On the Mechanism of the Pepsin-Catalyzed Hydrolysis of Sulfite Esters*

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ABSTRACT: To monitor the rate of inorganic sulfite formation during the course of the pepsin-catalyzed hydrolysis of diphenyl sulfite (DPS) and bis(*p*-nitrophenyl) sulfite (BNPS), we have employed *o*-chloranil (OCL) as a trapping agent. From a Lineweaver–Burk plot of data obtained at various initial substrate concentrations using OCL to trap the inorganic sulfite produced, a k_{cat} value of 5×10^{-2} sec^{-1} was measured at pH 2 and 25° for the hydrolysis of DPS catalyzed by pepsin. This was in reasonable agreement with the k_{cat} value of 4.3×10^{-2} sec^{-1} measured at the same pH by monitoring the release of phenol in the absence of OCL. Thus, under turnover conditions, as anticipated, there is no gross difference between the rates of phenol and inorganic sulfite liberation in the pepsin-catalyzed hydrolysis of DPS. In the presence of OCL, inorganic sulfite production in the pepsin-catalyzed hydrolysis of BNPS can be observed spectrophotometrically in the 420-nm region, while *p*-nitrophenol formation can be followed at 312.5 nm. Experiments at pH 1.5 in 10.3% CH_3CN performed on a stopped-flow spectrophotometer, using enzyme in excess over the BNPS concentration, gave identical values for the rates of *p*-nitrophenol and inorganic sulfite formation. This demonstrates that there is no accumulation of a covalent intermediate produced by the reaction of BNPS with pepsin in which preferential release of either *p*-nitrophenol or inorganic sulfite has occurred. Although we have been unable to obtain direct evidence for the existence of a covalent intermediate species in the pepsin-catalyzed hydrolysis of sulfite esters, the apparent inequality of the Michaelis constant for DPS hydrolysis with the constant measured for inhibition by DPS of enzymatic BNPS hydrolysis and the complexity of the k_{cat} *vs.* pH profile for BNPS hydrolysis represent indirect lines of evidence for the occurrence of such an intermediate. Furthermore, ^{18}O-labeling experiments described in the literature are consistent with the postulation of a covalent intermediate. Accordingly, as a working hypothesis, we are proposing a mechanism involving the production of covalent intermediates, in which an active-site carboxylate group in pepsin acts as a nucleophile, attacking the sulfur of the sulfite ester, and another active-site carboxyl group (in the protonated form) functions as a general acid catalyst assisting this reaction.

Several detailed mechanisms have been proposed for the reactions of pepsin with peptide substrates (Zeffren and Kaiser, 1967; Clement *et al.*, 1968; Hollands and Fruton, 1969; Knowles, 1970; Silver *et al.*, 1970). In contrast, there has been a dearth of mechanistic proposals for the pepsin-catalyzed hydrolysis of sulfite esters, due, in large measure, to the absence of information regarding the pH dependence of kinetic parameters, the meaning of the Michaelis constants, K_m, the rate of release of inorganic sulfite, and other such mechanistically significant points. Moreover, the relationship between the peptidase and sulfite esterase activities of pepsin has been somewhat unclear, especially since these two classes of substrates are structurally dissimilar.

Previous studies in this laboratory (May and Kaiser, 1969, 1971; Zeffren and Kaiser, 1968) have been mainly concerned with elucidating the kinetic behavior and pH dependence of the pepsin-catalyzed hydrolysis of DPS and BNPS.[1] Although such studies have provided interesting results, additional information is needed in order to formulate a detailed mechanism for these reactions. In particular, some knowledge of the nature of the intermediates formed during the course of such reactions is required. To this end, we have carried out a series of studies in this laboratory, the results of which are reported herein.

Experimental Section

Pepsin, twice recrystallized and lyophilized, was purchased from Worthington Biochemical Corp. (Lot No. PM-8BE, PM-718, PM-8JC, and PM-OAB). Enzyme solutions were prepared as described previously (May and Kaiser, 1971). Similarly, acetonitrile, deionized water, and stock solutions of DPS or BNPS were obtained or prepared as described elsewhere (May and Kaiser, 1969). Inorganic salts were the commercially available reagent grade materials, as were the organic solvents. Highly purified thionyl chloride was purchased from Matheson, and usually distilled successively from quinoline and linseed oil before use. Tetrachloro-*o*-benzoquinone (*o*-chloranil) was recrystallized four times from carbon tetrachloride and vacuum dried (mp 126.5–127.5). Tetrachlorocatechol monohydrate (Aldrich Chemical Co.) was recrystallized from benzene (under nitrogen) and dried under vacuum for 2 hr at 110° (mp 193–194°).

pH measurements were taken on a Radiometer pH meter, Model 4C, using various combined, concentric calomel glass electrodes. The meter was standardized with an appropriate standard buffer (Fisher Certified) before each determination. All melting points were taken on a Thomas-Hoover-type capillary apparatus and are uncorrected.

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[1] Abbreviations used are: diphenyl sulfite, DPS; bis(*p*-nitrophenyl) sulfite, BNPS; *o*-chloranil, OCL;c arbobenzoxy, Z; 2-(*N*-morpholinoethanesulfonic acid, MES.

Syntheses. The preparation of BNPS has been described previously (May and Kaiser, 1969).

DIPHENYL SULFITE (DPS). To 500 ml of dry ether was added 151 g (1.5 moles) of phenol (recrystallized from petroleum ether, 30–60°) and 121 ml (1.5 moles) of pyridine, and the solution was cooled to 0° in an ice–salt bath. Highly purified thionyl chloride (54 ml, 0.75 mole) (Matheson) dissolved in 60 ml of ether was slowly added to the cold, stirred solution over 1.5 hr. Stirring was continued at 0° for an additional 45 min, after which the solution was brought to room temperature. The precipitate of pyridine hydrochloride was filtered and washed with ether. The combined washings and filtrate were washed twice with water, dried over anhydrous Na_2SO_4, and concentrated on a rotary evaporator, leaving a pale yellow oil. Distillation gave 125 g of pure diphenyl sulfite: bp 142–144° (4 mm) (lit. (Bunton and Schwerin, 1966) bp 160, 8 mm). The infrared (ir) spectrum showed the characteristic sulfite absorption band at 8.6 μ.

