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BENZYLAMINE METABOLISM AT LOW O_2 CONCENTRATIONS

RELATIVE SENSITIVITIES OF MONOAMINE OXIDASE, ALDEHYDE DEHYDROGENASE AND HIPPURATE SYNTHESIS TO HYPOXIA

DEAN P. JONES
Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322, U.S.A.

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Abstract—The O_2 dependence of the metabolism of benzylamine to benzaldehyde, benzoate and hippurate was studied in isolated rat hepatocytes. The initial oxidation to benzaldehyde, catalyzed by monoamine oxidase, had an apparent K_{mO_2} value of 34 μM in cells and 40 μM in isolated rat liver mitochondria. The conversion of benzaldehyde to benzoate was essentially independent of O_2 concentration in spite of the dependence of the reaction upon cytosolic NAD^+. The conversion of benzoate to hippurate was half-maximal at 2.4 μM O_2 in hepatocytes and at about 0.5 μM O_2 in rat liver mitochondria. These values are consistent with the O_2 dependence of bioenergetic changes in these preparations and indicate that the O_2 dependence of hippurate formation is due to ATP availability for synthesis of benzoyl-CoA. These studies show that the three metabolic processes involved in benzylamine metabolism have markedly different dependences upon O_2 and that metabolism of benzylamine by monoamine oxidase is O_2 dependent over a physiologically important range.

Tissue hypoxia is an important component of pathologic conditions which are treated with a variety of therapeutic agents. Since many drug-metabolizing reactions require O_2 either directly as a substrate or indirectly because of their dependence upon cellular energetics [1], it is important to know which of the pathways are sensitive to O_2 insufficiency under physiological conditions. Previous studies with isolated rat hepatocytes have shown that oxidation by cytochrome P-450 and conjugation by the glucuronidation pathway are more sensitive to hypoxia than conjugation by the sulfation pathway [2, 3]. Both the oxidation and glucuronidation pathways are sensitive to O_2 supply *in vivo* in patients with chronic hypoxia (Kaplan *et al.*, unpublished observations).

In vitro studies with a variety of substrates have shown that the apparent K_{mO_2} for isolated monoamine oxidase [4–6] is in the range of normal physiological O_2 concentrations. Because of the potential differences between O_2 dependences of activities of isolated enzymes and their intracellular functions, we developed a model system with which enzyme function could be examined under low, steady-state O_2 concentrations in single cell suspensions [2, 7]. The purpose of the current study was to determine the intracellular O_2 dependence of monoamine oxidase. For this purpose, metabolism of the model substrate benzylamine was examined in suspensions of isolated rat hepatocytes. This substrate was considered suitable for these studies because it is readily permeable to cells, is rapidly metabolized, and allows simultaneous analysis of the O_2 dependence of two other reactions which are important in drug metabolism, viz. NAD^+-dependent dehydrodgenation and conjugation with glycine. Benzaldehyde is the product of the oxidation of benzylamine by monoamine oxidase and is metabolized principally by an NAD^+-dependent dehydrogenation which occurs in the cytosolic fraction [8]. The product of this dehydrogenation, benzoate, is metabolized mainly by conjugation with glycine to form hippurate [9]. This process occurs in the mitochondria and involves an ATP-dependent formation of a coenzyme A intermediate. The accumulation of these major metabolites, benzaldehyde, benzoate and hippurate, was measured by high-performance liquid chromatography.

The results of the current studies show that the three metabolic processes have very different dependences upon O_2 and consequently the metabolites obtained from benzylamine vary considerably as a function of O_2 concentration. The apparent cellular K_{mO_2} for benzylamine oxidation was found to be 34 μM. Because this value is similar to O_2 concentrations found in tissues *in vivo*, it suggests that oxidations that are catalyzed by monoamine oxidase may be dependent upon O_2 availability *in vivo*.

METHODS

Benzylamine, benzaldehyde, benzoic acid, hippuric acid, pargyline, collagenase and Hepes [4-(2-hydroxyethyl)-1-piperizinethane sulfonic acid] were purchased from the Sigma Chemical Co., St. Louis, MO. HPLC grade methanol was from the Baker Chemical Co., Phillipsburg, NJ. Doubly-distilled water was used for chromatography, and deionized water was used for media for cell preparations and incubations.

Isolated hepatocytes were prepared from male white rats [Kng:(SD) Br, King Animal Laboratories;

fed *ad lib.*] by perfusion of the liver with collagenase [10]. Cell viability was 95–99% as estimated by exclusion of 0.2% trypan blue. Incubations were performed at 37° in rotating round-bottom flasks in Krebs–Henseleit medium supplemented with 12.5 mM Hepes [10]. Low steady-state O_2 concentrations were established as previously described [3]. Incubations were terminated by addition of 0.5 ml of 0.3 M perchloric acid per ml of incubation mixture, and protein was removed by centrifugation.

