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Requirements for Stereospecificity in Hydrolysis by α-Chymotrypsin. Diethyl α-Acetamidomalonate[1]

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Structural requirements in the substrate for stereospecificity in hydrolysis by α-chymotrypsin are being studied. The hydrolysis of diethyl acetamidomalonate is catalyzed by α-chymotrypsin, in a reaction, apparently first order both in ester and in enzyme, which stops after hydrolysis of one ester group and leads to optically active sodium 2-acetamido-2-carbethoxy acetate. Consideration of this and other information indicates that the β-aryl substituent, characteristic of typical substrates for this enzyme, may be required for high reactivity and thus for chemical specificity but is neither necessary nor sufficient for stereospecificity. Conformational diastereomeric interactions of the acylamido group with groups on the enzyme may be responsible for stereospecificity.

Introduction

A number of molecules of type Ca, b, d, d have been shown to undergo enzymatic reactions stereospecifically at one site *d* in preference to the other. Experiments utilizing C-14 showed that in the Krebs cycle, both addition of acetate to oxaloacetic acid and dehydration of the citric acid so formed must proceed asymmetrically.[2-4] Early doubts[2] as to this were allayed by the suggestion that such symmetrical molecules would react asymmetrically provided that they made contact with the enzyme at three different sites, of which only one effected the chemical reaction.[5] These conditions did not appear unlikely, intermediate complexes[6a] with components making contact at several points[6b] having been long proposed in enzymatic reactions, and the subsequent experiments proved the formation and asymmetric utilization[3,4] of citric acid. Similarly, the reduction of acetaldehyde to ethanol and the oxidation of ethanol to acetaldehyde by DPN-yeast alcohol dehydrogenase with deuterium tracer were stereospecific, leading to one enantiomorph of CH_3CHDOH in the reduction, and in the oxidation, to transfer to DPN of that deuterium or hydrogen which had been added enzymatically to the aldehyde.[7] Also asymmetrically C-1 labelled glycerol was formed biologically from acetate in goats,[8] and this was converted asymmetrically in rats[8,9] to glucose labelled at C-3 and C-4, phosphorylation apparently occurring asymmetrically. The plausibility of the three point contact as an explanation for such asymmetric reactions appears to have led to its wide acceptance and to extension of its applicability to other enzymatic reactions. Thus, it has been said that optical specificity is in general dependent upon the enzyme and substrate making contact at three points or more[10] and that optical specificity might not be observed in the hydrolysis of lactate esters by chymotrypsin because the absence of the β-aryl group in this substrate would lead to absence of the three point contact.[11]

Although the Ogston suggestion provides a schematic account of how reactions may proceed asymmetrically, it has also been pointed out that the two sites *d* in molecules Ca,b,d,d are not sterically equivalent, since conversion of one to d′ leads to the D enantiomorph of Ca,b,d,d′ while identical conversion of the other leads to the L enantiomorph.[12] It is this difference which allows the Ogston picture to be a possible explanation, but it also requires that reactions of such a molecule with any optically active reagent (L′), affecting the site d, proceed through two different diastereomeric transition states—L′D and L′L and thus proceed at unequal rates at the two sites d. As the reactive functional group of the optically active reagent attacks a site d, other groups on the reagent interact differently with the groups a and b depending upon which of the sites d is being attacked. Asymmetric reaction of Ca, b, d, d with an enzyme or other optically active molecule may then be analyzed in the steric and steric-electronic terms of conformational analysis, *i.e.*, the same analysis as may be applied to the reactions of enantiomorphs with an enzyme or an optically active reagent, or to the reduction of a carbonyl group by an optically active Grignard reagent[13] or alkoxide.[14] This, to be sure, involves interactions or contacts among groups near the centers and developing centers of asymmetry, but such an analysis will be different in its implications from that derived from the now familiar three point contact picture.

It seemed desirable to us to study the structural requirements in the substrate for stereospecificity in some enzyme reactions, and, initially, to examine this in hydrolysis of substrates of structure Ca,b,d,d by α-chymotrypsin. The relation—or lack of it—between reactivity and stereospecificity, and the relation of structure to these two properties might give useful information about the catalytic processes

(1) (a) We are pleased to acknowledge support of this work by the Division of Research Grants, National Institutes of Health RG 4584, and Guggenheim and Fulbright Fellowships (to S.G.C.). (b) For a preliminary report, see S. G. Cohen and L. Altschul, *Nature*, **183**, 1678 (1959).

(2) H. G. Wood, C. H. Werkman, A. Hemingway and A. O. Nier, *J. Biol. Chem.*, **139**, 483 (1941).

