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Rate Enhancement Specificity with α-Chymotrypsin: Temperature Dependence of Deacylation†

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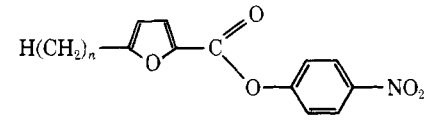
ABSTRACT: The relative rate of the hydrolysis of 2-(5-*n*-alkyl)furoyl-α-chymotrypsin reaches a maximum with the propyl derivative. The Arrhenius plots for the hydrolyses of the 2-furoyl-, 2-(5-ethyl)furoyl-, and 2-(5-*n*-propyl)furoyl-α-chymotrypsins display a discontinuity, while the plots obtained with the remaining furoyl derivatives 5-methyl, 5-*n*-butyl, and 5-*n*-amyl are linear. We conclude that the deacylation of the furoyl derivatives of α-chymotrypsin involves a minimum of two elementary reaction steps. Depending upon the reaction conditions, rate enhancement specificity appears to be either entropy or enthalpy controlled.

The protein α-chymotrypsin is useful for testing theories of rate enhancement, because it is one of the best understood enzymes. The crystal structure of this protein and of various derivatives has been determined (Mavridis et al., 1974; Blow and Steitz, 1970). The reaction pathway has been sufficiently described (Bender and Kezdy, 1965) for a reasonable chance of success in attempting the assignment of activation parameters to elementary reaction steps. The study of homologous substrate series allows the isolation of various factors controlling reaction rates, and a large number of series have been examined (e.g., Dupaix et al., 1973; Fife and Milstien, 1967; Marshall and Akgün, 1971). Therefore, a reasonable explanation of rate enhancement specificity (the variation of enzymatic turnover with different substrates) should be possible with this enzyme. But it is precisely here that a serious discrepancy is found in the literature. The reaction of esters and amides with chymotrypsin occurs through esterification of serine 195 on the enzyme, followed by hydrolysis of the acyl enzyme intermediate liberating free enzyme and acid. The turnover of ester substrates is generally limited by the latter, deacylation step. As a result, the deacylation rate constant can be determined with ester substrates either from steady-state kinetics, or from direct observation of the acyl enzyme decay. Bender and co-workers (1964), Kaplan and Laidler (1966), and Fife and Milstien (1967; with the straight-chain fatty acid series) have reported that rate enhancement specificity is entropy controlled—i.e., faster turnover rates between substrates are a reflection of more positive activation entropies—and that $\Delta H^{\ddagger}$ for different substrates remains relatively constant. Fife and Milstien (1967) simultaneously studied branched chain fatty acid derivatives, with which they observed compensatory changes between activation entropies and enthalpies, but enthalpy control of specificity; i.e., negative changes in both activation enthalpy and entropy accompany faster rates. Martinek et al. (1972) and Marshall and Chen (1973) also studied the straight-chain fatty acids and reported compensatory changes with activation enthalpy control of specificity. Finally, Cane and Wetlaufer (1966) studying the same straight-chain series reported compensatory changes with entropy control; i.e., positive changes in both activation enthalpy and entropy accompany faster rates.

Any successful predictive theory of rate enhancement specificity should include, or be based on, the calculation of activation enthalpies and entropies, and, thus, the resolution of this apparent discrepancy is important before a meaningful theory of chymotrypsin rate enhancement can be formulated. Since the different conclusions cited above may have arisen from the experimental difficulties inherent in accurate measurement of enzyme kinetics, we have reinvestigated this problem paying particular attention to the minimization of experimental errors. We chose to study the homologous series of *p*-nitrophenyl esters of 2-(5-*n*-alkyl)furoic acid



because of the properties of *p*-nitrophenyl 2-furoate hydrolysis described by Inward and Jencks (1965). The acylation of chymotrypsin by these esters is easily monitored from the

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absorbance of the released *p*-nitrophenolate ion. The subsequent deacylation can also be followed spectrophotometrically since the spectra of furoic acids and their corresponding esters differ in the ultraviolet. Moreover, substrate K_M's are low (less than 1 μM at 25 °C, neutral pH's), and acylation is much faster than the subsequent deacylation. For these reasons we have been able to utilize the following protocol in single turnover experiments. A small excess of chymotrypsin is mixed with substrate which is bound rapidly, and virtually completely. When the rapid release of *p*-nitrophenolate is completed, the reaction solution contains a mixture of free and acylated chymotrypsin, but no substrate ester. The hydrolysis of the acyl enzyme can then be observed directly as a pseudo-first-order ultraviolet absorbance change. Use of single turnover experiments eliminates many of the difficulties inherent in steady-state kinetics. For example, there is no need to determine substrate, enzyme, or product concentrations precisely; only the observed absorbance changes are required in the computation of first-order rate constants. Moreover, no corrections for the nonenzymatic hydrolysis of the *p*-nitrophenyl esters are required, and the extrapolations needed to determine V_{max}, and hence the deacylation rate, are avoided.

As we shall report here deacylation rates vary with the length of the alkyl side chain in a manner which suggests that reactivity is dependent on the volume of the acid. However, the Arrhenius plots obtained with some of the substrates indicate a minimum of two rate processes in the deacylation reaction. We shall discuss some possible explanations for this apparent complication and show how our results may explain the literature discrepancies cited above.

