Optical Properties and the Chemical Nature of Acyl-Chymotrypsin Linkages

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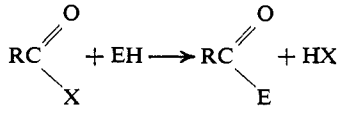
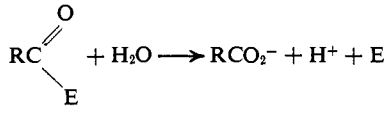
Abstract: On the supposition that the specific spectral properties of mono-β-arylacrylolyl enzymes are related to their special reactivity, the spectra and optical rotatory dispersions of α-chymotrypsin and some acyl derivatives have been examined and compared with the spectra and optical rotatory dispersions of a group of relevant small molecules. The reactivities of these acyl enzymes have previously been correlated with their catalytic functions in specific substrate reactions, and there are considerable presumptive arguments that the acyl–enzyme linkage is *via* a specific serine hydroxyl oxygen (an ester linkage). The spectral properties, especially the large red shifts on acylation, and chemical reactivities of these mono-β-arylacryloyl enzymes differ from those of small molecule model acylserine esters. On the basis of the comparisons of their optical properties, these differences are found to be due to an *endo*-energetic configurational change which takes place when the enzyme is acylated with the β-acryloyl substrates. It is found that the "native" β-acryloyl enzyme has a "*s-cis*" configuration in contrast to the small molecule model compounds which are predominantly in the "*s-trans*" configuration. The magnitude of the induced optical rotatory activity attributable to the transition associated with the absorption band of the β-acryloyl component of the acetylated enzyme, nmr measurements on model compounds, and other spectral information are shown to exclude other possible configurational changes such as out-of-plane skewing or *cis–trans* isomeration about the double bonds. Chemical perturbation (*via* enzyme substituents) of an otherwise conventional acyl–serine linkage is also considered and rejected as an unlikely origin of the observed spectral shifts.

A number of detailed studies of the chemical and spectral properties of aroyl chymotrypsins have been reported.[1-3] These aroyl enzymes can be prepared, virtually stoichiometrically, by reactions of the type illustrated in eq 1. Under appropriate conditions



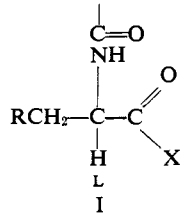
(1)

of pH, when the "leaving group" is strongly electron withdrawing, this specific monoacylation reaction may be sufficiently faster than the succeeding deacylation reaction (eq 2) for the acyl intermediate to be studied in



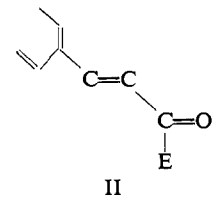
(2)

isolation. A variety of parallels can be drawn between the relatively slow pathways of acylation and deacylation exhibited with these aroyl acylating agents and the rapid turnover of specific substrates (I) of α-chymotrypsin. Although many of the monoaroyl-



I

chymotrypsin derivatives are sufficiently stable to be studied spectrophotometrically by conventional techniques, they are all nevertheless strikingly more reactive than the corresponding aroylalkyl esters. This comparison is of significance since denaturation of the monoacyl enzyme leads to the formation of an identifiable monoacylserine O-ester peptide.[3-5] The aromatic nature of the acyl-enzyme derivatives reported herein is of no particular chemical catalytic consequence; aliphatic acyl enzymes have similar chemical properties.[6,7] Due to its intense ultraviolet absorption, the aroyl group provides an indicator of the electronic environment at the site of covalent attachment to the enzyme. The effectiveness of this system as an electronic indicator can be greatly enhanced by extension of the π-resonance systems as in derivatives of type II or III because the intense $\pi-\pi^*$ transition occurs at longer wavelengths and so sufficiently outside the region of ultraviolet absorption of the enzyme to simplify the spectrophotometric measurements. In addition, equally energetic shifts result in larger shifts in wavelength because of the inverse nature of the relationship between energy and wavelength.

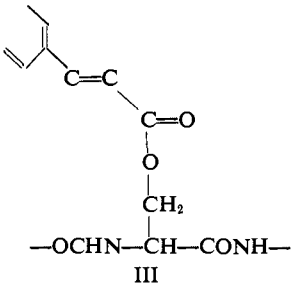


II

Denatured solutions of the mono-β-arylacryloyl-enzyme derivatives have similar chemical properties and identical ultraviolet and/or near-visible spectra when compared with model serine peptides containing

(1) M. L. Bender, G. R. Schonbaum, and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2540 (1962).
(2) M. Caplow and W. P. Jencks, *Biochemistry*, 1, 883 (1962).
(3) S. A. Bernhard, S. J. Lau, and H. Noller, *ibid.*, **4**, 1108 (1965).
(4) R. A. Oosterbaan, P. Kurst, J. Van Rotterdam, and J. A. Cohen, *Biochim. Biophys. Acta*, **27**, 557 (1958).
(5) H. Noller and S. A. Bernhard, *Biochemistry*, 4, 1118 (1965).
(6) M. L. Bender, *J. Am. Chem. Soc.*, **84**, 2540 (1962).
(7) A. K. Balls and H. N. Wood, *J. Biol. Chem.*, **219**, 245 (1956).

the corresponding acyl ester linkage (III).[3,4] On the other hand, the native derivatives have strikingly dif-



III

ferent chemical and spectral properties. The studies reported herein were undertaken as part of an attempt to investigate the relevance of these unusual spectral properties to the chemical catalytic process.

In order to clarify our objective in the present choice of experiments, it is well to restate a number of previously noted facts concerning the spectral properties of acyl enzymes of type II in the native and in the denatured (or degraded) state.

(1) The strong long-wavelength electronic transition of the acyl chromophore is always "red shifted" in the native acyl enzyme, relative to the denatured enzyme;[8] denatured derivatives are *always* identical in spectrum with small model O-acylserine-peptide derivatives and to proteolytically degraded O-acylseryl-enzyme fragments.[1,3]

(2) The same strong long-wavelength electronic transition in the native acyl-enzyme derivative is always comparable in intensity to that of the denatured or model compound derivatives.

(3) This spectral "red shift" which varies considerably from derivative to derivative ($\Delta\lambda$ can be as large as 30 mμ) is unexpected in view of the finding that this particular enzyme binding site resembles an environment of low polarity (a "hydrophobic" binding site); all aroyl chromophores thus far considered are "blue shifted" when transferred to solvents of low polarity. Arguments have been previously proposed to exclude the possibility that the red shift arises from a difference in "solvent polarity" surrounding the chromophore in the native *vs.* the denatured (unfolded) acyl enzyme.[8]

(4) All experimental measurements of the kinetics of unfolding, enzyme inactivation, and disappearance of the red shift in the acyl chromophore have failed to indicate any "uncoupling" in the three processes (the kinetics of the three processes are identical). Thus, the implication is that this red shift is a relevant indicator of the catalytic process.

