from alloxan or alloxanate grown cells failed to oxidize any of these compounds. Further, incubation of these extracts with the five membered compounds in no instance gave rise to glyoxylate.

Parabanic acid and oxaluric acid, in addition to 5-hydroxyhydantoin are split into urea and a two carbon fragment, by an enzyme which is constitutive, extracts of succinate or alloxanate grown cells splitting these compounds. The urea formed is then attacked by the urease present in crude extracts causing CO₂ evolution.

In the case of parabanic acid and oxaluric acid this accounts for the observed CO₂ production. There is no oxygen uptake as glyoxylate does not result, and the oxalic acid formed is not oxidized. With 5-hydroxyhydantoin, on the other hand, additional CO₂ production accompanied by oxygen uptake is observed as a result of further metabolism of glyoxylate (Gray, Gerhart, and Brooke, 1959a). This is consistent with the growth observed on these compounds. Extracts do not convert parabanic acid to 5-hydroxyhydantoin in the presence of reduced diphosphopyridine nucleotide.

Formation of urea and urease. Urease was found in crude cell extracts as a constitutive enzyme. The activity disappeared after the extracts were dialyzed more than 5 hr. Urea was also found to accumulate, albeit in less than theoretical amounts, in crude extracts metabolizing alloxanic acid. The accumulation was difficult to reconcile with the presence of a potent urease, until it was found that alloxanic acid was a powerful inhibitor of bacterial and jack bean urease (Gray, Gerhart, and Brooke, 1959b). Urea did not accumulate when 5-hydroxyhydantoin, parabanic acid, or oxaluric acid were being metabolized by extracts as these compounds do not inhibit urease.

Activity of fractionated extract. The 0.30 to 0.60 ammonium sulfate fraction differed from the crude cell free extract in several respects. It showed increased alloxanic decarboxylase activity (QCO₂ (N) = 12,000) and would not yield CO₂ with any compound tested except alloxanic acid. The enzyme had a broad pH optimum between 6.8 and 7.8. Unfortunately, the preparation retained the ability to split 5-hydroxyhydantoin to glyoxylate and urea. Although the latter reaction proceeded at a slower pace than the decarboxylation of alloxanic acid, it was nevertheless fast enough to foil attempts to isolate 5-hydroxyhydantoin and characterize it chemically. This fraction lacked urease and showed negligible activity against glyoxylate. Glyoxylate was removed 16 times faster when thiamine pyrophosphate and Mg²⁺ were added. A proposed pathway for the conversion of alloxanic acid to glyoxylic acid and urea is shown in Fig. 1.

Consideration of an alternative pathway. Although the evidence favors 5-hydroxyhydantoin as the first reaction product of alloxanic acid,
another reaction sequence could produce the same results. It is conceivable that alloxan acid could be split into mesoxalic acid and urea much in the same manner as barbituric acid is hydrolyzed to malonate and urea (Hayaishi and Kornberg, 1952; Lara, 1952). Decarboxylation of the mesoxalic acid would then yield glyoxylate, just as malonate is decarboxylated to acetate (Gray, 1952; Wolfe, Ivler, and Rittenberg, 1954; Hayaishi, 1953). Such a pathway, however, seems improbable for several reasons. Mesoxalic acid was not attacked by enzyme preparations containing alloxan acid decarboxylase, and although mesoxalic acid liberates CO₂ spontaneously upon occasions, this evolution of CO₂ was variable. For such a pathway to operate one would have to assume that what has been called alloxan acid decarboxylase is in reality an alloxanase followed by a spontaneous decarboxylation of mesoxalic acid. The spontaneous decarboxylation of mesoxalic acid is too erratic for this route of metabolism. It may be mentioned here, parenthetically, that extracts of alloxan acid grown cells would not attack barbituric acid, nor would the isolate grow on barbituric acid or related pyrimidines. In the course of these studies we did isolate several microorganisms which would grow on barbituric acid, but would not attack alloxan (alloxanate). Other workers had previously isolated organisms with similar growth characteristics (Wang and Lampen, 1952; Hayaishi and Kornberg, 1952). It would therefore appear that the abilities to attack alloxan acid and barbituric acid exist as independent functions.

**Uric acid metabolism.** The organism under study grew well on uric acid, allantoin, and allantoic acid but failed to grow on hydantoic acid. Extracts of uric acid grown cells attacked uric acid, allantoin, and allantoic acid, whereas extracts of succinate grown cells failed to attack any of these compounds. Extracts of allantoin grown cells attacked allantoin and allantoic acid, but were not back-adapted to attack uric acid. Furthermore, extracts of uric acid grown cells showed the same pattern of glyoxylate metabolism as extracts of cells grown on glyoxylate or glyoxylate precursors such as alloxan acid or glycic acid (Gerhart, Gray, and Brooke, 1959). Thus uric acid appears to be metabolized by way of an induced pathway involving first allantoin, then allantoic acid, and finally urea and glyoxylate acid. The only difference between this system and that reported for another aerobic microorganism by Campbell (1955) is the subsequent fate of the glyoxylate. The pathways for uric acid and alloxan acid metabolism would appear to converge at the glyoxylate level as shown in Fig. 2. Allanturic acid is an unstable compound and may be an intermediate since it is not known whether both nitrogen to carbon bonds in allantoic acid or 5-hydroxyhydantoic acid are broken simultaneously.

