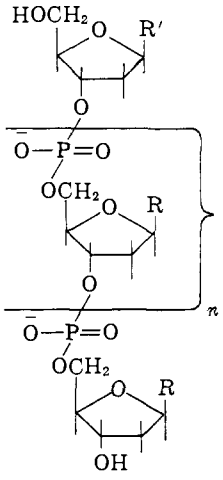
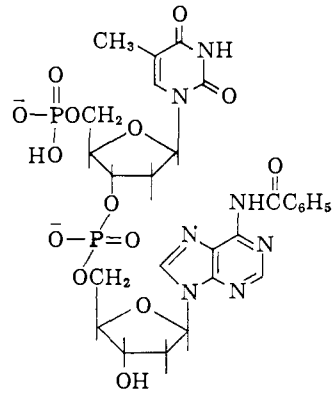
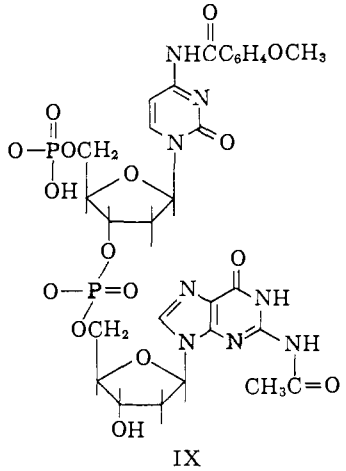
(d-TpApC)[5] emphasized the necessity for protecting the amino groups in the nucleosides.[3] The present communication outlines the approaches which have been developed successfully for the synthesis of oligo- and polynucleotides containing different nucleotides in predetermined sequences.



VII, R = cytosine
R′ = thymine



VIII



IX

Pentanucleotides.—5′-O-*p*-Methoxytritylthymidylyl-(3′→5′)-N-benzoyldeoxyadenosine (I) was prepared in 75% yield by condensation of N-benzoyl-3′-O-acetyldeoxyadenosine-5′ phosphate (0.7 mmole) with 5′-O-di-*p*-methoxytritylthymidine (1.5 mmoles) and a careful alkaline treatment. The thymidine trinucleotide (II) was prepared by acetylation of the trinucleotide (pTpTpT).[6] A mixture of pyridinium I (0.080 mmole), II (0.019 mmole), pyridinium Dowex-50 ion exchange resin (0.150 g.), dicyclohexylcarbodiimide (DCC) (250 mg.) in dry pyridine (0.5 ml.) and dimethylformamide (0.2 ml.) gave after 5 days at room temperature and subsequent chromatography on a DEAE-cellulose (carbonate) column the pentanucleotide (III) in 12% isolated yield. The latter was characterized by established chemical and enzymic methods.[3,4,6] In a parallel study, the condensation of N,O$^{3'}$-diacetyldeoxyguanosine-5′ phosphate with N-anisoyl-5′-O-di-*p*-methoxytrityldeoxycytidine[7] then a brief alkaline treatment gave IV which now was condensed with the protected deoxycytidine trinucleotide (V) by a procedure similar to that described above. After the removal of the protecting groups, pure deoxycytidylyl-(3′→5′)-deoxyguanylyl-(3′→5′)-deoxycytidylyl-(3′→5′)-deoxycytidylyl-(3′→5′)-deoxycytidine (VI) was isolated in 17% yield. The two examples cited demonstrate that suitable protecting groups are now available for the manipulation of all of the major deoxyribo-nucleosides and -nucleotides for polynucleotide synthesis.