Liver mitochondria were prepared as previously described [11] and incubated in 110 mM sucrose, 60 mM Tris·HCl, 60 mM KCl, 5 mM $MgCl_2$ and 0.5 mM EDTA, pH 7.4. Respiratory control values were between 5 and 9 for stimulation of respiration by 0.5 mM ADP in the presence of 5 mM succinate and 5 mM glutamate. Reactions were stopped by addition of acid as above.

Benzaldehyde, benzoate and hippurate acid were quantitated in protein-free extracts by high-performance liquid chromatography as described (D. P. Jones, *J. Chromat.*, in press). Briefly, this involves separation on a 5 μm reverse-phase C_{18} column (4.6 mm × 25 cm Ultrasphere-ODS, Beckman Instruments) with a methanol gradient (10–70%) in 1% acetic acid, pH 4.2, and containing 0.18% ethyl acetate. Metabolites were detected and quantitated by absorbance at 250 nm. Identification of products was consistent with the known characteristics of enzymes thought to function in this pathway [4–6, 8–9]. The formation of the compound identified as hippurate was dependent both upon benzoate and glycine concentrations in cell suspensions and was produced by mitochondrial preparations, but not by post-mitochondrial supernatant solutions. Low concentrations of benzoate in cell suspensions were nearly quantitatively converted to this product. Low concentrations of benzaldehyde in cell suspensions were converted in a time-dependent manner to the product identified as benzoate and subsequently to that identified as hippurate. In cell fractionation studies, the conversion of benzaldehyde to the product identified as benzoate occurred principally in the post-microsomal supernatant fraction and was stimulated 5- to 10-fold by addition of 1 mM NAD^+. Conversion of benzylamine to the product identified as benzaldehyde occurred in the mitochondrial fraction, and this conversion was essentially completely inhibited by pargyline in both mitochondrial and intact cell preparations (see below).

RESULTS

Benzylamine was metabolized rapidly by isolated hepatocytes under aerobic conditions with formation of benzaldehyde, benzoate and hippurate (Fig. 1). The rate of accumulation of the sum of benzaldehyde, benzoate and hippurate was 7.3 ± 1.6 nmoles/10^6 cells per min (N = 6). The accumulation of the individual products was essentially linear for 30 min in cells incubated with Krebs–Henseleit medium without glucose or any other organic metabolite. This indicates that the rates of metabolism of benzaldehyde and benzoate are nearly independent of the concentration of these metabolites in the incubation medium and suggests that intracellular

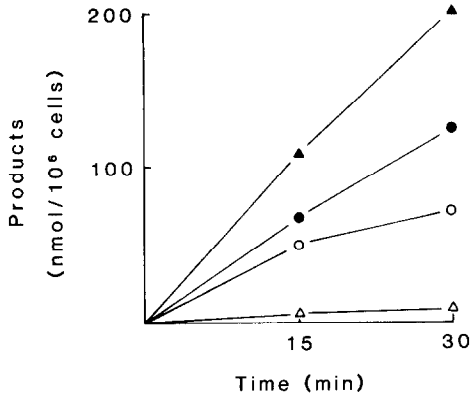

Fig. 1. Benzaldehyde, benzoate and hippurate formation by isolated hepatocytes incubated with 5 mM benzylamine. Cells (10^6/ml) were incubated under air at 37° in Krebs–Henseleit buffer, supplemented with 12.5 mM Hepes, pH 7.4. Samples were removed at indicated times, and the protein was removed by acid precipitation and centrifugation. Products were separated by HPLC and detected by the absorbance at 250 nm. Quantitation was obtained by integration and calculated relative to standards. Data are from one experiment which is typical of three. Key: benzaldehyde (○); benzoate (●); hippurate (△) and total products (▲). Total product formation was 7.3 ± 1.6 nmoles/10^6 cells per min (N = 6).

concentrations of these compounds are high relative to the K_m values for metabolic systems. Addition of 0.1 mM pargyline, an inhibitor of monoamine oxidase, gave greater than 90% inhibition of benzylamine oxidation. At lower concentrations of pargyline, the accumulation of benzaldehyde was decreased markedly, while benzoate accumulation was decreased to a lesser extent and hippurate formation was unaltered (Fig. 2). These results indicate that the system for conversion of benzoate to hippurate has a very high affinity for benzoate but a relatively low velocity under these conditions. The system for