Methods and Materials

The 5-*n*-alkylfuroic acids were synthesized by the following methods. 2-*n*-Alkylfurans were formylated using the procedure of Traynelis et al. (1957). The resulting 5-*n*-alkylfuran-2-carboxaldehydes were, without prior purification, oxidized to the corresponding acids with silver oxide. Approximately 20 mmol of the aldehyde was added to a mixture of 60 mmol of silver oxide in 250 ml of 5% (v/v) aqueous sodium hydroxide, and the reaction solution was stirred vigorously for approximately 65 h at room temperature. After filtration and concentration to ca. 60% of the original volume, the acid was precipitated out of solution by the slow addition of concentrated HCl at 5 °C. The acids were then recrystallized from petroleum ether, with an overall yield for the two steps of 50–60%. The melting points and ultraviolet absorption maxima were: 5-methylfuroic acid, 107–109 °C, 255 nm; 5-ethylfuroic acid, 92–94 °C, 256 nm; 5-propylfuroic acid, 62–64 °C, 257 nm; 5-butylfuroic acid, 71–73 °C, 257 nm; 5-amylfuroic acid, 59–61 °C 257 nm. Syntheses of the first three compounds in this series have been reported by others (Runde et al., 1930; Reichstein and Zschokke, 1932; Novitskii et al., 1966). The *p*-nitrophenyl esters were synthesized by the following procedure. Eight millimoles of the acid and nine millimoles of *p*-nitrophenol were dissolved in 50 ml of anhydrous ethyl ether. After the addition of 16 mmol of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, the reaction mixture was stirred at room temperature overnight. The ether solution was then filtered, washed with 0.05 M phosphate buffer, pH 7.0, dried over anhydrous magnesium sulfate, and evaporated to dryness. The solid residue was recrystallized from 95% ethanol. The final yields of the esters were 20–30%. The melting points, ultraviolet absorption maxima, and chemical composition (% C, % H, % N) were: *p*-nitrophenyl 2-furoate, 162–164 °C, 270 nm, (calculated, 56.66, 3.03, 6.01) 56.73, 3.03, 5.86; *p*-nitrophenyl 2-(5-methyl)furoate, 113–115 °C, 282 nm, (calculated, 58.30, 3.67, 5.67) 58.35, 3.70, 5.49; *p*-nitrophenyl 2-(5-ethyl)furoate, 74–76 °C, 283 nm, (calculated, 59.77, 4.24, 5.36) 59.36, 4.45, 5.33; *p*-nitrophenyl 2-(5-*n*-propyl)furoate, 65–67 °C, 283 nm, (calculated, 61.09, 4.76, 5.09) 61.08, 4.80, 5.49; *p*-nitrophenyl 2-(5-*n*-butyl)furoate, 62–64 °C, 287 nm, (calculated, 62.28, 5.23, 4.84) 61.97, 5.23, 5.26; *p*-nitrophenyl 2-(5-amyl)furoate, 79–81 °C, 287 nm, (calculated, 63.36, 5.65, 4.62) 63.11, 5.94, 5.12. The synthesis of the first of this series of esters has been reported previously (Bender et al., 1966). The esters were additionally characterized by the release of *p*-nitrophenol upon base hydrolysis using an extinction coefficient of 5.7×10^3 l. cm^{-1} mol^{-1} at 347 nm, the isosbestic point for the phenol and phenolate ion. By this criterion the esters were found to be 95–100% pure.

Kinetics of the enzyme-catalyzed hydrolysis of *p*-nitrophenyl 2-(5-*n*-alkyl)furoates were measured spectrophotometrically. Stock substrate solutions were prepared by dissolving 20 to 50 μmol of the *p*-nitrophenyl esters in 10 ml of acetonitrile. This stock was added to a solution of 5 μM α-chymotrypsin in 17.0 ml of buffer contained in a thermostated, 5.0-cm path length, spectrophotometer cell, which had been previously allowed to reach thermal equilibrium. The final substrate concentrations varied from 0.87 to 6.1 μM depending upon the experiment. The reaction was first monitored at 347 nm to follow the release of *p*-nitrophenol, and hence the acylation reaction. When no further absorbance change was observed, the wavelength was changed to 245 nm in order to monitor the deacylation of the 5-*n*-alkylfuroyl enzyme. The total absorbance change measured with a Cary 16 spectrophotometer ranged from approximately 0.01 to 0.1 absorbance unit, depending on the reaction conditions. The background absorbance was in the neighborhood of 0.5. The data for the deacylation reaction consisted of voltage readings as a function of time. These were fit with no weighting to the first-order equation:

$$A_t = A_\infty - \Delta A e^{-kt}$$

Steady-state reactions were also performed. Substrate at a final concentration of 1.0–30.0 μM was added to 0.5–1.0 μM α-chymotrypsin. The steady-state release of *p*-nitrophenol was monitored at 347 nm. Spontaneous hydrolysis rates were measured separately and subtracted from the enzymatic rates. The data pairs of weighted initial velocities (mol l.$^{-1}$ min^{-1}) and initial substrate concentrations were fit to the Michaelis–Menten equation. Alkaline saponification rates of the *p*-nitrophenyl 2-(5-*n*-alkyl)-furoates were measured in 10 mM borate, pH 10.5. The reaction was followed by the absorbance increase at 400 nm. By varying the buffer concentration, it was shown that borate does not catalyze the hydrolysis of these esters. When required, the concentration of active sites was determined by burst titration (Caplow and Jencks, 1962).

Apparent first-order rate constants for the deacylation of all of the 5-*n*-alkylfuroylchymotrypsins were measured as a function of pH at more than one temperature. Over the pH range studied, the rate profile resembled the titration curve of a single ionizing group with no apparent enzymatic activity at low pH's. The apparent pK_a associated with the profile as well as the pH independent rate constant, k', were calculated using the equation:

$$k_{obsd} = \frac{K_a}{K_a + (H^+)} k' \quad (1)$$

Data pairs of weighted k_{obsd} and pH were fit to this equation with the gradient expansion algorithm. At temperatures where the rate profile was not measured, the pK_a was estimated by plotting experimental pK_a's vs. the reciprocal of the absolute temperature and extrapolating or interpolating to the desired temperature on the straight line drawn through the points. In the absence of a measured rate profile, k' was determined as follows. Deacylation rate constants (k_{obsd}) were obtained in the region of pH 8.5 (0.2 M phosphate buffer) well above the estimated pK_a's. These observed rate constants were corrected to k' with eq 1 and the graphically computed pK_a's. Although the graphical procedure for the estimation of pK_a is subject to error, the inaccuracy introduced into the calculation of k' is small under our conditions. Since k_{obsd} was obtained at a sufficiently high pH, the corrections applied fell in the range of only 1–10%. The standard deviation of k', $\sigma_{k'}$, is related to those of k_{obsd} and K_a by:

$$\sigma_{k'} = \sigma_{k_{obsd}}{}^2\left[1 + \frac{(H^+)}{K_a}\right]^2 + \sigma_{K_a}{}^2\left[k_{obsd}\frac{(H^+)}{K_a{}^2}\right]^2 \quad (2)$$

For any reasonable estimate of σ_{K_a}, the second term in eq 2 is negligible. An error smaller than 0.5 pH unit in the estimate of pK_a can be shown to contribute an error of less than 2% to the computation of k' under our experimental conditions. We therefore felt it unnecessary to establish experimentally the pK_a for every temperature at which deacylation was measured.

First-order rate constants, steady-state rate constants, and the apparent equilibrium constant associated with k_{obsd} (eq 1) were determined from the experimental data by nonlinear, least-squares regression analysis using the gradient expansion algorithm (Bevington, 1969).

