enedioxyphenyl)-benzofuran (XIV) in 25 ml. of glacial acetic acid was cooled and treated with 0.5 ml. of concentrated nitric acid. The solution was stirred at room temperature for 0.5 hour and then diluted with 60 ml. of water. The precipitate (1.1 g.) was removed by filtration and dried. It was dissolved in 10 ml. of a 1:1 mixture of benzene and petroleum ether and was chromatographed on 100 g. (2 × 37 cm.) of acid-washed alumina using the same solvent mixture. The developing solvent was then changed to benzene and about 200 ml. of the eluate was collected. On concentration of the eluate, 711 mg. (66%) of 2-(2-methoxy-3,4-methylenedioxyphenyl)-3-nitrobenzofuran, m.p. 143–149°, was obtained. After one recrystallization from methano and one from cyclohexane, the product melted at 152–153°.

Anal. Calcd. for $C_{16}H_{11}NO_8$ (313.26): C, 61.34; H, 3.54; N, 4.47. Found: C, 61.65; H, 3.71; N, 4.98.

Permanganate Oxidation of 2-(2-Methoxy-3,4-methylenedioxyphenyl)-3-nitrobenzofuran (XV).—A mixture of 50 mg. of 2-(2-methoxy-3,4-methylenedioxyphenyl)-3-nitrobenzofuran (XV), 15 ml. of acetone, 120 mg. of potassium permanganate and 2 ml. of water was refluxed for one hour. The acetone was removed by distillation, and the residue was taken up in water. An aqueous solution of sodium bisulfite was added until the manganese dioxide had dissolved. An excess of solid potassium bicarbonate was added, and the solution was extracted twice with ether. On concentration of the ether extract, 29 mg. of a yellow oil was obtained. The aqueous phase was acidified and extracted four times with ether, and on concentration of the ether extract 16 mg. of a solid remained. The material, on recrystallization from alcohol–water, melted at 154–160°; there was no depression in the melting point of this compound on admixture with an authentic specimen of croweacic acid. The identity was also confirmed by paper chromatography, and by ultraviolet and infrared absorption spectra.

Alkaline Fusion of α-(2-Methoxy-3,4-methylenedioxyphenyl)-3-nitrobenzofuran (XV).—A mixture of 16 mg. of 2-(2-methoxy-3,4-methylenedioxyphenyl)-3-nitrobenzofuran (XV) 125 mg. of potassium hydroxide, and two drops of water was heated in a gold crucible at 230° for 10–15 minutes. The melt was dissolved in water, and the solution was acidified and extracted with ether. The ether solution was extracted with aqueous bicarbonate solution; the bicarbonate extract was acidified and then extracted with ether. Concentration of the ether solution gave a 4.8-mg. residue which was purified by sublimation at 60–80° (0.3 mm.). The sublimate melted at 154° (micro-sub-stage); there was no depression in the melting point of this compound on admixture with an authentic sample of salicylic acid.

RAHWAY, N. J.

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, THE JOHNS HOPKINS SCHOOL OF MEDICINE]

Imidazole Catalysis. VI.[1] The Intramolecular Nucleophilic Catalysis of the Hydrolysis of an Acyl Thiol. The Hydrolysis of n-Propyl γ-(4-Imidazolyl)-thiolbutyrate

BY THOMAS C. BRUICE

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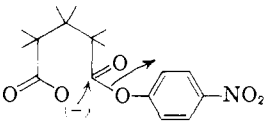
The intramolecular catalysis of the hydrolysis of the thiol–acyl bond of *n*-propyl γ-(4-imidazolyl)-thiolbutyrate (IV) has been studied. With the intramolecular assistance of the imidazolyl group this ester at *p*H values near neutrality undergoes solvolysis at between 10^6 and 10^7 times as fast as a normal thiol-ester. The possible relationship of this finding to the mechanism of action of acyl-thiolases is pointed out. The mechanism of the intramolecular catalysis has been shown to be similar to that for the hydrolysis of the phenyl esters of γ-(4-imidazolyl)-butyric acid which proceed *via* formation of a lactam intermediate.

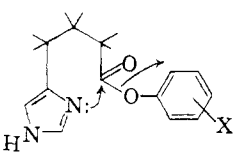
Introduction

It was pointed out a few years ago that intramolecular reactions might serve as worthwhile models for enzymatic processes.[2] Since this suggestion a number of studies involving intramolecular participation in the hydrolysis of ester and amide bonds have been reported in the literature (*cf.* ref. 1 for a detailed account). According to the present concepts of intramolecular catalysis, the large enhancement in rate of an intramolecular reaction as contrasted to a bimolecular reaction is due to a much less negative entropy of activation, the latter factor probably reflecting a smaller loss of translational entropy.[1,3]

