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-

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TABLE I

EFFECTS OF ACID AND SALT ON HYDROLYSIS RATES

Run	Compound	Temperature,[a] °C.	Initial concentration, M: PP or TP	Total added HCl	Added NaCl	Excess[b] HCl	k_1,[d] sec.$^{-1}$ × 10^6
1	PP	49.75	0.0234	0.1223	None	0.029	5.41 ± 0.06
2	PP	49.77	.0233	.1223	0.401	.029	4.24 ± .10
3	PP	49.76	.0232	.1427	None	.050	6.95 ± .16
4	PP	49.76	.0228	.1631	None	.072	9.15 ± .32
5	TP	42.07	.0219	.1274	0.300	.018	13.0 ± .4
6	TP	42.07	.0219	.1274	None	.018	16.9 ± .7
7	TP	42.07	.0219	.1529	0.300	.043	20.4 ± 1.3
8	TP	42.07	.0219	.1070	0.300	−.003[c]	8.7 ± 1.0

[a] Temperatures were held constant to ±0.005°. [b] [Total added HCl - 4PP salt] or [Total added HCl - 5TP salt]. [c] Molarity lacking to quite make [Added HCl] = 5[TP salt]. [d] Rate constants calculated analytically, with the observed average deviations from the mean.

marized in Table II. The average precision of rates was evaluated from duplicate experiments at each temperature. Arrhenius energies of activation (E^{Arr}) were calculated by the method of least squares from the log k_1 *vs.* $1/T$ data, and the other quantities of activation calculated in the standard way for the reference temperature 40.0°.

TABLE II

RESULTS OF RATE RUNS AT 40–50°

Initial concentrations: added HCl, 0.1273 M for PP, 0.1274 M for TP; added NaCl, 0.3000 M; PP,[a] 0.0236–0.0240 M, TP, 0.0219 M

Compound	Temp.,[b] °C.	k_1(sec.$^{-1}$) × 10^{-6}	At 40.0°: E^{Arr}[c] (kcal./mole)	$\Delta H^{\ddagger}$ (kcal./mole)	$\Delta F^{\ddagger}$ (kcal./mole)	ΔS^{*} (e.u.)
PP	39.80	1.51 ± 0.05	22.8	22.2	26.7	−14.5
PP	42.07	1.97 ± .03	(σ ±0.03)			±0.14
PP	44.79	2.75 ± .05				
PP	49.77	4.63 ± .10				
TP	39.59	9.55 ± .2	22.9	22.3	25.5	−10.3
TP	42.04	13.1 ± .6	(σ ±0.11)			±0.42
TP	44.71	16.0 ± .8				
TP	48.95	26.9 ± .6				

[a] As determined by initial titrations. [b] Temperatures held constant to be ±0.005°. [c] The indicated standard deviation (σ) values are obtained from deviations in the least squares treatment, and correspond to somewhat greater accuracy than would normally be warranted by the average percentage deviations in the k_1 values, namely, ±2.20% for PP and ±3.39% for TP rates.

Several interesting points emerge from the data of Table II. First, under the given catalytic conditions and with a constant level of inert salt present to minimize variations in ionic strength, the hydrolysis rates for TP *are faster by a factor of about 6.3* than those for PP. This difference in rates was a fortunate factor in the study of TP hydrolysis, since in at least the first half of a rate run no complications were introduced by any appreciable amount of PP hydrolysis accompanying residual TP hydrolysis.

This difference in rate constants does not arise from a difference in activation energies for TP and PP, since E^{Arr} values are constant within experimental error. Rather, the difference in rates (as directly reflected in *ca.* 1 kcal. difference in $\Delta F^{\ddagger}$ values) seems to stem directly from the difference of about 4 e.u. in the values of the entropy terms, $\Delta S^{\ddagger}$. Curiously, the entropy change in hydrolysis between initial and transition states is *less* for the more complex reactant TP, as compared to PP. This might indicate either a more favorable avenue of approach and orientation with respect to a given phosphorus atom for the nucleophilic agent water, in the transition state for TP as compared to PP, or perhaps simply reflect a difference in degree of solvation (and resulting stabilization) of the respective transition states as compared to their reactants.

It is to be noted in the above discussion that the quantities of activation were calculated in terms of total polyphosphate species in solution, without regard to the individual contributions of various ionic species to a given rate constant. Under the acidities used, this procedure would largely conform to rates determined by the neutral acids and their first ionization products. Further, the neglect of whatever temperature dependence is inherent in the equilibria involving these ionic species would be reflected in the absolute values of the calculated activation quantities. However, it is probable that the errors in over-all rate constants and activation quantities would be small and in the same direction for both TP and PP, preserving the relative comparisons of these quantities.

Experimental

Preparation of Materials.—Triply distilled water was used throughout these studies. The PP runs were made using Merck reagent grade sodium pyrophosphate decahydrate directly. Sodium triphosphate was furnished as an anhydrous powder by the Victor Chemical Works, and was recrystallized as the hydrate from water with a trace of added alcohol. The final product gave no detectable phosphate test,[9] and was checked for purity by complete acid hydrolysis and colorimetric phosphate determination.

