The Primary Specificity of α -Chymotrypsin. Acylated Amino Acid Esters with Normal Alkyl Side Chains¹

J. Bryan Jones,² Toyoki Kunitake,³ Carl Niemann,⁴ and George E. Hein⁵

Contribution No. 3196 from the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California. Received December 28, 1964

The α -chymotrypsin-catalyzed hydrolysis of a series of N-acetyl-L-amino acid esters of the general formula $CH_3CONHCH[(CH_2)_nH]CO_2CH_3$, where n = 1-6, has been examined. For this series, K_0 reaches a minimum and k_0 a maximum when n = 5. The method of interaction of acylated L-amino acid derivatives with chymotrypsin is discussed as well as the magnitude of the forces which can be attributed to the side chain. Each additional methylene group up to the optimum number contributes approximately 680-790 cal. to increased substrate binding and 500 cal. toward decreasing the free energy of activation of the rate-determining kinetic step. The data are incorporated into a general correlation between structure and α -chymotrypsin specificity.

Introduction

In recent years, a large number of compounds have been examined as substrates^{6,7} and inhibitors⁸ of α chymotrypsin-catalyzed reactions. The development

detailed analyses of data. This paper discusses the effect of alkyl substituents on the kinetics of α -chymotrypsin-catalyzed reactions for a specifically defined series of substrates.

Hein and Niemann¹¹ have suggested that many substrates or inhibitors of α -chymotrypsin can be considered as derivatives of the general formula $R_1R_2CHCOR_3$. All such compounds are characterized by four potentially different groups surrounding a single carbon atom—they are tetradentate.

If one considers an enzyme active site to consist of an asymmetric, tetradentate environment, complimentary to the potential groups on the substrate, then there are twelve possible modes of combination of any substrate with the enzyme surface.¹¹ Although some classes of compounds may combine with the enzyme in a number of ways, others are considered "limit" types7 which combine predominately in a single orientation. N-Acetyl-L-amino acid methyl esters, CH₃- $CONHCHRCO_2CH_3$, are considered to be limit types

Table I. α -Chymotrypsin-Catalyzed Hydrolysis of Some α -N-Acetyl-L-amino Acid Methyl Esters^a

Substrate, Ac-L- CHRCO2CH3	No. of expt. ^b	$[\mathbf{E}] \times 10^{3},$ μM^{c}	[S], m <i>M</i>	$K_{\mathfrak{o},d}$ m M	k_{6}^{d} , sec. ⁻¹	$k_0/K_0, M^{-1}$ sec. ⁻¹
$\mathbf{R} = \mathbf{C}_2 \mathbf{H}_5$	10 (1)	3.46	10.0-80.3	53.5 ± 2.6	1.05 ± 0.18	19.6
$\mathbf{R} = \mathbf{C}_4 \mathbf{H}_9$	8 (0), 8 (0)	0.839	1.61-15.2	6.70 ± 1.50^{e}	$2.70 \pm 0.17^{\circ}$	248
$\mathbf{R} = \mathbf{C}_5 \mathbf{H}_{11}$	8 (1), 8 (0)	0.315-0.407	0.660-5.87	1.64 ± 0.33^{e}	13.4 ± 0.80^{e}	8170
$\mathbf{R} = \mathbf{C}_{6}\mathbf{H}_{13}$	8 (0), 9 (0)	0.316-0.494	0.623-6.24	2.94 ± 0.45^{e}	6.23 ± 0.50^{e}	2120

^a In aqueous solution at 25.0°, pH 7.90, and 0.10 M in sodium chloride. ^b Number of experiments performed for evaluation of K₀ and k₀; number in parentheses refers to experimental results rejected by statistical reiterative evaluation procedure. • Based on a molecular weight of 25,000 and a nitrogen content of 16.5%. ^d Evaluated by a least-squares fit to the equation ([E][S]/ v_0) = (K_0/k_0) + ([S]/ k_0), as described in text. * Average of two results which agreed within the experimental error. The larger of the two calculated errors is reported.

