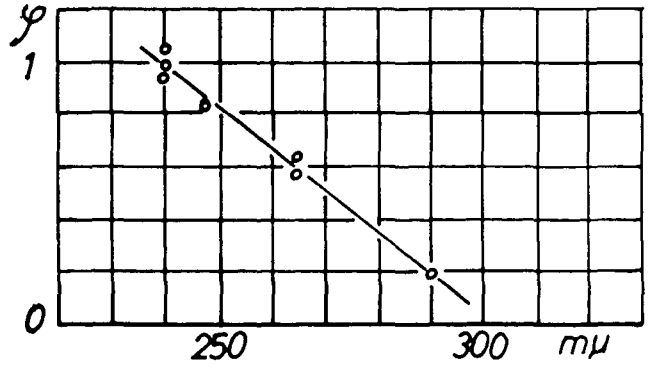


Fig. 5.—Quantum yields for the photochemical reversal of orotic acid dimer.

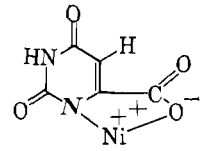
tion peak at 2800 Å, 2400 Å. irradiation achieved a back reaction of 95% to the original spectrum of orotic acid.

We also irradiated the orotic acid dimer chromatographically isolated. With this material reproducible quantum yields could not be obtained because of the unknown concentration of the orotic acid dimer resulting from the presence of paper contamination.

After standing for 20 hr. no alteration of the absorption spectrum could be observed in a 2×10^{-4} M solution of orotic acid maximally irradiated at 2900 Å.

Discussion

The pK_a values for orotic acid are 2.8, 9.45, and >13, respectively.[4] Uracil and thymine agree with the last two values. This indicates that the responsible group for our equimolar complexes between orotic acid and the divalent ions is the carboxyl group. In the pH range studied, hydroxide complexes like $Ni(OH)^+$ may be formed. One may assume complex formation between the metal hydroxide and the anion COO^- of orotic acid. A second type of complex formation is



Potentiometric experiments are necessary to decide between these two structures of complex formation.

The existence of the isosbestic point at 2940 Å. implies one defined equilibrium condition. The free energy of complex formation is of the order of 7 kcal./mole, indicating a weak bond between the metallic and the organic moiety.

Nickel complexes are stable against intense ultraviolet irradiation, presumably because of the quenching of the relevant excited state involved in the dimerization of orotic acid. In contrast, complexes with the diamagnetic Zn^{++} are weakly susceptible to ultraviolet light, the quenching being much weaker, and the dimerization having a quantum yield (measured in nitrogen) comparable to that found when irradiating free orotic acid in oxygen atmosphere.

The change in electronegativity, ionic radius, and atomic number does not seem to correlate with the red shift of the absorption peak. Further experiments are necessary to decide the nature of the quenching process.

Acknowledgment.—The author is indebted to Prof. M. Delbrück, at the California Institute of Technology, Pasadena, for helpful discussion and criticism of the manuscript.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND THE POLYMER RESEARCH INSTITUTE OF THE POLYTECHNIC INSTITUTE OF BROOKLYN, BROOKLYN, NEW YORK]

The Mechanism of Strong-Base-Initiated Polymerization of N-Carboxyanhydrides[1]

BY MURRAY GOODMAN AND URIEL ARNON[2]

RECEIVED MARCH 24, 1964

The strong-base-initiated polymerization of N-carboxyanhydrides, both N-substituted and unsubstituted, is shown to proceed by similar mechanisms. By using methylmagnesium bromide and 9-fluorenyllithium on the N-carboxyanhydride of γ-benzyl-L-glutamate it is shown that the initial step is an acid–base reaction. The proton attached to the nitrogen is abstracted, liberating the conjugate acid of the initiator anion and forming a low molecular weight peptide. By the use of 9-fluorenylpotassium and C-14 labeled sodium methoxide as initiators in NCA polymerizations, we have demonstrated that the resulting polymers do not contain the initiator anion as a terminal group. In N-substituted N-carboxyanhydrides, such as sarcosine and proline, the proton is abstracted from the α-carbon, as shown by the extensive racemization observed in the reaction between equimolar quantities of N-carboxy-L-proline anhydride and sodium methoxide. Evidence is presented that the propagation proceeds *via* an ionized carbamate group, as indicated both by the absence of free amino groups and amide ions in the polymerization solution, and by the strong-base characteristics exhibited in the polymerization of N-carboxy-γ-benzyl-L-glutamate anhydride initiated by sodium N-benzylcarbamate, which itself is not a strong base.

The mechanism of strong-base-initiated polymerization of N-carboxyanhydrides (NCA's), which was introduced and described by Blout and his co-workers,[3,4] has been for some years a subject of controversy.[5,6] As a result of their detailed investigation of the kinetics of strong-base-initiated polymerization of γ-benzyl-L-glutamate NCA, Idelson and Blout[4] proposed a mechanism which consisted essentially of an addition reaction by the initiator anion at the 5-position of the NCA ring, followed by ring opening and formation

(1) A preliminary report of this investigation was published in *Biopolymers*, **1**, 500 (1963).

(2) Submitted by U. Arnon in partial fulfillment of the requirements for the degree of Doctor in Philosophy, to the faculty of the Polytechnic Institute of Brooklyn.

(3) E. R. Blout and R. H. Karlson, *J. Am. Chem. Soc.*, **78**, 941 (1956).

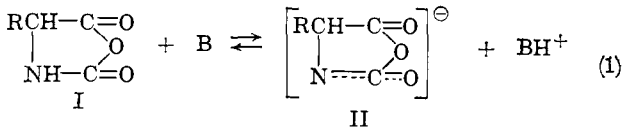
(4) M. Idelson and E. R. Blout, *ibid.*, **80**, 2387 (1958).