Rate Runs on PP.—The desired weights of sodium pyrophosphate and sodium chloride were added to a 250-ml. volumetric flask used as a reaction vessel, and dissolved in about 150 ml. of water. The necessary aliquot of standard acid was then added, the mixture thermostated (in a large water thermostat held constant to ±0.005°), and finally made to volume with previously thermostated water. The first 10-ml. aliquot was used to determine the initial PP concentration at time taken as t_0, using the Zn(II)–base titration essentially as described by Bell.[9] Subsequent aliquots were taken at regular intervals over roughly 60–75% of the reaction course, quenched in 100 ml. of water containing sufficient sodium acetate to neutralize free acid, and titrated for residual PP.

Rate Runs on TP.—Virtually the same preliminary operations were employed as those described above. Initial weights furnished initial TP concentrations, with subsequent TP concentrations at time t being evaluated as the difference between the initial value and the value for the orthophosphate concentration determined at t. The production of one orthophosphate from one TP during the first 50–70% of reaction was indicated by the excellent linearity of first order plots over this region. Analysis for phosphate was

carried out on each 10-ml. aliquot, after quenching by addition to a large volume of water plus sodium acetate, by dilution to the proper concentration range and application of the colorimetric method of Lowry and Lopez.[10] A Beckman spectrophotometer was used throughout the colorimetry, with the scheme calibrated at a wave length of 700 mμ.

Acknowledgment.—The suggestion of this overall project by Dr. Terrell L. Hill, and much useful discussion with Drs. Hill and Manual F. Morales are gratefully acknowledged.

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[CONTRIBUTION FROM THE DEPARTMENT OF ORGANIC CHEMISTRY, UNIVERSITY OF BRISTOL]

Mannose-Containing Polysaccharides. I. The Galactomannans of Lucerne and Clover Seeds[1]

BY P. ANDREWS, L. HOUGH AND J. K. N. JONES[2]

RECEIVED JANUARY 10, 1952

The galactomannans of lucerne and clover seed are found to be similarly constituted. Both polysaccharides are highly branched and contain D-galactopyranose end-groups combined with chains of 1,4- or 1,6-linked D-mannose residues, which are probably in the pyranose form.

Galactomannans are common constituents of the ungerminated seeds of leguminous plants, amounting in some cases to more than 40% of the total seed. They occur as mucilages in the endosperms of the seeds, from which they may be isolated by extraction with water. It is known that when the seeds germinate both the mucilage and the endosperm disappear, hence it is argued that the galactomannan is a food reserve polysaccharide.[3] An extensive investigation of the seeds of more than 160 species of legumes by Wise and Appling[4] and Anderson[5] has revealed that many of them contain galactomannans. The galactomannan of gum gatto (from *Ceratonia siliqua* seeds), commonly known as locust bean gum, is widely used in the textile, paper, food and other industries, and the purpose of examining so many other seeds was to find substitutes. The amounts of mucilaginous polysaccharide in the various seeds were found to vary greatly from one species to another; the highest yields of mucilage were obtained from carob seed (38% of the weight of the seed) and guar seed (35%). The composition of the galactomannans is also variable, although all those examined by these workers contained less galactose than mannose. For example, the molecular ratio of these sugars was 16:81 in *Sophora japonica*, whilst in guar seed galactomannan ("guaran"[6]) the figures were 38:58.5. Apparently, galactomannans derived from one species only are of variable composition, since the proportion of D-galactose to D-mannose in gum gatto has been variously reported as 16:84 by Hirst and Jones,[7] 20:80 by Smith[8] and Wise and Appling,[4] 27:73 by Spada[9] and 18:82 by Lew and Gortner.[10]

Two other galactomannans are known in which the ratios of the component hexoses are outside the range of those given above. Thus, the galactomannan of Fenugreek seed (*Trigonella foenum-graecum*) was deduced[11] from the optical rotation of the hydrolysis products, to contain D-galactose and D-mannose in the proportions 48:52, and Hirst, Jones and Walder[12] obtained from a sample of lucerne seed (*Medicago sativa*) a galactomannan ($[\alpha]_D$ + 89° in water) which is unique in that it contains more D-galactose than D-mannose, the ratio being 2:1. The latter polysaccharide was extracted from the milled seed with hot 10% sodium hydroxide solution. This paper describes the isolation in similar yield (*ca.* 5.5%) of a galactomannan ($[\alpha]_D$ + 118 ± 11°) from the seeds, *var. Provence*, by extraction with hot water only. (Further extraction of the seeds with alkali gave only another 1% of material, which contained some xylan.) Both this product and that of Hirst, Jones and Walder were purified by copper complex formation, followed by regeneration of the galactomannan with cold dilute mineral acid. The new preparation contains D-galactose and D-mannose in the proportions 4:5; the two polysaccharides are, therefore, differently constituted (see below). By the same method, a galactomannan ($[\alpha]_D$ + 78 ± 11°), containing D-galactose and D-mannose in the proportions 7:9, has been isolated from clover seed (*Trifolium pratense*).

Evidence concerning the structure of several galactomannans had earlier been obtained by studies of the methylated polysaccharides, and also by periodate oxidation techniques. From the fission products of methylated gum gatto, Hirst and Jones[7] identified 2,3,4,6-tetramethyl-D-galactose (1 part), 2,3,6-trimethyl-D-mannose (4 parts) and 2,3-dimethyl-D-mannose (1 part), whilst Smith[8] obtained precisely the same compounds, but in the proportions 1:2–3:1, respectively. Evidently the galactose in the gum is present in the pyranose form, occupying a terminal position, and is attached to a skeleton of mannose residues by 1,4- and/or 1,6-linkages Guaran[4-6] appears to have a similar

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