Activation entropies and enthalpies were computed using the equation:

$$k' = \frac{kT}{h} e^{-\Delta H^{\ddagger}/RT + \Delta S^{\ddagger}/R} \quad (3)$$

where k, h, and R are Boltzmann's, Planck's and the gas constant, respectively. For the alkaline saponification results, all data pairs of weighted k_{obsd} and weighted temperature were fit to eq 3 with a method described by Deming (1964), and using a program written in our laboratory. In this procedure $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are estimated by allowing fitting to both experimental variables, the observed rate constant k and T. In contrast, the gradient expansion algorithm, or other commonly employed procedures, allows for fitting to only one experimental variable. While the saponification data were adequately described by equation 3, all the enzymatic results were not, as will be described below. In these cases only portions of the temperature ranges studied were fit to the equation.

α-Chymotrypsin (lot CDS2CA) was purchased from Worthington Biochemical Corp. and was used with no further purification. Stock solutions of enzyme at pH 3.0 were used for all experiments, with the concentration determined from the absorbance at 280 nm (ϵ 5.0 × 10^4, Laskowski, 1961). Furoic acid was purchased from Eastman and recrystallized from benzene. 5-Methylfuran-2-carboxaldehyde, 2-ethylfuran, 2-*n*-propylfuran, 2-*n*-butylfuran, and 2-amylfuran were purchased from K & K Laboratories; 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride was obtained from Aldrich Chemical Company. Acetonitrile was distilled once and stored over molecular sieves. All other chemicals were obtained as reagent grade and used with no purification. All rate measurements were made in a Cary 16 spectrophotometer with a thermostated cell holder. The temperature was maintained within 0.1–0.4 °C depending on the conditions. All data analysis was performed on a Nova 1220 minicomputer.

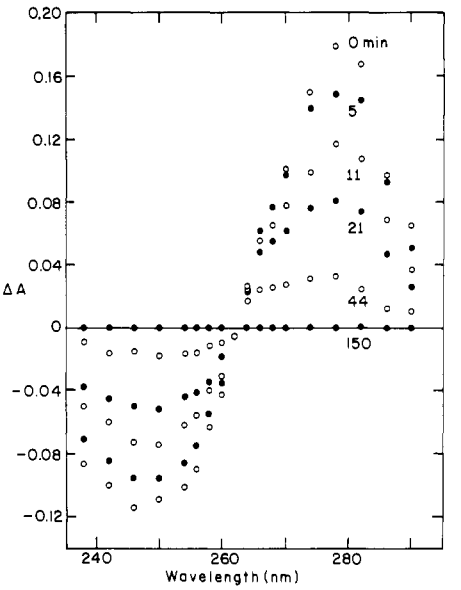

FIGURE 1: Deacylation of 2-(5-ethyl)furoylchymotrypsin. Spectral scans were initiated at the indicated times (scan time approximately 40 s). The difference spectra were computed by subtracting the spectrum obtained at 150 min from the spectra taken at earlier times. The initial concentrations of enzyme and substrate were 30 μM each; 0.2 M phosphate buffer, pH 8.2, μ = 0.6, 0.5% (v/v) acetonitrile.

Results

Inward and Jencks (1965) have reported that 2-furoyl-α-chymotrypsin is rapidly formed upon mixing chymotrypsin and *p*-nitrophenyl 2-furoate. The subsequent deacylation is far slower, and can be monitored by absorbance changes in the ultraviolet. We have verified that all the *p*-nitrophenyl 2-(5-*n*-alkyl)furoates up to a carbon chain length of five react in a similar fashion. In each case a slight excess of enzyme was mixed with substrate. When acylation was complete as measured by the *p*-nitrophenol absorbance at 347 nm, the ultraviolet region was scanned periodically. Typical difference spectra obtained with 5-ethyl-2-furoylchymotrypsin are shown in Figure 1. Of particular importance is the observed isosbestic point, suggesting only two spectrally distinct species. Similar difference spectra were obtained with all the furoyl substrates; peak maxima occurred between 265 and 280 nm, peak minima between 240 and 250 nm.

Using this same procedure, but monitoring at the single wavelength of 245 nm, reaction kinetics were determined. Absorbance increases at this wavelength obey a first-order rate relationship. Since the observed difference spectra are very close to the differences between the alkylfuroyl esters and their corresponding acids, we interpreted this first-order process as the hydrolysis of *n*-alkylfuroylchymotrypsin. Inward and Jencks (1965) have already made this interpretation for the case of *p*-nitrophenyl 2-furoate, and we have extended their observations to two additional substrates in the series. If the 245-nm absorbance change is due

Table I: Comparison of Deacylation Rate Constants Obtained in Steady-State Experiments and Spectral Observation at 245 nm.[a]

5-Alkyl Substituent	pH	V_M/E_0 (min^{-1})	K_M (μM)	k_{245} (min^{-1})
H-	7.00	0.052 (0.003)	0.69 (0.33)	0.0561 (0.0014)
CH_3-	7.11	0.0064 (0.0005)	<0.5	0.00625 (0.00006)
CH_3CH_2-	7.11	0.012 (0.0007)	<0.5	0.0121 (0.00074)

[a] The steady-state and first-order decay experiments were performed as described in the Methods section of the text. The reaction conditions were 0.2 M phosphate buffer, μ = 0.5, and 1.0% (v/v) or less acetonitrile. All reactions were run at 25 °C. The numbers given in parentheses are the estimated standard deviations of the calculated rate constants.

Table II: Deacylation Rate Constants Obtained from the Magnitudes of Burst Titrations and Steady-State Rates Compared with the Results of Spectral Observation at 245 nm.[a]

5-Alkyl Substituent	pH	k_{deacyl} (min^{-1}) Burst Titration	Steady State	k_{245}
CH_3-	7.34	0.00963 (0.00065)	0.0105 (0.0013)	0.00833 (0.00015)
CH_3CH_2-	7.34	0.0216 (0.00062)	0.0178 (0.0043)	0.0172 (0.00074)

[a] The rate constants were obtained as described in the text. The reaction conditions are the same as those given in Table I, except for the indicated pH's. The numbers in parentheses are estimated standard deviations.