(3) V. R. Potter and C. Heidelberger, *Nature*, **164**, 180 (1949).

(4) P. E. Wilcox, C. Heidelberger and V. R. Potter, THIS JOURNAL, **2**, 5019 (1950).

(5) A. G. Ogston, *Nature*, **162**, 963 (1948).

(6) (a) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913). (b) M. Bergmann and J. S. Fruton, *Advances in Enzymol.*, 1, 63 (1941).

(7) F. A. Loewus, F. H. Westheimer and B. Vennesland, THIS JOURNAL, **75**, 5018 (1953).

(8) P. Schambye and H. G. Woods, *J. Biol. Chem.*, **206**, 875 (1954).

(9) R. W. Swick and A. Nakao, *ibid.*, **206**, 883 (1954).

(10) (a) E. Smith, *Advances in Enzymol.*, **12**, 200 (1951); (b) K. Laidler, "The Chemical Kinetics of Enzyme Action," Oxford Press, New York, N. Y., 1958, pp. 172–173.

(11) I. Tinoco, *Arch. Biochem. Biophys.*, **76**, 148 (1958).

(12) (a) R. Altschul, P. Bernstein and S. G. Cohen, THIS JOURNAL, **78**, 5091 (1956); (b) P. Schwartz and H. E. Carter, *Proc. Natl. Acad. Sci., U. S.*, **40**, 499 (1954).

(13) H. S. Mosher and E. La Combe, THIS JOURNAL, **72**, 3994 (1950).

(14) W. von E. Doering and R. W. Young, *ibid.*, 72, 631 (1950).

and the active sites. Chymotrypsin was chosen because it shows high stereospecificity, reacting with L enantiomorphs[15,16]; kinetic studies have given detailed information about structure requirements in the substrate for high reactivity,[15] a β-aromatic substituent and an α-acylamido group being important; and specific proposals[17-19] for the mechanism of its action have been put forth. We are reporting at this time the results of a study of the action of α-chymotrypsin on diethyl acetamidomalonate (DEAM), a compound which lacks the β-aryl group which is characteristic of natural substrates for this enzyme.

Results and Discussion

Hydrolyses were carried out at 25° in water in the *p*H range 7.0–8.0, most of the runs at *p*H 7.8, and the reactions were followed essentially as described in the literature.[20] Since we wished to examine the products of the reaction, runs were carried to completion. The reactions stopped after consumption of one mole of alkali per mole of ester, indicating that the product would be the half-ester, α-acetamido-2-carbethoxyacetic acid. Results of some runs followed kinetically are summarized in Table I.

TABLE I

HYDROLYSIS OF DIETHYL α-ACETAMIDOMALONATE (DEAM) AT 25.2°

Exp. no.	*p*H	Buffer, mole/l. × 10^3	DEAM, mole/l.	Chymotr., mg./ml.	K_1, $min.^{-1}$
G9	7.68–7.95	0.67	0.0046	0.39	0.016 ± 0.001
G8	7.68–7.85	1.66	.0092	.39	.015
G6	7.60–7.90	3.33	.2000	.39	.016
G5	7.65–7.95	3.33	.0200	.50	.020
L10	7.60–7.90	3.33	.0200	1.10	.041
L13	7.70–7.90	16.1[a]	.063	1.69	.067
G7	7.8	3.33	.0200	0.00	~.0004

[a] Phosphate buffer, all other runs tris-hydroxymethylaminomethane (Sigma A.R.).

The concentration of substrate was varied from 0.0046 to 0.0637 *M* and the concentration of enzyme from 0.39 to 1.69 mg./ml., the times for half reaction being between 15 and 45 minutes. These concentrations of enzyme are similar to those used in the hydrolysis of methyl β-phenyl lactate and methyl β-phenyl-α-chloropropionate in 20% methanol[21] and to those used in the examination of compounds,[22] including diethyl α - benzylmalonate, which were reported not to be hydrolyzed by chymotrypsin. The hydrolysis of DEAM showed kinetics first order in ester to 75–85% reaction, with small increase in rate constant thereafter, presumably because of consumption of alkali in a side reaction. The first order constants were proportional to concentration of enzyme, the reaction appearing to be second order, first in both substrate and enzyme. Plots of the data at several enzyme concentrations are given in Fig. 1. The reactions are truly enzymatic, the rate in the absence of enzyme at *p*H 8 being less than 3% of that at the lowest concentration of enzyme. The Michaelis-Menten equation cannot be applied to these data. Perhaps if a detailed study of the first few per cent. of reaction were made that equation might be found to apply.