Alternative explanations for the red shift have been previously proposed,[1,3] but no clear-cut choice is

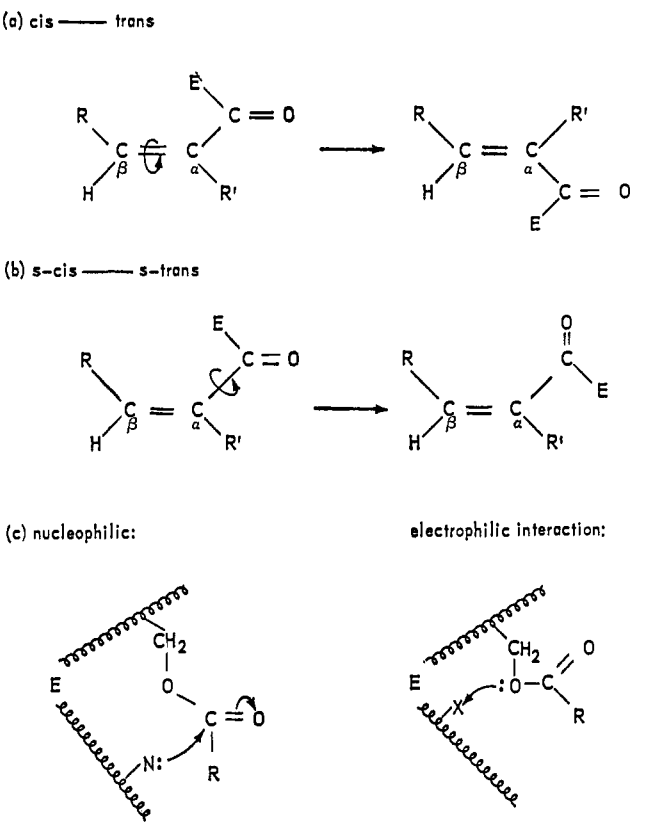


Figure 1. Potential mechanisms for the perturbation of the β-arylacryloyl-enzyme spectra: E = enzyme or peptide. The fact that the acryloyl group is shown linked to the enzyme or peptide before and after the isomerization is not intended to indicate necessarily that the isomerizations or out-of-plane rotations take place after acylation. The arrows are intended to indicate out-of-plane bending which for angles less than 180° results in skewed configurations.

evident from the existing experimental data. The alternatives previously proposed are as follows.

(1) The acyl enzyme is a "chemically perturbed" serine ester, the perturbation being of a type thus far not observed in model compound esters of this type.

(2) The native acyl–enzyme linkage is not *via* a serine O-ester linkage, but rather *via* an *active* acyl linkage which is transferred to the hydroxyl oxygen of serine upon denaturation of the acyl enzyme.

Extensive investigations of the denaturation process, under a variety of conditions of pH, have thus far demonstrated the invariant coupling of the red-shift disappearance to the denaturation process, and hence led us to reject as unlikely (although not impossible) the second of these alternatives. We chose, therefore, to consider the detailed electronic and stereochemical possibilities consistent with the former alternative. Three straightforward mechanisms for perturbing the spectra of esters of type III can be summarized as follows: (1) *cis–trans* isomerizations or "out-of-plane" rotations about the α,β double bond; (2) *s-trans–s-cis* isomerizations or rotation about the acryloyl carbon–carbon single bonds; (3) interactions with electrophiles at either carbonyl oxygen or interactions with nucleophiles at the carbonyl carbon *via* the constituent amino acid residues of the enzyme protein or *via* solvent. These mechanisms are summarized in Figure 1.

The present experiments are designed to explore the first two possibilities. Experimental data of two types

(8) Two recent studies[9] with somewhat conflicting results are most interesting in this respect. One by Kallos and Avatis would further emphasize the special requirement for explaining the red shift of the acyl derivatives since these authors have demonstrated from studies of tosyl derivatives that the solvent-dependent blue shift observed when the derivative is bound to the active site of the enzyme is directly related to the low polarity of the binding site. In the other, Westheimer, *et al.*, studied the photolysis products of diazoacetyl-chymotrypsin and conclude that water is present at the active site. If the latter result requires that the active site has a polar environment, then no special requirement exists for a spectral shift large enough to overcome the solvent effect.

(9) J. Kallos and K. Avatis, *Biochemistry*, 5, 1979 (1966); J. Shafer, P. Baronowsky, R. Laursen, F. Finn, and F. H. Westheimer, *J. Biol. Chem.*, 241, 421 (1966). We wish to thank the referee for calling the latter paper to our attention.

have been obtained from spectroscopic and optical rotatory dispersion studies of both model arylacryloyl compounds and of related acyl enzymes.

Experimental Section

Model Compounds and Reagents. Cinnamic acid, β-(2-furyl)-acrylic acid, methyl cinnamate, and ethyl β-(2-furyl)acrylate were purified commercial samples previously described.[3] β-(2-Furyl)-acrolein and α-methyl-β-(2-furyl)acrolein were obtained from K & K Chemical Co. The former was recrystallized from water and the latter redistilled under reduced pressure. α-Methylcinnamic acid (Aldrich Chemical Co.) was recrystallized from water and dried under vacuum. N-[β-(2-Furyl)acryloyl]imidazole was prepared as previously described.

N-Carbobenzyloxy-O-furylacryloyl-L-serinamide was prepared from a recrystallized sample of N-carbobenzyloxy-L-serinamide, the optical purity of which has been established; 0.01 mole of the amide was dissolved in 20 ml of cold (5°) pyridine and 0.01 mole of β-(2-furyl)acryloyl chloride[3] in 10 ml of tetrahydrofuran was slowly added. Solvent was removed after 5 min by flash evaporation at room temperature. The resultant gum was extracted with ethyl acetate, and the ethyl acetate solution was extracted exhaustively with aqueous phosphate buffer (0.5 *M*, pH 6.7). The ethyl acetate layer was then dried over Na_2SO_4, filtered, and made slightly turbid by the addition of Skellysolve B. On standing at 5° crystals formed. These were filtered, air dried, dissolved in 95% ethanol, recrystallized from hot ethanol–H_2O (~50% v/v), and dried in a vacuum oven at 40°; mp 158–160°; equiv of serine/358 g of product, 0.98; ϵ_M 2.6×10^4 (OD/*M* cm) at 303 mμ.

