Penicillanic Acid Sulfone: Interaction with RTEM β-Lactamase from *Escherichia coli* at Different pH Values†

Cemal Kemal‡ and Jeremy R. Knowles*

ABSTRACT: The interaction of the sulfone of penicillanic acid with the TEM-2 β-lactamase from *Escherichia coli* has been investigated as a function of pH between pH 7.0 and 9.6. The first-formed acyl-enzyme suffers one of three fates: deacylation, tautomerization to a bound enamine that transiently inhibits the enzyme, and a process (possibly transimination) that leads to enzyme inactivation. The observed changes in ultraviolet absorbance are consistent with the initially observed product of deacylation being the enamine tautomer (**4**) of the imine from malonsemialdehyde and penicillamine sulfinate. The same enamine can be generated nonenzymically from the sulfone at high pH. The transiently inhibited enzyme appears to be the same enamine attached to the enzyme by an ester linkage. The rather complex kinetic behavior can be deconvoluted by exploiting the effect of pH on the partitioning of the acyl-enzyme between deacylation and the transiently inhibited form of the enzyme. The pathways followed by penicillanic acid sulfone provide a model for the behavior of a number of other reagents that inactivate the β-lactamase.

In the preceding paper (Brenner & Knowles, 1981) we reported the details of the interaction of penicillanic acid sulfone (**1**) and of three 6-deuterated derivatives with the TEM-2 β-lactamase from *Escherichia coli*. These studies have led to the formulation of a mechanistic scheme [Scheme II of Brenner & Knowles (1981)] that accommodates the kinetic and spectroscopic features of the reactions that result in hydrolysis of the β-lactam and in enzyme inactivation. Important mechanistic information was provided by the behavior of the deuterated derivatives of penicillanic acid sulfone, the isotopic substitution resulting in a different partitioning of the first-formed acyl-enzyme intermediate.

In the present paper, we describe the consequences of changing the pH on the partitioning of the acyl-enzyme intermediate. The different pH dependences of the steps leading from the acyl-enzyme allow us further to delineate the interaction of penicillanic acid sulfone with the β-lactamase and to propose a generalized scheme for the action of this class of β-lactamase inactivators.

Experimental Procedures

Materials

Penicillanic acid sulfone (sodium salt) was a generous gift from Pfizer Inc. (as compound CP 45,899). Benzylpenicillin (Sigma Chemical Co.) was used as received. The following buffers were used for kinetic studies between pH 7 and 10.6: sodium phosphate (pH 7.0), *N*-ethylmorpholinium chloride (pH 7.5–9.0), sodium pyrophosphate (pH 9.0), and potassium carbonate (pH 9.6–10.6). Ionic strength was always maintained at 0.22 by the addition of KCl.

β-Lactamase used in these studies was the wild-type TEM-2 enzyme from *E. coli*, isolated and purified according to Fisher et al. (1978, 1980).

Methods

All kinetic measurements were done at 30 °C with a Perkin-Elmer 554 spectrophotometer. Measurements of pH were made at 30 °C with a Radiometer PHM64 pH meter.

Values of K_m and k_{cat} for benzylpenicillin between pH 7.0 and 9.6 were determined by monitoring its hydrolysis at 240 nm ($\Delta\epsilon$ = 570 M^{-1} cm^{-1}) or 250 nm ($\Delta\epsilon$ = 150 M^{-1} cm^{-1}) and treating the data by using the integrated Michaelis–Menten equation (Gutfreund, 1972). Values of k_{cat} for the hydrolysis of penicillanic acid sulfone were determined by monitoring its hydrolysis at 235 nm ($\Delta\epsilon$ = 1780 M^{-1} cm^{-1}). Typical concentrations of enzyme and substrate used were 100–750 nM and 50–270 μM, respectively.

Reactivation of β-lactamase that had been transiently inhibited by penicillanic acid sulfone was studied by two different methods. In the first approach, β-lactamase (300–700 nM) was incubated with sulfone in an ~10-fold (at pH 9) to ~400-fold (at pH 7) molar excess over enzyme. After the attainment of the steady state (<1 min, depending on the pH and the sulfone concentration), a portion (5–10 μL) of this solution was diluted into a solution of benzylpenicillin (1 mL, 2–4 mM) buffered at the desired pH, and the hydrolysis of the latter was monitored at 240 or 250 nm. Rate constants for reactivation were obtained by computer simulation of the absorbance vs. time data, using eq A10 of the Appendix. This method requires that the benzylpenicillin concentration remain at a saturating level during the course of reactivation of the transiently inhibited enzyme. Unfortunately, since K_m of benzylpenicillin is pH dependent (Table I), it is not feasible to meet this condition at pH >9 without increasing the benzylpenicillin concentration above 4 mM, to levels at which the absorbance (at wavelengths that can be used to follow the hydrolysis) is unacceptably large. Thus at pH 9.6, where 4 mM $\simeq K_m$, the reactivation rate cannot be obtained; at pH 9.0, where 4 mM $\simeq 6K_m$, the method works only marginally; only below pH 8.5, where 4 mM > $20K_m$, can accurate results be obtained. In the second method, the hydrolysis of penicillanic acid sulfone was monitored by following the absorbance increase at 280 nm, and the rate of enzyme reactivation was obtained from the decrease in absorbance at this wavelength after all the sulfone had hydrolyzed. This method exploits the fact that the transiently inhibited enzyme has a strong absorption at 280 nm. It is particularly useful at pH values above 8.5 where the contribution to the absorbance at 280 nm from the small molecule reaction products in solution is minimal (see Results and Discussion).

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‡ Present address: Department of Chemistry, University of Florida, Gainesville, FL 32611.

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Table I: K_m Values for Benzylpenicillin at Different pH Values[a]

pH	K_m (μM)
7.05	20
8.00	67
8.53	230
9.03	680
9.58	3200

[a] The buffers used were sodium phosphate (pH 7.05), *N*-ethylmorpholine (pH 8.00–9.03), and potassium carbonate (pH 9.58).

Generation of the enamine **4** nonenzymically from penicillanic acid sulfone in appreciable quantities requires that the hydrolysis of the sulfone to produce **4** be faster than the decomposition of **4**. This condition is met only at high pH. Thus in 0.5 N NaOH, the half-lives for the appearance and disappearance of the chromophoric product [λ_{max} 267 nm ($\epsilon \cong 20\,000$ M^{-1} cm^{-1})] from the sulfone were found to be ~0.3 and ~8.5 min, respectively. With this knowledge, stock solutions of **4** were prepared by dissolving the sulfone (~1 mg) in 0.5 N NaOH (0.1 mL), allowing hydrolysis to proceed at room temperature for ~2.5 min, and then rapidly cooling the reaction mixture to 0 °C.

