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Myeloperoxidase of the Leucocyte of Normal Human Blood. II. Isolation, Spectrophotometry, and Amino Acid Analysis*

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Received March 12, 1964

The first reported preparation of myeloperoxidase of A_{430}/A_{280} of 0.83 from the leucocytes of normal human blood donors was accomplished by column chromatography on XE-64 of extracts obtained from the crude enzyme precipitated from tryptic digests of frozen-thawed cells in 50% cold ethanol. The preparation was chemically characterized by Fe (0.093%) and N (14.3%), and by the following amino acid composition expressed as residues per molecule: lysine (11), histidine (4), arginine (35), aspartic acid (51), threonine (23), serine (21), glutamic acid (37), proline (31), glycine (26), alanine (26), valine (17), methionine (12), isoleucine (16), leucine (43), tyrosine (9), tryptophan (13), phenylalanine (17), and cystine as cysteic acid (13). The absorption spectra of the oxidized and reduced enzyme are the same as those described in the literature, but the pyridine hemochromogen spectrum is similar to that of formyl-diacetyl hemes and the hemochromogen of cytochrome oxidase. Solutions of alkaline dithionite convert the spectrum to a protohemochromogen type.

Procedures reported for the isolation of myeloperoxidase vary with the source of starting material. Summaries have been reported (Paul, 1963; Maehly, 1955). In the original preparation of the peroxidase from tubercular empyema (Agner, 1941) a mixture of ether, ammonium sulfate and water were used to precipitate the enzyme, which was dissolved in water and precipitated with alcohol after the sulfate was removed by precipitation with barium ions. The enzyme was then redissolved in water, and any insoluble material was recycled. More recently, Agner (1958) prepared crystalline myeloperoxidase from the cells of infected dog uteri which were digested at room temperature in ammoniacal ammonium sulfate and fractionally precipitated with ammonium sulfate. After purification, this product was passed through kieselguhr; material crystallizable from ammoniacal ammonium sulfate was obtained. In the case of rat chloroma, a tumor rich in myeloperoxidase, Schultz *et al.* (1957) prepared the crystalline enzyme (Schultz, 1958) by combining the preparation of crude enzyme described by Agner (1941) with the use of CG-50 (XE-64) Amberlite resin.

* This work was supported in part by grants (CA-3715 and CY-5152) from the National Institutes of Health, U. S. Public Health Service. The material presented here has been taken in part from a thesis by Herman W. Shmukler, submitted in partial fulfillment of requirements for the degree of Doctor of Philosophy in Biological Chemistry in the Graduate School of Hahnemann Medical College. The authors wish to express their gratitude toward Merck Sharp & Dohme Co. for making available the supply of white cells used in these experiments.

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Application of the above procedures to the isolation of the peroxidase from leucocytes of normal human blood, however, was found to be unsatisfactory. Treatment of normal white cells with trypsin, precipitation of the digest with alcohol, and extraction of the enzyme from the precipitate with phosphate buffer as described by Maehly (1955) proved to be a good starting point. In our hands this product was readily purified on the ion-exchange column (XE-64) previously described (Schultz *et al.*, 1957). In the present report a process is described which has been in use for 3 years, during which each week 300 ml of buffy coat of the blood of 300 donors was continuously processed. The enzyme content based on activity and cell counts of twenty-five such batches, as has been reported, varied over a wide range; but the average value was $2.13 \pm 0.14 \times 10^{-6}$ enzyme units per cell (Schultz and Kaminker, 1962).

The purpose of this report is to describe in detail the preparation of highly purified enzyme, having a ratio of A_{430}/A_{280} of 0.83 (equal to Agner's crystalline product), and to present hitherto unreported spectrophotometric properties of the enzyme and its chemical composition. Subsequent reports will deal with the nature of the heme peptides obtained from tryptic digests and preliminary data on primary structure.