β-(2-Furyl)acryloyl-α-chymotrypsin was prepared by the stoichiometric reaction of α-chymotrypsin with the acylimidazole as described previously.[3] The method involved the addition of microliter quantities of the acylimidazole to milliliter quantities of enzyme solution. It was therefore possible to measure spectra and optical properties of the enzyme solution before and after acylation, without transfer of sample from one cell to another. Denatured enzyme and denatured acyl enzyme were prepared in soluble form by the addition of concentrated (0.1 *M*) solutions of sodium dodecyl sulfate (to a final concentration of about 0.02 *M*).

Spectrograde organic solvents (Matheson Coleman and Bell Chemical Co.) were utilized throughout.

Spectrophotometric and Optical Rotatory Dispersion Measurements. All spectrophotometric studies were carried out in a Cary Model 14 spectrophotometer. Maximal slit widths were approximately 0.2 mm. Optical rotatory dispersion measurements were performed with the aid of a Cary Model 60 spectropolarimeter at spectral band widths of 20 A or less. Cylindrical quartz cells could be used interchangeably in the two instruments. Unless otherwise noted, optical densities in the polarimetric experiments did not exceed 1.0 OD. Depending on the stability of samples, the spectra of polarimetric samples were determined either concurrently (with aliquots of the same solutions) or by direct measurements in the polarimetric sample cells, after the polarimetric measurements were completed. The kinetics of deacylation of the acyl enzyme was measured spectrophotometrically as previously described.[1,3] Changes in polarimetric properties associated with deacylation were measured both at fixed wavelengths as a function of time and by repeated scanning of the wavelength region at regular intervals in the spectropolarimeter. All spectrophotometric measurements were made at 25° except for the kinetic studies which were run at 27°. Spectropolarimetric studies were all at 27°.

Results and Discussion

The spectral absorption and optical rotatory dispersion measurements were designed to explore two suggested origins of the red shift of acyl derivatives of the native enzyme. The spectral characteristics of a variety of β-acryloyl derivatives are summarized in Table I. These compounds have been selected with regard to the relevance of their chemical and electronic structure to these spectral shifts. The choice of the furylacryloyl and cinnamoyl derivatives is dicated by the steric and spectral characteristics which make them not only suitable substrates for the enzyme but, as noted earlier, peculiarly sensitive indicators of the chemical and physical effects of their environment.

