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# Distribution of Xanthine Oxidase and Xanthine Dehydrogenase Specificity Types Among Bacteria

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A diverse collection of xanthine-metabolizing bacteria was examined for xanthine-, 1-methylxanthine-, and 3-methylxanthine-oxidizing activity. Both particulate and soluble fractions of extracts from aerobically grown gram-negative bacteria exhibited oxidation of all three substrates; however, when facultative gram-negative bacteria were grown anaerobically, low particulate and 3-methylxanthine activities were detected. Gram-positive and obligately anaerobic bacteria showed no particulate activity or 3-methylxanthine oxidation. Substrate specificity studies indicate two types of enzyme distributed among the bacteria along taxonomic lines, although other features indicate diversity of the enzyme within these two major groups. The soluble and particulate enzymes from *Pseudomonas putida* and the enzyme from *Arthrobacter* S-2 were examined as type examples with a series of purine analogues differing in the number and position of oxygen groups. Each preparation was active with a variety of compounds, but the compounds and position attacked by each enzyme was different, both from the other enzymes examined and from previously investigated enzymes. The soluble enzyme from *Pseudomonas* was inhibited in a competitive manner by uric acid, whereas the *Arthrobacter* enzyme was not. This was correlated with the ability of *Pseudomonas*, but not *Arthrobacter*, to incorporate radioactivity from [2-$^{14}$C]uric acid into cellular material.

Enzymes capable of oxidizing free purine bases usually referred to as xanthine oxidase (EC 1.2.3.2), xanthine dehydrogenase (EC 1.2.1.37), or aldehyde oxidase (EC 1.2.3.1), depending on their properties, are widely distributed throughout nature. Isolation of the activities from diverse sources (6, 7, 17-19, 23) has revealed that these enzymes are all fundamentally similar with regard to molecular properties and prosthetic group content, although they may differ considerably in utilization of electron acceptors and the relationship to the economy of the cells from which they are obtained. Another common feature of these enzymes is the relatively broad specificity pattern for purine substrates. All of these enzymes are capable of oxidizing a collection of purines and related compounds and are capable of attacking, with one substrate or another, each of the three positions on the purine ring subject to oxidation (4, 6, 14, 24, 27).

Although a number of chemical features have been recognized or suggested in the analysis of the individual specificity patterns (1, 4, 15, 16, 22), no single chemical parameter can reliably serve as the basis of predicting reactivity with substrates for this class of enzymes since each enzyme source displays a unique pattern. The favored position of attack by an enzyme on a substrate with more than one oxidizable position may vary dramatically from one enzyme source to another. It would seem that a number of factors determine the individual specificity patterns and that these factors can vary independently. Thus, we may have the opportunity in comparing the substrate specificity patterns of these enzymes to trace the evolution of individual catalytic tendencies within the framework of the relatively stable broad pattern of specificity.

This study brings together our initial observations of the specificity pattern of a variety of bacterial purine-oxidizing preparations in an attempt to determine how diverse these patterns are within the bacteria and whether or not specific patterns can be correlated with taxonomic groups. In addition, we have performed a number of experiments designed to investigate the significance of the broad pattern of

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specificity and whether particular patterns could be identified with the physiological role the enzyme is presumed to play in a given cell.

There have been two major previous investigations of the specificity of bacterial xanthine-oxidizing enzymes. The first study was with a purified enzyme from *Clostridium cylindrosporum* (6) in which a kinetic analysis was performed with a limited but highly selected list of compounds. The second study was performed primarily with whole cell preparations of a strain of *Psuedomonas aeruginosa* (5, 11) where a more extensive list of substrates was examined, all at one comparable concentration. In the latter study, it was assumed that only one purine-oxidizing enzyme was involved and permeability factors were not excluded in accounting for the results. The clostridial enzyme showed a notable preference for oxidizing the 8 position of purine derivatives—a feature that may be related to what is believed to be the principal physiological action of the enzyme in the uric acid-fermenting cells, i.e., catalytic action at a position 8 of uric acid to bring about the formation of xanthine. On the other hand, based on an unusual activity with several analogues, it has been suggested that the enzyme from *Pseudomonas* may prefer position 2 of the purine ring for oxidative attack (5). These suggestions arising out of the previous studies are given as examples. Other features of these systems have been pointed out (5, 6, 11, 15) and there are more fragmentary studies in the literature on the specificity of the enzymes from other bacterial sources. We will attempt to bring this information together with our new observations in this paper.

## MATERIALS AND METHODS

**Collection of cultures.** As shown in Table 1, 13 of the 50 bacterial cultures used in this investigation had been previously taxonomically defined and were obtained from established culture collections. The remaining strains were all obtained in this laboratory either by enrichment on media containing purines by one of the procedures enumerated below or by screening of collections of selected groups of bacteria for the ability to metabolize xanthine. The latter collections had been assembled from diverse sources as a result of the activities of the students of the undergraduate microbiology course here at Irvine. The screening of fluorescent pseudomonads was facilitated by the use of agar disk auxanography (29), testing for the ability of the organism to form an appreciable colony on a disk containing only xanthine as an available source of carbon and nitrogen. Collections of members of the genera *Bacillus* and *Streptomyces* were screened for the ability to catabolize xanthine by observing their ability to produce clear zones around colonies in solid media containing precipitated xanthine. The observation of xanthine clearing was facilitated by the use of plates with 30 ml of an agar medium containing 0.01% xanthine, 0.01% yeast extract, and 0.1XS (see below) with an overlay of 3 ml containing the above medium plus 1% suspended xanthine.

In the choice of the established cultures, we benefited from an earlier survey of standard bacteria for xanthine-oxidizing activity (M. Figueroa, Ph.D. thesis, University of Kentucky, Lexington, Ky., 1967). Since most ordinary bacteria in culture collections have no measurable activity, the information provided in this thesis was useful in selecting standard bacterial strains.

Enrichment method I (Table 1), which usually resulted in the isolation of fluorescent pseudomonads, involved shaking of individual soil samples in mineral salt media containing 0.1% xanthine (or caffeine) as the sole source of carbon and nitrogen. A detailed description of the isolation of cultures by this method has been given (30). Enrichment method II was similar to the above procedure except for the inclusion in the medium of 0.01% yeast extract and treatment of the inoculated flask at 70°C for 10 min prior to incubation. Enrichment method III involved the inoculation of tubes filled with the same xanthine-containing medium except for the addition of 0.5% yeast extract. After incubation without shaking, material from these tubes was streaked on the same solid medium employed in method II. The plates were incubated anaerobically by the GasPak procedure (Baltimore Biological Laboratory). Various colonies developing under these conditions and showing clearing of xanthine were selected for isolation. *Veillonella* were isolated from human saliva by streaking samples from separate individuals on a lactate-containing medium (28) also using the GasPak procedure. Uric acid-requiring clostridia were isolated from soil samples on a tap-water medium containing 0.05% $K_2HPO_4$, 0.05% $MgSO_4$, and 1% uric acid (pH 7.5). After enrichment in stoppered bottles, samples from this medium were streaked on plates containing the same medium and incubated anaerobically as before.

**Taxonomy.** For convenience, the cultures used in this investigation were grouped in five major categories in Table 1, corresponding to the Results Tables 2, 5, 6, 7, and 8, respectively. Table 1 attempts to list some of the characteristic differences of the individual strains. The group properties are discussed below.

All members of group 1 which were isolated in this laboratory were gram-negative, polarly flagellated, obligately aerobic, oxidase-positive rods which produced a water-soluble fluorescent pigment but were pyocyanine negative and unable to hydrolyze gelatin or grow on threonine or *meso*-inositol as sole sources of carbon. Accordingly, these cultures were designated *Pseudomonas putida*. Other xanthine-utilizing species were encountered in the screening procedure, but those strains selected, based on favorable growth characteristics, turned out to be *P. putida*. The separate strains also showed characteristic colonial features not itemized in Table 1. In addition, group 1 included five standard strains of other species of *Pseudomonas*.