Homologous Polynucleotides Bearing a Different Terminal Nucleoside.—A mixture of N,O$^{3'}$-diacetyldeoxycytidine-5′ phosphate and N-anisoyldeoxycytidine-5′ phosphate was polymerized as described previously.[8] After 9 days at room temperature, the mixture was treated in dry dimethylformamide with an excess of 5′-O-di-*p*-methoxytritylthymidine (0.75 mmole of the nucleoside for the polymeric mixture derived from a total of 0.7 mmole of the protected deoxycytidine nucleotide). Work-up after three days, including ammoniacal and acidic treatments, gave the homologous d-T-(pC)$_n$-pC (VII) as the new series of major products which were isolated pure and characterized. This method is complementary to that described earlier[6] for preparation of polynucleotides of the type d-pT(pT)$_n$-pC by copolymerizing N,O$^{3'}$-diacetyldeoxycytidine-5′ phosphate and thymidine-5′ phosphate. The present principle has been used in this Laboratory for the binding of deoxyribopolynucleotides to cellulose by ester bond formation with the hydroxyl groups of the latter.[9]

Deoxyadenylate-thymidylate and Deoxyguanylate-deoxycytidylate Copolymers.—*β*-Cyanoethyl thymidine-5′ phosphate was prepared by reaction of pyridinium thymidine-5′ phosphate with DCC in the presence of hydroacrylonitrile. The condensation of N-benzoyl-3′-O-acetyldeoxyadenosine-5′ phosphate (0.59 mmole) with *β*-cyanoethyl thymidine-5′ phosphate (0.88 mmole) in the presence of DCC (1.1 g.) and pyridine (1 ml.), a careful alkaline treatment and chromatography afforded the protected dinucleotide (VIII) in 31% yield. The analogous reaction of N,O$^{3'}$-diacetyldeoxyguanosine-5′ phosphate (4 mmoles) with N-anisoyldeoxycytidine-5′ *β*-cyanoethyl phosphate (2 mmoles) gave the protected dinucleotide (IX) (0.9 mmole). The protected dinucleotide (VIII) (0.5 mmole) was treated in dry pyridine (0.75 ml.) with DCC (2 mmoles) for 6 days at room temp. Work-up inclusive of an ammoniacal treatment and chromatography on a DEAE-cellulose column gave d-pTpApTpA (10.7%), d-pTpApTpApTpA (5%), d-pTpApTpApTpApTpA (2%) and higher homologs (1.1%), from which after treatment with bacterial alkaline phosphomonoesterase the polynucleotides d-T-(pApT)$_4$pA and d-T-(pApT)$_5$pA were isolated pure. The polymerization of the protected dinucleotide (IX) similarly gave the polynucleotides d-pCpGpCpG, d-pCpGpCpGpCpG and d-pCpGpCpGpCpGpCpG although the extent of polymerization was less satisfactory. The polynucleotides herein described are being used in studies of polynucleotide interactions as well as in enzymatic studies.

(5) For the system of abbreviations see Chapter 5, ref. 2.
(6) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 675 (1961).
(7) H. Schaller and H. G. Khorana, *Chem. and Industry*, 699 (1962).
(8) H. G. Khorana, A. F. Turner and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 686 (1961).
(9) P. T. Gilham, *ibid.*, **84**, 1311 (1962).

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ACYL-ENZYME INTERMEDIATES IN THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF "SPECIFIC" SUBSTRATES. THE RELATIVE RATES OF HYDROLYSIS OF ETHYL, METHYL AND *p*-NITROPHENYL ESTERS OF N-ACETYL-L-TRYPTOPHAN[1,2]

Sir:

The stepwise process for the catalysis of hydrolytic reactions by α-chymotrypsin (eq. 1) has received con-

(1) This research was supported by Grant H-5726 of the National Institutes of Health.
(2) Paper XVIII in the series, The Mechanism of Action of Proteolytic Enzymes.