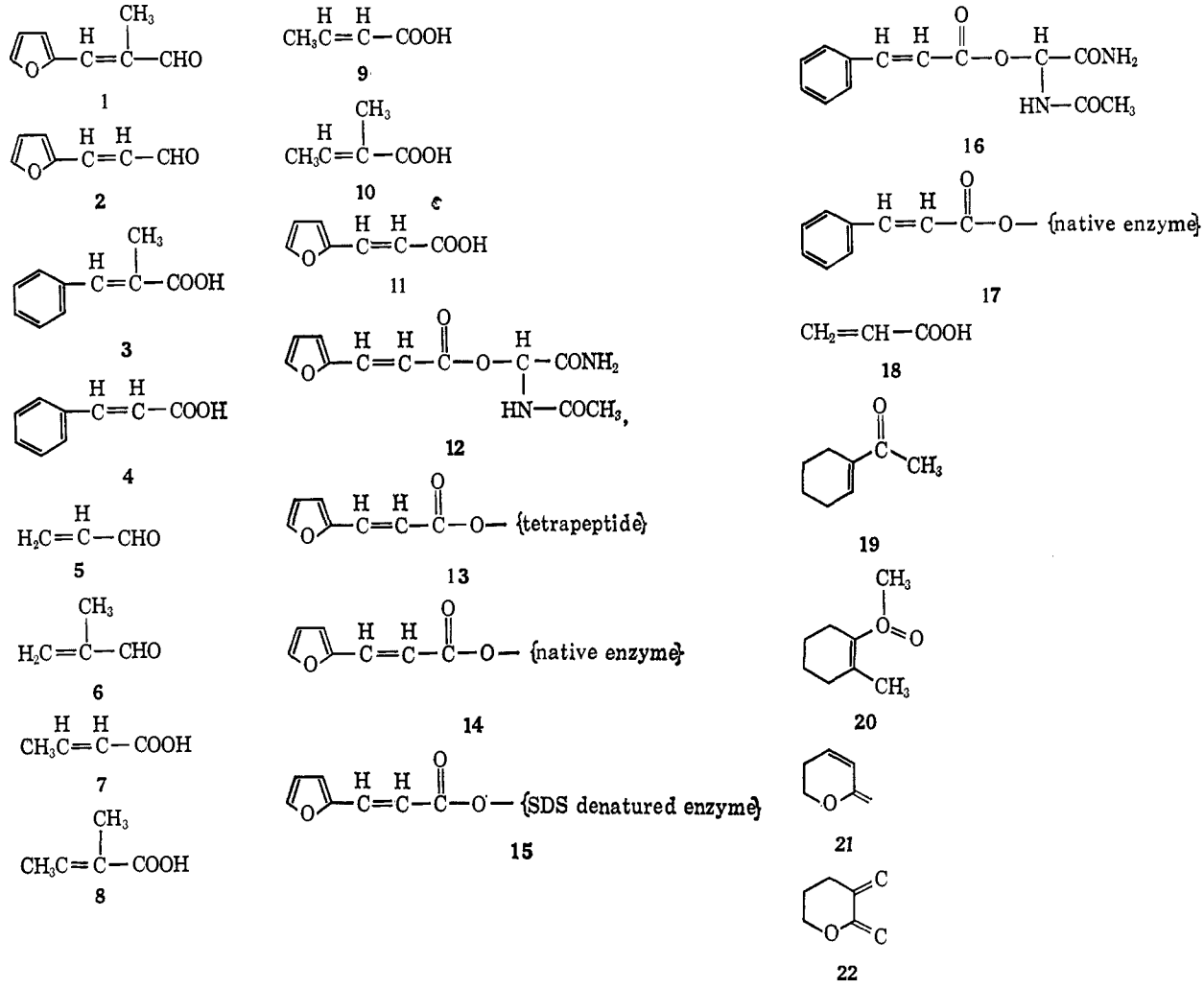
Table I

Compd	Solvent	λ_{max}, mμ	ϵ_{max}	Ref
α-Methyl-β-(2-furyl)acrolein (**1**)	CH_2Cl_2	316	2.80×10^4	*a*
β-(2-Furyl)acrolein (**2**)	CH_2Cl_2	315	2.86×10^4	*a*
α-Methylcinnamic acid (**3**)	CH_2Cl_2	271	1.45×10^4	*a*
Cinnamic acid (*trans*) (**4**)	CH_2Cl_2	279	2.17×10^4	*a*
Acrolein (**5**)	C_2H_5OH	207	1.12×10^4	*b*
α-Methylacrolein (**6**)	C_2H_5OH	216	1.10×10^4	*b*
trans-Methacrylic acid (**7**)	C_2H_5OH	205	1.40×10^4	*c*
α-Methyl-*trans*-methacrylic acid (**8**)	C_2H_5OH	213	1.25×10^4	*c*
cis-Methacrylic acid (9)	C_2H_5OH	205.5	1.35×10^4	*c*
α-Methyl-*cis*-methacrylic acid (**10**)	C_2H_5OH	216	0.90×10^4	*c*
β-(2-Furyl)acrylic acid (**11**)		308	2.5×10^4	*a*
N-Acetyl-O-furylacryloyl-serinamide (**12**)	H_2O	309		*d*
N-Carbobenzyloxythreonyl-(O-furylacryloyl)seryl-methionylalanylmethylate (**13**)	H_2O	308		*d*
Furylacryloyl derivative on native enzyme (**24**)	H_2O (pH 4.2)	320	1.92×10^4	*d*
Furylacryloyl derivative on SDS-denatured enzyme (**15**)	H_2O	309		*d*
N-Acetyl-O-cinnamoyl-serinamide (**16**)	H_2O	281	2.4×10^4	*d*
Cinnamoyl derivative on native enzyme (**17**)	H_2O (pH 4.2)	292	1.7×10^4	*d*
Acrylic acid (**18**)	C_2H_5OH	200	1.0×10^4	*c*
1-Acetylcyclohex-1-ene (*s-trans*) (**19**)		232	1.25×10^4	*e*
1-Acetyl-2-methylcyclohex-1-ene (**20**)		245	0.05×10^4	*e*
Hex-2-eno-S-lactone (**21**)	C_2H_5OH	205	1.07×10^4	*f*
5-Hydroxypent-1-ene-2-carboxylic acid lactone (**22**)	C_2H_5OH	220	0.57×10^4	*g*

[a] Our measurements. [b] W. F. Forbes and R. Shilton, *J. Am. Chem. Soc.*, **81**, 786 (1959). [c] A. T. Nielsen, *J. Org. Chem.*, **22**, 1539 (1957). [d] Ref 3. [e] H. H. Jaffé and M. Orchin, ref 18, p 421. [f] U. Eisner, J. A. Elvidge, and R. P. Linstead, *J. Chem. Soc.*, 1372 (1953). [g] E. R. H. Jones, T. Y. Shen, and N. E. Whiting, *ibid.*, 230 (1950).

We will consider first the possibility that the red shift results from "out-of-plane" bending about the α,β *double bond* or from *cis–trans* isomerization with respect to *this* bond and show from ultraviolet spectral data, nmr measurements, and measurements of induced optical activity that these mechanisms cannot be the origin of the red shift.

A. "Out-of-Plane" Bending about the α,β Double Bond. To demonstrate the effect of out-of-plane bending as a factor in the observed spectral shifts, we chose to examine the substituted derivatives α-methylfurylacrolein (**1**) and α-methylcinnamic acid (**3**) relative to their unsubstituted homologs furylacrolein (**2**) and cinnamic acid (**4**). The former two compounds would be expected to be skewed into a nonplanar (with respect to the conjugated system) configuration because of the effect of α-methyl substitution on the van der Waal's contacts (Figure 2). Note that in both cases the α-methyl carbon–*ortho* carbon (or oxygen) contact distance is about 2.5 A in the planar conformation. Nonbonded C–C contacts this short have never to our knowledge been observed in crystals. The ultraviolet spectra of these compounds (Figure 3) must be examined for perturbing effects other than the known red shift that occurs on α-methyl substitution (see, for example,



the spectral effect of α-methyl substitution in acrolein (**5**, **6**) and methacrylic acid (**7**, **8** and **9**, **10**—Table I). Note that, instead of the red shift, there is a large blue shift of λ_{max} in α-methylcinnamic acid derivatives and a lack of a red shift in the furoyl derivative. The effect is more pronounced in α-methylcinnamic acid, presumably due to the more unfavorable contacts with two *o*-carbons in the phenyl derivative relative to one carbon and one oxygen in the furyl case. It is clear that a large twist out of planarity about the double bond, as must occur in the two α-methyl derivatives,[10] is inconsistent with the observed spectra of the acyl enzymes. The enzyme derivatives of type II show no large decrease in extinction. More important, the shift in λ_{max} for the native acyl enzyme is in the wrong direction. In fact, the blue shift in λ_{max} due to out-of-plane distortion is undoubtedly even greater than that observed since, as we have seen, the α-methyl group contributes electronically (*ca.* 10 mμ) to an increase in λ_{max} over the homologous α-hydrogen derivative (see Table I). The effects of out-of-plane twisting in skewed dienes have been very extensively investigated in regard to the effect of skew on both the ultraviolet spectra[12,13] and the optical rotatory dispersion.[14]

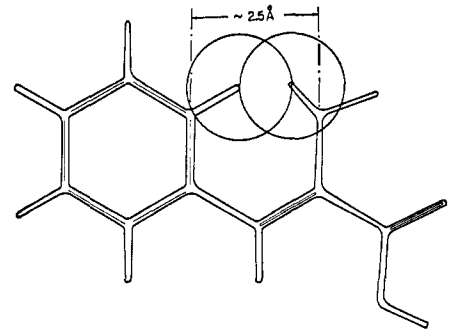


Figure 2. α-Methyl-*trans*-cinnamic acid drawn to scale in the *planar* conformation from Dreiding models, showing the van der Waals overlap of hydrogen atoms on the aromatic ring with those on the methyl group. Two of the three methyl hydrogens are eclipsed with respect to the plane of the ring. van der Waals radius of hydrogen = 1.00 A.

(10) As noted above, examination of space-filling models[11] of α-methylcinnamic acid indicated that the acrylic group would not maintain a planar configuration. This is in accord with the observed blue shift relative to cinnamic acid. To check this, we examined the nmr chemical shift of α-methylcinnamic acid relative to α-methylacrylic acid. On the basis of the coordinates taken from the models and the theory of the effect of aromatic ring currents on nmr chemical shifts (C. E. Johnson and F. E. Bovey, *J. Chem. Phys.*, **29**, 1012 (1958)), the α-methyl protons in the cinnamic acid derivative are expected to be 31 cps downfield with respect to those of the acrylic acid derivatives if the entire conjugated system is planar and *upfield* by about 5 cps if the system is skewed about 90° out of plane. The experimentally observed shift is 11 cps *upfield*, in excellent confirmation of the ultraviolet interpretation of nonplanarity. We wish to thank Mr. Robert Bradley of the National Institutes of Health for making these measurements.

(11) Pauling, Corey, and Koltun models developed by the Atomic Models Subcomittee of the Biophysics and Biophysical Chemistry Study Section of the National Institutes of Health.

(12) H. Suzuki, *Bull. Chem. Soc. Japan*, **35**, 1715 (1962).