(5) E. Katchalski and M. Sela, *Advan. Protein Chem.*, **13**, 243 (1958).

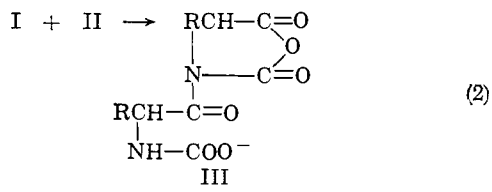
(6) C. H. Bamford and H. Block, "Polyamino Acids, Polypeptides and Proteins," Mark A. Stahmann, Ed., University of Wisconsin Press, Madison, Wis., 1962, p. 65.

of a carbamate anion. Propagation was envisaged as proceeding by a similar route, *i.e.*, addition of the negative carbamate ion at the 5-position of the next NCA molecule and ring opening with regeneration of the carbamate group. The unstable mixed anhydride formed in the reaction was assumed to lose carbon dioxide, resulting in formation of the peptide bond. The authors presented much evidence in support of this mechanism. By its very nature, however, it excludes tertiary amines and secondary amines with bulky substituents as initiators in polymerizations proceeding by this route. This was clearly recognized by the authors, who considered the exclusion justified in view of the different relationship between anhydride–initiator ratio (A:I) and degree of polymerization (DP) observed with these initiators, compared, for example, with the methoxide series.

On the basis of their work with tertiary amines, Bamford and his collaborators[6,7] have proposed an acid–base mechanism to account for their observation that only N-unsubstituted NCA's seem to be capable of undergoing polymerization in media such as pyridine. Reports to the contrary[8] were attributed by Bamford and his co-workers to the presence of impurities. The acid–base mechanism involves abstraction, by the initiator, of a proton from the nitrogen atom of the ring.



(1)



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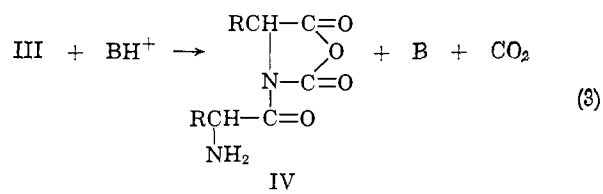
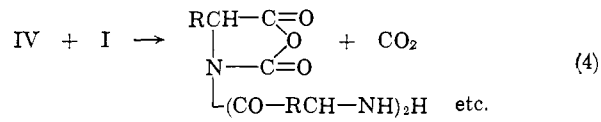


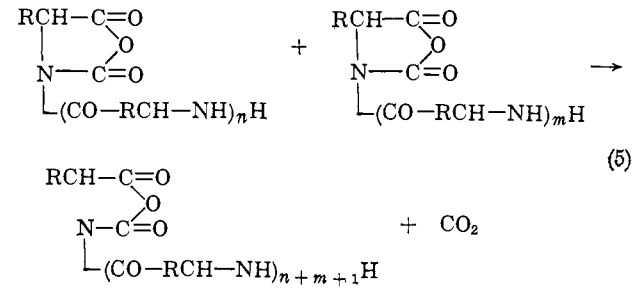
(3)

The resultant negative species (II) reacts with an additional molecule of monomer to yield III. This carbamate regains a proton from the conjugate acid of the initiator and loses carbon dioxide to form a species (IV) which can undergo propagation by the "primary amine mechanism,"[5] *i.e.*, nucleophilic addition at C-5 accompanied by ring opening, proton transfer, and loss of carbon dioxide with regeneration of the primary amine as the terminal group (reaction 4)



(4)

Since the growing chains are bifunctional, coupling may also occur.



(5)

Originally, initiation by strong bases such as hydroxides and methoxides was not included in this scheme. This would be difficult to envisage in view of the requirements of reaction 3. A carbamate ion would not be expected to abstract a proton from a conjugate acid such as methanol, although the possibility exists if an amide ion is formed by prior loss of carbon dioxide. Serious objections have been raised against this mechanism on the basis of the rates of polymerization encountered in strong-base- and tertiary-amine-initiated polymerizations. These rates are greater by two orders of magnitude compared with primary-amine-initiated reactions. Since the propagation is assumed to proceed *via* addition of a primary amine group, the difference in rates is difficult to explain, even if the coupling reaction is taken into account.

Another objection which can be brought against this scheme is that although doubts have been raised as to the possibility of initiation by tertiary amines in the case of N-substituted NCA's, this is not so when strong bases are used. Both L-proline and sarcosine NCA's have been polymerized by sodium methoxide initiation.[9] The kinetics of these polymerizations and the resultant molecular weights as functions of the A:I ratio are very similar to those obtained with γ-benzyl-L-glutamate NCA.[4] Thus, a similar mechanism seems to be operative in both cases.

In the Idelson-Blout mechanism, the initiator anion is presumed to become a terminal group of the polymer chain. This is not the case in the Bamford scheme, in which the anion should be recoverable in one form or another from the polymerization mixture after isolation of the product. Thus, by a judicious choice of initiators, we solved the problem of the nature of the initial reaction. We have also established the nature of the propagating species by investigating the three possibilities, *i.e.*, free amine, amide ion, or carbamate. The present work uses these chemical approaches rather than the kinetic approach for the elucidation of the mechanism of strong-base initiation.