TABLE I

THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF DERIVATIVES OF N-ACETYL-L-TRYPTOPHAN[a]

Derivative	k_{cat}, sec.$^{-1}$	K_m(app) × 10^5, M	k_2, sec.$^{-1}$	k_3, sec.$^{-1}$	Relative k_{OH^-} of acetate, M^{-1} sec.$^{-1}$	Rate-determining step
Amide[b]	0.036	500	*0.036*[f]	(30)[g]	1	Acylation
Ethyl ester[c]	26.9 ± 0.5	9.7	*480*	*29*[f]	2,750	Deacylation
Methyl ester[d]	27.7 ± 0.5	9.5	*500*	*29*	5,500	Deacylation
p-Nitrophenyl ester[e]	30.5 ± 1.2	0.2	*26,300*	*30.6*	315,000	Deacylation

[a] 25.0°; pH 7.0; 0.1 M phosphate buffer; 3.17% (v./v.) acetonitrile; $[E_0] = 8 \times 10^{-8}$ M measured by titration with N-*trans*-cinnamoylimidazole: G. R. Schonbaum, B. Zerner and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961); kinetic runs were carried out on a Cary 14 PM recording spectrophotometer and recording spectrophotometer equipped with a stopped-flow mixing device. [b] M.p. 194–194.5°; $[\alpha]$D +19.3° (methanol). All rotations here reported taken at 23–24°. [c] M.p. 110–111.5°; $[\alpha]$D +7.1° (ethanol). [d] M.p. 154–155°; $[\alpha]$D +13.8° (methanol). [e] M.p. 136–137°; $[\alpha]$D +0.47° (acetone); *Anal.* Calcd. for $C_{19}H_{17}N_3O_5$: C, 62.12; H, 4.67; N, 11.44. Found: C, 62.13; H, 4.87; N, 11.29. 1.00 mole $NO_2C_6H_4OH$/mole. This compound is essentially racemic. The enzymatic hydrolysis accounts for 50 ± 1% of the substrate added under the conditions used. The D compound irreversibly inhibits the enzyme in a first-order process, due to acylation of the enzyme (this acylation can be clearly seen at higher enzyme concentrations where the reaction of the L-compound is essentially instantaneous and the slow acylation of the D compound can be measured. Thus the kinetic equation for the DL system is $-dS/dt = (k_{cat}S[E_0]e^{-kdt})/K_m(app) + S)$ (K. J. Laidler, "The Chemical Kinetics of Enzyme Action," Oxford Univ. Press, London, 1958). If the enzyme is saturated with the substrate $V = k_{cat}[E_0]e^{-kdt}$. By plotting log V *vs.* t, straight lines are obtained whose intercepts at $t = 0$ yield $k_{cat}[E_0]$. At $[S_0]$ from 21 to 160 × 10^{-6} M, such plots are linear and yield essentially the same k_{cat}. [f] Calculated figures are shown in italics (see text). [g] Assumed.

siderable justification from investigations utilizing nitrophenyl esters and related compounds.[3] In eq. 1, ES′ represents the acyl-enzyme intermediate, P_1 the alcohol and P_2 the acid if the substrate is an ester.

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \qquad (1)$$
$$\qquad\qquad\qquad\qquad\quad + \\ \qquad\qquad\qquad\qquad\quad P_1$$

Critics, however, have pointed out that this mechanism may apply to a *p*-nitrophenyl ester, but not to a "specific" methyl ester or amide substrate. Conformity to eq. 1 was demonstrated unequivocally in the α-chymotrypsin-catalyzed hydrolysis of methyl cinnamate, by direct spectrophotometric investigation.[4] Criticism was still advanced since this compound, although a methyl ester, is not a "specific" substrate, since it contains a cinnamic acid moiety.

We have devised experiments to ascertain whether the kinetic behavior of "specific" substrates is consistent with eq. 1, by determining the catalytic rate constant, k_{cat} (eq. 2), of the α-chymotrypsin-catalyzed hydrolyses of the ethyl, methyl and *p*-nitrophenyl esters of a specific compound, N-acetyl-L-tryptophan. A theoretical analysis[5] of chymotrypsin kinetics predicts that the reactions of these three acetyl-L-tryptophan derivatives will show essentially identical catalytic rate constants. The catalytic rate constants for the ethyl, methyl and *p*-nitrophenyl esters shown in

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \qquad (2)$$

Table I are almost identical, although the rate constant for the amide is considerably lower. These data are internally consistent and also consistent with the large body of other kinetic data on chymotrypsin reactions, assuming all reactions in Table I proceed *via* eq. 1, with the rate-determining step noted there.

