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The Heat of Hydrolysis of *p*-Nitrophenylphosphate[1]

BY JULIAN M. STURTEVANT

RECEIVED AUGUST 9, 1954

The heat of hydrolysis of singly charged *p*-nitrophenylphosphate ion to form uncharged *p*-nitrophenol and dihydrogen phosphate is −6280 ± 100 cal. per mole at 25°. Comparison of this result with the heats of hydrolysis recently reported for adenosine triphosphate and inorganic pyrophosphate leads to conclusions which are strongly dependent on an arbitrary choice of reaction types to be compared. The variation with *p*H of the apparent heat of hydrolysis of the ester gives for the second ionization of *p*-nitrophenylphosphate $pK' = 5.4$ and $\Delta H = -2700$ cal. per mole.

Calorimetric determinations of the heats of hydrolysis of adenosine triphosphate[2] (ATP) and inorganic pyrophosphate[3] have recently been reported. In the present paper this work is extended to include a typical ester, *p*-nitrophenylphosphate. The hydrolysis of this ester, catalyzed by prostatic phosphatase,[4] has been studied over the *p*H range 3.6 to 5.8.

Experimental

The calorimetric procedure has been described in detail.[5] All measurements were carried out at 25.00 ± 0.05°. *p*H measurements were made with a Cambridge Model R meter, taking the *p*H of a 0.10 *M* acetic acid, 0.10 *M* sodium acetate solution to be 4.65.

Analytical Procedure.—*p*-Nitrophenol was determined by its absorption in alkaline solution at 400 mμ. Samples were diluted to 25 ml. with H_2O after the addition of 1 ml. of 10% Na_2CO_3, and the optical density in a 1-cm. tube measured in a Beckman DU spectrophotometer. Experiments with recrystallized *p*-nitrophenol gave unit optical density with 1.36×10^{-6} mole of *p*-nitrophenol in 25 ml.

Each calorimetric experiment was paralleled by a hydrolysis run in a glass container started at about the same time as the calorimetric experiment and using the same solutions. The amount of *p*-nitrophenol liberated was determined in the product of the reaction in glass, after deduction of the 1-4% of free *p*-nitrophenol (it was assumed that the pure ester shows no absorption at 400 mμ) found in the substrate solution after the several hours' standing at room temperature needed for thermal equilibration in the calorimeters. This procedure was adopted because it was found that 2-4% of the *p*-nitrophenol liberated was absorbed by the rubber in the calorimeters. It was assumed that the heat accompanying this absorption could be neglected.

Substrate.—Disodium *p*-nitrophenylphosphate ("Sigma 104") was purchased from Sigma Chemical Company, and was used without further purification. Hydrolysis of the ester as described above gave rise to a net liberation of 2.95 ± 0.02 (standard deviation) mmoles of *p*-nitrophenol per gram; if the *p*-nitrophenol found in the substrate solutions before hydrolysis is added to this figure it becomes 3.03 ± 0.02. A titration curve showed that 1.52 mmoles of HCl was required to half neutralize one gram of substrate. These figures agree well in showing that the neutralizable material present liberates the theoretical amount of *p*-nitrophenol; they correspond to a formula weight of 330 as compared to 263 for anhydrous disodium salt. The difference between these formula weights corresponds closely to $4H_2O$. However the salt decomposes on being heated so that moisture determinations were not made; it lost 13.5% (theoretical 19.6%) of its weight on evacuation to 1 mm. for several hours at room temperature. Each heat of reaction listed in Table I is based on the net liberation of *p*-nitrophenol observed in that experiment.

Results and Discussion

Hydrolyses were carried out in acetate buffers of constant total concentration of 0.05 *M*, at initial substrate concentrations of approximately 0.5 m*M*. Under these conditions, the reactions were closely zero order in substrate until well over half completion. The data for a typical pair of runs with the same starting solutions are shown in Fig. 1, in which the heat evolution in calories per liter of reacting solution is plotted against the time. The observed points deviate from the zero-order line during the first few minutes because of calorimetric lags, so that an extrapolation to zero time is necessary. It is to be noted that the intercept gives the total heat evolution *not* including the heat of mixing or the heat of any very rapid process immediately following the initiation of the reaction.

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[1] Presented before the Division of Biological Chemistry of the American Chemical Society, New York, N. Y., September, 1954.

[2] R. J. Podolsky, C. Kitzinger, T. H. Benzinger, J. M. Sturtevant and M. F. Morales, *Federation Proc.*, **13**, 112 (1954).