To date two intramolecular models appear to be of particular interest since they show a surprising kinetic similarity to the solvolysis of the enzyme ester complex. These are the mono-glutarate ester of *p*-nitrophenol[4] (I) and the phenyl esters of γ-(4-imidazolyl)-butyric acid (II).[1] The hydrolysis of I has been shown to proceed with anchimeric participation of the carboxyl anion, the rate constant being in the range of esteratic rates and about 10^2 slower than IIa. For the model IIa



I



IIa, X = *p*-NO_2
b, X = *m*-NO_2
c, X = *p*-Cl
d, X = H

the rate and its *p*H dependence curve are almost identical to that for the hydrolysis of the *p*-nitrophenyl acetate – chymotrypsin complex[5]—which also involves participation of an imidazolyl group. For the phenyl esters II the rate decreases as the substituents on the phenolic portion become more electron releasing but all the rates fall in the range of enzymic catalyzed hydrolytic reactions, the slowest studied (IId) having a rate constant larger than that for the hydrolysis of the phenyl acetate–wheat germ lipase complex.[6]

(1) For the previous paper in this series see: T. C. Bruice and J. M. Sturtevant, THIS JOURNAL, **81**, 2860 (1959).

(2) H. Morawetz and E. W. Westhead, *J. Polymer Sci.*, **16**, 273 (1955).

(3) M. L. Bender and M. C. Neveu, THIS JOURNAL, **80**, 5388 (1958).

(4) P. E. Zimmering, E. W. Westhead and H. Morawetz, *Biochem. Biophys. Acta*, **25**, 376 (1957).

(5) T. Spencer and J. M. Sturtevant, THIS JOURNAL, **81**, in press (1959).

(6) O. Gawron, C. J. Grelecki and M. Duggan, *Arch. Biochem. Biophys.*, **44**, 455 (1953).

The mechanism by which thiol-esters (particularly of CoA and glutathione) are enzymatically hydrolyzed and undergo transfer reactions is of great importance in biochemistry. The fact that the thiol-ester bond is no more subject to hydroxyl and hydronium ion catalysis than are oxygen esters,[7] coupled with the fact that the thiol-esters are thermodynamically less stable than oxygen esters in aqueous media suggests that they may be subject to rapid intramolecular catalysis by the proper nucleophile. In this paper the kinetics and mechanism for the hydrolysis of the *n*-propyl ester of γ-(4-imidazoyl)-thiolbutyric acid (IV) is presented. This represents the first instance of the facile intramolecular nucleophilic catalysis of a thiol–acyl bond.

Experimental

n-Propyl γ-(4-Imidazolium)-thiobutyrate *p*-Toluenesulfonate (IV).—γ-(4-Imidazolyl-butyric acid hydrochloride[1] (III) was finely ground and dried in an Abderhalden pistol. The dry acid (0.5 g., 0.0026 mole) was placed in a test-tube and thionyl chloride (0.21 ml., 0.003 mole) added. The test-tube was fitted with a drying tube and when the spontaneous reaction had subsided the contents were heated at 55° in a hot water-bath for 1 hr. To the acid chloride there was then added 1.83 ml. (0.02 mole) of *n*-propyl thiol and with constant shaking the reaction mixture was heated on the steam-bath until the evolution of HCl had been completed. To the oily residue was added 0.52 g. (0.003 mole) of freshly fused anhydrous *p*-toluenesulfonic acid. The whole was dissolved in chloroform and the solution boiled until the odor of neither HCl nor thiol was detectable. The chloroform solution was then concentrated to a small volume and carefully overlaid with anhydrous ether (excess). A seed crystal (prepared by rubbing a trace of the tosylate salt under ether) was introduced into the ether layer. The test-tube containing the two layers was then set aside. As the chloroform and ether layers diffuse the product crystallizes as white plates (1.0 g., 100%). For an analytical sample IV was recrystallized five times from methyl acetate–ether (charcoal) by the procedure previously described (0.38 g., 38%), m.p. 85°.

Anal. Calcd. for $C_{17}H_{24}O_4S_2N_2$: N, 7.29; S, 16.66. Found: N, 7.59; S, 16.67.

The thiol-ester is extremely hygroscopic and decomposes rapidly in air to liberate *n*-propyl thiol. For this reason the compound was distributed into smal vials which were used one by one for the kinetic studies.

N,O-Diacetyl-4-(2'-hydroxyethyl)-imidazole hydrochloride (VII) and methyl γ-(4-imidazolium)-butyrate *p*-toluenesulfonate (VIII) were prepared by literature procedures.[1]

Apparatus.—The determination of *p*H values was carried out with a model 22 Radiometer *p*H meter. All spectrophotometric studies were made with a model PMQII Zeiss spectrophotometer which was thermostated at 30° by circulating water from a constant temperature bath. To minimize temperature flux on mixing of reactants the room in which the spectrophotometric studies were made was kept at 29°. The autotitrator employed was a Radiometer type TTT la with a ⅋ Metrohm type X glass electrode and external calomel electrode leading to a ⅋ microtitration cell *via* a ⅋ salt bridge. The titration cell was kept at 15 ± 0.01° by means of a Precision circulating water-bath cooled by a refrigerating bath. Base added with time to maintain constant *p*H was recorded on a Fitzpatrick recorder which drove an Agla-micrometer syringe. Constant stirring was affected by means of a micro-stirring bar.

