Evaporation of the ether and treatment of the residue in methanol with phenyl isothiocyanate gave the bisphenylthiourea derivative, m.p. 108–111° after crystallization from benzene.

Anal. Calcd. for $C_{23}H_{25}N_5S_2$: C, 63.5; H, 5.8; N, 16.1; S, 14.7. Found: C, 63.7; H, 6.2; N, 15.9; S, 14.4.

N–CH₃ Determination.—Nicotine (50 μl.) was subjected to a standard Herzig–Meyer determination and the CH₃I generated was collected in 500 μl. of dry toluene cooled in a Dry Ice–acetone trap. Triethylamine (2 ml. of a 5% solution in absolute ethanol) was added and the mixture was allowed to stand at room temperature for 24 hr. Evaporation of the solvent *in vacuo*, crystallization of the residue two times from 1–1.5 ml. of isopropyl alcohol, and drying at 50° (10 μ) for 12 hr. yielded 28 mg. of pure methyltriethylammonium iodide, m.p. 297° dec.

Anal. Calcd. for $C_7H_{18}NI$: C, 34.5; H, 7.5; N, 5.8. Found: C, 34.7; H, 7.4; N, 5.8.

From scintillation counting of weighed samples of the methyltriethylammonium iodide, the following activities were obtained: root, sample 1, 336; sample 2, 370; aerial, 54. The 95% ethanol and *p*-dioxane were allowed to stand over sodium bisulfite for 24 hr. before being used to dissolve these samples. Under these conditions, the scintillation solutions remained colorless during the entire counting period and gave counting efficiencies of 72–74% with samples as large as 14 mg. Since the root nicotine had an activity of 17,160 and the aerial nicotine had an activity of 3010, the methyltriethylammonium iodide activities show that 2.0–2.2% of the activity of the root nicotine and 1.8% of the activity of the aerial nicotine was present in the N–CH₃.

Ultraviolet-Induced Dimerization of Free and Metal-Complexed Orotic Acid

BY A. HAUG

A red shift of the π–π* absorption band, arising from the interaction of orotic acid with paramagnetic and diamagnetic ions, is studied. With all ions used 1:1 complexes are formed. The behavior against ultraviolet radiation, as measured by dimerization of orotic acid, is somewhat different with the paramagnetic Ni⁺⁺ compared to the diamagnetic Zn⁺⁺. The orotic acid–Ni⁺⁺ (1:1) complex is stable toward intense irradiation with ultraviolet light in contrast to the corresponding Zn⁺⁺ complex. The quantum yield (measured in nitrogen) of the photochemical decomposition of the Zn⁺⁺ complex is comparable with the quantum yield when irradiating uncomplexed orotic acid in oxygen atmosphere

Introduction

The extent to which orotic acid (6-carboxy-2,4-dioxypyrimidine) is photochemically decomposed by irradiation with ultraviolet light depends distinctly upon the environmental medium. Beukers, *et al.*,[1] reported that the percentage of orotic acid, sensitive to the light of a germicidal lamp, increases from 14 to 37% when bubbling oxygen or nitrogen, respectively, through the solution. Further, orotic acid, first irradiated in a nitrogen atmosphere, is partially reconstituted on subsequent irradiation in oxygen. Replacing oxygen by paramagnetic ions, 10^{-3} *M* solution of Ni⁺⁺ or Cu⁺⁺ in 0.2 *M* HCl, brought about the same partial recovery of orotic acid. The authors concluded that paramagnetic molecules or ions partially inhibit the dimerization of orotic acid in solution. They further believed that the paramagnetic substances change the photochemical equilibrium between orotic acid and its dimer.

However, recent work of Johns, *et al.*,[2] studying the dimerization of thymine upon irradiation with monochromatic ultraviolet light, indicates that the photochemical equilibrium between thymine and its dimer is strongly dependent on the wave length. At 2350 Å. the equilibrium mixture is over 90% thymine, at 2750 Å. it is over 80% thymine dimer.