METHODS AND PROCEDURES

Nitrogen was determined by micro-Kjeldahl, Fe by the procedure described by Drabkin (1941), and the amino acids by the method of Spackman *et al.* (1958) using the Phoenix automatic amino acid analyzer. Tryptophan was assayed both spectrophotometrically

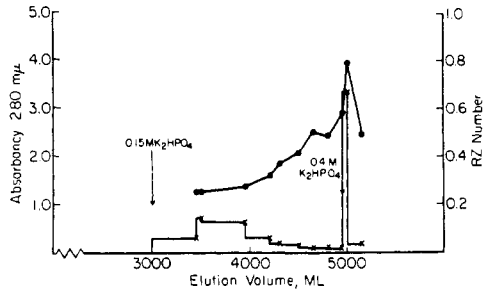

FIG. 1.—Chromatography of myeloperoxidase on XE-64 at alkaline *p*H. Fraction (d) (250 ml) of Table II was diluted with water to make the phosphate concentration 0.1 M, and passed through a fresh XE-64 column. The adsorbed enzyme was developed by the addition of K_2HPO_4 in the concentrations indicated by the arrows. The progress of the fractionation was followed by measuring the light absorption at 280 and 430 mμ. x- - - - -x A_{280}; ●——●, ratio of absorbance at 430 mμ to that at 280 mμ.

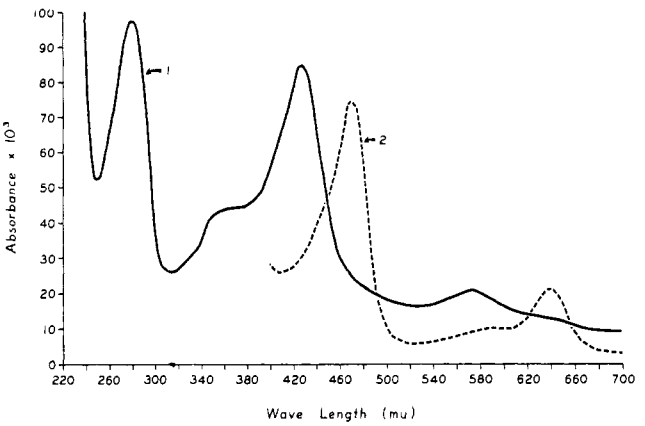

FIG. 2.—Absorption spectra of oxidized and reduced myeloperoxidase isolated from normal human leucocytes in 0.1 M phosphate buffer, *p*H 7.0. (1) Oxidized native enzyme; (2) after addition of sodium dithionite.

(Goodwin and Morton, 1946) along with tyrosine and also by the method of Spies and Chambers (1949). Peroxidase activity was measured by a modification (Schultz *et al.*, 1957) of the method originated by Chance (1955). An enzyme unit was taken as K equal to $\sqrt{1/t}$, where t is time in seconds required for the absorbance of the test solution to increase 0.050. Only t values between 6 and 26 seconds were used.

Spectrophotometry.—Measurements of the absorption spectra and absorbance readings were made in a Process and Instrument recording spectrophotometer which uses the optics of the Beckman DU spectrophotometer and has a scale expander which makes it possible to read 0–0.100 absorbance over a 30-cm. (12-in.) chart range. In this way visible spectra can be measured continuously on the same solution without dilution by changing the range to 0–1.0 absorbance, in the region of the violet and ultraviolet where the absorbance in hemeproteins is up to ten times that of the visible region. The particular range used is indicated on the left and right ordinates in the figures described. Dydinium glass was used to calibrate the wavelength scale.

Isolation of Myeloperoxidase from Leucocytes of Human Blood

Each batch of buffy coat from the white cells of 300 donors was treated with an equal volume of 0.01 M phosphate buffer (*p*H 7.0) containing 0.001 M Versene adjusted to *p*H 7.0, and the whole suspension was centrifuged at 17,000 rpm in a Spinco Model L preparative ultracentrifuge, using a No. 40 rotor. Washing and laking of the red cells was repeated until the cells were nearly white, in which form they could be frozen and stored at −20° until ready for use.