Kinetics. (A) Spectrophotometric.—All experiments were performed in aqueous solution employing 0.2 *M* phosphate or borate as buffer and the ionic strengths were adjusted to 1.4 *M* with KCl. The disappearance of the thiol-ester bond of IVa-b was followed at 232 mμ and the appearance of lactam VI was followed at 266 mμ. In preliminary experiments the appearance of lactam was determined at various wave lengths in the vicinity of its λ_{max} of 254 mμ. The value of 266 mμ was chosen as one at which perfect first-order kinetics were followed and the maximum molar extinction obtained. At wave lengths below 260 mμ the thiol-ester absorption overlaps that of the lactam. The disappearance of lactam could be followed at 254 mμ without complications. Since the hydrolysis of IV and VI followed strict first-order kinetics (Fig. 1) at all *p*H values the method of Guggenheim[7a] was employed for the determination of the rate constants. In practice the following procedure was employed. The buffer was equilibrated for 1 hr. at 30° in a constant temperature water-bath and the spectrophotometer cells were equilibrated at 30° within the cell compartment of the spectrophotometer. After adding the buffer to the cells the compound was introduced and mixed with the buffer in the following manner. A 20 hypodermic needle was introduced into the vial containing the ester and about 1-2 mg. of the ester pressed into the open end. The needle was next connected to a 1-ml. Tuberculin syringe which had previously been equilibrated at 30° in the buffer employed. At zero time the needle was introduced into the cell and the reactants mixed by pulling the buffer into the syringe and then discharging it back into the photometer-cell, this operation being repeated several times. The absorbance of the reaction mixtures was then determined after 15 sec. (time for bubbles to clear) and at regular time intervals of between 5 sec. and 5 min. (depending on the rate). It was possible to obtain by this means at least 25 points on the absorbance *vs.* time plot for the fastest reactions studied (Fig. 1.). In all cases the reactions were followed to 6-8 half-lives and the value of Δt for the Guggenheim plots was chosen as three half-lives.

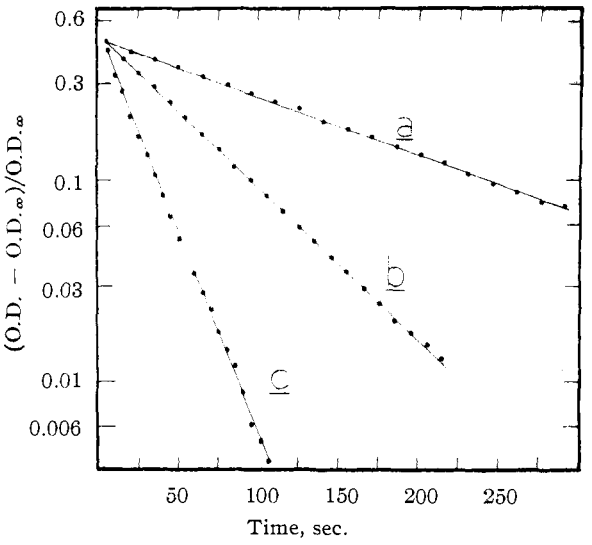


Fig. 1.—First-order plots for the lactamization of *n*-propyl γ-(4-imidazolyl)-thiolbutyrate at *p*H values of: (a) 6.5; (b) 6.95; and (c) 7.9. Reactions studied in phosphate buffer (0.2 *M*) at an ionic strength of 1.4 *M* provided by KCl and at a temperature of 30°. The reactions were followed by observing the change in absorbance at 232 mμ with time.

(B) **Titrimetric.**—For the titrimetric studies the ester was introduced into the microtitration cell (without weighing or prior solution). The titration cell contained 1.5 ml. of 0.1 *N* KCl thermostated at 15°. The compound dissolved immediately and after flushing the microtitration cell with nitrogen and adjusting the *p*H of the solution to the desired value a record of ml. base added to keep constant *p*H with time was obtained. The reactions were followed to at least 6–8 half-lives and the rate constants determined by the method of Guggenheim.[7a] Only those runs in which the standardization of the autotitrator before and after the reaction differed by 0.02 *p*H unit or less were calculated.

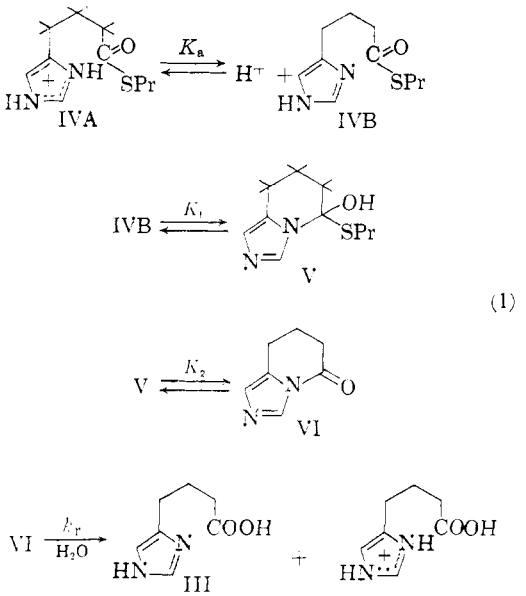
pK_a' determinations for the methyl ester VIII were made with the autotitration device described above and under conditions of ionic strength and temperature identical to the kinetic experiments. The value of the pK_a' was obtained by fitting the titration data to theoretical curves.

