[CONTRIBUTION FROM THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY]

Thermodynamic Study of Some Enzyme–Inhibitor Complexes of Chymotrypsin. II[1,2]

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The equilibrium constants for complex formation, of chymotrypsin with: I, N-acetyl-3,5-dibromo-D-tyrosine; II, 4-(4-hydroxy-3,5-dibromophenyl)-DL-3-acetaminobutanone-2; III, N-acetyl-β-(4-methoxy-3,5-dibromophenyl)-L-alanine; and for IV, chymotrypsinogen with N-acetyl-3,5-dibromo-L-tyrosine, have been determined at 5 and 20° and from *p*H 4 to 7.5. The standard free energy changes for formation of the complexes from enzyme and substrate, for all four systems, remain between two and three thousand calories over the entire *p*H range. Systems II and III had negative values for both entropy and enthalpy at *p*H 7.5. Systems I and IV had positive values for the entropy, and the enthalpy was small or 0. The equivalent weight of chymotrypsinogen is found to be 22,500. These results indicate that the changes in thermodynamic functions on complex formation may be correlated with the catalytic activity of the enzyme. The results are interpreted in terms of a theory of enzyme action.

Introduction

The first paper of this series[3] established, by direct measurement of the equilibrium constant of an enzyme–substrate complex, an apparent relationship between the catalytic properties of chymotrypsin and the apparent standard entropy and enthalpy changes for formation of the complex. These thermodynamic functions of complex formation, however, do not depend simply upon the primary forces between enzyme and substrate but also on such extraneous factors as the separation of parts of the enzyme and substrate surfaces from water, hydrogen ion equilibria and non-specific electrostatic effects. Since it is not possible at this time to determine directly the effect of these extraneous properties in the complex buffer systems used, it becomes necessary to compare systems which have very similar or predictable extraneous characteristics and differ only in their intrinsic binding properties in order to obtain information on the latter. To the extent that such comparisons are valid, the effect of ambiguous standard states and activities is also eliminated from consideration.

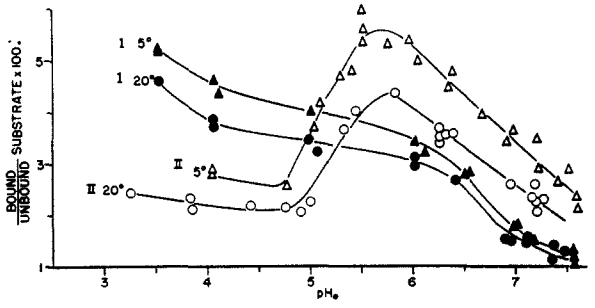

Fig. 1.—Amount of substrate bound by enzyme: I, N-acetyl-3,5-dibromo-D-tyrosine–chymotrypsin; II, 4-(4-hydroxy-3,5-dibromophenyl)-DL-acetaminobutanone-2-chymotrypsin; enzyme, 1%; substrate less than 10^{-4} *M*.

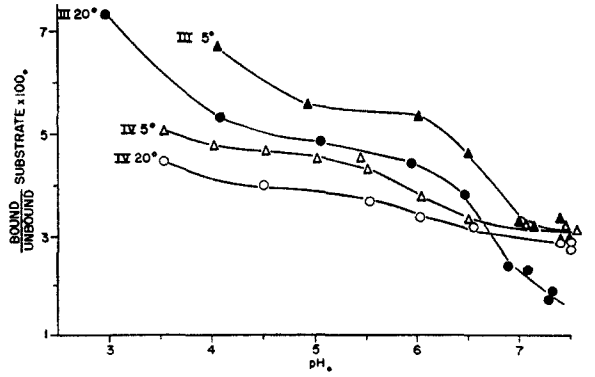

Fig. 2.—Amount of substrate bound by enzyme: III, N-acetyl-β-(4-methoxy-3,5-dibromophenyl)-L-alanine–chymotrypsin; IV, N-acetyl-3,5-dibromo-L-tyrosine–chymotrypsinogen; enzyme, 1%; substrate less than 10^{-4} *M*.

