CCD or paper chromatography. Proline is slightly high perhaps due to the overlapping NH_4Cl band. The primary objective in the experiments summarized in Table II was that of isolation and the necessary careful standardization of the analytical procedure which would give a closer approximation to theoretical recoveries was not done. The figures for the hydrochlorides resulted from a re-run and were expected to be somewhat low.

The hydrolysis products of tyrocidine A contain a volatile base in addition to amino acids. This base has been isolated as the hydrochloride and proved to be ammonia. Quantitatively, two ammonia molecules result from the minimum molecule of tyrocidine A.

Evidence against the presence of a free carboxyl group in tyrocidine A was obtained by a study of the corresponding acetyl derivative. The latter was prepared in the crystalline state and was found to be neutral in reaction. Hence there can be no free carboxyl group in the original polypeptide. The fact that tyrocidine A is insoluble in excess aqueous sodium carbonate but readily soluble in aqueous sodium hydroxide is consistent with the presence of a free phenolic hydroxy group but no carboxyl group in the molecule.

The molecular weight of tyrocidine A has been shown in an accompanying paper[8] to approximate 1270. This value is in agreement with the amino acid analyses of Table II and with the other analytical data reported in this paper. The peptide is entirely built up of amino acids or their primary amides. Furthermore, the following paper[8] reports that there are no free α-amino groups in tyrocidine A which is basic by virtue of the free δ-amino group of the ornithine residue. No free carboxyl group is present. It must therefore be a cyclic peptide with the γ- and β-carboxyl groups of glutamic and aspartic acids in the form of primary amides. A large ring with 20 carbon atoms and 10 nitrogen atoms appears most probable.

The amino acid residue formula of tyrocidine A can be written (D-phe.)$_2$(L-phe.), (L-val.), (L-tyr.), (L-leu.), (L-pro.), (L-ornithine), (L-glutamine) and (L-asparagine) which corresponds to the empirical formula $C_{66}H_{87}O_{13}N_{13}$. This formula has been confirmed by the preparation of derivatives.[8]

It would thus appear that the first objective stated in the introduction, the isolation of a pure polypeptide, has now been reached in a satisfactory manner. Also the question raised as to whether the 220 tube distribution apparatus is adequate for such a problem has been answered in the affirmative.

Acknowledgments.—We are indebted to Mr. D. Rigakos for the microanalyses and to Dr. J. R. Weisiger and Miss E. Jacobs for the amino nitrogen and amino carboxyl determinations. The technical assistance of Miss D. McNamara is also acknowledged.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

The Chemistry of Tyrocidine. II. Molecular Weight Studies

BY ALAN R. BATTERSBY[1] AND LYMAN C. CRAIG

RECEIVED FEBRUARY 15, 1952

A general approach to the determination of the molecular weights of polyfunctional substances is suggested. The method should prove particularly useful for the characterization of higher molecular weight substances and for additional criteria of purity. The procedure has been applied to tyrocidine A and has shown the value 1270 which is the minimum molecular weight derived by amino acid analysis, to be the true molecular weight.

An unexpected result was obtained when attempts were made to purify tyrocidine A by fractional crystallization from methanol containing dry HCl. The sample being studied had already been purified by countercurrent distribution (CCD)[2] until it gave a single band. However, on redistribution of the recrystallized material two well separated bands were obtained, one of which corresponded to tyrocidine A.

It was soon found that the new band contained a transformation product of tyrocidine A formed by conversion of $-CONH_2$ groups to $-COOCH_3$ groups. The conclusion that only *one* of the possible $-CONH_2$ groups present per molecule was involved could be deduced from interpretation of the distribution pattern. This result suggested a possible means of deriving the molecular weight of tyrocidine A and in fact a general approach to the derivation of molecular weights of polyfunctional substances. This paper presents the molecular weight studies with tyrocidine A.

(1) On leave of absence as a Commonwealth Fund Fellow from the University of St. Andrews, Scotland.

(2) A. R. Battersby and L. C. Craig, THIS JOURNAL, **74**, 4019 (1952).