(7) (a) P. N. Rylander and D. S. Tarbell, THIS JOURNAL, **72**, 3021 (1950); (b) B. K. Morse and D. S. Tarbell, *ibid.*, **74**, 416 (1952).

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

Results and Discussion

The mechanism of imidazolyl participation in the hydrolysis of IV, with all expected equilibria and intermediate products, is given in (1). The



(1)

rate of thiol-ester disappearance was followed by the decrease in absorbance of the thiol-acyl bond (232 mμ) and by the increase in absorption of VI (266 mμ). The rates of thiol-ester disappearance by these two criteria were in agreement at all *p*H values studied. In Table I there is recorded the rate constants determined at various *p*H values by following the disappearance of IV a–b.

TABLE I

DISAPPEARANCE OF THIOL ESTER (232 mμ) AS A FUNCTION OF *p*H (T = 30°, μ = 1.4)

*p*H	O.D.$_{t=0}$	k_{obs}, min.$^{-1}$	*p*H	O.D.$_{t=0}$	k_{obs}, min.$^{-1}$
6.15	2.4	0.189	6.95	2.07	1.40
	2.21	.192		1.67	1.72
	2.1	.191		1.67	1.57
	1.73	.214		1.43	1.53
	Av.	0.196 ± 0.008		0.885	1.24
6.05	2.00	0.126		Av.	1.50 ± 0.14
	1.61	.168	7.35	2.10	1.905
	1.52	.163		1.98	1.905
	Av.	0.153 ± 0.017		1.90	2.00
6.31	2.15	0.308		1.63	2.10
	1.60	.255		1.35	1.95
	1.51	.163		1.50	2.16
	1.41	.210		Av.	2.00 ± 0.06
	1.02	.230	7.5	2.15	2.30
	0.97	.230		1.54	2.44
	Av.	0.233 ± 0.031		Av.	2.37 ± 0.07
6.50	2.45	0.354	7.6	3.00	2.82
	2.10	.383		2.70	2.78
	1.70	.338		1.58	2.72
	Av.	0.358 ± 0.016		1.42	2.63
6.67	1.295	0.580		1.36	2.83
	1.048	.571		Av.	2.76 ± 0.06
	Av.	0.575 ± 0.005			

The first-order constants for the disappearance of IVa–b can be shown to be insensitive to bimolecular catalysis by the imidazolyl groups of IVb since the initial absorbances of the reaction mixtures at 232 mμ—an index of ester concentration at zero-time—are not correlatable to the determined rates. Employing the autotitrator (*p*H 6.0), catalysis of the hydrolysis of IVa–b could not be realized with imidazole or pyridine at concentrations up to $10^{-2}M$ and the rate of hydrolysis was insensitive to ionic strength between 0.1 and 1.0 M. Also, no hydroxide ion or phosphate buffer catalysis of the lactamization could be demonstrated spectrophotometrically. Therefore, the rate of thiol-ester disappearance in the *p*H range studied is dependent solely on the ratio of IVa to IVb. In equations 2 and 3, K_{app} is the

$$\frac{-\mathrm{d\,(ester)}}{\mathrm{d}t} = k_r\left[\frac{K_{app}}{K_{app}+a_H}\right](\mathrm{IVa} + \mathrm{IVb}) \quad (2)$$

$$k_{obs} = k_r\left[\frac{K_{app}}{K_{app}+a_H}\right] \quad (3)$$

apparent dissociation constant of the ester determined kinetically and a_H is the hydrogen ion activity here assumed to be that value determined by the glass electrode. From equation 2, a plot of $k_{obs} \times a_H$ *vs.* k_{obs} should be linear with the intercept on the k_{obs} ordinate of k_r and a slope of $1/K_{app}$. In Fig. 2, k_{obs} has been plotted *vs.* the product of $k_{obs} \times a_H$ and *vs.* *p*H. Included also in Fig. 2 are the rate constants for lactam appearance. The data supports equation 2; the value of k_r is 5.3 min.$^{-1}$ and K'_{app} is 7.55. The value of pK_a' for the corresponding $-OCH_3$ ester (VIII) was found, titrametrically, to be 7.36 at the same temperature and ionic strength as employed in the kinetic studies. Though IV cannot be titrated in water, VIII which is refractive to hydrolysis under these conditions can and the pK_a' of the latter should be that of IV.[1] The agreement between K_{app} and K'_a is sufficient to show that V (equation 1) is at a very low steady state concentration in the mechanism of the lactamization of IV[8] though it is not for the phenyl esters (IIa, b, c and d).[1]