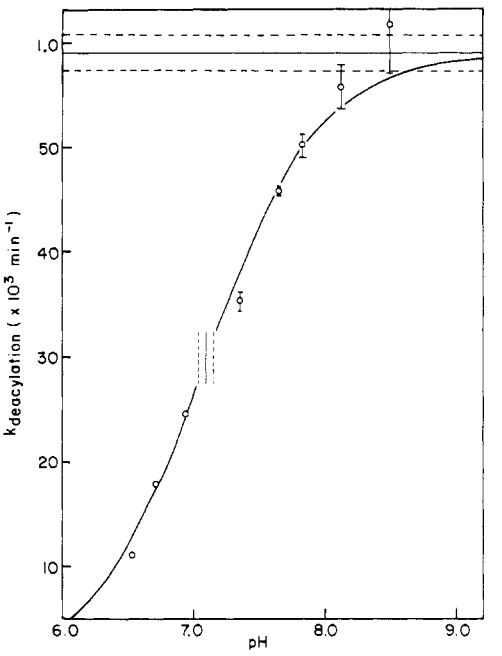

FIGURE 2: pH dependence of 2-(5-*n*-propyl)furoylchymotrypsin deacylation. The apparent first-order rate constants for deacylation were determined at 25 °C as described in the text. The bars associated with each point represent ±1 standard deviation. Where no bars are given, they are smaller than the point itself. The dashed lines represent ±1 standard deviation about the best fit values of the pK_a and the pH-independent rate constant: pK_a = 7.09 (0.06); k' = 59.2 (1.7) × 10^{-3} min^{-1}.

to the deacylation reaction, then the steady-state rate constant obtained with the *p*-nitrophenyl ester should be identical with the rate constant obtained in the single turnover experiment. As shown in Table I this is true with the first three substrates in the series. An additional test was based on the absorbance burst at 347 nm due to rapid release of *p*-nitrophenol during the initial acylation reaction. In the presence of saturating substrate, both the magnitude of the initial burst and the rate of the subsequent slower absorbance change due to steady-state turnover are directly proportional to the concentration of active and, hence, deacylated enzyme present at time zero. On this basis we measured the deacylation rates of the 5-methyl and 5-ethyl enzyme derivatives. After mixing a slight excess of enzyme with substrate, aliquots of the reaction mixture were removed at intervals to determine the burst magnitude and the steady-state turnover using saturating levels of the unsubstituted substrate *p*-nitrophenyl 2-furoate. First-order rate constants for the appearance of free enzyme were calculated with both of these variables and are compared in Table II with the rate constants obtained from the absorbance increase at 245 nm. The constants from the burst experiments are slightly larger than those from the 245-nm absorbance experiments, which may be due to the relative V_M's of the three compounds. Nonetheless, the results of the two experiments are sufficiently similar to support the interpretation that the 245-nm change is a direct measure of enzyme deacylation.

Our next concern was to show that the deacylation reaction was first order only in the concentration of acyl enzyme, and independent of all other components in the reaction mixture. At a total enzyme concentration of 5.0 μM, the deacylation rate constant of 2-furoylchymotrypsin remains constant within experimental error when the substrate, *p*-nitrophenyl 2-furoate, is varied from 0.87 to 6.1 μM (pH 6.20, 0.2 M phosphate, 25 °C). Under the same experimental conditions, but at a fixed substrate concentration of 2.0 μM, the deacylation rate is unchanged upon the addition of the first product, *p*-nitrophenol, and of the second product, 2-furoic acid, over the concentration ranges 0–87 μM, and 0–27 μM, respectively. Thus, *p*-nitrophenol and furoic acid neither stimulate nor inhibit deacylation under our experimental conditions; furthermore, the reverse reactions, acylation of enzyme by furoic acid and reformation of the *p*-nitrophenyl ester, do not make significant contributions to the observed rate constant. The independence of the rate constant on the initial substrate to enzyme concentration suggests that the concentration of free enzyme does not affect deacylation. We were particularly concerned that free chymotrypsin formed during the deacylation might interfere by catalyzing the hydrolysis of the remaining acylated enzyme, especially at the higher temperatures and pH's of the subsequent experiments. This concern appears to be unwarranted; at pH 8.60, 0.2 M phosphate, and 43.8 °C, the deacylation rate of 2-(5-ethylfuroyl)chymotrypsin remains unchanged when substrate is maintained at 4.0 μM and enzyme is varied from 4.2 to 12 μM. If significant autolysis of the acyl enzyme occurs, it is not seen in the measured rate constant. In two final control experiments we established that variation of the phosphate buffer concentration (and, hence, ionic strength) from 0.05 to 0.50 M or of the acetonitrile level (in which the substrate esters were dissolved) from 0 to 0.7% (v/v) does not affect the deacylation of 2-furoylchymotrypsin at pH 7.0 and 25 °C.

Table III: Deacylation Rate Constants—Apparent pK_a and Temperature Dependence.

5-Alkyl Substituent	pK_a, 25 °C[a]	$(k'/k_{OH})_{rel}$[b] 0 °C[c]	25 °C	50 °C[c]
H-	6.95 (0.07)	1.0	0.62	0.44
CH_3-	7.27 (0.05)	0.39	0.28	0.20
CH_3CH_2-	7.33 (0.06)	1.1	0.65	0.52
$CH_3(CH_2)_2$-	7.09 (0.06)	3.8	1.2	1.1
$CH_3(CH_2)_3$-	6.96 (0.09)	0.60	0.46	0.36
$CH_3(CH_2)_4$-		0.095	0.12[c]	0.15

[a] The pK_a's were computed from experimental data with eq 1 as described in the text. Estimated standard deviations are given in parentheses. [b] The enzymatic deacylation rate constant k' has been divided by the pseudo-first-order saponification constants, obtained with the corresponding *p*-nitrophenyl esters at pH 10.5; this corrects for the intrinsic reactivities of the furoyl residues. All corrected rate constants are reported relative to the computed deacylation constant for the furoyl enzyme ester at 0 °C. [c] All the entries at 0 and 50 °C, as well as that for the amyl derivative at 25 °C, were computed from the appropriate activation parameters in Tables IV and V.