Experimental

Partial Methanolysis of Tyrocidine A.—A solution of 1.09 g. of tyrocidine A hydrochloride in 12 cc. of methanol was cooled to 0° and a slow stream of dry HCl was passed into the ice-cold solution for about 10 sec. The solution, which was now about 0.1 *N* with respect to HCl, was concentrated *in vacuo* at a low temperature to about 7 cc., when crystallization began. It was permitted to stand at room temperature for 4 hr. after which 0.70 g. of colorless rods was collected.

At the time of the experiment this product was considered to be tyrocidine A of the highest purity but upon distribution of a 400-mg. portion of it to 720 transfers, pattern a of Fig. 1 was obtained. The system methanol, chloroform, 0.1 *N* HCl, volume proportions 2,2,1, was used.

The material in the right peak was recovered by evaporation of the solutions *in vacuo* at less than room temperature. The crystalline residue of tyrocidine A hydrochloride, 203 mg., was recrystallized from methanol–ether. It melted at 240–242° with decomposition. It was dried for analysis at 110° in high vacuum.

Anal. Calcd. for $C_{66}H_{87}O_{13}N_{13}$·HCl: C, 60.64; H, 6.79; N, 13.93; Cl, 2.71; OCH_3, 0. Found: C, 60.85; H, 6.80; N, 13.60; Cl, 2.71; OCH_3, 0.

The lower layer of the solution from tubes 85–125 was separated and the upper layer extracted thoroughly with fresh lower layer of the solvent system used for the distribution. Evaporation of the combined lower layers left 145 mg. of the methyl ester. Recrystallization from methanol-ether gave colorless needles which melted at 236–237° with decomposition. A mixed melting point with tyrocidine A hydrochloride showed no depression. It was dried for analysis in high vacuum at 110°.

Anal. Calcd. for $C_{67}H_{88}O_{14}N_{12}$·HCl: C, 60.86; H, 6.71; N, 12.73; N(amide), 1.06; OCH_3, 2.36; Cl, 2.68. Found: C, 60.62; H, 6.71; N, 12.8; N(amide), 1.07; OCH_3, 2.38; Cl, 2.65.

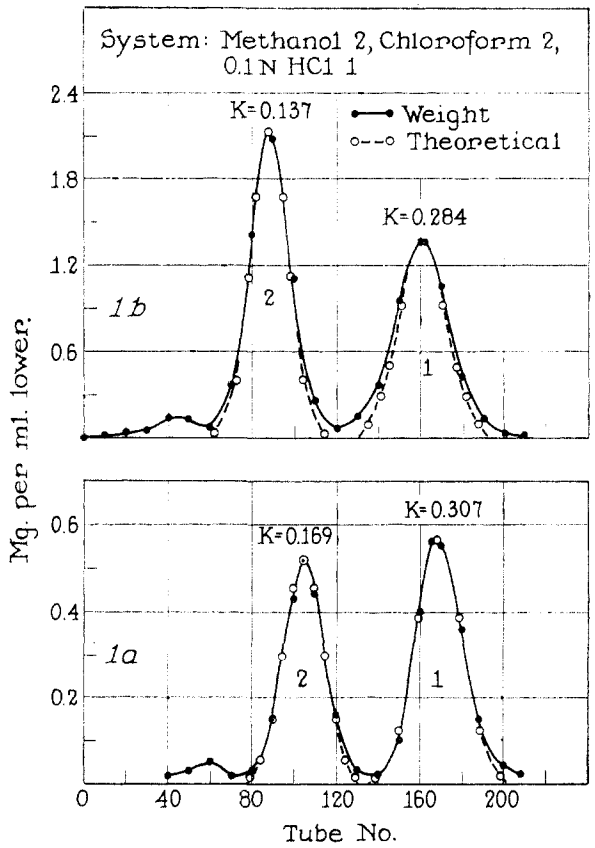


Fig. 1.—Distribution patterns of partial methylation products of tyrocidine A.

A second sample of tyrocidine A treated a little longer with methanol and HCl and distributed as above gave pattern b of Fig. 1. Here more of the ester has been formed. Complete hydrolysis of the methyl ester and paper chromatography of the hydrolysate gave a pattern of spots identical with that from tyrocidine A.