The rate of lactamization of the thiol-ester was also followed with the autotitrator. Because of the velocity of the reaction and the response time of the instrument the temperature had to be lowered from 30 to 15° for the autotitrator studies. At *p*H values between 5.5 and 6.7 the imidazolyl group of IV is primarily in the protonated form (IV-A). Thus, the lactamization step must result in the over-all loss of a fraction of a proton, while hydrolysis of VI would not alter the hydrogen ion activity. A plot of k_{obs} *vs.* $1/a_H$ was found to be essentially linear (Fig. 3)—as it should be in this *p*H range—and from the slope an apparent second-order rate constant for hydroxide ion catalysis can be calculated. The calculated value was found to be 2.18×10^6 l. mole^{-1} min.$^{-1}$ (15°). The second-order rate constant for the hydrolysis of methyl thiol acetate has been reported to be 1.54 l. mole^{-1} min.$^{-1}$ (25°).[6] Therefore, if the hydrolysis of IV were occurring with hydroxide ion catalysis the rate constant would be about 3×10^6 greater than that previously determined for the specific base catalysis of a closely analogous thiol-ester

(8) T. C. Bruice and G. L. Schmir, THIS JOURNAL, **81**, 4552 (1959).

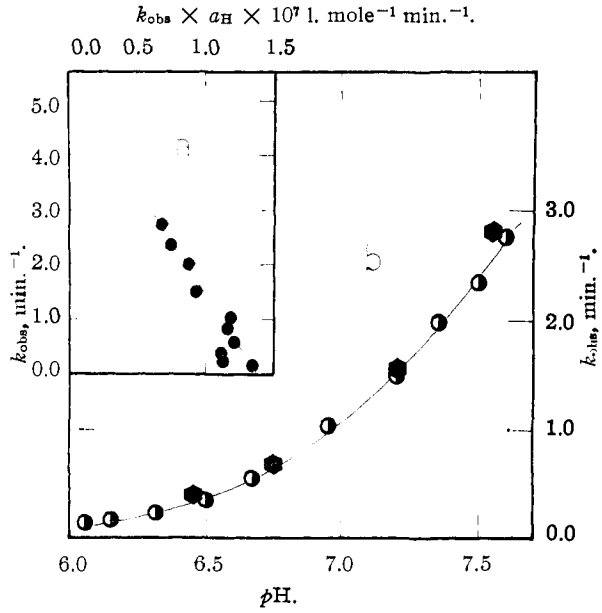


Fig. 2.—(A) Plot of k_{obs} *vs.* $k_{obs} \times a_H$ for the disappearance of the thiol-acyl bond of *n*-propyl γ-(4-imidazolyl)-thiolbutyrate. The intercept on the k_{obs} ordinant is the rate constant for IVb → V (5.3 min.$^{-1}$) and the slope is equal to $(K_{app})^{-1}$ (K_{app} = 7.55). (B) Plots of log k_{obs} *vs.* *p*H for the disappearance of thioacyl bond of (IVa-b) determined at 232 mμ (◑) and for the appearance of lactam determined at 266 mμ (●). Reactions run at 30° in aqueous phosphate buffer (0.2 *M*) at an ionic strength of 1.4 *M* provided by KCl.

bond. From the spectrophotometric studies (Fig. 2) the rate at full participation of the imidazolyl group is 5.3 min.$^{-1}$. To obtain a *pseudo* first-order rate constant of this magnitude for specific base catalysis of an aliphatic thiol-ester would require a calculated concentration of hydroxide ion of *ca.* 4 *M*. These comparative values illustrate the powerful intramolecular catalysis that may be realized by an imidazolyl group in the hydrolysis of a thiol-ester. The rate constant for the reaction of imidazole with ethyl thiol acetate has been reported to be 0.996 l. mole^{-1} min.$^{-1}$ (26.2°).[9] It should be noted that the second-order rate constant for the reaction of imidazole and hydroxide ion with ethyl thiol acetate are of the same order of magnitude. The failure of nucleophilicity to correlate with K_a' is strikingly illustrated by the fact that the K_a' of imidazole is 10^7 smaller than that of hydroxide ion. Apparently, aliphatic thiol-esters are much more susceptible to nucleophilic attack by nitrogen bases than by oxygen bases.[10] A similar but less marked preference has been reported for the phenyl ester, *p*-nitrophenyl acetate.[11] The knowledge of the susceptibility of a bond to preferential nucleophilic attack should serve the enzymologist well in attempts to elucidate the most probable modes of enzymic catalysis.

(9) M. L. Bender and B. W. Turnquest, THIS JOURNAL, **79**, 1652 (1957).

(10) T. C. Bruice, "Acyl Thiols," in "The Organic Chemistry of Sulfur," N. Kharasch, ed., Pergamon Press, London, 1959.

