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Initial Hydrogenation during Catabolism of Picric Acid by *Rhodococcus erythropolis* HL 24-2

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***Rhodococcus erythropolis* HL 24-2, which was originally isolated as a 2,4-dinitrophenol-degrading bacterium, could also utilize picric acid as a nitrogen source after spontaneous mutation. During growth, the mutant HL PM-1 transiently accumulated an orange-red metabolite, which was identified as a hydride-Meisenheimer complex of picric acid. This complex was formed as the initial metabolite and further converted with concomitant liberation of nitrite. 2,4,6-Trinitrocyclohexanone was identified as a dead-end metabolite of the degradation of picric acid, indicating the addition of two hydride ions to picric acid.**

Recent explorations of explosives at former production and manufacturing plants (6) revealed contamination of soil and groundwater by polynitroaromatics such as 2,4,6-trinitrotoluene. Although these chemicals were synthesized and dumped mainly during the Second World War, they are still present at high concentrations, indicating their resistance to microbial metabolism. Ammonium 2,4,6-trinitrophenoxide (ammonium picrate) was also used by the military as an explosive (12). Consequently, picric acid has also been found as a contaminant in soil and groundwater.

Erikson (4) was the first to observe a microbial attack on picric acid by *Micromonaspora* strains. Gundersen and Jensen (5) described the metabolism of picric acid by *Corynebacterium simplex* which was isolated from soil as a 4,6-dinitro-2-methylphenol-degrading organism. Bioconversion of picric acid by this strain was indicated by the formation of nitrite. Tabak et al. (15) observed a color change from yellow to orange-red in enrichment cultures with picric acid. Detailed knowledge about bacterial growth with picric acid or the degradative pathway of picric acid, however, is lacking. Only picramic acid was identified as a metabolite of picric acid (16).

Investigations of the aerobic microbial degradation of 2,4-dinitrophenol (2,4-DNP) by the *Rhodococcus erythropolis* strains HL 24-1 and HL 24-2 have demonstrated an enzymatic reduction of the aromatic nucleus with 4,6-dinitrohexanoate as a dead-end product (10). This indicated that a nucleophilic attack may play an important role during initial catabolism of 2,4-DNP. In the present paper, the corresponding primary catabolic reactions of picric acid by these organisms are described.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and measurement of growth. *R. erythropolis* HL 24-1 and HL 24-2 were originally isolated by their abilities to grow with 2,4-DNP as a nitrogen source. For growth of both strains in batch cultures, the mineral medium as described by Lenke et al. (10) contained 0.5 mM 2,4-DNP and 10 mM succinate. The mutant strain HL PM-1 was grown under the same conditions, and 2,4-DNP (0.5 mM) could be replaced by picric acid. Fluted Erlenmeyer flasks were incubated at 30°C on a rotary shaker at 150 rpm, and growth was determined photometrically by measuring A_{546}. Because an orange-red metabolite was formed during growth, corrections were made by determining the absorbance of the cell-free medium. Solid media were prepared by the addition of 1.5% agar to the mineral medium.

Analytical methods. Nitrite and ammonium ion concentrations were measured by photometric methods as described in the accompanying paper (10). Quantitative analysis of picric acid and metabolites was performed by a modification of the reverse-phase high-performance liquid chromatography (HPLC) method (10). To analyze picric acid, the Meisenheimer complex, and nitrite, the mobile phase was acetonitrile-water-H_3PO_4 (200:800:2.6 [vol/vol/vol]). To quantify 1,3,5-trinitropentane, each sample was incubated for 90 min with amidosulfonic acid (10 μl of a 2 M solution per 200-μl probe) and afterwards monitored by HPLC with a mobile phase of acetonitrile-water-H_3PO_4 (230:770:2.6 [vol/vol/vol]). In addition, the conversion of picric acid was followed by ion-pair chromatography with a 5 mM solution of tetrabutylammonium phosphate (Pic A; Waters, Milford, Conn.) in methanol-water (4:6 [vol/vol]).