Trypsin Digestion.—Preliminary tests were carried out to determine the optimal conditions for solubilization of the enzyme. The criterion of solubilization was the amount of activity found in the 0.1 M phosphate extract of the product precipitated from the digest at an alcohol concentration of 50% at 0–4°. Optimal extraction was obtained when 1 g of trypsin (1–300, Nutritional Biochemicals Corp.) was added to 100 ml of cells and incubated for 4 hours, as suggested by Maehly (1955). Up to 4-fold increase in enzyme activity was found in most cases (Schultz and Kaminker, 1962). In routine practice, therefore, cells stored in the frozen state were thawed at 37° and blended in the cold with four times their volume of 0.01 M phosphate buffer, *p*H 7.8; to this a clear solution of trypsin (1–300) was added in the proportion of 100 gm per 100 ml original cells and allowed to digest for 4 hours at 37° with occasional shaking.

Alcohol Precipitation.—Following digestion the flasks were immersed in a dry ice–acetone bath until the digest formed a water-ice slurry whose temperature was about 1°. Ethyl alcohol precooled to −15° was added slowly to prevent the temperature from rising above 4° until the concentration of alcohol was 50%. After the precipitate formed had been allowed to settle for 30 minutes, the suspension was centrifuged at 20,000 *g* for 10 minutes, and the precipitate was diluted in 0.1 M phosphate buffer (*p*H 7.4) to the original cell volume and stored frozen until used.

Extraction of the Alcohol Precipitate.—The suspension of the 50% alcohol-insoluble material in 0.1 M phosphate buffer was centrifuged at 20,000 *g* and the brown supernatant was decanted. A second extraction prepared in the same way, when added to the first, usually accounted for about 80% of the original activity. The insoluble residues were saved and stored in 1% Triton-K_2HPO_4 (0.1 M, *p*H 8) until sufficient material accumulated to make reprocessing worth while. The combined extracts were then chromatographed on Amberlite (XE-64) as previously described (Schultz *et al.*, 1957) with the following modifications.

Column Chromatography.—The elution procedure used for the absorbed enzyme from chloroma was based on increasing the concentration of buffer without a change in *p*H, which was kept at 7.0. In the case of the enzyme of the human leucocyte less than 40% yields of material were obtained having a ratio A_{430}/A_{280} of about 0.3 on the first cycle. The buffer concentration ranged from 0.2 to 0.4 M. When the enzyme was recycled on fresh resin using K_2HPO_4 instead of the sodium potassium phosphate buffers, considerable improvement in resolution and yield were obtained. This is seen in Figure 1. Table I contains an outline of the procedure and the yields at each step. Purity was measured in all cases as the ratio of the optical density at 430 to that at 280, which is 0.83 at 100%. This value is referred to here as the RZ[1] (Reinheit Zahl) number. Products having RZ of 0.7 or greater were collected from each of three to four batches, pooled, and recycled, using as an additional step in the elution process 0.2 M K_2HPO_4 buffer. Figure 2 illustrates such an elution pattern. Eluates of less than 0.4 RZ were saved and precipitated with ammonium sulfate after removing phosphate ions by dialysis;

[1] Abbreviations used in this work: RZ, Reinheit Zahl purity number.

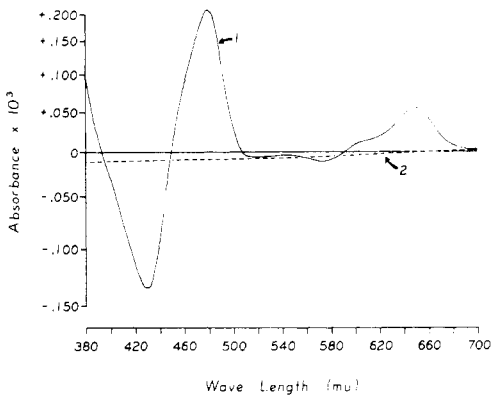

FIG. 3.—Reduced difference spectrum of myeloperoxidase of normal human leucocytes. The oxidized enzyme was placed in both the reference and sample cuvets and the baseline spectrum was obtained as seen in curve 2. A few crystals of sodium dithionite were added to the sample cuvet and the scan was repeated to obtain curve 1. This spectrum is the same as that found with the chloroma peroxidase of the rat (Schultz, 1958), and with rabbit leucocytes by Chance (1953).