This report is concerned with four such systems, one catalytically active and three different catalytically inactive types. The specific substrates (or inhibitors) are closely related to the original substrate, N-acetyl-3,5-dibromo-L-tyrosine, and in system IV, chymotrypsinogen is the protein, rather than chymotrypsin as in the first three systems. The substrates are: I, N-acetyl-3,5-dibromo-D-tyrosine; II, the ketone 4-(4-hydroxy-3,5-dibromophenyl)-DL-3-acetaminobutanone-2, where a methyl group has been substituted for the carboxyl-hydroxy group of the original substrate; III, N-acetyl-β-(4-methoxy-3,5-dibromophenyl)-L-alanine where the phenolic hydroxyl has been methylated; IV, the original substrate, N-acetyl-3,5-dibromo-L-tyrosine. Systems I, II and IV are catalytically inactive while system III should be catalytically active. This report is primarily concerned with the thermodynamic interactions at the labile bond, *i.e.*, the carboxylic C–OH. The closely related subject of the attractive forces is being investigated at the present time.

Results

As in the previous paper,[3] 1% protein and $<10^{-4}$ *M* substrate solutions were used. The results obtained are plotted in Figs. 1, 2 as the ratio of bound to unbound compound as a function of *p*H at 5° and at 20°. This function is inversely proportional to the equilibrium constant, $K = (E)(S)/(ES)$, for complex concentrations small compared with enzyme concentrations as is the case here. An examination of these curves reveals that, with the exception of the ketone, the shapes and magnitudes are fairly similar and that, in the *p*H region of enzymatic activity, binding decreases with increasing *p*H. Although the ratios of the amounts

(1) Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

(2) This paper was presented, in part, before the Biological Chemistry Division of the American Chemical Society, Milwaukee, Wis., April, 1952.

(3) D. G. Doherty and F. Vaslow, THIS JOURNAL, **74**, 931 (1952).

of substrate bound at 5° to that at 20° do not follow any consistent pattern below *p*H 5, above *p*H 6 two definite types of behavior emerge. For systems II and III this ratio becomes increasingly large with increasing *p*H similar to the original substrate system, while for systems I and IV, the ratio decreases in magnitude. The significance of the temperature variation is discussed later. The maximum binding of the ketone corresponds closely to the *p*H of the initiation of chymotrypsin action as given by Northrup,[4] and to the *p*H reported in early work as the isoelectric point. The significance of this behavior is not clear.

A plot of mg. of enzyme bound/mM. bound substrate as a function of the reciprocal substrate concentration for the N-acetyl-3,5-dibromo-L-tyrosine–chymotrypsinogen system is presented in Fig. 3. Extrapolation yields an equivalent weight of 22,500 for chymotrypsinogen which is in good agreement with recent physical measurements of the molecular weight.[5] Since there is only one binding position it is highly probable that this is the same position as the active center of chymotrypsin. In contrast to the evidence of the diisopropyl fluophosphate binding experiments, where no reaction was obtained with chymotrypsinogen,[6] this position is exposed. Thus the conversion of the precursor to the active enzyme probably involves only a minor change in the active center of the molecule.

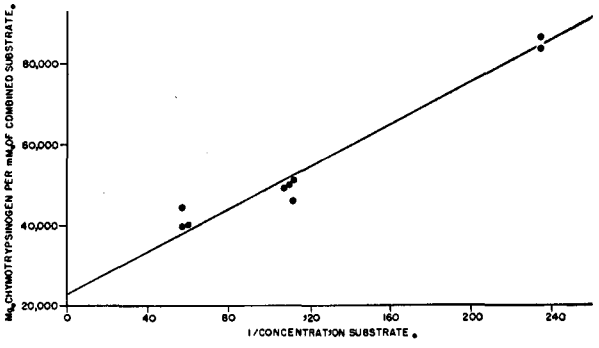

Fig. 3.—Equivalent weight of chymotrypsinogen.

Discussion

The standard free energy, entropy and enthalpy changes for the reaction, enzyme + substrate = enzyme–substrate complex, have been calculated at half *p*H unit intervals from *p*H 4 to *p*H 7.5. In this calculation, for lack of detailed information, both enzyme and substrate activities have been set equal to their concentration, and hydrogen ion equilibria have been neglected. The neglect of the hydrogen ion equilibria implies the definition of a new standard state for each *p*H value or the qualification that the results are apparent. The free energies vary between −3000 and −2000 cal./mole and the curves for all five systems are similar in magnitude and shape. However, in the variation