The rate of disappearance of **4** at different pH values was studied by adding a portion (1 μL) of a stock solution in 0.5 N NaOH, prepared as described above, to 1 mL of buffer, and monitoring the decay in absorbance at 267 or 280 nm. The first-order rate constants obtained did not depend on the age of the stock solution of **4**, so the products of the decomposition of **4** evidently do not influence the rate of its hydrolysis.

Results and Discussion

Recent studies with penicillanic acid sulfone (**1**) have shown that this compound is an inhibitor of the β-lactamase used in this work (Fisher & Knowles, 1980; Labia et al., 1980; Brenner & Knowles, 1981). The aim of the current study was to characterize the interaction of the sulfone with the enzyme, in the hope of detecting and identifying intermediates the structures of which might give us more insight into the mechanism of the inhibition of the enzyme. All of the kinetic parameters reported in this paper were obtained under conditions that limited the *irreversible* inactivation of the enzyme to <10% to avoid unnecessary complications. It should also be pointed out that the nonenzymic rate of hydrolysis of penicillanic acid sulfone is extremely slow, the half-life being >100 h between pH 2.6 and 9.0 (English et al., 1978) and ~20 h at pH 9.6. The complication of having to deal with both enzymic and nonenzymic rates of hydrolysis was therefore easily avoided in the pH region studied (pH 7.0–9.6).

Dependence of k_{cat} on pH. The enzyme-catalyzed hydrolysis of penicillanic acid sulfone was followed at 235 nm, where the products of hydrolysis absorb more strongly than the substrate [ϵ_{235nm}(products) = 2350 and ϵ_{235nm}(substrate) = 570 M^{-1} cm^{-1}]. Although for the most part the plots of A_{235nm} vs. time are very close to being linear (Figure 1), the initial part of the reaction is complex. This is clear from the changes in A_{280nm} immediately after the sulfone is mixed with the enzyme: there is initially a rapid increase in absorption at 280 nm, followed by the decay of this absorbance to a steady-state level (see below). The plot of A_{235nm} becomes linear at the same time that the A_{280nm} changes have stabilized. This takes a few seconds at pH 7 and ~5 min at pH 9. (The plots of A_{235nm} vs. time show clear lag phases at high pH, though this is not easily discernible on the scale on which Figure 1 is drawn.) For this reason, values of k_{cat} were obtained from the slopes of the A_{235nm} vs. time plots, *after* changes at 280 nm had stabilized. From these k_{cat} values (Table II), it may be seen that the rate of hydrolysis of the sulfone catalyzed by the β-lactamase is pH dependent, decreasing by ~1 order of magnitude for each unit of pH increase.

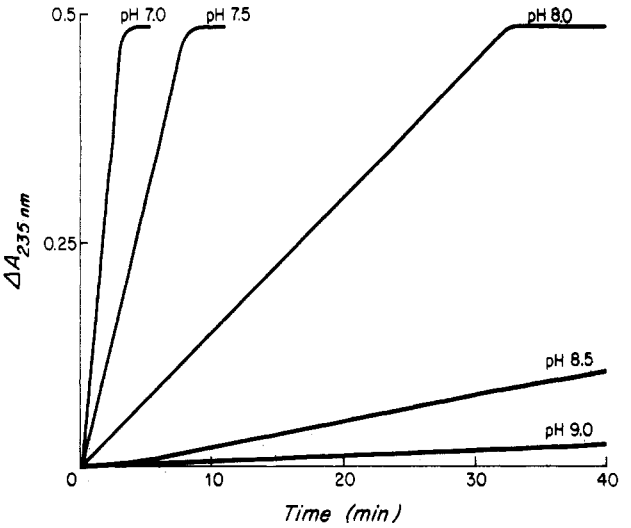


FIGURE 1: Time course of hydrolysis of penicillanic acid sulfone (0.27 mM) by β-lactamase (0.75 μM) as monitored by absorbance changes at 235 nm. The buffers used were sodium phosphate (pH 7.0), *N*-ethylmorpholine (pH 7.5–8.5), and potassium pyrophosphate (pH 9.0), all at a concentration of 10 mM (I = 0.22, adjusted with KCl).

Table II: Turnover Numbers for β-Lactamase-Catalyzed Hydrolysis of Penicillanic Acid Sulfone and First-Order Rate Constants for Reactivation of Transiently Inhibited Enzyme[a]

pH	k_{cat}[b] (s^{-1})	k_{react} (s^{-1})	k_{cat}/k_{react}
7.05	1.7	$(5.2 \pm 1.0) \times 10^{-2}$	33 ± 9
7.50	7.5×10^{-1}	$(3.4 \pm 0.3) \times 10^{-2}$	22 ± 5
8.00	1.7×10^{-1}	$(1.8 \pm 0.2) \times 10^{-2}$	9.4 ± 2.2
8.53	3.5×10^{-2}	$(9.0 \pm 0.5) \times 10^{-3}$	3.9 ± 0.8
9.03	7.7×10^{-3}	$(2.2 \pm 0.1) \times 10^{-3}$	3.5 ± 0.7
9.58	1.8×10^{-3}[c]	$\sim 5.3 \times 10^{-4}$	~3.4

[a] The buffers used are listed in Table I. [b] ±20%. [c] The slower equilibration of the first-formed enamine at higher pH values (k_1 of Table III) means that the steady-state reaction cannot be measured for ~30 min at this pH.

Formation and Breakdown of Transiently Inhibited Enzyme. When a sample of an incubation of β-lactamase and penicillanic acid sulfone (in 1:400 molar ratio) is diluted into a solution of benzylpenicillin, the rate of hydrolysis of the benzylpenicillin is time dependent, increasing from a value close to 0 at the time of mixing to a limiting velocity which is >95% of the velocity that would have been observed if the sulfone had been absent from the first incubation (Figure 2). Since the rate of benzylpenicillin hydrolysis measures the instantaneous concentration of free β-lactamase, this acceleration suggests that the sulfone is reversibly inhibiting the enzyme. At pH 7, the recovery of enzyme activity occurs rapidly ($t_{1/2} \simeq 13$ s), but the reactivation rate becomes slower at higher pH values (Figure 2). The rate constants for the reactivation process were derived both from computer simulation (see Appendix I) of the reactivation curves (such as are shown in Figure 2) and also by following the disappearance of the transiently inhibited enzyme at 280 nm after all of the sulfone had hydrolyzed (see below). These rate constants are listed in Table II.