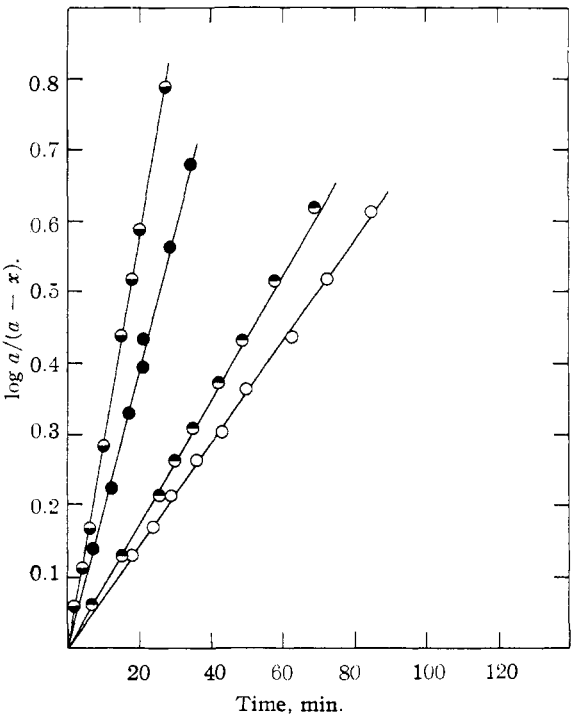
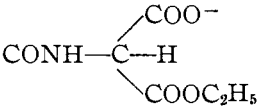


Fig. 1.—Chymotrypsin catalyzed hydrolysis of diethyl acetamidomalonate: ○, 0.020 *M* diethyl acetamidomalonate, 0.39 mg./ml. chymotrypsin; ◓, 0.020 *M* diethyl acetamidomalonate, 0.50 mg./ml. chymotrypsin; ●, 0.020 *M* diethyl acetamidomalonate, 1.10 mg./ml. chymotrypsin; ◒, 0.063 *M* diethyl acetamidomalonate, 1.69 mg./ml. chymotrypsin.

Polarimetric examination of the hydrolysis solution showed that an optically active product had been formed, indicating that the reaction had proceeded asymmetrically, leading to optically active 2-acetamido-2-carbethoxyacetate anion, $+CH_3$-



The product of run L13, in which one equivalent of alkali was consumed in about 60 minutes showed $\alpha_{obsd.}$ + 1.08°, corrected for α-chymotrypsin, + 1.30°, $[\alpha]^{23}D$ + 60°, calculated on the assumption of quantitative hydrolysis to the active half acid. The product racemized slowly in solution, presumably because of the readily enolized α-hydrogen, losing 20% of its activity in two days at *p*H 7.8. A second hydrolysis (L19), containing slightly lower concentrations of substrate, enzyme and buffer was complete in 3 hr. and led to $\alpha_{obsd.}$ + 1.16°, $[\alpha]^{23}D$ + 62°. The rotation is due to the formation of the indicated

(15) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).
(16) M. Brenner, E. Sailer and V. Kocher, *Helv. Chim. Acta*, **31**, 1908 (1948).
(17) F. Westheimer, *Proc. Natl. Acad. Sci. U. S.*, **43**, 969 (1957).
(18) L. W. Cunningham, *Science*, **125**, 1145 (1957).
(19) T. Spencer and J. M. Sturtevant, THIS JOURNAL, **81**, 1874 (1959).
(20) (a) G. W. Schwert, H. Neurath, S. Kaufman and J. E. Snoke, *J. Biol. Chem.*, **172**, 221 (1948). (b) T. H. Applewhite, R. B. Martin and C. Neiman, THIS JOURNAL, **80**, 1957 (1958).
(21) J. E. Snoke and H. Neurath, *Arch. Biochem.*, **21**, 351 (1949).
(22) J. E. Snoke and H. Neurath, *ibid.*, **21**, 437 (1949).

product since (a) the α-chymotrypsin has a rather low rotation which remains constant under the experimental conditions and (b) when ten times as much substrate was used with twice the concentration of enzyme (run L23) the observed rotation rose proportionately to the substrate, $\alpha_{obsd.}$ + 7.49°, $[\alpha]^{23}D$ + 63°. One cannot be certain whether the stereospecificity and the asymmetry in this process is complete, but the specific rotation is somewhat greater than that of some α-monosubstituted derivatives of malonic acid,[23] and our work on diethyl β-acetamidoglutarate[24] indicates that α-chymotrypsin is likely to be completely stereospecific in these reactions.