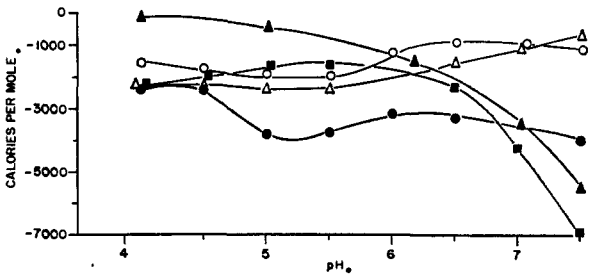

Fig. 4.—ΔH of formation of enzyme–substrate complex: △ = I, D-acid–chymotrypsin; ● = II, DL-ketone–chymotrypsin; ■ = III, L-methoxy acid–chymotrypsin; ○ = IV, L-acid–chymotrypsinogen; ▲ = L-acid–chymotrypsin.

of heat content with *p*H (ΔH, Fig. 4), two types of behavior become evident. In the *p*H region above 6.0, the ΔH of the catalytically active systems (*i.e.*, the original substrate, the methyl ether, and also the inactive ketone) becomes strongly negative with increasing *p*H. This is in direct contrast with the ΔH of the insensitive systems (*i.e.*, chymotrypsinogen and N-acetyl-3,5-dibromo-D-tyrosine) which increase toward zero. Thus the intrinsic binding forces, such as van der Waals and hydrogen binding (as distinct from entropy binding), increase strongly for the sensitive systems while decreasing for the insensitive ones. Kinetic inhibition studies[7] and Fisher–Hirshfelder–Taylor model studies indicate that the D-isomer fits the L-template reasonably well, being bound at the benzene ring and probably at two more points. Therefore, the difference in heats of formation must be due to a very accurate fit of the substrate as compared with an approximate fit of the inhibitor and the forces involved must be of a short range type. A similar factor is evident in the comparison of the enzyme with the zymogen. In the latter case some group appears to be blocking the close fit necessary for activity. It might be expected that, for the methyl ether and the methyl ketone, the work of separation of the methyl groups from water would be less than for the replaced phenolic and carboxylic hydroxyl groups, resulting in a more negative ΔH. Indeed, this observation is borne out for the methyl ether and partly for the methyl ketone. In view of the marked differences between D- and L-acid curves, the interpretation of the racemic ketone results is uncertain. However, since the DL-ketone does show to a small extent the changes characteristic of catalytically active substances, it seems likely that the L-ketone would show a considerably more negative entropy and enthalpy change and the D-ketone would resemble the D-acid. This is an assumption which will be checked as soon as the L-ketone can be prepared.

The results obtained from the entropy measurement ($-T\Delta S$ for T = 285°, Fig. 5) again show a segregation into two types of behavior. The sensitive systems again show increasing negative entropies of complex formation while the insensitive ones show positive entropies of complex formation. With respect to the isomers, N-acetyl-3,5-dibromo-L-and-D-tyrosine, the translational entropy losses must be identical. The entropy loss due to the

(4) J. H. Northrup, M. Kunitz and R. M. Herriot, "Crystalline Enzymes," 2nd Ed., Columbia Univ. Press, New York, N. Y., 1948.

(5) E. L. Smith, D. M. Brown and M. Laskowski, *J. Biol. Chem.*, **191**, 639 (1951).

(6) E. F. Jansen, M. D. F. Nutting, R. Jong and A. K. Balls, *ibid.*, **179**, 189 (1949).

(7) H. T. Huang and Carl Niemann, THIS JOURNAL, **73**, 3223 (1951).

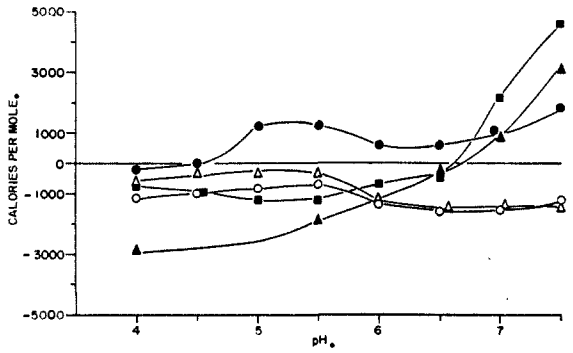

Fig. 5.—$T\Delta S$ of formation of enzyme–substrate complex: △ = I, D-acid–chymotrypsin; ● = II, DL-ketone–chymotrypsin; ■ = III, L-methoxy acid–chymotrypsin; ○ = IV, L-acid–chymotrypsinogen; ▲ = L-acid–chymotrypsin.