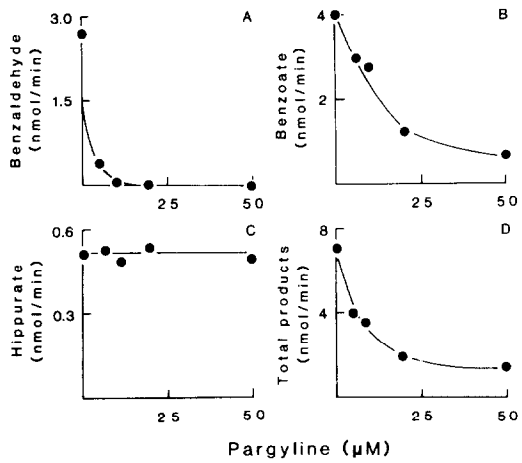

Fig. 2. Effect of pargyline on product formation from benzylamine. Cells were incubated and products assayed as described in Fig. 1 with addition of pargyline at the concentrations indicated. Data are from one experiment which is typical of three.

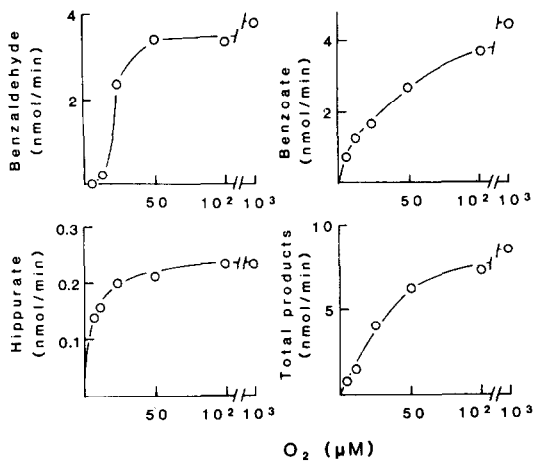

Fig. 3. Effect of O_2 concentration on product formation from benzylamine. Incubations and assays were performed as described in Fig. 1 with O_2 controlled at various steady-state values [3]. Data are from one experiment which is typical of six.

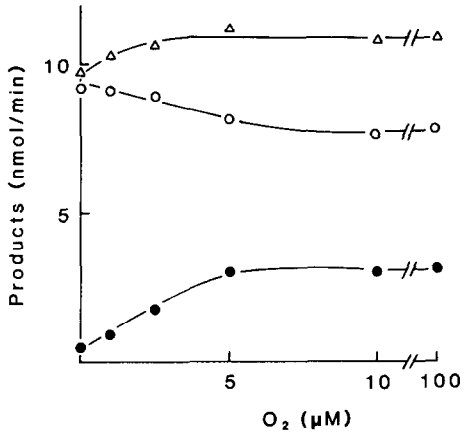

Fig. 4. Effect of O_2 concentration on product formation from 2 mM benzaldehyde. Cells were incubated and products analyzed as described in Fig. 3, except that benzaldehyde was added instead of benzylamine and 1 mM glycine was also included. Data are from one experiment which is typical of three. Key: benzoate (○); hippurate (●); and total products (△). Total product formation at 200 μM O_2 was 8.5 ± 3.1 nmoles/10^6 cells per min (N = 3).

benzaldehyde oxidation has a high affinity for benzaldehyde and also a high relative velocity.

A similar complexity of metabolite accumulation was found as benzylamine oxidation was varied by controlling O_2 concentration (Fig. 3). Total product formation from benzylamine was half-maximal at 34 ± 4 μM O_2 (N = 5). At O_2 concentrations below 20 μM, very little benzaldehyde was detected. At higher O_2 concentrations, a nearly constant rate of benzaldehyde accumulation occurred. Because total product accumulation increased over this O_2 concentration range, the rate of benzaldehyde oxidation must increase in relation to the increased rate of benzylamine oxidation. Benzoate was the major metabolite under most conditions, and its accumulation had approximately the same O_2 concentration dependence as the total product formation. Hippurate formation was relatively insensitive to O_2 concentration over this range and had a P_{50} value below 5 μM. Addition of 1 mM glycine stimulated hippurate formation to a maximal rate of 2–3 nmoles/10^6 cells per min. Under these conditions, benzoate accumulation was correspondingly lower and the P_{50} value for hippurate formation was somewhat higher (data not shown). Thus, the benzylamine oxidation by monoamine oxidase appears to be the reaction in the pathway which is most sensitive to O_2 deficiency. However, the relative accumulation of products is also determined by the relative rates and O_2 dependences of the subsequent reactions.