Experimental

Measurement of CO_2 Evolution.—The progress of the polymerizations was followed by measuring the rate of carbon dioxide evolution, using the method developed by Patchornik and Shalitin.[10] The gas was swept out of the polymerization solution by a stream of nitrogen which was passed through a purification train in which water and carbon dioxide were removed and the gas was saturated with the vapor of the solvent used in the polymerization. The gas was absorbed in a 3:3:1 mixture of dioxane–ethanol–benzylamine containing a few drops of thymol blue indicator solution, into which was inserted the tip of a buret containing a standardized methanol solution of sodium methoxide. A measured excess of base was maintained in the solution at all

(7) D. G. H. Ballard and C. H. Bamford, *J. Chem. Soc.*, 381 (1956).

(8) L. Bilek, J. Derkosch, H. Michl, and F. Wessely, *Monatsh. Chem.*, **84**, 717 (1953).

(9) (a) G. D. Fasman and E. R. Blout, *Biopolymers*, **1**, 3 (1963); (b) *ibid.*, **1**, 99 (1963).

(10) A. Patchornik and Y. Shalitin, *Anal. Chem.*, **33**, 1887 (1961).

times. Additional titrant was added when the equivalence point was reached, as denoted by a color change from blue to green.

Preparation of N-Carboxyanhydrides.—N-Carboxy-γ-benzyl-L-glutamate anhydride (NCA) was prepared by the method described by Blout and Karlson.[3] The product was recrystallized twice from methylene chloride and five times from ethyl acetate–hexane. All handling, including recrystallizations, of this and other NCA's, was carried out in an efficient drybox.

N-Carboxysarcosine anhydride was prepared by phosgenation of a suspension of free-base sarcosine in dioxane.[9b] The product was purified by recrystallizing twice from boiling chloroform–hexane and twice from ethyl acetate–hexane.

N-Carboxy-L-proline anhydride was prepared by the method of Kurtz, *et al.*[11] This product was recrystallized twice from ethyl acetate–hexane.

Solvents.—Dioxane was purified by the method of Vogel[12] and stored over lithium aluminum hydride, from which it was fractionally distilled immediately prior to use. Tetrahydrofuran was stored over solid potassium hydroxide and fractionally distilled from lithium aluminum hydride immediately before use. Ethyl acetate was purified by the method of Fieser[13] and stored over calcium hydride from which it was fractionally distilled prior to use. Hexane and methylene chloride were refluxed over and fractionally distilled from phosphorus pentoxide. The same procedure was used for benzene, with lithium aluminum hydride as the drying agent.

Initiators and Reagents.—Sodium methoxide solutions, used both for initiation and for carbon dioxide measurements, were prepared by dissolving clean sodium metal in absolute methanol. The solutions were standardized by addition of a measured volume to distilled water and titration with standard hydrochloric acid to the phenolphthalein end point.

Carbon-14 labeled sodium methoxide initiator solution was prepared in the following manner: 1 mc. of anhydrous $C^{14}H_3OH$ (specific activity 25 mc./mmole[14]) was diluted with 30 ml. of abolute methanol. The resultant, highly radioactive, alcohol (1 ml.) was further diluted with 30 ml. of methanol, the liquid was cooled by immersion of the container in a Dry Ice–acetone bath, and a small piece of clean sodium metal was added. The base was standardized as described above. Radioactive standardization was carried out by adding a measured volume of the solution to ethanol (both quantities equal to those subsequently used for initiation and for precipitation of the polymer, respectively, the measurement being repeated for each polymerization). A definite volume (1 or 2 ml.) of the resultant solution was mixed with an equal volume of the phosphor solution, consisting of 0.5% of 2,5-diphenyloxazole in toluene. The measurements were carried out at −10° using an EKCO 612 well type scintillation counter and an EKCO N530 scaler at PM voltage 1150 v. and bias 12.5 v.

Methylmagnesium bromide was prepared by passing a slow stream of methyl bromide through a thoroughly dried flask containing tetrahydrofuran and magnesium turnings. The entire system was protected from the atmosphere by a blanket of prepurified nitrogen. A slight residue of the metal was allowed to remain in the reagent solution at the end of the reaction. The clear solution was standardized by addition of a measured volume to a measured excess of standard hydrochloric acid, and subsequent titration with sodium hydroxide to the phenolphthalein end point.

A commercial solution of *n*-butyllithium in hexane (supplied by the Foote Chemical Company, New Johnsonville, Tenn.) was used after filtration through a fine filter to remove lithium hydroxide. The reagent was standardized in the same manner as methylmagnesium bromide.

9-Fluorenyllithium was prepared as follows[15]: fluorene (obtained from the Eastman Kodak Company, Rochester, N. Y.) was dissolved in dioxane and mixed with the above mentioned hexane solution of *n*-butyllithium at 80°, approximately equimolar amounts being used. The mixture, which immediately assumed the characteristic orange-brown color of the product, was heated for about 1 hr. The resultant crystals of 9-fluorenyllithium were collected by filtration and washed twice with dioxane to remove hexane and unreacted fluorene and *n*-butyllithium. The reagent was stored under dry dioxane, in which it is partially soluble. The supernatant saturated solution was used in the experiments. It was standardized by addition of a measured volume to moist ethanol and titration of the resulting solution with standard hydrochloric acid to the phenolphthalein end point.

9-Fluorenylpotassium was prepared as follows[16]: A solution of fluorene (4.2 g., 25.3 mmoles) in 1,2-dimethoxyethane (about 40 ml.) was heated to 70°, and a slight excess (1.0 g., 25.6 mmoles) of clean potassium metal was added. The temperature was maintained until nearly all the potassium was consumed, after which the red-brown solution was cooled, filtered, and standardized in the same manner as 9-fluorenyllithium. The over-all fluorene content (including unreacted starting material) was found by diluting a measured volume of the reagent solution with a known amount of dioxane and determining the height of the 300 mμ peak in the ultraviolet spectrum (measured on a Cary 14 recording spectrophotometer, using a 0.01-cm. quartz cell).

