BIOORGANIC CHEMISTRY 9, 273–287 (1980)

Comparative Aspects of Hydroxamic Acid Production by Thiamine-Dependent Enzymes

MICHAEL D. CORBETT AND BERNADETTE R. CHIPKO

Rosenstiel School of Marine and Atmospheric Science, University of Miami, 4600 Rickenbacker Causeway, Miami, Florida 33149

Received March 30, 1979

The reactions of 4-chloronitrosobenzene with pyruvate decarboxylase and transketolase were investigated by use of a new high-pressure liquid chromatography method to determine any differences between these two enzymes with respect to hydroxamic acid production. In addition to the previously established difference in the type of hydroxamic acid produced by the two enzymes, several new and interesting differences in their reaction with nitrosoaromatics were discovered. Most notable was the finding that pyruvate decarboxylase gave 4-chlorophenylhydroxylamine as the major product from 4-chloronitrosobenzene, while transketolase did not produce any detectable hydroxylamine. A redox mechanism was proposed to account for arylhydroxylamine production by pyruvate decarboxylase. This redox mechanism can also explain hydroxamic acid production by pyruvate decarboxylase; however, a previously proposed nucleophilic reaction mechanism occurring simultaneously could not be totally disproven. Either of the two mechanisms is equally likely for transketolase action in view of the present evidence. Another major difference between these enzymes is that the rate of 4-chloronitrosobenzene conversion was found to be much faster for pyruvate decarboxylase than for transketolase when each enzyme was subjected to its own optimal reaction conditions. Transketolase displayed typical enzyme saturation kinetics with 4-chloronitrosobenzene with a K_m of 0.31 mM and V_{max} of 0.033 μmol ml^{-1} min^{-1} unit^{-1} relative to 5 mM D-fructose 6-phosphate as sugar substrate. On the other hand, the reaction with pyruvate decarboxylase was first order in 4-chloronitrosobenzene with a combined rate constant of 2.0 min^{-1} unit^{-1} ml.

INTRODUCTION

In 1974 we reported a novel chemical reaction that resulted in the conversion of aromatic C-nitroso compounds **2** to *N*-arylhydroxamic acids **4** (*1*). This reaction occurred when nitrosobenzene **2a** was reacted with α-hydroxyethylthiamine, an adduct of thiamine considered to be analogous to the "active acetaldehyde" intermediate **1a** of pyruvate decarboxylase (*2*). Subsequently, we demonstrated that the thiamine-dependent enzymes, pyruvate decarboxylase (*3*) and transketolase (*4*), also converted nitrosoaromatics to hydroxamic acids. The mechanism that was proposed at that time (*1*) is totally consistent with Breslow's mechanism for thiamine-catalyzed reactions (*5, 6*), and is depicted in Fig. 1. The hydroxamic acid product resulting from pyruvate decarboxylase is of the *N*-acetyl type **4**, while that resulting from transketolase is of the *N*-glycolyl type **5**. Most recently, we demonstrated the conversion of 4-chloronitrosobenzene **2b** to *N*-(4-

0045-2068/80/030273-15$02.00/0

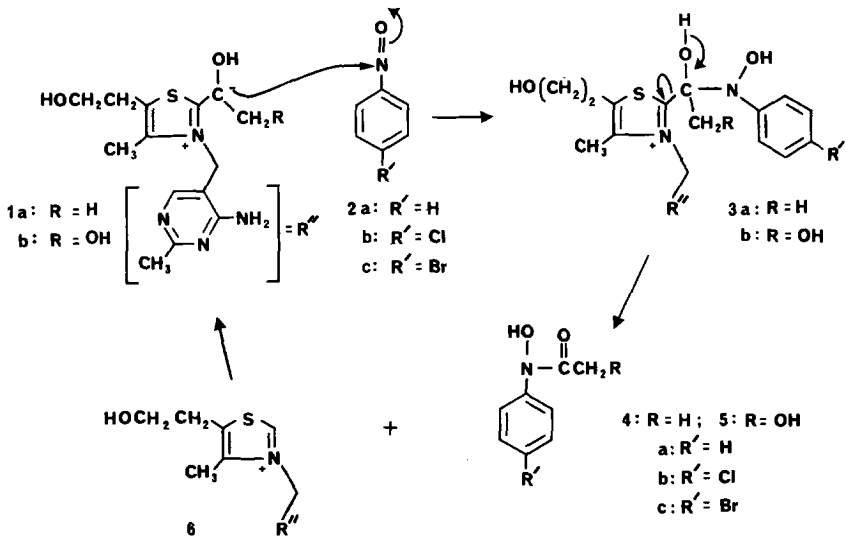

FIG. 1. Nucleophilic mechanism for hydroxamic acid production by the action of thiamine-dependent enzymes on nitrosoaromatics.

chlorophenyl)glycol-hydroxamic acid **5b** by subcellar fractions of rat liver (*7*). Such a conversion of nitrosoaromatics is an expected action of transketolase present in liver homogenates.

We have reinvestigated these enzymatic reactions with a new high-pressure liquid chromatography (hplc) technique developed in our laboratory (*8*). This new analytical method allowed for a direct analysis of incubation mixtures without the need for relatively drastic procedures that included solvent extraction and evaporation. As a result we now report the intermediate formation of an arylhydroxylamine during the reaction of pyruvate decarboxylase with nitrosoaromatics. This observation has required us to partially modify our originally proposed mechanism. Furthermore, pyruvate decarboxylase and transketolase behave in considerably different fashion with respect to arylhydroxylamine formation.