[3] N. S. Ging and J. M. Sturtevant, THIS JOURNAL, **73**, 2454 (1951).

[4] The enzyme preparation was kindly supplied by Dr. Gerhard Schmidt, of the Boston Dispensary.

[5] A. Buzzell and J. M. Sturtevant, THIS JOURNAL, **73**, 2454 (1951).

TABLE I

THE HEAT OF HYDROLYSIS OF *p*-NITROPHENYLPHOSPHATE AT 25°

Run	*p*H	Ionic strength, moles/l.	$[S]_0$, moles/l. $\times 10^3$	K_m, moles/l. $\times 10^3$	$-\Delta H_{app}$, cal./mole of PNP liberated	$x_1\Delta H_1$, cal./mole	$x_2\Delta H_2$, cal./mole	$-x_3\Delta H_3$, cal./mole	$-x_4\Delta H_4$, cal./mole	$-\Delta H$, cal./mole
4A	5.85	0.046	0.447	0.107	4040	290	80	0	1920	6330
4B	5.85	.046	.447	.109	4090	290	80	0	1920	6380
2A	5.37	.040	.854	.135	4830	100	20	0	1260	6210
2B	5.37	.040	.854	.139	4720	100	20	0	1260	6100
3A	5.27	.039	.430	.066	4830	80	20	0	1110	6040
3B	5.27	.039	.430	.071	5010	80	20	0	1110	6220
5A	4.76	.025	.585	.068	5790	20	0	0	480	6290
5B	4.74	.025	.585	.064	5780	20	0	0	470	6270
6A	4.32	.014	.462	.051	6120	10	0	10	160	6300
6B	4.32	.014	.462	.051	6170	10	0	10	160	6350
7A	3.82	.0050	.573	.056	6240	0	0	30	70	6340
7B	3.72	.050	.497	.051	6240	0	0	40	50	6330
8B	3.62	.0025	.520	.053	6320	0	0	50	50	6420
									Mean	6280 ± 100

The usual Michaelis–Menten formulation[6] of enzyme kinetics leads to the expression

$$V = -\frac{d[S]}{dt} = \frac{k_3[E]_0[S]}{K_m + [S]} \quad (1)$$

where [S] is the substrate concentration, $[E]_0$ the total enzyme concentration, K_m the Michaelis–Menten constant, and t the time. The slope of the zero-order line in Fig. 1 is evidently equal to $k_3[E]_0$. This information is not reported here because variable losses of enzyme activity took place during the thermal equilibration in the calorimeters, so that no absolute measure of enzyme concentration was available. However, the value of K_m can be evaluated as the substrate concentration at which the reaction rate is equal to half its maximum value, provided equation 1 is not invalidated by the relatively large concentration of reaction products present at the end of the zero-order portion of the reactions. Graphical evaluation of K_m is illustrated in Fig. 1, and the values obtained are recorded in column 5 of Table I. Substrate concentrations were based on the amount of *p*-nitrophenol liberated. Below *p*H 5.3, K_m appears to be constant at about 6.0×10^{-5} *M*; the data are insufficiently detailed to allow a certain conclusion that the apparent rise at higher *p*H values is real. Runs 7A and 7B show that K_m is unchanged by an increase in ionic strength from 0.005 to 0.05 *M*.

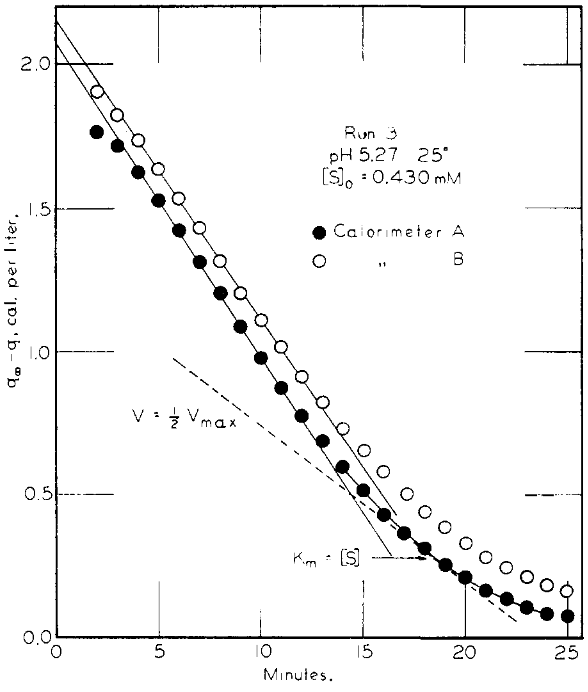

Fig. 1.—The kinetics of heat evolution in the hydrolysis of *p*-nitrophenylphosphate at 25°.