When benzylpenicillin hydrolysis was used to monitor the reactivation process, the concentration of intact sulfone [which has a K_i of 0.8 μM at pH 7.0 (Brenner & Knowles, 1981)] after dilution into the substrate solution was always low enough

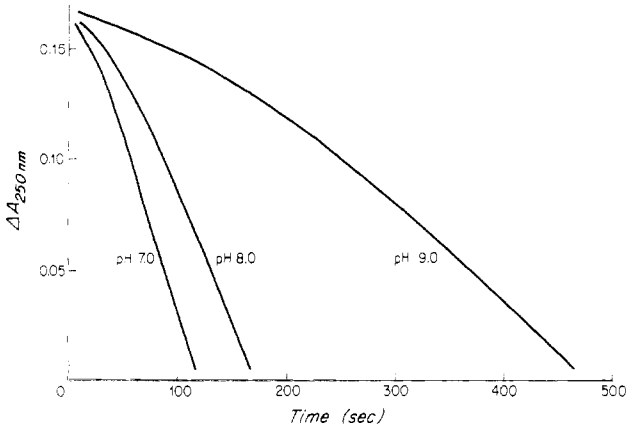


FIGURE 2: pH dependence of rate of reactivation of transiently inhibited β-lactamase as monitored by benzylpenicillin hydrolysis at 250 nm. The transiently inhibited enzyme was generated at pH 9 by adding a 14-fold molar excess of penicillanic acid sulfone to β-lactamase (0.66 μM), a portion (10 μL) of this solution being added to a solution (1 mL) of benzylpenicillin (4 mM) buffered at the desired pH. (For the experiment at pH 9.0, the final enzyme activity was measured by assay of a solution that had been incubated in buffer for 30 min. This was done to avoid excessive depletion of the assay substrate during the slow reactivation at this pH.)

so that its effect as a competitive inhibitor was negligible. At each pH, the rate of reactivation was independent of the time of incubation of the sulfone with the enzyme. The identity of the transiently inhibited enzyme was the same at all pH values studied, as indicated by experiments where the inhibited enzyme was generated at one pH and then added to solutions of benzylpenicillin at another pH. The rate of reactivation depended only on the pH of the benzylpenicillin assay solution and not on the pH at which the transiently inhibited enzyme was generated. After the establishment of the steady-state reaction between enzyme and sulfone at each pH, 90 ± 10% of the enzyme was in the transiently inhibited form, as determined by extrapolation of the reactivation curve (e.g., Figure 2) back to the time of the addition of enzyme to the benzylpenicillin solution.

Number of Turnovers Required to Reach Steady State. Since at the steady state the enzyme is nearly all tied up in the transiently inhibited form and since at each pH the turnover number (k_{cat}) is greater than the rate constant for the reactivation of the transiently inhibited enzyme (k_{react}), the kinetic pathway must be branched, and a greater than stoichiometric amount of sulfone must be required to tie up the enzyme in the transiently inhibited form. We expect, therefore, a pre-steady-state "burst" of product formation when sulfone is added to enzyme. The predicted stoichiometry of this burst may be calculated by dividing k_{cat} by k_{react} (see Table II). The values of k_{cat}/k_{react} show not only that the burst is more than stoichiometric but also that the burst size is pH dependent. The burst size decreases from ~33 at pH 7 to ~4 at pH 8.5 and does not change much as the pH is increased further (Table II). These results suggest that the pre-steady-state reaction is not simple, and we therefore decided to measure the burst size directly by determining the amount of product formed during the pre-steady-state period. This was accomplished by monitoring the product formation at 280 nm. The results of these studies, which yield much more information than just the burst size, are discussed below.

Absorbance Changes at 280 nm. When the A_{280nm} of incubations of β-lactamase and penicillanic acid sulfone is followed as a function of time at pH 8.0, 9.0, and 9.6, the results shown in Figures 3–5, respectively, are obtained. When the initial sulfone concentration is higher than the enzyme

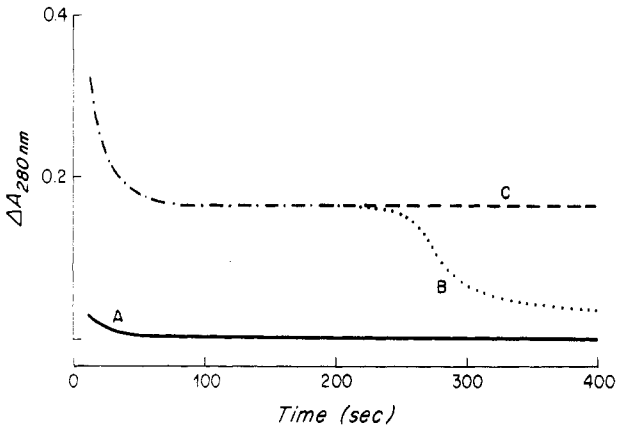


FIGURE 3: Absorbance changes at 280 nm upon β-lactamase-catalyzed hydrolysis of penicillanic acid sulfone in 0.1 M *N*-ethylmorpholinium chloride buffer, pH 8.0. The enzyme concentration was 3.4 μM. Curves A–C were obtained with equimolar concentration and 56- and 112-fold molar excesses of sulfone, respectively.

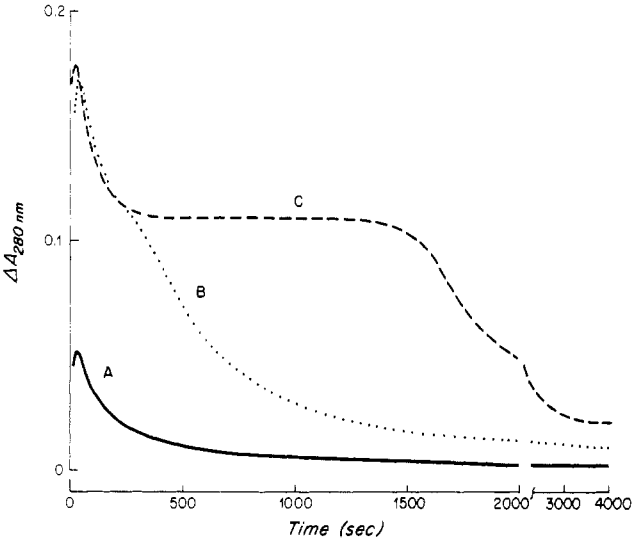


FIGURE 4: Absorbance changes at 280 nm upon β-lactamase-catalyzed hydrolysis of penicillanic acid sulfone in 0.25 M *N*-ethylmorpholinium chloride buffer, pH 9.0. The enzyme concentration was 3.5 μM. Curves A–C were obtained with equimolar concentration and 5.8- and 14.4-fold molar excesses of sulfone, respectively.