Conversion of picric acid by resting cells of *R. erythropolis* HL 24-1, HL 24-2, and HL PM-1. Resting-cell experiments with *R. erythropolis* HL 24-1, HL 24-2, and HL PM-1 under aerobic and anaerobic conditions were carried out as described in the accompanying paper (10). Cells were grown with succinate (10 mM) as a carbon and energy source. Ammonia (2 mM), 2,4-DNP (0.5 mM), or picric acid (0.5 mM) served as a nitrogen source. The conversion of picric acid was followed by HPLC, and the amount of nitrite was estimated photometrically. Because the formation of a colored metabolite during the conversion of picric acid interfered with this assay, a nitrite-free reference of each probe was prepared by the addition of 10 μl of 2 M amidosulfonic acid.

Isolation and identification of 1,3,5-trinitropentane. Resting cells of *R. erythropolis* HL 24-1 were used for the accumulation of bioconversion products of picric acid. Cells pregrown with 2,4-DNP and succinate (three times with 1,000-ml mineral medium in 3-liter fluted Erlenmeyer flasks) were harvested, resuspended twice in 500 ml of phosphate buffer (optical density of 3 to 4), and incubated at 30°C with 0.5 mM picric acid. The turnover of picric acid was followed by HPLC. In order to obtain larger amounts of metabolites,

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after total conversion of picric acid, the cells were harvested, resuspended twice in fresh buffer (500 ml), and incubated again with picric acid (0.5 mM). The cell-free culture fluids were combined, acidified to pH 2 with amidosulfonic acid, and incubated for 90 min at room temperature. Finally, the conversion product (1,3,5-trinitropentane) was extracted with chloroform. The chloroform extracts were combined, dried over $MgSO_4$, and evaporated to dryness. 1,3,5-Trinitropentane was purified by semipreparative HPLC with acetonitrile-water-trifluoroacetic acid (500:500:1) as the mobile phase. 1,3,5-Trinitropentane was crystallized from methanol (13). ^1H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were obtained as described in the accompanying paper (10). Positive-ion chemical ionization spectra were recorded on a mass spectrometer (MAT 8430; Finnegan, Bremen, Germany).

Conversion of picric acid with sodium borohydride. The conversion of picric acid with sodium borohydride was carried out as described by Severin and Schmitz (14). The isolated salt was further converted to 1,3,5-trinitropentane by acid catalysis (13). Amidosulfonic acid instead of acetic acid was used to perform this reaction. For the preparation of the Meisenheimer complex of picric acid, a 20 mM solution of picric acid was adjusted to a pH of 10 by NaOH. Small portions of an aqueous solution of sodium borohydride (20 mg/ml) were carefully added. The formation of an orange-red product was observed by HPLC. This colored solution was used for further investigations.

RESULTS

Picric acid as growth substrate. Neither of the original isolates, *R. erythropolis* HL 24-1 and HL 24-2, was able to utilize picric acid as the sole source of nitrogen. When induced for the degradation of 2,4-DNP, however, picric acid was metabolized by resting cells of both strains. Nitrite was identified as a metabolite. These observations indicated that the enzymes of the pathway adopted for the degradation of 2,4-DNP also attack picric acid. After strain HL 24-2 was streaked on agar plates containing 0.5 mM picric acid and 10 mM succinate in mineral medium, single colonies appeared, which evidently could use picric acid as a nitrogen source. In addition to growth, a color change from yellow to orange-red around the colonies was observed. These colonies (at least 3 to 4 mm in diameter after 7 to 10 days of incubation) appeared at a frequency of 2×10^{-5}. From one of these colonies, the derivative strain HL PM-1 was selected for further investigations. Experiments with resting cells of *R. erythropolis* HL 24-2 or HL PM-1 pregrown with ammonia and succinate showed that catabolic activity toward picric acid could be induced only in the derivative strain HL PM-1. Obviously, by spontaneous mutation this organism had acquired the ability to induce a catabolic activity which converts 2,4-DNP and picric acid to the same metabolites as the wild-type organism.