Inorganic Sulfite Trapping Studies. PRELIMINARY STUDIES. Preliminary studies on the reaction of *o*-chloranil with Na_2SO_3 were performed on a Cary 15 spectrophotometer to determine whether the reaction is fast, stoichiometric, and accompanied by a large absorbance change at a convenient wavelength, so that it could be used to monitor the formation of inorganic sulfite in the hydrolysis of sulfite esters. In a typical experiment, 300 μl of acetonitrile was added to 3 ml of buffer or enzyme solution (pH 2.0) in a spectrophotometer cuvette. Then 25 μl of a 0.0281 M solution of *o*-chloranil in acetonitrile was added, and the spectrum of the resulting solution was recorded. Finally, 5 μl of a 0.0204 M aqueous Na_2SO_3 solution was added, and the spectrum recorded immediately. Similar experiments were performed using various amounts of Na_2SO_3 corresponding to 5–300% of the concentration of *o*-chloranil and in the presence of various amounts of pepsin. In all cases where *o*-chloranil was in excess the addition of Na_2SO_3 caused an immediate decrease in absorbance by the amount expected from reaction of an exactly stoichiometric amount of *o*-chloranil. When the *o*-chloranil was completely reduced by addition of a slight excess of Na_2SO_3, the spectrum of the final solution was identical with that of an authentic sample of tetrachlorocatechol. Pertinent spectral data at pH 2 are as follows: *o*-chloranil, $\epsilon_{350} = 2466$, $\epsilon_{390} = 1417$, $\epsilon_{410} = 983$; tetrachlorocatechol, λ_{max} 297 nm (ϵ_{max} 2200). The latter does not absorb above 340 nm. The isosbestic points were 300, 294, and 242 nm. Each of the above values is the average of at least eight determinations.

Kinetics of the Trapping Reaction. The kinetics of the reaction of *o*-chloranil with Na_2SO_3 in acid solution were followed spectrophotometrically using a Durrum-Gibson stopped-flow spectrophotometer. In a typical experiment, a mixture of 5 ml of pH 2 phosphate buffer plus 100 μl of a 2×10^{-3} M aqueous solution of Na_2SO_3 was allowed to react with a mixture of 5 ml of 0.01 N HCl, 1.25 ml of acetonitrile, 50 μl of 2 N HCl, and 100 μl of a 0.0317 M stock solution of *o*-chloranil. The solutions were charged into the respective syringes of the stopped-flow instrument and allowed to equilibrate to 25.0° for about 10 min. The reaction was followed at various wavelengths and was repeated with varying concentrations of *o*-chloranil.

Hydration of SO_2. In preliminary experiments, performed on a Cary 15 spectrophotometer, a given amount of Na_2SO_3 was added to solutions of progressively increasing acidity, in order to determine the absorption characteristics of SO_2. It was found that SO_2 absorbs strongly in the 280-nm region (in 6 N HCl, λ_{max} 282–284 nm (ϵ_{max} 1600)), and that this absorption decreases markedly as the solution is made less acidic.

Studies on the rate of the $SO_2 \rightleftarrows SO_3^{2-}$ interconversion were performed on a Durrum-Gibson stopped-flow spectrophotometer at 278 nm. Solutions of Na_2SO_3 in 1 N HCl were allowed to react with either (a) 0.9 N NaOH or (b) 1 N NaOH, to initiate the reaction. The final pH of the reaction solution in expt a was 1.37, and that in expt b was 11. The considerable heat of mixing of the NaOH and HCl solutions caused some complicating deflections observed on the oscilloscope, but careful control runs, in which the Na_2SO_3 was omitted, showed that these deflections were not evident on very fast time scales ($\leq$10 msec/div). On the 0.5-msec/div time scale, a distinct loss of absorbance with time could be observed, an event which was not seen when the Na_2SO_3 was omitted. Although this time scale is too fast for the determination of the rate constants for the $SO_2 \rightleftarrows SO_3^{2-}$ interconversion with the stopped-flow spectrophotometer, this result clearly indicates that this reaction is extremely rapid.

Analysis for Inorganic Sulfate Formed in the Reaction of o-Chloranil with Na_2SO_3. ISOLATION OF SULFATE. Sodium sulfite (0.2377 g, 1.89 mmoles) (analytical reagent grade) was dissolved in 225 ml of a stirred 50% (v/v) acetonitrile-deionized water solution, after which approximately 25 drops (0.75 ml) of 12 N HCl was added. *o*-Chloranil (0.52 g, 2.12 mmoles) was dissolved in 5 ml of acetonitrile and centrifuged at 2000 rpm for about 10 min, and then 4.5 ml of the clear solution was added to the stirring Na_2SO_3–HCl mixture. The resultant solution had a slightly reddish color due to the small excess of *o*-chloranil. Deionized water (100 ml) was added, and the solution was then washed three times with small portions of ether. The aqueous layer was heated to boiling, and 10 ml of a 0.2 M $BaCl_2$ solution was slowly added, with stirring, to give a white precipitate. The precipitate was allowed to settle, another 3 ml of $BaCl_2$ was added (no additional cloudiness), and the warm solution was stirred and then allowed to stand for 45 min. The hot plate was then removed, and the solution was allowed to stand overnight. The solution was then filtered through a weighed, fine porosity sintered-glass funnel, and the flask and solid were rinsed five times with hot water. After washing three times with 2-ml portions of acetonitrile, the solid was dried for 3 hr under vacuum at 111°, and weighed. The yield was 0.3875 g of $BaSO_4$ (88%).

Anal. Calcd for $BaSO_4$: S, 13.74. Found: S, 13.73. The ir spectrum was identical with that of an authentic sample.

Treatment of Data. First-order plots were generally constructed on semilogarithmic paper directly from photographs of stopped-flow traces, since transmittance changes of $\leq$5% are generally proportional to absorbance changes. In cases where infinity transmittance values could not be recorded directly on the oscilloscope (*e.g.*, the first part of a biphasic reaction), they were obtained from Kézdy plots (Kézdy *et al.*, 1958), and then used to construct first-order plots from the raw data.[2] Occasionally, due to large background absorbances, the transmittance changes recorded on the oscilloscope were not strictly proportional to absorbance changes, and the points of the oscilloscope trace were converted to a table of absorbances at given time intervals using a Monroe Epic 3000 calculator. In all cases, at least two, and often three, photographs from a given set of runs were ana-

[2] Since several plausible infinity values can usually be obtained from each Kézdy plot, several first-order plots were constructed, and the one which gave the best line was retained.

lyzed, and the first-order rate constants averaged to give the retained value. In addition, care was always taken to adjust the oscilloscope sweep time scale suitably, so that reactions could be followed for approximately 4 half-lives.

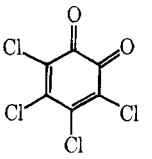
DPS Hydrolysis. The rate of the acid-catalyzed hydrolysis of DPS in 1 N HCl was measured in parallel experiments by following (a) release of phenol and (b) release of inorganic sulfite using *o*-chloranil as a trapping agent. In expt a 50 μl of a 0.0107 M DPS stock solution was added to a mixture of 3 ml of 1 N HCl and 220 μl of acetonitrile, and the release of phenol was followed spectrophotometrically at 269 nm for 10 min. In expt b the same amount of DPS was added to a mixture of 3 ml of 1 N HCl, 200 μl of acetonitrile, and 20 μl of a 0.039 M solution of *o*-chloranil in acetonitrile, and the release of inorganic sulfite was monitored by following the loss of absorbance due to the quinone at 350 nm. The first-order rate constants were obtained by plotting the data according to the method of Kézdy *et al.* (1958).