of rapid kinetic methods^{9,10} has resulted in the accumulation of a vast body of data. Unfortunately, it has been impossible to interpret most of this information in terms of quantitative relations between structure and reactivity. Recent advances in our understanding of the general nature of the specificity of α -chymotrypsin7.11 permit more definitive experiments and more

- (1) Supported in part by a grant from the National Institutes of Health, U. S. Public Health Service.
- (2) Department of Chemistry, University of Toronto, Toronto, Canada.
- (3) Department of Organic Synthesis, Faculty of Engineering, Kyushu University, Fukuoka, Japan.
- (4) Deceased, April 29, 1964.
- (5) Department of Chemistry, Boston University, Boston 15, Mass.
 (6) R. J. Foster and C. Niemann, J. Am. Chem. Soc., 77, 2378 (1955).
 (7) G. E. Hein and C. Niemann, Proc. Natl. Acad. Sci. U. S., 47, 1341 (1961).
- (8) R. A. Wallace, A. N. Kurtz, and C. Niemann, Biochemistry, 2, 824 (1963).
- (9) M. Dixon and E. C. Webb, "Enzymes," Academic Press Inc., New York, N. Y., 1958, Chapter 2
- (10) T. H. Applewhite, R. B. Martin, and C. Niemann, J. Am. Chem. Soc., 80, 1457 (1958).

for all cases where R is a large alkyl or a simple aromatic derivative.

These compounds also are representative members of the general class of acylated amino acid derivatives which can be employed to measure the primary specificity of α -chymotrypsin toward isolated peptide-like linkages.

Kinetic data on some N-acetyl methyl esters of L-amino acids with alkyl or benzyl side chains have already been reported.^{12,13} The present study completes the first systematic series of homologous compounds whose mode of interaction with the enzyme can be ascribed predominantly to a specific limit type.

Results

Table I lists the kinetic constants and pertinent experimental data for the compounds reported in this

- (12) J. P. Wolf, III, and C. Niemann, Biochemistry, 2, 18, 493 (1963).
- (13) J. B. Jones and C. Niemann, ibid., 1, 1093 (1962); 2, 498 (1963).

⁽¹¹⁾ G. E. Hein and C Niemann, ibid., 84, 4495 (1962).

Table II. Kinetic Constants for the α -Chymotrypsin-Catalyzed Hydrolysis of α -N-Acetyl-L-amino Acid Methyl Esters^a

^a In aqueous solution at 25.0°, pH 7.90, and 0.10 *M* in sodium chloride. ^b See ref. 14. ^c See ref. 15. ^d H. R. Waite and C. Niemann, *Biochemistry*, **1**, 250 (1962). ^e J. B. Jones, unpublished work. ^f J. B. Jones and C. Niemann, *Biochemistry*, **2**, 498 (1963).

study. Table II lists the kinetic constants for the homologous series of N-acylated L-amino acid esters, $CH_3CONHCHRCO_2CH_3$, where R ranges from H to $n-C_6H_{13}$. Other pertinent compounds are also listed. The effect of increasing side chain on both kinetic constants is plotted in Figures 1 and 2.



Figure 1. Variation of K_0 with length of side chain.

Figure 3 plots both logs K_0 and k_0 as a function of chain length. If it is assumed that K_0 values approximate equilibrium constants¹⁴ or a combination of equilibrium constants, then the logarithmic values are proportional to the free energy of binding $\Delta F = -2.3$ $RT \log K_0 \cong -(1350 \text{ cal.}) \log K_0$ for the experimental conditions. From the change in $\log K_0$ with increasing chain length, one can calculate that each additional methylene group contributes 890 ± 450 cal., if the values from C-1 to C-5 are used, or 680 ± 290 cal., if only the values from C-2 through C-5 are used. Likewise, one can estimate the contribution of each methylene group to increase the rate. For the linear portion, C-2 through C-5, each additional methylene group contributes 500 \pm 170 cal. to lower the activation energy for the rate-determining step represented by k_0 .