release of bound water from the enzyme might be expected to be the reverse of that observed, the more tightly bound L-isomer releasing more water than the D-isomer. Since the D-isomer is probably bound at three points, it would lose its rotational entropy similarly to the L-isomer. This consideration would also apply to the entropy losses due to internal rotations. Therefore the source of the 12 e.u. difference between the antipodes must lie in the vibrational entropies of the complexes. An explanation can be offered depending on the catalytic properties of the system and based on the theoretical calculations of Stearn.[8] Stearn has shown that if a bond such as a C–N were brought up to a dipole or single charge with the proper orientation, then a relatively small expenditure of potential energy would serve to lower materially the activation energy for breakage of the original bond and the formation of a new bond. Thus the negative potential energy of the active type complexes serves to bring the labile bond up against a charged point or dipole on the enzyme surface. The active center of the enzyme then consists of two parts: (1) an attractive center determining the specificity and orientation of the substrate and (2) a charge center activating the labile bond which would be repulsive in potential. If the two centers were not on a rigid framework, then the enzyme freedom would be restricted by the potentials applied at two separate points and the entropy accordingly reduced as has been found experimentally. It seems less likely that the difference could be caused by the loss of entropy associated with a three-point fit of the inhibitor as compared with a close fit of the substrate to the specificity determining center. The evidence for the repulsive nature of the activating center is obtained from the ketone data. The parallel of the thermodynamic potentials of this compound to that of sensitive compounds implies that similar forces are acting. Since the methyl group could not be attracted by a polar enzyme group but could be sterically repelled, the potential appears to be repulsive. If the hydroxy or methyl were replaced by hydrogen then the repulsion and the entropy change should be very much less and hence this compound should be very strongly bound, *i.e.*, it might be a very good inhibitor. This aldehyde is now being prepared as an important test of the theory. Outside of the enzymatically active *p*H region, no attempt has yet been made to explain the complicated variations of the thermodynamic functions other than to say that ionizations of several groups and changes of protein configuration are probably involved.

(8) A. E. Stearn, *J. Gen. Physiol.*, **18**, 301 (1935).

Experimental

Procedures and Materials.—The methods utilized for the binding studies were those previously reported.[3] Chymotrypsin and chymotrypsinogen were prepared from frozen bovine pancreas according to the method of Kunitz and Northrup.[9]

N-Acetyl-β-(4-methoxy-3,5-dibromophenyl)-L-alanine.—N-Acetyl-3,5-dibromo-L-tyrosine ethyl ester[3] (4.1 g.) was dissolved in 50 ml. of methanol, cooled in ice and an ether solution of diazomethane added in portions to a permanent yellow color. This solution was evaporated *in vacuo* to a sirup, taken up in 50 ml. of methanol and de-esterified by the addition of 10 ml. of 1 *N* sodium hydroxide. After 30 minutes, 11 ml. of 1 *N* hydrochloric acid was added and the mixture was diluted with water to turbidity. Scratching induced crystallization and the product was filtered after standing overnight in the ice-box; yield 3.8 g., m.p. 172–173°, $[\alpha]^{22}D$ +151.5° (*c*, 0.5% in 1 *N* NaOH).

Anal. Calcd. for $C_{12}H_{13}NO_4Br_2$ (395.1): C, 36.5; H, 3.3; N, 3.5; Br, 40.4. Found: C, 36.4; H, 3.3; N, 3.4; Br, 40.3.

DL-4-(4-Hydroxyphenyl)-3-acetaminobutanone-2.—This compound has been previously prepared by Levene and Steiger[10] and Dakin and West.[11] Although both groups were in agreement on analytical figures, a difference in melting point (163–166° for Levene and Steiger and 135° for Dakin and West) has never been explained. Preparation of this compound by both procedures yielded an identical product with a melting point of 165–167° which was raised to 166–168° by recrystallization from methanol.

Anal. Calcd. for $C_{12}H_{15}O_3N$ (221.2): C, 65.0; H, 6.8; N, 6.3. Found: C, 64.9; H, 6.8; N, 6.3.