Sodium N-benzylcarbamate was prepared by using the carbon dioxide absorption apparatus and solution described above. "Bone-dry" carbon dioxide (obtained from the Matheson Company, East Rutherford, N. J.) was passed into the benzylamine solution, which was then titrated with sodium methoxide until the color of the thymol blue indicator changed from yellow to green. The process was repeated until most of the benzylamine had been consumed. The methanol and ethanol present in the solution were removed by distillation, leaving a precipitate of the carbamate over dioxane. The mixture was transferred to a drybox and filtered, and the product was washed several times with tetrahydrofuran, followed by hexane. The sodium N-benzylcarbamate was then dried and dissolved in absolute methanol. It was standardized by adding a measured volume to an excess of dilute sulfuric acid in the apparatus usually used for polymerizations and determining the amount of carbon dioxide evolved.

Methyl iodide was used either in the pure state or in the form of a dioxane solution, depending on the quantities required. No purification of the compound was carried out.

Methyl methacrylate (containing a stabilizer) was distilled from cuprous chloride at room temperature and an absolute pressure of 13 mm. of mercury, and collected in a flask immersed in a Dry Ice–acetone bath. The distillation was carried out shortly before the material was to be used, and the frozen methyl methacrylate was kept at the Dry Ice temperature until needed, when it was rapidly brought to room temperature and immediately introduced into the polymerization mixture.

Polymer Isolation.—Poly-γ-benzyl-L-glutamate was isolated by decantation of the polymerization mixture into about 40 volumes of absolute ethanol, with vigorous stirring. The fibrous precipitate was filtered, dissolved in the minimum amount of dioxane, and the solution filtered and lyophilized.

Polysarcosine was isolated by pouring the polymerization mixture into anhydrous ethyl ether, filtering, washing with ether, and lyophilizing a slurry of the polymer in dioxane.

Molecular Weight Determination.—The molecular weights of the poly-γ-benzyl-L-glutamate polymers were determined, where needed, by viscometry. An Ubbelohde viscometer with a solvent flow time of 248.6 sec. was used. The solvent was dichloroacetic acid. The molecular weights were calculated using the expression: $[\eta] = 2.78 \times 10^{-5} M^{0.87}$, established by Doty and his coworkers.[17]

Measurement of Methane Evolution.—In experiments using methylmagnesium bromide, the methane evolved in the reaction with γ-benzyl-L-glutamate NCA was measured in a gas buret connected to the outlet of the polymerization flask. Atmospheric pressure in the system was achieved by means of a flask of mercury which could be raised or lowered to the desired height. A tube of "Indicarb" (supplied by the Fisher Scientific Company, New York, N. Y.) was attached to the system between the polymerization flask and the gas buret to absorb carbon dioxide evolved in the reaction. During these experiments, the reflux condenser of the polymerization apparatus was cooled by ethanol at a temperature of −78° to prevent the vapor pressure of the solvent from affecting the readings. (Because of the relatively large

(11) J. Kurtz, G. D. Fasman, A. Berger, and E. Katchalski, *J. Am. Chem. Soc.*, **80**, 393 (1958).

(12) A. I. Vogel, "Practical Organic Chemistry," Longmans, Green and Co., London, 1956, p. 177.

(13) L. F. Fieser, "Experiments in Organic Chemistry," D. C. Heath and Co., Boston, Mass., 1955, p. 287.

(14) The material was prepared by the California Corporation for Biochemical Research, Los Angeles, Calif.

(15) W. E. Goode, F. H. Owens, R. P. Fellmann, W. H. Snyder, and J. E. Moore, *J. Polymer Sci.*, **46**, 317 (1960).

(16) G. W. H. Scherf and R. K. Brown, *Can. J. Chem.*, **38**, 2450 (1960).

(17) P. Doty, J. H. Bradbury, and A. M. Holtzer, *J. Am. Chem. Soc.*, **78**, 947 (1956).

volume between the surface of the solution and the adsorbent tube, not all the carbon dioxide could be eliminated, and the readings were therefore usually higher than calculated from the theoretical amount of methane.)

Results and Discussion

Initiation. Methylmagnesium Bromide and 9-Fluorenyllithium.—As has been mentioned above, one most promising approach to the problem of the initiation step involves the use of initiators whose fate can be ascertained. Grignard and related organometallic reagents yield the original hydrocarbon when they undergo an acid–base reaction, and it was expected that this type of conjugate acid, *e.g.*, methane, would hardly take part in a reaction such as (3) above. Moreover, the appearance of the hydrocarbon could be easily detected: in the case of methane, the amount of gas liberated could be measured by simple volumetric methods, while in the case of the fluorene derivative, the fate of the hydrocarbon could be determined by measurement of the ultraviolet spectrum of the polymer. Fluorene and its derivatives possess a strong absorption peak in the ultraviolet (ϵ 10,000 at 300 mμ); thus, failure to detect this absorption in the spectrum of the polymer would tend to disprove the addition mechanism.

When γ-benzyl-L-glutamate N-carboxyanhydride was treated with catalytic amounts of either of these two reagents, a total of between 2 and 2.5 moles of carbon dioxide was evolved for every mole of reagent, after which the evolution ceased. Where methylmagnesium bromide was used, methane was evolved instantaneously and quantitatively. In the case of 9-fluorenyllithium, the characteristic red-brown color of the reagent disappeared immediately upon addition of the reagent to the NCA solution. In both cases, no polymer was formed. These observations seemed to indicate that an acid–base reaction occurred, and the fact that polymerization failed to ensue may be attributed to the nature of the cationic counter ion. Idelson and Blout[4] reported that the polymerization initiated by lithium methoxide, although it did take place, proceeded at a much slower rate than with all the other alkali metal methoxides.