Partial Substitution with 1-Fluoro-2,4-dinitrobenzene (FDNB).—A solution of 250 mg. of tyrocidine A hydrochloride in 3 cc. of 95% ethanol, was diluted with a solution of 80 mg. of sodium bicarbonate in 3 cc. of water. A solution of 72 mg. of FDNB in 4 cc. of 95% ethanol was then added and the mixture was allowed to stand for 18 hr. at room temperature. After acidification with 1 *N* HCl the reaction mixture was evaporated to dryness *in vacuo*, then twice more after adding absolute alcohol. The residue was extracted with a mixture of chloroform and ethanol to remove the organic material from the inorganic salts. The extract was evaporated to 0.5 cc. and the desired product precipitated by addition of a large excess of dry ether. The ether solution was discarded since it contained only a small amount of ether soluble oil.

The precipitate was distributed in a system formed by chloroform, benzene, methanol and 0.01 *N* HCl in the volume proportions 10, 20, 23 and 7, respectively. At 218 transfers the pattern of Fig. 2 was obtained.

The solid recovered from Band 1 proved to be unchanged tyrocidine A. The yellow solid, N-DNP-tyrocidine A, recovered from Band 2, tubes 134–160, was crystallized from methanol acidified with HCl. Fifty-one mg. of yellow needles was obtained which were recrystallized. The substance was dried *in vacuo* at 130° for analysis.

Anal. Calcd. for $C_{72}H_{89}O_{17}N_{15}$: C, 60.16; H, 6.25; N, 14.63. Found: C, 59.70; H, 6.10; N, 14.55.

With the Beckman quartz spectrophotometer at 350 mμ an optical density of 1.82/cm. was found for a methanolic solution containing 0.191 mg./cc. Bands 4 and 5 correspond to those in Fig. 3.

Complete Substitution with 1-Fluoro-2,4-dinitrobenzene.—A solution of 0.12 g. of tyrocidine A hydrochloride, 0.6 g. of sodium bicarbonate, 4 cc. of 95% ethanol and 3 cc. of water was treated with a solution of 0.30 cc. of FDNB in 2 cc. of 95% ethanol and shaken for 4 hr. at room temperature. The precipitated solid was collected and washed with dry chloroform and ethanol until all the organic material had been leached out. The filtrate was made slightly acid with HCl and evaporated to dryness. It was taken up in dry chloroform and filtered to remove the remaining inorganic material. The filtrate was evaporated and the residue was distributed in the system used in the preceding experiment. At 220 transfers the pattern of Fig. 3 was obtained.

The solutions in tubes 23–42 were evaporated and the residue was recrystallized from aqueous methanol. Yellow needles of the N:O-disubstituted DNP-derivative weighing 68 mg. were obtained which melted at 292–296° with decomposition. The substance was dried at 110° for analysis.

Anal. Calcd. for $C_{75}H_{91}O_{21}N_{17}$: C, 58.45; H, 5.72; N, 14.86. Found: C, 58.44; H, 5.59; N, 14.78.

With the Beckman quartz spectrophotometer an optical density of 1.895/cm. was found at 350 mμ for a methanolic solution containing 0.195 mg./cc.

Isolation of δ-DNP-Ornithine from N:O-Disubstituted DNP-Tyrocidine A.—Two grams of this DNP-tyrocidine A, dissolved in 40 cc. of glacial acetic acid, was treated with 80 cc. of concentrated HCl and 30 cc. of water and heated under reflux for 30 hr. The solution was evaporated to dryness *in vacuo* and the residue dissolved in 30 cc. of water. After 24 hr. at 4° the slightly soluble O-DNP-tyrosine hydrochloride was filtered off. The filtrate was made alkaline with ammonia, saturated with butanol and extracted four times with twice its volume of *s*-butanol saturated with water. Evaporation of the combined butanol extracts left a yellow residue which was dissolved in the minimum volume of a mixture of 5 volumes of *s*-butanol and 2 volumes of 3% aqueous ammonia. This solution was run onto a 60 cm. × 5 cm. column of powdered cellulose (Solka-Floc) which had been thoroughly washed with the solvent system. The yellow band of DNP-ornithine was washed through the column with the above solvent system and after removal of ammonia from the appropriate fraction by evaporation, was isolated as the crystalline monohydrochloride, m.p. 217–218° with decomposition.