The mutant strain *R. erythropolis* HL PM-1 was able to utilize picric acid as the sole source of nitrogen also in batch cultures (Fig. 1). Characteristically, picric acid was consumed by strain HL PM-1 within 7 h, whereas only relatively small amounts of succinate were utilized during this period. Obviously, picric acid seemed to delay the utilization of succinate. Nitrite, liberated during turnover of picric acid, evidently served as a nitrogen source during this second period of growth with succinate. At the same time, when nitrite was eliminated, the above-mentioned orange-red metabolite transiently accumulated and a second rather unstable metabolite was detected by HPLC in the culture fluid.

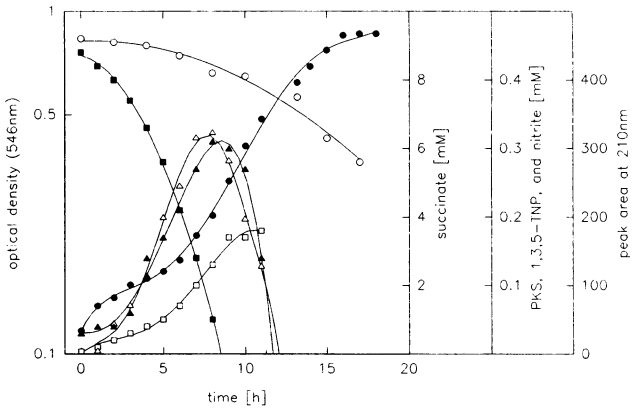

FIG. 1. Growth of *R. erythropolis* HL PM-1 with picric acid (0.5 mM) as a nitrogen and succinate (10 mM) as a carbon source. The culture was incubated in a 500-ml fluted Erlenmeyer flask on a rotary shaker at 30°C. Increase in cell density (●) was determined photometrically at 546 nm. Concentrations of picric acid (PKS [■]), the Meisenheimer complex (peak area at 210 nm [△]), 1,3,5-trinitropentane (1,3,5-TNP [□]), and succinate (○) were measured by HPLC, and nitrite (▲) release was quantified photometrically.

Metabolites of picric acid. Resting cells of *R. erythropolis* HL 24-1, HL 24-2, or the mutant strain HL PM-1, grown with 2,4-DNP and succinate, metabolized picric acid and liberated more than 2 mol of nitrite from 1 mol of picric acid (maximum specific activities of the cells were 17 to 22 μmol/min/g of protein, depending on the strain used). By ion pair chromatography, two additional metabolites were detected; one of these compounds (retention volume, 12.88 ml) exhibited a characteristic UV-visible light absorption at 420 and 490 nm which was correlated with the observed orange-red of the culture fluid. This metabolite (A) only transiently accumulated (Fig. 1). The second metabolite (X) had a retention volume of 3.01 ml. HPLC analysis by an acidic solvent system (pH 2) revealed only one well-defined signal (retention volume, 5.17 ml) corresponding to the orange-red metabolite (A). An additional less-characteristic peak corresponding to metabolite X distinguished itself by a strong tailing. Obviously, metabolite X was unstable under acidic conditions. In order to elucidate its fate, the cell-free culture fluid was incubated with amidosulfonic acid (pH 1), thereby destroying the accumulated nitrite. After 90 min, two new products were distinguished, exhibiting retention volumes of 4.62 ml (product B) and 12.43 ml (product Z). Upon addition of NaOH (pH 7.5), metabolite A was regenerated from product B, indicating a pH-dependent equilibrium between these compounds. While B was identified as the protonated form of A, product Z was generated from metabolite X by an irreversible acid-catalyzed reaction. Careful analysis of the time course of the acid-catalyzed turnover of X to Z showed an initial rapid formation of an intermediate product Y (retention volume, 6.52 ml) followed by a considerably slower conversion of Y to Z. The final product Z was quite stable under acidic conditions.