There was no evidence to suggest that uric acid grown cells could metabolize uric acid by way of alloxan. Extracts of uric acid or allantoin grown cells had no alloxan acid decarboxylase activity.

**DISCUSSION**

The route suggested for the catabolism of alloxan acid is of interest in that it is the first

![Schema for degradation of alloxan or alloxan acid to glyoxylate acid and urea.](image)

**Fig. 1.** Schema for degradation of alloxan or alloxan acid to glyoxylate acid and urea.

![Schema for convergence of alloxan and uric acid metabolism.](image)

**Fig. 2.** Schema for convergence of alloxan and uric acid metabolism.
schema presented for the complete breakdown of the spontaneous decomposition product of the pyrimidine, alloxan. There are cogent reasons for believing that 5-hydroxyhydantoin is the first product formed from alloxanic acid, but this compound did not accumulate to an extent that would permit isolation and preparation of derivatives. Furthermore, it was difficult to detect small quantities by paper chromatography because of its feeble acidity, weak ultraviolet absorption, and the lack of specific microtests which would distinguish it from related compounds. To our knowledge, this compound has never been implicated in a biological system before, and the only extant report on its chemistry is that of Biltz and Kobel (1921). Further studies on the chemistry of this compound, especially with the aim of obtaining a specific method for identifying small quantities, would be useful.

The basic reason for this study of alloxanic acid metabolism in bacteria was to provide a model for the breakdown of this compound which might serve as a guide in studying its fate in other forms of life, and in addition, provide further knowledge and methods for the evaluation of the hypothesis that diabetes may result from the endogenous formation of alloxan from normal metabolites in the body.

The fact that our bacterium was isolated on alloxanic acid and was also capable of growing on uric acid, the precursor implicated most frequently as a progenitor of alloxan, gave us the unusual opportunity of determining whether uric acid could in fact be metabolized by way of alloxan. The results indicate quite clearly that a conventional pathway exists for uric acid catabolism and that growth on this compound or its first degradation product, allantoin, does not induce the enzyme alloxan carboxylase, characteristically formed in cells grown on alloxanic acid or on neutral solutions of alloxan. This supports the findings of Lee and Stettin (1952) who could not find labeled alloxan in rats which had been injected with uric-1,3-N\(^{14}\) and carrier alloxan.

It cannot be assumed that a pathway for a given compound in bacteria would be the same in mammalian tissue, but it would be of interest to see whether mammalian tissues contain alloxanic acid decarboxylase and whether the whole animal can produce C\(^{14}\)O\(_2\) from alloxanate prepared from alloxan labeled in the C\(_4\) or C\(_6\) position (which is to say, from alloxanic acid labeled in the C\(_4\) and the carboxyl position). Such an attack on alloxanic acid would not be inconsistent with the findings of Lee and Stettin (1952) that alloxan and alloxanic acid are catabolized in the rat and rabbit through unidentified intermediates consisting principally of substituted urea compounds. These workers agreed with Seligson and Seligson (1951) that the major excretion products are derived from alloxanic acid.

Since the presence of alloxanic acid decarboxylase in microorganisms may turn out to be a general index of prior experience with alloxan, it should be possible to screen various compounds, such as uric acid, dialuric acid, isodialuric acid, and barbituric acid, and test the idea that bacteria can form alloxan from various precursors and thereby precipitate diabetes.

ACKNOWLEDGMENTS

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SUMMARY

Alloxanic acid (or alloxan, which is converted to alloxanic acid on neutralization) served as a sole source of carbon, nitrogen, and energy for the growth of an isolate obtained by soil enrichment technique. Results of growth studies, application of simultaneous adaptation, and experiments with cell extracts and fractions show that this microorganism has an inducible pathway for the catabolism of alloxanic acid. Alloxanic acid is first attacked by a specific decarboxylase, which has been partially purified. Data are presented which suggest that the decarboxylation product is 5-hydroxyhydantoin, a cyclic form of allanturic acid. This compound is then hydrolyzed to form glyoxylic acid and urea. Nitrogen for growth is supplied by the ammonia liberated by the action of a constitutive enzyme, urease.

Uric acid is converted to glyoxylylate and urea in this organism by way of the conventional aerobic sequence involving allantoin and allantoic acid. No evidence was obtained to support the hypothesis that uric acid might be metabolized.
by way of the diabetogenic pyrimidine, alloxan. Knowledge of the route established for the catabolism of alloxanic acid, however, should permit one to evaluate this hypothesis with respect to other potential precursors of alloxan.

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