EXPERIMENTAL

Yeast pyruvate decarboxylase, yeast transketolase, thiamine pyrophosphate, sodium pyruvate, xylulose 5-phosphate, and fructose 6-phosphate were obtained from Sigma Chemical Co. 4-Chloronitrosobenzene was prepared as previously described (*9*). All other reagents employed were of ACS-certified purity. The hplc analyses were performed on a system composed of Waters Associates Models 6000A pump, U6K septumless injector, and 440 absorbance detector. The chromatographic column was a Waters μBondapak C_{18} equipped with a guard column containing C_{18} Bondapak. Solvent systems were aqueous methanol mixtures as described under the analytical procedure below.

Prior to use, both the pyruvate decarboxylase and transketolase were treated with thiamine pyrophosphate and Mg^{2+} to insure that the enzymes were fully activated. This was achieved by combining 8 vol of the $(NH_4)_2SO_4$ suspension of yeast pyruvate decarboxylase with 1 vol of 20 m*M* thiamine pyrophosphate and 1 vol of 20 m*M* $MgSO_4$ (*10*), and by dissolving 1 mg (25 units) of crystalline yeast transketolase in 5.0 ml of pH 7.6, 0.05 *M* Tris · HCl containing thiamine pyrophosphate and $MgCl_2$ at 2 m*M* concentrations each. Enzyme activities were determined spectrophotometrically as previously described by pyruvate decarboxylase (*10*) and transketolase (*11*).

Pyruvate decarboxylase incubations. Working solutions of pyruvate decarboxylase were prepared by dilution of the $(NH_4)_2SO_4$ suspension of enzyme containing 2 m*M* thiamine pyrophosphate and 2 m*M* $MgSO_4$ with 0.05 *M*, pH 6.0, KH_2PO_4 buffer such that the enzyme concentration was 3.0 or 0.6 units/ml. Incubations were then conducted by adding 1 vol of one of the enzyme solutions to 9 vol of 0.05 *M* KH_2PO_4 buffer at the appropriate pH, and which contained sufficient sodium pyruvate, thiamine pyrophosphate, $MgSO_4$, and 4-chloronitrosobenzene such that the desired concentrations were attained upon dilution with the enzyme solution. The buffer with cofactors and substrates was equilibrated to 28°C prior to initiation of the reaction by the addition of enzyme. Aliquots of 1.0 ml were taken at timed intervals and quenched by rapid addition to 1.0 ml of methanol precooled to −20°C. Analysis of each aliquot was performed by the injection of 10-μl samples onto the hplc system described below.

A study of the effect of 4-chloronitrosobenzene concentration upon the initial rate of the reaction was conducted over a concentration range of 0.25 m*M* to 0.75 m*M*. The enzyme concentration was 0.06 unit/ml with 0.46 m*M* $MgSO_4$, 0.24 m*M* thiamine pyrophosphate, and 91 m*M* sodium pyruvate in 0.05 *M*, pH 6.0, KH_2PO_4 at 28°C. Aliquots were taken at 30 sec, and the experiment was repeated three times at each substrate concentration.

Studies of the effect of pH, sodium pyruvate concentration, and time course of the reaction were conducted at 28°C by employing the more concentrated working enzyme solution with 0.05 *M* KH_2PO_4 buffer at the appropriate pH with 91 m*M* sodium pyruvate and 0.28 m*M* 4-chloronitrosobenzene. No additional $MgSO_4$ or thiamine pyrophosphate was added to the buffer, since these components present in the more concentrated working enzyme solution gave final concentrations of 0.017 m*M* $MgSO_4$ and 0.017 m*M* thiamine pyrophosphate in the incubations. The study of the effect of sequential addition of 4-chloronitrosobenzene upon the reaction was conducted as for the time course study beginning with 0.28 m*M* substrate followed by addition of sufficient substrate to reestablish this same concentration at 5 and 10 min. The assumption was made that the previously added 4-chloronitrosobenzene had been consumed at these times. The trapping study was conducted in a manner similar to the time course with 0.30 m*M* 4-chloronitrosobenzene; however, 1.5 m*M* aniline was also present in the reaction. Sampling and analysis were conducted in the usual manner over a 30-min incubation period, with particular effort made to detect acetanilide in the reaction product.

Transketolase incubations. The working solution of transketolase was prepared

as described above and contained approximately 5 units/ml of transketolase activity (*11*) and sufficient cofactors. Incubations were then conducted by adding 1 vol of the transketolase solution to 9 vol of 0.10 *M* tris · HCl at the appropriate pH, and which contained either 5 m*M* D-fructose 6-phosphate or 5 m*M* D-xylulose 5-phosphate, and sufficient 4-chloronitrosobenzene such that the desired reaction concentrations were attained upon addition of the enzyme to initiate the reaction. The incubations were carried out at 28°C, and 1.0-ml aliquots were taken at timed intervals, quenched by rapid addition to cold methanol, and analyzed as described below.

A study of the effect of 4-chloronitrosobenzene concentration upon the initial rate of the reaction was conducted over a concentration range of 0.125 to 0.75 m*M*. The enzyme concentration was determined to be 0.42 unit/ml with 0.2 m*M* thiamine pyrophosphate, 0.2 m*M* $MgCl_2$ and 5 m*M* D-fructose 6-phosphate in pH 7.4 (at 28°C), 0.10 *M* Tris · HCl buffer. Aliquots were taken at 2 min and the entire experiment was repeated two times.

Studies of the effect of pH, time course, and substitution of D-xylulose 5-phosphate were conducted at 28°C on 0.25 m*M* 4-chloronitrosobenzene with transketolase activity of about 0.5 unit/ml. Aliquots were taken at 2 min for analysis. A study of the sequential addition of 4-chloronitrosobenzene was performed in a manner similar to that employed for pyruvate decarboxylase.