TABLE 1. *Cultures used*

| Main group | Laboratory strain designation | Applicable taxon | Source and distinguishing characteristics of strain |
|---|---|---|---|
| 1 | 40 | *Pseudomonas putida* | Enrichment method I on caffeine, will grow on 0.5% caffeine (30) |
| 1 | 22 | *Pseudomonas putida* | Enrichment method I on caffeine |
| 1 | A-B | *Pseudomonas putida* | Enrichment method I on xanthine |
| 1 | A-BS | *Pseudomonas putida* | As for strain A-B, separate isolate |
| 1 | H | *Pseudomonas putida* | As for strain A-B, separate isolate |
| 1 | E | *Pseudomonas putida* | Originally isolated on asparagine; found to metabolize xanthine by screening procedure |
| 1 | M | *Pseudomonas putida* | As for strain E, separate isolate |
| 1 | 4-6 | *Pseudomonas putida* | As for strain E, separate isolate |
| 1 | 3-2 | *Pseudomonas putida* | As for strain E, separate isolate |
| 1 | 8-3 | *Pseudomonas putida* | As for strain E, separate isolate |
| 1 | 15667 | *Pseudomonas acidovorans* | ATCC 15667 |
| 1 | 45 | *Pseudomonas aeruginosa* | ATCC 17423 |
| 1 | 35 | *Pseudomonas aureofaciens* | ATCC 17415 |
| 1 | 382 | *Pseudomonas cepacia* | ATCC 17759 |
| 1 | 78 | *Pseudomonas testosteroni* | ATCC 11996 |
| 2 | 8750 | *Alcaligenes faecalis* | ATCC 8750 |
| 2 | A | *Escherichieae* | By enrichment method III, MR+, VP−, Lac−, Cit−, $H_2S$−, nonmotile |
| 2 | C | *Escherichieae* | By enrichment method III, MR+, VP−, Lac+, $H_2S$+: growth factors required; occasional motile cell, flagella insertion not observed but motion characteristic of this group |
| 2 | G-S | *Klebsielleae* | By enrichment method III, MR−, VP+, Lac−, $H_2S$+: growth factors required; highly motile, peritrichous flagellation |
| 2 | K-12 | *Escherichia coli* | *Escherichia coli* K-12, strain 3110, obtained from D. L. Wulff, University of California, Irvine. |
| 2 | SM | *Serratia marcescens* | Isolated at Irvine from grass clippings; prodigiosin produced at room temperature, but not at 37°C |
| 3 | SF | *Nocardia* | Isolated as a minor colony type by method III; later found not to develop anaerobically; colony described in text |
| 3 | GF | *Nocardia* | As for SF except will grow anaerobically and gives a positive oxidase test |
| 3 | 10 | *Arthrobacter* | Isolated by enrichment method II; yellow translucent smooth colonies; coryneform filaments and pseudobranched clusters breaking up and rounding up in old culture |
| 3 | S-2 | *Arthrobacter* | Similar to strain 10, except oxidase negative and smaller cells; by enrichment method II |
| 3 | NR | *Arthrobacter* | As for S-2, separate isolate |
| 3 | 16-4 | *Arthrobacter* | By enrichment method II; larger than previous strains with greater tendency towards and more stable pseudobranching; colorless |
| 3 | 12 | *Arthrobacter* | More uniform rods than previous strains; occasional motile cell; gram positive but more easily decolorized; oxidase positive |
| 3 | 16 | *Arthrobacter* | Similar to strain 12 but tendency to form pairs of almost uniform rods |
| 4 | 393 | *Lactobacillus caseae* | ATCC strain 393 |
| 4 | 8-15 | *Bacillus* sp. | Obtained by dilution of pasteurized soil on complex media, later found to degrade xanthine by screening; obligate aerobe, no growth factor requirements but will not use xanthine as sole carbon source; long rods or filaments no swelling with endospore formation; motile with peritrichous flagella |

TABLE 1—*Continued*

| Main group | Laboratory strain designation | Applicable taxon | Source and distinguishing characteristics of strain |
|---|---|---|---|
| 4 | 10-5B | *Bacillus* sp. | Obtained as for strain 8-15; obligate aerobe; nonmotile filament with terminal spore and swelling; growth factors required |
| 4 | W168 | *Bacillus subtilis* | Strain W168 obtained from E. W. Nester, University of Washington, Seattle; C. Kusyk, Ph.D. thesis, University of North Carolina, 1960 |
| 4 | 3-19 | *Streptomyces* sp. | Obtained as an air contaminant on xanthine-containing media; aerial mycelia flexuous and spores white; vegetative mycelia appear to produce water-soluble yellow pigment |
| 4 | 19-5B | *Streptomyces* sp. | Isolated from soil by dilution on complex media and later found to utilize xanthine by screening; like 3-19 except aerial mycelia smaller and no yellow pigment |
| 4 | 6-5C | *Streptomyces* sp. | Obtained as for 19-5B; dark grey highly coiled aerial mycelia |
| 4 | 4-15 | *Streptomyces* sp | Obtained as for 3-19; brown flexuous aerial mycelia |
| 5 | G | *Clostridium acidiurici* | Obtained by enrichment from soil |
| 5 | A | *Clostridium acidiurici* | As for strain G, separate isolate |
| 5 | B | *Clostridium acidiurici* | Separate isolate |
| 5 | R | *Veillonella alcalescens* | See text |
| 5 | C | *Veillonella alcalescens* | See text |
| 5 | J1 | *Veillonella alcalescens* | See text |
| 5 | D2 | *Veillonella alcalescens* | See text |
| 5 | D1 | *Veillonella alcalescens* | See text |
| 5 | 221 | *Veillonella alcalescens* | Originally *Micrococcus lactilyticus* 221 obtained from collection of lyophilized cultures, Department of Microbiology, University of Washington |
| 5 | 228 | *Peptococcus aerogenes* | Obtained from University of Washington collection of lyophilized culture as *Micrococcus aerogenes* 228; same as ATCC 14963 |

Group 2 (Table 1) consisted of those remaining gram-negative rods which were capable of growth in air but did not belong to *Pseudomonas*. The three facultative members which were obtained by enrichment were all oxidase, phenylalanine deaminase, and urease negative and apparently belonged to the *Enterobacteriaceae*. However, they could not be unequivocally placed in a specific genus based on the results of several diagnostic tests included in Table 1, and the individual strains did not correspond with the descriptions of any recognized species. Accordingly, we have placed them only in a tribe. Apparently, organisms obtained by enrichment on xanthine and belonging to this general group may not fit the established, detailed taxonomic patterns. Furthermore, it is recognized that the established taxonomy is not always satisfactory for the detailed classification of some isolates from nonclinical material(8).

Group 3 consisted of nine strains of gram-positive rods which correspond in morphological characteristics to the group of bacteria which include *Corynebacterium, Arthrobacter, Mycobacterium,* and *Nocardia*. Based primarily on the morphological characteristics recorded for the individual strains in Table 1, we have placed these organisms in several genera, although a wide range of morphological variation was encountered. However, these genera do not seem completely defined (8), some of our strains have some anomalous properties, and we have not attempted to obtain a species designation. In addition to the finding that all of these strains require some growth factor(s) when grown primarily on xanthine, all of these rods exhibited a pleomorphism or coryneform character. There was a tendency of all of these cultures designated *Arthrobacter* to produce more or less rounded or spherical cells upon continued incubation in various media, and this effect was more pronounced on media containing xanthine. The cultures designated as *Nocardia* were highly filamentous branched structures during early colony development. The mature colony was yellow and translucent with subsurface growth which appeared fuzzy or filamentous at the edge and did not break up if a needle was passed over the surface. If the agar was broken up, the colony yielded a suspension of pleomorphic cells similar to the young arthrobacters.

Group 4 contained all the remaining gram-positive bacteria, and all can be unambiguously assigned to a genus based on distinctive morphological and physiological properties. The morphological and

physiological characteristics of the anaerobes (group 5) all corresponded closely to the literature descriptions. However, differences in the tendency of the different *Veillonella* isolates to clump or "diffuse" in submerged culture containing 0.1% agar suggested that the strains have different surface features.

**Growth and preparation of enzyme sources.** A method previously described for aerobic growth of *P. putida* in 1-liter volumes was routinely used in this investigation (30). Generally a 0.1× dilution of the mineral salts solution previously described was supplemented with 0.1% purine substrate as a sole source of carbon and nitrogen. In some instances, 0.01 to 0.1% of yeast extract was added to the above media to satisfy growth factor requirements or enhance growth. Unless otherwise noted, the purine substrate disappeared during the growth and was responsible for much of the growth of the organism on these complex media. Individual growth requirements and specialized media are presented in Table 1 and accompanying discussion. Anaerobic growth was obtained by use of stoppered 1-liter bottles filled with media. Deviations from these procedures in individual experiments are indicated in Tables 2, 5, 6, 7, and 8.

Methods previously described for the harvesting of cells, the preparation of extracts, the measurement of protein, and in particular the preparation of cell-free particles from the crude sonic extracts by differential centrifugation and the use of sucrose layers were applied as described earlier (30).

**Assay of enzymes.** The ferricyanide-linked xanthine dehydrogenase assay was used for the determination of the enzyme in this investigation exactly as previously described (30). In the case of the extracts from *Pseudomonas*, the reaction with xanthine was linear with time up to 80% substrate exhaustion, but some deviations were noted in this range with the methylated substrates, resulting in activation or inactivation of the enzyme depending on the preparation and substrate (see Discussion). For the purpose of this investigation the following method was used to determine the activity with methylated substrates. A minimum of 0.2 unit of enzyme, determined with xanthine, was added to the reaction mixture containing the methylated substrate. Readings were made at 30-s intervals over a period of 4 min. A unit of enzyme is that amount which catalyzes the reduction of 1 μmol of ferricyanide per min in the presence of xanthine.