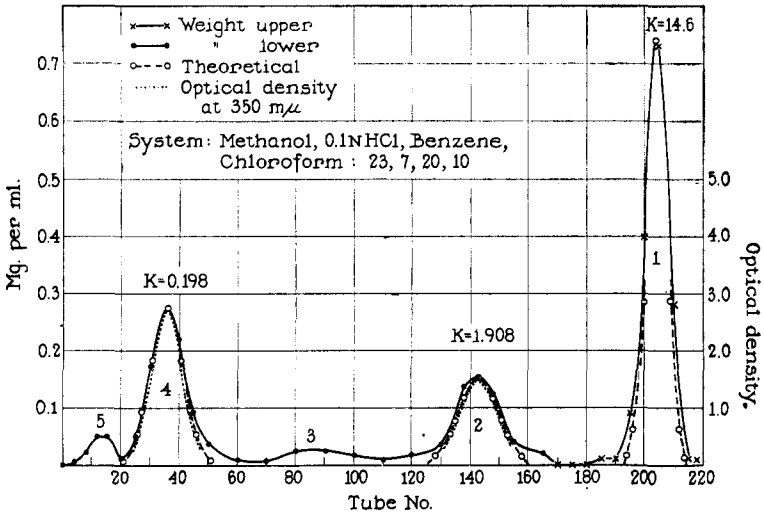
Anal. Calcd. for $C_{11}H_{14}O_6N_4$·HCl: CO_2-N_2, 4.18. Found: CO_2-N_2, 4.12.

Discussion

The problem of the determination of molecular weights for polypeptides of molecular size approximately 1,000 or more has never been satisfactorily solved. Such approaches as freezing point depression, diffusion and sedimentation in the ultracentrifuge have not given results in agreement. With these methods, the state of aggregation of the molecule probably is a confusing factor. In general this type of substance shows a strong tendency to associate and tenaciously holds solvent of crystallization.

The structural chemist in dealing with fairly simple substances has often estimated the amount of some functional group in the molecule, for example methoxyl, amino or carboxyl groups, to confirm the value for the molecular weight derived

by physical methods. However, it is necessary to know the number of the estimated functional groups in each molecule in order to calculate the molecular weight. With complex substances, this knowledge has not been readily gained. It now appears that CCD can be of great assistance in this connection and thus the range over which the method can be used has been considerably extended.



Ffg. 2.—Distribution patterns of partial DNP-substitution products of tyrocidine A.

A partition ratio, K, for purposes of this discussion can be regarded as an over-all balance of hydrophobic and hydrophilic properties. Any change made in the solute molecule which releases or covers a polar group will have a striking effect on the partition ratio. When more than one such group is involved, each will produce its separate contribution to the shift in the partition ratio. Therefore, the various stages of substitution in a polyfunctional molecule should be readily separable by CCD. Moreover, from probability considerations, a substitution reaction carried only part way to completion and interrupted when a considerable fraction of unsubstituted material still remains, should yield appreciable amounts of the mono- and disubstituted derivatives even in the event that rate considerations should favor higher substitution. If the monosubstituted derivative reacts about one hundred times faster, or more, than the original substance, then it may be difficult to detect the former unless a very large excess of substance over substituting reagent can be used. This is not always practicable and an alternative is to study a second partial reaction involving different functional groups in the molecule to obtain confirming evidence. With either of the reactions used in this work it is unlikely that a monosubstituted polypeptide would react much faster than the original substance; the chances that both reactions vastly favor high substitution are negligible.

When the products of such a partial substitution (or hydrolysis, methanolysis, etc.) are distributed, a series of bands should result. If only one functional group is present to react, two bands should appear, with two groups, three bands, etc. One band will be the unsubstituted material, the one nearest to it should always be the mono, the disubstituted derivative should be nearest to the mono, etc. Thus analysis for the substituting group in the various bands permits calculation of molecular weight since from the over-all picture the number of the substituents is clearly revealed.