TABLE I

APPLICATION OF ONE-COLUMN TECHNIQUE TO THE ISOLATION OF MYELOPEROXIDASE FROM LEUCOCYTES[a]

Fraction	Volume (ml)	RZ	Enzyme Activity (units)	Recovery (%)
Extracts from EtOH precipitate	900		166,000	
Unretained eluate 0.15 M phosphate, *p*H 7.0	900		7,000	4.2
1	250		980	0.6
2	250			
0.15 M K_2HPO_4				
1	500	0.07		
2	450	0.05	1,350	0.8
3	110	0.12	1,700	1.1
4	460	0.10	22,000	13.3
5	450	0.10	3,600	2.2
6	600	0.10	7,800	4.7
7	490	0.22	8,200	4.9
8	500	0.45	7,100	4.3
0.3 M K_2HPO_4				
1	250	0.75	116,000	69.8
2	250	0.16	2,900	1.7

[a] White blood (300 ml) cells were processed as described in the text. 900 ml of the solubilized ethanol fraction was chromatographed at *p*H 7.0, 0.1 M phosphate buffer on 51 × 51-mm (2 × 2-in.) XE-64. The column was washed with 500 ml of 0.15 M phosphate buffer, *p*H 7.0, 4000 ml of 0.15 M K_2HPO_4, and 500 ml of 0.3 M K_2HPO_4. Fractions were collected in the volumes indicated in the table. RZ is the value of A_{430}/A_{280} reported to be 0.83 for crystalline enzyme (Agner, 1958). A unit of enzyme activity is described in text under Methods.

subsequent chromatography as described raised the RZ to 0.7, at which point the material was combined with material of like purity for final purification.

RESULTS

Composition.—The analysis we made on three preparations had A_{430}/A_{280} ratios of 0.83 and represented products obtained over a 9-month period. For preparations A, B, and C the Fe contents were 0.093, 0.092, and 0.093%, respectively, and N contents were 14.48, 14.45, and 14.4%. All values are on a moisture-free basis. No ether-soluble lipids could be obtained by continuous extraction on a 10-mg sample using a micro soxhlet for a period of 48 hours. The unhydrolyzed protein yielded not more than 3% carbohydrate (J. Schultz and H. W. Shmukler, unpublished data).

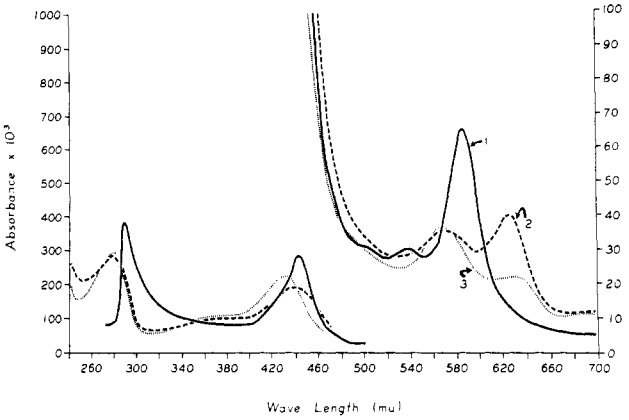

FIG. 4.—Hemochromogen spectra of myeloperoxidase. Enzyme (0.28 mg/ml) in (1) 30% pyridine in 0.5 M NaOH, 1.5 N NH_4OH, and (3) in 0.2 M phosphate buffer, *p*H 7.0. The left ordinate is read for the Soret and ultraviolet spectra and the right ordinate for the visible band. The reduced spectra of (1) and (3) are in Fig. 5.

Spectrophotometry.—The spectra of the oxidized and reduced enzyme are seen in Figure 2; the difference spectra of the reduced enzyme are shown in Figure 3 and the hemochromogen spectra in Figures 4 and 5. The experimental conditions are described in the legends.