Preparations from several diverse strains of the bacteria investigated did not give linear assays with xanthine (*Serratia, Alcaligenes, Lactobacillus,* and *Bacillus subtilis*). The specific activities were, nevertheless, reproducibly estimated from initial rate data. The rapid loss of activity under the conditions of assay with all of these enzymes was due to an inactivation of the enzyme related to the addition of the substrate since fresh substrate did not restore lost activity and the preparations were reasonably stable in the concentrated extracts and when diluted in buffer in the absence of the substrate. Part of the difficulty encountered with these strains may be due to the low specific activity which limited the assay to lower total units of enzyme due to the large amount of extract protein required. When enzyme from more active sources was diluted to this degree during assay, some nonlinearity was observed.

The same conditions were employed when organic dyes and other artificial electron acceptors were used for the assay of the enzyme except for substitution at the previously indicated concentration (30) for the ferricyanide. Previously used instrumental changes for these dyes and methods for calculating the concentrations were employed (31). Anaerobic conditions, which were always used with the phenazine, indophenol, and viologen dyes, were obtained as previously described (31). The oxygen-linked assay (oxidase activity) was calculated exactly as previously described (30); activities with nicotinamide adenine dinucleotide (NAD) were determined under the same condition at 340 nm. Specific activities are expressed in terms of micromoles of electron acceptor disappearance. Data with one-electron acceptors (ferricyanide, viologen dyes) should be divided to compare with two-electron acceptor (indophenol, oxygen, phenazine) data in terms of the micromoles of substrate (electron donor) disappearance.

**Analytical procedures and radioactive methods.** [2-$^{14}$C]uric acid (8 mCi/mmol) and [8-$^{14}$C]xanthine (9.2 mCi/mmol) were obtained from Calbiochem. [8-$^{14}$C]adenine was obtained from ICN and was diluted with unlabeled adenine to give 10.6 mCi/mmol for use in our experiments. In general, procedures used earlier in this laboratory for scintillation counting were employed (30). Radioactivity after urease treatment was determined after exposure of a sample to excess urease followed by acidification with acetic acid and brief heating. Ammonia was determined by nesslerization; urea was determined by nesslerization after urease treatment.

A spectrophotometric method for determining the positions of oxidation by xanthine oxidase and dehydrogenase on unsubstituted and monooxygenated purine substrates has been extensively described in the literature (3, 10, 13, 17, 29). We have made similar determinations with several of our sources of activity using this approach and taking advantage of the spectral data previously employed. The exceptional properties of our systems will be described in the text. The success of this method depends on the observed absence of uricase activity with the *Pseudomonas* preparations (30). The *Arthrobacter* extract contains uricase and the preparation of *Arthrobacter* enzyme used in these experiments was obtained free of uricase by repetitive precipitation of the enzyme activity with ammonium sulfate at 30 to 40% saturation.

## RESULTS

**Xanthine-oxidizing enzymes from *Pseudomonas*.** The xanthine dehydrogenase activity of cell-free extracts prepared from nine individual isolates of *P. putida* are shown in Table 2. A fivefold variation in the level of specific activity was observed in extracts from all of these strains when they were grown on xanthine or its metabolic precursor, caffeine. Almost as much variation in specific activity was encoun-

TABLE 2. *Xanthine dehydrogenase activities of aerobic pseudomonads*[a]

| Species | Strain | Growth conditions[a] | Sp act with xanthine (μmol/min per mg of protein) | Relative % activity | | Ratio of 1MX activity to 3MX activity |
|---|---|---|---|---|---|---|
| | | | | 1MX | 3MX | |
| *P. putida* | 40 | Various conditions[b] | 0.2(0.12–0.30) | 35(18–65)[b] | 41(20–78)[b] | 0.85(0.41–1.6)[b] |
| *P. putida* | E | 0.5%X | 0.087 | 16 | 30 | 0.54 |
| *P. putida* | E | 0.1%X | 0.151 | 29 | 78 | 0.49 |
| *P. putida* | E | 0.1%X | 0.368 | 24 | 51 | 0.47 |
| *P. putida* | M | 0.1%X | 0.033 | 100 | 51 | 2.0 |
| *P. putida* | H | 0.1%X | 0.37 | 25 | 46 | 0.5 |
| *P. putida* | A-B | 0.1%X | 0.76 | 26 | 85 | 0.5 |
| *P. putida* | A-BS | 0.1%X | 0.073 | 48 | 16 | 3.0 |
| *P. putida* | 4-6 | 0.1%X | 0.055 | 62 | 60 | 1.03 |
| *P. putida* | 3-2 | 0.1%X | 0.041 | 12 | 83 | 0.14 |
| *P. putida* | 3-2 | 0.1%X + 0.1%YE | 0.120 | 21 | 53 | 0.40 |
| *P. putida* | 8-3 | 0.1%X (young) | 0.039 | 135 | 70 | 1.8 |
| *P. putida* | 8-3 | 0.1%X (old) | 0.043 | 90 | 84 | 1.1 |
| *P. putida* | 22 | 0.1%X | 0.198 | 59 | 48 | 1.2 |
| *P. aureofaciens* | 36 | 0.1%X + 0.01%YE | 0.043 | 43 | 44 | 0.98 |
| *P. cepacia* | 382 | 0.1%X + 0.01%YE | 0.044 | 39 | 36 | 1.08 |
| *P. testosteroni* | 78 | 0.1%X + 0.01%YE | 0.0164 | 28 | 26 | 1.08 |
| *P. acidovorans* | 15667 | 0.1%X + 0.01%YE | 0.0192 | 42 | 25 | 1.68 |
| *P. aeruginosa* | 45 | 0.1%X + 0.01%YE | 0.0098 | 113 | 62.5 | 1.81 |

[a] The following abbreviations are used: X, xanthine; YE, yeast extract; 1MX, 1-methylxanthine; 3MX, 3-methylxanthine. All media includes 0.1× mineral salts given in text. Strains grown on yeast extract-containing media grew very poorly on xanthine alone.

[b] The data values given for strain 40 are the average of 20 different conditions as defined in Fig. 1. The values in parentheses are the extreme range observed with the 20 extracts.

tered when extracts were prepared from separate batches of cells of the same strain (see strains 40 and E, Table 2).

Table 2 also records the relative rate of oxidation of 1-methylxanthine and 3-methylxanthine and the ratio of these activities. Every extract prepared from a species of *Pseudomonas* displayed activity towards both compounds and this seems to be a characteristic of the enzyme from the genus. However, there were extensive variations in these ratios, not only within the collection as a whole but in the different preparations from the same strain. Thus, it would appear that with strain 40 either the quality of the enzyme is subject to variation or there are several enzymes of different or overlapping specificity (see Discussion).

The 20 extracts of strain 40 used to summarize the activity of this organism towards xanthine in Table 2 were obtained from cells in several different conditions of growth (Fig. 1). It was found that a low 1-methylxanthine/3-methylxanthine oxidation ratio was characteristic of cells taken within 1 day of the attainment of stationary growth whereas a relatively high ratio was characteristic of cells maintained 3 or more days in the stationary phase. In contrast to the progressive change in the 1-methylxanthine/3-methylxanthine oxidation ratio, the specific activity with xanthine or the presence of high or low values for the general utilization of the methylxanthine substrates

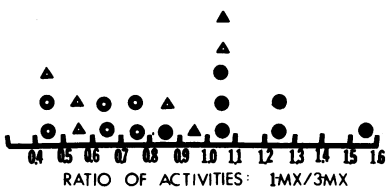


FIG. 1. *Distribution of the ratio of 1-methylxanthine activity to 3-methylxanthine activity obtained with 20 extracts of Pseudomonas putida 40 obtained after several conditions of growth. Each symbol represents the activity of a separate extract preparation. Symbols: ○, activities with extracts from younger xanthine-grown cells; ●, older xanthine-grown cells; △, younger caffeine-grown cells; ▲, older caffeine-grown cells.*

did not vary during this time period in any consistent manner.