Since the readings of optical rotation were rather high, it seemed desirable to attempt to follow the enzymatic hydrolysis polarimetrically. A solution of DEAM (*ca.* 0.1 *M*) and the enzyme was prepared in a high concentration (1 *M*) phosphate buffer at *p*H 8.05 so that the reaction could be followed to completion in a polarimeter tube without the customary addition of alkali necessary in the presence of low concentration buffer. The reaction appeared to proceed satisfactorily, but the specific rotation rose only to $[\alpha]^{23}D$ + 44° in 36 minutes and then fell to half this value in about 2.5 hr. and all optical activity was lost in less than 24 hr. It seems likely that the high concentration of buffer led to a fairly rapid general acid catalyzed racemization of rate about one-fourth that of the hydrolysis.

In a larger scale experiment 4.35 g. of the ester was hydrolyzed by 0.10 g. of chymotrypsin in 2 hr. Aliquots were dialyzed against water and the specific rotations observed in the dialysates were slightly low, $\alpha^{23}D$ + 53°, possibly because the *p*H was not controlled well during this hydrolysis. Aliquots of the dialysates were lyophilized, leading quantitatively to the expected amounts of sodium 2-acetamido-2-carbethoxy acetate. These solids could be redissolved and their rotations redetermined, indicating very little (*ca.* 4%) further loss in activity. An infrared spectrum of this salt in D_2O was identical with that of an authentic racemic sample in the amide–carboxyl–ester region. Treatment of the salt with cold concentrated hydrochloric acid led to the free acid, 2-acetamido-2-carbethoxyacetic acid in 80% yield, identified by decomposition and mixed decomposition point with an authentic sample, neutralization equivalent and infrared spectrum.

With respect to isolation of the optically active free acid it may be noted that 2-acetamido-2-carbethoxy acetic acid is a fairly strong acid, k_a *ca.* 10^{-2}, that it is largely dissociated in dilute solution and that the racemization is effectively catalyzed by acid. A hydrolysate, L23, was brought to *p*H 3.85 and observed in the polarimeter, a half-life of about 20 minutes being found for the racemization. Similarly a dialysate, L24, when brought to this *p*H showed a rapid racemization which was essentially stopped by return to neutrality. Since a dilute solution of this acid has a *p*H of about 2, very rapid racemization would be expected. The product of an enzymatic hydrolysis, L25, was dialyzed, the dialysate was treated at 0° rapidly with Dowex-50 ion exchange resin in its acid form, quickly filtered and frozen and lyophilized. The 2-acetamido-2-carbethoxyacetic acid was obtained in 58% yield and when examined in ethanol in a polarimeter had half of the specific optical activity which had been in the hydrolysate. It racemized in this solution with a half life of 25–30 minutes. Another portion of the optically active acid was dissolved between *p*H 6 and 9 and this solution showed a specific rotation 70% of that of the hydrolysate indicating about 30% racemization during dialysis, treatment with the ion exchange resin, filtration, drying and redissolving. The active acid had a melting-decomposition point not depressed by mixture with the *dl* acid, this having been observed in other optically active derivatives of malonic acid.[23] The infrared spectrum of the active acid in chloroform was identical with that of an authentic *dl* sample.

Diethyl acetamidomalonate is hydrolyzed slowly but a symmetrically by α-chymotrypsin. The β-aryl group, characteristic of typical substrates for this enzyme is, in fact, not necessary for stereospecificity. In addition, when present in other substrates it is not sufficient to lead to stereospecificity, since α-chymotrypsin appears to hydrolyze *d* and *l* methyl β-phenyl-α-chloropropionate[21] at equal rates and it hydrolyzes both *d* and *l* methyl β-phenyllactate,[21] to be sure at unequal rates, the *l*-isomer considerably more rapidly than the *d*. It may also be noted, as has been pointed out[17] that Michaelis constants and inhibitor constants for this enzyme are independent of stereochemistry provided that the compounds contain an aromatic residue.[15] Thus the β-aryl group, important in formation of the Michaelis complex is perhaps necessary for *chemical specificity*, *i.e.*, for high reactivity, but is neither necessary nor sufficient for, and is only secondarily related to stereospecificity. Where α-chymotrypsin shows high stereospecificity, there is commonly an acylamido group present at a center of asymmetry or, as in the malonate, at a developing center of asymmetry; a hydroxyl group at the center of asymmetry is less effective, while chlorine is ineffective. It appears that steric interactions of a conformational diastereomeric character, probably involving hydrogen bonding, of the acylamido group with a group on the enzyme lead *via* a preferred orientation in a transition state or intermediate to the stereospecificity. Other compounds now being studied will give information about the allowable distances between the acylamido group and the hydrolyzing function and between the acylamido group and the center of asymmetry.