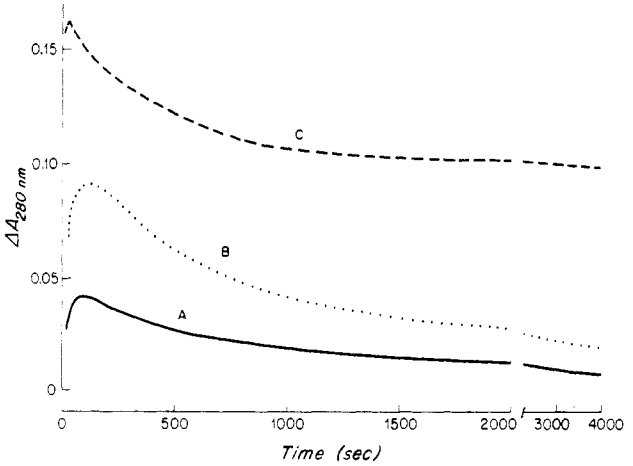
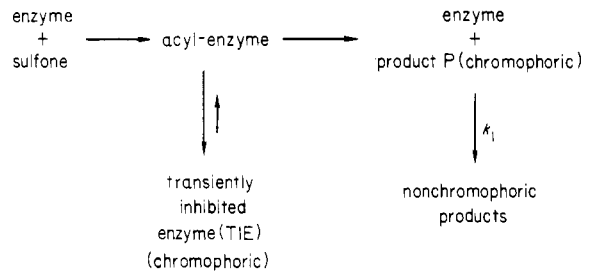


FIGURE 5: Absorbance changes at 280 nm upon β-lactamase-catalyzed hydrolysis of penicillanic acid sulfone in 0.17 M potassium carbonate buffer, pH 9.6. The enzyme concentration was 3.5 μM. Curves A–C were obtained with equimolar concentration and 2.5- and 24-fold molar excesses of sulfone, respectively.

concentration, these plots are clearly multiphasic. There are four distinct phases: an initial increase in A_{280nm} to a maxi-

Scheme I: Outline Pathway To Accommodate Changes in A_{280nm} Shown in Figures 3-5



mum, a decrease to a steady-sate level, the maintenance of the steady-state level, and a final decrease almost to the base line. These four phases are discussed in turn, according to the pathway shown in Scheme I.

Both the initial increase in A_{280nm} and the ensuing decrease are fast at pH 8.0, and the maximum cannot be observed with a conventional spectrophotometer (see Figure 3). At pH 9.0, however, the tail end of the increase can be seen (Figure 4). If the sulfone concentration is raised above the highest shown in Figure 4 (curve C has a sulfone concentration of 50 μM), to 140 μM, the maximum can no longer be detected, suggesting that at pH 9.0, $K_s > 50$ μM. The ensuing decrease in A_{280nm} does not depend on the sulfone concentration, and the explanation for faster achievement of the A_{280nm} maximum at high sulfone concentrations cannot be sought in the events following the initial burst. At yet higher pH values (pH 9.6, Figure 5), the initial maximum in A_{280nm} is reached more slowly, which may reflect an increase in K_s with increase in pH (though the longer time required to reach the maximum is at least partly due to a decrease in the rate of the ensuing absorbance decay at higher pH values). Such an increase in K_s would parallel the behavior of the K_m for benzylpenicillin (see Table I).

It is evident from the results shown in Figure 4 that the maximum *value* of A_{280nm} is saturatable with respect to the sulfone. Thus, a 5.8- and a 14.4-fold molar excess of sulfone produce about the same maximum, as does a 40-fold excess of sulfone (data not shown). This saturation behavior suggests that the attainment of the maximum absorbance at 280 nm signals the completion of the conversion of the enzyme to the transiently inhibited form. That is, as far as the enzyme species are concerned, steady state is arrived at about the time that A_{280nm} reaches a maximum. From the A_{280nm} observed when the molar ratio of enzyme to sulfone is unity, we may estimate the number of sulfone molecules consumed during the burst when the sulfone is added in excess. This provides an independent measure of the number of turnovers required to convert all of the enzyme to its transiently inhibited form. The burst sizes thus calculated are ~10, ~3.5, and ~3.5, at pH 8.0, 9.0, and 9.6, respectively, which are in excellent agreement with the k_{cat}/k_{react} ratios at these pH values listed in Table II. The initial increase in A_{280nm} represents the rapid production of a number of molecules of chromophoric product (P, Scheme I), while the enzyme accumulates into the transiently inhibited form.

The second phase of the reaction, in which the A_{280nm} decays to a steady-state level, is described exactly by the first-order rate law, and rate constants of 7.2×10^{-2}, 1.3×10^{-2}, and 2.2×10^{-3} s^{-1} were obtained for the reactions at pH 8.0, 9.0, and 9.6, respectively. What does this decay represent? Consideration of the amount of sulfone required to tie up the enzyme in the transiently inhibited form suggests an obvious answer to this question. Since during the pre-steady-state portion of the enzymic reaction a burst of product P absorbing at 280 nm is observed (Figures 3–5), the *decay* of this absorbance could represent the decomposition of the product resulting in the loss of the 280-nm chromophore (k_1 in Scheme I). Convincing evidence that this is indeed the case has been obtained by generating the putative chromophoric product P nonenzymatically (see below) and comparing its rate of disappearance with the rate of decay of the A_{280nm} maximum in the enzyme-catalyzed reaction. The first-order rate constants for these two processes were found to be identical at each pH value. Since in the enzyme-catalyzed reaction at the steady state the chromophoric product is being made continuously (via k_{cat}) while that produced during the pre-steady-state burst reaction is disappearing (via k_1), one may question whether the observed decay of the A_{280nm} should reflect only the rate of disappearance of the product. That this is true is shown in Appendix II.

During the third phase of the reactions shown in Figures 3–5, there is a substantial absorbance at 280 nm at the steady state, some of which is due to free product (P) in solution and some to the transiently inhibited enzyme (TIE). At pH 9.0, the burst size is 3.5 molar equiv, and at the point where all of the enzyme is in the transiently inhibited form (i.e., when A_{280nm} is maximal), $[TIE] = [E]_{total}$ (i.e., the concentration of transiently inhibited enzyme, [TIE], is equal to the total enzyme concentration) and $[P] = 2.5[E]_{total}$. The maximal value of A_{280nm}, $(A_{280nm})_{max}$, is therefore given by eq 1. When

$$(A_{280nm})_{max} = [E]_{total}\epsilon_{TIE} + 2.5[E]_{total}\epsilon_P \quad (1)$$

the A_{280nm} drops to a steady-state level, [TIE] still equals $[E]_{total}$ and $[P]_{ss} = k_{cat}[E]_{total}/k_1$ (see Appendix II). The steady-state value of A_{280nm}, $(A_{280nm})_{ss}$, is then

$$(A_{280nm})_{ss} = [E]_{total}\epsilon_{TIE} + k_{cat}[E]_{total}\epsilon_P/k_1 \quad (2)$$

The only two unknowns in eq 1 and 2 are ϵ_{TIE} and ϵ_P. Substituting the known values of $[E]_{total}$, k_{cat}, k_1, $(A_{280nm})_{ss}$, and $(A_{280nm})_{max}$, ϵ_{TIE} and ϵ_P are found to be $(2.4 \pm 0.3) \times 10^4$ and $(1.3 \pm 0.2) \times 10^4$ M^{-1} cm^{-1}, respectively. A similar calculation using the data obtained at pH 9.6 yields $\epsilon_{TIE} \simeq 2.0 \times 10^4$ and $\epsilon_P \simeq 1.2 \times 10^4$ M^{-1} cm^{-1}, in gratifying agreement with the results from the experiments at pH 9.0. In further support of this analysis is the fact that P, generated nonenzymatically, has an independently measured extinction coefficient of $(1.30 \pm 0.05) \times 10^4$ M^{-1} cm^{-1} at 280 nm (see below).