Analytical method. Each aliquot taken from the above experiments was analyzed for 4-chlorophenylhydroxylamine and either *N*-(4-chlorophenyl)acetohydroxamic acid or *N*-(4-chlorophenyl)glycolhydroxamic acid depending upon the enzyme under investigation. Additional analyses were performed on aliquots from the time-course study to determine the amount of unreacted 4-chloronitrosobenzene. In all cases, 10-μl volumes were injected onto the hplc system and quantitation was achieved by comparison of component peak heights to the peak heights of authentic standards (*8*). All determinations for the hydroxylamine and both hydroxamic acids were achieved on a 30-cm × 3.9-mm i.d. C_{18} μBondapak column employing 0.01% desferal mesylate (Geigy Chemical Co.) in 50% MeOH buffered to pH 3.5 with 0.01 *M* KH_2PO_4 as the solvent at a flow rate of 1.5 ml/min. Absorbance at 254 nm was recorded with the detector output generally at either 0.01 or 0.02 auf depending upon expected peak height. For the quantitative determination of unreacted 4-chloronitrosobenzene the same column was utilized but with the solvent consisting of 80% MeOH at a flow rate of 1.5 ml/min. Absorbance at 313 nm was recorded with detector output ranging from 0.05 to 0.01 auf. All data obtained from this hplc method were converted from nanograms of each component present in the 10-μl injection to either nanomoles or micromoles of each component present in the 1.0-ml aliquot taken from the incubations.

RESULTS

All analyses for the products produced by both pyruvate decarboxylase and transketolase action on 4-chloronitrosobenzene were conducted by hplc methods.

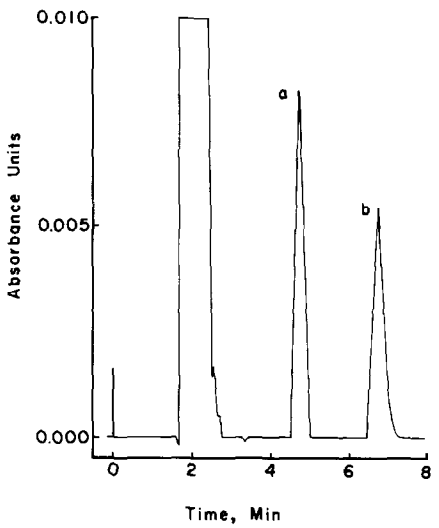

FIG. 2. Typical hplc chromatogram obtained by the use of 0.01% desferal mesylate in pH 3.5, 50% MeOH as solvent on C_{18} μBondapak. (a) 4-Chlorophenylhydroxylamine, **7b**; (b) *N*-(4-chlorophenyl)acetohydroxamic acid, **4b**.

A solvent system consisting of 0.01% desferal mesylate in buffered 50% methanol resulted in retention times of 4.5 min for 4-chlorophenylhydroxylamine **7b**, 5.0 min for *N*-(4-chlorophenyl)glycolhydroxamic acid **5b**, and 6.5 min for *N*-(4-chlorophenyl)acetohydroxamic acid **4b**. With this solvent system the substrate, 4-chloronitrosobenzene **2b**, eluted as a broad peak in 14 min. An example of a typical hplc chromatogram is illustrated in Fig. 2. For studies that required the determination of unreacted 4-chloronitrosobenzene, a second solvent system consisting of 80% methanol enabled rapid and sensitive determination of the substrate, which eluted in 6.5 min. The products of the enzymatic reactions could not be determined with this latter solvent system for previously reported reasons (*8*). Quantitative determination of each of the products and of unreacted substrate was achieved by measurement of peak heights and comparison to peak heights of authentic standards chromatographed under identical conditions. The identification of 4-chlorophenylhydroxylamine as a product in pyruvate decarboxylase incubations was confirmed by combining hplc eluates containing the peak, and then comparing the uv spectrum of this product to that of authentic 4-chlorophenylhydroxylamine. The identification of the hydroxamic acid products from these enzymatic incubations has been previously reported (*3*, *4*).

In the absence of pyruvate decarboxylase, 4-chloronitrosobenzene was stable in the presence of a large excess of sodium pyruvate over a broad pH range of from 5.0 to 7.2. Upon addition of pyruvate decarboxylase a rapid reaction occurred with the production of 4-chlorophenylhydroxylamine **7b** and *N*-(4-chlorophenyl)acetohydroxamic acid **4b**. The course of this reaction under optimal conditions is illustrated in Fig. 3. The most notable aspect of this reaction was the fact that hydroxylamine formation was more rapid than hydroxamic acid produc-

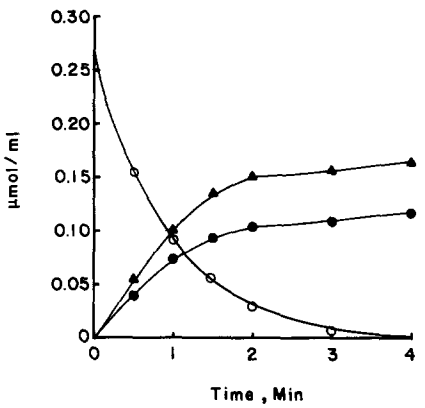

FIG. 3. The reaction of 4-chloronitrosobenzene with pyruvic acid and pyruvate decarboxylase as a function of time. The incubation was conducted in 10 ml of pH 6.0, 0.10 *M* KH_2PO_4 containing 0.91 mmol sodium pyruvate, 2.8 µmol 4-chloronitrosobenzene, 0.17 µmol $MgSO_4$, 0.17 µmol thiamine pyrophosphate, and 3 units of pyruvate decarboxylase. Aliquots of 1.0 ml each were quenched with methanol at the indicated time and analyzed as described under Experimental. 4-Chloronitrosobenzene (○); *N*-(4-chlorophenyl)acetohydroxamic acid (●); 4-chlorophenylhydroxylamine (▲).