Experimental[25]

Diethyl acetamidomalonate (Matheson) was crystallized from benzene–petroleum ether, m.p. 96.5–98°, reported[26] 96°.

2-Acetamido-2-carbethoxyacetic Acid.—Diethyl acetamidomalonate (21.7 g., 0.1 mole) was suspended in 200 ml. of 1:1 ethanol:water and 10 ml. of 0.1 *M* disodium hydrogen phosphate. Sodium hydroxide (110 ml., 0.1 *N*) was added slowly with stirring so that the solution was not alkaline to

(23) E. Fischer and F. Brauns, *Ber.*, **47**, 3181 (1914).

(24) S. G. Cohen and E. Khedouri, *Nature*, **186**, 75 (1960).

(25) Melting points are uncorrected; elementary analysis is by Dr. S. M. Nagy, Massachusetts Institute of Technology.

(26) L. Berlinguet, *Can. J. Chem.*, **33**, 1119 (1955).

phenolphthalein for longer than 30 seconds. Stirring was continued for 30 minutes, the volume was reduced to 50 ml. in vacuum without heating, and 20 ml. of cold (−5°) 12 *N* hydrochloric acid was added. Slight evolution of carbon dioxide occurred. The precipitated acid was collected and dried 16.8 g., 89%, m.p. 132–133° dec., and crystallized from benzene containing a little ethanol, 14.4 g., m.p. 140–141°, reported[27] 140–141.5°, neutralization equivalent 189, calculated 189.

The neutralization equivalent was determined in 0.1 *M* lithium chloride with use of a Beckman model G *p*H meter, a titration curve being obtained at the same time. The initial *p*H of an $8.99 \times 10^{-3} M$ solution of the half ester was 2.25 corresponding to $K_a = 9.3 \times 10^{-3}$ and indicating complete dissociation under conditions of the hydrolysis, *p*H 7.8.

α-Chymotrypsin, salt free, Worthington Biochemical Co., lot no. 576–81 was used in the kinetic runs. A salt free preparation from Armour, lot no. 823, was used in some of the optical rotation replicate runs. The enzyme was stored in a freezer at −15°. It was placed in a desiccator for 1 hr. at room temperature before being used.

The solution of chymotrypsin in 10^{-3} *M* buffer consumed 0.1 *N* alkali at 25° at the rate of 1.9 microliters per hour per mg.

Kinetic Experiments.—Reactions were carried out in a small beaker clamped in a container through which was circulated thermostatted water which maintained the reaction temperature at 25.2 ± 0.2°. The beaker contained a Teflon coated magnet for stirring and the reaction solution into which dipped the *p*H electrodes and the microburet. Reactions were followed either with a Beckman model G *p*H meter, 0.1 *N* sodium hydroxide being added with manual control from a Gilmont microburet pipet, or with a Radiometer Titrator, type TTTla, 0.4 *N* sodium hydroxide being added from an automatic buret.

A freshly prepared solution of α-chymotrypsin in buffer was placed in the reaction beaker and adjusted to *p*H 7.8 by addition of standard alkali. An aliquot of 10^{-3} *M* diethyl acetamidomalonate in buffer was added and stirring was started, this being taken as time zero. Volume of added alkali was recorded as a function of time, readings being made at the initial *p*H. For the slower reactions rate constants were determined from the slopes of the plots of log $(V_{calcd.}/V_t)$ *vs.* time. For the fastest reactions with enzyme, measurements were made at constant time intervals, and first order rate plots were obtained from the Guggenheim form of the first order rate law.[28]

Optical Activity of Product. (a) Run L13.—DEAM, 0.211 g. (0.97 mmole), was dissolved in 8.0 ml. of water in the reaction beaker, 5.0 ml. of a solution containing 26 mg. of chymotrypsin and 2.5 ml. of 0.1 *M* phosphate was added, stirring was started, the solution was kept at *p*H 7.8 by the *p*H-stat. One equivalent of alkali (2.30 ml., 0.428 *N*, 0.98 mmole) was consumed in 50 minutes, at which time the reaction essentially stopped; presumed concentration of half ester 0.184 g./16.8 ml., 10.9 mg./ml. based on quantitative yield. The solution was examined in a Zeiss-Winkel polarimeter, $\alpha_{obsd.}$ + 1.08°; a solution of chymotrypsin made up to the same concentration, and with buffer, as in the experiment, led to $\alpha_{obsd.}$ − 0.22°, corrected $\alpha_{obsd.}$ + 1.30°, $[\alpha]^{23}D$ + 60°, 1.5 hr. after the reaction started. After 44 hr. the observed rotation had fallen to + 0.86°, corrected, + 1.08°.