DISCUSSION

In the past year over 10 × 10^6 enzyme units of activity were processed in this laboratory by the procedures described; the yield was 3.5 × 10^6 units of material having A_{430}/A_{280} ratios of 0.8 or higher, about 35% on an activity basis. Although the rat chloroma enzyme gave a ratio of 300 enzyme units per mg, the product here was 1000 units per mg; the actual weight of enzyme was about 3.5 g.

While the ratio of the absorbance of the Soret band to absorbancy of protein in the preparations reported here is the same as that of the dog enzyme (Agner, 1958), and the spectra of the oxidized and reduced proteins are identical, there is a difference in the iron values. Agner's dog preparation contained 0.078% Fe and his previously reported value of the Fe content of a preparation from human empyema was 0.1% (Agner, 1941). He attributed this variation either to the difference in purity or to a species difference. Since our rat chloroma peroxidase had an Fe content of 0.07% (Schultz, 1958; Schultz and Rosenthal, 1959), and the present value is 0.093%, we believe the differences in these values to reflect a species difference.

The amino acid content is of interest especially in regard to the basic amino acids. The principal basic amino acids in hemoglobin are histidine and lysine, while that in cytochrome *c* is lysine; in myeloperoxidase the principal basic amino acid is arginine. In the case of crystalline horseradish peroxidase (Worthington, RZ 3.0) of highest purity the basic amino acids are evenly divided between lysine and arginine.[2] Tryptophan, which is generally of limited distribution in proteins, is relatively high in myeloperoxidase.

Although Agner reported a N value of 17.15% in the case of the enzyme of human empyema, no report for

[2] Unpublished experiments in this laboratory.

TABLE II
AMINO ACID COMPOSITION OF MYELOPEROXIDASE[a]

Amino Acid	Preparation A (mols/g-atom Fe)	Preparation B (mols/g-atom Fe)	Average (mols/g-atom Fe)	Residue Weight (g)	Total N Atoms (no)
Lys	10	12	11	1,410	22
Hist	4	4	4	549	12
NH_3	54	64	59	1,003	59
Arg	35	35	35	5,537	140
$CySO_3H$	13	13	13	1,966	13
Asp	52	49	51	5,870	51
Thr	24	22	23	2,325	23
Ser	21	21	21	1,829	21
Glu	35	38	37	4,777	37
Pro	30	32	31	3,010	31
Gly	25	26	26	1,485	26
Ala	25	26	26	1,849	26
Val	17	17	17	1,685	17
Met	13	10	12	1,454	12
Ileu	16	16	16	1,811	16
Leu	40	45	43	4,868	43
Tyr	8	8	8	1,306	8
Phe	16	17	17	2,502	17
Tyr (spec)	10	10	10		
Try (spec)	13	12	13	2,421	26
Subtotal			473	47,657	610
Weight of heme "porphyrin"				550	4
Total				48,207	614

[a] All values are in terms of moles amino acid per g-atom Fe. The iron content of preparation A was 0.093%, and of B, 0.092%; total N by micro-Kjeldahl = 14.48% and 14.45%. When the total number of N atoms as seen in last column is multiplied by 14 and divided by weight of enzyme (60,000) per g-atom of Fe, one obtains a value of 14.33, which accounts for all of the N as determined by micro-Kjeldahl method. The amino acids were determined after digestion in 6 N HCl at 105° in sealed tubes. After removal of the HCl, the digest was diluted so that the equivalent of 0.584 mg of enzyme was placed on each column. In addition, tyrosine and tryptophan values were obtained by spectrophotometric means (see text); this is indicated by (spec) in the table. After 72 hours of hydrolysis only threonine and serine showed losses; the figures indicated were calculated to zero time by extrapolation of the 24- and 72-hour data. Cystine and cysteine combined are represented by the cysteic acid determined after oxidation of the protein in accordance with Schram *et al.* (1954). Invariably a peak appeared in different amounts before the phenylalanine and tyrosine peaks, which averaged about 4 leucine equivalents per mole. These were not included in the N figures, for no significant change in other peaks was seen after 72 hours of hydrolysis.