DL-4-(4-Hydroxy-3,5-dibromophenyl)-3-acetaminobutanone-2.—To a cold solution of 2.2 g. of 4-(4-hydroxyphenyl)-3-acetaminobutanone-2 in 20 ml. of pyridine, 6.4 g. pyridine perbromide·HBr was added in portions with cooling over a 15-minute period. The precipitated pyridine·HBr was filtered off and the solution evaporated *in vacuo* to a sirup which was taken up in ethyl acetate. This solution was washed with ice-cold dilute HCl, water and extracted with 0.5 *N* NaOH. Acidification of the alkaline extract gave a crystalline product which was recrystallized from aqueous alcohol; yield 2.8 g., m.p. 133–134°.

Anal. Calcd. for $C_{12}H_{13}O_3NBr_2$ (379.1): C, 51.6; H, 3.5; N, 3.7; Br, 42.1. Found: C, 51.5; H, 3.4; N, 3.7; Br, 42.0.

Radioactive N-Acetyl-3,5-dibromo-D-tyrosine.—N-Acetyl-D-tyrosine (70 mg.) was brominated by the same procedure for the L-isomer.[3] The sirup obtained after evaporation of the acetic acid *in vacuo* was taken up in 1 ml. of 1 *N* hydrochloric acid and 1 ml. of ethyl acetate. The ethyl acetate solution was washed with water and extracted with 1 *N* sodium hydroxide. The alkaline extract was washed with ethyl acetate and a crystalline product obtained by acidification. The crystals were centrifuged and recrystallized, first from ethyl acetate–ligroin mixture and finally from water; m.p. 122–123°.

Radioactive N-Acetyl-β-(4-methoxy-3,5-dibromophenyl)-L-alanine.—An ethyl acetate solution of radioactive N-acetyl-3,5-dibromo-L-tyrosine[3] was treated with an excess of diazomethane in ether. After evaporation of the solvent *in vacuo* the sirupy ester was hydrolyzed with 1 *N* sodium hydroxide for one hour, acidified and extracted with ethyl acetate. A crystalline product was obtained by reëxtraction with 1 *N* sodium hydroxide and acidification of the alkaline extract; m.p. 172–173°.

(9) M. Kunitz and J. H. Northrup, *ibid.*, **18**, 433 (1935); **19**, 991 (1936).

(10) P. A. Levene and R. E. Steiger, *J. Biol. Chem.*, **74**, 689 (1927).

(11) H. D. Dakin and R. West, *ibid.*, **78**, 91 (1928).

Radioactive DL-4-(4-Hydroxy-3,5-dibromophenyl)-3-acetaminobutanone-2.—Radioactive pyridine perbromide·HBr was prepared by the distillation of labeled Br_2 from 125 mg. of pile bombarded NH_4Br into 90 mg. of pyridine·HBr in 0.3 ml. of glacial acetic acid. The acetic acid solution was warmed to dissolve the precipitate, cooled, the crystalline radioactive perbromide centrifuged off, washed with ligroin and dried. Unbrominated ketone equal to one-third the weight of the radioactive perbromide was dissolved in 0.2 ml. of pyridine and the perbromide added slowly, allowing time for decolorization between each addition. The solution obtained was evaporated *in vacuo* to dryness and the residue purified in a manner similar to the other radioactive derivatives; m.p. 133–134°.

OAK RIDGE, TENNESSEE

[CONTRIBUTION FROM THE RESEARCH LABORATORY OF THE GENERAL ELECTRIC COMPANY]

High Molecular Weight Polymethylene

BY SIMON W. KANTOR AND ROBERT C. OSTHOFF

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A polymethylene of molecular weight of 3.3 million was prepared by the catalytic decomposition of diazomethane. This polymer is highly crystalline and has a crystal melting point of 132°. This temperature is close to the convergence temperature for straight-chain paraffins. Some of the physical properties of the polymethylene are described and a mechanism of the polymerization is presented.

There has been considerable theoretical interest in ascertaining the physical properties of a very high molecular weight straight-chain hydrocarbon.[1,2] We have succeeded in preparing a polymethylene of *ca.* three million molecular weight by the catalytic decomposition of diazomethane in ether solution and we have determined some of its properties.

In a typical experiment, a 500-ml. solution of 10–11 g. of diazomethane in diethyl ether, prepared from N-nitrosomethylurea,[3] was treated with about 0.1 ml. of diethyl ether–boron trifluoride complex at 0°. Immediately the violent exothermic reaction liberated nitrogen and precipitated 3.7 g. of a white waxy polymeric solid.