(b) **L23.**—A solution of 64 mg. of α-chymotrypsin in 5 ml. of water and 1 ml. of 0.1 *M* phosphate was brought to *p*H 7.8 and to it was added, over a period of 1 hr., 2.17 g. (0.01 mole) of diethyl acetamidomalonate, alkali (0.49 *N*) being added as needed to maintain *p*H, 19.75 ml., 97% reaction in 2.25 hr., total volume 32 ml. The solution was examined in the polarimeter $\alpha_{obsd.}$ + 7.49°, $[\alpha]^{23}D$ + 63°. The solution was brought to *p*H 3.85, the observed rotation falling to about 4.0° when next observed, 8 minutes after the *p*H was changed. Readings were taken as follows in the polarimeter, the first number in each set being time (minutes), the second the observed rotation: 10, 3.48°; 22, 2.31°; 30, 1.61°; 38, 1.17°; 46, 0.93°; 54, 0.62°; 62, 0.41°; 70, 0.31°; 82, 0.20°; 90, 0.14°; 1400 (∞), 0.0.

(c) **L15.**—A blank solution was prepared from 0.379 g. (2.00 mmole) of 2-acetamido-2-carbethoxyacetic acid and 0.028 g. of α-chymotrypsin in 19 ml. of 1 *M* phosphate buffer, *p*H 7.3 and examined in a polarimeter, $\alpha_{obsd.}$ − 0.24°, 2 dm. tube. This was applied as a correction in each of the following readings. The reaction solution was prepared by addition of diethyl acetamidomalonate, 0.435 g. (2.00 mmole) to a solution of 0.028 g. of α-chymotrypsin in 18 ml. of 1 *M* phosphate buffer, *p*H 8.05. The ester dissolved slowly, over a period of 30 minutes, and the first polarimetric reading was made after 35 minutes, $\alpha_{obsd.}$ + 1.61°, rising to a maximum of + 1.75° in 37 minutes, $[\alpha]^{23}D$ + 44°, falling to + 1.13°, 76 minutes; + 0.82°, 109 minutes; + 0.55°, 148 minutes; + 0.47°, 185 minutes; + 0.21°, 220 minutes; − 0.01°, 22 hr.

Product Isolation. (a) **L24.**—Diethyl acetamidomalonate, 4.35 g. (0.0200 mole), was added over a period of 10 minutes to a solution of 0.101 g. of α-chymotrypsin in 18 ml. of 0.011 *M* phosphate buffer brought to *p*H 7.8. The *p*H was maintained by addition of 1.195 *N* sodium hydroxide, 17.2 ml. in 2 hr., total volume 42 ml. In this run the *p*H reached 5.4 and 9.5 for brief periods while the automatic buret was being refilled. One 20-ml. and two 10-ml. portions were each dialyzed against 20-ml. porifons of water for 7 hr. (Preliminary experiments had indicated that equilibrium was reached in 4 hr.) The dialysates showed rotations of (i) + 4.78°, (ii) + 3.18°, (iii) + 3.06°, corresponding to $[\alpha]^{23}D$ of + 53°. Two 10 ml. aliquots from dialysates (i) and (ii) were frozen and dried over night at 0.05 mm., leading to residues of 0.528 g. and 0.337 g., 104% and 100%, respectively, of the sodium salt of 2-acetamido-2-carbethoxyacetic acid. These residues were made up quantitatively to 10 ml. in water, and the optical rotations were redetermined, $\alpha_{obsd.}$ + 4.52° and $\alpha_{obsd.}$ + 3.06°, 4% loss in activity during lyophilization. The reconstituted sample (ii), 0.337 g. of the sodium salt, 9 ml. of dialysate (iii), corresponding to 0.304 g., and 5 ml. of (i) corresponding to 0.264 g. were combined, frozen and dried. The residue was taken up in 3 ml. of water, cooled in brine and acidified with 12 *N* hydrochloric acid cooled to −12°. 2-Acetamido-2-carbethoxyacetic acid was collected, washed with 12 *N* hydrochloric acid, acetone at −30° and cold petroleum ether, 0.65 g. (0.00344 mole), 80% yield, m.p. 138.5–140° dec., mixed m.p. 141–142° dec.; recrystallized from benzene, neutralization equivalent, 188.8, calculated 189.2.

Anal. Calcd. for $C_7H_{11}O_5N$: N, 7.40. Found: N, 7.43.