Up to this point, all data were obtained with extracts that had been sufficiently centrifuged to remove particle-associated activity. We previously demonstrated that a cell-free particle fraction from crude sonic extracts of *P. putida* strain 40 possesses xanthine oxidase, but not xanthine dehydrogenase activity (30). Table 3 includes data on the activity of particles prepared from strain 40 and *Pseudomonas acidovorans*. Our data suggest that the other species of *Pseudomonas* contain a similar fraction, but the procedure, which was perfected for strain 40, did not give preparations from the other

TABLE 3. *Summary of activities of cell-free bacterial preparations towards xanthine with various electron acceptors*[a]

| Major group | Distinguishing taxon | Strain | Prepn | Growth medium and conditions | Sp act with: | | | | Relative activity | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Ferricyanide | Oxygen | NAD | Other | 1MX | 3MX |
| 1 | *P. putida* | 40 | Supt | | 0.2 | 0.003 | 0.051 | 2.7(IP); 0.003(BV) | – | – |
| 1 | *P. putida* | 40 | Part[b] | | ND | (0.033) | ND | – | 19 | 56 |
| 1 | *P. putida* | H | Supt | | 0.37 | 0.0045 | – | – | – | – |
| 1 | *P. aeruginosa* | 45 | Supt | | 0.0098 | 0.00010 | (0.0023) | – | 76 | 21 |
| 1 | *P. aeruginosa* | 45 | Supt | | 0.0098 | (0.00010) | 0.0023 | – | 280 | 43 |
| 1 | *P. aureofaciens* | 36 | Supt | | 0.0431 | (0.00032) | 0.0015 | – | 64 | 64 |
| 1 | *P. cepacia* | 382 | Supt | | 0.004 | (0.00026) | 0.0073 | – | 31 | 33 |
| 1 | *P. testosteroni* | 78 | Supt | | 0.0164 | (0.00026) | 0.0063 | – | 43 | 26 |
| 1 | *P. acidovorans* | 15667 | Supt | | 0.0192 | (0.00046) | 0.0029 | – | 47 | 26 |
| 1 | *P. acidovorans* | 15667 | Part[b] | | ND | (0.0175) | ND | – | 35 | 46 |
| 2 | *Escherichieae* | A | Supt | Aerobic | 0.23 | 0.0040 | ND | – | – | – |
| 2 | *Escherichieae* | C | Supt | Aerobic | 0.87 | 0.0029 | ND | – | – | – |
| 2 | *Escherichieae* | A | Part | Aerobic | 0.20 | 0.030 | ND | – | – | – |
| 2 | *Escherichieae* | A | Part | Anaerobic | 0.12 | 0.024 | ND | – | – | – |
| 2 | *Alcaligenes* | 8750 | Supt | | 0.033 | ND | (0.316) | 0.52(IP) | 42 | 38 |
| 2 | *Serratia* | SM | Supt | | ND | (0.0036) | 0.0142 | – | 87 | 48 |
| 3 | *Arthrobacter* | S2 | Supt | Aerobic | 2.8 | 0.89 | ND | 7.5(IP); ND(BV) | – | – |
| 3 | *Arthrobacter* | 10 | Supt | Aerobic | 2.7 | 0.57 | ND | – | – | – |
| 3 | *Arthrobacter* | NR | Supt | Aerobic | 2.1 | 0.47 | – | – | – | – |
| 3 | *Arthrobacter* | 16-4 | Supt | Aerobic | 0.038 | 0.0038 | – | – | – | – |
| 3 | *Nocardia* | GF | Supt | Aerobic | 0.78 | 0.25 | – | – | – | – |
| 3 | *Nocardia* | GF | Supt | Anaerobic | 0.054 | 0.018 | – | – | – | – |
| 3 | *Nocardia* | SF | Supt | Aerobic | 0.78 | 0.25 | – | – | – | – |
| 4 | *Lactobacillus* | 393 | Supt | | 0.0062 | ND | ND | ND(IP); 0.004(PMS) | 234 | ND |
| 4 | *Bacillus* | 8:15 | Supt | | 0.031 | ND | (0.064) | – | 95 | ND |
| 4 | *Bacillus* | 10:5 | Supt | | 0.092 | ND | (0.053) | – | 80 | ND |
| 4 | *Bacillus* | W168 | Supt | | 0.0124 | ND | (0.033) | 0.134(IP) | 140 | ND |
| 4 | *Streptomyces* | 3:19 | Supt | | 0.011 | ND | (0.0032) | – | 33 | ND |
| 4 | *Streptomyces* | 4:15 | Supt | | 0.0005 | ND | (0.0004) | – | 24 | ND |
| 4 | *Streptomyces* | 19:5b | Supt | | 0.0078 | ND | (0.0040) | – | 25 | ND |
| 4 | *Streptomyces* | 6:5c | Supt | | 0.0030 | ND | (0.056) | – | 23 | ND |
| | *Penicillium*[c] | M | | | 0.38 | – | – | – | 86 | ND |
| | *Penicillium*[c] | | | | – | – | – | 0.25(PMS) | 50 | ND |

[a] Unless otherwise indicated, growth conditions are the same as in Tables 2, 5, 6, and 7 for group 1, 2, 3, and 4 organisms, respectively. In addition to the abbreviations used in Table 2, the following abbreviations are used: Supt, supernatant enzyme fraction; Part, cell-free particulate fraction; NAD, nicotinamide adenine dinucleotide; IP, 2,6-dichlorophenol-indophenol; BV, benzyl viologen; PMS, phenazine methosulfate; ND, activity not detected; –, activity not measured. The methylxanthine data at the right of the table were determined in experiments using the electron acceptor whose data are included in parentheses in the same line of data.

[b] The particle preparations from the pseudomonads contained approximately 19% as much protein as obtained in the supernatant fraction prepared from the same amount of sonically extracted cells.

[c] The *Penicillium* was obtained during the course of these investigations as an air contaminant giving clearing of xanthine on solid media.

species which could be shown to be completely free of whole cells and whole cell activities and the data have not been included. It is of interest that the particles from the two strains examined exhibit activity towards both 1-methylxanthine and 3-methylxanthine and thus share this unique property with the enzyme in the extract supernatants. However, we will show later that the substrate specificity of the particles differs significantly from that of supernatant enzyme. Although the supernatant enzymes of all species of *Pseudomonas* examined utilize NAD as an electron acceptor, the particles will not reduce this acceptor (Table 3). We have previously discussed the apparent inability of the particles to utilize ferricyanide as an acceptor for xanthine oxidation (30). Extensive sonic extraction of the particles does not release any detectable soluble activity.

Table 4 presents data on the level of xanthine dehydrogenase activity of extracts of strain 40 grown on xanthine, hypoxanthine, and 6,8-dioxypurine. We have been unable to grow the organism on purine, 2-oxypurine, adenine, or guanine as sole carbon sources. No soluble (or particulate) xanthine-oxidizing activity can be

obtained from cells of this organism grown on succinate and ammonia. However, the latter result depends on the use of an inoculum which has not been grown on the purines since the induced state is somewhat persistent. The finding that all three activities are similarly induced by each substrate (Table 4) is consistent with the view that a single enzyme with relatively broad substrate specificity is responsible for the growth of the organism on all three compounds. However, the particulate activity associated with these cells is also active with the same substrates (see Fig. 4), and the relationship of the two sources of activity needs further clarification. The inability to grow on adenine may reflect difficulties by the organism in concentrating (see Table 10 and associated discussion) as well as in deaminating the material. Adenine is not a substrate of the xanthine dehydrogenase. Suspensions of xanthine-grown cells of this organism when examined under the conditions of the spectrophotometric assay (see Fig. 4) were found to be unable to oxidize purine or 2-oxypurine at appreciable rates. The latter result suggests that neither the particulate nor the soluble activity is exposed on the surface of the cell. Previous observations of the activity of xanthine-grown whole cells towards the methylated xanthines (30) have supported the view that both sources of xanthine-oxidizing activities are located behind the permeability barrier of the cell.

TABLE 4. *Activities with three purines of Pseudomonas putida 40 extracts, prepared from cells grown on the three different purines*

| Growth medium | Sp act (μmol/min per mg of protein) (ferricyanide assay) with: | | |
|---|---|---|---|
| | Xanthine | Hypoxanthine | 6,8-Dioxypurine |
| 0.1% Xanthine | 0.22 | 0.16 | 0.05 |
| 0.1% Hypoxanthine | 0.11 | 0.09 | 0.04 |
| 0.1% 6,8-Dioxypurine | 0.17 | 0.12 | 0.05 |

**Other gram-negative xanthine-oxidizing enzymes from aerobic and facultative bacteria.** Table 5 shows the xanthine dehydrogenase activity of the cell-free extract supernatants of all the remaining gram-negative organisms studied in this investigation and capable of aerobic development. These organisms all display the pattern of 1-methylxanthine and 3-methylxanthine utilization observed above with the pseudomonads. Several of these organisms were shown to contain cell-free particle-associated activity in the sonic extracts and these activities, in contrast to the preparations from *Pseudomonas*, were able to reduce ferricyanide. In contrast to the extracts from the pseudomonads, the extracts from the facultative organisms obtained by enrichment on xanthine were unable to utilize NAD as an electron acceptor (Table 3).

