Studies of the Mechanism of Biotin Catalysis

Michael Caplow

Contribution from the Department of Biochemistry, Yale University, New Haven, Connecticut.[1] *Received June 28, 1965*

2-Imidazolidone has been used as an analog of the coenzyme biotin in an attempt to elucidate the enzymatic mechanism by which this cofactor is carboxylated and subsequently transfers a carboxyl group to a nucleophilic agent. Studies of the carboxylation reaction were carried out by studying the reaction of 2-imidazolidone with activated acyl compounds which serve as models of ATP-activated bicarbonate. No reaction was detected with p-nitrophenyl acetate, acetylimidazole, or acetyl-3-methylimidazolium chloride, indicating that the ureido nitrogen atom of biotin is not highly nucleophilic. Model studies of the transcarboxylation from an N-carboxybiotin intermediate have been carried out with N-carboxy-2-imidazolidone and several of its derivatives. An infrared assay has been developed for the study of the rate of decarboxylation of N-carboxy-2-imidazolidone, and evidence has been adduced indicating that this reaction is unimolecular. The kinetics of the reaction of nucleophiles with esters of N-carboxy-2-imidazolidone have been investigated. The rates of reaction of anionic nucleophiles are increased up to 40-fold by 0.8 M calcium chloride; calcium catalysis is not observed for the reaction of neutral amines. The results are interpreted as involving coordination of the metal with the tetrahedral intermediate and transition state leading to this intermediate, which results in a lowering of the energy barrier to reaction. The metal-ion catalysis has been discussed with reference to the recent findings with pyruvate carboxylase, which indicate that a metal ion is involved in the transcarboxylation reaction from an N-carboxybiotin intermediate.

Enzymatic carboxylation reactions involving biotin have been demonstrated to proceed in two steps as outlined in eq. 1 and 2.[2] In the initial step in this

$$\text{ATP} + \text{HCO}_3 + \text{biotin–enzyme} \overset{\text{Mg}}{\rightleftharpoons} \text{CO}_2\text{–biotin–enzyme} + \text{ADP} + \text{P}_i \quad (1)$$

$$\text{CO}_2\text{–biotin–enzyme} + \text{acyl coenzyme A} \rightleftharpoons \text{CO}_2\text{–acyl coenzyme A} + \text{biotin–enzyme} \quad (2)$$

sequence, biotin reacts with ATP-activated bicarbonate to form 1′-N-carboxybiotin. This intermediate subsequently acts as a carboxylating agent for an acyl coenzyme A. Biotin appears to function as a nucleophilic catalyst in these reactions, and the utilization of this cofactor may provide the enzyme with certain catalytic advantages as compared with a path involving the direct reaction of an acyl coenzyme A with ATP-activated bicarbonate. An attempt has been made to determine the special properties of biotin which might make it effective as a nucleophilic catalyst in these reactions by studying the biotin analog, 2-imidazolidone.

The results obtained in a study of metal ion catalysis of the reaction of nucleophiles with esters of N-carboxy-2-imidazolidone suggest that biotin may provide a coordination site necessary for effective utilization of a metal ion for transcarboxylation from an N-carboxybiotin intermediate. These studies appear to be especially relevant in the light of recent studies with pyruvate carboxylase, which indicate that this enzyme is a metalloenzyme in which the metal is involved in the transcarboxylation step from N-carboxybiotin.[3]

Experimental Section

Materials. N-Methoxycarbonyl-2-imidazolidone (m.p. 178–179°; lit.[4] m.p. 180°) was prepared by refluxing 6.88 g. of 2-imidazolidone in 80 ml. of chloroform with 12.4 ml. of freshly distilled methyl chloroformate for approximately 40 hr. The material was evaporated to dryness under vacuum and recrystallized twice from water. N-Phenoxycarbonyl-2-imidazolidone (m.p. 190–193°; lit.[4] m.p. 189°) was synthesized by the method of Schaeffer and Bhargava.[4] *Anal.*[5] Calcd: C, 58.24; H, 4.89; N, 13.59. Found: C, 57.93; H, 4.88; N, 13.66. N-*p*-Nitrophenoxycarbonyl-2-imidazolidone was synthesized by refluxing 4.23 g. of *p*-nitrophenyl chloroformate with 1.72 g. of 2-imidazolidone in 24 ml. of chloroform for 8 hr. The reaction mixture was evaporated to dryness under vacuum and recrystallized from ethanol–water, approximately 8:1, yielding a product with m.p. 228–230°. *Anal.*[5] Calcd.: C, 47.81; H, 3.61; N, 16.73. Found: C, 47.91; H, 3.69; N, 16.48. N-Carboxy-2-imidazolidone containing deuterium in the 3-position was synthesized immediately before use by mixing 377 mg. (2.60 mmoles) of N-methoxycarbonyl-2-imidazolidone, which had been equilibrated with a 15-fold molar excess of deuterium oxide, with 2.60 mmoles of potassium deuteroxide in a final volume of 2.6 ml. of deuterium oxide. Saponification was allowed to proceed for at least 10 min. at room temperature before the material was used. Acetylimidazole was synthesized by the method of Boyer[6] and acetyl-3-methylimidazolium chloride, m.p. 119–121°, by the method of Wolfenden and Jencks.[7] *p*-Nitrophenyl acetate was a gift from Dr. G. L. Schmir. Deuterium chloride was synthesized by the dropwise addition of phosphorus oxychloride to deuterium oxide, followed by distillation. Potassium deuteroxide was synthesized by evaporating to dryness a solution containing potassium hydroxide and a large molar excess of deuterium oxide. Deuterated monobasic potassium phosphate was prepared in a similar manner. All aqueous solutions were made up in boiled water within

(1) Supported by grants from the National Institutes of Health (GM 11820-01 and 02, and PHS 1-SO1-FR-05058-01).

(2) (a) F. Lynen, J. Knappe, and E. Lorch, *Proc. Intern. Congr. Biochem. 5th, Moscow*, 1961, **4**, 225 (1963); (b) Y. Kaziro and S. Ochoa, *J. Biol. Chem.*, **236**, 3131 (1961).

(3) M. C. Scrutton, M. F. Utter, and A. S. Mildvan, *Federation Proc.*, **24**, 285 (1965).

(4) H. J. Schaeffer and P. S. Bhargava, *J. Pharm. Sci.*, **53**, 137 (1964).

(5) Analysis by the Scandinavian Microanalytical Laboratory, Herlev, Denmark.

(6) J. H. Boyer, *J. Am. Chem. Soc.*, **74**, 6274 (1952).

(7) R. Wolfenden and W. P. Jencks, *ibid.*, **83**, 4390 (1961).

48 hr. of use and were protected from carbon dioxide contamination by tight stoppering or by using a drying tube containing Indicarb (Fisher Scientific Co.) as a carbon dioxide absorbent. Sodium hydroxide solutions were made up by dilution of a saturated solution. All other materials were reagent grade and were recrystallized or distilled before use. Water and deuterium oxide were glass distilled.

Rate Measurements. Visible and ultraviolet rate measurements were carried out in Teflon-stoppered quartz cuvettes in a Zeiss PMQII spectrophotometer equipped with a thermostated brass block maintained at 25°. Infrared spectral measurements were done with a Beckman I.R. 5 spectrophotometer, kindly provided by Professor J. S. Fruton. Calcium fluoride infrared cells of calibrated path length were obtained from the Perkin-Elmer Co. pH measurements were made with a Radiometer TTT 1 with a PHA 630 scale expander using a Radiometer G202B or G2222B electrode, except in the study of the temperature dependence of the rate of decarboxylation of N-carboxy-2-imidazolidone, where a G202C electrode was used. Calibration of the meter was performed with standard buffers obtained from the Beckman Instrument Co. and was carried out with the buffers maintained at the same temperature as the reaction mixture to be examined. Values of pD in 99% deuterium oxide were obtained from the relationship pD = pH + 0.40, which was demonstrated to hold within less than 0.02 unit with a Radiometer G202C electrode at 10 and 35°. This relationship has been found to hold at 25° by other workers.[8,9] Fife and Bruice[9] have reported that the correction factor for converting apparent pH to pD varies with temperature (from 0.35 at 10° to 0.47 at 35°). We are unable to account for this discrepancy.

The decarboxylation of N-carboxy-2-imidazolidone was carried out by mixing 2.5 ml. of a solution of saponified N-methoxycarbonyl-2-imidazolidone (*vide supra*) with 0.5 ml. of deuterium oxide and 0.05 ml. of a 1 *M* solution of deuterated monobasic potassium phosphate, in a water-jacketed glass vessel equipped with a magnetic stirrer. The pH of the reaction mixture was rapidly brought to the desired level by the addition of 5.8 *M* DCl (approximately 0.13 ml. is required for the reaction at pH 7.0), and the zero time for the reaction was taken at this point. The pH of the reaction mixture was kept from deviating from the initial pH by more than 0.02 unit during the first 2 half-lives of the reaction by the addition of 5.8 *M* DCl by a Radiometer SBR2/SBU1/TTA31 titration assembly. The rate of reaction was followed by making spectrophotometric measurements on aliquots of the reaction mixture which were quenched by dilution with 20 μl. of 1.8 *M* KOD to a final volume of 0.20 ml. in a calibrated test tube made from 4-mm. i.d. glass tubing. Six points were taken during the first 2 half-lives of the reaction, and the total volume of DCl added during this period was less than 0.16 ml. No correction was made for this dilution of the reaction mixture. Removal of aliquots after the first 2 half-lives frequently resulted in a negative pH excursion of approximately 0.2 unit. Evidently, removal of aliquots causes a slight inflow of DCl and the reduced reaction volume and substrate buffering allow for a significant pH change. The absorbance of the quenched reaction mixtures was measured at the wave length of maximal absorbance in the 5.85–5.90-μ region in a 0.025-mm. path length calcium fluoride cell using an identical cell containing pure deuterium oxide as a blank. The absorbance of the deuterium oxide at this wave length was approximately 0.15 when measured against air. Care was taken to prevent moving the wave length setting of the spectrophotometer during the spectral measurements since the absorbance is critically dependent upon the wave length in the 5.90-μ region. The total absorbance change in the course of these reactions is approximately 0.6, and the reactions followed first-order kinetics for at least 2 half-lives. A check was made on the calibration of the pH meter at the end of most kinetic runs, and the largest deviation found was 0.04 unit, except for one reaction at 10° where the meter had drifted 0.10 unit during the reaction.