Isolation and identification of 1,3,5-trinitropentane. To isolate product Z, 2,4-DNP-induced cells of *R. erythropolis* HL 24-1 were incubated with picric acid. After total conversion of the substrate, the cell-free culture fluid was acidified with amidosulfonic acid and incubated for 90 min. Product Z

TABLE 1. Characteristic signals from the chemical ionization mass spectrum of 1,3,5-trinitropentane

Mass (*m/z*)	Intensity (% of basic peak)	Fragment ion
225	95.7	$MH + NH_3\rceil^+$
178	100	$(MH + NH_3)^+ - HNO_2$
176	37.3	$MH - HNO\rceil^+$
163	67.7	$(MH + NH_3)^+ - 2\ HNO$
147	21.2	$(MH + NH_3)^+ - HNO - HNO_2$
131	63.4	$MH - H_2O, - HNO_2\rceil^+$
129	72.7	$MH - HNO_2, - HNO\rceil^+$
114	74.5	$MH - 2\ HNO_2\rceil^+$

was extracted from the acidified culture fluid with chloroform. B or A was not extracted by this procedure. After evaporation of the chloroform, a nearly colorless oil was obtained. Crystallization from methanol (13) resulted in the formation of colorless needles with a melting point of 38 to 39°C (corrected).

In order to identify the isolated product Z, a chemical ionization mass spectrum (ammonia as reagent gas) was recorded. The mass spectrum revealed an ion mass of *m/z* 225, which corresponds to the ammonium cluster of the molecular ion (207). The elimination of HNO_2 resulted in a major peak at *m/z* 178 (2). The characteristic signals of the fragmentation pattern of product Z are shown in Table 1. The molecular ion and the fragment ions revealed the following formula: $C_5H_9N_3O_9$. As shown for 2,4-DNP, reduction of the aromatic ring must have occurred.

For final identification of product Z, ^1H and ^{13}C NMR spectra were recorded. The integrals of the resonance lines of the ^1H NMR spectrum clearly showed the presence of nine aliphatic protons. The nine protons of the compound appeared as three multiplets, integrating to twice four protons and one proton, respectively. This clearly indicated a highly symmetric structure. Two of the multiplets resonated at δ 4.81 ppm (pseudoseptet [see below]) and between δ 4.46 and δ 4.60 ppm. The low field shift of both signal groups indicates that they are bound to nitro-substituted carbon atoms (8). The third multiplet, apparently due to two methylene groups, appeared between δ 2.54 and δ 2.74 ppm. This, together with the data of the mass spectrum, indicates that product Z is 1,3,5-trinitropentane.

1,3,5-Trinitropentane can be considered an analog of 4,6-dinitrohexanoate, a compound which has been shown to be a metabolite of 2,4-DNP catabolism (10). ^1H NMR spectra of both 1,3,5-trinitropentane and 4,6-dinitrohexanoate showed a pseudoseptet corresponding to a nitro-substituted tertiary carbon atom. In the case of the symmetric 1,3,5-trinitropentane, this signal group can easily be explained as a triplet of triplets, a ^3J coupling between 3-H and the identical 2-H_A, 4-H_A (gauche configuration) of 9.1 Hz, and a ^3J coupling between 3-H and the identical 2-H_B, 4-H_B (anticonfiguration) of 4.4 Hz. Because of the relation of the coupling constants of 2:1, two signals appeared as one signal, giving rise to a pseudoseptet with relative intensities of 1:2:3:4:5:4:3:2:1. The pseudoseptet observed in the ^1H NMR spectrum of 4,6-dinitrohexanoate as well can now be explained as a triplet of triplets, giving further evidence for the postulated structure.

The ^{13}C NMR decoupled magnetic resonance data are in accordance with the postulated structure of 1,3,5-trinitropentane (Table 2; Fig. 2). Three signals due to five sp^3 carbon atoms appeared in the spectrum. Two of these signals

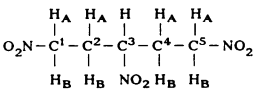

FIG. 2. Chemical structure for data in Table 2.