Tsubomura and Mulliken[3] investigated spectrophotometrically the extra absorption caused by oxygen bubbling into organic solvents like ethanol, dioxane, or benzene. They found that new absorption bands occur as a result of charge-transfer interaction between oxygen as an electron acceptor and the organic solvent as an electron donor. Theoretically they demonstrated that this electron donor–acceptor interaction may also produce the enhancement of the singlet–triplet absorption bands by oxygen.

Having in mind these two effects mentioned above (wave length dependence of the photochemical equilibrium and the enhancement of transition probabilities of the singlet–triplet transitions) the present research was carried out in the hope of explaining more clearly the mechanism of dimerization of orotic acid.

Experimental

Materials.—Orotic acid was purchased from the Aldrich Chemical Co., Milwaukee, Wis. It was purified by recrystallizing it from water. All other chemicals used (NiSO₄·6H₂O, ZnSO₄·7H₂O, CoSO₄·7H₂O, CuSO₄·5H₂O, Ni(CHO₂)₂·2H₂O, Hg(C₂-H₃O₂)₂ were obtained from E. Merck, Darmstadt, or Th. Schuchardt, Munich, respectively. Compressed nitrogen and oxygen gas was purchased from the Linde Co.

Doubly distilled water from a quartz column was stored in polyethylene bottles. Solutions of orotic acid were freshly prepared immediately prior to use.

Apparatus.—Irradiation experiments were carried out with a high flux ultraviolet monochromator, first constructed at the California Institute of Technology, copied and further improved at our Institute. The samples were irradiated at room temperature in a Teflon stoppered Beckman cuvette (1 cm. depth, 3 ml. vol.). Nitrogen or oxygen, respectively, was passed in through a small glass tube fixed in an aperture of the stopper and out through a second small gap. Before flushing the filled Beckman cell with nitrogen, the gas was bubbled through a stock solution of orotic acid for several hours. The nitrogen was deoxygenated in an alkaline pyrogallol solution and subsequently passed through a column of distilled water to prevent evaporation loss in the Beckman cell. During irradiation, stirring of the solution was achieved by a constant flow of nitrogen or oxygen, respectively, into the upper part of the cuvette, which was not exposed to the impinging ultraviolet beam.

Absorption spectra were measured with a Cary Model 15 or Zeiss PMQ 2 spectrophotometer; pH was measured using a pH meter 22 from Radiometer Inc., Copenhagen. Descending paper chromatography was performed on Whatman 3 mm. paper in 2-propanol-NH₃-H₂O, 7:1:2 (v./v.). The molar extinction coefficients were taken from Chargaff and Davidson.[4]

(1) R. Beukers and W. Berends, *Biochim. Biophys. Acta*, **38**, 573 (1960); R. Beukers, J. Vjlstra, and W. Berends, *Rec. trav. chim.*, **78**, 879, 883 (1959).

(2) H. E. Johns, S. A. Rapaport, and M. Delbrück, *J. Mol. Biol.*, **4**, 104 (1962).

(3) H. Tsubomura and R. S. Mulliken, *J. Am. Chem. Soc.*, **82**, 5968 (1960).