Studies of the rate of saponification of ethyl acetate, N-methoxycarbonyl-2-imidazolidone, and N-phenoxycarbonyl-2-imidazolidone and some studies with tolyl acetate were done with the Hestrin alkaline hydroxylamine assay.[10a] Aliquots (1 ml.) of the reaction mixture were mixed with an equal volume of a freshly prepared solution containing two parts of 3.5 *M* NaOH and one part of 4.0 *M* hydroxylammonium chloride. Reaction was allowed to proceed for 1.0 min. with ethyl acetate and tolyl acetate and for 0.5 min. with N-methoxycarbonyl- and N-phenoxycarbonyl-2-imidazolidone. Ferric chloride solution (2 ml.), 20% in 1.4 *M* HCl, was added and the optical density at 540 mμ was measured in 5.0-cm. cells after 10 min. with the acetate esters and after 1.0 or 5.0 min. with the imidazolidone compounds.

Spectrophotometric measurements of the rate of reaction of nucleophiles, at pH values above neutrality, with *p*-nitrophenyl acetate and *p*-nitrophenoxycarbonyl-2-imidazolidone were measured at 400 mμ and reactions of the latter compound in more acidic solutions were measured at 330 mμ. *p*-Tolyl acetate disappearance was followed at 295 mμ in alkaline solutions and at 280 mμ at pH values below 10. Reactions of acetylimidazole and 1-acetyl-3-methylimidazolium chloride were followed at 245 mμ. The reactions of the latter compound were studied by the method described by Wolfenden and Jencks.[7] Reactions were initiated by the addition of a small amount of substrate dissolved in purified acetonitrile to the temperature-equilibrated reaction mixtures; mixing was accomplished by inversion. In the reactions of hydroxide ion, piperidine, and triethylamine in the presence of calcium chloride, a very slight turbidity was observed prior to the addition of substrate. The optical density produced by this turbidity was 5–20% of the total optical density change observed in the reaction. However, the optical density remained constant prior to the addition of substrate, and stable end points for the

(8) P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960).
(9) T. H. Fife and T. C. Bruice, *ibid.*, **65**, 1079 (1961).

(10) (a) S. Hestrin, *J. Biol. Chem.*, **180**, 249 (1949). (b) Preliminary experiments with 1′-N-*p*-nitrophenoxycarbonylbiotin indicate that the rate constant for saponification of this compound is identical with that of N-*p*-nitrophenoxycarbonyl-2-imidazolidone. (c) Calculated from the second-order rate constant in the rate expression $v = k(RCOO^-)(H_3O^+$ or $D_3O^+)$. The acid concentration was determined from the pH or pD and $K_w = 10^{-14}$.

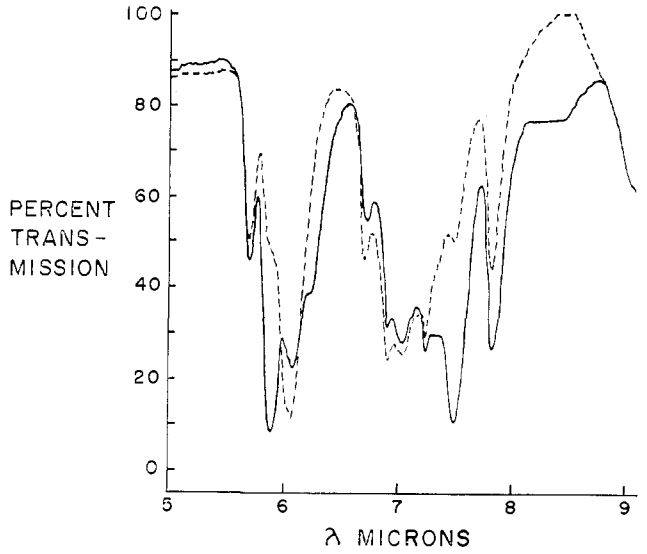


Figure 1. Infrared spectra of N-carboxy-2-imidazolidone before (solid line) and after (dotted line) decarboxylation at pD 7.4. The preparation of the sample is described in the Experimental Section.

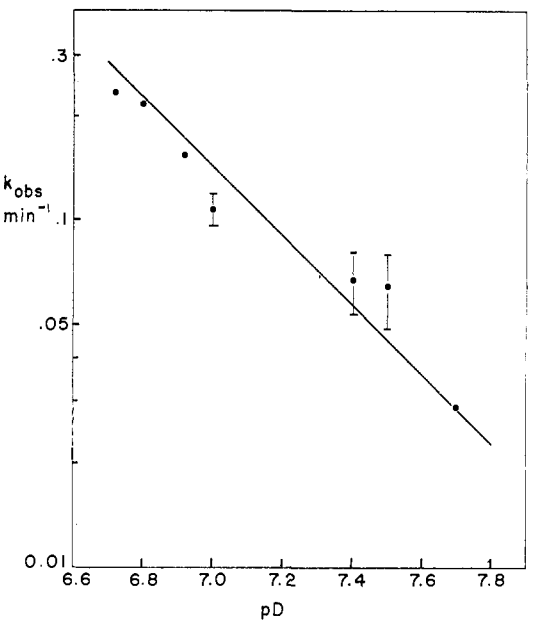


Figure 2. Rate of decarboxylation of N-carboxy-2-imidazolidone as a function of pD at 25°. Vertical bars represent the average deviation in cases where more than one determination was made. The slope of the line is 1.0.

reactions were observed indicating that the turbidity has no effect on the optical measurements used to record rates.

Results

Studies of the Nucleophilicity of 2-Imidazolidone. Attempts to observe a reaction between 2-imidazolidone and *p*-nitrophenyl acetate, acetylimidazole, or acetyl-3-methylimidazolium chloride were not successful (Table I). Although imidazole catalysis of the hydrolysis of acetylimidazole was observed, no catalysis of the reaction of imidazolidone was detected. No reaction could be observed between acetamide and acetylimidazole; addition of 1.0 *M* acetamide resulted in a 16% decrease in the rate of disappearance of acetylimidazole in a reaction mixture containing 2.5×10^{-4} *M* acetylimidazole and 0.4 *M* imidazole at pH 7.68.

Table I. Rates of Reaction of Imidazolidone with Activated Acyl Compounds at 25°

Substrate	pH	Imidazolidone, *M*	Imidazole, *M*	k_{obsd}, min.$^{-1}$
p-Nitrophenyl acetate[a]	7.73 ± 0.05	...	...	2.8×10^{-4}
	7.68 ± 0.01	0.98	...	2.6×01^{-4}
Acetylimidazole[b]	7.56	...	0.033	1.7×10^{-2}
	7.58	1.0	0.033	1.0×10^{-2}
	7.69	...	0.40	4.4×10^{-2}
	7.63	1.9	0.40	3.5×10^{-2}
1-Acetyl-3-methylimidazolium chloride[c]	4.53	...	...	3.25
	4.67	1.33	...	3.27
	4.60	3.27	...	3.26

[a] Substrate 5×10^{-5} *M*, 14% acetonitrile, 0.027 *M* γ-collidine buffer. [b] Substrate 2.5×10^{-4} *M*, 3% acetonitrile. [c] Approximately 0.03 mg. of substrate/ml. of reaction mixture, 0.03 *M* acetate buffer.

Decarboxylation of N-Carboxy-2-imidazolidone. A serious limitation to the use of ordinary manometric procedures for the study of rates of decarboxylation is the slow rate of evolution of dissolved gas. The rate of evolution of carbon dioxide from acidified sodium carbonate at 0.5° was found to be first order for at least 2 half-lives, with a half-life of approximately 60 sec., using a Warburg manometer shaken at 228 oscillations/min. A spectral assay was therefore developed for the study of the decarboxylation of N-carboxy-2-imidazolidone. The infrared spectra of N-carboxy-2-imidazolidone and the product of decarboxylation are given in Figure 1. A significant change in absorbance at 5.9 μ, which is related to the carbonyl stretching frequency of the starting material, is observed and the decrease in absorbance at this wave length was used to follow the rate of reaction. Absorbance at this wave length was found to follow Beer's law with concentrations of N-carboxy-2-imidazolidone to 0.8 *M*, and the molar extinction coefficient at this wave length is 1.7 for a 0.025-mm. light path. Absorbance by potassium carbonate, which is one of the ultimate products of the decarboxylation, is not observed at 5.9 μ. A study of the stoichiometry of the reaction was carried out, and the decarboxylation of 2.0 mmoles of N-carboxy-2-imidazolidone at pD 7.4 in deuterium oxide resulted in the uptake of 1.31 mmoles of deuterons after the reaction had proceeded for approximately 5 half-times. The amount of acid consumed for maintaining constant pH does not accurately reflect the stoichiometry of the reaction since it does not account for carbon dioxide that has been hydrated. It is therefore necessary to titrate the bicarbonate, and it was found that 0.34 mmole of acid was required to adjust the pD to 2.6. The total uptake of deuterons is therefore 1.65 mmoles, which is in reasonable accord with the predicted uptake of 1 mole of acid per mole of N-carboxy-2-imidazolidone undergoing decarboxylation. The decarboxylation of N-carboxy-2-imidazolidone is acid catalyzed, and the log of the rate is approximately linear with pD in the pD range 7.7–6.8 (Figure 2). The most rapid rate

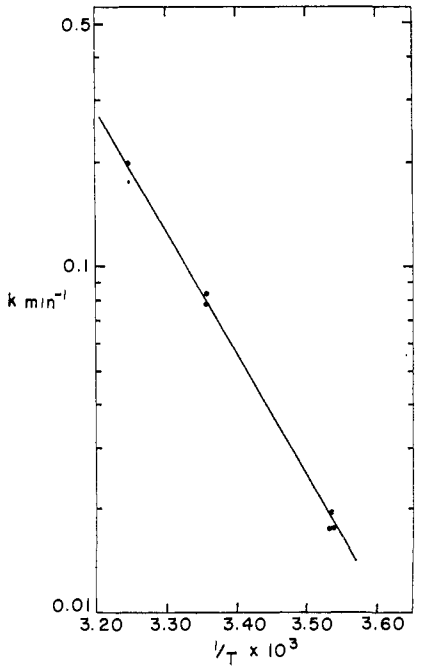


Figure 3. Arrhenius plot of the rate of decarboxylation of N-carboxy-2-imidazolidone at pD 7.4.