Table IV: Activation Parameters for the Deacylation of 2-(5-*n*-Alkyl)furoylchymotrypsins.[a]

5-Alkyl Substituent	Single Process	High Temp	Low Temp
		$\Delta H^{\ddagger}$ (kcal/mol)	
H-		16.92 (0.24)	15.79 (0.52)
CH_3-	17.24 (0.24)		
CH_3CH_2-		18.21 (0.34)	15.78 (0.28)
$CH_3(CH_2)_2$-		19.84 (0.68)	12.19 (0.61)
$CH_3(CH_2)_3$-	17.42 (0.71)		
$CH_3(CH_2)_4$-	20.89 (0.51)		
		$\Delta S^{\ddagger}$ (cal/(mol deg))	
H-		−14.61 (0.77)	−18.23 (1.75)
CH_3-	−17.27 (0.81)		
CH_3CH_2-		−12.47 (1.11)	−20.52 (0.94)
$CH_3(CH_2)_2$-		−6.10 (2.18)	−31.47 (2.09)
$CH_3(CH_2)_3$-	−15.89 (2.28)		
$CH_3(CH_2)_4$-	−7.00 (1.64)		

[a] Activation parameters were calculated as described in the text. For the three substrates which gave nonlinear Arrhenius plots, "high-temperature" $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ were calculated from rate constants obtained above 28 °C. The "low-temperature" values were calculated with the constants obtained at lower temperatures. The figures in parentheses are the estimated standard deviations.

The rate of chymotrypsin deacylation varies similarly with pH for all 2-(5-*n*-alkyl)furoyl derivatives of the enzyme; the pH–rate profile obtained with 2-(5-*n*-propyl)furoylchymotrypsin is presented in Figure 2 as an example. To obtain a pH-independent rate constant, we must either work at a pH well above the pK_a of the observed transition, or we must determine the pK_a in order to calculate this constant with eq 1. In order to avoid high pH's (because of the increased lability of free enzyme), we chose the second alternative. Although the experimental pK_a, calculated from data such as those presented in Figure 2, is assigned to a functional group on the protein, histidine 57 (Bender and Kezdy, 1965), it is known that this constant depends on the ester substrate used (e.g., Dupaix et al., 1973). This is observed with the *p*-nitrophenyl *n*-alkylfuroate series as well (Table III). While the interpretation of this observation can only be conjectured, the practical result is immediately evident; the pK_a must be known for every acyl enzyme derivative at every temperature at which k', the pH-independent rate constant, is to be obtained. In order to avoid an excessive amount of work, the following compromise procedure was used. The deacylation pK_a for each substrate was determined at only a few temperatures. For all other temperatures, the pK_a was estimated by interpolation or extrapolation as described in the previous section. Since deacylations were studied near pH 8.5, the use of these estimated pK_a's in the calculation of pH-independent rate constants introduces negligible errors.

The pH-independent, deacylation rate constants were determined with every substrate, except the amyl derivative, over the temperature range of approximately 10–45 °C. The deacylation of 2-(5-amyl)furoylchymotrypsin is slow, and measurements below 30 °C were judged unreliable so that data were obtained with this derivative only at the higher temperatures. The Arrhenius plots for the methyl, *n*-butyl, and amyl derivatives are linear, but a distinct discontinuity is seen with the *n*-propyl, ethyl, and unsubstituted derivatives. A comparison of the *n*-propyl and *n*-butyl results are presented in Figure 3. The deviation from linearity lies well outside the estimated experimental errors, with the standard deviation of the rate constants at a level of 5% for the worst case. We also considered that the temperature

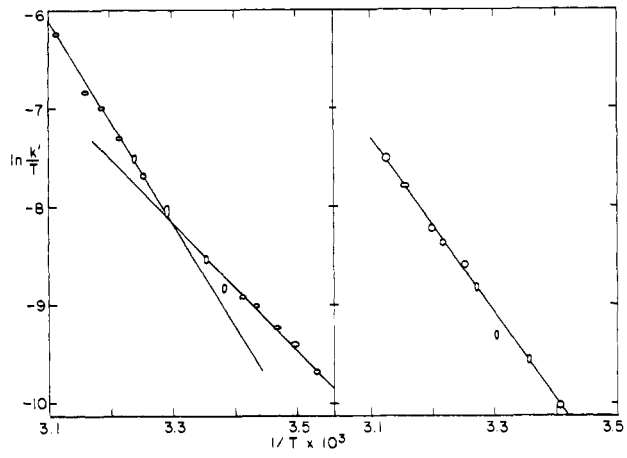

FIGURE 3: Temperature dependence of α-chymotrypsin deacylation. pH-independent rate constants were determined as a function of temperature as described in the text. The estimated standard deviation of each point is represented by the magnitude of the surrounding oval. (A) 2-(5-*n*-Propyl)furoylchymotrypsin; (B) 2-(5-*n*-butyl)furoylchymotrypsin.

data might be fit to a curved Arrhenius plot; i.e., $\Delta C_p^{\ddagger} \neq 0$. A regression analysis was performed on the basis of this assumption. The difference in slopes for the two linear portions of the ethylfuroyl and furoyl data is sufficiently small that a curved Arrhenius plot cannot be ruled out on the basis of statistical considerations. However, the propylfuroyl results clearly do not fit such a scheme and can only be interpreted in terms of a discontinuity. On the principle of interpretive economy, the results of all three were fit to a model of two activation parameter sets. In the three cases of nonlinear behavior, the apparent break in the curve occurs between 20 and 30 °C. The activation enthalpy, $\Delta H^{\ddagger}$, and entropy, $\Delta S^{\ddagger}$, were determined in the methyl, *n*-butyl, and amyl cases by fitting the rate constants obtained above 28 °C to eq 3. For the remaining three compounds, two sets of activation parameters were calculated, one above and one below the apparent discontinuity in the Arrhenius plot (the

Table V: Activation Parameters for the Alkaline Saponification of *p*-Nitrophenyl 2-(5-*n*-Alkyl)furoates at pH 10.50.[a]

5-Alkyl Substituent	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (cal/(deg mol))	k (min^{-1})[b]
H-	19.06 (0.28)	−6.33 (0.92)	0.549 (0.0042)
CH_3-	19.52 (0.31)	−7.05 (1.03)	0.179 (0.0014)
CH_3CH_2-	19.35 (0.29)	−7.63 (0.94)	0.175 (0.0010)
$(CH_3)_2CH_2$-	19.59 (0.22)	−7.01 (0.73)	0.156 (0.0004)
$(CH_3)_3CH_2$-	19.23 (0.31)	−8.24 (1.01)	0.160 (0.0016)
$(CH_3)_4CH_2$-	19.33 (0.22)	−8.04 (0.74)	0.151 (0.0011)

[a] The activation parameters were obtained as described in the text. The numbers in parentheses are estimated standard deviations. These parameters have not been corrected for hydroxide concentration to a standard state, nor for contributions due to the temperature dependence of the buffer ionization. [b] Obtained at 36 °C.

"low" and "high" temperature processes, respectively). The results are presented in Table IV.