Since band 1 of pattern a Fig. 1 was found to be unchanged tyrocidine A, band 2 on the basis of the above reasoning is interpreted as a monomethyl ester. From the methoxyl value (2.35%) a molecular weight of 1300 is indicated. The amide nitrogen was found to be 1.06%, a value approximately half that found (2.01%) for the original peptide. Methanolysis therefore converted one of the $-CONH_2$ groups to a $-COOCH_3$ group. Other analytical data are also in line with this conclusion.

In an accompanying paper[2] the quantitative amino acid composition of tyrocidine A was reported. From these results a minimum molecular weight of 1270 for the peptide could be derived which thus agrees well with the actual molecular weight derived here. Little doubt now remains in regard to the molecular size of tyrocidine A, even though Pederson and Synge[3] found higher values by diffusion.

As a further study of the method and to confirm the result from the methanolysis the Sanger reagent, FDNB[4] was used for a similar approach to molecular weight determination. Such a reagent has a number of advantages and gives colored bands when an amino group is substituted. The amount of substituent can be determined spectroscopically.

Tyrocidine A, as is shown later, has only two functional groups which can be attacked by FDNB,

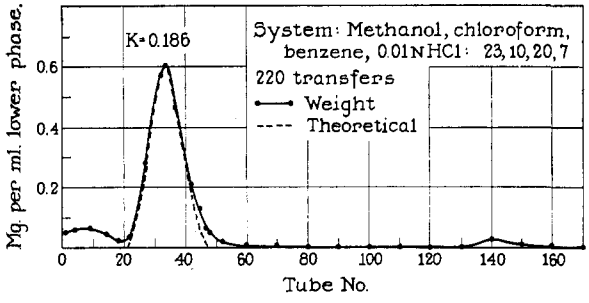


Fig. 3.—Distribution patterns of total DNP-substitution products of tyrocidine A.

the basic terminal amino group of the ornithine residue and the phenolic hydroxyl of the tyrosine residue. It was, therefore, interesting to find that at a certain stage of the reaction with FDNB the pattern shown in Fig. 2 was obtained.

(3) K. O. Pederson and R. L. M. Synge, *Acta Chem. Scand.*, **2**, 408 (1948).

(4) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

Band 1 was colorless and contained the unchanged tyrocidine A. Band 2 was yellow in color. By the argument used earlier, bearing in mind that O-DNP-derivatives of tyrosine are practically colorless whilst all N-DNP-derivatives of primary amines are bright yellow, band 2 is interpreted as the monosubstituted N-DNP-tyrocidine A. N-DNP-derivatives exhibit a broad absorption band whose maximum approximates 350 mμ. Depending somewhat on the solvent used, the molecular extinction coefficient at 350 mμ is in the range[5] 15,000 to 16,000. Assuming arbitrarily a value of 15,500, the molecular weight of the solute in band 2 was found by absorption measurements to be 1620. This is somewhat higher than the theoretical value which is 1436 for the mono DNP-derivative of a peptide of molecular weight 1270. However, it is sufficiently accurate to confirm completely that the true molecular weight of tyrocidine A is the minimum value of 1270 derived from amino acid and elementary analyses.

That the solute in band 2 is in fact N-DNP-tyrocidine A was proven by hydrolyzing it completely with 6 *N* HCl followed by chromatography of the products on paper. The pattern of spots obtained was the same as that given by hydrolyzed tyrocidine A itself, save that the hydrolysate of the DNP-derivative yielded no spot corresponding to ornithine. Instead, a new spot, yellow before spraying with ninhydrin, was found. The position of this new spot corresponded to a control of the δ-DNP-derivative of ornithine.

Band 4 of Fig. 2 contained the completely substituted DNP-tyrocidine A which was made in larger yield by treating tyrocidine A with an excess of the Sanger reagent. Distribution of the products gave the pattern shown in Fig. 3. When the molecular weight of this N:O-disubstituted tyrocidine A was calculated from the extinction at 350 mμ, again using 15,500 as the molecular extinction coefficient of the N-DNP chromophore, a value of 1600 was obtained. This calculation has neglected the contribution of the O-DNP residue to the absorption at 350 mμ which we have found to be 15% of that of the N-DNP residue, a value in agreement with Sanger's work.[5] Correcting for this, the molecular weight of the fully substituted derivative was found to be 1840. The molecular weight of the N:O-disubstituted DNP-derivative of a peptide of molecular weight 1270 is 1603. This result gives added support to the value derived above for the true molecular weight of tyrocidine A.