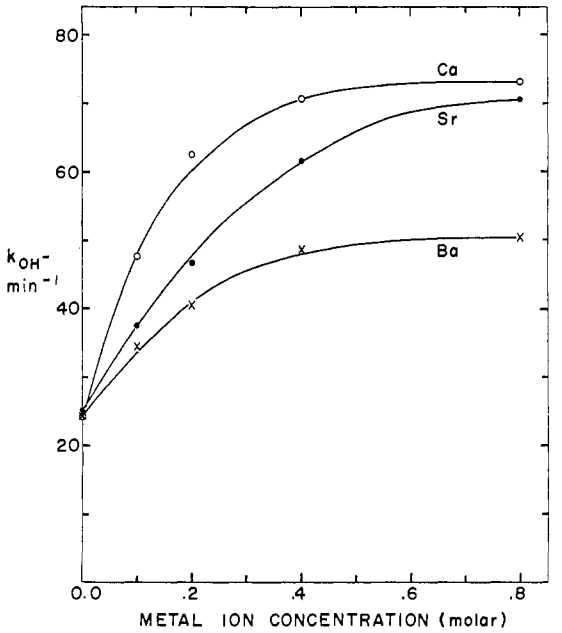


Figure 4. Effect of metal ion concentration on the rate of saponification of N-*p*-nitrophenoxycarbonyl-2-imidazolidone at 25°. Rates expressed as second-order rate constants obtained by dividing the observed pseudo-first-order rate constant by the added hydroxide ion concentration. Hydroxide ion concentrations employed were 0.0106, 0.0109, and 0.00778 *M* in the strontium, barium, and calcium reactions. Each point is the average of two rate measurements which deviated by less than 10%.

that was measured was at pD 5.31 ± 0.06 where the rate constant, obtained in single measurement, is 3.6 × 10^{-1} min.$^{-1}$ at 2°. The rate of decarboxylation at pD 7.4 was studied as a function of temperature in the range 10–35° and the results obtained are given in Figure 3. Values of ΔH^* and ΔS^* are 15.4 kcal./mole and +13.9 e.u., respectively.[10c] The results obtained in a single experiment indicate that the rate is not affected when the 0.016 *M* phosphate buffer present in the reaction mixtures is omitted.

Nucleophilic Reactions of N-Methoxycarbonyl-, N-Phenoxycarbonyl-, and N-p-Nitrophenoxycarbonyl-2-imidazolidone. Second-order rate constants for the saponification of N-methoxycarbonyl-, N-phenoxycarbonyl-, and N-*p*-nitrophenoxycarbonyl-2-imidazolidone along with the rate constants for the corresponding acetate esters are given in Table II.[10b] The rates

Table II. Rates of Saponification of N-Methoxycarbonyl-, N-Phenoxycarbonyl-, and N-*p*-Nitrophenoxycarbonyl-2-imidazolidone and the Corresponding Acetate Esters at 25°

Compound	k, M^{-1} min.$^{-1}$
N-Methoxycarbonyl-2-imidazolidone	0.83[a]
Methyl acetate	10.7[b]
N-Phenoxycarbonyl-2-imidazolidone	3.0[c]
Phenyl acetate	82[b]
N-*p*-Nitrophenoxycarbonyl-2-imidazolidone	27[d]
p-Nitrophenyl acetate	570[e]

[a] Ionic strength maintained at 0.34 with KCl, 2.0% methanol, hydroxide concentration 0.043–0.34 *M*, substrate 6.6 × 10^{-3} *M*. [b] From A. Skrabal and A. M. Hugetz, *Monatsh.*, **47**, 2 (1926). [c] Ionic strength maintained at 0.2 with NaCl, 2.0% acetonitrile, hydroxide concentration 0.017–0.034 *M*, substrate 1 × 10^{-3} *M*. [d] Ionic strength 1.2 with NaCl, 0.7% acetonitrile, hydroxide concentration 0.013–0.026 *M*, substrate 6.8 × 10^{-5} *M*. [e] J. F. Kirsch and W. P. Jencks, *J. Am. Chem. Soc.*, **86**, 837 (1964).

for the imidazolidone substrates are 13 to 25 times as slow as those of the corresponding acetate compounds. The fact that the product of the reaction does not give a positive hydroxamic acid color when treated with alkaline hydroxylamine proves that the product no longer contains an ester function. Reaction of hydroxide ion with N-methoxycarbonyl-2-imidazolidone therefore occurs with the side chain, rather than the ureido carbonyl group. Furthermore, the product of saponification decarboxylates readily in acid (*vide supra*) and does not have the infrared spectral properties of sodium methyl carbonate, indicating that C–N bond cleavage does not occur. Ninhydrin assay[11] of the product of saponification indicates that less than 1% of the product is ethylenediamine, which would be expected to be formed in the course of the ninhydrin assay if the reaction of hydroxide occurs with both carbonyl groups. It is interesting to note that Schaeffer and Bhargava[4] have found that nucleophiles react with esters and anilides of N-carboxy-2-imidazolidone at the ureido carbonyl group. These studies were, however, carried out in nonaqueous solutions at elevated temperatures.

Studies of the effect of barium, calcium, and strontium ions on the rate of saponification of N-*p*-nitrophenoxycarbonyl-2-imidazolidone are graphically illustrated in Figure 4. Pseudo-first-order rates were observed for all of these reactions, and the rate is dependent upon the metal ion concentration at low concentrations and is invariant at a high metal ion concentration. The maximum rate enhancement is 2.1-fold for barium chloride, 2.8-fold for strontium chloride, and 3.3-fold for calcium chloride, while the con-

(11) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961, p. 1309.

Table III. Rates of Reaction of Amines with N-*p*-Nitrophenoxycarbonyl-2-imidazolidone at 25°[a]

Nucleophile	pK_a[b]	pH	Concn., M[c]	$CaCl_2$, M	k_2, M^{-1} min.$^{-1}$[d]	k_{CaCl_2}/k_{KCl}
Piperidine	11.22[e]	11.66 ± 0.03	0.03–0.08	0.0	71	0.8
		11.66 ± 0.03	0.03–0.08	0.8	57	
Methoxyamine	4.60[f]	5.29	1.0	0	0.015	
		5.13 ± 0.02	0.5–1.0	0.8	0.015	1.0
Morpholine	9.18[g]	8.60–9.95	0.667	0	4.08	
		8.31–9.60	0.667	0.8	3.35	0.8
		10.36 ± 0.02	0.333–0.667	0	4.17	
		10.03 ± 0.01	0.333–0.667	0.8	2.57	0.6
Imidazole	6.95[h]	7.96 ± 0.01	0.5–1.0	0	0.047	
		7.72 ± 0.02	0.5–1.0	0.8	0.061	1.3
Triethylamine	10.65[e]	11.47 ± 0.05	0.067–0.167	0	0.12	
		11.24 ± 0.04	0.067–0.40	0.8	*i*	...
Hydroxylamine	5.97[j]	6.89–7.42	0.079–0.39	0	1.5	
		6.62–7.10	0.079–0.39	0.8	2.1	1.4

[a] Ionic strength maintained at 2.4 with KCl or $CaCl_2$, 0.7% acetonitrile, substrate 3.2–5.6 × 10^{-5} M. [b] Acid dissociation constants are assumed to be identical at constant ionic strength with potassium or calcium chloride. [c] Sum of the concentration of all species. No correction has been made for changes in concentration as a result of complex formation with calcium ions. [d] For the reaction of the free base. [e] H. K. Hall, *J. Am. Chem. Soc.*, **79**, 5441 (1957). [f] T. C. Bissot, R. W. Parry, and D. H. Campbell, *ibid.*, **79**, 796 (1957). [g] Determined by partial neutralization in the presence of 2.4 M KCl. [h] T. C. Bruice and G. L. Schmir, *J. Am. Chem. Soc.*, **80**, 148 (1958). [i] Not detectable. [j] Dissociation constant from W. P. Jencks, M. Caplow, M. Gilchrist, and R. G. Kallen, *Biochemistry*, **2**, 1313 (1963). Addition of 0.035 M triethylamine had no effect on the rates.

centrations at which half-maximal rate enhancement is observed are approximately 0.10, 0.15, and 0.20 M for calcium, barium, and strontium chloride. A similar calcium ion dependence of the rate of saponification was observed with N-phenoxycarbonyl-2-imidazolidone. No attempt was made in these studies to maintain the hydroxide ion concentration constant with changes in metal ion concentration, and obviously significant variations occur as a result of incomplete dissociation of a metal–hydroxide complex. Equilibrium constants for this reaction are not known for the conditions used in these studies, but use of the equilibrium constant for $CaOH^+$ formation obtained by Bates and co-workers[12] indicates that the hydroxide ion concentration falls approximately 90% by the addition of 0.8 M calcium ions. The effect of calcium ions on the rate of saponification of *p*-nitrophenoxycarbonyl-2-imidazolidone at constant pH is given in Figure 5, which shows that the rate is dependent upon the calcium ion concentration to 0.8 M; the rate is increased 20-fold by 0.8 M calcium chloride under these conditions. In studies of the saponification of N-*p*-nitrophenoxycarbonyl-2-imidazolidone where the hydroxide ion activity was not kept constant with changes in the calcium concentration and the hydroxide ion concentration was varied from 0.001 to 0.01 M, the rate was found to be proportional to the first power of the total hydroxide ion concentration.