Discussion

A structural modification from the glycine derivative $(\mathbf{R} = \mathbf{H})$ to the corresponding alanine derivative $(\mathbf{R} = \mathbf{CH}_3)$ provides the most dramatic variation in kinetic constants. For α -chymotrypsin-catalyzed reactions, K_0 is usually interpreted to be a measure of binding energy, independent of k_0 . In that case, no matter what type of interaction is envisaged between substrates and the enzyme active site, it is clear that the alanine derivative cannot interact as well with the



Figure 2. Variation of k_0 with length of side chain.

enzyme as can the glycine derivative. Since the alanine derivative is nevertheless hydrolyzed at a specific rate which is almost 100 times $[k_0(ala)/k_0(gly) = 98]$ that of the glycine derivative, it is reasonable to assume that the alanine derivative is bound to the enzyme in a productive mode. The additional methyl group probably provides sufficient bulk to prevent the alanine derivative from assuming a number of orientations in the acylamido, $\rho_{1,1}$ binding region. The substrate activation observed for N-acetylglycine methyl ester¹⁵ supports the view that this compound disports itself in a different fashion at the active site.

(15) J. P. Wolf, III, R. A. Wallace, R. L. Peterson, and C. Niemann, Biochemistry, 3, 940 (1964).

⁽¹⁴⁾ C. Niemann, Science, 143, 1287 (1963).

The α -methyl group in L-alanine is probably insufficient to orient the compound "correctly" at the active site, that is, so that $R_1-\rho_1$, $R_2-\rho_2$, and $R_3-\rho_3$ interactions predominate. However, this interaction mode should become increasingly significant as the size of the R₂ moiety is increased. Other binding modes will not be altered greatly by this monofunctional structural change. Therefore, the data for the α -Nacylated L-amino acid esters discussed here refer to a series of compounds which differ only in the degree of interaction with the ρ_2 -subarea on the active site.

Examination of the data immediately reveals that increasing chain length causes a variation in both kinetic constants, k_0 and K_0 . This is in contrast to the observations of Hofstee¹⁶ who studied the α -chymotrypsin-catalyzed hydrolysis of a series of normal alkyl esters of the phenolic hydroxyl on salicylic acid. He noted that K_0 remained essentially constant while k_0 increased with increasing fatty acid chain length from propionic through heptanoic acid.¹⁶ This difference emphasizes the necessity for detailed knowledge of the specificity of an enzyme before data can be theoretically interpreted. The difference in results noted here strongly suggests that phenolate esters and acylated amino acid esters interact with different subregions of the active site of α -chymotrypsin. The implications of this conclusion for the use of *p*-nitrophenyl acetate as a model substrate of α -chymotrypsin^{17,18} cannot be fully assessed at present.

Addition of methyl groups, up to an optimum for 2-acetamidoheptanoic acid methyl ester, causes an increase in binding energy of approximately 700 cal. for each methylene group. Two sources for this energy suggest themselves. Perhaps the simplest explanation involves the removal of the organic molecule from the aqueous phase into a microorganic phase on the enzyme surface. This has been variously called an "apolar"19 or hydrophobic^{20, 21} bond. It has been specifically considered by Canady and co-workers for binding of inhibitors to α -chymotrypsin.²² This is essentially a ground-state phenomenon which attributes the binding energy due to hydrocarbon portions to their inherent incompatibility with an aqueous environment. The same type of explanation has been discussed in slightly different language by Bernhard and Gutfreund.23 A second type of explanation considers the possible effects of modifications in the enzyme conformation resulting from the interaction of a portion of the substrate molecule with the active site.24,25 A native protein structure is usually stabilized by a considerable number of intramolecular forces.²⁶ Any interaction which causes a structural rearrangement may be