tion. At each sampling time during the reaction the sum on a molar basis of the nitroso substrate, hydroxylamine, and hydroxamic acid was consistently found to be between 95 and 100%. This feature of substrate accountability was found to be characteristic of the pyruvate decarboxylase reaction regardless of the conditions

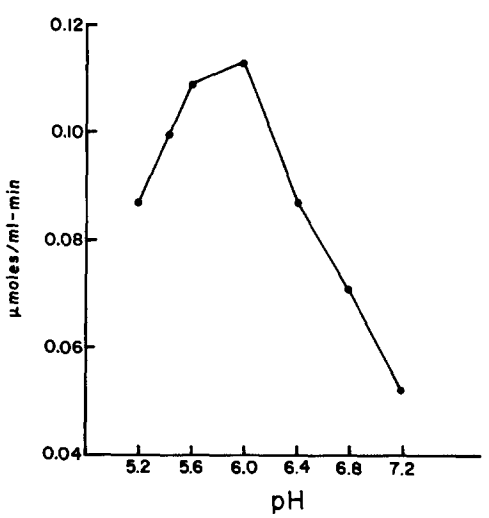

FIG. 4. The effect of pH upon the initial rate of reaction of 4-chloronitrosobenzene with pyruvic acid and pyruvate decarboxylase. Incubations were conducted in 2.0 ml of 0.05 *M* KH_2PO_4 at the indicated pH and which contained 0.18 mmol sodium pyruvate, 0.56 µmol 4-chloronitrosobenzene, 0.034 µmol $MgSO_4$, 0.034 µmol thiamine pyrophosphate, and 0.6 unit of pyruvate decarboxylase. A 1.0-ml aliquot was quenched with methanol after 30 sec incubation time and analyzed as described under Experimental. The curve represents the molar sum of the rates of formation of 4-chlorophenylhydroxylamine and *N*-(4-chlorophenyl)acetohydroxamic acid.

employed. At no time were any additional products, including the azoxy product, detected in this reaction.

To determine the possibility that the hydroxylamine might be produced by chemical or enzymatic hydrolysis of the hydroxamic acid product, the hydroxamic acid was incubated with pyruvate decarboxylase under the usual conditions, but in the absence of 4-chloronitrosobenzene. The hydroxamic acid was completely stable to such conditions, as neither the hydroxylamine nor the nitroso compound could be detected at any time during this incubation. The hplc analysis also indicated no detectable change in hydroxamic acid concentration throughout an incubation period of 1 hr.

The rate of hydroxylamine and hydroxamic acid production was directly related to the amount of enzyme employed in the reaction. The rate was independent of sodium pyruvate concentration under the usual reaction conditions, but did become directly related to pyruvate concentration when the latter was decreased below 50 m*M*. The pH profile (Fig. 4) for the pyruvate decarboxylase reaction paralleled that known for the usual metabolic function of this enzyme, which has an optimal pH of 6.0 (12). A kinetic analysis of the pyruvate decarboxylase reaction with 4-chloronitrosobenzene at the optimal pH and pyruvate concentration indicated that saturation of the enzyme by the nitroso substrate did not occur up to the maximal allowable reaction concentration of 4-chloronitrosobenzene. The solubility of the nitroso substrate limited studies to a maximum concentration of 0.8 m*M*. Up to this concentration the enzymatic reaction was linear with respect to nitroso concentration. The second-order rate constants were computed to be 1.4 ± 0.1 min^{-1} $unit^{-1}$ ml for hydroxylamine formation and 0.6 ± 0.07 min^{-1} $unit^{-1}$ ml for hydroxamic acid formation.

A trapping experiment was conducted under the conditions described in Fig. 3, except that 1.5 m*M* aniline was present in the incubation mixture. At no time during the incubation period was acetanilide detected by the hplc method (*8*). Attempts to use a synthetically prepared arylhydroxylamine as a trapping agent were made; however, it was found that upon mixing 4-chloronitrosobenzene with 4-bromophenylhydroxylamine in the absence of enzyme, a rapid metathetical chemical reaction occurred. Thus, any hplc observation of *N*-(4-bromophenyl)acetohydroxamic acid in an enzymatic competition experiment utilizing 4-chloronitrosobenzene as substrate and 4-bromophenylhydroxylamine as competitor would not be conclusive.

The stability of pyruvate decarboxylase to 4-chloronitrosobenzene was investigated by employing the technique of sequential addition of nitroso substrate to an enzymatic incubation. Assay for pyruvate decarboxylase activity by a standard method (*10*) was not considered to be applicable since the effects of the nitroso substrate and its products on standard assay conditions are unknown. Figure 5 indicates that pyruvate decarboxylase was stable in the presence of the nitroso substrate and its products, at least initially and for concentrations totaling less than 0.5 m*M*. The rate of enzymatic conversion following the third addition of 4-chloronitrosobenzene was obviously less than the initial rate of reaction (Fig. 5).