Anal. Calcd. for $(CH_2)_x$: C, 85.7; H, 14.3; N, 0.0. Found: C, 85.5; H, 14.4; N, 0.26.

This polymer could be dissolved in boiling benzene, toluene and xylene. The nitrogen content of a sample of the material reprecipitated from xylol solution was *ca.* 0.1%.

The molecular weight of this polymer was determined by measuring the intrinsic viscosity in xylol solution at 132°. The data are summarized in Table I.

TABLE I

VISCOSITY OF XYLOL SOLUTIONS OF POLYMETHYLENE

C(g./l.)	η_{rel}	η_{sp}/C
0.25	1.469	1.875
0.50	2.220	2.440
1.0	3.002	3.002
1.25	5.03	3.22

$[\eta] = 1.7 \pm 0.1$

The value of the intrinsic viscosity $\lim_{c \to 0} \eta_{sp}/C$ was obtained by a linear extrapolation of the graph of η_{sp}/C against C in the usual fashion. From the intrinsic viscosity (1.7 ± 0.1) and the Staudinger constants reported by Harris,[4] we obtained a molecular weight of $3.3 \times 10^6 \pm 0.4 \times 10^6$. This value is 100 to 1,000 times greater than that of the polymethylene previously obtained from diazomethane (reported molecular weights: 22,000,[4] 1,800–2,000,[5] *ca.* 20,000[6]).

In order to obtain the melting point of the polymer, thermal analyses in the temperature range of 25–213° were carried out with a copper–constantan thermocouple and a recording potentiometer. The results indicated a crystal melting point of 132° (rate of warming, 0.5°/min.) and a freezing point of 124° (rate of cooling, 0.5°/min.). The lower freezing point is not too surprising since polymer melts tend to supercool. Examination of the polymer under crossed Nicols indicated that the crystallites began to disappear at about the same temperature. However, the bulk viscosity remained very high until a temperature of *ca.* 200° was attained. A sample of the polymer maintained at 150° in an air oven for 165 hours did not flow under its own weight in spite of considerable discoloration and degradation.

We feel that the melting point of 132° is close to the convergence temperature for straight-chain paraffins considering the very high molecular weight of this polymethylene. This temperature compares favorably with the frequently quoted convergence temperature of 137°[2] which was derived from thermodynamic data.

An X-ray investigation of strips of the polymer indicated essentially the same crystal structure as commercial polyethylene but showed possibly an even higher degree of crystallinity than that found in previously reported cases.

Samples of the polymer were pressed into thin sheets at 230° and 10,000 lb./in.² pressure. Strips of this sheet had a tensile strength of 4,900 lb./in.² and an elongation at break of 500%. This value is about 2,000 pounds greater than that obtained with the commercially available polyethylenes[7] and is indicative of the greater molecular weight and lower degree of branching of the polymer prepared by the authors.

The electrical properties of this polymethylene are listed in Table II in which ϵ represents the dielectric constant and tan δ represents the power factor.

TABLE II

Frequency	tan δ	ϵ
60	0.0008	2.38
300	.0003	2.38
1000	.0001	2.38

The breakdown voltage was found to be *ca.* 2,760 volts/mil for a sheet 0.015 in. in thickness. All these dielectric properties are comparable to those of commercial polyethylenes.[7]

Mechanism of the Polymerization.—The polymerization of diazomethane could simply be represented as the combination of methylene diradicals, $\cdot CH_2 \cdot$ formed by loss of nitrogen from CH_2N_2. The growing diradical chain could then

(1) K. H. Meyer and A. van der Wyk, *Helv. Chim. Acta*, **20**, 1313 (1937).

(2) H. Mark, *J. Applied Phys.*, **12**, 41 (1941).

(3) "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1943, p. 165.

(4) I. Harris, *J. Polymer Science*, **8**, 353 (1952).

(5) H. Meerwein, *Angew. Chem.*, **60**, 78 (1948).

(6) G. D. Buckley, L. H. Cross and N. H. Ray, *J. Chem. Soc.*, 2714 (1950).

(7) C. E. Schildknecht, "Vinyl and Related Polymers," John Wiley and Sons, Inc., New York, N. Y., 1952, pp. 512, *et seq.*