The variation of deacylation rates among the 2-(*n*-alkyl)furoylchymotrypsins may be ascribed to differences both in the inherent reactivity of the esters toward hydrolysis, and in the rate enhancement specificity of the enzyme. The following procedure was used to account for the contribution of the first of these effects to the experimentally obtained activation parameters. Since the linear free energy relationship for the deacylation of para- and meta-substituted benzoylchymotrypsins is similar to that for the alkaline saponification of para- and meta-substituted *p*-nitrophenyl benzoates (Caplow and Jencks, 1962), it is reasonable to assume that saponification of the *p*-nitrophenyl 2-(5-*n*-alkyl)furoate series is a valid reference system for the enzymatic deacylation. The saponification of all six substrates was studied over the temperature range of approximately 20–45 °C in 10 mM borate buffer, pH 10.50 (measured at 25 °C). Hydrolysis rates were dependent on pH, but not on the concentration of buffer, eliminating the possibility of specific base catalysis. Arrhenius plots of the pseudo-first-order rate constants for all esters were linear; the $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ determined directly with eq 3 are presented in Table V. No attempt was made to correct these parameters for the heat of ionization of the buffer since an identical correction would be introduced with each substrate and only relative values are required. The saponification results were then used to calculate activation parameters (related by an additive, but unknown, constant to the parameters of enzymatic rate enhancement specificity) by taking the difference between enzymatic and saponification results.

Discussion

Hofstee (1959) and Marshall and Akgün (1971) reported that the relative deacylation rate constants of fatty acid acyl-α-chymotrypsins increase with chain length, reach a maximum, and then decrease. Qualitatively similar trends have been observed by Dupaix et al. (1973) with phenyl and indolyl series, and now by us with the 2-(5-*n*-alkyl)furoyl-α-chymotrypsins (Table III). A reasonable relationship between the results from the four laboratories becomes evident when relative deacylation rates for each of the series are plotted against the volumes of the acids (Figure 4). The rate enhancement specificities in the four series correlate reasonably well with the molecular size of the acyl group, suggesting that specificity is controlled, at least in part, by the geometrical arrangement of the acyl group. This conclusion of Marshall and Akgün (1971) is supported by our results. Even the slight difference between the indolyl and the other three series may be accounted for in terms of molecular geometry. It is seen with space filling models that indoleacetic acid (the first of the indolyl series) cannot assume an overall shape similar to those of the acids in the other series. However, indoleproprionic acid, with its additional methylene group, can assume an appropriate shape.

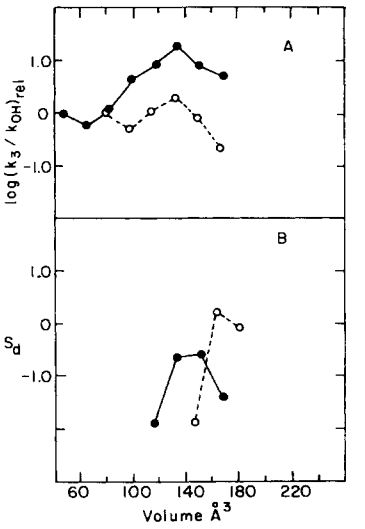

FIGURE 4: Relative deacylation rate constant vs. molecular volume. The volumes in Å^3 are calculated for the acyl moiety ($RCOO^-$) of acyl-α-chymotrypsin using the atomic volumes of Edward (1970). The rate constants were obtained at 25 °C. (A) (●) $RCOO^-$ = $H(CH_2)_nC(=O)O^-$; n = 1 through 8; data obtained from Marshall and Akgün (1971). (○) $RCOO^-$ = $H(CH_2)_nC_4H_2OC(=O)^-$; n = 0 through 5; data obtained from our results. (B) All data were obtained from Dupaix et al. (1973) and are presented in terms of the specificity constant S_d as calculated by these authors. (●) $RCOO^-$ = $C_6H_5(CH_2)_nC(=O)O^-$; n = 1 through 4. (○) $RCOO^-$ = $C_6H_6N(CH_2)_nC(=O)O^-$; n = 1 through 3.

It is remarkable that for these compounds, which are otherwise so different, molecular volume appears to be so important in rate enhancement specificity. Bender, Kezdy, and Gunter (1964), Page and Jencks (1971), and one of us (Klapper, 1973) have presented related theories in which the volume of the substrate relative to that of the active site plays a critical role in rate enhancement as expressed by catalytic entropies of activation. It was proposed in the three discussions that the enzyme functions in part by restricting the molecular motions of the substrate in the active site. The results collected in Figure 4 are consistent with this proposal. However, the more critical test of this proposal, whether the observed specificity is entropy controlled, cannot be made since the observed discontinuity in the Arrhenius plot obtained with three of the six furoyl derivatives strongly suggests that deacylation is composed of more than one elementary reaction. We are convinced, moreover, that the discontinuity in the temperature dependence is not an artifact.

We have ruled out a number of trivial explanations for the observed discontinuities. Since rate constants were calculated from the direct observation of deacylation, and not indirectly from turnover experiments, errors arising from the steady-state treatment cannot be invoked. Varying every component in the reaction mixture does not affect the deacylation rate constant. Thus, explanations based on product inhibition or stimulation, interaction between free and acyl enzyme, or reaction reversibility are not plausible.

The fact, which remains to be explained, that the discontinuity in the Arrhenius plot is observed for only three of the substrates itself tends to rule out possibility of a systematic error. Moreover, other laboratories have also reported similar discontinuities: Kaplan and Laidler (1966) with *N*-benzoyl-D- and -L-alanine methyl esters; Glick (1971) with *N*-acetyl-L-tyrosine ethyl ester; and Wedler et al. (1975) with *N*-acetyl-L-phenylalanine methyl ester, *N*-acetyl-L-leucine methyl ester, and *N*-benzoyl-L-alanine methyl ester. We believe that the nonlinearity observed by us and others is real, occurs with both specific and nonspecific substrates, and is more common than has been previously recognized. For example, examination of data published by Bender et al. (1964, Figure 1) suggests a discontinuity, overlooked by the authors, in the Arrhenius plot obtained for the hydrolysis of *trans*-cinnamoyl-α-chymotrypsin.