The time-course plots of the pyruvate decarboxylase reaction with 4-chloronitrosobenzene illustrated by Figs. 3 and 5 are typical of enzymatic reactions in

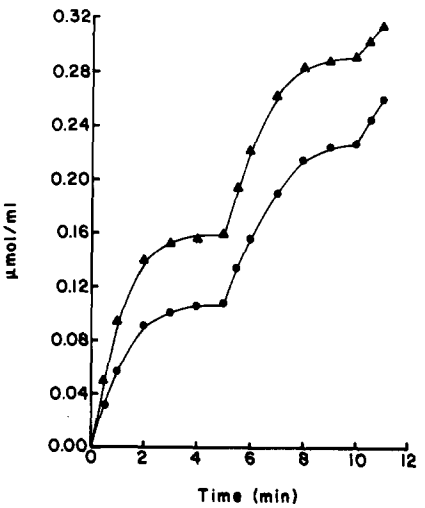

FIG. 5. The effect of sequential addition of 4-chloronitrosobenzene upon pyruvate decarboxylase. The incubation was conducted in the same manner as for the time course study except that at $t = 5$ and 10 min additional 4-chloronitrosobenzene was added to reestablish the concentration of this substrate at 0.28 m*M*. *N*-(4-Chlorophenyl)acetohydroxamic acid (●); 4-chlorophenylhydroxylamine (▲).

which substrate depletion has occurred. Under the conditions employed the reactions were complete within 5 min. Total consumption of nitroso substrate had occurred at this time with a resultant production of *N*-(4-chlorophenyl)acetohydroxamic acid in yields of 35–40% and 4-chlorophenylhydroxylamine production accounting for the remainder of the original substrate.

In the absence of transketolase, 4-chloronitrosobenzene was found to be stable in the presence of pH 7.4 Tris · HCl buffer containing D-fructose 6-phosphate, $MgCl_2$, and thiamine pyrophosphate. Upon addition of transketolase the conver-

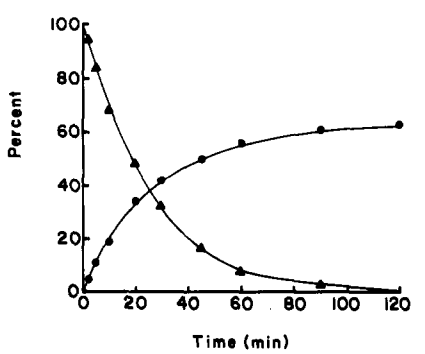

FIG. 6. The reaction of 4-chloronitrosobenzene with D-fructose 6-phosphate and transketolase as a function of time. The incubation was conducted in 10 ml of pH 7.4, 0.10 *M* Tris · HCl containing 50 µmol fructose 6-phosphate, 2.5 µmol 4-chloronitrosobenzene, 2.0 µmol $MgCl_2$, 2.0 µmol thiamine pyrophosphate, and 5 units of transketolase. Aliquots of 1.0 ml each were quenched with methanol at the indicated time and analyzed as described under Experimental. 4-Chloronitrosobenzene (▲); *N*-(4-chlorophenyl)glycolhydroxamic acid (●).

sion of nitroso substrate to *N*-(4-chlorophenyl)glycolhydroxamic acid was readily followed by hplc analysis. No trace of 4-chlorophenylhydroxylamine was detected under any of the conditions employed for transketolase incubations. The substitution of D-xylulose 5-phosphate for fructose 6-phosphate gave similar results, except that the rate of hydroxamic acid production was approximately twice as fast. All further studies were conducted with D-fructose 6-phosphate, because of the expense and limited purity of commercially available D-xylulose 5-phosphate.

The course of the irreversible reaction of 4-chloronitrosobenzene and transketolase with fructose 6-phosphate as the sugar substrate is illustrated in Fig. 6. Like the case for pyruvate decarboxylase, the sequential addition of nitroso substrate indicated that transketolase was stable to the usual reaction concentrations of 4-chloronitrosobenzene. The final percentage conversion of nitroso substrate to glycolhydroxamic acid was consistently found to be about 60–65% regardless of the amount of nitrosoaromatic initially added to the enzymatic incubation. We have been unable to determine the fate of the remaining 35–40% of nitroso substrate.

In addition to studies that eliminated the possibility that 4-chloronitrosobenzene might undergo chemical reactions with the keto-sugar substrates, it was also demonstrated that the nitroso compound was stable in the presence of glyceraldehyde 3-phosphate, erythrose 4-phosphate, and ribose 5-phosphate, which are expected products from the normal reactions of transketolase. It occurred to us that a possible reaction between either the keto-sugar substrates or aldose products with 4-chlorophenylhydroxylamine might account for both the failure to observe hydroxylamine formation and also the failure to obtain a material balance. Subsequently, hplc analysis was employed to determine the possible reaction products between 4-chlorophenylhydroxylamine and each of these sugars in Tris · HCl buffer. The only reaction that was observed was the oxidation of the hydroxylamine to 4-chloronitrosobenzene, and this process was nearly quantitative. No other products were detected in these reactions and we concluded that it was not chemical reactions of the hydroxylamine that prevented the attainment of a material balance. Furthermore, the oxidation of the hydroxylamine to the nitroso group did not occur rapidly enough with any of the sugars to explain the absence of 4-chlorophenylhydroxylamine production during the reaction of 4-chloronitrosobenzene with transketolase.

In a final attempt to detect hydroxylamine production by transketolase, the reaction was investigated over the pH range of 6.2 to 8.5. The optimal pH for hydroxamic acid production (Fig. 7) corresponds to the pH optimum of transketolase in normal biochemical reactions (*13*). A 30% inhibition of hydroxamic acid production was observed when a phosphate buffer was employed in place of the usual Tris buffer. This is in agreement with the known inhibition of phosphate buffers on transketolase activity (*13*). For all pH values investigated in the two different buffer systems, no 4-chlorophenylhydroxylamine was detected.