The fourth phase of the reactions shown in Figures 3–5 describes two events: the disappearance of the product P present at steady state (via k_1) and the disappearance (reactivation) of the transiently inhibited enzyme. From independent experiments it is known that at a given pH between 7.0 and 9.0, the disappearance of P is faster than that of the transiently inhibited enzyme (see below). Since (a) P disappears faster than the transiently inhibited enzyme, (b) $[P]_{ss} < [TIE]$ at about pH 8.5 (see eq A13 of Appendix II, and Tables II and III), and (c) $\epsilon_P < \epsilon_{TIE}$, the rate constant for the disappearance of the transiently inhibited enzyme may be calculated, above pH 8.5, from the decay of A_{280nm} at the end of the reaction. At pH 9.0, the rate constant obtained this way (2.2×10^{-3} s^{-1}) is in excellent agreement with the k_{react} obtained by monitoring reactivation by benzylpenicillin hydrolysis (see Table II). At pH 9.6 the latter method could not be used (see Experimental Procedures), and the reactivation rate constant (~5.3×10^{-4} s^{-1}) was obtained only from the final A_{280nm} changes of Figure 5.

Nature of Transiently Inhibited Enzyme and First-Formed Chromophoric Product. The normal product from the β-lactamase-catalyzed hydrolysis of a penam substrate is the

Table III: First-Order Rate Constants for Disappearance of Chromophoric Product from Penicillanic Acid Sulfone

pH	buffer concn (M)	k_{obsd}[a] (s^{-1})	k_i[b]
7.05[c]	0.1	2.9×10^{-1}	
7.10[c]	0.01	1.8×10^{-1}	
8.00[d]	0.1	8.6×10^{-2}	7.2×10^{-2}
8.00[d]	0.01	6.8×10^{-2}	
8.53[d]	0.1	3.0×10^{-2}	
9.02[d]	0.25	1.3×10^{-2}	1.3×10^{-2}
9.58[e]	0.17	2.8×10^{-3}	2.2×10^{-3}
9.58[e]	0.01	1.4×10^{-3}	
10.60[e]	0.10	6.8×10^{-4}	
12.0		2.7×10^{-4}	

[a] Determined from the fall in A_{280nm} in a product sample generated by brief incubation of penicillanic acid sulfone with 0.5 N NaOH (see Experimental Procedures). [b] Determined from the fall in A_{280nm} to the steady-state level, in the enzyme-catalyzed reactions shown in Figures 3–5. [c] Sodium phosphate buffer. [d] *N*-Ethylmorpholinium chloride buffer. [e] Sodium carbonate buffer.

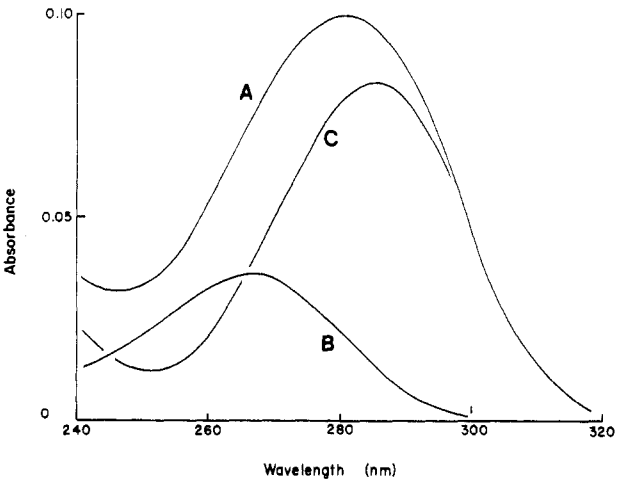


FIGURE 6: Spectrum of transiently inhibited β-lactamase at pH 9.0. (A) Difference spectrum of penicillanic acid sulfone plus β-lactamase, measured after the decay of A_{280nm} to a steady-state level. The sample cell contained β-lactamase (2.94 μM) and penicillanic acid sulfone (175 μM) in 0.25 M *N*-ethylmorpholinium chloride buffer, pH 9.0. The reference cell contained an identical concentration of the enzyme in the same buffer. (B) The spectrum of enamine **4**. The steady-state concentration of **4** (under the conditions of spectrum A) was calculated to be 1.8 μM by using eq A13 of Appendix II, and an independently determined spectrum of curve B scaled appropriately. (C) The spectrum of the transiently inhibited enzyme, obtained by subtracting curve B from curve A. This spectrum has $\lambda_{max} \simeq 286$ nm and $\epsilon \simeq 29\,000$ M^{-1} cm^{-1}.

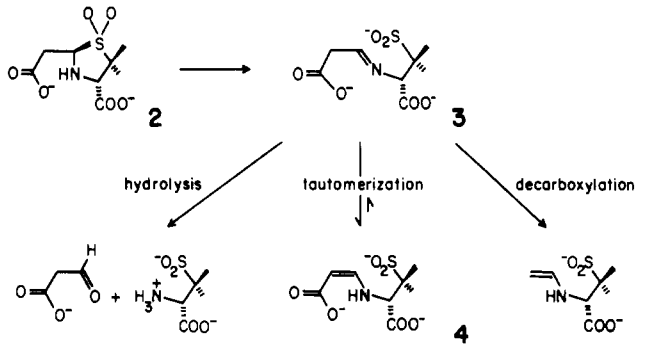
corresponding penicilloic acid, and, by analogy, the product from the enzyme-catalyzed reaction of penicillanic acid sulfone, **1**, might at first sight be formulated as **2** (Scheme II). Since, however, thiazolidine sulfones that bear no substituent on nitrogen, such as **2**, suffer rapid ring scission (Woodward et al., 1949), the imine **3** (Scheme II) should better describe the reaction product. Yet **3** is itself a labile species, which can hydrolyze, tautomerize to the enamine **4** [this is expected to be a very facile process: see, e.g., Guthrie & Jordan (1972)], or decarboxylate. These processes are illustrated in Scheme II. The formation of β-(penicillamine sulfinate) acrylate derivatives such as **4** from nucleophilic attack on penam sulfones has been known for a long time (Woodward et al., 1949; see also Cartwright & Coulson (1979)], and such compounds typically absorb strongly around 260–300 nm with large extinction coefficients ($\epsilon \simeq 10\,000$–$30\,000$ M^{-1} cm^{-1}) (Ostercamp, 1970; Greenhill, 1977). The pathways shown in Scheme II are supported by the transient appearance of a chromophoric species having λ_{max} near 280 nm when penicillanic acid sulfone is treated with base (see below), by the identification of the sulfinate of penicillamine (Brenner & Knowles, 1981), and by the identification of the sulfinate of penicillamine and phenoxyacetylglycinaldehyde from the hydrolysis of 6β-(phenoxyacetamido)penicillanic acid sulfone [C. Kemal, experiments quoted in Fisher et al. (1981)].