TABLE 2. ^1H NMR and ^{13}C NMR decoupled magnetic resonance data for 1,3,5-trinitropentane

Nucleus	Chemical shift (ppm) for R = H
1-H_A, H_B	4.81 (multiplet)
5-H_A, H_B	4.81 (multiplet)
2-H_A, H_B	2.54–2.74 (multiplet)
4-H_A, H_B	2.54–2.74 (multiplets)
3-H	4.46–4.60 (multiplet)
C-1	70.62
C-2	30.32
C-3	81.57
C-4	30.32
C-5	70.62

exhibited a low field shift because of the high anisotropic effect of the nitro substituents (9).

Further evidence for the destruction of picric acid by the addition of hydride ions comes from observations of Severin and Schmitz (14) and Severin and Adam (13). They described the conversion of picric acid with sodium borohydride, yielding a stable salt (Fig. 3 [S]) as a result of the addition of two hydride ions. Under acidic conditions, this salt was further converted to 1,3,5-trinitropentane. A sample of 1,3,5-trinitropentane (melting point, 28°C [uncorrected]) prepared by this procedure showed the same chromatographic properties as the product Z biologically obtained after acid treatment (melting point, 38 to 39°C [corrected]). If the acid-catalyzed formation of 1,3,5-trinitropentane from the stable salt (S; retention volume, 0.94 ml) was followed by HPLC, an intermediary product (ill-defined peak; retention volume, 3 to 4 ml) which exhibited the same chromatographic properties as metabolite X was detected. As observed with the biologically generated product, a direct precursor of 1,3,5-trinitropentane (Z; retention volume, 12.43 ml) is product Y (retention volume, 6.52 ml), which transiently emerged during the acid-catalyzed reaction.

Identification of the orange-red metabolite (A). Upon careful addition of sodium borohydride to picric acid, a color change from yellow to orange-red was observed, indicating the chemical formation of a hydride-Meisenheimer complex of picric acid. HPLC analysis showed that under these conditions, the chemically obtained product had the same retention volume (5.17 ml) and UV-visible light spectrum (λ_{max}, 420 and 490 nm) as the orange-red metabolite A of picric acid (5.19 ml; λ_{max}, 420 and 490 nm). Upon acidification with amidosulfonic acid, a product was immediately generated (retention volume, 4.62 ml; absorption maxima at 435 nm) which was also formed from metabolite A under acidic conditions (product B [see above]).

Mass balance of picric acid degradation. As described above, two additional metabolites besides nitrite, the orange-red Meisenheimer complex (metabolite A) and metabolite X, were formed from picric acid by resting cells of *R. erythropolis* HL 24-1, HL 24-2, and HL PM-1. The latter metabolite X was quantified indirectly by calculating the

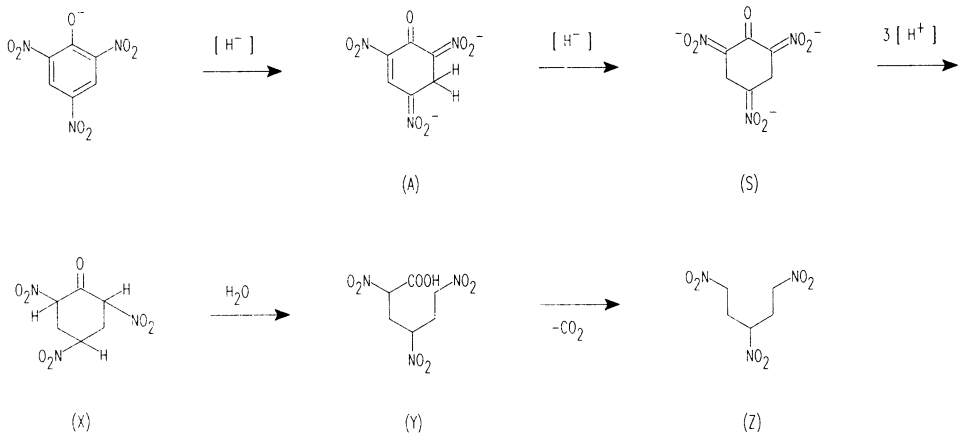

FIG. 3. Hypothetical mechanism of the formation of 1,3,5-trinitropentane. Product Y and product Z were formed by acid treatment of the culture fluid.