To follow the pepsin-catalyzed hydrolysis of DPS using the sulfite-trapping system, the following procedure was employed. Phosphate buffer at pH 2.00, μ = 0.05, containing 6% acetonitrile, was prepared and employed for the preparation of pepsin solutions in the usual manner (May and Kaiser, 1969, 1971). To 3 ml of enzyme solution was added the appropriate amount of acetonitrile to give the desired final percentage of organic solvent. The mixture was placed in the cell compartment of the Cary 15, which was flushed with a constant stream of nitrogen and equilibrated to 25.0 ± 0.2°, and the base line was zeroed. The desired amount of *o*-chloranil in acetonitrile was then added, and the absorbance of the solution recorded. The instrument was then switched to expanded scale, an appropriate neutral density screen was placed in the reference compartment, and the reaction was initiated by substrate addition. The solution was mixed and then a gentle stream of nitrogen was bubbled into the cell through a capillary tube for about 20 sec. After covering the cell with a ground-glass stopper, recording was commenced and the reaction followed at 390 nm under nitrogen for about 20 min. Infinity values were calculated from the amount of DPS added and the measured absorbance of *o*-chloranil in the cell. Reactions were carried out at pH 2.00 ± 0.02, in 10.5% acetonitrile. The nitrogen which was bubbled into the cell was passed through glass wool, H_2SO_4, NaOH, and $CaCl_2$ immediately before use. The pH of the reaction mixture was checked immediately after completion of each kinetic run.

BNPS Hydrolysis. The rates of both *p*-nitrophenol formation and inorganic sulfite formation during the pepsin-catalyzed hydrolysis of BNPS were followed spectrophotometrically in the presence of *o*-chloranil using a Durrum-Gibson stopped-flow spectrophotometer. In a typical experiment at pH 1.50, the substrate syringe was filled with a solution prepared in the following manner: 5 ml of distilled, deionized, and degassed water, equilibrated to 25°, 1.15 ml of acetonitrile, 150 μl of 2 N HCl, and 85 μl of a 0.031 M solution of *o*-chloranil in acetonitrile were mixed in a small flask and equilibrated to 25° in a water bath. Then 110 μl of a 1.75×10^{-3} M stock solution of BNPS was added and the mixture shaken vigorously, and immediately placed in the appropriate syringe of the stopped-flow instrument. Experiments at pH 2 were performed in the same manner, except that only 50 μl of 2 N HCl was added to the substrate syringe solution. In all cases, the final reaction mixture contained 10% acetonitrile. *p*-Nitrophenol formation was followed at 312.5 nm and sulfite liberation at 420–430 nm.

Results

One approach to the problem of monitoring the rate of formation of inorganic sulfite during the course of the pepsin-catalyzed hydrolysis of sulfite esters is to employ a suitable trapping agent.[3] Ideally, such a substance should react selectively with liberated inorganic sulfite at a rate which is much faster than the enzymatic reaction, should undergo a sizeable absorbance change at a convenient wavelength upon reaction, and should in no way affect the enzymatic reaction. Preliminary studies seemed to indicate that *o*-chloranil (OCL, I) was a suitable substance for this purpose. Using techniques



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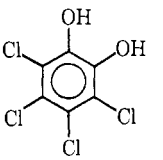


II

which are described in the Experimental Section, the following facts regarding the reaction of OCL with inorganic sulfite were established. (1) The reaction is stoichiometric and is complete within the time required to mix the reactants and initiate recording on the Cary 15 spectrophotometer (approximately 10 sec). (2) The products of the reaction are tetrachlorocatechol (II) and SO_4^{2-}. (3) The reaction is apparently acid catalyzed and does not proceed readily in deionized water. (4) The reaction is accompanied by a large absorbance decrease in the 400-nm region, where the background absorption of pepsin, sulfite esters, phenol, and *p*-nitrophenol (in acid solution) is minimal.

As a first test of this system, the rate of hydrolysis of DPS in 1 N HCl at 25° was measured in parallel experiments by following (a) release of phenol (no OCL present) and (b) release of inorganic sulfite using OCL as a trapping agent. The pseudo-first-order rate constants measured by these two methods were 2.75×10^{-3} and 2.77×10^{-3} sec^{-1}, respectively. However, in a similar set of experiments in the presence of pepsin, the rate of the enzymatic reaction in the presence of OCL, measured at 350 nm, was several times slower than the rate of phenol formation measured in the absence of OCL. This result indicated that either inorganic sulfite is liberated more slowly than phenol in the enzymatic reaction or that OCL inhibits the enzymatic reaction. Unfortunately, due to the large background absorption of *o*-chloranil near 270 nm, it was impossible to measure directly the rate of phenol formation in the presence of OCL in order to distinguish between these two possibilities. Therefore, a systematic investigation of the pepsin-catalyzed hydrolysis of DPS in the presence of OCL was undertaken.

Figure 1 presents a Dixon plot for the inhibition of the pepsin-catalyzed hydrolysis of DPS by OCL at pH 2. In all cases, the reaction was followed by monitoring release of inorganic sulfite using *o*-chloranil as a trapping agent. It is evident from Figure 1 that *o*-chloranil inhibits the pepsin-catalyzed hydrolysis of DPS with $K_I = 4.5 \times 10^{-4}$ M. The intercept on the E_0/v_0 axis of a Lineweaver–Burk plot for this reaction gives a value of k_{cat} of about 5×10^{-2} sec^{-1}, which is similar to that which we have obtained for DPS hydrolysis in

[3] The words inorganic sulfite are used here to describe the sulfur-containing species liberated during the hydrolysis of sulfite esters, whether they be SO_2, SO_3^{2-}, or HSO_3^-.

5.13% CH_3CN (k_{cat} = 4.3 × 10^{-2} sec^{-1}, May and Kaiser, 1969). Thus, these studies indicate that *o*-chloranil inhibits the pepsin-catalyzed hydrolysis of DPS, and that there is no gross difference between the rates of inorganic sulfite and phenol liberation in this reaction. It must be kept in mind that the extrapolation necessary to determine k_{cat} from the Lineweaver–Burk plot is quite great since solubility considerations limited the maximum attainable initial substrate concentration to about 16% of the apparent K_m value. This means that there is considerable uncertainty in the k_{cat} value obtained from this plot and we can, therefore, only say that there is no *gross* difference between the rates of phenol and inorganic sulfite formation. This uncertainty also precludes a clear determination from Figure 1 as to whether the mode of inhibition is purely competitive or somewhat mixed.[4]