(4) E. Chargaff and J. N. Davidson, "The Nucleic Acids I," Academic Press, Inc., New York, N. Y., 1955, p. 113.
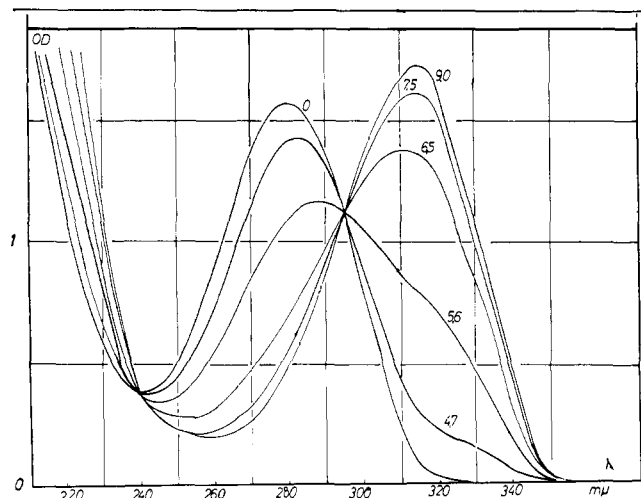
Fig. 1.—Absorption spectrum of an equimolar solution (2 × 10^{-4} M) of orotic acid and nickel ions at different pH. The curve O is the absorption spectrum of free orotic acid at pH 3.
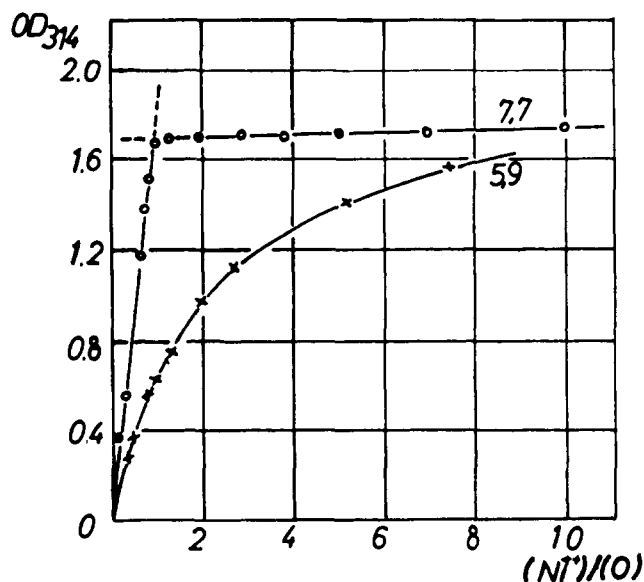

Fig. 2.—Optical density at 3140 Å. as a function of the ratio of the concentrations of nickel ions and orotic acid at two different pH values.

Analysis of the Orotic Acid–Metal Ion (1:1) Spectrum.—We found that orotic acid and metal ions form equimolar complexes. In this case the equilibrium constant between orotic acid and the metal ion may be written as

$$K = \frac{[OM]}{[O][M]}$$

In the spectral range between 2400 and 3600 Å. the absorption of the metal salt added to a solution of orotic acid is negligible, even if added in excess. The equilibrium constant, K (l./mole), may be determined from the Benesi–Hildebrand equation[5,6]

$$\frac{[O]}{D - D_0} (\epsilon_{OM} - \epsilon_0) = 1 + \frac{1}{K[M]}$$

Here, [O] and [M] are the molar concentrations of total orotic acid and of metal ion, respectively. D is the optical density of the mixture at wave length λ, D_0 is the optical density of the free orotic acid at wave length λ; ϵ_0 and ϵ_{OM} are the molar extinction coefficients of orotic acid and the complex, respectively.

The plot of $[O]/(D - D_0)$ vs. $1/[M]$ gives a straight line. The intercept on the ordinate is $1/(\epsilon_{OM} - \epsilon_0)$, and the intercept on the abscissa yields $-K$.

(5) H. A. Benesi and J. H. Hildebrand, *J. Am. Chem. Soc.*, **71**, 2703 (1949).

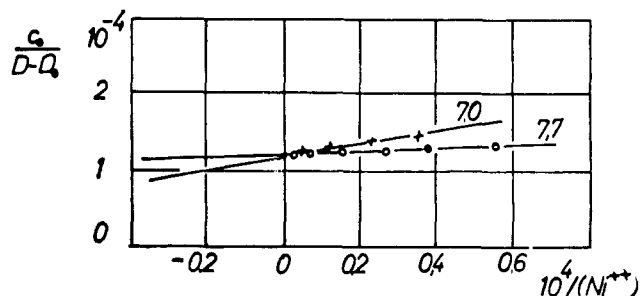(6) G. Briegleb, "Elektronen–Donator–Acceptor–Komplexe," Springer-Verlag, Berlin, 1961.