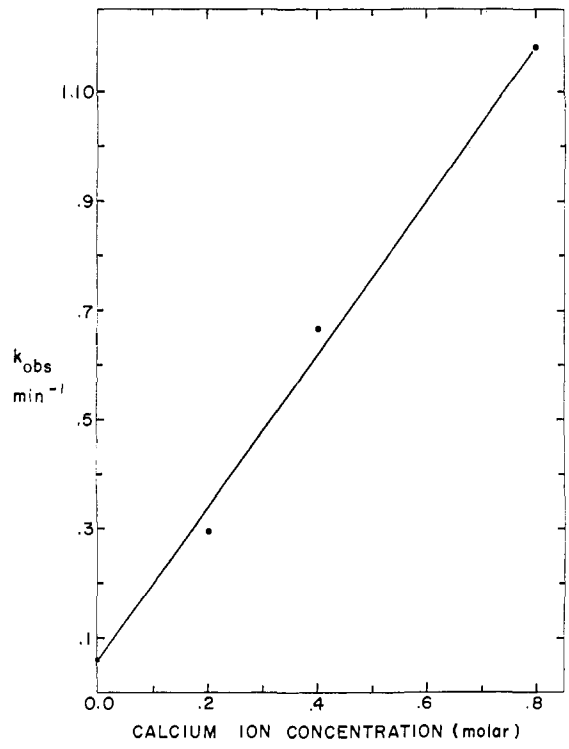


Figure 5. Dependence of the rate of saponification of N-*p*-nitrophenoxycarbonyl-2-imidazolidone on the calcium ion concentration at constant pH. Reactions at 25°, pH 11.24 ± 0.03, except for one reaction in 0.8 M calcium chloride in which the pH was 11.35. Ionic strength maintained at 2.4 with KCl, 0.7% acetonitrile, 0.1 M triethylamine buffer. Each point is the average of two rate measurements in which the deviation was less than 10%. The reaction of triethylamine is negligible under these conditions (Table III).

Attempts to demonstrate calcium catalysis of the reaction of several amine nucleophiles with *p*-nitrophenoxycarbonyl-2-imidazolidone were not successful, and the results obtained with triethylamine, piperidine, morpholine, methoxyamine, hydroxylamine, and imidazole are given in Table III. Catalysis of the reaction of the peroxide anion, the phenoxide ion, and the mercaptoacetate anion was observed, and the results obtained in these studies are given in Table IV which shows that the rates of reaction of these anions are increased from 4- to 22.8-fold in 0.8 M calcium chloride. As seen in Table IV, the rate of reaction of the peroxide anion is approximately proportional to the calcium concentration to 0.8 M.

The reaction of hydroxylamine with *p*-nitrophenoxycarbonyl-2-imidazolidone (Table III) is virtually unchanged by the addition of 0.8 M calcium chloride at

(12) R. G. Bates, V. E. Bower, R. G. Canham, and J. E. Prue, *Trans. Faraday Soc.*, **55**, 2062 (1959).

Table IV. Effect of Calcium Chloride on the Rates of Reaction of Several Nucleophilic Agents with N-*p*-Nitrophenoxycarbonyl-2-imidazolidone[a]

Nucleophile	pH	pK[b]	Concn.,[c] M	$CaCl_2$, M	k, M^{-1} min.$^{-1}$[d]	k_{CaCl_2}/k_{KCl}
Phenol[e]	10.35–10.36	10.0	0.05–0.10	0.0	1.39	
	10.36–10.40		0.05–0.10	0.8	5.57	4.0
Hydrogen peroxide[f]	8.30–8.36	10.19	0.006–0.018	0.0	172	
	8.33		0.018	0.1	497	2.9
	8.29–8.32		0.006–0.018	0.4	1600	9.3
	8.27–8.36		0.006–0.018	0.8	3910	22.8
Mercaptoacetate[g]	8.20–8.26	10.3	0.027–0.053	0.0	8.2	
	8.30		0.027–0.054	0.8	125.	15.3

[a] Ionic strength maintained at 2.4 by the addition of KCl when necessary, temperature 25°, substrate concentration 3.3×10^{-5} M. [b] See footnote *b* of Table III. [c] See footnote *c* of Table III. [d] For the reaction of the conjugate base. [e] See footnote *g* of Table III. [f] Reactions in 0.067 M Tris buffer, pK from A. J. Everitt and G. J. Minkoff, *Trans. Faraday Soc.*, **49**, 410 (1953) (measured at ionic strength 2.3). [g] Reactions in 0.067 M Tris buffer, pK from J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. I, Academic Press Inc., New York, N. Y., 1958, p. 465.

approximately neutral pH where the reactive species is the neutral hydroxylamine molecule, but increases markedly at alkaline pH where the hydroxylamine anion is probably the reactive species. The pH dependence of the rate of hydroxylaminolysis, which has been corrected for the reaction of neutral hydroxylamine, is given in Figure 6, and the reaction of hydroxylamine with *p*-nitrophenoxycarbonyl-2-imidazolidone follows the rate law

$$v = k_1(S)(NH_2OH) + k_2(S)(NH_2OH)(OH) \quad (3)$$

where $k_2(NH_2OH)(OH) = k_2'(NHOH^-)$. The values for k_1 are 2.1 M^{-1} min.$^{-1}$ and 1.5 M^{-1} min.$^{-1}$ while k_2 is 43×10^4 M^{-2} min.$^{-1}$ and 1.1×10^4 M^{-2} min.$^{-1}$ [13] in the presence and absence of 0.8 M calcium chloride. The rate constant for the specific-base-catalyzed reaction of hydroxylamine is increased 39-fold by 0.8 M calcium chloride. It is not possible to determine k_2' without knowledge of the acid dissociation constant of hydroxylamine since $k_2' = k_2K_w/K_a$, where $K_w = (H)(OH)$ and $K_a = (NHOH^-)(H)/(NH_2OH)$. The product of the hydroxylamine reaction was not determined and may be either the N- or O-acylhydroxylamine compound.[14]

The rate of hydroxylaminolysis of *p*-nitrophenoxycarbonyl-2-imidazolidone is unchanged by the addition of 0.2 M magnesium chloride at pH 7.9 (using 0.056 M hydroxylamine) and is increased 30% by the addition of 0.4 M cupric chloride at pH 6.67 (with 0.23 M hydroxylamine). Lithium chloride (1.3 M) has little effect on the rate of saponification, and the rate of reaction of 0.016 M hydroxide is increased 10% as compared to the rate in potassium chloride.

Calcium chloride has relatively little effect on the rate of reaction of nucleophilic agents with acetate esters (Table V). Unlike the reaction with *p*-nitrophenoxycarbonyl-2-imidazolidone, there is little variation with different nucleophiles in the magnitude of the calcium stimulation which ranges from 0 to 4.7-fold with the acetate compounds (Table V), as compared to from −0.4- to 39-fold stimulation observed in the reaction of the same nucleophiles with the *p*-nitrophenoxycarbonyl-2-imidazolidone. Calcium stimulation of the reaction of piperidine with tolyl acetate is similar to that observed with anionic nucleophiles, which differs from the *p*-nitrophenoxycarbonyl-2-imidazolidone reactions where the effects of calcium on the rate is very different for anionic and neutral nucleophiles. The results obtained with ethyl acetate are similar to those obtained by Bell and Waind,[15]

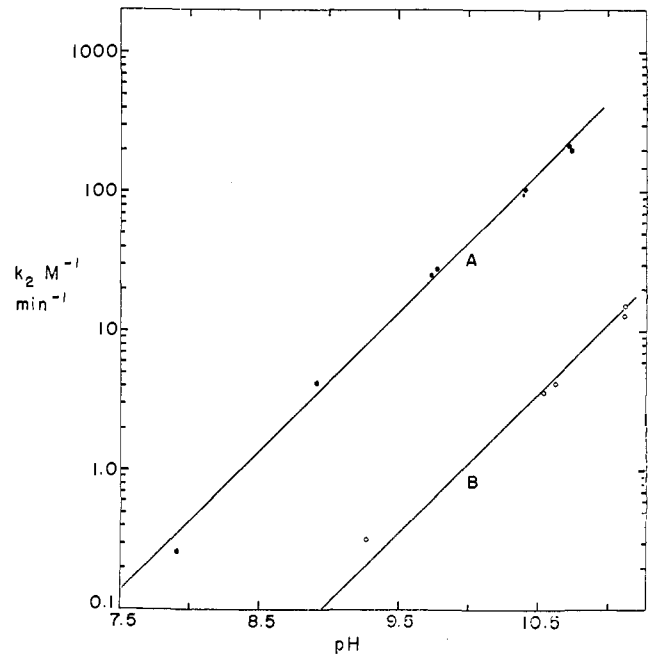


Figure 6. Rate of reaction of hydroxylamine with N-*p*-nitrophenoxycarbonyl-2-imidazolidone as a function of pH. Rates have been corrected for the reaction of neutral hydroxylamine. Reactions at 25°, ionic strength 2.5, 0.035 M triethylamine buffer, hydroxylamine concentration 0.198 M for reactions in KCl and 0.016–0.198 M for reactions in $CaCl_2$. Curves A and B describe the reactions in 0.8 M $CaCl_2$ and KCl, respectively. The correction for the neutral reaction of hydroxylamine was made from experiments summarized in Table III.

who observed no change in the rate of saponification with calcium concentrations to 0.16 M. It is interesting to note that calcium ions slightly decrease the rate of saponification of acetate esters when the hydroxide ion activity is not kept constant, but acts as a weak catalyst when decreases in the hydroxide ion activity are obviated by carrying out the reaction at constant pH.

(13) Hydroxide ion is expressed in terms of activity and was determined from the pH and $K_w = 10^{-14}$.

(14) W. P. Jencks, *J. Am. Chem. Soc.*, 80, 4581, 4585 (1958).

(15) R. P. Bell and G. M. Waind, *J. Chem. Soc.*, 1979 (1950).