- (16) B. H. J. Hofstee, Biochim. Biophys. Acta, 24, 211 (1957).
 (17) B. S. Hartley and B. A. Kilby, Biochem. J., 50, 672 (1952).
 (18) H. Neurath and B. S. Hartley, J. Cellular Comp. Physiol., 54,
- Suppl. 1, 179 (1959).
- (19) F. Karush, Advan. Immunol., 2, 1 (1962).
- (20) H. Kauzmann, Advan. Protein Chem., 14, 1 (1959).
 (21) R. Lumry, The Enzymes," Vol. 1, P. Boyer, H. Lardy, and K. Myrbäck, Ed., Academic Press Inc., New York, N. Y., 1959, p. 175.
- (22) J. L. Miles, D. A. Robinson, and W. J. Canady, J. Biol. Chem., 238, 2932 (1963).
- (23) S. A. Bernhard and H. Gutfreund, Progr. Biophys. Biophys. Chem., 10, 115 (1960).
- (24) D. E. Koshland, Jr., Federation Proc., 21, 1031 (1962).
- (25) A. Platt and C. Niemann, Proc. Natl. Acad. Sci. U. S., 50, 817 (1963).
- (26) H. Scheraga, "Protein Structure," Academic Press Inc., New York, N. Y., 1962.



Figure 3. Effect of length of side chain on log K_0 and log k_0 .

expected to be endothermic. It is possible, however, that such rearrangements permit new or stronger intramolecular interactions or intermolecular interactions with polar portions of the substrates so that the net result is exothermic.

The apolar bond undoubtedly plays an important role in enzyme-substrate interaction. It is unfortunate that more information is not available at present which permits an experimental evaluation of this parameter. Since the major factor considered is the variation in the ground state of the reactant molecule, a number of possible experimental methods can be used for this evaluation.^{22,27} It is unfortunate that discussion of these topics is still limited by the inadequacy of appropriate data for model systems. We currently know more about the kinetics of the reactions of acylated amino acid esters than about their solubilities!

It is unlikely, however, that this single factor can account completely for the differential specificity of enzymes for homologous series of substrates. One can expect that ground-state parameters would vary in a monotonic manner. The addition of each methylene group should decrease water solubility. The data for the binding of *n*-alkyl- α -N-acetyl-L-amino acids to the enzyme do not support this view. The addition of one more methylene group to norleucine actually causes an increase in the dissociation constant, K_0 .

A more striking reason, however, for concluding that differences in ground states do not completely explain the variations in K_0 values encountered is that antiparallel alterations are observed in K_0 and k_0 values. As can be seen in Figure 3, in the series of acylated amino acid esters with aliphatic residues, there is a good correlation between K_0 and k_0 values. For a limit case in which only productive binding modes are considered, any energy factor which only considers the enzyme as a different solvent phase cannot explain the systematic variation in k_0 with structure. Any ground-state phenomenon which involves the interaction of the substrate with solvent leaves each of the substrates in a similar energetic situation once it has been removed from the aqueous phase. It is possible,

(27) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids, and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943.

of course, that the K_0 variation is not only kinetically independent, but also entirely unrelated to the variation in k_0 . However, the parallel variation in these values makes this suggestion at least aesthetically unpleasing.

The second factor mentioned previously, the alteration of enzyme configuration by substrates, offers an appealing additional property. The p_2 -region of the enzyme may contain an amino acid residue which contains both a hydrocarbon residue and a functional group. Interaction with the substrate may modify the position of this residue so that it partakes more or less in new hydrogen bonds or ionic interactions, which in turn enhance the catalytic property of the enzyme. The cause of the low k_0 values observed for α -chymotrypsin-catalyzed hydrolysis of N-methyl-substituted amino acid substrates^{28,29} has not yet been determined. A crucial hydrogen bond interaction may be involved rather than a steric factor. This view is supported by the decreased k_0 values observed when an NH group is replaced by a CH₂ group.⁷