A number of observations made in our study of DPS hydrolysis using OCL as a trapping agent for inorganic sulfite deserve special mention. (1) It was necessary to follow the DPS reaction under nitrogen to retard what appeared to be a complicating slow reoxidation of newly formed tetrachlorocatechol. This reoxidation appeared as a slow absorbance increase and deviation from isosbesticity when a solution of partially reduced OCL was allowed to stand in air. Under nitrogen, this reaction was not a complicating factor during the time period required for measurement of the enzymatic initial rate (15–20 min). (2) In the presence of 10^{-4} M pepsin at pH 2, a solution of OCL showed a slow definite increase in absorbance, possibly indicative of a slow oxidation of a group (or groups) on the enzyme. However, at the pepsin concentrations used in the DPS studies (approximately 7 × 10^{-6} M) this was not a complicating factor. Similarly, the rate at which this phenomenon occurred was much too slow to affect the stopped-flow studies with BNPS described below. (3) In this system, *o*-chloranil is slowly being reduced by liberated inorganic sulfite, and its concentration is therefore decreasing. Also, it is likely that the product tetrachlorocatechol inhibits pepsin to a different degree than does *o*-chloranil. Generally, since the reaction was followed to only 15–20% completion of the hydrolysis of DPS, and *o*-chloranil was in substantial excess, the concentration of inhibitor was essentially constant and this was not a serious complication. However, this situation may be responsible for the scatter in points corresponding to relatively low inhibitor concentrations along the lower line in Figure 1, where the percentage change in OCL concentration is greatest.

BNPS Hydrolysis. When a given amount of BNPS is added to a solution containing *o*-chloranil and excess pepsin at pH 2, an immediate decrease in OCL absorbance by the amount expected from complete reaction of *all* of the inorganic sulfite liberated during BNPS hydrolysis is observed. This result indicates that inorganic sulfite liberation during the pepsin-catalyzed hydrolysis of BNPS is a rapid process, and stopped-flow techniques must be employed for its study. However, before investigating this system further, we first undertook a study of the kinetics of the trapping reaction itself, in order to determine whether it proceeds substantially more rapidly than the pepsin-catalyzed hydrolysis of BNPS.

After several unsuccessful attempts to determine a simple first-order rate constant for the reaction of *o*-chloranil with

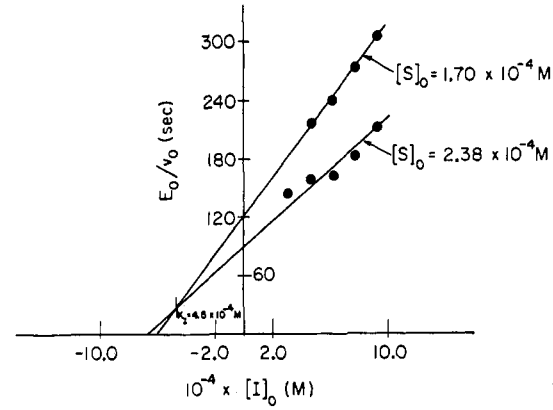


FIGURE 1: Dixon plot for the inhibition of DPS hydrolysis by *o*-chloranil. The reaction was followed by monitoring the release of inorganic sulfite using *o*-chloranil as a trapping agent.

inorganic sulfite in acid solution, it became apparent that this reaction is quite complex. Judging from stopped-flow oscilloscope traces generated in the 390- to 410-nm region, there are three successive phases. The first of these is extremely rapid, and is apparently complete within about the first 10 msec after mixing (pH 2, OCL = 2 × 10^{-4} to 4 × 10^{-4} M, 10.5% CH_3CN). Due to the limitations of our instrument, as well as the mixing characteristics of the solvents used (20% CH_3CN in one syringe, aqueous solution in the other), it was not possible to study the kinetics of this phase. However, it is clearly many times faster than the enzymatic reaction at moderate pepsin concentrations (see below). The second phase exhibits first-order kinetics, and has an observed first-order rate constant of approximately 20 sec^{-1}, which is essentially independent of the concentration of *o*-chloranil in the range 1.2 × 10^{-4} to 3.5 × 10^{-4} M.[5] The third phase also exhibits first-order kinetics and has an observed first-order rate constant of 2.8 sec^{-1}.

Figure 2 presents photographs of stopped-flow oscilloscope traces obtained from following the last phase of the trapping reaction at various wavelengths at pH 2. The time scale of these traces is such (0.1 sec/div) that the deflection at the beginning of each trace represents the relatively rapid second phase of the trapping reaction, and the slower deflection which follows represents the third phase. The observed first-order rate constants obtained from several of these photographs are listed in Table I. The identity of these k_{obsd} values indicates that we are indeed observing the same reaction at these various wavelengths. Inspection of the photographs in Figure 2 reveals that the intermediate and the product tetrachlorocatechol have an isosbestic point near 312.5 nm, and that the absorption of the intermediate becomes very small above 410 nm. (Tetrachlorocatechol does not absorb above 340 nm; see Experimental Section.) Photographs taken at closely spaced intervals near 410 nm indicate that the absorption of the intermediate declines rapidly in this region, and small deflections can sometimes be observed with the relatively large slit openings used (about 1 mm). There is some uncertainty in the quoted wavelengths for the isosbestic points

[4] In Figure 1, purely competitive inhibition requires that the lines intersect at a height of $1/k_{cat}$. Schlamowitz *et al.* (1968) present several examples of the effects of various types of inhibitors on plots such as that in Figure 1, and also find that inhibition by various organic compounds which are structurally similar to I and II is slightly mixed.

[5] The experimental traces were usually analyzed by constructing Kézdy plots (Kézdy *et al.*, 1958) since infinity values could not be obtained directly. Also, the occurrence of the first very rapid phase caused deviations in the measurements of the second phase at the beginning of the traces. These factors caused considerable scatter in the value of k_{obsd} obtained for the second phase in various studies (18–24 sec^{-1}).

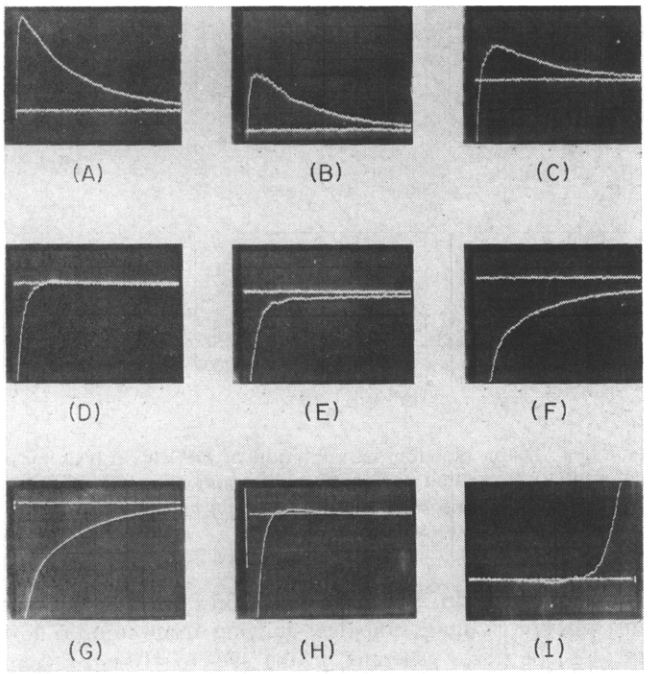


FIGURE 2: Photographs of stopped-flow oscilloscope traces for the last phase of the trapping reaction at various wavelengths. All runs were performed with the same reactant concentrations, and the deflections at the beginning of each picture represent the more rapid preceding phase. Wavelengths and time scales for the traces are as follows: A, 294 nm, 0.1 sec/div; B, 300 nm, 0.1 sec/div; C, 305 nm, 0.1 sec/div; D, 312 nm, 0.1 sec/div; E, 315 nm, 0.1 sec/div; F, 325 nm, 0.1 sec/div; G, 370 nm, 0.1 sec/div; H, 409 nm, 0.1 sec/div; and I, 410 nm, 0.05 sec/div. The oscilloscope transmittance scale is the same for all traces (2% full scale transmittance change). Photograph I has been inadvertently inverted.