Unlike pyruvate decarboxylase, transketolase did display saturation kinetics with 4-chloronitrosobenzene before the solubility limit of this substrate was reached (Fig. 8). The usual double-reciprocal plot of the data in Fig. 8 indicated

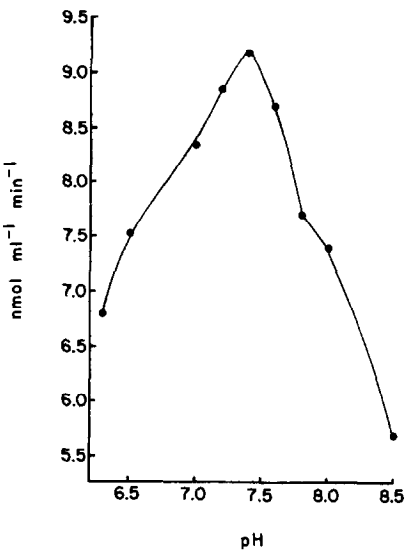

FIG. 7. The effect of pH upon the initial rate of reaction of 4-chloronitrosobenzene with D-fructose 6-phosphate and transketolase. Incubations were conducted in 2.0 ml of 0.10 *M* Tris · HCl at the indicated pH and which contained 10 μmol fructose 6-phosphate, 0.5 μmol 4-chloronitrosobenzene, 0.4 μmol $MgCl_2$, 0.4 μmol thiamine pyrophosphate, and 1 unit of transketolase. A 1.0-ml aliquot was quenched with methanol after 2.0 min incubation time and analyzed as described under Experimental. The curve represents the amount of *N*-(4-chlorophenyl)glycolhydroxamic acid produced in 1.0 min.

the K_m for 4-chloronitrosobenzene relative to fructose 6-phosphate to be 0.31 m*M* and the V_{max} to be 0.014 μmol min^{-1} ml^{-1} with a transketolase activity of 0.42 unit/ml. The turnover number for 4-chloronitrosobenzene by transketolase was computed to be 111 min^{-1} under the conditions employed.

DISCUSSION

Our previous investigations of the enzymatic conversion of nitrosoaromatics to hydroxamic acids were performed with tlc and spectrophotometric methods (*3, 4*). Until the present study we had been unable to utilize hplc as a quantitative tool for hydroxamic acid analysis because of the pronounced tailing of these compounds on hplc columns of all types. Very recently we discovered that the addition of desferal mesylate to the chromatographic solvent system eliminated chemisorption-induced tailing of the hydroxamic acid peaks (*8*). This new chromatographic system allows for the quantitation of aromatic hydroxamic acids at concentrations of at least 1 μg/ml in enzymatic reaction mixtures. This high degree of sensitivity eliminated the need for solvent extraction and evaporation steps in the workup procedure. As a result we were able to elucidate more accurately the interaction of thiamine-dependent enzymes with nitrosoaromatics.

In the present study 4-chloronitrosobenzene was employed as a model aromatic nitroso compound with which to investigate the enzymatic reactions. Qualita-

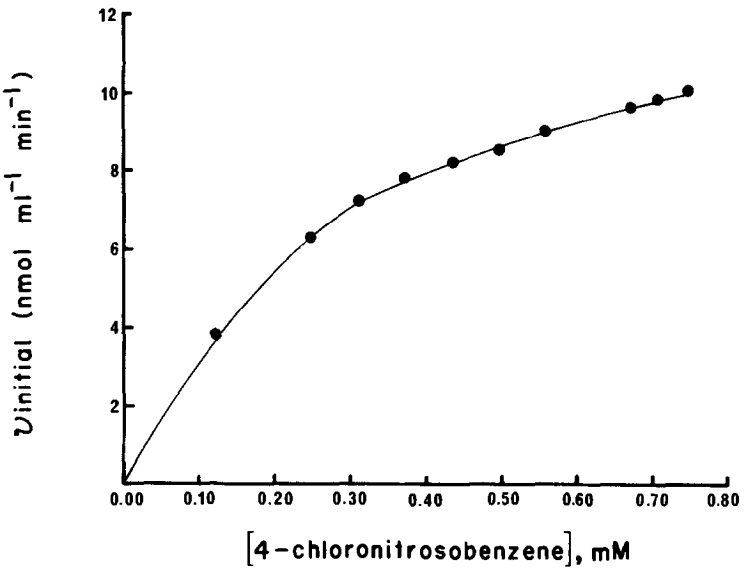

FIG. 8. The effect of substrate concentration upon the initial rate of conversion of 4-chloronitrosobenzene to *N*-(4-chlorophenyl)glycolhydroxamic acid by transketolase. Incubations were conducted in 2.0 ml of pH 7.4, 0.10 *M* Tris · HCl containing the indicated concentration of 4-chloronitrosobenzene, 10 μmol fructose 6-phosphate, 0.4 μmol $MgCl_2$, 0.4 μmol thiamine pyrophosphate, and 0.84 unit of transketolase. A 1.0-ml aliquot was quenched with methanol after 2.0 min incubation time and analyzed as described under Experimental. Each datum point is the average of three individual experiments.

tively identical results were obtained with nitrosobenzene, 4-bromonitrosobenzene, and 4-methylnitrosobenzene. For quantitative investigations the 4-chloro analog was chosen as the model compound on the basis of its physical properties. 4-Chloronitrosobenzene is substantially less volatile than nitrosobenzene, a property that results in minimal losses during its incubation in aqueous buffers. On the other hand, 4-chloronitrosobenzene is sufficiently soluble in aqueous buffers to allow for investigations to be conducted in reasonably high concentrations.

The most significant discovery was the observation of 4-chlorophenylhydroxylamine as the major product from the action of pyruvate decarboxylase on the nitroso substrate. This discovery and the fact that no hydroxylamine was produced during transketolase incubations constitute a major difference between these enzymes.

Since the mechanism proposed in Fig. 1 cannot account for the observed arylhydroxylamine formation by pyruvate decarboxylase, we now feel that a more likely mechanism is one involving a redox interaction between the nitroso functional group and the active acetaldehyde intermediate **1a**. Such a mechanism is presented in Fig. 9. This interaction results in the production of the hydroxylamine functional group in close proximity to oxidized enzyme intermediate **8a**. This oxidized intermediate has been previously proposed to be formed by inorganic oxidants (*14*) and is known to be a good acylating intermediate (*15*). Transfer of the acyl group to the nearby hydroxylamine **7** would result in the

hydroxamic acid **4**, while simple hydrolysis would allow the hydroxylamine **7** to be released into the solution. In view of our observation that the hydroxylamine is the predominant product of the enzymatic reaction, it is apparent that simple hydrolysis of the oxidized intermediate **8a** to acetic acid is the predominant pathway.