(13) N. L. Allinger and N. A. Muller, *J. Am. Chem. Soc.*, **86**, 2811 (1964).

(14) E. Charney, *Tetrahedron*, **21**, 3127 (1965).

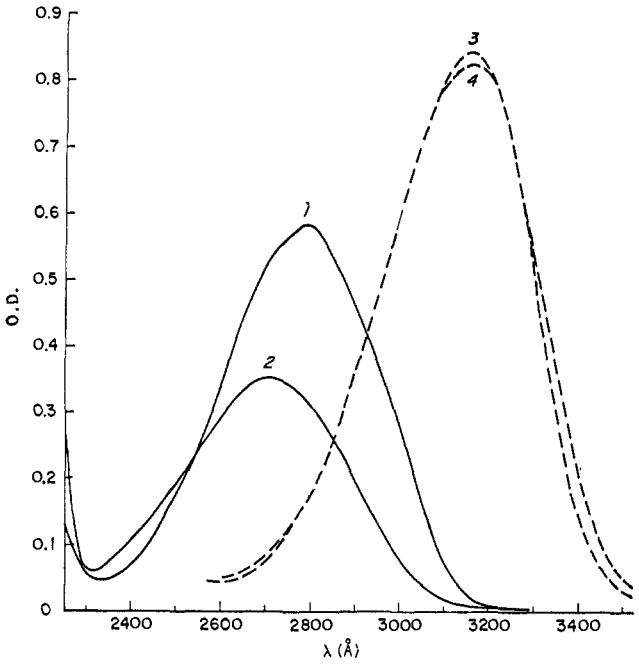


Figure 3. Ultraviolet spectra of acryloyl derivatives: (1) *trans*-cinnamic acid, 0.253×10^{-3} *M*; (2) α-methylcinnamic acid, 0.253×10^{-3} *M*; (3) furylacrolein, 0.296×10^{-3} *M*; (4) α-methylfurylacrolein, 0.296×10^{-3} *M*. All spectra were taken in 1-cm cells; solvent CH_2Cl_2.

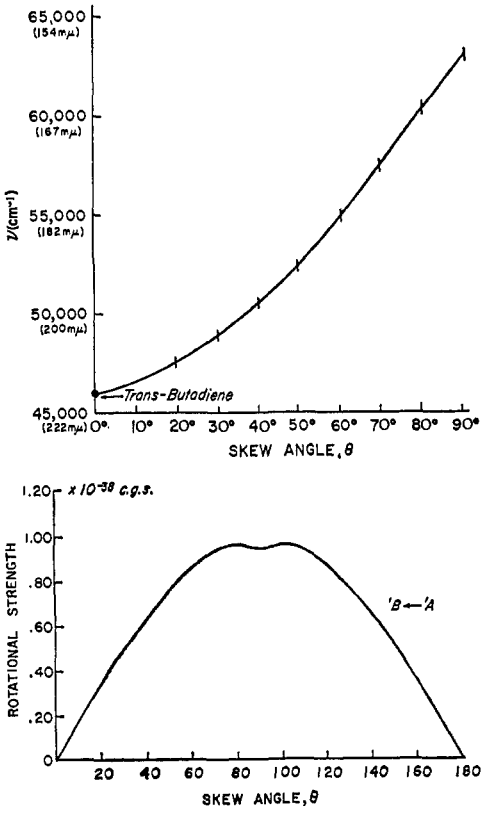


Figure 4. Optical absorption and rotatory activity of nonplanar conjugated dienes calculated from Hückel molecular orbitals. (a) Dependence on skew angle of the long-wavelength $\pi \rightarrow \pi^*$ transition of a conjugated diene. (b) Dependence on the skew angle of the rotational strength of the long-wavelength $\pi \rightarrow \pi^*$ transition of a conjugated diene (positive rotational strength corresponding to right-handed helical configuration, only, is shown).

Although a large out-of-plane skew can be ruled out solely on the basis of the foregoing considerations

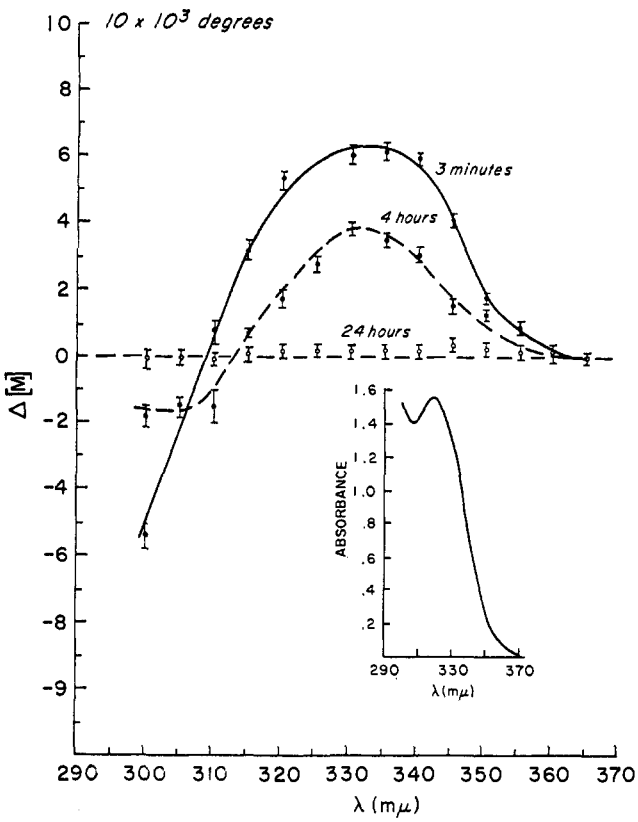


Figure 5. Induced molar rotation $[[\Delta M] = [M](\text{FAI} + \alpha\text{-chymotrypsin}) - [M](\alpha\text{-chymotrypsin})]$ of acetylated enzyme complex on native enzyme at pH 4.30, 27°; (FAI) 0.0142 mg/ml; (α-chymotrypsin) 2.61 mg/ml. Insert: absorption spectrum 3 *min* after addition of FAI.