It is also possible to interpret the variations in K_0 and k_0 with structure simply on the basis of a variation in the interaction of the hydrocarbon moiety with a nonpolar residue at the active site. The data suggest that an optimum size for substrate, rather than total hydrophobic interaction, determines maximum k_0 and minimum K_0 values. It is interesting to note that an extended $n-C_6H_{13}$ chain is longer than a benzyl or hexahydrobenzyl group while an $n-C_5H_{13}$ chain is approximately the same length as the two cyclic groups. Acylated amino acid esters containing $n-C_5H_{13}$, benzyl, or hexahydrobenzyl side chains exhibit the lowest K_0 and highest k_0 values observed in this entire series, although they should differ considerably in the extent of hydrophobic interaction with the enzyme.

The increase in rate for each methylene group, 500 \pm 170 cal./mole, is very similar to the value calculated from Hofstee's data by Lumry, 21 -650 cal./mole. The similarity is particularly striking because Hofstee observed the maximum rate with *m*-carboxyphenylheptanoate (1), while in the present series, the maximum rate was observed with 2-acetamidoheptanoic acid methyl ester² (II). These compounds have identical hydrocarbon side chains. For aliphatic salicylates, the minimum K_0 value was shifted to the octanoic acid ester, but a maximum in k_0 was still observed for salicyl heptanoate.30

$$CH_{3}CH_{2}CH_{2}CH_{2}CH_{2}CO_{2}-C_{6}H_{4}-CO_{2}-I$$

$$I$$

$$CH_{3}CH_{2}CH_{2}CH_{2}CH_{2}CH_{-}CO_{2}CH_{4}$$

$$NH$$

$$CO$$

$$CO$$

$$CO$$

$$CH_{3}$$

$$II$$

The fatty acid esters probably failed to show significant variations in K_0 values because the predominant binding mode does not involve interaction of the ali-

- (28) S. Kuk-Meri and N. Lichtenstein, Biochim. Biophys. Acta, 25, 182 (1957).
- (29) R. L. Peterson, K. W. Hubele, and C. Niemann, Biochemistry, 2,942 (1963).
- (30) B. H. J. Hofstee, Biochim. Biophys. Acta, 32, 182 (1959).

phatic side chain with the ρ_2 -locus. They most efficiently associated with the enzyme by an interaction of the aromatic group with one or more subsites. This interpretation is consistent with the low k_0 values observed by Hofstee. It is also consistent with the characteristically low K_0 and k_0 values of N-benzovlvs. N-acetyl-substituted amino acids, for compounds with small side chains.³¹

The suggestion that amino acid side chains function both to bind substrate and to enhance rate by modification of the conformation due to a polar group suggests the possibility of exploring for this polar function with suitably substituted side chains. The negligible splitting of lysyl peptide bonds by chymotrypsin is particularly noteworthy. The size of the side chain is identical with that of 2-aminoheptanoic acid. Whether the lack of reactivity is primarily due to poor binding (high K_0) or low reactivity (low k_0) has not yet been ascertained.

Experimental³²

N-Acetyl-L- α -aminobutyric Acid Methyl Ester. The method of Brenner and Huber³³ was used to esterify 5.0 g. of acetyl-L- α -aminobutyric acid.

Evaporation of the reaction mixture yielded an oil which was dissolved in chloroform and washed successively with 30 ml. of 1 M aqueous sodium carbonate and 30 ml. of water. The chloroform layer was dried over magnesium sulfate and evaporated. The resulting solid was recrystallized from isopropyl ether to yield 3.5 g. (64%) of the desired product, m.p. 39° , $[\alpha]^{25}D - 75.1 \pm 0.4^{\circ} (c \ 2.2 \ H_2O).$

Anal. Caled. for C₇H₁₃NO₃ (159.2): C, 52.8; H, 8.2; N, 8.8. Found: C, 52.6; H, 8.3; N, 8.8.