9-Fluorenylpotassium.—In view of the fact that polymerization was not observed with 9-fluorenyllithium, which we attributed to the cation rather than to the anion, the potassium derivative of fluorene was prepared and used as an initiator. The initiator consisted of a 1,2-dimethoxyethane solution of 9-fluorenylpotassium with a basicity corresponding to 0.725 *M* and a total fluorene content (determined by ultraviolet measurement) of 0.767 *M*. Successful polymerizations were observed. The A:I ratio used was 64, yielding a polymer with a degree of polymerization of 180. The polymerization followed first-order kinetics after an initial autocatalytic period, similar to the phenomenon observed in sodium methoxide-initiated polymerizations. Figure 1 depicts the ultraviolet spectra of the polymer and of the residue from the filtrate after precipitation of the polymer, respectively.

If the resultant polymer contained one fluorenyl residue at the starting end of each chain, the optical density at 300 mμ would have been 0.152 under the conditions of the measurement. As may be seen in Fig. 1, the absorbance at this wave length is zero. The spectrum is in fact identical with that obtained from

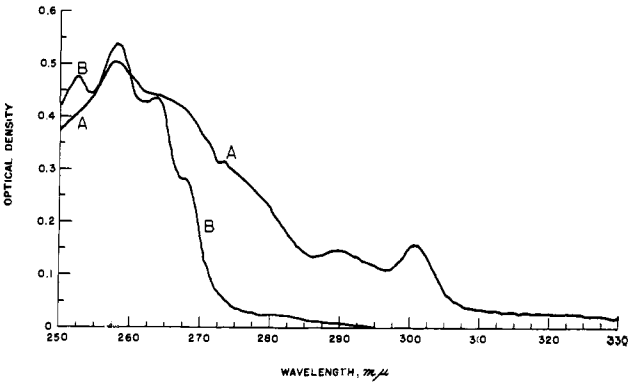
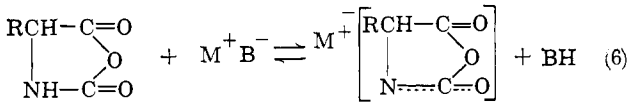
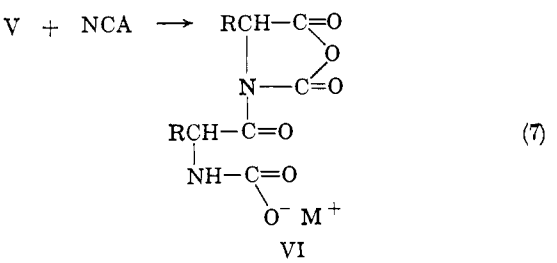


Fig. 1.—Ultraviolet spectra of poly-γ-benzyl-L-glutamate system initiated with 9-fluorenylpotassium (A:I = 64): (A) residue remaining from removal of solvent from filtrate used to isolate polymer; (B) isolated polymer of poly-γ-benzyl-L-glutamate.

samples prepared by sodium methoxide and triethylamine initiation. All of the fluorene was recovered in the filtrate following precipitation of the polymer, as calculated from the data shown in Fig. 1A.

These observations are consistent with the following acid–base reaction scheme as originally proposed by Bamford and Block[6]





However, when M^+B^- is a compound such as methylmagnesium bromide, 9-fluorenyllithium, *n*-butyllithium, etc., reaction of V with NCA to yield VI (reaction 7) is very slow because of the nature of the counter ion.

Radioactive Sodium Methoxide.—Although the results obtained with 9-fluorenylpotassium may be regarded as conclusive insofar as the choice between the addition and the acid–base mechanism is concerned, we felt that in view of the unusual nature of the initiator, confirmation was necessary. Furthermore, if an acid–base scheme is postulated in which the hydrogen attached to the nitrogen atom of the NCA ring is abstracted as a proton, the problem of N-substituted N-carboxyanhydrides remains unsolved.

The first difficulty was removed by using radioactively labeled sodium methoxide as the initiator. The results are summarized in Table I.

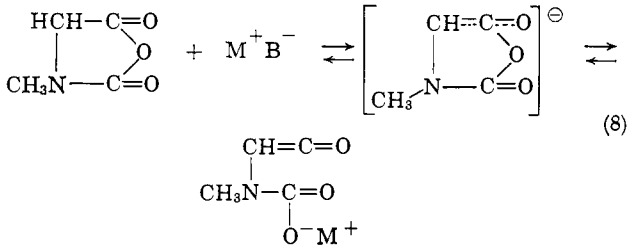
TABLE I
INITIATION BY RADIOACTIVE SODIUM METHOXIDE

NCA	A:I	DP of the polymer	% Radioactivity in polymer
γ-Benzyl-L-glu	60	490	0.7
γ-Benzyl-L-glu	30	131	0.6
Sarcosine	60	Not measured	None

These results clearly indicate that the initiation indeed proceeds *via* an acid–base mechanism, and this

is the case for both types of NCA. In view of the result obtained with sarcosine NCA, it is probable that the residual radioactivity found in poly-γ-benzyl-L-glutamate is due to ester interchange, a result to be expected in view of the relatively prolonged contact time between the polymer and the radioactive compound.

It is obvious from the structure of an NCA such as that derived from sarcosine that only the α-carbon atom of the amino acid possesses hydrogen atoms sufficiently activated to undergo acid–base reactions. Since sarcosine is optically inactive, it is difficult to demonstrate the occurrence of such a reaction which is similar in many respects to the well known Claisen-type reactions between activated hydrogens and strong bases represented by the following equation showing a ketene–carbamate initial product.