since large slit openings were generally used and since we did not recalibrate the monochromator of our stopped-flow instrument after its receipt from the Durrum Instrument Corp.

With this information about the trapping system in hand, it is apparent that inorganic sulfite liberation during the course of the pepsin-catalyzed hydrolysis of BNPS can be monitored above 410 nm. In this region, the slow phase of the trapping reaction is not a problem, and the experimental conditions can be adjusted so that the initial phases of the trapping reaction are much faster than the enzymatic reaction. In addition, the liberated *p*-nitrophenol does not absorb in this region (acid solution), and the only observable transmittance changes arise from reduction of OCL. Thus, the kinetic situation which is established is that of series first-order reactions, and provided that the induction period has been passed, the rate of OCL reduction will equal the rate of inorganic sulfite liberation due to the enzyme-catalyzed hydrolysis of BNPS.

The results of the previously described experiments with DPS establish that *o*-chloranil (and possibly tetrachlorocatechol as well) inhibits pepsin-catalyzed hydrolyses. Therefore, the rate of *p*-nitrophenol formation during the pepsin-catalyzed hydrolysis of BNPS was measured in the presence of the same amount of *o*-chloranil employed in the inorganic sulfite trapping studies on this system, in order to allow a direct comparison between the results of these two sets of experiments. *p*-Nitrophenol formation was followed at 312.5 nm, in order to avoid complications from the slow phase of the trapping reaction (see above). Since at this wavelength the $\Delta\epsilon$ for *p*-nitrophenol formation ($\approx$15,000) is much greater than the $\Delta\epsilon$ for the loss of OCL absorption ($\approx$1500) in our system,

TABLE I: Observed Rate Constants for the Slow Phase of the Trapping Reaction at Various Wavelengths.[a]

Wavelength (nm)	k_{obsd} (sec^{-1})
294	2.83
297	2.88
305	2.97
350	2.83
370	2.79

[a] All data gathered at 25°, pH 2, in 10.6% acetonitrile. The concentration of *o*-chloranil in the final reaction mixture was 2.48×10^{-4} M. All runs obeyed a first-order rate law.

the observed rate of change of the absorbance is essentially a reflection of the rate of *p*-nitrophenol formation.

For experiments in which the rates of *p*-nitrophenol and inorganic sulfite formation during the pepsin-catalyzed hydrolysis of BNPS at pH 1.50 in 10.3% CH_3CN were measured, the enzyme solution was made up in phosphate buffer, and the final reaction solution was 9.1×10^{-5} M in pepsin and 2×10^{-4} M in *o*-chloranil. Duplicate experiments gave k_{obsd} values of 2.78 and 2.60 sec^{-1} for *p*-nitrophenol formation (312.5 nm) and k_{obsd} values of 2.61 and 2.86 sec^{-1} for inorganic sulfite liberation (420 nm).[6] The reactions followed first-order kinetics for at least 3–4 half-lives, a fact which confirms our prediction that under these experimental conditions no kinetic complications should be encountered. It is evident thus that under these conditions, the rates of inorganic sulfite and *p*-nitrophenol formation during the pepsin-catalyzed hydrolysis of BNPS are equal.

There is one additional aspect of these inorganic sulfite trapping studies which warrants further comment. All of our studies on the nature of this system have been carried out using acidic solutions (near pH 2) of Na_2SO_3, to which the following equilibria apply (Eigen *et al.*, 1961; Weast, 1967; Cotton and Wilkinson, 1966).

$$SO_2 + xH_2O \rightleftharpoons H_2SO_3;\ K \ll 1$$

$$SO_2 + xH_2O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HSO_3^- + H^+$$

$$\downarrow\uparrow$$

$$SO_3^{2-} + H^+$$

$$K_1 = \frac{[HSO_3^-][H^+]}{[\text{total dissolved } SO_2] - [HSO_3^-] - [SO_3^{2-}]} = 1.3 \times 10^{-2} \quad (1)$$

$$K_2 = \frac{[H^+][SO_3^{2-}]}{[HSO_3^-]} \approx 1 \times 10^{-7} \quad (2)$$

Thus, in these solutions, the sulfur-containing species actually present are mainly HSO_3^- and SO_2. On the other hand, it is possible that the sulfur-containing species initially liberated during the pepsin-catalyzed hydrolysis is SO_2, which then

[6] At 420 nm a subsequent slow, first-order reaction involving inorganic sulfite release was also observed. This phenomenon is discussed below.

TABLE II: Results of Stopped-Flow Studies on the Slow Reaction Observed during the Pepsin-Catalyzed Hydrolysis of BNPS in Phosphate Buffer at pH 1.50.[a]

Expt No.[b]	$10^4 \times$ E (M)	k_{obsd} (sec^{-1})
1a	1.60	0.30
1b	1.60	0.29
2a	1.20	0.31
2b	1.20	0.30
3	0.80	0.29
4a	0.40	0.31
4b	0.40	0.31

[a] All data gathered at 25°, 0.4% CH_3CN. Ionic strength of the reaction solution was 0.05. The reaction was followed at 325 nm. [b] Runs with the same reference number (*e.g.*, 2a and 2b) were performed in parallel with the same solutions.