N-Acetyl-L-norleucine Methyl Ester. Esterification of 6.0 g. of L-norleucine with thionyl chloride in methanol³³ yielded the ester. The crude product was acetylated with 7.2 g. of acetyl chloride in chloroform at 0° in the presence of 13.0 g. of potassium carbonate. The mixture was stirred overnight, 100 ml. of water was added, and the layers were separated. The chloroform layer was washed with 50 ml. of water and dried over magnesium sulfate. Evaporation gave an oil. This oil was distilled to give a crystalline product, b.p. 94° (0.3 mm.), m.p. 46-47°, $[\alpha]^{25}D$ -44.3 ± $0.8^{\circ}(c\ 2,\ H_2O).$

Anal. Calcd. for C₉H₁₇NO₃ (187.2): C, 57.7; H, 9.2; N, 7.5. Found: C, 57.5; H, 9.2; N, 7.7.

N-Acetyl-L-2-aminoheptanoic Acid Methyl Ester. The racemic amino acid was prepared by condensation of *n*-amyl bromide with ethyl acetamidomalonate as directed by Albertson.³⁴ The crude product obtained in 82% yield after decarboxylation had a melting point of 278–283°. Acetylation with hot acetic anhydride gave N-acetyl-DL-2-aminoheptanoic acid, m.p. 105.7-107° (lit.³⁵ m.p. 106°).

The acetylated product was esterified by the method of Brenner and Huber³³ and treated as above. Re-

- (32) Melting points are corrected. Microanalyses by Spang Micro (32) McHung points are concreted. Microanalyses by objective analytical Laboratory, Ann Arbor, Mich.
 (33) M. Brenner and W. Huber, *Helv. Chim. Acta*, 36, 1109 (1953).
 (34) N. H. Albertson, *J. Am. Chem. Soc.*, 68, 450 (1946).

 - (35) N. F. Albertson, ibid., 72, 1396 (1950).

⁽³¹⁾ J. B. Jones, C. Niemann, and G. E. Hein, Biochemistry, submitted for publication.

crystallization from hexane and treatment with Norit-A gave a pure product, m.p. $61-62^{\circ}$.

The DL-amino acid derivative was resolved with α -chymotrypsin. Solution of 7.7 g. of the acylated amino acid ester in 1500 ml. of water and addition of 0.05 g. of α -chymotrypsin resulted in hydrolysis. Standardized sodium hydroxide solution was added, keeping the pH between 7 and 8. After 45 min. sodium hydroxide corresponding to 49% hydrolysis had been added. The reaction mixture was extracted with three 200-ml. portions of chloroform. The cloroform extract was dried over magnesium sulfate and evaporated. The resulting oil was recrystallized from petroleum ether (b.p. 30-60°) to yield N-acetyl-D-2-aminoheptanoic acid methyl ester, m.p. 27.5-28.5°, $[\alpha]^{24}D - 23.6°$ (c 1.5, MeOH).

Anal. Calcd. for $C_{10}H_{19}NO_3$ (201.2): C, 59.7; H, 9.5; N, 7.0. Found: C, 60.1; H, 9.1; N, 7.1.

The aqueous layer from the resolution was acidified with HCl and evaporated *in vacuo*. The organic residue was dissolved in ethyl acetate and extracted into aqueous sodium bicarbonate. The resulting aqueous layer was again acidified and extracted with ethyl acetate. Evaporation of the organic solvent yielded 1.4 g. of white powder. This was recrystallized from water to give 1.05 g. of white needles, m.p. 112° (lit.³⁶ m.p. 108°).

The L-acid was re-esterified as described above. The product was recrystallized from hexane and ethyl

(36) R. Marshall, S. M. Bernbaum, and J. P. Greenstein, J. Am. Chem. Soc., 78, 4636 (1956).

acetate to give white needles, m.p. $26-27^{\circ}$, $[\alpha]^{25}D$ 22.6 ± .5° (c 2, MeOH).

Anal. Found: C, 59.6; H, 9.2; N, 6.9; ash, 0.44.