In spite of the fact that the redox mechanism of Fig. 9 can account for the observed products, we could not eliminate the possibility that a simultaneous but slower nucleophilic process, shown in Fig. 1, was also occurring to produce hydroxamic acid. We attempted to trap the oxidized intermediate **8a** with a suitable nucleophile, such as aniline, which would produce a readily detectable product. Our inability to detect acetanilide in such pyruvate decarboxylase incubations suggests either that the hydroxylamine is a much stronger nucleophile than aniline, or that the proximity of the newly generated hydroxylamine **7b** to the oxidized intermediate **8a** at the enzyme active site precludes any possible competition by aniline. Although this attempt to prove the intermediacy of the oxidized intermediate **8a** failed, we still feel that the mechanism in Fig. 9 must be the dominant, if not exclusive, pathway for the reaction between nitrosoaromatics and pyruvate decarboxylase, since it readily accounts for both products. The nucleophilic mechanism in Fig. 1 cannot account for hydroxylamine production.

The redox mechanism in Fig. 9 is similar to enzymatic reaction mechanisms proposed by Christen (*16*). Both pyruvate decarboxylase (*17*) and transketolase (*18*) have been shown to undergo paracatalytic enzyme modification upon treatment with certain inorganic oxidants and redox indicator dyes. It has been proposed that the oxidation of intermediates, such as active acetaldehyde **1a** or active glycolaldehyde **1b**, will produce reactive intermediates **8a** and **8b** at the enzyme active site, which can then acylate nearby groupings and induce a modification in the enzyme. The important parallel to our work is the proposed

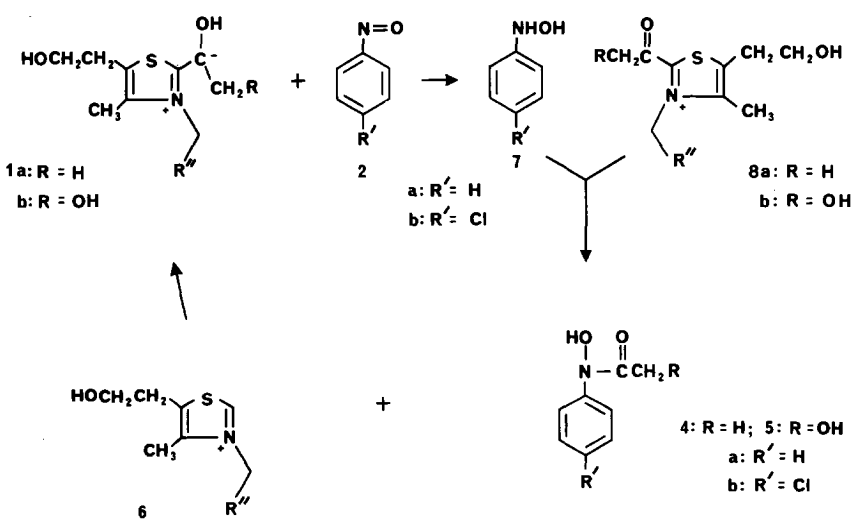

FIG. 9. Redox mechanism for hydroxamic acid and hydroxylamine production by the action of thiamine-dependent enzymes on nitrosoaromatics.

oxidized enzyme intermediates such as **8a** and **8b**. Unlike all other oxidants employed in such studies, the reduced (hydroxylamine) form of the nitroso oxidant can itself react with the oxidized enzyme intermediate **8** giving rise to hydroxamic acids. We have not pursued the possibility that our reaction might induce paracatalytic modification of these thiamine-dependent enzymes, since our interest has been on the mechanism for the production of potentially toxic hydroxamic acids. However, Fig. 5 illustrates the relative stability of pyruvate decarboxylase during sequential treatments with 4-chloronitrosobenzene. In spite of the potential for nitroso compounds to induce enzymatic denaturation through reaction with sulfhydryl groups (*19*), the pyruvate decarboxylase activity, as indicated by hydroxylamine and hydroxamic acid production in Fig. 5, was surprisingly stable up to the third addition of 4-chloronitrosobenzene. Before the observed decrease in enzyme activity apparent in Fig. 5 could be attributed to paracatalytic modification, some rather rigid conditions must be proven (*16*).

The production of 4-chlorophenylhydroxylamine by pyruvate decarboxylase was not a result of simple hydrolysis of the hydroxamic acid product, since the latter was found to be completely stable to the incubation conditions. This finding also proved that this enzymatic reaction is irreversible. Similar studies with transketolase also proved that hydroxamic acid production was irreversible for that enzyme.

Our inability to detect the transketolase-catalyzed conversion of nitrosoaromatics to the hydroxylamine at the 1% limit of detection suggests that the nucleophilic mechanism presented in Fig. 1 is operative for this enzyme as originally proposed. Unfortunately, the redox mechanism presented in Fig. 9 cannot be ruled out for transketolase, since it is possible that following the redox reaction, a very rapid reaction occurs between the arylhydroxylamine **7** and active glycolate **8b** to give the glycolhydroxamic acid **5**. Unlike pyruvate decarboxylase, perhaps there exists no opportunity for the active site-generated hydroxylamine to escape from transketolase in detectable quantities. This latter possibility and the possibility that the two enzymes produce hydroxamic acids by totally different mechanisms pose a question that may remain unanswered for some time, as we have been unable to design an experiment that would prove either of these possibilities.