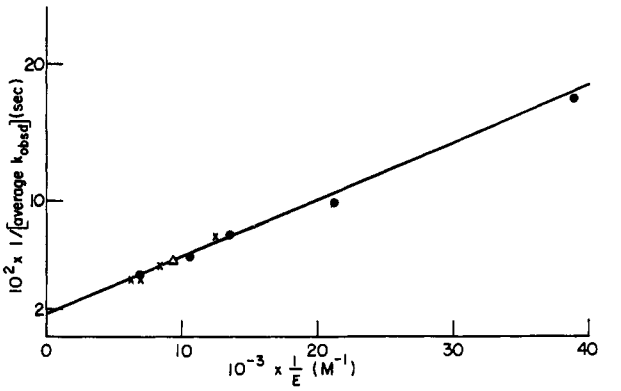


FIGURE 3: Plot of $1/[\text{av } k_{obsd}]$ *vs.* 1/[E] for the pepsin-catalyzed hydrolysis of BNPS at pH 1.50, 25.0 ± 0.1°, in 0.4% acetonitrile. Points are from the average of k_{obsd} values measured at each enzyme concentration. (●) Data gathered in HCl–NaCl solution; (X) data gathered in phosphate buffer; (△) data gathered in $HClO_4$ solution. The line is a computer calculated, weighted least-squared fit of the raw data to the reciprocal form of the equation $k_{obsd} = k_{cat}[E]/[K_m + E]$ (May and Kaiser, 1971). Data in phosphate and $HClO_4$ solutions are for the rapid enzymatic reaction only (see text).

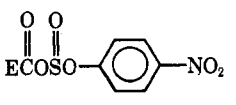
reacts with water to give HSO_3^-. Since this reaction involves formation of a sulfur–oxygen bond, it was conceivable to us that it might be relatively slow and could therefore be a complicating factor. For this reason, we attempted to measure the rate of hydration of SO_2 using the pH-jump technique described in the Experimental Section. We found that the apparent rate of this reaction was far too fast to study using conventional stopped-flow techniques. Eigen *et al.* (1961) have studied this reaction using relaxation methods and have found that the values of the rate constants k_1 and k_{-1} in the above scheme are 2×10^9 M^{-1} sec^{-1} and 3.4×10^6 sec^{-1}, respectively.

Solvent Effects on BNPS Hydrolysis. During the course of a study of the pH dependence of the rate of the pepsin-catalyzed hydrolysis of BNPS (May and Kaiser, 1971), we noticed that in some solvents deviations from first-order kinetics could be observed toward the end of the reaction when *p*-nitrophenol formation was followed at 325 nm. This phenomenon was first noted in phosphate buffer at pH 2.0. However, in this solvent the deviations were quite small, generally appeared only after 3 half-lives, and were most apparent in reactions with relatively low enzyme concentrations. These considerations made it extremely difficult to determine whether or not this effect was real, since there are inherent inaccuracies in the stopped-flow method. For example, with the greatly expanded oscilloscope scale used in these studies (full scale deflection of $\leq 5\%$ transmittance change) there was often considerable background noise in the traces. In addition, the relative error in the points of first-order plots constructed from stopped-flow traces is always greatest toward the end of the reaction where small differences between similar transmittance values must be taken.

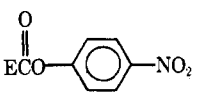
As the pH–rate study was extended to more acidic solutions of phosphate buffer, it became apparent that these deviations were indicative of a real phenomenon. At pH 1.75 the reactions showed substantial deviations from first-order kinetics, and at pH 1.50, the stopped-flow traces were distinctly biphasic. These observations promoted us to repeat all of the studies on the pepsin-catalyzed hydrolysis of BNPS below pH 2.75 using HCl–NaCl–H_2O solutions of the appropriate pH and ionic strength. In these latter solutions, the enzymatic reactions were strictly first-order and no subsequent slow reactions were observed. Although these are not strictly "buffered" solutions, careful adjustment of the HCl content of the substrate syringe allowed the reactions to be run at constant pH (±0.02 pH unit). Experiments performed in phosphate buffer at pH 2.75 and above were not repeated since no complicating solvent effects were apparent under these conditions.

A thorough investigation of this phenomenon using the usual stopped-flow techniques revealed the following facts. (1) When the pepsin-catalyzed hydrolysis of BNPS was repeated at pH 1.50 in an $HClO_4$ solution a biphasic reaction was again observed. However, the second phase in this solvent proceeds much more slowly than in phosphate at the same pH. (k_{obsd} in $HClO_4$ is approximately 0.01 sec^{-1}.) (2) In phosphate buffer at pH 1.50, the first and second phases of the reaction can be easily separated, and the kinetic behavior of each phase analyzed. The slow phase exhibits strict first-order kinetics, and as shown in Table II, the rate constants obtained, k_{obsd}, are independent of the concentration of enzyme in the reaction solution. (3) The first phase of the reaction at pH 1.50 exhibits first-order kinetics, and its rate is dependent on enzyme concentration. Moreover, as is evident from Figure 3, the k_{obsd} values for this phase at various initial enzyme concentrations in both $HClO_4$ and phosphate are in excellent agreement with those predicted from data gathered in an HCl–NaCl solution at the same pH. From these results, it is evident that the rate of the slow phase is independent of enzyme concentration but is dependent on the nature of the solvent. On the other hand, the first phase is a true enzyme-catalyzed process, and is unaffected by the nature of the solvent.

To establish whether the slow reaction represents the buffer-catalyzed decomposition of a covalent intermediate species, such as III or IV, formed at the active site of pepsin during the enzymatic reaction, the following set of experiments was conducted. An $HClO_4$ solution of pH 1.50 was placed in the cell compartment of a Cary 15 spectrophotometer, and a given amount of BNPS was added. The slow nonenzymatic



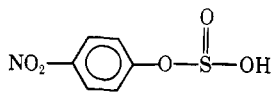
III



IV

hydrolysis of the ester was followed at 318 nm for 30–60 sec. Recording was then interrupted, a small amount of pepsin rapidly added with stirring, and recording resumed. An immediate "burst" of absorbance by the amount expected for complete hydrolysis of all the added BNPS was observed. The experiment was repeated with the addition of a different amount of pepsin, and the same result was obtained. In both cases, the concentration of BNPS in solution was at least ten times greater than the concentration of pepsin. This result clearly indicates that the enzyme is able to turn over many times within the time required to mix the reactants and initiate recording on the Cary 15 (about 10 sec). Thus, the slow reaction observed in the stopped-flow experiments in this solvent cannot be decomposition of a covalent intermediate species at the active site of the pepsin.

A possible explanation for the existence of the slow reaction is that it represents the buffer-catalyzed hydrolysis of the half-ester species, V. Since we have established that the pepsin-catalyzed hydrolysis of BNPS results in concurrent liberation of inorganic sulfite and *p*-nitrophenol, if V is present, it must be formed during the acid-catalyzed hydrolysis of BNPS, which is proceeding relatively slowly in the substrate syringe of the stopped-flow instrument. Presumably, when the contents of the enzyme and substrate syringes are mixed, all of the unreacted BNPS is rapidly scavenged by the enzyme, leaving only *p*-nitrophenol and a small amount of unhydrolyzed V. That half-ester species analogous to V are intermediates in the acid-catalyzed hydrolysis of various sulfite esters has been suggested by several investigators (see, for example, Bunton and Schwerin, 1966).