Chymotrypsin catalyses can be characterized as nucleophilic reactions.[3] Toward the nucleophile hydroxide ion, the relative reactivities of *p*-nitrophenyl, methyl, and ethyl esters at 25° are in the ratio 100:2:1; toward imidazole, the relative reactivities are more disparate. The fact that the three esters of N-acetyl-L-tryptophan react at essentially identical rates[6] can most readily be explained by postulating a stepwise process involving the rate-determining decomposition of a common intermediate containing the N-acetyl-L-tryptophanyl group. Such an intermediate may be most simply identified as N-acetyl-L-tryptophanyl-α-chymotrypsin.[7]

In chymotrypsin reactions following eq. 1, $(k_2/k_3 + 1) = K_m/K_m(app)$.[8] If acylation is rate-controlling ($k_2 << k_3$), $K_m = K_m(app)$; if deacylation is rate-controlling ($k_2 >> k_3$), $K_m(app) = K_m(k_3/k_2)$. The difference in $K_m(app)$ between the tryptophan ester and amide substrates cannot be explained on structural grounds since the specificity resides in the tryptophan residue of these compounds. These differences can be explained readily by postulating that the real K_m's of all these compounds are similar, that the K_m of the amide reaction is the only real K_m (acylation rate-controlling), and the K_m's of the ester reactions are apparent K_m's whose values are diminished by the ratio (k_3/k_2) (deacylation rate-controlling).[3]

Using the relationship $(k_2/k_3 + 1) = K_m/K_m(app)$,[8] the assumptions that $K_m^{Amide}(app) = K_m^{Amide}$ and $K_m^{Amide} = 2.9K_m^{Ester}$ for all esters,[9] and the relationship $k_2 = k_{cat}(1 + k_2/k_3)$,[8] the calculations of k_2 and k_3 in Table I were made. The calculated k_3 values of the three esters are internally consistent: the calculated k_2 values of the four compounds parallel closely the nucleophilic order as determined by the corresponding alkaline rate constants.

The conclusions of this communication are further substantiated by the effect of pH on the catalytic rate constants of "specific" substrates, and on the rate constants of acylation (k_2) and deacylation (k_3) of poor substrates of chymotrypsin reported another communication.[10]

(3) For references, see M. L. Bender, *J. Am. Chem. Soc.*, **84**, 2582 (1962).

(4) M. L. Bender and B. Zerner, *ibid.*, **83**, 2391 (1961); **84**, 2550 (1962).

(5) B. Zerner, to be published.

(6) In the α-chymotrypsin-catalyzed hydrolyses of ethyl, methyl and *p*-nitrophenyl hippurate, a variation of catalytic rate constant of over ten-fold is observed, indicating, among other things, that the enzymatic process is capable of distinguishing among these esters.

(7) H. Gutfreund and B. R. Hammond, *Biochem. J.*, **73**, 526 (1959), measured the catalytic rate constants for the α-chymotrypsin-catalyzed hydrolyses of N-benzoyl-L-tyrosine amide, N-benzoyl-L-tyrosine ethyl ester and N-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester. They observed that the catalytic rate constant of the *p*-nitrophenyl ester is only 50% larger than that of the ethyl ester, and concluded that all three reactions proceed according to equation 1. Unfortunately, the comparison is not valid, for a benzyloxycarbonyl derivative was compared with a benzoyl derivative. This defect was pointed up when we synthesized N-benzyloxycarbonyl-L-tyrosine ethyl ester and compared it to the corresponding *p*-nitrophenyl ester. The catalytic rate constants of these two compounds differ by a factor of *ca.* 20, and therefore do not exhibit the identity of rates demonstrated here, possibly for steric reasons. Gutfreund and Hammond, on the basis of their erroneous (although reasonable) extrapolation, concluded that in the α-chymotrypsin-catalyzed hydrolysis of N-benzoyl-L-tyrosine ethyl ester both acylation and deacylation are rate-controlling while we conclude that in the hydrolysis of N-acetyl-L-tryptophan ethyl ester deacylation is almost entirely rate-controlling. It would appear that the reaction of the former compound also proceeds with deacylation rate-controlling.