Fig. 3.—Benesi–Hildebrand lines for the equimolar complex of orotic acid and nickel ions at two different pH values.

Determination of the Quantum Yield.—Irradiation of orotic acid yields as photoproduct its dimer characterized by a cyclobutane ring structure. The quantum yield was determined from the initial rate of reaction[2]

$$\left(\frac{dD_{tot}}{dL} \right)_{L = 0} = -A \epsilon_0' C[O]$$

$$A = 2.303 \times 10^3 \epsilon_\lambda \varphi_\lambda$$

Here D_{tot} represents the optical density at 2800 Å. (the wave length at which the photochemical reaction was followed), A is the reaction rate constant, $C[O]$ is the molar concentration of orotic acid at the start of irradiation, ϵ_0' and ϵ_λ are the molar extinction coefficients of orotic acid at 2800 Å. and at the wave length of irradiation, respectively, φ_λ is the quantum yield at wave length λ, L represents the total average incident radiation flux.

Results

Spectra of an Equimolar Solution of Orotic Acid and Nickel Ions.—Figure 1 represents the spectra of an equimolar, 2 × 10^{-4} M, solution of orotic acid and nickel ions at different pH values. The reference cell contained water and the corresponding amount of $NiSO_4$. At longer wave lengths, up to 6000 Å., there was no further absorption peak noticeable at the low concentrations tested. At 2940 Å. there exists a sharply defined isosbestic point. The point at 2400 Å. does not seem to be an isosbestic one. In the pH range between 2 and 9 the change in the optical density of free orotic acid at 3140 Å. may be neglected; Fig. 1 lists the spectrum of orotic acid (O) at pH 3.

Equimolar Complex Between Orotic Acid and Nickel Ions.—At constant pH value (without buffer and in 0.02 M Tris buffer) we measured the optical density at 3140 Å. for varying concentrations of Ni^{++}. Figure 2 shows a plot of the optical density at 3140 Å. of a 2.2 × 10^{-4} M solution of orotic acid at pH 7.7 against increasing Ni^{++} concentration. The experimental data clearly indicate a complex of orotic acid and nickel ions in the molar ratio 1:1. Measurements at pH values below 7 did not yield a curve with a sharp corner at this molar ratio. There exists a more or less gradual increase in optical density as the nickel ion concentration is increased, thus indicating a dissociable complex. The attempt to suppress this dissociation by 0.2 M KCl was unsuccessful. Figure 2 shows the plot of an experiment at pH 5.9. Adding nickel formate instead of nickel sulfate gave an identical result.

Equilibrium Constants.—In Fig. 3 are plotted two Benesi–Hildebrand lines with pH as parameter. The stability constants K are 1.5 × 10^4, 7.9 × 10^4, 13 × 10^4, and 35 × 10^4 l./mole for pH values of 7.0, 7.7, 8.0, and 9.0, respectively, the ionic strength kept constant with 0.1 M KCl. The K values are rather

inaccurate because of the small slope of the Benesi–Hildebrand lines. Stability constants measured in a medium not stabilized by KCl agreed with those mentioned above. The free energy of complex formation may be calculated from the equation $\Delta F = RT \ln K$. Taking as an average $K \approx 2 \times 10^5$, the free energy amounts to 7 kcal./mole (22°). The molar extinction coefficient at 3140 Å. is ϵ_{max} 8500 as calculated from the intercept of the ordinate.

Adding one drop of 0.1 M EDTA to 10 ml. of a 10^{-4} M solution of orotic acid–Ni^{++} (1:1) at pH 8 immediately destroys the complex as shown spectrophotometrically.