Since there is now good evidence for an acyl-enzyme intermediate in the catalytic cycle of the β-lactamase (Fisher et al., 1980) and for acylation of a unique serine residue by β-bromopenicillanic acid (Knott-Hunziker et al., 1979), by quinacillin sulfone (Fisher et al., 1981), and probably by 6-chloropenicillanic acid sulfone (Cartwright & Coulson, 1980), it seems reasonable to conclude that the transiently inhibited form of the enzyme is an ester (on an enzyme serine residue) of the enamine **4**. The interaction of the β-lactamase with penicillanic acid sulfone is thus analogous to the alcoholysis of other penam sulfones: after attack of the alcohol on the β-lactam, the thiazolidine ring opens up, leading to the formation of an ester of β-(penicillamine sulfinate) acrylate. The absorption spectrum of this species is derived in Figure 6. It should be noted that on the basis of the unusual spectral characteristics of the β-aminoacrylate chromophore [$\lambda_{max} \sim 280$ nm ($\epsilon \simeq 20\,000$ M^{-1} cm^{-1})], the transiently inhibited enzymes formed by the interaction of clavulanic acid with the TEM-2 β-lactamase (Charnas et al., 1978) and with the β-lactamase from *Staphylococcus aureus* (Cartwright & Coulson, 1979) have been assigned analogous structures.

Nonenzymic Generation of Enamine 4 and Its pH-Dependent Rate of Hydrolysis. From the foregoing discussion it will be clear that the immediate product (**2** or **3**) arising from the hydrolysis of penicillanic acid sulfone will have only a transient existence and that some of it, at least, should be converted to the enamine **4**. On the basis of the behavior of analogous compounds, however, **4** is expected to be rather short-lived and have a half-life of seconds to minutes at neutral pH (Guthrie & Jordan, 1972; Dixon & Greenhill, 1974). Since in the pH range of 2.6–9.6 the half-life of penicillanic acid sulfone in the absence of enzyme is measured in days, there is no hope of accumulating **4** as a product in this pH range. The hydrolysis of the parent sulfone should, however, be base catalyzed, and it seemed likely that at high pH the rate of hydrolysis of the sulfone (to yield **4**) would become faster than the rate of hydrolytic fragmentation of **4**. This expectation was reinforced by indications (Dixon & Greenhill, 1974) that the stability of **4** should *increase* with increasing pH. The hydrolysis of penicillanic acid sulfone was therefore investigated in 0.5 N NaOH. As expected, the rate of hydrolysis of the β-lactam is quite fast at this pH ($t_{1/2} \simeq 20$ s), and the immediate hydrolysis product disappears at a slower rate ($t_{1/2} \simeq 8.5$ min) than the rate of its formation. This product has spectral properties entirely consistent with the structure of **4** [λ_{max} 267 nm ($\epsilon \simeq 20\,000$ M^{-1} cm^{-1})]. The rate of disappearance of the chromophoric product, generated from penicillanic acid sulfone in 0.5 N NaOH, has been studied as a function of pH and buffer concentration (Table III). Buffer catalysis is not strong, the rate rising <2-fold on increasing the buffer concentration 10-fold at pH 7.0 and 8.0.

That the product generated from sulfone in NaOH is identical with the first product released in the enzyme-catalyzed reaction is indicated by the following: (a) the rates of disappearance of the two materials are essentially identical at pH 8.0, 9.0, and 9.6; (b) the extinction coefficients of both species are the same at 280 nm; (c) the two materials each decompose to yield products having the same pH-dependent

Scheme II



spectral characteristics [at pH 7, λ_{max} 224 nm (sh) (ϵ = 3150 M^{-1} cm^{-1}); at pH >12, λ_{max} = 257 nm (ϵ = 16 500 M^{-1} cm^{-1}); p$K_a \simeq 11$]. In summary, these results indicate that enamine **4** is the immediate observable product from the enzyme-catalyzed hydrolysis of penicillanic acid sulfone.

Mechanism of Reaction of β-Lactamase with Penicillanic Acid Sulfone. The results presented in this paper must now be examined with respect to the outline scheme proposed earlier on the basis of the behavior of the 6-deuterated analogues of penicillanic acid sulfone (Brenner & Knowles, 1981). We have suggested that attack by serine-70 of the β-lactamase on the β-lactam carbonyl group leads to a tetrahedral intermediate that collapses to give the enzyme-bound imine (A in Scheme II of the preceding paper). This acyl-enzyme may suffer deacylation to the imine **3** (Scheme II) which very rapidly tautomerizes to the chromophoric enamine **4**, as discussed above. Tautomerization of the acyl-enzyme competes with deacylation and leads to the *bound* enamine B (Scheme II of the preceding paper), which is the transiently inhibited enzyme characterized above. The only alternative candidate for the acyl-enzyme A is the acyl-enzyme corresponding to **2** (Scheme II) in which the thiazolidine ring is still intact. If this were the structure of the acyl-enzyme, the transiently inhibited enzyme (B) would have to be formed either by a β elimination across C-6 and C-5 or via the imine corresponding to **3**. The latter is preferred, both because opening of thiazolidine sulfones to the imine is extremely facile whereas β elimination of aliphatic sulfones is much less so (Wallace et al., 1963), and also because our deuterium labeling results require that such an elimination would have to proceed with syn stereochemistry (Brenner & Knowles, 1981). Further, the kinetic consequences of deuterium substitution in penicillanic acid sulfone are inconsistent with the β-elimination pathway for the generation of the transiently inhibited enzyme, B (Brenner & Knowles, 1981).

The only question that remains relates to the pathway by which native enzyme is regenerated from the transiently inhibited species, B (Scheme II of the preceding paper). Three paths must be considered. The first (pathway i) involves a reversal of the tautomerization step (i.e., B → A, in Scheme II of the preceding paper) to form the imine A that then deacylates, the second (pathway ii) involves the hydrolysis of the imine before deacylation of the ester of malonsemialdehyde, and the third (pathway iii) involves the direct deacylation of B. Each of these paths is consistent with the slow recovery of enzyme activity: in (i) and (ii) the conversion of B to A is thermodynamically unfavorable, and in (iii) the deacylation of an α,β-unsaturated ester (B) will be slower than that of a saturated ester (A).