A portion of the original dialysate (i) was brought to *p*H 3.75 by 0.1 ml. of 1 *N* hydrochloric acid, the observed rotation falling initially from + 4.78° to + 2.97°; 2.73°, after 2 minutes; 2.42°, 6 minutes; 1.27°, 40 minutes; 0.53°, 75 minutes. The solution was brought to *p*H 7.2 by 0.6 ml. of 1.12 *N* NaOH, $\alpha_{obsd.}$ + 0.25°, 91 minutes; + 0.25°, 103 minutes.

(b) **L25.**—Diethyl acetamidomalonate, 2.43 g. (0.0112 mole), was suspended in 10 ml. of water and 3 ml. of 0.1 *M* Na_2HPO_4, α-chymotrypsin, 0.070 g., was added, and *p*H was maintained by addition of 0.5 *M* NaOH, 22.78 ml. (0.0114 mole) being added in 2 hr. The solution was filtered and washed into a 50 ml. volumetric flask and the rotation was determined $\alpha_{obsd.}$ + 4.05°, corr. + 4.27° $[\alpha]^{23}D$ + 50°. The temperature had risen to 38°, and local *p*H may have been high during this run. A 25-ml. portion (i) and a 20 ml. portion (ii) were each dialyzed against 25 ml. portions of water at 10° for 20 hr. leading to $\alpha_{obsd.}$ of + 2.03° and + 1.96°. Dialysate (i) was treated for 45 seconds with 5 ml. of Dowex 50 ion exchange resin, acid form, filtered into a receiver cooled in Dry Ice–acetone, frozen and dried at 0.05 mm. for 19 hr., leading to 0.303 g., 58% yield of optically active 2-acetamido-2-carbethoxyacetic acid, m.p. 133–135° dec., mixed m.p. with *dl* sample, 133–137° dec., m.p. of *dl* sample, 139–140° dec., all determined simultaneously. A portion, 0.100 g., was diluted to 10.2 ml. in ethanol and observed in a polarimeter. The initial reading, *ca.* 5 minutes, was $\alpha_{obsd.}$ + 0.50°, $[\alpha]^{23}D$ 25°, falling as follows, 9 minutes, 0.41°; 15 minutes, 0.30°; 45 minutes, 0.17°; 60 minutes, 0.10°; 95 minutes, 0.05°. A second part of the acid, 0.193 g. (1.02 mmole), was added in portions to 5 ml. of H_2O and 2 ml. of 0.1 *M* Na_2HPO_4, *p*H being maintained between 6–9 by addition of 0.5 *M* sodium hydroxide, 2.06 ml., 1.03 mmole, and the rotation was determined $\alpha_{obsd.}$ + 1.45, $[\alpha]^{23}D$ ~35°.

Infrared spectra were determined on a Perkin-Elmer Model 21, spectrophotometer. Optically active monethyl α-acetamidomalonate (experiment L25) showed in chloroform absorption bands at 2.95–3.35μ (w), 5.73 (s), 5.96 (s), 6.65 (m), 7.05 (w), 7.33 (w), 7.55 (m), 7.85 (m), 8.45 (m).

(27) J. M. Swan, *Austral. J. Sci. Res.*, **A5**, 711 (1952).

(28) E. A. Guggenheim, *Phil. Mag.*, (7) **2**, 538 (1926).

The synthetic racemic monoester had the same absorption spectrum. Optically active sodium monoethyl α-acetamidomalonate (experiment L24) showed, in D_2O, absorption bands at 2.95–3.05 μ (w), 5.82 (m), 6.18 (s), 6.78–6.90 (w), 7.25 (m), 7.34 (m), 7.55 (w). The synthetic racemic salt had the same absorption spectrum.

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Reaction of Pyridoxal-5-phosphate with Aminothiols

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A study of the reaction between pyridoxal-5-phosphate and aminothiols was prompted by the observation that pyridoxal-5-phosphate is released in the form of a complex with cysteine when muscle phsphorylase *a* is incubated in a cysteine buffer at *p*H 6.8. In aqueous media over a wide range of *p*H values the 4-formyl group of pyridoxal-5-phosphate reacts with the sulfhydryl group of an addend that contains no amino group to form a highly dissociable thiohemiacetal; it also reacts with the amino group of an addend that contains no sulfhydryl group to form a highly dissociable Schiff base. When both functional groups are present in suitable proximity on the same addend, the product is a relatively stable complex containing a thiazolidine ring. The second order constants describing the rates of complex formation between pyridoxal-5-phosphate and both cysteine and its ethyl ester have been determined as a function of *p*H and compared with those found for the same reactions with 5-deoxypyridoxal. The results are believed to bear upon the bonding of pyridoxal-5-phosphate in muscle phosphorylase.