and of the observed spectra of native acyl enzymes, smaller skew angles cannot be summarily dimissed on this basis. In the case of small angles, however, the recent interpretation of the ultraviolet spectra[12,13] and the optical rotatory dispersion[14] of skewed conjugated systems offers some help. The effect of skew angle on λ_{max} is shown schematically in Figure 4a. For small skew angles a blue shift is predicted, but the shift is small enough so that structural environmental effects may have stronger influences on the position of maximum absorption. The possibility of out-of-plane skew of the chromophoric group in the native acyl enzyme can, however, be tested more quantitatively by comparing the observed optical activity with that predicted from theoretical considerations. Rotational strength is expected to be very similar to that calculated for skew dienes (Figure 4b). It is easy to see that, although only small spectral shifts are expected, the rotational strength undergoes a large change even for small angles. The optical rotatory dispersion of α-chymotrypsin and β-(2-furyl)acryloyl-α-chymotrypsin in native and denatured (sodium dodecyl sulfate) states was therefore measured. A notable difference was observed between the native enzyme and the native acyl enzyme (Figure 5). Note that this difference in rotatory dispersion disappears when the enzymes are denatured (Table II). The "Cotton effect" induced by furylacryloylation corresponds approximately in wavelength to that predicted on the basis of the measured absorption band due to the furylacryloyl chromophore of the native enzyme (Figure 5, insert). The induced rotatory

Table II. Optical Activity of Acyl Complexes

Expt	Complex	Rotation[a] (1-cm path length), deg
I[a,b]	α-Chymotrypsin (2.61 mg/ml)	−0.0740 ± 0.0004
II[a,b]	α-Chymotrypsin (2.61 mg/ml) plus N-β-(2-furyl)acryloyl-imidazole (0.0167 mg/ml)	−0.0690 ± 0.0004
III[b]	α-Chymotrypsin (2.70 mg/ml) denatured with SDS	−0.0650 ± 0.0004
IV[b]	α-Chymotrypsin (2.70 mg/ml) plus N-β-(2-furyl)acryloyl-imidazole (0.0133 mg/ml) denatured with SDS	−0.0650 ± 0.0004
V[c]	N-CBZ-*o*-furylacryloyl-L-serinamide (5.6×10^{-5} *M*)	0.000 ± 0.0004

[a] Rotations measured at 3350 A, the peak of induced activity in solution II, the native acyl–enzyme complex. The activity of solution II was measured 3 min after the addition of the acryloyl compound. In a separate experiment at pH 6.24 the maximum induced activity was found to be from 20 to 25% higher than that obtained in expt II. I and II were measured at pH 4.30, III and IV at pH 4.24. [b] Measured on the Cary 60 spectropolarimeter at the University of Oregon, for which we wish to thank Professor John Schellman. [c] Measured on the Cary 60 spectropolarimeter at the National Institutes of Health.

dispersion is related to the catalytic site configuration as indicated by the fact that this induced rotatory dispersion disappears with time (Figure 5), presumably *via* a process related to the deacylation of the acyl enzyme (and regeneration of the native enzyme).[15] Additional evidence for this comes from the measurement of the optical activity of the same chromophore acylated to a model serine peptide, N-carbobenzyloxy-L-serinamide. As indicated in Table II, no induced optical activity within the noise limited detectability of the instrument (approximately ±0.0004° under the conditions of measurement) is observed. Induced Cotton effects of the type observed with the native acyl enzyme can have two origins. (a) The chromophoric group itself remains planar, *i.e.*, the nuclear positions retain an essentially planar configuration but the charge distribution is distorted by the asymmetric environment of the enzyme to give rise to induced optical activity in the transitions of the chromophore. This effect will generally be very much smaller than the second type. (b) The chromophoric group itself skews into a nonplanar configuration. It is this type which gives rise to the optical activity observed in the ultraviolet transitions of, for example, the steroid dienes.[16] Although there is apparent qualitative consistency between the observed rotatory dispersion properties and the "skewed double bond hypothesis," the quantitative measure of the "trough" of the "Cotton effect" (Table II) is inconsistent with this hypothesis. Small out-of-plane twists are associated with very much larger troughs in the rotatory dispersion. The magnitude of the expected optical activity may be calculated using the simple approximation that the order of the activity will be that expected from a skew diene (or larger since the conjugated groupings are more extended in the cinnamoyl and furylacroyl compounds). On this basis it is calculated[14] that the observed optical activity (molar amplitude = 12×10^3 degrees/*M* dm (Table II)) cannot come from configurations skewed more than 3 or 4°, hardly enough to account for significant perturbations in the absorption spectra. We conclude therefore that there is no significant out-of-plane twisting about the α,β double bond in the native acyl enzyme.

B. Planar *cis–trans* Isomerization about the α,β Double Bond. This possibility can be eliminated from a consideration of the spectra of model compounds (Table I). For example, the wavelength of maximum absorption of *cis*- and *trans*-methacrylic acids (**7**, **9**) differ by only 0.5 mμ, and while this is at 205 mμ, it is still energetically *much* smaller than the 11-mμ shifts observed in the cinnamoyl–native enzyme complex (**17**) (about 120 cm^{-1} compared to about 1400 cm^{-1} for the latter). The shift to α-methyl-*cis*-methacrylic acid (**10**) from the *trans* configuration (**8**) is accompanied by a small shift of 3 mμ to the red (somewhat more energetic than the unsubstituted acrylic acid), but, more generally, where the unsaturated system extends over more than two bonds shifts to the blue are encountered. For example, the long-wavelength band of *cis*-stilbene is about 280 mμ compared to about 295 mμ for the *trans* compound, and in 1,4-diphenylbutadiene, the conversion from the *trans,trans* configuration to the *cis,trans* is accompanied by a blue shift of about 17 mμ.[17] The experimental evidence is in full qualitative accord with either simple molecular orbital or free-electron theory,[18] which predicts *increasing* red shifts with *increasing trans* extension of conjugated systems.

C. *s-trans–s-cis* Isomerization about the Acryloyl Carbon–Carbon Single Bond. Spectral data relating to *cis–trans* isomerization of esters of this type are limited. Nevertheless, a number of pertinent studies have been reported.

Referring first to spectral investigations, we note that the methyl ketones **19** and **20**, which are known from infrared studies[19] to be respectively in the planar *s-trans* and *s-cis* configuration, have their long-wavelength strong absorption (presumably the $\pi \rightarrow \pi^*$) at 232 and 245 mμ, respectively, a shift of about 2280 cm^{-1} to the red. In the case of the lactones **21** and **22** in which the nominal double bonds are respectively *s-trans* and *s-cis*, the peaks of the same strong absorption band are at 205 and 220 mμ, a 3230-cm^{-1} shift to longer wavelengths,[20] and while a small part of this

(15) It should be noted that vicinal dissymmetry is sufficient for induced optical activity, a condition which is fulfilled when the isolated substrate molecule is bound to a site in fairly rigid orientation (see, for example, D. D. Ulmer, T.-K. Li, and B. L. Vallee, *Proc. Natl. Acad. Sci. U. S.* 47, 1155 (1961), and the results of a recent investigation of dye–polymer optical activity by K. Yamaoka and R. Resnik, *J. Phys. Chem.*, 70, 4051 (1966)). Thus it is not a necessity that the optical activity disappears even if the helical or otherwise rigidly oriented structure of the protein is destroyed. Nevertheless, it does appear likely that the destruction of the secondary or tertiary structure is likely to wash out all or most of the optical activity by increasing the symmetry of the environment or destroy it completely by releasing the bound substrate from its rigid orientation. In fact the disappearance of the induced optical activity, as is the case here, with SDS denaturation of the α-chymotrysin, may be used to draw the tentative conclusion that the bound substrate has been released from its rigid configuration with respect to the protein.