Because of the asymmetry of the absorption band at 3140 Å. we used for the calculation of the oscillator strength the relation[6]

$$f = 1.62 \times 10^{-8} \epsilon_{max}(\bar{V}_{max} - \bar{V}_1)$$

Here $\bar{V}_{max}$ and $\bar{V}_1$ are the wave numbers at 3140 Å. and at the long wave length side at half-band width of the absorption band, respectively. Taking the absorption band measured at pH 8 we obtain $f = 0.23$. This value indicates a strongly allowed transition, very similar to the original transition.

Structural Analogs of Orotic Acid and Different Metal Ions.—We could not detect any change of the absorption spectrum adding nickel ions to a 10^{-4} M solution of uracil or 1,3-dimethyluracil. Taking the ester butyl orotate no change of the absorption spectrum could be observed upon adding nickel ions.

Changing the metal cation a shift in the absorption peak of the orotic acid complex resulted. The experimental data are listed in Table I; some pertinent data on the metal ions used are listed too.

TABLE I

POSITION OF THE ABSORPTION PEAK OF OROTIC ACID–METAL ION, AND PERTINENT DATA OF THE PROPERTIES OF METAL IONS USED[a]

	Ni^{++}	Co^{++}	Cu^{++}	Zn^{++}	Hg^{++}
λ_{max}, Å.	3140	3130	3120	3060	3050
Ionic radius, Å.	0.69	0.73	0.72	0.74	1.10
Electronegativity	1.8	1.7	1.8	1.5	1.9

[a] J. D'Ans and E. Lax, "Taschenbuch für Chemiker und Physiker," Springer-Verlag, Berlin, 1943, p. 113; F. F. Nord, "Advances in Enzymology," Interscience Publishers, Inc., New York, N. Y., 1959, p. 157.

Irradiation of Orotic Acid with Monochromatic Ultraviolet Light.—Figure 4 represents the quantum yields when irradiating in nitrogen atmosphere a 10^{-4} M solution of orotic acid in phosphate buffer (pH 7) with monochromatic ultraviolet light. Within error of measurements there was no difference in quantum yields when doing the same experiments at pH 2. The quantum yields clearly increased with the concentration of the orotic acid used. Figure 4 shows the plot of the dependence of the quantum yield for a 10^{-3} M solution of orotic acid in phosphate buffer (pH 7) and nitrogen.

We were not able to calculate quantum yields when irradiating orotic acid in 0.2 M HCl and nitrogen atmosphere. In particular, at the start of irradiation there was an "explosive" change of the optical density at 2800 Å., probably because of photochemical chlorination of the heteroaromatic ring. Therefore, further irradiation in 0.2 M HCl was not carried out.
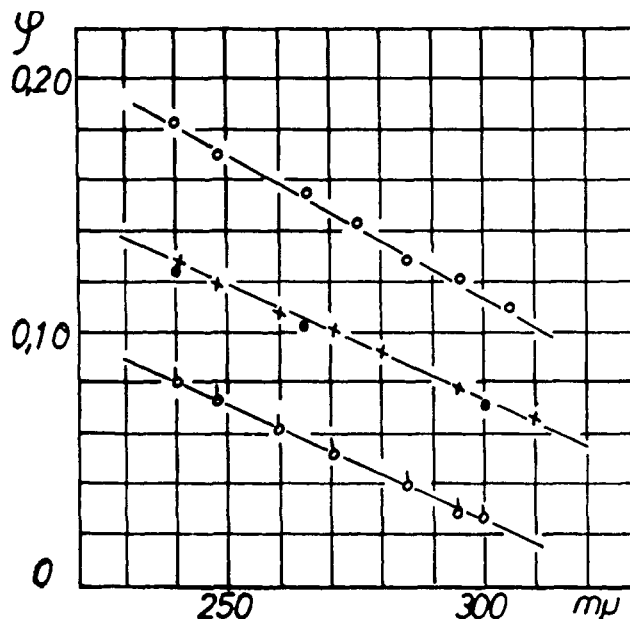

Fig. 4.—Wave length dependence of the quantum yields for dimerization of orotic acid in a 10^{-4} M solution (X—X—X), 10^{-3} M solution (O—O—O), in a solution containing nickel ions at the molar ratio 1:1 at pH 3.5 (●—●—●), and finally a solution containing an excess in nickel ions 1:8 at pH 3.5 (◊—◊—◊), respectively. All measurements were done in a nitrogen atmosphere.