(11) T. C. Bruice and R. Lapinski, THIS JOURNAL, **80**, 2265 (1958).

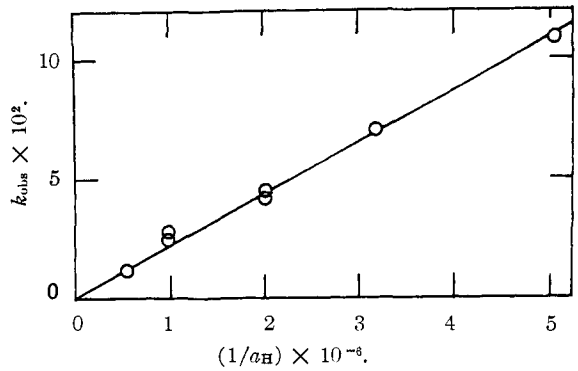


Fig. 3.—Plot of $(a_H)^{-1}$ *vs.* k_{obs} for the lactamization of IVa-b. Rate constants were determined by the appearance of hydronium ions with time (T = 15°, μ = 0.1 with KCl).

The increase in efficiency of intramolecular catalysis over bimolecular catalysis has been expressed as the ratio of the intramolecular rate constant divided by the bimolecular rate constant (time^{-1}/l. mole^{-1} time^{-1} = moles l.$^{-1}$).[3] This ratio, which is the effective concentration of reactants in the bimolecular reaction necessary to realize a pseudo first-order rate equal to that for the intramolecular reaction, is a most crude measure since it is seldom possible to compare pairs of reactions in which the bases are of the same pK_a' and the bonds undergoing scission are identical. However, applying this criterion to the lactamization of IV the necessary concentration of imidazole required to realize a pseudo first-order rate constant for N-acetylimidazole formation of 5.3 min.$^{-1}$, with ethyl thiolacetate, would be 53 *M*. This ratio compares favorably to that for the intramolecular catalysis of the hydrolysis of the phenyl ester IId as compared to the intermolecular catalysis of the hydrolysis of phenyl acetate by imidazole (73 *M*)[1,12]—comparisons for the other phenyl esters of III cannot be made because of a change in mechanism.[1]

At *p*H values approaching neutrality the over-all rate of conversion of IVa–b to III is controlled by the rate of hydrolysis of the intermediate lactam VI (Table II). However, at acidic *p*H values and *p*H values above 10.6 the expulsion of the thiol would be rate limiting. In the solvolysis of the esters IIa, b, c and d the intermediate lactam VI was not identified.[1] We have now shown (Table II) that an intermediate absorbing at 254 mμ with a rate of hydrolysis comparable to that for the product from IV is formed in the instance of the hydrolysis of IIa. Thus, the hydrolysis of the esters II also proceed through VI.

The susceptibility of VI to general and specific base-catalyzed hydrolysis has been compared to that of N,O-diacetyl-4-(2′-hydroxyethyl)-imidazole (VII), which is an alcyclic N-acetylimidazole possessing an alkyl group in the 4-position. Electronically the N-acyl bonds of VI and VII should be comparable. In borate buffers VI and VII were found to be hydrolyzed at rates independent of buffer concentration. Plots of $1/a_H$ *vs.* k_{obs} for the hydrolytic reactions are given in Fig. 4. The values of k_{OH} determined from the plots (assuming

(12) T. C. Bruice and G. L. Schmir, *ibid.*, **79**, 1663 (1957).

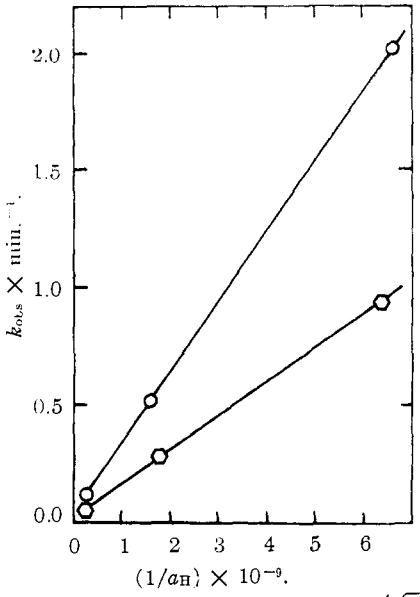


Fig. 4.—Plots of k_{obs} for the hydrolysis of VI (⬭) and VII (O) *vs.* $(a_H)^{-1}$. Reactions were run in 0.2 *M* borate buffer at 30° and the ionic strength was 1.4 *M* provided by KCl.