First, it is clear that (ii) is not the major pathway. The ϵ_{280nm} value of the chromophoric product (**4**) generated by the enzyme agrees with that of the product generated by the action of NaOH on the sulfone. While it is not known whether the conversion of the sulfone to **4** by NaOH is quantitative, the large calculated value of ϵ ($\sim$20 000 M^{-1} cm^{-1}), *based on the sulfone*, strongly suggests that the conversion to **4** occurs in high yield. It is likely, then, that **3** is the major, if not the only, product released from the enzyme and that **3** rapidly tautomerizes to give **4**.

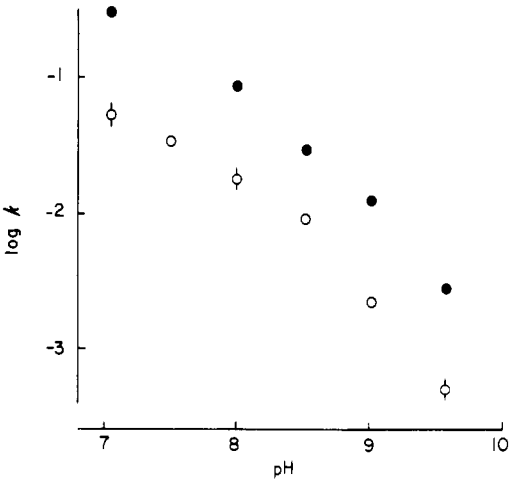


FIGURE 7: Rate constants for hydrolysis of enamine **4** (Scheme II) (●) and for reactivation of transiently inhibited β-lactamase B (Scheme II of the preceding paper) (○).

Of the two remaining possibilities, pathway iii is the hydrolysis of the α,β-unsaturated ester and pathway i involves tautomerization of the enamine to the imine followed by the normal deacylation route. The reactivation reaction is faster at low pH (Table II), and the pH dependence of this process is very similar to that of the fragmentation of **4** as measured by k_{obsd} (Table III). These data are plotted together in Figure 7. Since the enamine **4** hydrolyzes via the corresponding imine **3** (Guthrie & Jordan, 1972; Dixon & Greenhill, 1974), the close similarity of the pH dependences of the decomposition of **4** and of the transiently inhibited enzyme B (Scheme II of the preceding paper) suggests that B, too, fragments via prior tautomerism to the imine A, which then deacylates. It thus appears that pathway i is the favored one and that both the fragmentation of **4** and the decomposition of B proceed through an imine intermediate, the formation of which is (at least partially) rate determining. If the transiently inhibited enzyme deacylated directly, we should not, a priori, expect it to exhibit a pH–rate profile so similar to that observed for the disappearance of the enamine **4**.

The mechanistic scheme developed in this and the preceding paper to account for the behavior of penicillanic acid sulfone with the β-lactamase appears to be a paradigm for a number of β-lactamase inactivators, such as clavulanic acid and the sulfones of quinacillin and methicillin. These penam analogues share with penicillanic acid sulfone many of the kinetic and spectroscopic features outlined here, and it seems likely that in part, they follow similar pathways. Attack of a serine hydroxyl group at the β-lactam carbonyl yields a tetrahedral intermediate that may collapse to an acyl-enzyme in which the thiazolidine (or in the case of clavulanate, oxazolidine) ring has opened, yielding an imine. This imine may suffer a number of fates, amongst which are hydrolysis (regenerating free enzyme), tautomerism (reversibly forming a transiently stable inhibited enzyme), and further attack by enzyme nucleophiles (resulting in irreversible enzyme inactivation). The relative importance of these different pathways depends, of course, on the precise chemistry involved, and the partitioning ratios can be changed by such alterations as isotopic substi-

tution (Brenner & Knowles, 1981), pH variation (this paper), and peripheral structural changes [e.g., the sulfones of quinacillin and phenoxymethylpenicillin (Fisher et al., 1981)]. While these effects may radically change the behavior of a given penam derivative in terms of its interaction with the purified β-lactamase in vitro, the relationship between these interactions and the utility of β-lactamase inactivators as synergists with β-lactam antibiotics in vivo is less clear. The local concentration of an inactivator that can be achieved in vivo may well be inadequate to inactivate the β-lactamase irreversibly, and the synergistic effects of β-lactamase inactivators may derive more from their ability to preoccupy the β-lactamase (by forming the transiently inhibited enzyme) for long enough to allow the antibiotic access to its target.

Acknowledgments

We are grateful to the Pfizer group for a generous gift of penicillanic acid sulfone (CP 45,899) and to Daniel Brenner for helpful suggestions.

Appendix

(I) The kinetics of the hydrolysis of a good substrate in the presence of an enzyme sample that is slowly recovering catalytic activity are described by eq A1 and A2

$$E' \xrightarrow{k_{react}} E \tag{A1}$$

$$E + S \rightleftharpoons E{\cdot}S \xrightarrow{k_{cat}} E + P \tag{A2}$$

where E′ is the transiently inhibited enzyme, k_{react} is the first-order rate constant for the conversion of E′ to active enzyme, S is a good substrate, and k_{cat} is the turnover number for S. In general, after dilution of a solution of E′ into a solution of S, some of the enzyme will be in the transiently inhibited form, E′, and some in the fully active form, E. At any time t, the hydrolysis of S will be catalyzed by the active enzyme present

$$[E]_t = [E] + [E]_0 \tag{A3}$$

where E is the active enzyme generated from E′ and E_0 is the active enzyme present at $t = 0$. As long as S is at saturating concentrations, the reaction velocity at any instant is

$$-d[S]/dt = k_{cat}[E]_t \tag{A4}$$

Now, since

$$[E] = [E']_0(1 - e^{-tk_{react}}) \tag{A5}$$

we may write

$$[E]_t = [E']_0 + [E]_0 - [E']_0e^{-tk_{react}} \tag{A6}$$

from which

$$-d[S]/dt = k_{cat}([E']_0 + [E]_0) - k_{cat}[E']_0e^{-tk_{react}} \tag{A7}$$

Since $[E']_0 + [E]_0$ equals the total enzyme present, eq A7 may be written

$$-d[S]/dt = V_{max} - k_{cat}[E']_0e^{-tk_{react}} \tag{A8}$$

where V_{max} is the velocity attained after all of the transiently inhibited enzyme has reactivated. Equation A8 may be integrated to

$$[S]_0 - [S] = V_{max}t + [(E']_0k_{cat}/k_{react})(e^{-tk_{react}} - 1) \tag{A9}$$

where $[S]_0$ is the concentration of substrate at $t = 0$. Rearranging eq A9, we obtain

$$\ln([S]_0 - [S] - V_{max}t + [E']_0k_{cat}/k_{react}) = \ln([E']_0k_{cat}/k_{react}) - tk_{react} \tag{A10}$$

If the left-hand side of eq A10 is plotted against t, a line with a slope equal to k_{react} should be obtained. To do this requires assigning a value for $[E']_0k_{cat}/k_{react}$. A computer program was written to allow variation of this term until the best least-squares fit to eq A10 was obtained. Note that in the term $[E']_0k_{cat}/k_{react}$, k_{cat} is known, $[E']_0$ may be estimated from the initial slope of the progress curve, and an approximate value for k_{react} may be estimated from the time required for complete reactivation of E′. If eq A10 correctly describes the reactivation process, the natural logarithm of the derived value of $[E']_0k_{cat}/k_{react}$ should equal the intercept of the line from eq A10. This was found to be the case, the deviation of this intercept from the derived value being always <1%.