Table V. Effect of Calcium Chloride on the Rate of Reaction of Several Nucleophilic Agents with Acetate Esters at 25°

Nucleophile	Substrate	pH	Concn., M[a]	$CaCl_2$, M	k, M^{-1} min.$^{-1}$	k_{CaCl_2}/k_{KCl}
Hydroxide	Ethyl acetate[b]		1.69×10^{-2}	0.0	7.4	
			1.69×10^{-2}	0.2	6.8	0.9
Hydroxide	*p*-Tolyl acetate[c]		6.96×10^{-3}	0	44	
			6.96×10^{-3}	0.4	34	0.8
			6.96×10^{-3}	0.8	32	0.7
Hydroxide	*p*-Nitrophenyl acetate[d]		9.4×10^{-4}	0	350	
			9.4×10^{-4}	0.4	268	0.7
Hydroxide	*p*-Nitrophenyl acetate	11.26 ± 0.04		0	403[e]	
		11.25 ± 0.02		0.8	1690[e]	4.2
Hydroxide	*p*-Tolyl acetate	11.73 ± 0.02		0	79[f]	
		11.76 ± 0.07		0.8	137[f]	1.7
Phenol	*p*-Nitrophenyl acetate	10.37 ± 0.01	0.05–0.10	0	56[g,h]	
		10.37 ± 0.04	0.05–0.10	0.8	56[g,h]	1.0
Mercapto-acetate	*p*-Nitrophenyl acetate	8.28 ± 0.01	0.027–0.053	0	270[g,i]	
		8.27 ± 0.02	0.027–0.053	0.8	642[g,i]	2.4
Hydrogen peroxide	*p*-Nitrophenyl acetate	8.33 ± 0.02	0.006–0.018	0	5010[g,j]	
		8.34 ± 0.05	0.006–0.018	0.8	18,400[g,j]	3.7
Piperidine	*p*-Tolyl acetate	11.73 ± 0.02	0.20–1.2	0	2.3[g,k]	
		11.76 ± 0.07	0.27–0.93	0.8	7.9[g,k]	3.4
Hydroxylamine	*p*-Tolyl acetate	7.35 ± 0.08	0.098–0.49	0	0.34[l]	
		7.07 ± 0.04	0.098–0.49	0.8	0.42[l]	1.2
Hydroxylamine	*p*-Tolyl acetate	7.35 ± 0.08	0.098–0.49	0	2.74[m]	
		7.07 ± 0.04	0.098–0.49	0.8	4.12[m]	1.5
Hydroxylamine	*p*-Tolyl acetate	10.60–11.26	0.04	0	68,000[n]	
		10.18–10.85	0.04	0.8	320,000[n]	4.7

[a] See footnote *c* in Table III. [b] Ionic strength maintained at 0.6 with NaCl or $CaCl_2$, substrate concentration 6.4×10^{-4} M, 1% acetonitrile. [c] Ionic strength maintained at 2.4 with KCl or $CaCl_2$, substrate 5×10^{-4} M, 0.5% acetonitrile. [d] Ionic strength 1.2 with NaCl or $CaCl_2$, substrate concentration 6.7×10^{-5} M, 0.7% acetonitrile. [e] Reaction in 0.3% acetonitrile, 2.9×10^{-5} M substrate, ionic strength 2.4 with KCl or $CaCl_2$, 0.05–0.10 M triethylamine buffer. Hydroxide rate determined by extrapolation to zero buffer concentration and expressed in terms of hydroxide ion activity determined from the pH and $K_w = 10^{-14}$. [f] Reaction in 0.2–1.2 M piperidine buffer, ionic strength maintained at 2.4 with KCl or $CaCl_2$, substrate concentration 5.3×10^{-5} M, 0.3% acetonitrile. Rates obtained by extrapolation to zero buffer concentration and expressed in terms of hydroxide ion activity determined from the pH and $K_w = 10^{-14}$. [g] See footnotes *b* and *d* in Table III. Ionic strength maintained at 2.4 with KCl or $CaCl_2$, substrate concentration $5.3–6.7 \times 10^{-5}$ M, 0.7% acetonitrile. [h] $pK = 10.0$, determined by partial neutralization in 2.4 M KCl. [i] See footnote *g* in Table IV. [j] See footnote *f* in Table IV. [k] Ionic strength 2.4 with KCl or $CaCl_2$, 0.3% acetonitrile, $pK = 11.22$ (see footnote *e* in Table III). [l] Ionic strength maintained at 2.8 with $CaCl_2$ and/or KCl, 1% acetonitrile, 8×10^{-5} M substrate, pK for hydroxylammonium chloride 5.97 (given in footnote *j* in Table III). Rate constant defined in eq. 4 of text as k_1. [m] Same experimental conditions as in footnote 1, rate constant defined in eq. 4 as k_2, units are M^{-2} min.$^{-1}$. [n] Reaction in 2.7×10^{-3} M triethylamine buffer, substrate 2.7×10^{-5}, 0.2% acetonitrile, ionic strength 2.4 with KCl or $CaCl_2$. Reaction rates corrected for the reactions of neutral hydroxylamine (see footnotes *l* and *m*) and hydroxide ion; this correction amounted to less than 6% in all cases. Rate constants are values for k_3 as defined in eq. 4. Hydroxide ion is expressed as activity determined from the pH and $K_w = 10^{-14}$, units for the rate constants are M^{-2} min.$^{-1}$.

The reaction of hydroxylamine with tolyl acetate follows the rate law given in eq. 4; values for the rate

$$v = k_1(S)(NH_2OH) + k_2(S)(NH_2OH)^2 + k_3(S)(NH_2OH)(OH) \quad (4)$$

constants for these reactions in the presence and absence of 0.8 M $CaCl_2$ are given in Table V. The k_2 term in the rate is not observed with *p*-nitrophenoxycarbonyl-2-imidazolidone or with *p*-nitrophenyl acetate, but is observed with phenyl acetate[16a] which resembles tolyl acetate in the respect that both compounds have relatively poor leaving groups. Calcium ions have little effect on the reaction of any of the different hydroxylamine species with tolyl acetate.

Discussion

The function of biotin in enzyme-catalyzed carbon dioxide transfer reactions can be described as involving the ureido nitrogen atom as a nucleophilic catalyst. The sequence of reactions is described in eq. 1 and 2. If biotin is to function as an effective nucleophilic catalyst in these reactions it would be predicted that the coenzyme will have certain special properties which make it first, highly susceptible to carboxylation by ATP-activated bicarbonate and second, highly reactive as a carboxyl-transfer agent once it has been carboxylated. A study was therefore made in which analogs of the reactants in eq. 1 and 2 were used to determine if these predictions are correct.

Model Studies of the Carboxylation of Biotin. A study has been made of the reaction of 2-imidazolidone with activated acyl compounds. 2-Imidazolidone was chosen as a biotin analog since this compound is identical with the portion of the biotin molecule that undergoes carboxylation and it appears to contain all of the components of the coenzyme that might play a significant role in a nonenzymatic study. Experiments utilizing the entire biotin molecule are presently under way to test this hypothesis.[10b] Analogs of ATP-activated bicarbonate were used since the precise structure of the intermediate that reacts with biotin is not known.[16b] It appears reasonable to assume that one of the most prominent features of this intermediate is a

(16) (a) W. P. Jencks and J. Carriuolo, *J. Am. Chem. Soc.*, 82, 675 (1960). (b) The carboxylation of biotin is formulated as a reaction between biotin and ATP-activated bicarbonate rather than as a reaction of ATP-activated biotin and bicarbonate because of the bicarbonate *and* inorganic phosphate requirement for the propionyl carboxylase catalyzed exchange of ADP into ATP.[31b] M. F. Utter and M. C. Scrutton have recently observed a bicarbonate and inorganic phosphate independent exchange of ADP into ATP catalyzed by pyruvate carboxylase (private communication); however, the rate of this reaction is only 0.2–0.4% of the rate of carbon dioxide fixation and a kinetic analysis suggests that the exchange reaction is not on the main kinetic pathway for carbon dioxide fixation.

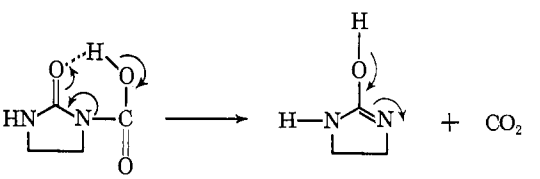
highly reactive carbonyl group, and several compounds which have this property were therefore studied.

No significant reaction of 2-imidazolidone with *p*-nitrophenyl acetate, acetylimidazole, and acetyl-3-methylimidazolium chloride could be observed. Considerations of the dependence of the rate on basicity for the reaction of nucleophiles with *p*-nitrophenyl acetate[17] indicate that no reaction will be observed with a nucleophile with a pK of approximately 1.0 unless the compound is unusually reactive.[18] Although imidazole catalysis of the reaction of water with acetylimidazole has been observed (ref. 19 and Table I), no catalysis of the reaction of imidazolidone or acetamide was detected. Interpretation of these results is limited since the dependence of general base catalysis on the basicity and structure of the nucleophile has not been elucidated fully. Jencks and Carriuolo[19] have obtained evidence for imidazole catalysis of the reaction of ammonia with acetylimidazole, indicating that a nucleophile less acidic than water can be activated by a general base catalyst. The failure to observe a reaction between imidazolidone and acetyl-3-methylimidazolium chloride further illustrates the low nucleophilicity of this compound. In the pH range 1–6 the major path for hydrolysis of acetyl-3-methylimidazolium chloride is by reaction of water[7] (pK = −1.7), and analysis of a Brønsted plot[17] for the reaction of nucleophiles with acetylimidazolium predicts that the specific rate for a compound with a pK of 1.0 should be reasonably similar to that observed with 55 M water. It is, of course, not possible to come to any definite conclusions about these results because of the possible intrusion of nonspecific solvent effects in the highly concentrated solutions used in this study.