The structure which has been assigned above to the solute in band 4 was confirmed by complete hydrolysis and paper chromatography of the hydrolysate. The pattern of spots differed from that given by hydrolyzed tyrocidine A only in that the former pattern lacked the spots corresponding to ornithine and tyrosine. However, two new spots were found which corresponded to the O-DNP-tyrosine and δ-DNP-ornithine.

A larger quantity of the N-O-disubstituted DNP-tyrocidine A was completely hydrolyzed and the crystalline DNP-ornithine monohydrochloride was isolated from the hydrolysate by partition chromatography on powdered cellulose. The DNP-residue was shown to be attached to the δ- and not the α-amino group of ornithine by the ninhydrin–CO_2 method. It follows from this and the paper chromatography above, that no α-amino group is free in tyrocidine A, the basic group in the molecule being the δ-amino group of ornithine. Furthermore, the phenolic hydroxyl group of tyrosine is free in the original peptide.

In a previous communication[6] the molecular weight of gramicidin S was derived by a procedure similar to that described above. It is thus quite apparent that the order of magnitude of the molecular weight can be derived reliably in this way, but that the question of the accuracy of the method remains to be established. The difficulty here is that although the DNP-derivatives of amino acids and of several simple peptides have fairly constant molecular extinction coefficients at 350 mμ, the DNP residue when incorporated into a large polypeptide evidently does not have exactly the same absorption. In the cases of the DNP-derivatives of tyrocidine A, polypeptin, bacitracin A[7] and gramicidin S the DNP residue absorbs a little less strongly than do simple DNP-derivatives. Since we now know the molecular weight of these substituted polypeptides beyond question, a value of about 14,500 can be calculated for their molecular extinction coefficient at 350 mμ. From this the values 1520 and 1720 for the mono and disubstituted derivatives, of tyrocidine A, respectively, can be calculated. As more DNP-derivatives of polypeptides are examined, it should be possible to say whether the slightly weaker absorption noted above is a general phenomenon or not. Until more information is available on the subject, molecular weight values derived from DNP-derivatives of polypeptides using a molecular extinction coefficient of 14,500 should not be regarded as giving the true value closer than to ±10%.

Finally it may be mentioned that the distribution patterns obtained by partial substitution reactions for molecular weight studies can contribute greatly as added evidence that the polypeptide to be used as a starting point for structural studies is homogeneous. If the bands containing the various derivatives are clear cut and agree with the theoretical curves, then the chances of the presence of appreciable impurity with functional groups available for reaction but with the same *K* as the peptide, are greatly reduced.

The two small bands 3 and 5 of Fig. 3 were suspected in this connection. On complete hydrolysis and paper chromatography they showed spots similar to those derived from the hydrolysates of bands 2 and 4, respectively. However, when the partial substitution of tyrocidine A was accomplished by a short reaction time (15 min.) but with a large excess of FDNB bands 2 and 4 came out clearly but 3 and 5 were scarcely present. The conclusion was therefore reached that 3 and 5

(5) F. Sanger, *Biochem. J.*, **45**, 563 (1949).

(6) A. R. Battersby and L. C. Craig, THIS JOURNAL, **73**, 1887 (1951).

(7) The quantitative amino acid spectrum of polypeptin and bacitracin A and molecular weight has been studied in a similar manner and will be reported shortly.

are formed by side reactions due to the long reaction in alkaline solution and do not indicate impurity in the peptide preparation.

Acknowledgments.—The microanalyses were made by Mr. D. Rigakos. The technical assistance of Miss D. McNamara and Miss E. Jacobs is also acknowledged.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE NAVAL MEDICAL RESEARCH INSTITUTE]

The Energetics of Acid-catalyzed Hydrolysis of Triphosphoric and Pyrophosphoric Acids[1]

BY S. L. FRIESS

RECEIVED DECEMBER 22, 1951

In the acid-catalyzed hydrolysis of triphosphoric and pyrophosphoric acids, a direct dependence of first-order rate constants on the concentration of excess hydrochloric acid present is noted for each reaction. Both reactions show a negative salt effect with respect to added sodium chloride. Under nearly equivalent conditions, the first stage of acid-catalyzed hydrolysis of triphosphoric acid is approximately 6 times faster than that for pyrophosphoric acid. This effect is shown to be caused by a difference of 4 e.u. in entropies of activation for the two hydrolyses. The reactions are further characterized by equal values (*ca.* 22.8 kcal./mole) of their energies of activation.