An analysis of the kinetics with which these two enzymes react with 4-chloronitrosobenzene resulted in the discovery of another major difference between these enzymes. On a normalized basis pyruvate decarboxylase is much more reactive toward nitroso substrates than is transketolase.

There is strong evidence that the rate-limiting step for the normal biochemical reaction of pyruvate decarboxylase is the final step, that is, dissociation of acetaldehyde from the enzyme (*20*). We suspect that 4-chloronitrosobenzene might facilitate this dissociation of acetaldehyde from the enzyme by either of the potential mechanisms, although the redox mechanism (Fig. 9) seems most likely. Oxidation of active acetaldehyde **1a** to an intermediate such as **8a** by a nitrosoaromatic is likely. Hydrolysis of an "abnormal" enzyme intermediate such as **8a** would be expected to occur much more rapidly than hydrolysis of active acetaldehyde **1a** (*21, 22*). In fact, **8a** has been referred to as active acetate and is

known to be a high-energy compound (*15, 23*). An analysis of our rate data shows the sum of the second-order rate constants, based on enzyme units, is 2.0 ± 0.2 min^{-1} $unit^{-1}$ ml for the combined processes of hydroxylamine and hydroxamic acid production by pyruvate decarboxylase. Although the conditions employed in the determination of these constants cannot be identical to those employed to define a unit of pyruvate decarboxylase activity (10), we feel that this sum of the constants suggests the possibility that nitrosoaromatics act to increase the rate of this last step and thus to increase the overall rate of pyruvate turnover by pyruvate decarboxylase. This possibility is under investigation.

Transketolase reacted relatively slowly with 4-chloronitrosobenzene. The turnover number computed for this nitroso substrate was an order of magnitude lower than the turnover numbers for the normal biochemical reactions catalyzed by transketolase (*13*).

The reactions of pyruvate decarboxylase and transketolase with nitrosoaromatics are similar in that optimal reactivity is achieved under conditions known to be optimal for the normal function of each enzyme. This is best illustrated by the pH dependency studies. The differences between these enzymes, aside from the type of hydroxamic acid produced, are much more interesting. The rate with which each enzyme reacts with 4-chloronitrosobenzene compared to the potential rate of interaction differs considerably for the two enzymes. The most intriguing difference between pyruvate decarboxylase and transketolase is the possibility that these enzymes interact with nitrosoaromatics by totally different mechanisms.

ACKNOWLEDGMENTS

This work was supported by Grant CA 21992 from the National Cancer Institute, and by Research Career Development Award ES 00038 to M.D.C. from the National Institute of Environmental Health Sciences, DHEW.

REFERENCES

1. M. D. CORBETT, *Bioorg. Chem.* **3**, 361 (1974).
2. H. W. GOEDDE, H. INOUYE, AND H. HOLZER, *Biochim. Biophys. Acta* **50**, 41 (1961).
3. M. D. CORBETT, J. E. CAHOY, AND B. R. CHIPKO, *J. Nat. Cancer Inst.* **55**, 1247 (1975).
4. M. D. CORBETT AND B. R. CHIPKO, *Biochem. J.* **165**, 263 (1977).
5. R. BRESLOW, *J. Amer. Chem. Soc.* **80**, 3719 (1958).
6. J. ULLRICH, Y. M. OSTROVSKY, J. EYZAGUIRRE, AND H. HOLZER, *Vitam. Horm.* **17**, 365 (1970).
7. D. G. BADEN, M. D. CORBETT, AND B. R. CHIPKO, *Bioorg. Chem.* **9**, 231–237 (1980).
8. M. D. CORBETT AND B. R. CHIPKO, *Anal. Biochem.* **98**, 169 (1979).
9. M. D. CORBETT, B. R. CHIPKO, AND D. G. BADEN, *Biochem. J.* **175**, 353 (1978).
10. J. ULLRICH, "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, Eds.), Vol. 18a, pp. 112–113. Academic Press, New York, 1970.
11. S. SAITOU, T. OZAWA, AND I. TOMITA, *FEBS Lett.* **40**, 114 (1974).
12. D. E. GREEN, D. HERBERT, AND V. SUBRAHMANYAN, *J. Biol. Chem.* **138**, 327 (1941).
13. E. RACKER, "The Enzymes" (P. D. Boyer, H. Lardy, and R. Myrbäck, Eds.); Vol. 5, pp. 397–406. Academic Press, New York, 1961.

14. L. O. KRAMPITZ, I. SUZUKI, AND G. GREULL, *Fed. Proc.* **20,** 971 (1961).
15. C. P. NASH, C. W. OLSEN, F. G. WHITE, AND L. L. INGRAHAM, *J. Amer. Chem. Soc.* **83,** 4106 (1961).
16. P. CHRISTEN, "Methods in Enzymology," (W. B. Jakoby and M. Wilchek, Eds.), Vol. 46, pp. 48–54. Academic Press, New York, 1977.
17. M. J. HEALY AND P. CHRISTEN, *Biochemistry* **12,** 35 (1973).
18. P. CHRISTEN, M. COGOLI-GREUTER, M. J. HEALY, AND D. LUBINI, *Eur. J. Biochem.* **63,** 223 (1976).
19. P. EYER, *Chem. Biol. Interact.* **24,** 227 (1979).
20. M. H. O'LEARY, *Biochem. Biophys. Res. Commun.* **73,** 614 (1976).
21. K. DAIGO AND L. J. REED, *J. Amer. Chem. Soc.* **84,** 659 (1962).
22. G. E. LIENHARD, *J. Amer. Chem. Soc.* **88,** 5642 (1966).
23. R. BRESLOW, *Ann. N.Y. Acad. Sci.* **98,** 445 (1962).