To examine this in greater detail, the O_2 dependences of benzaldehyde and benzoate metabolism were studied. Formation of benzoate from added benzaldehyde (2 mM) was more rapid (Fig. 4) than the maximal rate from added benzylamine (see above). The higher rate may be due to a higher benzaldehyde concentration which was needed to maintain linear rates of metabolism for 15 min because of the rapid rate of benzaldehyde evaporation. With this high initial concentration of benzaldehyde, benzyl alcohol formation (about 2 nmoles/10^6 cells per min) was also observed. Consequently, a portion of the benzoate formation may be due to a low affinity system in which benzaldehyde functions as the electron acceptor. With benzylamine as substrate, no benzyl alcohol formation was detected.

The rate of benzoate formation from benzaldehyde was essentially independent of O_2 concentration, and since benzoate was the major product under these conditions, the rate of metabolism was also O_2 independent (Fig. 4). However, hippurate formation (Fig. 4) had an O_2 concentration dependence similar to that found with benzylamine and also that found with 2 mM benzoate (Fig. 5). The P_{50} value for hip-

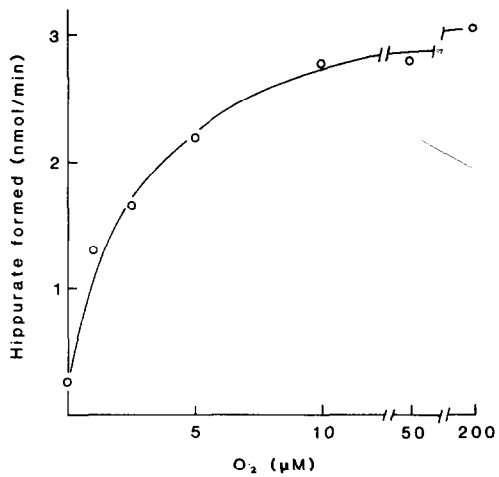

Fig. 5. O_2 dependence of hippurate formation from benzoate in isolated hepatocytes. Cells were incubated and products analyzed as described in Fig. 3, except that benzoate was included instead of benzylamine and 1 mM glycine was also included. Data are from one experiment which is typical of three. Rate of hippurate formation at 200 μM O_2 was 2.9 ± 0.2 nmoles/10^6 cells per min (N = 3).

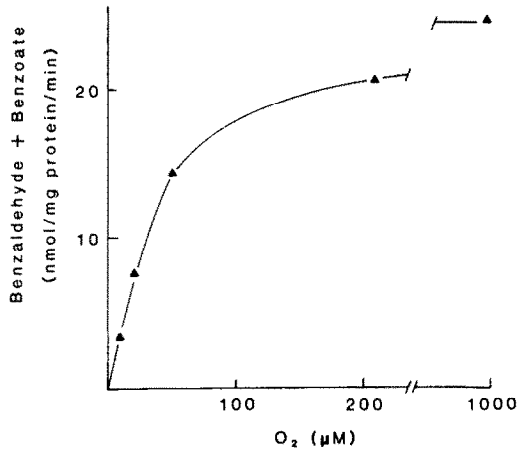

Fig. 6. O_2 dependence of benzylamine metabolism by isolated rat liver mitochondria. Mitochondria were incubated with 5 mM benzylamine at about 1 mg protein/ml in the presence of 0.5 mM ADP and 5 mM succinate. The product formation was principally benzaldehyde; a small and variable fraction of benzoate and hippurate was also formed and is also included in the expression of total products. Data are from one experiment which is typical of three preparations. Rate of product formation at 1 mM O_2 was 25.4 ± 3.1 nmoles per mg protein per min (N = 3).

purate formation was 2.4 ± 0.2 μM O_2 (N = 3) with either benzaldehyde or benzoate as substrate.

In suspensions of isolated rat liver mitochondria, benzylamine was metabolized mostly (>90%) to benzaldehyde, with little accumulation of either benzoate or hippurate. This is in agreement with previous studies which showed that benzaldehyde is metabolized principally by a cytosolic NAD^+-dependent system and that mitochondria oxidize benzaldehyde only at a slow rate. The O_2 dependence of benzylamine oxidation (sum of benzaldehyde, benzoate and hippurate) was similar to that for isolated hepatocytes and had a P_{50} value of 40 μM (Fig. 6).