If it is assumed that the acylation of 2-imidazolidone is similar to the carboxylation of biotin by an activated form of bicarbonate, the results obtained in these studies suggest two requirements for enzymatic carboxylation. First, the carboxylating agent is probably more activated than any of the substrates examined in this study and second, some form of basic catalysis is probably required for carboxylation of the nitrogen atom.[20] Cordes and Ogata, *et al.*,[21] have recently studied the reaction of urea with acetaldehyde, which is similar to the carboxylation reaction described in eq. 1. It is interesting that the reaction occurs with the neutral amine and the rate, at neutral and acidic pH, is first order with respect to both amine and aldehyde. This contrasts with the dimerization of acetaldehyde where the reaction requires hydroxide ion catalysis for significant reaction rates and the rate-limiting step, at high aldehyde concentrations, is the formation of the carbanion nucleophile by C–H bond cleavage.[22] It appears reasonable to make a comparison of the reactions urea–acetaldehyde, acetaldehyde–acetaldehyde, with the reactions biotin–activated bicarbonate, acyl coenzyme A–activated bicarbonate since the two sets of reactions involve nucleophilic attack on an activated carbonyl group by either a urea nitrogen atom or a saturated carbon atom. The results obtained with acetaldehyde suggest that a major advantage of utilizing biotin as an initial acceptor of activated bicarbonate is that the reaction does not involve the slow cleavage of a C–H bond, which would be the case if an acyl coenzyme A derivative were the acceptor. Hydrolysis of the ATP-activated bicarbonate compound is thereby probably minimized. The formation of N-carboxybiotin from activated bicarbonate may well result in the formation of a less reactive carboxylating agent (*vide infra*); however, it may also result in the formation of a more discriminating carboxylating agent that is stable enough to react with an acyl coenzyme A derivative. There is considerable evidence supporting the hypothesis that less reactive compounds show increased selectivity in their reactions.[23]

Decarboxylation of N-Carboxy-2-imidazolidone. The results obtained in a study of the decarboxylation of N-carboxy-2-imidazolidone are similar to those obtained by Wood, *et al.*,[24] in a study of the rates of decarboxylation of N-carboxybiotin–oxaloacetate transcarboxylase and related compounds. Quantitative comparison with the earlier results is, however, not possible since deuterium oxide rather than water was used in the studies reported here. Although differences in acidity can be corrected for by comparison of pH and pD, values of the pK of the carboxyl group are not known in the two solvents and, since the rate appears to depend upon the extent of protonation of this group, comparison is not possible without this information.

The rate of decarboxylation of N-carboxy-2-imidazolidone in the neutral pH range increases with increasing acidity. These results are consistent with a mechanism in which the reactive species is neutral N-carboxy-2-imidazolidone, or a dipolar ion in which the negative charge is on the carboxyl group and the positive charge is on the ureido carbonyl group or the tertiary nitrogen atom. Since protonation of the carboxyl group would be expected to hinder decarboxylation, reactions involving the neutral carboxylic acid are only observed with highly acidic carboxylic acid or with carboxylic acids containing a basic center near the site of decarboxylation.[25] The proximity of the ureido system in N-carboxy-2-imidazolidone probably accounts for the decarboxylation of the neutral compound. A possible mechanism for the decarboxylation



(5)

(17) W. P. Jencks and J. Carriuolo, *J. Am. Chem. Soc.*, 82, 1778 (1960).

(18) The pK for the conjugate acid of imidazolidone does not appear to have been determined, and the value given here was obtained by assuming that this compound has acidic properties similar to those of N-methylurea, which has a pK estimated at 0.8: N. F. Hall, *ibid.*, 52, 5115 (1930).

(19) W. P. Jencks and J. Carriuolo, *J. Biol. Chem.*, 234, 1280 (1959).

(20) The reaction of biotin with ATP-activated bicarbonate may well occur with the enolic rather than the ketonic form of biotin. Although the enol would not be expected to be present in high concentrations, this species may be the initial product of transcarboxylation and may react prior to, or in concert with, ketonization.

(21) (a) Dr. E. H. Cordes, private communication; (b) Y. Ogata, A. Kawasaki, and N. Okumura, *J. Org. Chem.*, 30, 1636 (1965).

(22) R. P. Bell, *J. Chem. Soc.*, 1637 (1937).

(23) G. S. Hammond, *J. Am. Chem. Soc.*, 77, 334 (1955).

(24) (a) H. G. Wood, H. Lochmüller, C. Riepertinger, and F. Lynen, *Biochem. Z.*, 337, 247 (1963); (b) J. Knappe, Abstracts of the Sixth International Congress of Biochemistry, New York, N. Y., Vol. V, 1964, p. 355.

(25) E. S. Gould, "Mechanism and Structure in Organic Chemistry," Henry Holt and Co., Inc., New York, N. Y., 1959, pp. 348, 349.

is given in eq. 5. The mechanism, as written, is unimolecular, and the following considerations support this scheme.

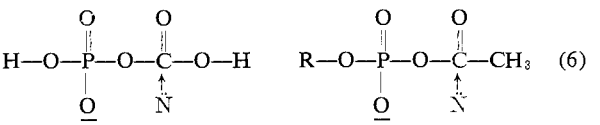
Decarboxylation of N-carboxy-2-imidazolidone by a bimolecular pathway could possible involve nucleophilic attack by either hydroxide ion or water. The observed pH dependence of the decarboxylation rules out a significant contribution to the rate by reaction of hydroxide ion with the neutral or anionic form of N-carboxy-2-imidazolidone. The pH rate data do not, however, provide any information about the possible participation of a water molecule as a nucleophilic agent. It does, however, appear to be possible to rule out this mechanism by considering the reaction of water with N-methoxycarbonyl-2-imidazolidone. The reaction of water with this compound would be expected to be similar to the reaction of water with the neutral carboxy substrate and, since water does not react at a measurable rate with the ester, it seems reasonable to conclude that the same is true of the carboxy compound.

Analysis of the entropy of activation is frequently useful for distinguishing unimolecular and multimolecular reactions since this parameter is generally zero or slightly positive for unimolecular processes. The activation parameters for the decarboxylation of N-carboxy-2-imidazolidone were therefore measured, and values for ΔH^* and ΔS^* are 15.4 kcal./mole and +13.9 e.u., respectively. The corresponding values, calculated from the results reported by Wood and coworkers,[24] for the decarboxylation of an N-carboxybiotin–oxaloacetate transcarboxylase complex are $\Delta H^* =$ 23.2 kcal./mole and $\Delta S^* = +40.3$ e.u.[10c] Since the enthalpy for dissociation of the carboxyl group is generally near 0,[26] it seems reasonable to assume that no correction need be made for variation of the fraction of the substrate present in the acidic form with variations in temperature. The value for ΔS^* for the decarboxylation of N-carboxy-2-imidazolidone is significantly positive and, therefore, suggestive of a unimolecular mechanism. It is not possible to evaluate the significance of the ΔS^* for decarboxylation of the N-carboxybiotin–oxaloacetate transcarboxylase complex since it is not perfectly clear that this parameter is related only to the decarboxylation.

While the above considerations do not conclusively rule out a bimolecular pathway for decarboxylation of N-carboxy-2-imidazolidone, the experimental evidence is most satisfactorily accounted for by assuming a unimolecular process. It is interesting to note that the formation of several carbamic acids has been shown to proceed by reaction of the amine with carbon dioxide rather than carbonic acid.[27] Considerations of microscopic reversibility indicate that the reverse reaction proceeds by a unimolecular pathway in which carbon dioxide rather than carbonic acid is the product.

Reaction of Nucleophiles with N-Methoxycarbonyl-, N-Phenoxycarbonyl-, and N-p-Nitrophenoxycarbonyl-2-imidazolidone. Studies of the rates of saponification of N-methoxycarbonyl-, N-phenoxycarbonyl-, and N-*p*-nitrophenoxycarbonyl-2-imidazolidone indicate that the rates are 13 to 25 times slower than those of the corresponding acetate esters. It is interesting that saponification of the methyl ester proceeds without C–N bond cleavage. This would also be expected to be true with the other compounds, both of which have very much better leaving groups, and has been demonstrated spectrophotometrically with the *p*-nitrophenyl ester. Knappe[24b] has suggested that the catalytic efficiency of biotin is related to the "electron-attracting effect of the ureido-type system (which) causes an increase in the electrophilic character of the carboxy carbon atom facilitating transfer of the carboxyl... group to nucleophilic substrates." The results obtained in these studies are not consistent with this hypothesis. The rate of saponification is a measure of the extent of carbonyl polarity produced by electron-withdrawing groups in the acyl portion of esters.[28] Since the rates for the imidazolidone substrates are slower than those containing the electron-*donating* methyl group, *i.e.*, the acetate esters, electrophilic activation by the mechanism described by Knappe appears unlikely. N-*p*-Nitrophenoxycarbonyl-2-imidazolidone was found to be less reactive than *p*-nitrophenyl acetate with all of the nucleophiles investigated.

Since the structure of ATP-activated bicarbonate is not known, it is not possible to make an accurate quantitative estimate of the relative ability of this compound and N-carboxybiotin to donate a carboxyl group to a nucleophilic agent. The product formed in the activation of bicarbonate by ATP may be carbonyl phosphate, and although this compound has not been demonstrable, it may be possible to make an estimate of the carboxyl-transfer ability of this intermediate by considering the acyl-transfer ability of an acyl phosphate. As seen in eq. 6, these two reactions are quite closely related.