Introduction

When aldehydes react with amines highly dissociable Schiff bases are formed; when they react with mercaptans thiohemiacetals are formed that are also highly dissociable. Schubert[1] described a relatively stable product containing a thiazolidine ring that was formed when aldehydes react in aqueous solution with those aminothiols in which the two functional groups are in suitable proximity. Heyl, *et al.*,[2] studied the products formed when pyridoxal reacts in organic solvents with twenty-three amino acids; only with three, namely cysteine, histidine and penacillamine, was condensation with ring closure observed. These reactions were not carried out in aqueous solutions or with other 4-formyl pyridine analogs; nor were the products characterized spectrophotometrically.

Extensive studies of the absorption spectra of 3-hydroxypyridine analogs have been made by Harris, *et al.*,[3] Peterson and Sober,[4] and Metzler and Snell.[5] The latter authors discussed changes in spectra associated with ionization of the phenolic hydroxyl group, loss of the proton from the pyridinium nitrogen and introduction of a formyl group at position 4. They pointed out the marked similarities between the spectra of pyridoxal-5-phosphate and 5-deoxypyridoxal. Similarities in spectra are to be expected since the structures differ only in the substituents at position 5, namely a methylene phosphate group in the former, a methyl group in the latter. Importantly, neither of these groups can form a hemiacetal with the formyl group at position 4, in contrast with the 5-hydroxymethyl group of pyridoxal. Unlike pyridoxal, which at *p*H 7.0 shows little absorbancy near 380 mμ, the other 4-formyl analogs mentioned have bands with high absorbancies and maxima at or near this wave length. This fundamental difference is attributed to the predominance of the hemiacetal structure of pyridoxal,[6] in which the carbonyl group is not free.

Studies of the mechanism whereby pyridoxal-5-phosphate functions as coenzyme in the decarboxylation of amino acids have not revealed the direct participation of the essential phosphate group in this type of reaction.[7] More recently pyridoxal-5-phosphate was shown by Baranowski, *et al.*,[8] to be a prosthetic group in muscle phosphorylase, an enzyme that catalyzes a totally different type of reaction. Cori and Illingworth[9] demonstrated that it restores activity to the apoenzymes of both phosphorylases *a* and *b*. Its phosphate group does not exchange either with that of glucose-1-phosphate or with inorganic phosphate in the phosphorolytic reaction.[10] The bonding of pyridoxal-5-phosphate to the apoenzyme has been partially elucidated by Fischer, *et al.*,[11] who showed the formyl carbon to be bonded to the protein both through the ε amino group of a lysyl residue and also through a second group designated as X. The present paper is primarily concerned with model complexes that are formed in aqueous solutions when pyridoxal-5-phosphate and 5-deoxypyridoxal react with aminothiols. It is suggested that in certain respects these complexes simulate those native to muscle phosphorylase.

Advantage is taken of changes in the absorption spectra of pyridoxal-5-phosphate and 5-deoxypyridoxal as they react with aminothiols. In aqueous solutions the carbonyl carbon of both of these pyridine analogs is sp^2-bonded, in contradistinction to that of pyridoxal, which is sp^3-bonded as a consequence of hemiacetal formation.[6] Absorption spectra at *p*H 7.0 of the sp^2-bonded carbonyl car-

(1) N. P. Schubert, *J. Biol. Chem.*, 111, 671 (1935); *ibid.*, 114, 341 (1936).

(2) D. Heyl, S. A. Harris and K. Folkers, THIS JOURNAL, **70**, 3429 (1948).

(3) S. A. Harris, T. J. Webb and K. Folkers, *ibid.*, **62**, 3198 (1940).

(4) E. A. Peterson and H. A. Sober, *ibid.*, **76**, 169 (1954).

(5) D. E. Metzler and E. E. Snell, *ibid.*, **77**, 2431 (1955).

(6) D. Heyl, E. Luz, S. A. Harris and K. Folkers, *ibid.*, **73**, 3430 (1951).

(7) S. Mandeles, R. Koppelman and M. E. Hanke, *J. Biol. Chem.*, **209**, 327 (1954).

(8) T. Baranowski, B. Illingworth, D. H. Brown and C. F. Cori, *Biochim. Biophys. Acta*, **25**, 16 (1957).

(9) C. F. Cori and B. Illingworth, *Proc. Natl. Acad. Sci., U. S.*, **43**, 547 (1957).

(10) B. Illingworth, H. S. Jansz, D. H. Brown and C. F. Cori, *ibid.*, **44**, 1180 (1958).

(11) E. H. Fischer, A. B. Kent, E. R. Snyder and E. G. Krebs THIS JOURNAL, **80**, 2906 (1958).