(16) A. Moscowitz, E. Charney, U. Weiss, and H. Ziffer, *J. Am. Chem. Soc.*, **83**, 4661 (1961); E. Charney, H. Ziffer, and U. Weiss, *Tetrahedron*, **21**, 3121 (1965); R. Deen and H. Y. C. Jacobs, *Koninkl. Nedl. Akad. Wetenschap. Proc. Ser. C*, **64**, 313 (1961).

(17) J. H. Pinckard, B. Willie, and H. Zechmeister, *J. Am. Chem. Soc.* **70**, 1938 (1948).

(18) See, *e.g.*, the discussion in H. H. Jaffé and M. Orchin, "Theory and Applications of Ultraviolet Spectroscopy," John Wiley and Sons, Inc., New York, N. Y., 1962.

(19) R. L. Erskin and E. S. Waight, *J. Chem. Soc.*, 3425 (1960).

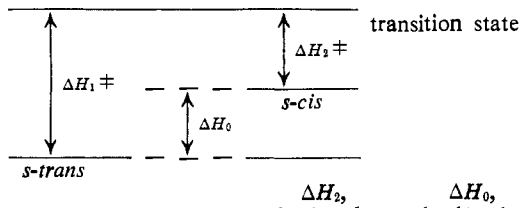
(20) This spectral shift is frequently referred to for configurations of the type of compounds **21** and **22** as due to differences between endocyclic and exocyclic configurations. Without taking up the general

very large change may be due to the hyperconjugative effect of an extra carbon substituent, certainly the major part arises from the relative ground and electronically excited state stabilities of the *s-trans* and *s-cis* configurations.[13]

Studies of the conjugated dienes, both experimentally and theoretically,[21,22] have also shown the *s-trans* → *s-cis* configurational change is accompanied by large red shifts.

One of the most interesting related studies is that of deGroot and Lamb[23] who studied rotational isomers of a number of acrylic molecules by ultrasonic relaxation. Table III summarizes these data on cinnamaldehyde and furylacrolein. Compare the difference in ground-state energy of these compounds with the data of Bernhard, *et al.*,[3] on the spectral shifts of cinnamoyl and furoyl derivatives upon formation of the acyl enzymes, respectively 1400 and 1140 cm^{-1} (309 → 320 mμ and 281 → 292 mμ). The relative energy differences ($\Delta E_{cinnamyl}/\Delta E_{furylacryloyl}$) are 1.23, determined from the spectral shifts, and 1.25, determined from the ultrasonic relaxation studies. The observed red shift is hence consistent with the postulate of *cis–trans* isomerization about the ester *single* bonds. Moreover, the observed thermodynamic stability of the acyl enzyme[24] is consistent with that expected from a *trans–cis* isomerization between absorbed (*s-trans*) ester substrate and covalent (*s-cis*) acyl enzyme ester.

Table III[a]

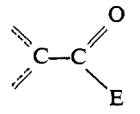


	ΔH_2, kcal/mole	ΔH_0, kcal/mole
Cinnamoylaldehyde	5.62	1.5
Furoylacrolein	5.10	1.2

[a] Data from ultrasonic relaxation studies, ref 16.

The hypothesis of *s-trans* → *s-cis* isomerization is supported by two other facts about native acyl enzymes.

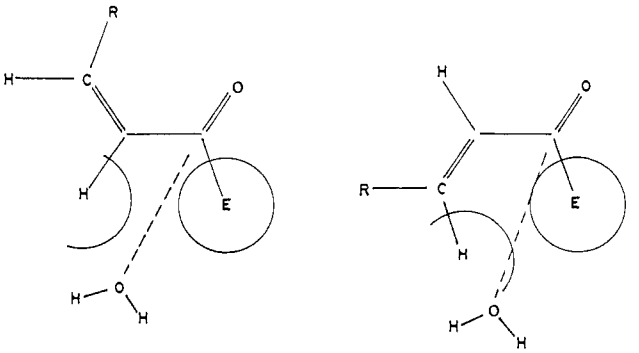
(1) Although native acyl enzymes of type II are all significantly red shifted relative to the corresponding alkyl esters (III), acyl enzymes of type IV (in which λ_{max} and ϵ_{max} are comparable, *e.g.*, benzoyl-, *meta*- and *para*-substituted benzoyl-, and 2-furoylchymotrypsins) are only slightly, or not at all, red shifted.[2] In the latter



IV

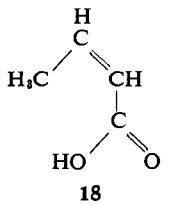
acyl enzymes there is either no distinction (benzoyl and *para*-substituted benzoyl) or little distinction (2-furoyl and *meta*-substituted benzoyl) between the *s-cis* and *s-trans* configurations; hence there should be no spectral shift.

(2) Acyl enzymes of type IV are kinetically far more stable toward hydrolysis than are acyl enzymes of type II.[2,24,25] This kinetic stability is not a consequence of electronic contributions ("substituent effects") from the acyl moiety.[26] Acetyl- and cinnamoylchymotrypsins are hydrolyzed at comparable rates. Benzoylchymotrypsin is hydrolyzed nearly two orders of magnitude more slowly. If the native acyl enzyme is always in the *s-cis* configuration so as to allow for attack by water (*i.e.*, if the *s-cis* configuration is of importance in the catalytic pathway), the branched planar β,β′,α configuration in IV may block the nucleophilic attack



by water. In this regard it is interesting to note the nonplanar (L) configuration at the α-carbon atom of specific substrates (I) (a requirement for very rapid catalysis), indicative, if the "*cis*-ester" hypothesis is correct, of a highly critical stereospecificity for nucleophilic attack.

questions of this attribution, it should be sufficient to point out that, for this case, the strong perturbation which might be expected from lactone formation is observed to give a much smaller effect than the *s-trans* → *s-cis* (endo- → exocyclic) transformation. Thus *s-trans*-β-methacrylic



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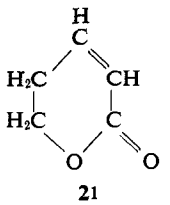


21

acid (**18**) has λ_{max} 205.5 mμ (ε 1.35 × 10^4) while the *s-trans* lactone **21** has λ_{max} 205 mμ (ε 1.07 × 10^4).