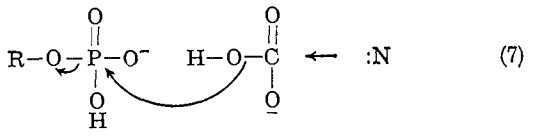
(6)

The successful correlation of the rates of reaction of hydroxide ion and imidazole with acyl compounds[29] suggests that the saponification rate may be useful for estimating the carboxyl-transfer ability of possible forms of ATP-activated bicarbonate. Thus, it may be possible to estimate the reactivity of the carbonyl group of carbonyl phosphate with an acyl coenzyme A derivative from measurements of the saponification rate of an acyl phosphate. This information is readily available for acetyl phenyl phosphate, which is believed to undergo saponification by a bimolecular pathway with a rate constant (corrected to 25°) of 125 M^{-1} min.$^{-1}$.[30] Similar reasoning may be used to provide information about the reactivity of an acyl coenzyme A derivative with a protonated N-carboxybiotin intermediate. While the rate constant for the reaction of hydroxide ion with a protonated N-carboxybiotin intermediate is not available, the rate constant for the reaction of hydroxide with the related compound, N-methoxycarbonyl-2-imidazolidone, is 0.8 M^{-1} min.$^{-1}$. The very much greater reactivity of the acyl phosphate is consistent with the hypothesis that an acyl coenzyme A

(26) J. T. Edsall and J. Wyman, Table IV, footnote *g*, p. 452.
(27) A. Jensen, M. B. Jensen, and C. Faurholt, *Acta Chem. Scand.*, 6, 1073 (1952).
(28) W. P. Jencks and J. Carriuolo, *J. Am. Chem. Soc.*, 83, 1743 (1961).
(29) See Table II, footnote *e*.
(30) G. DiSabato and W. P. Jencks, *J. Am. Chem. Soc.*, 83, 4400 (1961).

compound could be expected to be very much more reactive with a carbonyl phosphate intermediate than with N-carboxybiotin.

Kaziro and Lynen and co-workers[31] have suggested that the activation of bicarbonate by ATP occurs as a concerted process with dehydration of the bicarbonate occurring simultaneously with nucleophilic attack of the biotin nitrogen atom. This is described in eq. 7,



(7)

where R represents the ADP portion of ATP.[32] The carboxylating agent in this case is equivalent to some species intermediate between carbon dioxide and bicarbonate, depending upon the amount of C–O bond cleavage that has taken place when nucleophilic attack occurs. Considering the enormous reactivity of carbon dioxide with nucleophilic agents (the rate constant for reaction with hydroxide ion is 510,000 M^{-1} min.$^{-1}$ [33]), it seems reasonable to assume that, even if little C–O bond cleavage has occurred when nucleophilic attack takes place, the carboxylating species formed in this reaction would be expected to be very highly activated.

While these arguments are seriously limited by the fact that the substrates used for comparison may not be entirely appropriate, it does appear that the formation of N-carboxybiotin from ATP-activated bicarbonate results in the formation of a less reactive carboxylating species. The possible catalytic advantages afforded an enzyme by utilizing this pathway have been discussed above, and the remainder of this work will be concerned with a discussion of experimental data which suggest ways by which an N-carboxybiotin intermediate may be prevented from undergoing unimolecular decomposition, and how it may be converted to an effective carboxylating agent.

Metal Ion Catalysis. Metal ion catalysis of the reaction of nucleophiles with esters of N-carboxy-2-imidazolidone has been observed. The rate increases range from −0.4- to 40-fold, and depend upon the nature of the nucleophile. Calcium, and in one case barium and strontium, were used in these studies since even with the most reactive substrate studied most reactions must be carried out at alkaline pH, where almost all metal ions are made insoluble.

Studies of the effect of calcium ions on the rate of saponification of *p*-nitrophenoxycarbonyl-2-imidazolidone indicate that the rate is initially dependent upon the calcium concentration and levels out at high concentrations (Figure 4). The results are consistent with a mechanism involving the formation of an activated calcium–substrate complex and an inactive calcium–hydroxide complex.[34] The rate law for reaction by this pathway is

$$v = k_1(\mathrm{S})_f(\mathrm{OH})_f + k_2(\mathrm{CaS})(\mathrm{OH})_f \qquad (8)$$

where the subscript f refers to the uncomplexed concentration. The calcium dependence of the rate, where $(\mathrm{Ca}) \gg (\mathrm{S})$, $K_2 = (\mathrm{Ca})(\mathrm{S})_f/(\mathrm{CaS})$, $K_1 = (\mathrm{Ca})(\mathrm{OH})_f/(\mathrm{CaOH})$, and $(\mathrm{OH})_{\mathrm{total}} = (\mathrm{OH})_{\mathrm{free}} + (\mathrm{CaOH})$, is

$$\frac{k_{\mathrm{obsd}}}{(\mathrm{OH})_{\mathrm{total}}} = \frac{k_1K_1K_2}{[(\mathrm{Ca}) + K_1][(\mathrm{Ca}) + K_2]} + \frac{k_2K_1(\mathrm{Ca})}{[(\mathrm{Ca}) + K_1][(\mathrm{Ca}) + K_2]} \qquad (9)$$

When k_2 and k_2/K_2 are greater than k_1, *i.e.*, when calcium catalysis is significant, eq. 9 predicts that the rate is dependent upon the calcium concentration when $(\mathrm{Ca}) < K_1$ and K_2, is independent of the calcium concentration when $(\mathrm{Ca}) > K_1$ or K_2, and decreases with increasing calcium concentration when $(\mathrm{Ca}) > K_1$ and K_2. Consideration of reasonable values for K_2 and measurements of K_1 under somewhat different experiment conditions[12] indicate that the leveling out of the rate is related to the calcium concentration becoming greater than K_1. It is not possible to determine the constant k_2 in eq. 9 without knowledge of K_1 and K_2.

At constant pH the rate expression for saponification is

$$v = k_1'(\mathrm{S})_f + k_2'(\mathrm{CaS}) \qquad (10)$$

where $k_2' = k_2(\mathrm{OH})_f$ and $k_1' = k_1(\mathrm{OH})_f$. The calcium ion dependence of the rate is

$$k_{\mathrm{obsd}} = \frac{k_2'(\mathrm{Ca})}{K_2 + (\mathrm{Ca})} + \frac{k_1'K_2}{K_2 + (\mathrm{Ca})} \qquad (11)$$

Equation 11 predicts that the rate is linear with respect to the calcium concentration when $(\mathrm{Ca}) < K_2$ and either decreases or becomes independent of the metal concentration when $(\mathrm{Ca}) > K_2$. As seen in Figure 5, the rate of saponification at constant pH is linear with respect to the calcium concentration up to 0.8 *M*, indicating that K_2 is greater than 0.8 *M*. The rate of saponification of *p*-nitrophenoxycarbonyl-2-imidazolidone at constant pH is increased 20-fold by 0.8 *M* calcium chloride.

The reactions of anionic nucleophiles with *p*-nitrophenoxycarbonyl-2-imidazolidone are catalyzed by calcium chloride; catalysis is either absent or not significant with neutral amines. This is best illustrated by comparing the effect of calcium on the reaction of hydroxylamine at neutral pH where reaction occurs with the uncharged amine, and at alkaline pH where the nucleophile is anionic. The neutral reaction is unchanged while the rate of the anionic reaction is increased 40-fold by the addition of 0.8 *M* calcium. Attempts to demonstrate a reaction of the morpholine anion (Table III) were not successful.

Calcium has relatively little effect on the rates of reaction of nucleophiles with *p*-tolyl acetate or *p*-nitrophenyl acetate (Table V). These reactions were usually carried out under conditions identical with those used for the study of the imidazolidone compounds and the results indicate that a part of the imidazolidone

(31) (a) Y. Kaziro, L. F. Hass, P. D. Boyer, and S. Ochoa, *J. Biol. Chem.*, 237, 1460 (1962); (b) Y. Kaziro, E. Leone, and S. Ochoa, *Proc. Natl. Acad. Sci. U. S.*, 46, 1319 (1960); (c) F. Lynen, J. Knappe, E. Lorch, G. Jütting, E. Ringelmann, and J. P. Lachance, *Biochem. Z.*, 235, 123 (1961).

(32) An alternate mechanism for carboxylation involving an initial phosphorylation of the ureido carbonyl group could be included in this scheme by assuming that R in eq. 7 represents the enolic form of biotin. Calvin and Pon have suggested a somewhat similar mechanism: *J. Cellular Comp. Physiol.*, 54 (Suppl. 1), 51 (1959).

(33) J. A. Sirs, *Trans. Faraday Soc.*, 54, 201 (1958).

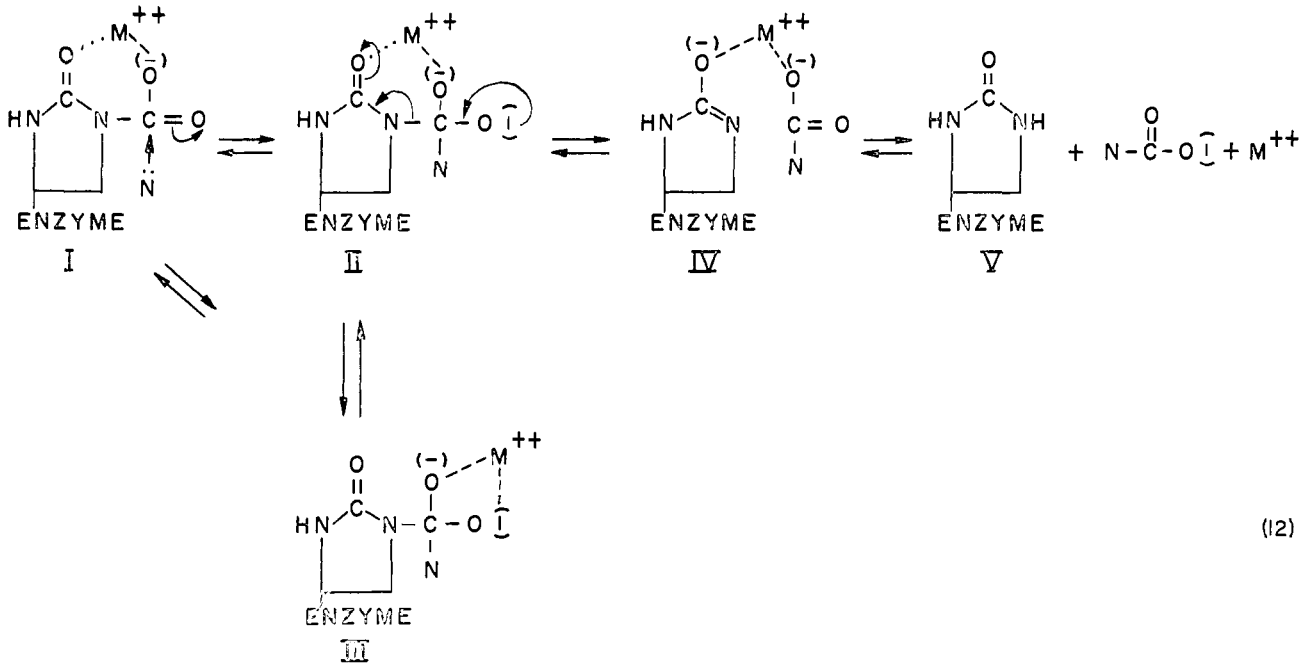
(34) The $CaOH^+$ complex appears to be inactive as a base in the specific-base-catalyzed decomposition of diacetone alcohol: R. P. Bell and J. E. Prue, *J. Chem. Soc.*, 362 (1949).

moiety is required for metal ion catalysis. The small rate effects observed in the presence of calcium may be related to an increase in the activity coefficient of the ester.