(8) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956).

(9) The ratio of K_I's of N-acetyl-D-tryptophan amide and N-acetyl-D-tryptophan isopropyl ester is 2.9.[3] The calculated values of k_2 are inversely proportional to the assumed value of this number.

Thus there exists an imposing body of kinetic evidence which requires that the mechanism of hydrolysis of "specific" substrates by α-chymotrypsin follow eq. 1.

(10) M. L. Bender, G. E. Clement, F. J. Kézdy and B. Zerner, *J. Am. Chem. Soc.*, **85**, 358 (1963).

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THE APPARENT MOLAR VOLUME OF SODIUM IN AMMONIA AT −45°[1]

Sir:

We have previously[2] reported measurements of the apparent molar volume, V, of sodium in ammonia at 0°; V, defined as (volume of solution − volume of solvent)/g. atoms Na, is nearly independent of concentration, being 56.3 ml. mole^{-1} at 0.35 M and about 2 ml. mole^{-1} greater at 0.009 M. The change is but little outside of experimental error. Evers and Filbert[3] have reported similar measurements at −45°; they found V to decrease slowly from 62 ml. mole^{-1} at 0.2 M to 60 at 0.050 M and steeply to 53 at 0.040 M, and then to rise steeply from 53 at 0.023 M to 59 at 0.018 M, 64 at 0.012 M and 65 at 0.0036 M. They have stated their accuracy to be better than 3%; however, it would be expected that the probable errors of such measurements in dilute solution would be roughly inversely proportional to the concentration.

The dissimilarity of these two reports has led us to perform measurements at −45° also; the procedure was essentially as previously described. A stirred methyl alcohol bath was used, cooled by a system described elsewhere[4] controlled by a Hallikainen controller and electrical heater and monitored with a platinum thermometer, Mueller bridge, and recorder. The dilatometer bulbs were sealed together, rather than being assembled with waxed joints. Results are given in Table I; over-all uncertainties are estimated separately for each run. Within experimental accuracy, V is constant at 60.7. Agreement with the data of Evers and Filbert at the higher concentrations is satisfactory, but there is no evidence for a minimum or any considerable change at lower concentrations.

TABLE I

APPARENT MOLAR VOLUME OF Na IN NH_3 AT −45°

C (mole liter^{-1})	V (ml. mole^{-1})	C (mole liter^{-1})	V (ml. mole^{-1})
0.1640	60.7 ± 0.2	0.0276	$61.5^{+1.0}_{-1.5}$
.0940	60.7 ± 0.2		
.0493	59.8 ± 1.0	.0269	61.2 ± 0.8
.0428	$59.7^{+1.0}_{-0.5}$	.0234	59.9 ± 0.8
		.0161	60.4 ± 1.2
.0388	60.0 ± 0.7	.0114	59.4 ± 1.2
.0317	61.4 ± 1.2	.0058	59.9 ± 3.0

It may be noted that V decreases from −45 to 0°; for aqueous solutions of electrolytes at ordinary temperatures, V increases with increasing temperature.