(8)

This reaction is similar to that postulated for N-unsubstituted NCA's.

Obviously, an optically active N-substituted NCA would undergo racemization in this type of reaction. Since in most cases only catalytic quantities of strong base are used to initiate the polymerization, racemization occurring in the single terminal residues of long-chain molecules is undetectable even after hydrolysis of the resultant polyamino acid. To overcome this difficulty, L-proline NCA was allowed to react with an equimolar amount of sodium methoxide, the product was treated with 5 *N* hydrochloric acid for 24 hr. at 130°, and the optical rotation was measured. Control solutions were treated in a similar manner. The results are tabulated in Table II.

TABLE II
EFFECT OF SODIUM METHOXIDE ON THE OPTICAL ROTATION OF L-PROLINE NCA AND L-PROLINE

Compound	Base	Molar ratio anhydride to base	$[\alpha]^{25}D$, deg. (5 *N* HCl)
L-Proline NCA[a]	$NaOCH_3$	1	−14.6
L-Proline NCA[a]	$NaOCH_3$	100	−33.5
L-Proline NCA[b]	None	...	−34.0
L-Proline[b]	None	...	−40.0
L-Proline[c]	...	...	−52.4

[a] Samples were treated with 5 *N* HCl at 130° for 24 hr. following reaction with base. [b] These controls were also subjected to acid hydrolysis conditions as described in *a*. [c] The amino acid was simply dissolved in 5 *N* HCl and the optical rotation measured.

Thus, although some racemization occurred in all the samples as a result of the drastic acid treatment, the only sample to undergo extensive racemization was that which had been allowed to react with an equimolar quantity of sodium methoxide. Thus, while the hydrogen atom on the α-carbon of the N-substituted amino acid NCA seems to be sensitive to both acidic and basic conditions, there is no doubt that the preliminary treatment with strong base results in nearly total racemization, *i.e.*, to the extent of about 72% in this case.

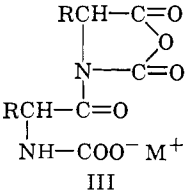
Propagation.—In the mechanism proposed by Bamford and Block,[6] the first step in the propagation consists of the abstraction of a proton from the conjugated acid of the initiator by compound III, which simultaneously loses carbon dioxide and is transformed into IV (reaction 3). We have already presented our objections to this scheme. Nevertheless, we felt that the absence of a free amino group should be positively proven before we sought other *propagation* mechanisms.

Addition of Methyl Iodide to the Polymerization Mixture.—The absence of free amine in the reaction mixture was demonstrated by the use of methyl iodide as a "scavenger." Alkyl iodides are well known to react rapidly and quantitatively with amines, and therefore the injection of methyl iodide into the polymerization solution should cause rapid and complete inhibition of the polymerization, if free amine were the active carrier of the reaction. (Additional work on amine-initiated polymerization of NCA's by J. Hutchison of our laboratory has shown that methyl iodide significantly reduces the rate of polymerization.)

The iodide was added in a quantity corresponding to the amount of sodium methoxide used as initiator, since it was assumed that this represented the maximum possible concentration of free amine in the solution. The reagent was added after 25% conversion. When no effect whatsoever could be observed, an additional quantity of methyl iodide, 3.5 times larger than the first portion, was injected at the 40% conversion point. Again no effect could be observed.

In a similar experiment, the amount of iodide added was somewhat in excess of the total quantity of N-carboxyanhydride (NCA) remaining in the solution at the time of addition, *i.e.*, in very large excess as far as the presumed concentration of amine is concerned. The course of the polymerization was once again unaltered. Thus it is obvious that the addition of methyl iodide has no effect at all on the reaction.

Addition of Methyl Methacrylate to Polymerization Mixtures.—If compound III is the actual initiating

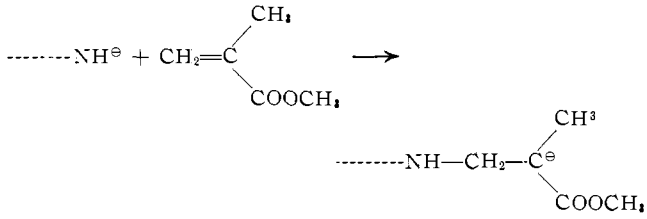


III

species, two possibilities arise for the propagation mechanism. The carbamate can attack a new molecule of monomer at the C-5 position forming a mixed anhydride which subsequently decomposes to the peptide bond (as proposed by Idelson and Blout[4]), or it may lose carbon dioxide to yield an amide ion which adds to the next molecule of NCA (as proposed by the same authors in their electron-transfer mechanism). The second alternative seems *a priori* less plausible in view of the highly basic nature of amide ions. Such strong bases, even more than methoxides, would tend to abstract protons from NCA molecules rather than to undergo addition reactions, and the result would be chain transfer with all the consequences entailed by this phenomenon. Furthermore, free amine would be formed, and this has been shown not to be the case.

Nevertheless, an attempt was made to disprove directly the presence of amide ions in the polymerizing solution. We recognize of course that such ions, if present, would react even more rapidly than free amine with methyl iodide.