amount of 1,3,5-trinitropentane (product Z) formed by acid treatment. 1,3,5-Trinitropentane turned out to be a dead-end metabolite, whereas the orange-red Meisenheimer complex was further converted by the cells, with concomitant liberation of nitrite (Fig. 4).

After picric acid and the transiently formed Meisenheimer complex had been consumed by the cells, the organic nitrogen was quantitatively recovered, partly as nitrite (HL 24-1, 62% ± 1%; HL 24-2, 78% ± 1%; HL PM-1, 80% ± 1%). The remainder of organic nitrogen was found in the culture fluid as metabolite X. The nitrogen balance of the three experiments shows that no other nitrogen-substituted metabolite was formed in significant amounts during conversion of picric acid.

However, picric acid was also converted by *R. erythropolis* HL 24-1, HL 24-2, or HL PM-1 under anaerobic conditions, with a rather low level of activity (specific activity, 4 to 6 μmol/min/g of protein). Such a low level of activity has also been observed for the metabolism of 2,4-DNP under anaerobic conditions (10). Under these conditions, another metabolite besides nitrite and metabolite X was formed from picric acid. It was identical with 4,6-dinitrohexanoate, which was identified as a metabolite of 2,4-DNP (10). Careful analysis of the time course of the anaerobic conversion of picric acid showed the intermediate formation of rather small amounts of 2,4-DNP as a precursor of 4,6-dinitrohexanoate. Obviously, reductive elimination of nitrite from picric acid under anaerobic conditions generates 2,4-DNP, and further reduction yields 4,6-dinitrohexanoate.

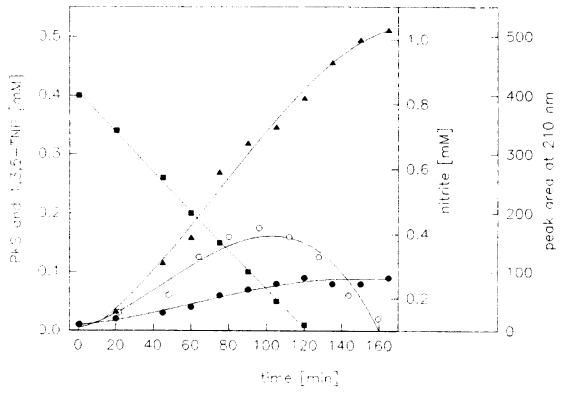

FIG. 4. Conversion of picric acid by resting cells of *R. erythropolis* HL PM-1. Resting cells were obtained by growth in mineral medium with picric acid (0.5 mM) and succinate (10 mM). The cells were harvested, resuspended in phosphate buffer (optical density at 546 nm of 1.3), and incubated at 30°C with 0.5 mM picric acid on a rotary shaker. The concentrations of picric acid (PKS [■]), the Meisenheimer complex (peak area at 210 nm [○]), and 1,3,5-trinitropentane (1,3,5-TNP [●]) were determined by HPLC. The amount of nitrite (▲) was estimated photometrically.

DISCUSSION

The 2,4-DNP-degrading bacterium *R. erythropolis* HL 24-2 acquired the ability to utilize picric acid as a nitrogen source by spontaneous mutation. In contrast to the original strain, HL 24-2, this mutant, designated HL PM-1, can induce the enzymes of 2,4-DNP catabolism also in the presence of picric acid. Present data indicate that an analogous catabolic sequence is involved in the metabolism of both substrates, 2,4-DNP and picric acid.