It is also of interest to note that both the volume and absorption spectra[5] of sodium–ammonia solutions are essentially constant through a concentration range where the magnetic, electrochemical, and thermochemical properties change drastically. Becker, Lindquist, and Alder[6] proposed the equilibria $(1/2)M_2 = M = M^+ + e^-$ to explain the conductance and magnetic properties. Gold, Jolly and Pitzer[7] proposed that the intermediate non-conducting paramagnetic species M is an ordinary ion-pair and that M_2 is a quadrupolar ionic assembly. However, dissociation of salt ion-pairs in ammonia produces a decrease of 20 to 30 ml. mole^{-1} in V[1].

(1) This work was performed under the auspices of the U. S. Atomic Energy Commission.

(2) S. R. Gunn and L. G. Green, *J. Chem. Phys.*, **36**, 363 (1962).

(3) E. C. Evers and A. M. Filbert, *J. Am. Chem. Soc.*, **83**, 3337 (1961).

(4) S. R. Gunn, *Rev. Sci. Instruments*, **33**, 880 (1962).

(5) M. Gold and W. L. Jolly, *Inorg. Chem.*, **1**, 818 (1962).

(6) E. Becker, R. H. Lindquist and B. J. Alder, *J. Chem. Phys.*, **25**, 971 (1956).

(7) M. Gold, W. L. Jolly and K. S. Pitzer, *J. Am. Chem. Soc.*, **84**, 2264 (1962).

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SIGMOID AND BELL-SHAPED pH-RATE PROFILES IN α-CHYMOTRYPSIN-CATALYZED HYDROLYSES. A MECHANISTIC CORRELATION[1]

Sir:

The thesis enunciated earlier, that all chymotrypsin-catalyzed hydrolyses follow eq. 1, has been substantiated by spectrophotometric and kinetic evidence.[2,3] On experimental and theoretical grounds the two steps of acylation and deacylation (k_2 and k_3) were postulated

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \qquad (1)$$
$$\qquad\qquad\qquad\qquad\quad + \\ \qquad\qquad\qquad\qquad\quad P_1$$

to be mechanistically equivalent, the enzymatic components of their transition states being identical.[3] On this basis, the same pH dependence might be expected in all chymotrypsin steps and reactions. However, some chymotrypsin-catalyzed reactions show bell shaped pH-rate profiles while others show sigmoid pH-rate profiles.[4]

The pH dependencies of the rate constants of the two *individual* steps (k_2 and k_3) (eq. 1 and Table I) indicate that pH-rate profiles of *deacylation* obey a *sigmoid* curve, while the pH-rate profile of the only *acylation* step observed over a sufficient pH range follows a *bell-shaped* curve.

The pH dependencies of the *catalytic rate constants* (turnover) (eq. 2 and Table II) of this research and literature data may be correlated (on the basis of Table I) with the pH dependence of a rate-controlling acylation or deacylation step. In the hydrolyses of acetyl-L-tryptophanamide and acetyl-L-tryptophan ethyl ester, acylation and deacylation, respectively, were shown to be rate-controlling.[2] The respective bell-shaped and sigmoid pH dependencies of these reactions are in complete accord with such a designation. The steric and electronic similarity of acetyl-L-tryptophanamide to the other amides and hydroxamides in Table II suggests that they should exhibit acylation rate-controlling steps and therefore bell-shaped pH-rate profiles, as found experimentally (the other amide "bells" do not, however, reflect k_{cat}.). The similarity of the three ethyl ester substrates in Table II suggests that they should exhibit rate-controlling deacylation steps and sigmoid pH-rate profiles, as found experimentally. All these results are self-consistent and lead one to

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \qquad (2)$$

(1) This research was supported by Grant H-5726 of the National Institutes of Health. Paper XIX in the series, The Mechanism of Action of Proteolytic Enzymes.

(2) B. Zerner and M. L. Bender, *J. Am. Chem. Soc.*, **85**, 356 (1963).

(3) M. L. Bender, *ibid.*, **84**, 2582 (1962).

(4) These observations apply to reactions in which K_m (or K_m(app)) has been separated from the appropriate rate constant.