Metal ion activation of N-carboxy-2-imidazolidone esters may result from coordination of the metal with the ureido carbonyl group and the anionic oxygen atom of a tetrahedral intermediate formed by nucleophilic attack on the ester bond. The similarity of the structure of the transition state and the tetrahedral intermediate would be expected to result in appreciable coordination of the metal with the activated complex. This coordination would be expected to stabilize the transition state and thereby lower the energy barrier for reaction. Bell and Waind[15] have proposed a similar role for calcium in the saponification of ethyl acetate. The requirement that the nucleophile is anionic for metal ion catalysis may be related to the fact that the tetrahedral intermediate initially formed with anionic nucleophiles is negatively charged, while it has no net charge with neutral nucleophiles. Since calcium chelation is predominantly an electrostatic interaction,[35] it would be expected that coordination with both the tetrahedral intermediate and the transition state leading to the intermediate would be greatest with structures having a negative charge.

Metal ion catalysis may also involve coordination of the metal with the ureido and ester carbonyl groups of the substrate. Coordination would be expected to polarize the ester group and thereby make it more susceptible to nucleophilic attack. The requirement that the nucleophile must be anionic for effective catalysis can be explained by the fact that a calcium–substrate complex is cationic and may be unusually reactive with negatively charged nucleophiles. The rates of reaction of anionic nucleophiles with acetylimidazolium are 20–60 times faster than predicted from studies of the reactions of these nucleophiles with the neutral *p*-nitrophenyl acetate molecule.[36] However, it should be noted that there are relatively few cases in which simple electrostatic interactions play a significant role in catalysis. Furthermore, calcium chelation is predominantly an electrostatic phenomenum and would not be expected to be very significant with the neutral ester.

Scrutton and co-workers[3] have recently found that pyruvate carboxylase is a metalloenzyme containing a manganese ion for each of four enzyme-bound biotin molecules. Evidence has also been obtained that indicates that the metal ion is involved solely in the transcarboxylation step from the N-carboxybiotin intermediate. In the light of this information, it appears reasonable to make a comparison between the role of the metal ion in transacylation of N-carboxyimidazolidone esters and enzymatic transcarboxylation from N-carboxybiotin. A possible mechanism for metal ion catalysis of transcarboxylation is given in eq. 12. The first product



(12)

formed in this sequence is a chelate containing the enzyme-bound metal ion, the ureido carbonyl group, and the side chain carboxyl group (structure I). Stiles[37] has suggested an identical chelate for N-carboxybiotin, but dismissed this suggestion on the grounds that the enzymatic process had not been shown to require a metal ion. The fact that metal ion catalysis of reactions of N-carboxy-2-imidazolidone esters has been accounted for by postulating an intermediate chelate resembling I provides some support to the possible existence of this structure in the enzymatic reaction. This type of chelate is similar to those formed by acetoacetic acid, and its formation would be expected to polarize the carboxyl group and activate it for nucleophilic attack. What is probably equally important is that formation of this chelate would be expected to prevent unimolecular decarboxylation, as described in eq. 5. This is supported by the results obtained in a study of the reaction of nitroparaffins with magnesium methyl carbonate,

(35) R. J. P. Williams, *Enzymes*, **1**, 391 (1959).
(36) J. Gerstein and W. P. Jencks, *J. Am. Chem. Soc.*, **86**, 4655 (1964).
(37) M. Stiles, *Ann. N. Y. Acad. Sci.*, 88, 332 (1960).

where the metal ion appears to increase the rate of reaction and drives the reaction to completion by preventing decarboxylation of the product.[37,38] Metal ions inhibit nonenzymatic decarboxylation of Schiff bases formed between amino acids and pyridoxal.[39] The reaction of a nucleophile with structure I may result in the formation of a carbonate-like chelate (structure III) with the oxygen atoms of a tetrahedral intermediate, and the metal ion could conceivably lower the energy barrier for this reaction by chelation with a transition state resembling the tetrahedral intermediate. Breakdown of a chelated tetrahedral intermediate requires reorganization of the chelate so that the electrons of the oxygen atom are made available to expel the imidazolidone group, and this is shown in eq. 12 in the conversion of structure III to II. Breakdown of the tetrahedral intermediate with C–N bond cleavage is facilitated by the metal ion since the leaving group is an analog of a neutral enol rather than an amine anion. As discussed above, saponification of N-methoxycarbonyl-2-imidazolidone proceeds without C–N bond cleavage, indicating that it is more difficult to displace the amine anion than methoxide ion. Structure III may not be an obligatory intermediate in these reactions, in which case structure II is formed directly from I.

(38) (a) M. Stiles and H. L. Finkbeiner, *J. Am. Chem. Soc.*, **81**, 505 (1959); (b) K. J. Petersen, *Acta Chem. Scand.*, **3**, 676 (1949).
(39) G. D. Kalyankar and E. E. Snell, *Biochemistry*, **1**, 594 (1962).

Acknowledgments. The author is indebted to Mrs. Patricia Messore for expert technical assistance and to Professor W. P. Jencks for a most helpful discussion.

Communications to the Editor

Aromatic Substitution. XXIV.[1] The Alkylation of Toluene and Benzene *via* Diazotization of Amines with Nitrosonium Salts

Sir:

Pearson, *et al.*,[2] recently reported the isopropylation of benzene, toluene, and *p*-xylene *via* diazotization of isopropylamine with butyl nitrite in the presence of equimolar amounts of a carboxylic acid. Using competitive reaction conditions they found $k_{toluene}/k_{benzene} = 0.8$ and $k_{p\text{-}xylene}/k_{benzene} = 0.6$. It was suggested that the unusual reactivity ratios (toluene and *p*-xylene reacting *slower* than benzene) are a consequence of a solvent cage effect (the aromatic hydrocarbons being an essential part of the solvent system). The relative electronic and chemical properties of the aromatic hydrocarbons could not explain the observed data, nor was it possible to suggest that the observed relative rates reflect comparative reactivities of the aromatic substrates in kinetically controlled competitive reactions. Consequently doubt was expressed in the validity of any activity sequences obtained from competition reactions in nonpolar organic solvents.

The above findings represented a serious challenge to all previous investigations dealing with relative reactivities of aromatics in alkylation and related electrophilic aromatic substitution systems based on competition experiments. In view of our previous interest in competitive rate determinations of benzene and alkylbenzenes in Friedel–Crafts type alkylations[3] we felt obliged to extend our investigations to the alkylation of benzene and toluene *via* diazotization of amines.

(1) Part XXIII: G. A. Olah, J. A. Olah, and N. A. Overchuk, *J. Org. Chem.*, **30**, 3373 (1965).
(2) D. E. Pearson, Ch. V. Breder, and J. C. Craig, *J. Am. Chem. Soc.*, **86**, 5054 (1964).
(3) G. A. Olah, S. J. Kuhn, and S. H. Flood, *ibid.*, 84 1688 (1962); G. A. Olah, S. H. Flood, S. J. Kuhn, M. E. Moffatt, and N. A. Overchuk, *ibid.*, **86**, 1046 (1964); G. A. Olah, S. H. Flood, and M. E. Moffatt, *ibid.*, **86**, 1060 (1964).

In order to carry out the alkylation of aromatics *via* diazotization of amines our work was not primarily directed to reproduce the conditions reported by Pearson, *et al.*,[2] where alkylation was only a minor reaction, the major product being propylene and isopropyl acetate (or related esters). We tried to find a more suitable way to carry out alkylations *via* diazotization of amines. We found that when aromatic hydrocarbons were allowed to react with amines and stable nitrosonium salts, like $NO^+BF_4^-$, $NO^+PF_6^-$, $NO^+SbF_6^-$, $NO^+AsF_6^-$, and $NO^+HSO_4^-$, alkylation *via* diazotization takes place and the corresponding alkylated aromatics are obtained. The reactions can be carried out in organic solvents like nitromethane and acetonitrile. As the size of the anion generally affects the solubility, it was found that the hexafluorophosphate salt, for example, is much more soluble than the tetrafluoroborate and it was used preferentially in present investigations.

$$ArH + RNH_2 + NO^+PF_6^- \longrightarrow ArR + N_2 + H_2O + HPF_6$$

Competitive alkylations of benzene and toluene were carried out by adding to a solution of 0.125 mole each of benzene and toluene and 0.05 mole of nitrosonium hexafluorophosphate (Ozark Mahoning Corp., Tulsa, Okla., purified from $NO_2^+PF_6^-$ impurity by washing with benzene) in 50 g. of nitromethane (acetonitrile) a solution of 0.025 mole of the amine in 20 g. of nitromethane. The addition was carried out over a period of 20 min., while the reaction mixture was vigorously stirred and kept in a constant temperature bath at 25°. The reaction mixture was then quenched with excess ice–water and neutralized, and the organic layer was washed, separated, and dried over anhydrous magnesium sulfate and analyzed by gas–liquid partition chromatography (using a high sensitivity Perkin-Elmer Model 226 gas chromatograph equipped with a 150-ft. open tubular (capillary) column coated with *m*-bis(*m*-phe-