An interesting result was obtained when several of the facultative members were grown

TABLE 5. *Xanthine dehydrogenase activity of preparations from other gram-negative organisms (group 2, Table 1)*

| Genus or tribe | Strain | Growth conditions and prepn[a] | Sp act (μmol/min per mg) (ferricyanide assay) with: | | | Ratio of activities (1MX/3MX) |
|---|---|---|---|---|---|---|
| | | | Xanthine | 1MX | 3MX | |
| *Escherichieae* | A | Aerobic, 0.1%X + 0.1%YE | 0.87 | 0.36 | 0.56 | 0.64 |
| | A | Aerobic, 0.1%X + 0.1%YE (Part)[b] | 0.20 | 0.044 | 0.11 | 0.41 |
| | A | Aerobic, 0.1%UA + 0.1%YE | 0.14 | 0.049 | 0.078 | 0.63 |
| | A | Aerobic, YE only | 0.060 | 0.015 | 0.028 | 0.54 |
| | A | Anaerobic, 0.1%X + 0.1%YE | 0.047 | 0.039 | 0.014 | 2.1 |
| | A | Anaerobic, 0.1%X + 0.1%YE (Part)[b] | 0.123 | 0.041 | 0.070 | 0.58 |
| | A | Anaerobic, 0.1%UA + 0.1%YE | 0.038 | 0.026 | 0.013 | 2.0 |
| | A | Anaerobic, YE only | 0.0315 | 0.0103 | 0.0088 | 1.2 |
| *Escherichieae* | C | Aerobic, 0.1%X + 0.1%YE | 0.23 | 0.11 | 0.15 | 0.73 |
| | C | Aerobic, YE only | 0.038 | 0.0083 | 0.014 | 0.59 |
| | C | Anaerobic, 0.1%X + 0.1%YE | 0.036 | 0.047 | 0.0045 | 1.04 |
| | C | Anaerobic, YE only | 0.020 | 0.0068 | 0.0049 | 1.38 |
| *Klebsielleae* | G-S | Aerobic, 0.1%X + 0.1%YE | ND | ND | ND | |
| | G-S | Aerobic, YE only | ND | ND | ND | |
| | G-S | Anaerobic, 0.1%X + 0.1%YE | 0.11 | 0.16 | 0.0068 | 23.6 |
| | G-S | Anaerobic, YE only | 0.0037 | 0.0019 | 0.0005 | 40.5 |
| *Alcaligenes* | 8750 | Aerobic, 0.1%X + 0.1%YE | 0.033 | 0.037 | 0.037 | 1.0 |

[a] The preparations are supernatant extract fractions unless it is specifically stated that the preparations are cell-free particles. Abbreviations used are defined in previous tables; UA, Uric acid.

[b] The particle preparations from aerobic cells and from anaerobic cells contain 48 and 27%, respectively, as much protein as the soluble extract prepared from the same amount of sonic extract.

under anaerobic conditions. In that situation there was a major shift in the ratio of methylxanthine utilization toward that observed with gram-positive and obligately anaerobic organisms, that is, towards increased utilization of 1-methylxanthine and the exclusion of 3-methylxanthine (Table 5). This shift was accompanied by a change in the distribution of the xanthine dehydrogenase in the soluble and particulate fractions obtained from the extract (Table 5) and in the size distribution of enzymatic activity in extract supernatants revealed by gel chromatography (Fig. 2). It is possible that the latter distribution was effected by the relative instability of the particulate activity and by its tendency to elute from the particles. It is of interest that, if the particle preparations of Table 5 were heated briefly at 50°C, there was a release of xanthine-oxidizing activity to the supernatant fraction. The highly unstable activity so released retained the original ratio of activities towards the methylxanthines. It would appear that there are two enzymes produced by this organism, one under aerobic conditions and one predominating under anaerobic conditions. The interrelationship, regulation, and distribution of these several activities is complex and needs further investigation.

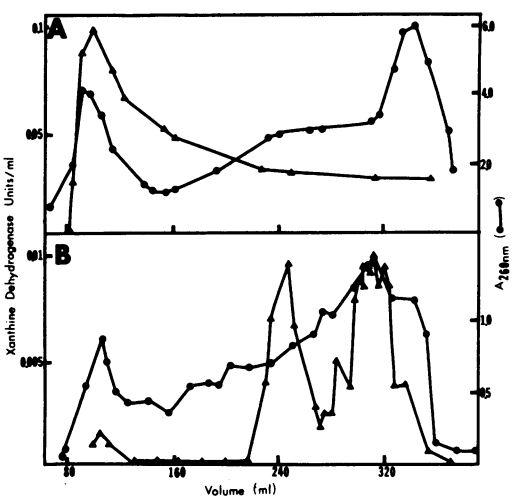


FIG. 2. *Elution profile of xanthine dehydrogenase (ferricyanide assay) using cell-free extracts of strain A (Escherichieae sp.) from a column (2.5 by 65 cm) of Sepharose 4B. The same column was used for both runs and eluted in an identical manner with 0.1XS as the buffer. Column A: 5 ml of cell-free extract of strain A grown aerobically with xanthine (Table 5) and containing 440 mg of protein was applied to the column; yield of activity, 25%. Column B: 5 ml of cell-free extract of strain A grown anaerobically with xanthine (Table 5) and containing 110 mg of protein was applied to the column; yield of activity, 20%. Symbols: ▲, xanthine dehydrogenase units per milliliter; ●, absorbance at 260 nm.*

**Xanthine-oxidizing activities of gram-positive aerobic and facultative bacteria.** In contrast to the previous results in which some 3-methylxanthine activity was associated with the xanthine dehydrogenase activity of every preparation of aerobically grown gram-negative bacterium examined, the remaining organisms in this investigation, which consisted of all gram-positive bacteria and obligate anaerobes, were found to give xanthine dehydrogenase-containing preparations devoid of detectable activity with 3-methylxanthine.

Table 6 records the oxidizing activity of members of *Nocardia* and *Arthrobacter* towards xanthine and 1-methylxanthine. The arthrobacters with yellow colonies (strains 10, NR, and S-2) when grown on xanthine gave the highest specific activities with xanthine encountered in this investigation, implying that they may be excellent sources of xanthine oxidase. This suggestion has been confirmed by our finding (J. S. Downard and C. A. Woolfolk, unpublished results) that a homogeneous preparation of the enzyme can be obtained from extracts of S-2 after only a 20-fold purification.

It is of interest that 3-methylxanthine does not inhibit the enzyme when added to the xanthine assay at the usual substrate levels, suggesting that it does not bind effectively to the enzyme site. On the other hand, 1-methylxanthine is almost as good as or significantly better than xanthine as a substrate under assay conditions with this group of organisms. Also, this group of enzymes is exceptionally active with oxygen when compared with the other organisms in this study, and the absence of NAD-linked activity with the several extracts examined from this group suggest that these enzymes may be appropriately referred to as oxidases. On the other hand, it is clear that ferricyanide directly interacts with the enzyme, rather than merely titrating the uric acid produced, since slightly enhanced rates of the reaction are obtained under anaerobic conditions with this electron acceptor.

Table 7 contains the results obtained with the remaining gram-positive cultures capable of growth in air (group 4, Table 1) all of which belonged to well-defined genera. Very low levels of xanthine dehydrogenase were found with the extracts from the strain of *Lactobacillus*. Also, activity was observed with phenazine methosulfate under anaerobic conditions (Table 3). This is consistent with the previous report of very low levels of the activity with this organism when coupled with tetrazolium dyes (32). 1-Methylxanthine was a superior sub-

TABLE 6. *Xanthine dehydrogenase activities of cell-free extracts of the Arthrobacter and Nocardia*[a]

| Genus | Strain | Growth conditions | Ferricyanide-linked dehydrogenase activities (μmol/min per mg of protein) with: | |
|---|---|---|---|---|
| | | | Xanthine | 1-Methylxanthine |
| *Arthrobacter* | 10 | 0.1%X + 0.01%YE | 2.7(1.9) | 2.7 |
| | 10 | X + 0.1%YE | 0.9 | 0.95 |
| | 10 | UA + 0.01%YE | 0.5 | 0.42 |
| | 10 | UA + 0.1%YE | 0.4 | – |
| | 10 | 0.1%YE only | ND | – |
| *Arthrobacter* | S-2 | X + 0.01%YE | 2.8 | 2.8 |
| | S-2 | UA + 0.01%YE | 0.25 | 0.25 |
| | S-2 | 0.1%YE only | 0.0098 | 0.011 |
| *Arthrobacter* | NR | X + 0.01%YE | 2.1 | 2.1 |
| *Arthrobacter* | 16-4 | X + 0.01%YE | 0.038 | 0.030 |
| *Arthrobacter* | 12 | X + 0.01%YE | 0.049 | 0.026 |
| *Arthrobacter* | 16 | X + 0.01%YE | 0.0068 | 0.0041 |
| | 16 | X + 0.1%YE | 0.0089 | – |
| | 16 | 0.1%YE only | ND | – |
| | 16 | UA + 0.1%YE | 0.06 | – |
| | 16 | Hypoxanthine + 0.1%YE | 0.05 | – |
| *Nocardia* | G-F | X + 0.01%YE | 0.78 | 1.3 |
| | G-F | X + 0.1%YE | 0.053 | 0.053 |
| | G-F | YE only | 0.103 | 0.107 |
| | G-F | X + 0.01%YE, anaerobic | 0.054 | 0.053 |
| | G-F | YE only, anaerobic | ND | – |
| *Nocardia* | S-F | X + 0.01%YE, aerobic | 0.75 | 0.75 |
| | S-F | X + 0.01%YE, anaerobic | 0.095 | 0.093 |

[a] Unless otherwise indicated, incubation was aerobic and the purines were added to the media at 0.1%. For abbreviations, see Tables 2 and 3.