N content of the dog enzyme was given. In the data reported here, the preparation contained 14.4% N, all of which can be accounted for in terms of the N contributed by the constituent amino acid residues (Table II). The presence of substances other than amino acids is obvious. Thus, studies on unhydrolyzed material indicated no change in weight after extensive extraction with lipid solvents, and less than 3.0% carbohydrate and ash were found. Preliminary data indicate that the preparations are homogeneous in the ultracentrifuge. A sedimentation constant of about 7.3 S was found by Agner. An iron content of probably at least 2 Fe atoms per molecule was found.

In his extensive studies on verdoheme and verdoglobins, Lemberg (1951) was particularly intrigued by the spectrophotometric properties of myeloperoxidase because of certain similarities of its spectrum to that of choleglobin, a product of unknown structure derived from hemoglobin (Foulkes *et al.*, 1951). Absorbance of myeloperoxidase in the red resembled that of reduced myeloperoxidase; and like choleglobin, myeloperoxidase could be converted to a red product whose

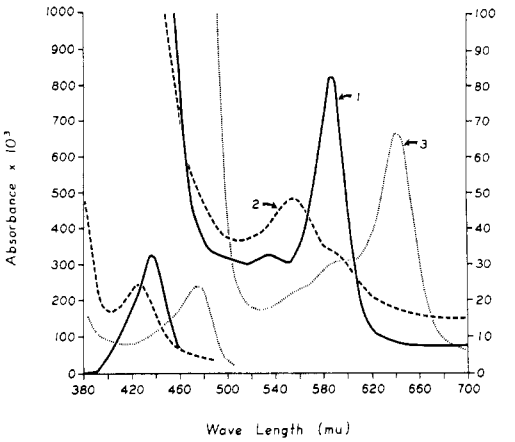

FIG. 5.—Hemochromogen spectra of myeloperoxidase. Curve 1 represents the reduced pyridine hemochromogen of Fig. 5; curve 2 shows the spectrum of alkaline dithionite red derivative (see text); and curve 3 represents the native enzyme treated with dithionite, that is, the reduced spectrum of Fig. 5. These curves demonstrate that myeloperoxidase can be converted to products exhibiting the spectra of the formyl heme-pyridine and protoheme-pyridine hemochromogens reflecting the nature of its prosthetic group. The ordinate at the left is absorbance at (0–1.00) × 10^3) and at the right is (0–0.100) × 10^3. The scale for the visible spectrum is at the right and that for the Soret and ultraviolet absorption spectra are at the left ordinate.

hemochromogen resembled protoheme. Thus in Figure 5 the alkali- and dithionite-treated materials are seen to have a Soret band and an α band in the region of protohemochromogen. Of equal interest in this connection is the pyridine hemochromogen in Figures 4 and 5, where the α band is at 586 mμ, not much different from the 583 mμ found for pyridine hemochromogens for crystalline and highly purified preparations of cytochrome oxidase (Yonetani, 1961, Okunuki et al., 1958). The spectrum bears a remarkable resemblance to the action spectrum of Atmungsferment redrawn by Paul (1963) from Warburg's data. These findings indicate that the prosthetic group of myeloperoxidase is different from any of the known hemoproteins, even lactoperoxidase, where the ratio of the absorbance of the Soret band to the absorbance of the protein is of the same order of magnitude as in myeloperoxidase, and where the linkage to the peptide chain is an ester and the pyridine hemochromogen is of the protoheme type (Hultquist and Morrison, 1963). The α band of pyridine hemochromogen of diacetyldeuterohemin is at 582 mμ (Paul, 1959). However, the fact that the pyridine hemochromogen is similar to that of the formyl hemes and diacetyl heme, in the case of myeloperoxidase, indicates at least the presence of highly polar side chains. In addition to these findings one might add that there is a deepening of the green color of the enzyme when in alkaline solution of a *p*H greater than 10. This is reflected in the spectra in 1.5 M NH_4OH seen in Figure 4, where a marked increase in absorption of 630 is noted, with no change in the absorption at 570 mμ. Dialysis of the NH_4OH solution restores enzyme activity and restores the original spectrum, but when 1 N sodium hydroxide is used instead of NH_4OH such reversals do not take place. Of interest in this connection is that acidification of the NaOH solution results in a liberation of hydrogen sulfide, indicative of a labile sulfur in the molecule.[1]