N-Acetyl-\alpha-aminocaprylic Acid Methyl Ester. Commercial α -aminocaprylic acid (Calbiochem) was esterified and acetylated as described above to give the racemic product, m.p. 74–75.5°. The DL-ester was resolved with α -chymotrypsin as indicated for 2-aminoheptanoic acid and the L-acid was re-esterified. The D-ester was obtained after recrystallization from hexane, m.p. 57–58.5°, $[\alpha]^{25}D - 20.8 \pm 1.0°$ (c 2, MeOH). The L-ester was also recrystallized from methanol, m.p. 55.5–57°, $[\alpha]^{25}D - 18.5 \pm 1.0°$ (c 2, MeOH).

Anal. Calcd. for $C_{11}H_{21}NO_3$ (215.3): C, 61.4; H, 9.8; N, 6.5. Found: C, 61.5; H, 9.6; N, 6.5.

Kinetic Studies. The procedure was identical with that described previously.^{10,37} All experiments were conducted in aqueous solutions at 25.0°, pH 7.90 \pm 0.10, and 0.10 *M* with respect to sodium chloride. The enzyme preparation was crystalline, bovine, saltfree α -chymotrypsin, Armour Lot No. T-97207. The primary data were analyzed with a Datatron 220 digital computer, programmed as described earlier.³⁸

Acknowledgment. The authors wish to thank Dr. Werner A. Mukatis for his assistance in the preparation of this manuscript.

(37) T. H. Applewhite, H. Waite, and C. Niemann, *ibid.*, **80**, 1465 (1958).

(38) H. I. Abrash, A. N. Kurtz, and C. Niemann, *Biochim. Biophys.* Acta, 45, 378 (1960).

The Effect of Magnesium Ion on the Secondary Structure of Deoxyribonucleic Acid

John W. Lyons¹ and Leonard Kotin

Contribution from the Department of Chemistry, Washington University, St. Louis, Missouri. Received October 29, 1964

The effect of magnesium ion (Mg^{2+}) on the thermal and phase stability of deoxyribonucleic acid (DNA) has been studied by means of spectrophotometric and nuclear magnetic resonance techniques with the following conclusions: (1) Mg^{2+} interacts with DNA at the phosphate sites only. (2) Whereas aqueous solutions of the pure magnesium salt of DNA are relatively resistant to thermal denaturation, their thermal stability is reduced in the presence of added $MgCl_2$. (3) The proposed mechanism for the precipitation of DNA with an excess of Mg^{2+} is that site-bound magnesium forms ionic links between separated DNA strands through -P-O-Mg-O-P complexes leading to the exposure of the bases to solvent. This is followed by hydrophobic base-base interaction leading to large aggregates and, finally, phase separation.

(1) Correspondence should be addressed to the Research Department, Inorganic Chemicals Division, Monsanto Co. St. Louis, Mo. 63166.

In a recent report^{2a} dealing with the properties of the magnesium salt of deoxyribonucleic acid (MgDNA) we showed that, in the absence of added simple salt, MgDNA is much more resistant to thermal denaturation than is NaDNA. We also reported that when $MgCl_2$ is added to previously heat-treated and quenched (denatured) MgDNA a precipitate forms, whereas no precipitate is observed with native MgDNA. Moreover, if native MgDNA is heated in the presence of MgCl₂ a precipitate appears at a temperature lower than the melting temperature, $T_{\rm m}$, of the pure MgDNA solution. In experiments in which the activity coefficient of magnesium ion was measured in MgDNA-MgCl₂ mixtures,^{2b} we have found that magnesium ion is bound to an extent greater than 1 mole of Mg^{2+} to 2 moles of P(DNA); i.e., some magnesium ion is ren-

(2) (a) J. W. Lyons and L. Kotin, J. Am. Chem. Soc., 86, 3634 (1964);
(b) J. W. Lyons and L. Kotin, *ibid.*, 87, 1670 (1965).