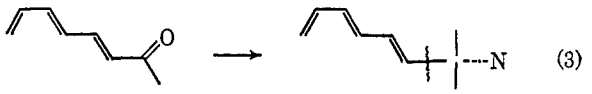
Conclusions

The spectral investigations reported here support the hypothesis that the acylation of α-chymotrypsin with acryloyl substrates is accompanied by a *s-trans* to *s-cis* configurational change about the acryloyl single bond. The possibility of chemical perturbation other than structural has not been experimentally examined, but some of the spectral data have a bearing on this ques-

(21) R. S. Mulliken and C. A. Rieke, *Rept. Progr. Phys.*, **8**, 231 (1941).
(22) N. Allinger and C. Tai, *J. Am. Chem. Soc.*, **87**, 2081 (1965).
(23) M. S. deGroot and J. Lamb, *Proc. Roy. Soc.* (London), **A242**, 36 (1957).
(24) M. L. Bender, G. R. Schonbaum, and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2562 (1962).
(25) In connection with the mechanism of chymotrypsin activity, T. C. Bruice [*J. Polymer Sci.*, **49**, 101 (1961)] has previously suggested the possible requirement of a *trans–cis* change in the O-ester configuration, similar, and possibly related mechanistically, if not spectroscopically, to the conformational change described here.
(26) S. A. Bernhard, E. Hershberger, and J. Keizer, *Biochemistry*, **5**, 4120 (1966).

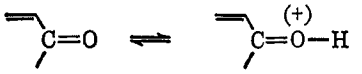
tion.[27] If, as seems likely from these results, the endoenergetic configurational change does occur, then any model of the active site of α-chymotrypsin must accommodate this requirement.

(27) External chemical modification of the ester linkage: none of the above-mentioned data excludes the possibility of chemical interaction somewhere in the proximity of the ester linkage. From the known properties of esters, aqueous solvent, and amino acid residues of the protein, the plausible loci of chemical interaction are at either of the two ester oxygens or at the carbonyl carbon. Nucleophilic interaction at the (electrophilic) carbonyl carbon can be rejected as a significant contribution, since such attack would effectively decrease the intensity and blue shifts. To a rough approximation, this type of interaction would transform the spectrum of a cinnamoyl derivative to that of a styrene derivative (eq 3). Such an acyl-enzyme derivative would be virtually



(3)

unobservable spectroscopically. Electrophilic interaction at oxygen is, however, a distinct possibility. The most obvious electrophilic agent is, of course, a proton (eq 4). Indeed, the spectra of the N-methylamides of cinnamic and furylacrylic acids in strongly acid solutions (approximately 1 *M* HCL)[28] are red shifted to precisely the same wavelengths as



(4)

the corresponding acryloyl enzymes. This result is not unexpected, since protonation of N-methylamides is known to occur at the carbonyl oxygen,[29] a process which would lead to electron delocalization at the C–N bond and hence to a spectrum essentially the same as that observed in the corresponding aldehydes and ketones. The spectra of the protonated furoyl and benzoyl-N-methylamides are similarly red shifted. No such large red shift is observed, however, with the corresponding furoyl and benzoyl enzymes.[2] Moreover, protonation of this type (eq 4) is seemingly unlikely at or near neutrality, where the spectra of the acyl enzymes have been measured. The possibility that hydrogen bonding at carbonyl oxygen, rather than complete proton transfer, is the origin of the red shift cannot be ruled out. No significant red shifts have been noted, however, in the spectra of cinnamoyl and furylacryloyl esters in the presence of very strong hydrogen-bond donors (relative to the corresponding spectra in pure H_2O).[3]

(28) S. A. Bernhard and S. J. Lau, unpublished results.

(29) A. Berger, A. Loewenstein, and S. Meiboom, *J. Am. Chem. Soc.*, 81, 62 (1959).

The Mechanism of Polymerization of N-Carboxyanhydrides in Dimethylformamide. Evidence of the Presence of Cyclic Terminals in Polymers Obtained by Strong Base Initiation

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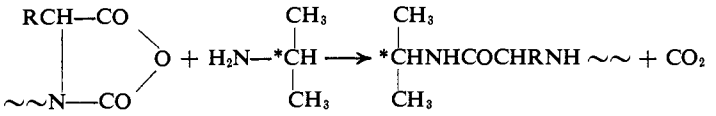
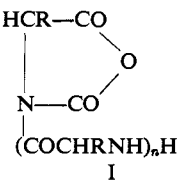
Contribution from the Institute of Organic Chemistry, University of Padua, Padua, Italy, and VIII Sez. Centro Nazionale di Chimica Macromolecolare, Padua, Italy. *Received December 28, 1966*

Abstract: γ-Benzyl-L-glutamate N-carboxyanhydride was polymerized in DMF using C^{14}-labeled amines as initiators. All the radioactivity was incorporated in the polymers obtained by C^{14}-isopropylamine initiation, indicating that normal primary amine polymerization is operative in this case. No radioactivity was found in the polymers when C^{14}-methyldiisopropylamine was used as the initiator. The Bamford mechanism is operative in this case. Using C^{14}-diisopropylamine both mechanisms are simultaneously operative. Polymers prepared by initiation with unlabeled diisopropyl- and methyldiisopropylamine and treated with an excess of C^{14}-labeled isopropylamine exhibit considerable radioactivity. This radioactivity must be due only to reaction between the labeled amine and cyclic terminal present in the polymers.



On the basis of extent of the radioactivity incorporated in polymers "killed" with C^{14}-labeled isopropylamine, the number of polymer molecules containing cyclic terminals, *i.e.*, formed *via* Bamford mechanism, was estimated. It was found that cyclic terminals deactivate *after* the end of the polymerization.

In previous papers[1,2] we presented evidence of the existence of bifunctional intermediates in the polymerization of N-carboxyanhydride (NCA) in dimethylformamide (DMF) initiated by strong bases. We found that when the polymerization mixture at 95% conversion is concentrated, a marked increase in molecular weight of the polymer is observed. We interpreted this fact assuming that coupling between bifunctional species occurs during concentration. These species are



I

formed if initiation and propagation occur *via* "active monomer" mechanisms, as suggested by Bamford[3] and Szwarc.[4]

(1) A. Cosani, G. D'Este, E. Peggion, and E. Scoffone, *Biopolymers*, 4, 595 (1966).

(2) E. Peggion, E. Scoffone, A. Cosani, and A. Portolan, *ibid.*, 4, 695 (1966).

(3) C. H. Bamford and H. Block, in "Polyamino Acids, Polypeptides, and Proteins," M. A. Stahmann Ed., University of Wisconsin Press, Madison, Wis., 1962, p 65.

(4) M. Szwarc, *Advan. Polymer Sci.*, 4, 1 (1965).