Irradiation of a 10^{-4} M solution of orotic acid in phosphate buffer (pH 7) and oxygen atmosphere gives a dramatic alteration in quantum yield. The quantum yield was found to be $\varphi = 0.0024$ and is independent of the wave length in the range $2400 \leq \lambda < 3100$ Å.

Compared to a 10^{-4} M solution of free orotic acid there seems to be no change in quantum yield irradiating a 10^{-4} M solution of orotic acid–Ni^{++} (1:1) at pH 3.5 (without buffer) and in nitrogen atmosphere. As an example three such measurements are plotted in Fig. 4. Increasing the nickel ion concentration lowers the quantum yield measured under the same experimental conditions. Such an experiment is plotted for the molar ratio 1:8.

Irradiating at 2850 or 3140 Å. a 10^{-4} M solution of orotic acid–Ni^{++} (1:1) at pH 7.7 (no buffer) in nitrogen or oxygen atmosphere no change of the absorption spectrum could be observed, even with large doses of ultraviolet radiation, i.e., $\varphi < 10^{-4}$.

Doing the analogous experiment with orotic acid–Zn^{++} (1:1) at pH 7.7 (no buffer) instead of nickel ions, the quantum yield is determined as φ (3060 Å.) $= 0.003$ in nitrogen; in an oxygen atmosphere the quantum yield is lower by a factor of 30. After maximally bleaching (75% bleach) a 10^{-4} M solution of orotic acid–Zn^{++} at pH 7.7, subsequent irradiation at 2400 Å. causes a partial return (50%) of the original extinction coefficient of the complex.

Irradiation of Orotic Acid Dimer with Monochromatic Ultraviolet Light.—To determine the quantum yields for the photochemical splitting of the cyclobutane ring of orotic acid dimer we maximally irradiated a 2×10^{-4} M solution of orotic acid in phosphate buffer (pH 7) and nitrogen atmosphere at 2900 Å. To the solution thus treated ultraviolet light of different wave lengths was applied. The quantum yields in Fig. 5 were calculated from the increase of the absorp-
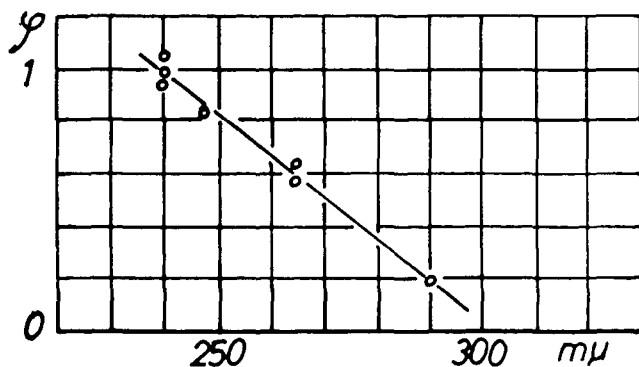
Fig. 5.—Quantum yields for the photochemical reversal of orotic acid dimer.

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 $2 \times 10^{-4} M$ 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
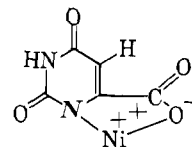
Potentiometric experiments are necessary to decide between these two structures of complex formation.

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.

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

BY MURRAY GOODMAN AND URIEL ARNON[2]

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]

(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).

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

(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.