Ionic amides, such as lithium amide and sodamide, have been used with success in the anionic polymerization of vinyl monomers such as methyl methacrylate.[15,19] This material can thus serve as a "scavenger" for free amide ions. The primary reaction envisaged as the result of the addition of methyl methacrylate to a solution of an N-carboxyanhydride undergoing polymerization by an amide ion mechanism is as follows



This carbanion can react further by various pathways, either with additional NCA or with more of the vinyl monomer. In all possible cases, the effect of the polymerization of the N-carboxyanhydride is either inhibition of the reaction itself or disruption of the helical conformation of the poly-α-amino acid chain. The latter phenomenon would cause a decrease in the absolute value of the characteristic criteria of helicity, the slope (b_0) of the Moffitt equation[20] describing the optical rotatory dispersion of polypeptides, and the mean residue rotation measured at the trough (233 mμ) of the Cotton effect in the ultraviolet rotatory dispersion.[21,22]

The addition of methyl methacrylate was carried out at various stages of the polymerization and under varying conditions, *i.e.*, at room temperature and at $-78°$ (since the anionic polymerization of vinyl compounds is more rapid and efficient at low temperatures). The results were identical and consistent in all cases, and an example is illustrated in the kinetic curve of Fig. 2A.

As may be seen from Fig. 2A, the addition of methyl methacrylate to the polymerizing mixture had no effect at all on the rate of the reaction. The yield of polymer, based on the amount of NCA used, was 97% after purification, a value which is entirely normal. The DP of the polymer was 700, and the k_p calculated from this and the slope of the kinetic curve had a value of 2.7 l. mole^{-1} sec.$^{-1}$. The helical content of the polymer was determined as described above, the values obtained being $-13{,}070°$ for the mean residue rotation at the 233 mμ Cotton effect and $b_0 = -591$, both values indicating essentially full helicity.

It is thus clear that methyl methacrylate does not enter into any of the reactions possible in the case of its anionic initiation by an amide anion at the active chain end of the growing polypeptide. This leads to the conclusion that free amide ions are not present in the polymerizing solution and are therefore not involved in the mechanism of propagation of N-carboxyanhydrides.

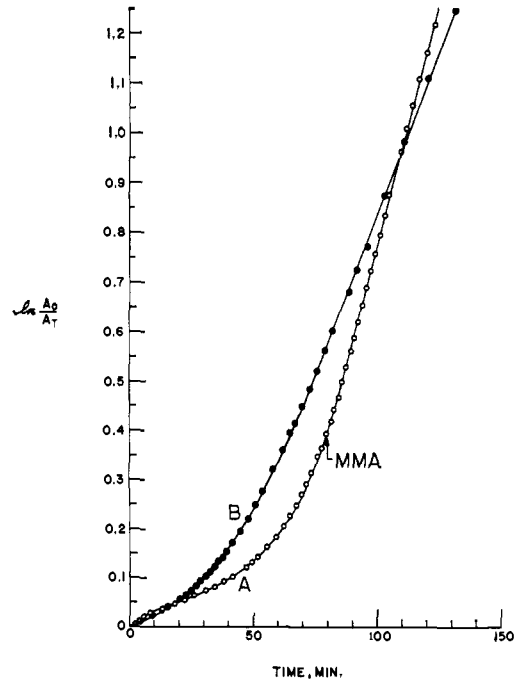
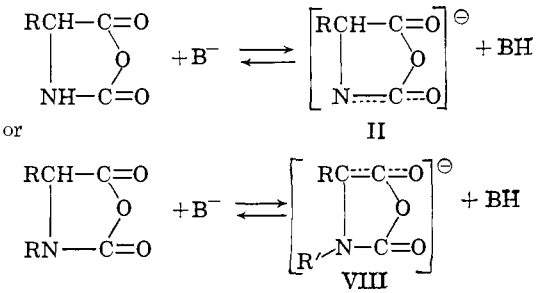


Fig. 2.—Kinetic curves describing polymerization of γ-benzyl-L-glutamate N-carboxyanhydride (temperature of polymerization 18°): (A) initiator sodium methoxide (A:I = 90); after one-third conversion, undiluted methyl methacrylate monomer added in large excess of N-carboxyanhydride; (B) initiator sodium N-benzylcarbamate (A:I = 100).

Initiation by Sodium N-Benzylcarbamate.—Since the absence of both free amine and amide ions has been demonstrated, the only reasonable alternative for the structure of the active chain end is an ionized carbamate group. Bearing in mind that carbamates are by no means strong bases, were the polymerization to proceed at a rate similar to that observed with strong bases we would possess reasonable evidence for the carbamate propagation mechanism.[4] The kinetic curve shown in Fig. 2B supports this proposal completely since the kinetics are identical with those observed with the γ-benzyl-L-glutamate NCA initiated by sodium methoxide (Fig. 2A).

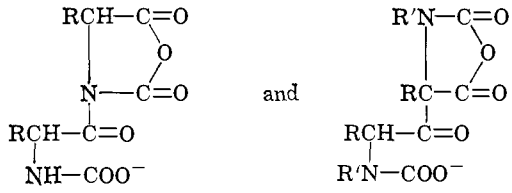
Conclusions

The investigation described in this report leads to the conclusion that, regardless of the nature of the N-carboxyanhydride, the initial reaction between typical monomers and a strong base does not involve carbonyl additions of any kind, but is a pure acid–base reaction in which the NCA plays the role of the acid, as originally proposed by Bamford and Block.[6] The particular proton abstracted from the monomer depends on the type of NCA involved. A hydrogen atom attached to

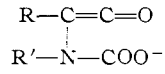


(18) W. C. E. Higginson and N. S. Wooding, *J. Chem. Soc.*, 760 (1952).
(19) N. S. Wooding and W. C. E. Higginson, *ibid.*, 1178 (1952).
(20) P. Urnes and P. Doty, *Advan. Protein Chem.*, **16**, 401 (1961).
(21) W. Kuhn, *Ann. Rev. Phys. Chem.*, **9**, 417 (1958).
(22) N. S. Simmons, C. Cohen, A. G. Szent-Gyorgyi, D. B. Wetlaufer, and F. R. Blout, *J. Am. Chem. Soc.*, **83**, 4766 (1961).

the nitrogen of the ring is more acidic than one attached to the α-carbon and is thus preferentially abstracted. If the NCA is N-substituted, the α-carbon will lose a proton in a Claisen-type reaction. The result in either case is the formation of strongly basic, negatively charged species (II and VIII) which attacks an additional molecule of the monomer at the 5-position, causing opening of the ring and formation of a carbamate group.