$K_w = 10^{-14}$) are 2.98×10^4 l. mole^{-1} min.$^{-1}$ for VII and 1.44×10^4 l. mole.$^{-1}$ min.$^{-1}$ for VI. The value of k_{OH} for N-acetylimidazole has been reported to be 1.9×10^4 l. mole^{-1} min.$^{-1}$.[13]

TABLE II

THE RATE OF DISAPPEARANCE OF II (254 mμ) AS A FUNCTION OF *p*H

*p*H	Source[a]	k_{obs}, min.$^{-1} \times 10^2$		Buffer, 0.2 *M*
6.67	T	1.19		Phosphate
		1.25	1.22 ± 0.03	Phosphate
7.0	T	1.14		Phosphate
		1.19	1.18 ± .01	Phosphate
	N	1.14		Phosphate
		1.11	1.12 ± .02	Phosphate
		1.10		Phosphate
7.5	T	1.50		Phosphate
		1.48	1.49 ± .01	Phosphate
7.8	T	1.86		Phosphate
		1.94		Phosphate
		1.92	1.91 ± .03	Phosphate
8.45	T	4.95		Borate
		5.04		Borate
		5.06	5.02 ± .04	Borate
9.25	N	27.4		Borate
		28.8		Borate
		27.9	28.0 ± .5	Borate
9.8	N	82.9		Borate
		93.9		Borate
		93.2		Borate
		93.2	93.3 ± .3	Borate

[a] The symbols T and N refer to the ester employed for the formation of VI (*i.e.*, thiol-ester and *p*-nitrophenyl ester, respectively). At higher *p*H values the nitro ester was employed because its rate of lactamization is forty times faster than that of V (*cf.* ref. 1.).

The rate of nucleophilic attack (*p*H 7.0) of VI by phosphate, 5.3×10^{-2} l. mole^{-1} min.$^{-1}$, was also found to be slower than that for VII, 2.47×10^{-1} l. mole^{-1} min.$^{-1}$ (Fig. 5).

(13) W. Jencks, *J. Biol. Chem.*, in press for 1959.

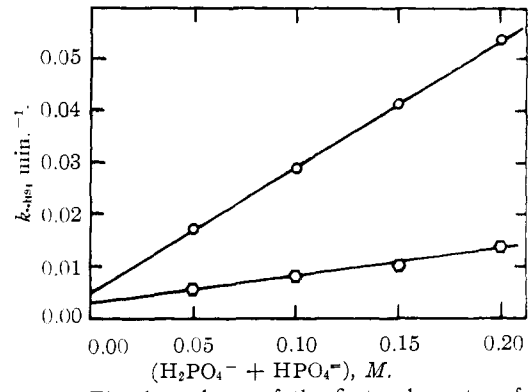


Fig. 5.—The dependence of the first-order rates of disappearance of VI (O) and VII (⬭) on the concentration of phosphate (*p*H 7.0, μ = 1.4 provided by KCl, T = 30°).

Since N-acetylimidazoles are quite susceptible to attack by thiols,[18] the possibility of external return *via* K_2 (equation 1) was investigated. The fact that the disappearance of thiol-ester (266 mμ) is first order to completion indicates that no mass action effect is operative.[15]

Employing the autotitrator and at *p*H 6.0, T = 15°, the addition of *n*-propyl mercaptan up to $10^{-2}M$ had no effect on the rate of hydrolysis. To determine if the rate was sensitive to thiol at any concentration, mercaptoethanol was employed. The latter has the advantage of being miscible with water. At a concentration of 0.2 *M* in thiol and at *p*H 6.0 the hydrolysis of the ester could be completely inhibited. At lower concentrations hydrolysis took place, but at rates greatly reduced from those observed without added thiol. The determination of an actual equilibrium constant by this method was abandoned because at the concentrations of thiol necessary to observe a mass-action effect the glass electrode was damaged and had to be discarded after every few experiments. For this reason reasonably reproducible rate constants could not be obtained. In any event the value of K_2 must be quite small and operationally negligible under the conditions of no added thiol.[16]

Possible Biochemical Significance.—The results of this investigation suggest that for those enzymes responsible for the specific hydrolysis of thiol esters[17-22] a very simple mechanism is plausible.

(15) O. T. Benfey, E. D. Hughes and C. K. Ingold, *J. Chem. Soc.*, 2488 (1952); S. Winstein, E. Clippinger, E. Fainberg, A. H. Heck and G. C. Robinson, THIS JOURNAL, **78**, 328 (1956).

(16) Added thiol could actually have several effects on the rate of the over-all hydrolysis, two of these being: (1) the external-return or mass-action effect, and (2) since mercaptans, disulfides and other sulfur compounds decrease the absorbance of acyl thiols at 232 mμ (W. W. Kielley, E. R. Stadtman and L. B. Bradley, "Glutathione," Academic Press, Inc., New York, N. Y., 1954, p. 57), a yet undescribed complex could be formed with the ester that would slow its rate of hydrolysis.

(17) O. K. Behrens, *J. Biol. Chem.*, **141**, 503 (1944).

(18) E. Racker, *ibid.*, **190**, 685 (1951).

(19) J. Gergely, P. Hele and C. V. Ramakrishnan, *ibid.*, **198**, 323 (1952).

(20) S. Kaufman, C. Gilvary, O. Cori and S. Ochoa, *ibid.*, **203**, 869 (1953).