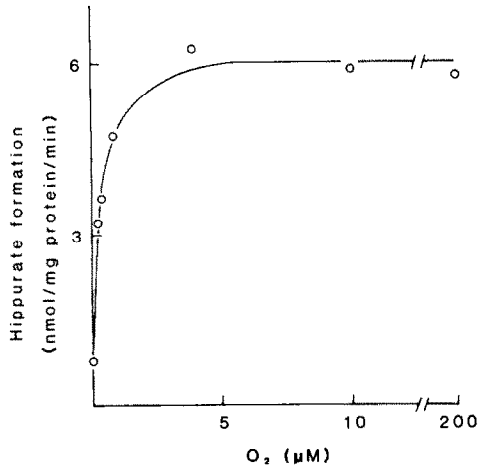

Fig. 7. O_2 dependence of hippurate formation from benzoate by isolated rat liver mitochondria. Mitochondria were incubated as described in Fig. 6 with 2 mM benzoate instead of benzylamine. Data are from one experiment which is typical of three preparations. Rate of hippurate formation at 200 μM O_2 was 6.2 ± 0.4 nmoles per mg protein per min (N = 3).

Benzaldehyde oxidation was unaltered by varying O_2 concentration from 0 to 200 μM in incubations of either isolated mitochondria or post-mitochondrial supernatant solutions with or without NAD^+ (1 mg/ml).

Benzoate conjugation to form hippurate had a P_{50} value below 1 μM O_2 (Fig. 7), a value well below that for isolated hepatocytes. Because of the technical difficulty of obtaining reliable steady-state O_2 concentrations below 1 μM, this value can only be approximated, but appears to be about 0.5 μM.

DISCUSSION

The apparent cellular K_{mO_2} for benzylamine oxidation by isolated hepatocytes occurs within the same O_2 concentration range which is present in liver *in vivo* (average value about 35 μM; [12]). This corresponds to previous studies of the isolated enzyme [4–6] and suggests that therapeutic agents metabolized by monoamine oxidase may also be O_2-dependent *in vivo*. However, this sensitivity to hypoxia is in contrast to the relative insensitivity of benzaldehyde and benzoate metabolism to hypoxia.

Benzaldehyde oxidation may occur by either aldehyde oxidases (i.e. requiring O_2 as electron acceptor) or aldehyde dehydrogenases (i.e. requiring NAD^+ as electron acceptor). The aldehyde oxidase reaction is O_2 dependent because of the requirement for O_2 as substrate. Because the current studies show that the rate of the oxidation in cells, mitochondria and post-mitochondrial supernatant solutions is essentially independent of O_2 concentration, it appears that O_2 is not a direct requirement for benzaldehyde oxidation in liver cells. Consequently, it appears likely that the oxidation is catalyzed by a dehydrogenase such as the NAD^+-dependent enzyme in the cytosolic fraction [8]. If this enzyme is quantitatively important in the metabolism, the lack of inhibition at low O_2 concentrations, in spite of the decreased NAD^+/NADH rates during hypoxia [13], indicates that sufficient NAD^+ is available for the reaction even under anaerobiosis. Under this condition, electrons cannot be transferred to the mitochondrial electron transport chain, and the NAD^+ pool is not sufficient to support the reaction so that other electron acceptors must be involved. Since benzyl alcohol is formed under these conditions, the oxidation of benzaldehyde to benzoate under anaerobic conditions appears to be supported in part by the function of benzaldehyde as an electron acceptor.

The O_2 dependence of hippurate synthesis is likely to be due to the requirement for ATP in the synthesis of benzoyl-CoA. The P_{50} for this reaction in cells is the same as that for drug sulfation [3] and indicates that the intramitochondrial ATP availability is similar to the cytosolic availability under these hypoxic conditions. The lower P_{50} value for hippurate formation in isolated mitochondria corresponds to the lower K_m for mitochondrial respiration [14].

The different O_2 dependences of the reactions of benzylamine metabolism and the corresponding variation in metabolite production indicate that the rela-

tive rates of product formation may be a sensitive indicator of tissue oxygenation. Thus, empirical calibration of product formation *in vivo* might provide a useful non-invasive measure of hypoxia. Compounds that are metabolized in specific organs or tissues may consequently provide tissue-specific measures of oxygenation.

The recent observation that acetaminophen metabolism is increased by administration of O_2 to chronically hypoxic patients (Kaplan *et al.*, unpublished observations) and the current results that benzylamine oxidation is O_2-dependent at physiological O_2 concentrations in hepatocytes indicate that tissue hypoxia may be an important factor in determining metabolism *in vivo*. However, additional *in vitro* experiments with other drugs, as well as more extensive *in vivo* studies, will be required to clarify the specific conditions under which altered drug metabolism during hypoxia is of therapeutic importance.

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