[1] For discussion of the possible significance of this observation see Rajagopalan and Handler (1964), *J. Biol. Chem.* *239*, 1509, who discuss similar observations on other proteins.

The explanation of these phenomena will be more evident from the data to be presented at a later date in detail on the nature of the heme and porphyrin of the enzyme, which has been suggested in preliminary reports (Schultz *et al.*, 1961; Schultz *et al.*, 1964).

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Decomposition of Carbamylphosphate in Aqueous Solutions*

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Received April 6, 1964

In a detailed investigation of the mechanism of the decomposition of carbamylphosphate, the first-order rate constants for the release of orthophosphate from the monoanion and dianion (occurring predominately between *p*H 2–4 and 6–8, respectively) are similar but different. There is no significant effect of buffer concentration or ionic strength on the decomposition of either of these ionic species. This rules out a general acid or base catalysis and suggests that charge formation is not involved in the rate-determining step. There is also an acid-catalyzed reaction (below *p*H 2) and a base-catalyzed reaction (above *p*H 8). The rate of the base- (and acid- [M. Halmann, A. Lapidot, and D. Samuel, 1962, *J. Chem. Soc.*, 1944]) catalyzed decomposition, in contrast to the mono- and dianion decomposition, is increased significantly by raising the ionic strength of the solution. The pK_1 and pK_2 of carbamylphosphate have been found to be 1.1 and 4.9. A *p*H profile of ^{18}O incorporation into the orthophosphate formed during decomposition of carbamylphosphate in $H_2{}^{18}O$ showed maximal incorporation (92%) at *p*H 3.0, where the monoanion represents 97.5% of the ionic species present, with a symmetrical decrease on raising or lowering the *p*H from 3.0. These data show that P—O bond cleavage occurs with the monoanion, whereas C—O bond cleavage occurs with both the dianion and neutral species. Ammonia release is not first order between *p*H 4 and 6 because the cyanate formed from the decomposition of carbamylphosphate dianion is only slowly hydrolyzed to ammonia. For this reason ammonia release cannot be used to follow the rate of carbamylphosphate decomposition. When carbamylphosphate hydrolysis is carried out between *p*H 4 and 6 in the presence of azide, no increase in rate is observed although carbamylazide accumulates. Since the *p*H curve of carbamylazide formation reflects carbamylphosphate dianion concentration, it has been concluded that azide is trapping cyanate formed from the dianion and not carbamyl cation as suggested by Halman and co-workers (*vide supra*). Our data support different mechanisms for the hydrolysis of the monoanion and dianion of carbamylphosphate involving P—O and C—O bond cleavage, respectively, and suggest that these reactions are monomolecular and may be facilitated by six-membered ring structures.

The importance of carbamylphosphate as a required intermediate for the biosynthesis of arginine, urea, and the pyrimidine ring, as well as its participation in other enzymatic reactions (Jones, 1963) warrants a rather detailed knowledge of its stability in aqueous solutions. Initial studies of the nonenzymatic decomposition of carbamylphosphate in water (Jones and Lipmann, 1960) showed that the rate of the release of orthophos-

* This work was supported in part by grants from the National Science Foundation (GB-885) and from the National Institutes of Health (CA-03658). A preliminary report of this work was presented at the American Chemical Society meetings held in New York City in September, 1963.

† This work will be submitted for partial fulfillment of the requirements for the Ph.D. degree at Brandeis University. Predoctoral Fellow of the National Institutes of Health (1962–64).

‡ Contribution No. 303.