(II) Consider an enzyme-catalyzed reaction that produces a chromophoric product, P, which decomposes nonenzymically (after release into free solution) with a first-order rate constant k_1. Now, if in a pre-steady-state burst reaction, an amount of P is produced such that its concentration is higher than the steady-state level, it will decay according to

$$-d[P]/dt = k_1[P] - V_{max} \tag{A11}$$

where V_{max} is the zero-order rate constant for the enzyme-catalyzed production of P. Integration of eq A11 gives

$$[P] = \{(k_1[P]_0 - V_{max})e^{-k_1t} + V_{max}\}/k_1 \tag{A12}$$

where $[P]_0$ is the concentration of P after the burst. [This corresponds to the maxima in the plots of A_{280nm} vs. time, shown in Figures 3–5.] When the chromophore has reached a steady-state level, $d[P]/dt = 0$ and the concentration of P at the steady state ($[P]_{ss}$) is

$$[P]_{ss} = V_{max}/k_1 = k_{cat}[E]_{total}/k_1 \tag{A13}$$

From eq A13 and A12, we obtain

$$\ln([P] - [P]_{ss}) = \ln\{(k_1[P]_0 - V_{max})/k_1\} - k_1t \tag{A14}$$

and a plot of $\ln([P] - [P]_{ss})$ vs. t yields k_1. In practice, $A_{280nm} - (A_{280nm})_{ss}$ is used in place of $[P] - [P]_{ss}$, the proportionality constant appearing in the intercept of the semilogarithmic plot, rather than in the slope (from which k_1 derives).

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Structure of Transcriptionally Active and Inactive Nucleosomes from Butyrate-Treated and Control HeLa Cells†

Patricia A. Egan and Beatriz Levy-Wilson*

ABSTRACT: Nuclei from butyrate-treated or control HeLa cells were separated into micrococcal nuclease sensitive and resistant chromatin. Those regions most sensitive to the nuclease, amounting to some 10% of the chromatin, consisted mainly of mononucleosomes with equimolar amounts of the inner histones H2A, H2B, H3, and H4, very little H1, and equimolar amounts of the two small high-mobility group (HMG) proteins, HMG-14 and -17. Both in butyrate-treated and in control cells, these nuclease sensitive monomers were some 5–7-fold enriched in DNA sequences which are transcribed into cytoplasmic polyadenylated RNA, while resistant monomers are depleted in the same sequences. Electrophoretic analyses of the transcriptionally active mononucleosomes revealed heterogeneity. Several subcomponents were resolved when monomers of butyrate-treated or control cells were electrophoresed at low ionic strength. Active monomer subcomponents differ in their molar content of HMG-14 and -17, in their content of H1 and A24, and in the length of their DNA. Some minor differences between nucleosomes of butyrate-treated and control cells were observed.

Sodium butyrate, at millimolar concentrations, promotes a number of morphological and biochemical changes in a variety of mammalian cell lines without causing cell death. These changes include growth inhibition and morphological alterations in HeLa cells, Chinese hamster ovary (CHO) cells, and neuroblastoma cells (Ginsberg et al., 1973; Wright, 1973; Henneberry & Fishman, 1976), increases in tyrosine hydroxylase (Prasad & Sinha, 1976) and adenylate cyclase activity in neuroblastoma cells, and induction of erythroid differentiation in erythroleukemic cells (Leder & Leder, 1975). Sodium butyrate also induces the hyperacetylation of histones, especially H3 and H4. This effect results from the inhibition of the enzyme histone deacetylase (Candido et al., 1978; Vidali et al., 1978; Sealy & Chalkley, 1978). However, this is reversed upon the removal of sodium butyrate, and the cells regain their normal morphology, and their histones return to the nonacetylated state (Vidali et al., 1978).

Recent work by Reeves & Cserjesi (1979) indicates that sodium butyrate affects the patterns of gene expression in cultured Friend erythroleukemic cells. Thus, 24 h after treatment of these cells with sodium butyrate, not only do the histones become hyperacetylated but also there occurs an induction of the expression of 3800 new unique gene products not found in control cells. In addition, two-dimensional gel electrophoretic analysis of the butyrate-treated cells shows that these cells synthesize many new species of proteins that are undetectable in control cells. These changes are reversed once the fatty acid is removed. These finding suggest that histone hyperacetylation may be involved in the biochemical mechanisms promoting new genomic expression in these cells.

Motivated by these interesting observations, we addressed the question as to whether significant alterations occur in the structure of transcriptionally active and inactive chromatin after exposure of HeLa cells to sodium butyrate. To this end, we chose to investigate the following issues: First, we attempted to fractionate chromatin from HeLa cells into transcriptionally active and inactive moieties. We then characterized in detail the structure and properties of transcriptionally active nucleosomes from control HeLa cells and made further careful comparisons of these characteristics with those of nucleosomes obtained from butyrate-treated cells. Ultimately, we wish to elucidate whether sodium butyrate can alter qualitatively or quantitatively the sets of genes which are found in an active conformation in these human neoplastic cells.

Materials and Methods

Cell Cultures. HeLa cells, strain S3, were grown in minimum Eagle's media, supplemented with 7% bovine calf serum. Penicillin at 100 units/mL and streptomycin sulfate at 100 μg/mL were also added. Growth was at 37 °C under 5% CO_2 (sodium bicarbonate buffer), in roller bottles (Corning), to a density of 5×10^7 cells/bottle.

Preparation of Nuclei. We followed the procedure of Milcarek et al. (1974) with some modifications. In brief, cells were first chilled on ice for 5 min, scraped from the bottles, and spun 10 min at 2000 rpm in a Sorvall GSA rotor. The cell pellet was resuspended in 10 volumes of buffer 1 (10 mM NaCl, 40 mM Tris, pH 8.3, and 1.5 mM $MgCl_2$), supplemented with poly(vinyl sulfate) at 25 μg/mL and spermine at 35 μg/mL. The cells were lysed by the addition of Nonidet P-40 to 0.5% for 2 min on ice. Nuclei were harvested by

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