V

This hypothesis is supported by the observation that in phosphate buffer (pH 1.50) inorganic sulfite liberation during the pepsin-catalyzed hydrolysis of BNPS, as measured by the *o*-chloranil trapping reaction, is biphasic. In duplicate determinations, the value of k_{obsd} obtained for the slow reaction was 0.27 sec^{-1} which is in good agreement with the k_{obsd} values for *p*-nitrophenol formation listed in Table II.

In addition, the fact that the rate of decomposition of the species which may be V varies with the nature of the solvent could reflect the relative nucleophilicities of various ions toward the half-ester. Thus, perchlorate is a much less effective catalyst than phosphate at pH 1.50. Similarly, the observation that the reaction in phosphate becomes more rapid with increasing pH may be a reflection of the fact that $H_2PO_4^-$ is a more effective catalyst than is H_3PO_4.

We wish to emphasize that our suggestion that the slow reactions seen in the phosphate and perchlorate solutions are due to the decomposition of V should be regarded only as a working hypothesis and by no means as a definite conclusion.[7] In any event, our results do clearly indicate that the slow reaction is a solvent-associated phenomenon in which the active site of pepsin is not involved. Further experiments to elucidate the nature of the slow reactions are currently in progress in our laboratory.

It is noteworthy that in MES buffer at pH 4.95 and 5.36, what appeared to be a slow absorbance increase with time could be observed subsequent to the enzymatic hydrolysis of BNPS. However, it was difficult to discern whether this was a real phenomenon or simply an artifact, since the deflections were very small ($\leq 1\%$ transmittance change) and the "reaction" was very slow (apparent half-life ≈ 60 sec). Against the possibility that this could be the slow decomposition of a covalent intermediate formed from the reaction of the enzyme with the substrate at these relatively high pH values, a substrate in excess burst experiment analogous to that performed in phosphate at pH 1.50 was carried out. As before, an immediate burst in absorbance by the amount expected for complete hydrolysis of all the BNPS present was observed, indicating that a slowly decomposing intermediate species had not been formed. Thus, if this slow reaction is real, it may indicate that MES is a rather ineffective catalyst for the decomposition of V.

Discussion

Besides the results of the experiments described, those of related studies with sulfite ester substrates must be taken into account in formulating a plausible mechanism for the pepsin-catalyzed hydrolysis of sulfite esters. Moreover, since much mechanistically significant information about pepsin has been obtained in studies using peptide substrates, the relationship between the peptidase and sulfite esterase activities of this enzyme must also be considered.

The following evidence exists that, despite the disparity in their structure, both the peptide and sulfite ester substrates of pepsin react at the same site on the enzyme. (1) Reid and Fahrney (1967) found that treatment of pepsin with diazoacetyl-DL-norleucine methyl ester in the presence of Cu(II) ions not only inactivates the enzyme toward hemoglobin and synthetic peptide substrates (Rajagopalan *et al.*, 1966) but also causes the enzyme to lose its activity in catalyzing the hydrolysis of diphenyl sulfite and methyl phenyl sulfite. Similarly, reaction of pepsin with either α-*p*-dibromoacetophenone, a compound which generally causes some loss of pepsin's peptidase activity, or α-diazo-*p*-bromoacetophenone, a compound which can cause total loss of pepsin's peptidase activity (Erlanger *et al.*, 1967), drastically reduces the enzyme's activity toward diphenyl sulfite (S. W. May and L. R. Kaplan, unpublished observations). (2) The specific peptide substrate Z-Phe-Tyr competitively inhibits the hydrolysis of diphenyl sulfite (Reid and Fahrney, 1967). (3) AcPhe inhibits the sulfite esterase and the peptidase activity of pepsin to the same extent.

The bell-shaped k_{cat}/K_m *vs.* pH profile found for the pepsin-catalyzed hydrolysis of BNPS (May and Kaiser, 1971) closely resembles those obtained for the hydrolysis of neutral peptide substrates (Cornish-Bowden and Knowles, 1969; Denburg *et al.*, 1968). In terms of the kinetic and chemical modification data available in the literature and the usual interpretation of such k_{cat}/K_m *vs.* pH profiles, a plausible hypothesis is that two carboxyl groups on the free enzyme, one in its acidic form and the other in its basic form, are involved in each process.[8] Moreover, since sulfite esters are known to

[7] An objection which might be raised against our suggestion is that it requires a small but significant buildup of V during the buffer-catalyzed hydrolysis of BNPS. Yet, the observed rate of the "slow" reaction is so much more rapid than that of the buffer-catalyzed hydrolysis of BNPS that such a buildup might not be expected. A final judgment of the merits of our suggestion must await a detailed investigation of the nature of the intermediates formed during the nonenzymatic hydrolysis of BNPS and of the effects of nucleophiles and buffer ions on this reaction.

[8] The evidence for the participation of two active-site carboxyl groups in pepsin's action on peptide substrates has been summarized recently by Knowles (1970).

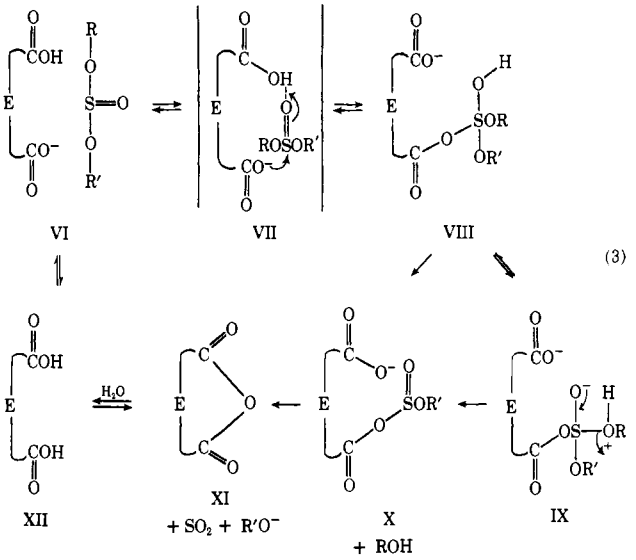
be highly reactive toward various carboxylic acids (Iselin *et al.*, 1957; Schwyzer *et al.*, 1960), the notion that carboxyl groups in the active site of pepsin are involved in the enzyme-catalyzed hydrolysis of these compounds is very reasonable.

The results of the present study with the inorganic sulfite trapping system have established that *p*-nitrophenol and inorganic sulfite are liberated concurrently during the pepsin-catalyzed hydrolysis of BNPS at pH 1.50. This is a mechanistically important conclusion, since it seemed possible that sulfite could remain bound to the enzyme and then be liberated in a subsequent slower step. We have also found that there is no gross difference between the rates of phenol and inorganic sulfite liberation during DPS hydrolysis at pH 2.00. However, this latter result could have been anticipated, since the experiments were carried out under turnover conditions.