We propose that a minimum of two elementary reactions composes the hydrolysis of the acyl enzyme, and that differences in mechanisms need not be invoked to explain linear Arrhenius plots obtained in some cases, and nonlinear in others. Utilizing all of the experimental activation parameters in a compensation plot supports the latter contention. Relative activation enthalpies and entropies were calculated from the entries of Tables IV and V as described in the previous section. All the points in the resulting graph of $\Delta\Delta H^{\ddagger}$ vs. $\Delta\Delta S^{\ddagger}$ appear to fall onto two straight lines (Figure 5). The first passes through the "high" temperature parameters for the unsubstituted, the ethyl, and the *n*-propyl derivatives, as well as the activation parameters of the methyl and *n*-butyl derivatives. The second line passes through the "low" temperature points, and the methyl, *n*-butyl, and amyl points. Thus, the Arrhenius plots obtained with the methyl and *n*-butyl derivatives may appear linear, either because experimental errors mask a small difference between "high" and "low" temperature results, or the discontinuities occur at a sufficiently low temperature that we would not have observed them. The hydrolysis of the amyl-chymotrypsin derivative appears as a "low" temperature process in spite of the fact that this reaction was observed only above 30 °C; i.e., were it to occur, the discontinuity in the Arrhenius plot would be above the highest temperature we used with this derivative.

This interpretation of the compensation plot is based on the assumption that the activation parameters obtained with a homologous series of compounds, all passing through the same rate-limiting elementary reaction step, will yield a linear free-energy plot. Since the reverse statement is clearly not valid, the results can be taken as consistent with the interpretation but not yet definitive. This does not, however, affect the remaining discussion which is independent of the argument just presented. As we mentioned in the introductory section, workers in different laboratories have come to three different conclusions regarding rate enhancement specificity in the deacylation of chymotrypsin: (i) constant activation enthalpy—specificity, entropy controlled; (ii) variation in both activation entropies and enthalpies—specificity, entropy controlled; and (iii) variation in both activation entropies and enthalpies—specificity, enthalpy controlled. The reactivity of the 2-(5-*n*-alkyl)furoyl-α-chymotrypsin series decreases in the order $CH_3(CH_2)_2- > CH_3CH_2- \geq H- \geq CH_2(CH_2)_3- > CH_3- > CH_3(CH_2)_4-$ (Table V). Almost the identical order is observed in Figure 5 on moving along the "low" temperature line from left to right, and along the "high" temperature line in the opposite direction. (The differences in the reactivity order and the position of the points in Figure 5 may arise from ambiguity in interpreting the single set of activation parameters for the CH_3- and $CH_3(CH_2)_3-$ derivatives.) Moving from left to right in Figure 5 is equivalent to moving toward more positive $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$. Thus, the rate enhancement specificity is enthalpy controlled along the "low" temperature line. In the opposite direction $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ are more negative, and, thus, rate enhancement specificity is entropy controlled along the "high" temperature line. In other words, at lower temperatures rate enhancement specificity would appear to be enthalpy driven, and entropy opposed, while the opposite would appear to hold at higher temperatures. The apparent conflict in the literature may, therefore, be due to the fact that Arrhenius plot discontinuities were overlooked, so that the conclusions drawn depended on the distribution of tempertures over which data were collected.

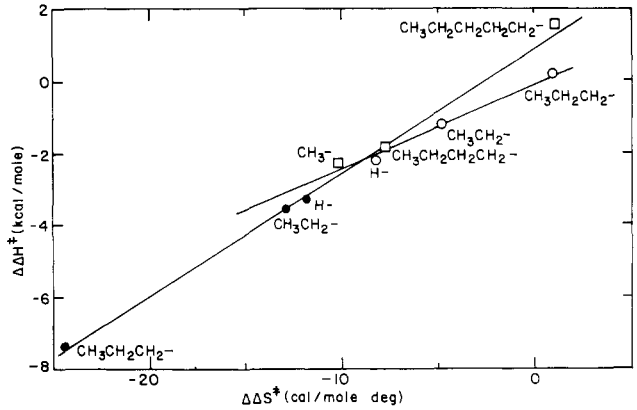

FIGURE 5: Compensation plot for α-chymotrypsin deacylation. Activation enthalpies and entropies were calculated as explained in the text: "low" temperature activation parameters (●); "high" temperature activation parameters (○); activation parameters from linear Arrhenius plots (□). The slopes of the two curves are T_c = 345 °C, and T_c = 233 °C.

Some consideration may be given to the kinetic events responsible for the observed Arrhenius plot discontinuities. We can suggest three minimal classes of postulates: (i) there are two forms of the acyl enzyme, both of which turn over

$$\begin{array}{ccc} EA & \overset{k_1}{\rightleftharpoons} & E + \text{acid} \\ \Updownarrow & & \\ EA' & \overset{k_2}{\rightleftharpoons} & E + \text{acid} \end{array}$$

(If this explantion is to be valid, then the equilibrium between the two forms must be more rapid than the rates of deacylation: we have observed only one kinetic process under all experimental conditions.); (ii) the hydrolysis of the acyl enzyme involves the obligatory intermediate X

$$EA \overset{k_1}{\rightleftharpoons} X \overset{k_2}{\rightleftharpoons} E + \text{acid}$$

(iii) two parallel reactions occur with different mechanisms

$$EA \begin{array}{l} \overset{k_1}{\rightleftharpoons} E + \text{acid} \\ \underset{k_2}{\rightleftharpoons} E + \text{acid} \end{array}$$

Each of these schemes could explain the observed results. While we cannot choose definitively between these three general formulations, we have collected data which make some specific proposals unreasonable.

There are a number of experimental observations which

may be fit into the first of the above three categories, two acyl enzyme forms in rapid equilibrium: thermal denaturation, suggested by Kaplan and Laidler (1966) in explanation of the Arrhenius plot discontinuity they observed; polymerization of α-chymotrypsin at slightly basic pH (Pandit and Rao, 1974); a salt-induced conformational shift (Royer et al., 1969); and a proton mediated equilibrium which occurs near pH 9 (Kim and Lumry, 1971; Mavridis et al., 1974), which may be linked to the ionization of the α-amino group on isoleucine-16 (McCoon et al., 1968). We believe that none of these possibilities would serve as a valid explanation of our observations.