TABLE 7. *Xanthine dehydrogenase activities of cell-free extracts of other gram-positive bacteria grown aerobically*[a]

| Genus | Strain | Growth medium | Ferricyanide-linked dehydrogenase activity (μmol/min per mg of protein) with: | |
|---|---|---|---|---|
| | | | Xanthine | 1-Methylxanthine |
| *Lactobacillus* | 393 | 0.1%YE + complex additions[b] | 0.0062 | 0.0170 |
| *Bacillus* | 10-5B | 0.1%X + 0.1%YE | 0.092 | 0.039 |
| *Bacillus* | 8-15 | 0.1%X + 0.1%YE | 0.031 | 0.036 |
| *Bacillus* | W168 | 0.1%X + 0.1% sodium lactate | 0.0124 | 0.0135 |
| *Streptomyces* | 19-5B | 0.1%X | 0.0078 | 0.020 |
| *Streptomyces* | 6-5C | 0.1%X | 0.0030 | 0.0037 |
| *Streptomyces* | 3-19 | 0.1%X | 0.011 | 0.010 |
| *Streptomyces* | 4-15 | 0.1%X | 0.0005 | 0.0009 |

[a] For abbreviations, see Table 2.
[b] The medium for the *Lactobacillus* included 1% peptone, 1% peptonized milk, and 40% by volume of filtered tomato juice.

strate with this enzyme source under two assay conditions (Tables 3 and 7). The three diverse strains of species of *Bacillus* had patterns of substrate and electron acceptor utilization similar to each other, as did the four strains of *Streptomyces* (Tables 3 and 7). All seven of the strains of *Bacillus* and *Streptomyces* gave relatively high 1-methylxanthine-oxidizing activities when compared with xanthine when the ferricyanide assay was used. When the oxygen-linked assay was performed (which amounts to a drop in substrate concentration at which the comparison is made), the relative activity of the four strains of *Streptomyces* with 1-methylxanthine dropped considerably, whereas the same activity with each of the three strains of *Bacillus* remained high (compare Table 3 with Table 7). Although saturation experiments were not

performed with all strains and substrates in these investigations, the suggestion arises from these data that the *Streptomyces* enzymes may be uniformly less avid in the binding of 1-methylxanthine relative to xanthine than the three strains of *Bacillus*. The extract from *Streptomyces* 3-19 oxidizes 6,8-dioxypurine at 53% the rate of xanthine under the condition of the standard NAD-linked assay and similar results were obtained with the other extracts of *Streptomyces*. Although these are not unusual findings for xanthine-oxidizing activities, they are of interest with regard to the recent reports of unusual purine-oxidizing capabilities of noninduced whole cells of this organism (26, and see Discussion).

**Xanthine-oxidizing activities of anaerobic bacteria.** It is of interest that all of the enzymes from the obligate anaerobes (which included gram-positive and gram-negative organisms) were found to have no detectable activity with 3-methylxanthine, whereas 1-methylxanthine was utilized as effectively or more effectively than xanthine under the conditions of the assay (Table 8). The observations with the three clostridia are in agreement with the previous observations with a purified enzyme from this source (6). Although a very slow rate of reduction of indophenol was observed previously in the presence of 3-methylxanthine, it could not be determined if the compound was slowly oxidized. The possibility that 3-methylxanthine might be oxidized at rates less than 1% that of xanthine by our preparations of clostridial enzymes cannot be eliminated. Furthermore, we have not determined the electron acceptor specificity of our anaerobic preparations. The purified enzyme from *Clostridium* has been shown to utilize oxygen and NAD very slowly (6), and the enzyme from *Veillonella* utilized oxygen slowly and NAD not at all. The latter enzyme has been shown to utilize ferridoxin as an electron acceptor, and the reduced form of this carrier potentially could serve as an electron donor in the reduction of uric acid by these enzymes (24).

TABLE 8. *Xanthine dehydrogenase activities of cell-free extracts of anaerobic bacteria*

| Genus | Strain | Ferricyanide-linked dehydrogenase activity (μmol/min per mg) with: | |
|---|---|---|---|
| | | Xanthine | 1-Methylxanthine |
| *Peptococcus* | 228 | 0.76 | 0.91 |
| *Veillonella* | 221 | 0.064 | 0.074 |
| *Veillonella* | D-1 | 0.047 | 0.058 |
| *Veillonella* | D-2 | 0.32 | 0.49 |
| *Veillonella* | J-1 | 0.29 | 0.40 |
| *Veillonella* | C | 0.13 | 0.19 |
| *Veillonella* | R | 0.073 | 0.084 |
| *Clostridium* | B | 0.76 | 1.0 |
| *Clostridium* | A | 1.1 | 1.1 |
| *Clostridium* | G | 0.6 | 0.6 |

**Studies related to the physiological roles of the several types of xanthine-oxidizing enzymes.** In view of the earlier suggestions that a major function of the anaerobic enzymes may involve a reduction of uric acid, the possibility was considered that the methylxanthine specificity pattern observed with the aerobic gram-positive bacteria might be a reflection of such a capability. Accordingly, experiments were performed to determine whether differences existed between examples of the two types of aerobic bacteria (*P. putida* 40 and *Arthrobacter* S-2) in the ability to incorporate [2-$^{14}$C]uric acid (Table 9). The labeled carbon in these experiments was converted through catabolism either to $CO_2$ or urea. The internal carbons which are the actual sources of carbon and energy for the cells were not labeled. With uric acid, there was little or no incorporation of the intact molecule into the cells of either type of xanthine-oxidizing organisms when the organisms were grown under vigorous aeration. With xanthine, there was some incorporation of radioactivity into the cells. This is as expected since the compound is well known to be utilized in the synthesis of nucleic acids. However, much less incorporation was observed in the organisms that oxidize xanthine than in a standard culture of *Escherichia*. It is possible that active catabolism of the xanthine by a cell reduces the effectiveness of the cell in incorporating the molecule intact.

Table 10 presents some additional data relating to the incorporation of lack of incorporation of radioactive adenine, xanthine, and uric acid into the different types of cells. When the purines were added as supplements to the medium, but not as the principal catabolite, *Escherichia* and *Arthrobacter* were effective in the incorporation of adenine, but *Pseudomonas* and *Nocardia* were ineffective. In contrast to the results obtained with *Escherichia*, xanthine was not effectively incorporated into any of the purine-degrading bacteria when incubated under aerobic conditions. Effective incorporation of xanthine and, more interestingly, significant incorporation of uric acid were observed with *Pseudomonas*, but not the gram-positive bacteria, when the growth of the organism was limited by the availability of molecular oxygen. The counts incorporated from uric acid were found to be precipitable with trichloroacetic acid and the counts so precipitated

TABLE 9. *Incorporation and degradation of radioactive purines by several strains of bacteria grown with vigorous aeration*[a]

| Culture medium | Organism used | cpm | | | Cells (mg [dry wt]) | Residual substrate (mM) (xanthine or uric acid) | Ammonia (mM) | Urea (mM) |
|---|---|---|---|---|---|---|---|---|
| | | Culture medium | After urease treatment | Cells | | | | |
| 0.1% Uric acid | Uninoculated | 138,200 | | | | 7.47 | 0.7 | 0 |
| | *Pseudomonas putida* 40 | 64,700 | 7,300 | 25 | 1.7 | <0.1 | 16.8 | 7.9 |
| | *Arthrobacter* S-2 | 59,640 | 7,700 | 190 | 2.6 | 6.1 | 4.8 | 5.2 |
| | *Nocardia* G-F | 134,700 | 19,200 | 0 | 1.4 | <0.1 | 0.7 | 15.4 |
| 0.1% Xanthine | Uninoculated | 163,900 | | | | 6.4 | 1.2 | 0 |
| | *Pseudomonas putida* 40 | 90,700 | | 141 | 1.31 | <0.1 | 12.0 | 5.4 |
| | *Arthrobacter* S-2 | 79,692 | 4,671 | 318 | 2.4 | (<0.1) | 4.4 | 5.4 |
| | *Nocardia* G-F | 166,400 | | 391 | 2.3 | <0.1 | 1.2 | 5.4 |
| 0.1% Xanthine + 0.1% glucose | *Escherichia coli* K-12 | 163,900 | | 1,020 | 1.2 | | | |

[a] All media employed here were supplemented with 0.03% Casamino Acids (Difco) and 1-$\mu$g/ml quantities of the following vitamins: folic acid, biotin, nicotinic acid, pantothenic acid, riboflavin, thiamine, pyridoxal, and vitamin $B_{12}$. This satisfied the growth requirements of all strains without providing purines. Growth of all the organisms except *Escherichia* was largely dependent on the purine addition. Each medium containing the indicated amount of nonlabeled purine was supplemented with an amount of the same labeled purine (see text) to provide the counts per minute indicated for the uninoculated medium. Unless otherwise stated, all data in this table refer to 10 ml of culture medium. Residual substrates were determined spectrophotometrically on medium supernatant and represent maximum values.