If our hypothesis that the relatively slow, solvent associated reaction observed in $HClO_4$ and phosphate solutions represents decomposition of the half-ester species, V, is correct, it follows that the immediate products of the *enzymatic* hydrolysis of BNPS are not a molecule of *p*-nitrophenol and a free molecule of V. This is a consequence of the fact that the observed rates of liberation of both *p*-nitrophenol and inorganic sulfite during the pepsin-catalyzed hydrolysis of BNPS are much faster than the rate of the solvent-catalyzed hydrolysis of V. Thus, either such a species is not formed at all during the enzymatic reaction, or, if it is formed, it remains bound to the enzyme and decomposes in a very rapid, enzyme-catalyzed reaction.

During the course of our studies with sulfite ester substrates of pepsin, we have been unable to obtain direct evidence for the existence of a covalent intermediate species like III during the course of the pepsin-catalyzed hydrolysis of sulfite esters. However, our observations on the inequality of the apparent Michaelis constant for the hydrolysis of DPS and the inhibition constant for the inhibition of the hydrolysis of BNPS by added DPS (May and Kaiser, 1969) together with those on the complexity of the k_{cat} *vs.* pH profile for BNPS hydrolysis represent indirect lines of evidence which militate for the existence of such an intermediate. Moreover, inspection of the literature reveals that despite a 10-fold difference in K_m, the k_{cat} values for methyl phenyl sulfite and methyl *p*-bromophenyl sulfite at pH 4 are nearly equal (Reid, 1968; Stein and Fahrney, 1968). This suggests that the rate-determining step in these hydrolyses may be the breakdown of a common intermediate analogous to III. In addition, we have found that acetate ion, which is capable of reacting with BNPS to form a reactive intermediate similar to III, is an extremely effective catalyst for BNPS hydrolysis (May, unpublished observations). Finally, the ^{18}O-labelling experiments of Stein and Fahrney (1968) are consistent with the postulation of such an intermediate species.

As a working hypothesis, the following mechanism based on the postulated formation of covalent intermediates is suggested for the pepsin-catalyzed hydrolysis of sulfite esters (eq 3). This mechanism is consistent with all of the known data for these reactions. According to the mechanism, the reactive form of the enzyme (VI) is that in which one of the two active-site carboxyl groups is in its anionic form and the other is in its un-ionized form.[9] Whether formation or decomposition of the intermediate anhydride species is rate-determining will depend on the nature of the R and R′ groups in the sulfite ester substrates. An example of a step analogous to the proton transfer shown in going from VIII to IX, which serves to convert RO^- to a more favorable leaving group, is seen in the nonenzymatic hydrolysis of phosphate monoesters (Bruice and Benkovic, 1966). The proximity of the carboxylate group to the mixed-anhydride function in X greatly facilitates the nucleophilic displacement reaction which results in the formation of XI. Ample precedent for postulating this type of reaction exists in the hydrolysis of succinate



(3)

and phthalate monoesters (Jencks, 1969). Finally, it is possible that tetrahedral species like VIII and IX are not formed as discrete intermediates and that the anhydride X is produced directly from VII in a concerted process.

[9] The possibility exists that enzyme–substrate complexes obtained from both VI and XII lead to the formation of products in the pepsin-catalyzed hydrolysis of BNPS (May and Kaiser, 1971). If this is true, a mechanism similar to that in eq 3 can be readily written for the breakdown of the complexes produced from XII.

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The Role of Pyridoxal Phosphate in the B_{12} Coenzyme-Dependent D-α-Lysine Mutase Reaction*

Colin G. D. Morley† and Thressa C. Stadtman‡

ABSTRACT: D-α-Lysine mutase catalyzes the interconversion of D-α-lysine and 2,5-diaminohexanoate (2,5-DAH), a reaction involving a shift of the ϵ-amino group of lysine to the adjacent carbon atom. The mutase consists of two dissimilar protein moieties, a cobamide protein (E_1) and a sulfhydryl protein (E_2). Two of the cofactors required for the mutase reaction, B_{12} coenzyme and pyridoxal phosphate (PLP), are bound by the E_1 protein. This protein, in the absence of E_2, catalyzes a PLP- and Mg^{2+}-dependent exchange of hydrogen at position 6 of D-lysine with water. Both the hydrogen-exchange and the overall mutase reaction are inhibited in parallel by known PLP inhibitors. Available evidence suggests that PLP is directly involved in the catalysis of the amino group migration.

The B_{12} coenzyme-dependent D-α-lysine mutase complex that catalyzes reaction 1 exhibits an absolute requirement for pyridoxal phosphate (Morley and Stadtman, 1970). A similar

$$\underset{\text{NH}_2}{\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\underset{\text{NH}_2}{\text{CH}}\text{COOH}} \rightleftharpoons \underset{\text{NH}_2}{\text{CH}_3\text{CH}}\text{CH}_2\text{CH}_2\underset{\text{NH}_2}{\text{CH}}\text{COOH} \quad (1)$$

reaction catalyzed by L-lysine 2,3-aminomutase (Chirpich *et al.*, 1970), which does not require a B_{12} coenzyme, is activated by pyridoxal phosphate, ferrous iron, and *S*-adenosylmethionine.

Some studies reported in the present communication indicate that in the D-α-lysine mutase reaction pyridoxal phosphate may play a role in the catalytic reaction *per se*. Furthermore, it is suggested that the amino group which replaces the abstracted hydrogen may migrate as a pyridoxal phosphate derivative.

Materials

D-α-Lysine mutase was purified either as the two separated dissimilar proteins, a cobamide protein (E_1) and a sulfhydryl protein (E_2) (Stadtman and Grant, 1971), or as a complex of the two proteins (Morley and Stadtman, 1970).

Pyridoxal 5′-phosphate and pyridoxal were purchased from Nutritional Biochemicals Corp. Pyridoxamine 5′-phosphate and pyridoxine 5′-phosphate were obtained from Dr. M. Flavin. Methyl pyridoxal phosphate was a generous gift from Dr. E. Helmreich, University of Würzburg, Würzburg, Germany. The pyridoxal antagonists, L-1-aminoproline and D-1-aminoproline, were prepared as described by Klosterman *et al.* (1967). Isonicotinic acid hydrazide was purchased from Eastman Kodak. Tritiated water (100 mCi/g) and DL-lysine-6-*t* (8.7 mCi/mmole) were purchased from New England Nuclear.

Methods

Routine assays of D-α-lysine mutase activity were performed as described previously (Morley and Stadtman, 1970). Radioactivity was determined in a Beckman LS-250 liquid scintillation system using 2,5-diphenyloxazole (0.8%) in toluene as counting fluid.

Tritium-Exchange Techniques. Exchange of tritium from H_2O-*t* into amino acids was carried out by incubating the appropriate mixture for 10 hr at 37°, acidifying with $HClO_4$, and passing the supernatant solution, after removal of denatured protein, over a Dowex 50-H^+ × 4 pad (1 × 2 cm). The Dowex was washed with water and the amino acids were eluted with NH_4OH (12%). The eluate was evaporated to dryness and redissolved in 0.5 ml of water, and aliquots were assayed for radioactivity.

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