The thermal transition temperature of α-chymotrypsin is greater than 60 °C at pH 8 (Lumry and Biltonen, 1969), and greater than 50 °C at pH 8.5 (our observation). These temperatures are much higher than those at which the discontinuities are observed, between 20 and 30 °C. The protein concentrations used in our experiments are much lower than those at which there is evidence for polymerization (Kim and Lumry, 1971; Pandit and Rao, 1974). Our kinetic results indicate no protein concentration dependence, an additional argument against enzyme polymerization as an explanation of the observed discontinuities. The hydrolytic rate with 2-furoyl-α-chymotrypsin remains unchanged over a wide range of phosphate buffer concentration, and hence ionic strength; this suggests that a salt dependent conformational equilibrium cannot be invoked in explanation. Finally, were the observed discontinuities due to a proton dependent equilibrium, then the shape of the Arrhenius plot would be expected to change with pH, becoming linear at higher and lower pH's. We have, however, still observed the discontinuity at pH 10.0 in the hydrolysis of 2-(5-*n*-propyl)furoylchymotrypsin, and the "high" and "low" temperature thermodynamic parameters are identical, within experimental error, to the pH 8.5 results (Table IV): "high", $\Delta H^{\ddagger}$ = 18.4 (1.0) kcal/mol, $\Delta S^{\ddagger}$ = −11.1 (3.4) cal/(deg mol); "low", $\Delta H^{\ddagger}$ = 12.95 (0.39) kcal/mol, $\Delta S^{\ddagger}$ = −29.0 (1.3) cal/(deg mol).

Thus, if an equilibrium between enzyme forms is to be invoked, it will most likely not be one of the four just considered. In the present absence of delimiting data, any molecular rearrangement may still be postulated. But one suggestion is particularly intriguing in light of the recently reported results of Kerr et al. (1975). Rather than considering protein conformational changes, we would like to propose that the furoyl residue may lie in more than one position within the active site region and still undergo hydrolysis. If reactivity varies with position, and if the positional equilibrium is temperature dependent, then this model would be plausible. Currently, however, we have no direct evidence to support this idea of semiproductive binding modes.

We have also sought evidence for the obligatory participation of a reaction intermediate. The results have been negative. All the reactions we studied were strictly first order, and the hydrolysis of 2-(5-ethyl)furoyl-α-chymotrypsin proceeds with an isosbestic point, evidence for only two spectrally distinct species (Figure 2). These observations are, however, insufficient to rule out the occurrence of a kinetically significant intermediate. Finally, we have no evidence for or against the possibility of two parallel, but mechanistically different pathways, although we do not favor such an interpretation.

Irrespective of the molecular interpretation, the observed discontinuity has a practical result. We began this study in order to obtain the activation parameters for an elementary, enzyme mediated reaction step. These parameters are essential if one wishes to construct a quantitative theory of enzymatic rate enhancement. However, our data indicate that the deacylation of α-chymotrypsin is more complex than was originally believed. Until we can determine unambiguously the source of the two or more rate processes which we now postulate, we cannot obtain the elementary activation parameters desired. It is interesting to note that, even though chymotrypsin has been so extensively studied, we have managed to isolate one new facet of this protein which remains to be explained. It is clear that carefully constructed experiments on the temperature dependence of enzyme-catalyzed reactions can uncover new information, which may ultimately prove useful in explaining the mechanism(s) of enzyme catalysis.

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Optimal Conditions and Specificity of Interaction of a Distinct Class of Nonhistone Chromosomal Proteins with DNA†

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ABSTRACT: A subclass of nonhistone chromatin proteins with high DNA affinity has been isolated from rat liver. The interaction of the isolated proteins with DNA in vitro was characterized utilizing a nitrocellulose filter binding technique. The temperature, time, concentration, ionic strength, and pH dependence were characterized. Optimal interaction was observed at 0.19 M NaCl, pH 7.5, with a protein to DNA ratio of 13 (w/w). Equilibrium and kinetic competition experiments indicated that these proteins interact optimally with A-T rich and single-stranded DNA. The data also suggest that these proteins might affect the helix-coil transition of DNA.

Interaction of proteins with DNA is a requisite for numerous genome regulatory functions in both bacterial and viral systems (von Hippel and McGhee, 1972). Clearly, such interactions must also be important in the functioning of the eukaryotic chromosome. However, the number and types of interactions may in fact be much larger since the eukaryotic chromosome, or chromatin as it is operationally defined, consists of a supramolecular complex of DNA, RNA, and histone and nonhistone proteins, the latter being termed the NHCP[1]. This complexity and the lack of specific biological and/or biochemical assays have impeded the elucidation of the roles of these macromolecules in chromatin function. Increasingly, however, the DNA binding properties of both histone and NHC proteins have been utilized as a specific probe of possible biological roles for these proteins. In particular, studies have shown that NHCP bind to homologous and heterologous DNA (van den Broeck et al., 1973; Patel and Thomas, 1973), to low and high C_0t DNA (Allfrey et al., 1973), and to intermediate C_0t DNA (Sevall et al., 1975). Also, some of the NHCP exhibit specificity of binding only to DNA from the species from which the NHCP were originally isolated (Kleinsmith et al., 1970; Teng et al., 1971; Kostraba et al., 1975).

We reported previously on the isolation and partial characterization of a subclass of rat liver NHCP which exhibited high affinity for DNA and deoxynucleohistone (Patel, 1972). This group of proteins, referred to as APNH[1], is comprised predominately of two molecular weight species, approximately 19 000 and 16 000 daltons. Initial experiments showed that APNH bound to DNA to a greater extent than other NHP fractions (Patel and Thomas, 1973; Thomas et al., 1973). These experiments also suggested both partial species and single-strand specificity. In this study, the DNA binding properties of APNH were examined in more detail employing a nitrocellulose filtration technique as an assay of DNA-protein complexes (Riggs et al., 1968; Riggs and Bourgeois, 1968; Riggs et al., 1970a). The primary advantages of this technique were its rapidity, sensitivity, and its requirement of very little DNA and protein. This assay was exploited extensively to determine the optimal conditions for the DNA-APNH interaction and to determine various binding parameters which serve as a measure of the specificity of the interaction.

Methods

(*a*) *Buffers and Media.* Luria broth is 1% bactotryptone-0.5% yeast extract-0.5% NaCl, pH 7. K media is M9 buffer (Adams, 1959) which is 1% casamino acids, 1% glucose, 10^{-4}% thiamine, and either 2 μg (K + 2) or 10 μg (K + 10) thymidine per milliliter. SB buffer is 0.19 M NaCl-0.01 M Tris-HCl-0.001 M EDTA, pH 7.5. SSC is 0.15 M NaCl-

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[1] Abbreviations: NHCP, nonhistone chromatin proteins; NHP, nonhistone proteins; APNH, nonhistone chromatin proteins with high DNA affinity; EDTA, ethylenediaminetetraacetate; HAP, hydroxylapatite; PEG, poly(ethylene glycol); Me_2SO, dimethyl sulfoxide.