The identification of a hydride-Meisenheimer complex (A) of picric acid as an initial metabolite of picric acid unequivocally shows that aerobic catabolism is initiated by a nucleophilic rather than by an electrophilic attack of the aromatic ring. This can be rationalized by the highly electrophilic character of the nitro substituents which make the aromatic ring less susceptible to oxygenases. In contrast, the addition of nucleophilic ions like hydroxide, methoxide, and hydride to polynitroarenes is favored, resulting in the formation of colored, so-called Meisenheimer complexes. This is a well-known chemical reaction of this class of compounds (1, 3, 11).

That the *R. erythropolis* strains HL 24-1 and HL 24-2 and the derivative strain HL PM-1 can actually transfer hybride ions to picric acid is supported by the detection of 1,3,5-trinitropentane as a major product (product Z in Fig. 2) in the acidified culture fluid. Characteristically, 1,3,5-trinitropentane (Z) was also identified as a product of the chemical reduction of picric acid with $NaBH_4$ as a hydride donor (13, 14). 2,4,6-Trinitrocyclohexanone (X) as the initial product is readily converted into 1,3,5-trinitropentane (Z) under acidic conditions. Its formation from picric acid must be based on the transfer of two hydride ions rather than one. According to the reaction scheme suggested by Fig. 2, the initial

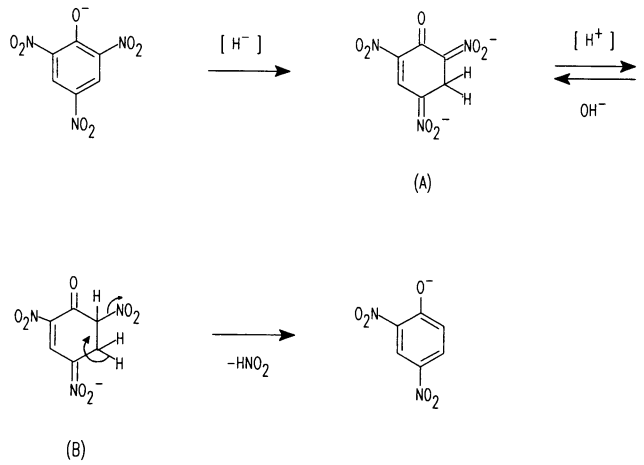

FIG. 5. Proposed mechanism for nitrite elimination from picric acid.

catabolic reaction of picric acid is the formation of the Meisenheimer complex (metabolite A) by the addition of one hydride ion. This hydride-Meisenheimer complex can be mimicked by the addition of one hydride ion from sodium borohydride. The Meisenheimer complex must also be a precursor of the "stable salt" isolated by Severin and Schmitz (14). This product (S) is generated from picric acid by the addition of two hydride ions. Under physiological conditions (pH 7.4), S is not detectable. It is protonated, yielding 2,4,6-trinitrocyclohexanone (metabolite X). Ring opening and decarboxylation of X yielding 1,3,5-trinitropentane are favored at low pH values (2 to 3) and must be considered nonbiological reactions.

A corresponding mechanism can readily explain the formation of the dead-end metabolite 4,6-dinitrohexanoate from 2,4-DNP by the addition of two hydride ions and hydrolysis of the intermediate 4,6-dinitrohexanone (10).

Whereas transfer of two hydride ions gives rise to dead-end metabolism of picric acid, the addition of one hydride ion generates the hydride-Meisenheimer complex and must be important for the utilization of picric acid aerobically (Fig. 5). The hydride-Meisenheimer complex (A) or its protonated form (B) regenerates aromaticity by the elimination of nitrite and formation of 2,4-DNP. Actually, 2,4-DNP is a metabolite of picric acid. Obviously, this mechanism predominates and allows partial degradation and utilization of picric acid as a nitrogen source. Whether only one equivalent of nitrite from picric acid is liberated by the hydride addition mechanism or 2,4-DNP as a metabolite passes the same reaction sequence for further nitrite elimination and total degradation is the subject of current investigations.

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