(21) W. W. Kielley and L. B. Bradley, *ibid.*, **206**, 327 (1954).

(22) H. J. Strecker, P. Mela and H. Waelsch, *ibid.*, **212**, 223 (1955).

Thus, if an acyl-thiol were absorbed onto a protein so that the steric relationship of the ester-bond to an imidazolyl group of histidine (or other appropriate nucleophile) was analogous to that in IV then the protein *would* undergo acylation at an "enzymic" rate. The value of V_m for the hydrolytic reaction and its *p*H dependence would depend on the rate of the intercomplex nucleophilic displacement, the rates of acid and base catalysis of acyl-protein hydrolysis and the pK_{app} of the nucleophile involved. If the process were no more efficient than the model studied here the rate of the acylation step would still be 10^6 greater than for the hydrolysis of a thiol-ester in water at neutrality. Furthermore, if an imidazolyl group were the nucleophile then the protein involved would be a specific acyl-thiol hydrolase because neither the methyl ester nor the amide of III undergo hydrolysis at room temperatures.[1]

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Synthetic Polysaccharides. IV. Preparation of Carboxyl Derivatives of Polyglucose

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Synthetic polyglucose has been oxidized with sodium periodate to the dialdehyde derivative of polyglucose which, by further oxidation with different amounts of chlorous acid, has been converted to a series of derivatives containing different amounts of carboxyl groups.

The preparation of polyglucose sulfates was reported in the third paper of this series.[1] These highly branched polyanions were useful models to study macromolecular interactions with basic or amphoteric proteins.[2] For example, polyglucose sulfate preparations of different molecular weight and different degree of substitution had different enzyme inhibitory potency.[3]

Derivatives having known amounts of weaker dissociating anionic groups, such as carboxyl groups, but retaining the high molecular weight and the unique highly-branched structure of the polyglucose[4] have been sought for further studies. These carboxyl derivatives have the advantage in certain biological applications of being less toxic than the sulfates, which are strong anticoagulants.[1,5]

This paper reports the preparation of a series of non-dialyzable polyglucose carboxyl derivatives which have different carboxyl content. The derivatives were prepared by oxidation of polyglucose first with periodate ion to an aldehyde derivative, and then with different amounts of chlorous acid further converting a percentage of the aldehyde groups to carboxyl groups.[6]

The polyglucose was prepared by one of the previously reported polycondensation methods.[7] It was not fractionated, it had a highly branched structure[4,8] and an intrinsic viscosity of 0.06; a number average molecular weight of 13,000 was indicated by the reducing end group method.[7] The polyglucose, however, contained only 15% of a lower molecular weight fraction which dialyzed through cellophane in forty hours. This latter experiment showed that about 85% of the polymer had substantially higher molecular weight than 13,000. In fact, indication from previous measurements[4] was that the majority of such a polymer had a higher molecular weight than 30,000. The preparation was therefore highly polydisperse.

To choose the length of oxidation a preliminary periodate oxidation was carried out under previously employed experimental conditions[8] (0.03 *M* periodate, 2°, in the dark). The amounts of periodate consumed and of formic acid liberated were measured at intervals. These values were generally similar to those obtained from previous determinations on similar polyglucoses.[8] Most of the oxidation took place rapidly within the first 24 hours, and although after this time the amounts of periodate consumed and formic acid released still increased, the rate of increase was much lower and approximately linear. An oxidation period of 48 hours was selected for the preparative work, and the periodate concentration was increased to 0.188 *M* in order to reduce the volume.

Again the oxidation was followed by measuring the periodate consumed and the formic acid released (Fig. 1). After 48 hours of oxidation, 30% non-dialyzable product was obtained. It contained 63% dialdehyde and 24% unoxidized glucose. Apparently over-oxidation at a few sites of the polymer chain caused random scission which increased considerably the amount of dialyzable product.

Our figure of 63% for the dialdehyde content of the non-dialyzable fraction of periodate oxidized polyglucose has to be taken with some reservation. In the determination we heated a sample of the aldehyde with *p*-nitrophenylhydrazine and measured the intensity of the color of the insoluble *p*-

(1) J. W. Wood and P. T. Mora, THIS JOURNAL, **80**, 3700 (1958).
(2) P. T. Mora and B. G. Young, *Nature*, **181**, 1402 (1958).
(3) P. T. Mora and B. G. Young, *Arch. Biochem. Biophys.*, **82**, 6 (1959).
(4) P. T. Mora, *J. Polymer Sci.*, **23**, 345 (1957).
(5) E. London, R. S. Theobald and G. D. Twigg, *Chemistry & Industry*, 1060 (1955).
(6) *Cf.* B. T. Hofreiter, I. A. Wolff and C. L. Mehltretter, THIS JOURNAL, **79**, 6457 (1957).
(7) P. T. Mora and J. W. Wood, *ibid.*, **80**, 685 (1958).
(8) P. T. Mora, J. W. Wood, P. Maury and B. G. Young, *ibid.*, **80**, 693 (1958).