TABLE 10. *Incorporation of small supplements of radioactive purines into the cells of several strains of bacteria grown primarily on succinate*

| Radioactive supplement and growth conditions | Organism used | cpm associated with the cells from 10 ml of growth medium | mg (dry wt) of cells from 10 ml of growth medium |
|---|---|---|---|
| Adenine (vigorous aeration) | *Escherichia coli* K-12 | 4,986 | 2.9 |
| | *Nocardia* G-F | 2,987 | 3.9 |
| | *Arthrobacter* S-2 | 11,100 | 6.4 |
| | *Pseudomonas putida* 40 | 619 | 1.3 |
| Xanthine (vigorous aeration) | *Escherichia coli* K-12 | 11,700 | 1.1 |
| | *Nocardia* G-F | 404 | 4.2 |
| | *Arthrobacter* S-2 | 41 | 6.6 |
| | *Pseudomonas putida* 40 | 0 | 0.9 |
| Uric acid (vigorous aeration) | *Escherichia coli* K-12 | 26 | 1.6 |
| | *Nocardia* G-F | 77 | 4.5 |
| | *Arthrobacter* S-2 | 207 | 6.9 |
| | *Pseudomonas putida* 40 | 0 | 1.1 |
| Xanthine (oxygen limitation) | *Nocardia* G-F | 17,225 | 1.6 |
| | *Arthrobacter* S-2 | 256 | 0.8 |
| | *Pseudomonas putida* 40 | 13,300 | 0.8 |
| Uric acid (oxygen limitation) | *Nocardia* G-F | 237 | 0.8 |
| | *Arthrobacter* S-2 | 80 | 1.5 |
| | *Pseudomonas putida* 40 | 3,518 | 0.8 |

[a] Media employed contained 0.1XS (mineral salts), 1% sodium succinate, 0.03% Casamino Acids (Difco), vitamins (Table 9), and where indicated approximately 150,000 cpm of the radioactive purine per 10 ml (see text). Oxygen limitation was achieved in following manner: 10 ml of medium was inoculated and sealed in vials containing 10 ml of additional air space. These vials were shaken until a small amount of stationary growth was observed. The vials were opened and resealed and shaken as before. The process was repeated several times until the indicated final growth was achieved.

were extractable with water-saturated phenol. These results suggest that the incorporation is into the nucleic acids via the reduction of the uric acid. The only known catalytic activity that could accomplish this would be xanthine dehydrogenase operating in the reverse direction, as has been suggested, as a functional activity with anaerobic enzymes (6). Whether *Pseudomonas* contains an electron donor of sufficiently high redox potential to accomplish this process efficiently is unknown. Perhaps the incorporation is only the result of the dismutative activity of xanthine dehydrogenase involving uric acid with more reduced purines formed de novo (6, 24, 31).

Table 9 also contains some stoichiometric observations that demonstrate differences in the metabolism of the purines by the bacteria employed. The data with *Norcardia* are consistent with a breakdown of the uric acid to 2 mol of urea and suggest a lack of urease. Thus, the organism may not be able to effectively utilize the purine as a nitrogen source. However, the growth factor requirement of the organism when growing on purines is not satisfied by the addition of ammonia to the medium. On the other hand, the strains of *Pseudomonas* and *Arthrobacter* employed apparently excrete one-half the [2-$^{14}$C]uric acid radioactivity as urea, the other half presumably being converted to carbon dioxide. We have previously reported that *Pseudomonas* 40 lacks urease (30). The results are consistent with the equilibration of the 2 and 8 position of uric acid by the known action of uricase (9) followed by a pathway in which only 1 mol of urea is produced, as was previously reported (25).

A xanthine dehydrogenase functionally designed to reduce uric acid under some circumstances of cell growth (see Table 10 and accompanying discussion) might be expected to be inhibited effectively by this compound when studied in the direction of xanthine oxidation. In consideration of the incorporation studies reported above, it is of interest that the enzyme from *Pseudomonas* is inhibited in a competitive manner by uric acid ($K_i = 2 \times 10^{-4}$; $K_m = 2.5 \times 10^{-4}$), whereas the enzyme from *Arthrobacter* is apparently insensitive to this compound (Fig. 3). Uric acid is also found to inhibit this enzyme when NAD and 2,6-dichlorophenol-indophenol are used as the electron acceptors at levels consistent with the above-quoted $K_m$ and $K_i$ values. Thus, the inhibition observed in Fig. 3 is apparently not dependent on the use of an electron acceptor of high redox potential. The enzyme from *Arthrobacter* will not couple to the viologen dyes used to demonstrate the inhibition which occurs with the enzyme from *Pseudomonas* (Table 3) due to uric acid.

**Patterns of oxidation of purine and monooxypurines by several of the xanthine-oxidizing preparations.** We referred above to the earlier suggestion that the xanthine-oxidizing activity from *Pseudomonas* might show a tendency for oxidation of purines at position 2 (5) whereas the xanthine dehydrogenase from the *Clostridium* tends to oxidize position 8 (6). Although it is known that the enzyme from *Veillonella* oxidizes hypoxanthine at position 6 (28), the latter enzyme is also known to oxidize purine at position 8 (24). We felt that it would be of some interest to examine this situation with examples of the enzymes from aerobes studied here. Possibly, there would be a correlation of the methylxanthine specificity pattern with the tendency for oxidation of a particular position of the purine ring.

The activities associated with the cell-free particles of *Pseudomonas* 40 possessed a markedly different specificity pattern than that observed with the soluble enzyme from that organism (compare Fig. 4A with 4B). Relative to xanthine, the other substrates were more rapidly oxidized by the particles and hypoxanthine was oxidized at two positions, both reactions being more rapid than the oxidation of xan-

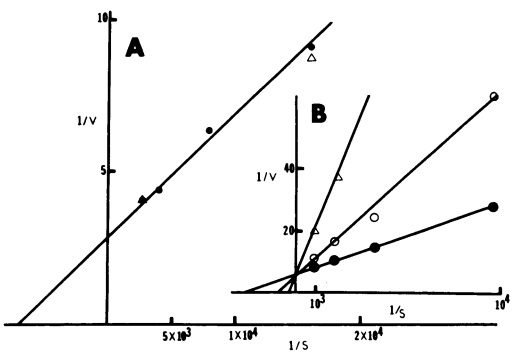


FIG. 3. *Effect of uric acid on several bacterial xanthine-oxidizing preparations. (A) Purified Arthrobacter S-2 enzyme (see text) was employed. Except for the indicated concentrations of xanthine, standard assay conditions were employed with 0.5 mM 2,6-dichlorophenol-indophenol as the electron acceptor. Each assay contained 0.075 unit (determined by the ferricyanide method) of enzyme. Symbols: ●, xanthine only; △, xanthine plus 5 × 10$^{-4}$ M uric acid. (B) Cell-free extracts of Pseudomonas putida 40 containing 1.2 units of xanthine dehydrogenase were used for each assay with 0.2 mM benzyl viologen as the electron acceptor. Symbols: ●, xanthine only; ○, xanthine plus 4 × 10$^{-4}$ M uric acid; △, xanthine plus 10$^{-3}$ M uric acid.*

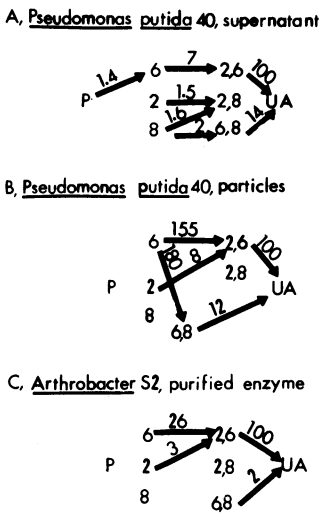


FIG. 4. *Pattern of three xanthine-oxidizing preparations with purine and its oxygenated derivatives. Abbreviations: P, purine; 2, 2-oxypurine; 6, hypoxanthine; 8, 8-oxypurine; 2,6, xanthine; 2,8, 2,8-dioxypurine; 6,8, 6,8-dioxypurine; UA, uric acid. Compounds not associated with an arrow were found not to be oxidized by the spectrophotometric technique. Compounds associated with an arrow were oxidized to the indicated products. The rate of each individual reaction is indicated by the number above the arrow and is expressed as percent relative to the rate observed by the same preparation for the oxidation of xanthine.*

thine itself. The latter conclusion was based on the finding that the particle preparations gave significant initial increases at 262 nm when hypoxanthine was used as a substrate (not observed when the supernatant enzyme was examined). This result is consistent with some 6,8-dioxypurine accumulation since hypoxanthine and xanthine possess an isosbestic point at the wavelength employed here and uric acid absorbs significantly less. 6,8-Dioxypurine would be expected to accumulate under these conditions due to its relatively slow rate of utilization. On the other hand, a simultaneous decrease at 249 nm was observed where 6,8-dioxypurine and hypoxanthine have an isosbestic point and uric acid is more absorptive. This would only be consistent with the production of some xanthine. Purine does not appear to be oxidized by the particles at a detectable rate since no spectral changes were observed when the particles were incubated with this substrate.