Alternatively, structure VIII may simply open to give a ketene–carbamate form which could then follow the propagation mechanism



The propagation reaction proceeds by the mechanism proposed by Idelson and Blout,[4] *i.e.*, formation of mixed anhydrides followed by loss of carbon dioxide, and regeneration of a carbamate group at the active chain end.

Acknowledgment.—We wish to thank the National Science Foundation for its generous support of this research under Grant No. GB 256.

COMMUNICATIONS TO THE EDITOR

Oxidation by Molecular Oxygen. I. Reactions of a Possible Model System for Mixed-Function Oxidases[1,2]

Sir:

In 1954, Udenfriend and co-workers[3] described a nonenzymatic system (composed of ascorbic acid, ferrous or ferric ions, and molecular oxygen) which caused hydroxylation of aromatic compounds. Several authors[4] have proposed that the hydroxylating agent in this system is the hydroxyl radical. However, a careful examination of the isomeric products formed clearly indicates that the hydroxyl radical is not the hydroxylating species when oxygen is the oxidant.[5-7]

The Udenfriend reaction has been considered as a possible model for phenylalanine hydroxylase which is a mixed-function oxidase.[8] Since other mixed-function oxidases with similar characteristics cause hydroxylation of saturated hydrocarbons and epoxidation of olefins,[8,9] we have looked at the reactions of the model system with a simple saturated hydrocarbon and an olefin.[10] The results are shown in Table I. The significant data in the table are the relative amounts of products formed under the varying conditions and not the absolute amounts since the solvent (acetone) can also react and ascorbic acid is oxidized by O_2 in the absence of other substrates.

TABLE I

OXIDATION OF CYCLOHEXANE AND CYCLOHEXENE

	Conditions[a]	Products from cyclohexane: Cyclohexanol, yield (mg.)[b]	Cyclohexanone, yield (mg.)[b]	Cyclohexene oxide, yield from cyclohexene (mg.)[b]
1	Undenfriend reaction,[c] 1.1 mmole of ascorbic acid, 0.04 mmole of Fe^{++}	2.1	0.7	1 to 2
2	Same as (1) but with 50 μg. of catalase[c]	2.4	0.5	1 to 2
3	Same as (1) but without O_2[d]	<0.1	<0.1	<0.1
4	Same as (3) plus 1.0 mmole of H_2O_2[d]	<0.1	<0.1	<0.1
5	Fenton reaction, 1.0 mmole of H_2O_2, 1.0 mmole of Fe^{++}	*ca.*2 (plus other products)	*ca.*2	2 to 3 (plus other products)

[a] All reactions were carried out in a heterogeneous mixture containing 31.5 ml. of 0.058 *M* acetate buffer (pH 4.5), 30 ml. of acetone, and 5 ml. of cyclohexane or cyclohexene. Separate experiments were performed to obtain the oxidation products of cyclohexane and cyclohexene. [b] Analyzed by gas chromatography. [c] Shaken under an atmosphere of air for 2 hr. [d] Shaken under an atmosphere of N_2 for 2 hr.

With the complete system (1, see Table I) both cyclohexanol and cyclohexanone are formed from cyclohexane, and cyclohexene oxide is formed from cyclohexene. Excess catalase has no effect on the amounts of products formed (2). If ascorbic acid is omitted from these experiments, then no products are formed. If O_2 is omitted (3) no products are formed. Also,

(1) Presented at the 146th National Meeting of the American Chemical Society, Denver, Colo., Jan., 1964; Abstracts of Papers, Division of Biological Chemistry, p. 13A.

(2) This investigation was supported by PHS research grant GM-09585 from the Division of General Medical Sciences, Public Health Service.

(3) S. Udenfriend, C. T. Clark, J. Axelrod, and B. B. Brodie, *J. Biol. Chem.*, **208**, 731 (1954).

(4) See, for example, R. Breslow and L. N. Lukens, *ibid.*, **235**, 292 (1960), and R. R. Grinstead, *J. Am. Chem. Soc.*, **82**, 3472 (1960).

(5) R. O. C. Norman and G. K. Radda, *Proc. Chem. Soc.*, 138 (1962).

(6) G. A. Hamilton and J. P. Friedman, *J. Am. Chem. Soc.*, **85**, 1008 (1963), and unpublished results.

(7) It is important to distinguish between the Udenfriend reaction with O_2 as the oxidant and the reaction with H_2O_2 as the oxidant. The original workers[3] concluded that the two systems gave similar products but later work has shown that when H_2O_2 is the oxidant the hydroxyl radical is the hydroxylating agent, but some different species is involved when O_2 is the oxidant.[5,6]

(8) H. S. Mason, *Advan. Enzymol.*, **19**, 128 (1957).

(9) For a recent review see M. Hayano and J. W. Foster in "Oxygenases," O. Hayaishi, Ed., Academic Press, Inc., New York, N. Y., 1962, pp. 181, 241.

(10) Other investigators [11,12] have studied the reaction of the Udenfriend system with aliphatic carbon–hydrogen bonds but the systems studied were complex and insufficient controls were done to delineate the course of the reaction.

(11) M. Chvapil and J. Hurych, *Nature*, **184**, 1145 (1959).

(12) A. Cier, C. Nofre, and A. Revol, *Compt. rend.*, **247**, 542, 2486 (1958); *ibid.*, **250**, 2638 (1960).