In connection with studies on the acid-catalyzed hydrolysis of adenosine triphosphate, it was of some interest to obtain corresponding data on the catalyzed hydrolyses of triphosphoric (TP) and pyrophosphoric (PP) acids.

Previous work[2-5] on the rates of pyrophosphate hydrolysis has indicated an approximately linear dependence of first-order rate constants (calculated for the single species $H_4P_2O_7$ or its first ionization product $H_3P_2O_7^-$) with respect to the acidity of solution, in the low *p*H range. This behavior is in marked contrast to a very abrupt decrease in rate amounting to virtual cessation of hydrolysis, in neutral or basic solution. Also, very limited data by Abbott[4] pointed to a factor of about 2.6 as the increase in rate for the acid-catalyzed reaction over a ten degree interval, in the temperature range of 75–100°.

Brief studies[6,7] on the kinetics of hydrolysis of triphosphate salts have been limited to neutral and basic media, and reveal a hydrolysis rate increasing markedly with increase in temperature and decreasing abruptly with increase in *p*H. In these studies, water and dilute base were used as solvents, over the temperature range 60–100°.

In the present work an attempt was made to study the hydrolysis of TP and PP under parallel reaction conditions, with a moderate (and roughly constant) degree of acid catalysis corresponding to the enzyme catalysis observed for adenosine triphosphate, and at temperatures not too far from those employed by enzyme systems. Results obtained under these conditions might subsequently have a bearing on the interpretation of the more complicated enzymatic processes.

A set of preliminary runs was designed to obtain some measure of the effect of added acid and inert salt on the hydrolysis rates of PP and TP. The following represent the stoichiometric equations involved.

$$\underset{(PP)}{H_4P_2O_7} + H_2O \longrightarrow 2H_3PO_4 \qquad (1)$$

$$\underset{(TP)}{H_5P_3O_{10}} + H_2O \longrightarrow H_3PO_4 + H_4P_2O_7 \qquad (2)$$

In the runs on TP, the initial rates were uncomplicated by additional hydrolysis from the PP resulting in the primary step (2), since it will be shown that hydrolytic step (2) is intrinsically faster than (1). Rates were followed in (1) by evaluation of PP concentration as a function of time, using a titrimetric procedure developed by Britske and Dragunov[8] and by Bell.[9] In the runs employing reaction (2), the concentration of orthophosphate was followed by the colorimetric technique of Lowry and Lopez,[10] which precludes any further hydrolysis of the PP and TP present during the analysis time. The results of this portion of the work are summarized in Table I. In these runs HCl was used to generate the free PP and TP species from their sodium salts, together with excess HCl as catalyst, and NaCl was used to observe the magnitude of a representative salt effect.

It is seen from Table I on comparing runs 1, 3 and 4 and also 5, 7 and 8 that the addition of excess HCl beyond that required to produce the fully acidic PP and TP species produces an almost linear increase in the value of the first-order rate constant k_1. Also, from runs 1 and 2, and 5 as compared to 6, a negative salt effect on rate is to be noted, with the addition of 0.40 and 0.30 *M* NaCl causing percentage decreases in rate constant of 22 and 23% for PP and TP, respectively, at the catalyst levels indicated.

Following the isothermal measurements (Table I), the hydrolysis rates of the two polyphosphates were studied as a function of the temperature, over the interval 40–50°. The data are sum-

(1) The opinions in this paper are those of the author and do not necessarily reflect the views of the Navy Department.

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(7) R. N. Bell, *Ind. Eng. Chem.*, **39**, 136 (1947).

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(9) R. N. Bell, *Anal. Chem.*, **19**, 97 (1947).

(10) O. H. Lowry and J. A. Lopez, *J. Biol. Chem.*, **162**, 421 (1946).