On the other hand, purine was oxidized at the 6 position by the *Pseudomonas* supernatant activity since there was a decrease at 265 nm and a simultaneous increase at 300 nm corresponding to the production of uric acid in a sequence of reactions in which the first step was largely rate determining. If the purine had been oxidized appreciably at either of the other available positions, there would have been an increase in absorption at 317 nm due to the accumulation of 2,8-dioxypurine which is refractory. The two-way oxidation of 8-oxypurine was indicated by the finding that there is some increase at 317 nm but also a simultaneous increase at 295 nm (isosbestic point for 8-oxypurine and 2,8-dioxypurine) which was abolished by addition of uricase. 2-Oxypurine was oxidized by the supernatant preparation from *Pseudomonas* since the compound gave an additional progressive increase at 270 nm where all potential products are more absorptive, but no detectable change at 317 nm (isosbestic point with 2,8-dioxypurine). Incubation of 2-oxypurine with the particles resulted in the eventual loss of the absorption at 317 nm with the production of uric acid, so no 2,8-dioxypurine was produced by the latter source. Similar methods were used to establish the pattern of oxidation with the enzyme from *Arthrobacter*. The enzyme from the gram-positive organism shows a more restricted action towards the compounds examined. The nonreactive compounds did not inhibit the enzyme and apparently do not bind effectively to it.

## DISCUSSION

**Comparison of the substrate specificity pattern of the different bacterial xanthine-oxidizing preparations.** In this study, we wished to consider the stability of the bacterial patterns of specificity observed with xanthine dehydrogenase in comparing one source with another. For this purpose, we assembled a collection of bacteria including representatives of many diverse groups and attempted to include a number of independent examples of each group. We focused on the use of the 1-methylxanthine and 3-methylxanthine as secondary substrates for the enzymes since Bergman and co-workers previously suggested that these substrates discriminate effectively between the classical milk enzyme, which utilizes 1-methylxanthine but not 3-methylxanthine as substrates, and the enzyme from *P. aeruginosa*, which was reported to utilize 3-methylxanthine but not 1-methylxanthine (5, 11). We extended the observation with 3-methylxanthine to include all aerobically grown gram-negative sources of the enzyme included in this investigation. However, we found all of these sources to be able to utilize 1-methylxanthine – the latter compound serving as a substrate for every

source of xanthine-oxidizing activity we examined. We do not believe that this difference is due to differences between our strains of bacteria and those of the previous investigators (one possibility), but rather to difficulties in the determination of activity with the methylated substrates with ordinary whole cells (30) and to a tendency of certain preparations of the enzyme to be relatively unstable in the presence of 1-methylxanthine (see Materials and Methods).

All of the enzymes investigated from gram-positive bacteria were found to be unable to utilize 3-methylxanthine as substrates, at least at relative rates that we could detect (1% or less than that of xanthine). We also find it highly interesting that obligately anaerobic bacteria (including those strains considered to be gram negative) share the pattern of methylxanthine utilization observed with the gram-positive strains and that the pattern observed with facultative gram-negative bacteria switches to the gram-positive pattern of methylxanthine utilization when the bacteria are grown anaerobically (Fig. 2; Table 5). Based on these findings, gram-negative bacteria grown aerobically could be considered to contain a biologically unique form of xanthine dehydrogenase since all other sources of the enzyme including eucaryotic sources in the literature and the one eucaryotic organism examined here (Table 3) are unable to utilize 3-methylxanthine appreciably.

Although consideration of the methylxanthine oxidation patterns observed with the different preparations permitted the classification of the enzymes into two groups corresponding to the main subdivisions of true bacteria, a comparison of the electron acceptor capabilities of the sources of the enzyme revealed considerable variation within each of the two groups (see Table 3). Both gram-negative bacteria and gram-positive bacteria contained activities which, based on these data, would be appropriately referred to as xanthine oxidase on the one hand or xanthine dehydrogenase on the other. With the single exception of the activity we detected in *Serratia*, these features are quite stable when the comparison is being made with two members of the same genus or tribe. For example, the enzyme from *Arthrobacter* differs from the soluble enzymes from other gram-positive bacteria in that oxygen but not NAD will serve effectively as the electron acceptor. Among the gram-negative bacteria, *Pseudomonas* seems to possess two activities: a particulate oxidase and a soluble dehydrogenase. On the other hand, the comparable soluble enzyme from facultative gram-negative groups seems ineffective in the utilization of NAD.

As explained in Results, it was felt that there might be some correlation of the position of oxidation on oxygenated purines with the methylxanthine substrate pattern. Our results have not supported such a view (Fig. 4). Each of the new enzyme sources examined displays a pattern different from the two bacterial patterns previously described (6, 24) and from the milk enzyme (3, 4). Thus, the methylxanthine oxidation pattern may be a more stable feature of the bacterial enzymes than the pathways of purine and monopurine oxidation. The latter patterns permit further subdivisions in classification of the enzyme according to specificity. It must be stated, however, that it is not known how variable these features will be since only a single example has been used of each of the five groups of bacteria which have so far been examined in this manner.

Another attempt to correlate xanthine dehydrogenase function with the methylxanthine pattern (explained in Results) was also unsuccessful in that we were able to measure the incorporation of uric acid into the cells of *Pseudomonas* 40 under appropriate conditions but not into *Arthrobacter*. This latter finding was, however, correlated with the sensitivity of the xanthine dehydrogenase from the organisms to uric acid as an inhibitor. The recent finding that the xanthine dehydrogenase from *Pseudomonas multivorans* (23) was not sensitive to inhibition by uric acid suggests that the latter feature (and possibly also the ability to reduce uric acid) may be one of the more variable features of the enzymes. On the other hand, the possibility that a second function unrelated to xanthine dehydrogenase may be involved in the incorporation of uric acid has not been ruled out.

**Consideration of the possibility of multiple xanthine-oxidizing enzymes with overlapping specificity.** Probably because of the influence of the extensive substrate specificity studies with the milk xanthine oxidase and other related purified enzymes, there has been a tendency to consider the oxidation of all purine analogues by each new preparation containing xanthine dehydrogenase activity as due to the activity of a single enzyme. This may not always be the case and the possibility that there may be several enzymes of differing or overlapping specificity should be considered. There is evidence for such a situation in *Aspergillus* (21), and the aldehyde oxidase-xanthine oxidase pair from mammalian sources can be considered to be another example (14). There can

be very specific enzymes involved in the oxidation of purines as evidenced by our previous study of 2-oxypurine dehydrogenase from *Peptococcus aerogenes* 228 (31). It was recently reported that there is a noninducible hypoxanthine-oxidizing activity (which produces but does not oxidize 6,8-dioxypurine) widely distributed in *Streptomyces* (26) in addition to a more typical inducible xanthine-oxidizing activity. However, we have not been able to demonstrate the noninducible activity with cells or extracts of the strains of *Streptomyces* employed here using our methods. Since the original observations were all made with whole cells, the possibility that the effects are due to permeability changes cannot be discounted. It would be interesting to determine the pattern of oxidation of purines with the cell-free preparations of *Streptomyces* as in Fig. 4. However, the enzyme does not utilize molecular oxygen at a detectable rate (Table 3), and separation followed by chemical analysis is required for this determination.

We do not know if the collection of activities associated with the cell-free particles of *Pseudomonas* (Fig. 4) is due to a single enzyme of broad specificity or to a collection of individual activities. The point is highly interesting and worthy of further investigation. The activity associated with the extracts from *Arthrobacter* can be assumed to be due to a single activity since the purified activities will be shown later to be homogeneous (Downard and Woolfolk, unpublished results). We have suggested that the facultative gram-negative organisms may contain one or the other of two enzymes depending on the condition of growth. It would, thus, be tempting to apply this explanation to the soluble extract of *Pseudomonas* 40 where considerable variability in the methylxanthine ratio (Fig. 4) was encountered. However, we have investigated this situation in some detail (C. A. Woolfolk, unpublished results). While it would not be appropriate to present all this information here, the results suggest that there are two forms of the enzyme related to the level of aggregation (monomer and dimer) with differing but overlapping specificities for the methylxanthine substrates and that these two forms are interconvertible under certain conditions. Thus, in this situation it would seem to be unnecessary to postulate two genetically different xanthine dehydrogenases to account for the variability in the data.

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