The observed heats of hydrolysis, in calories per mole of *p*-nitrophenol liberated, are listed in column 6 of Table I. There is an increase of over 50% between *p*H 5.85 and 3.62, which is to be attributed to ionization effects. At low *p*H the reaction is essentially

$$O_2N{\cdot}C_6H_4{\cdot}OPO_3H^-(aq) + H_2O = O_2N{\cdot}C_6H_4OH(aq) + H_2PO_4^-(aq);\ \Delta H \quad (2)$$

In addition, the following ionization reactions must be considered

$$O_2N{\cdot}C_6H_4OH + AcO^- = O_2N{\cdot}C_6H_4O^- + HOAc;\ \Delta H_1 \quad (3)$$

$$H_2PO_4^- + AcO^- = HPO_4^= + HOAc;\ \Delta H_2 \quad (4)$$

$$H_3PO_4 + AcO^- = H_2PO_4^- + HOAc;\ \Delta H_3 \quad (5)$$

$$O_2N{\cdot}C_6H_4{\cdot}OPO_3H^- + AcO^- = O_2N{\cdot}C_6H_4{\cdot}OPO_3^= + HOAc;\ \Delta H_4 \quad (6)$$

where $\Delta H_i = \Delta H_i' - \Delta H_{HOAc}$, $\Delta H_i'$ being the heat of ionization of the corresponding species, and ΔH_{HOAc} that of acetic acid. The first ionization of *p*-nitrophenylphosphate is neglected because it probably has such a low *pK* that it does not appreciably affect the results. The ionization of acetic acid is included since the solutions were strongly enough buffered to prevent a change of *p*H during the reaction. The ΔH of reaction (2) is given by

$$\Delta H = \Delta H_{app} - x_1\Delta H_1 - x_2\Delta H_2 + x_3\Delta H_3 + x_4\Delta H_4 \quad (7)$$

where x_1 is the fraction of *p*-nitrophenol in the form of the ion $O_2N{\cdot}C_6H_4O^-$ at the *p*H of the experi-

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[6] See, for example, J. S. Fruton and S. Simmonds, "General Biochemistry," John Wiley and Sons, Inc., New York, N. Y., 1953, pp. 241 ff.

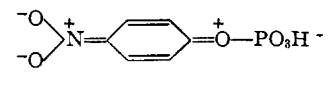
ment, and the other x's have corresponding significance. The x's may be calculated from the corresponding pK' values. The ionization corrections in columns 7–10 of Table I were calculated using the constants given in Table II. pK_4' and $\Delta H_4'$ were selected to secure the best constancy of the ΔH values listed in column 11 of Table I. After this selection had been made, a titration curve of p-nitrophenylphosphate was determined by titration with HCl, and from this curve pK_4' was found to be 5.38. Uncertainties in the other constants, particularly in pK_1' and $\Delta H_1'$, are such as to render $\Delta H_4'$ uncertain to the extent of approximately ±150 cal. per mole. The pronounced effect of the p-nitrophenyl group on the second ionization constant of phosphoric acid, a lowering of 1.5 pK units, is interesting. Simple ester groups in general produce a lowering of approximately half this amount or less,[7] in spite of the statistical factor of 0.3 pK unit favoring phosphoric acid. It is reasonable to attribute a part of the additional lowering in the present case to a small contribution from the resonance form

TABLE II

CONSTANTS EMPLOYED IN CALCULATING IONIZATION CORRECTIONS

i	pK_i'	ΔH_i, cal./mole	Reference
1	7.1	+5500	*a*
2	6.9	+ 820	*b*
3	2.1	−1770	*b*
4	5.4	−2700	*c*
ΔH_{HOAc} = −90 cal. per mole			*b*

[a] M. Bender and J. M. Sturtevant, THIS JOURNAL, **69**, 607 (1947). [b] H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," 2nd edition, Reinhold Publ. Corp., New York, N. Y., 1950, p. 514. [c] This paper.



which lowers the electron density in the vicinity of the dissociable proton. The fact that the entropy of ionization of p-nitrophenylphosphate (−34 entropy units per mole) is more positive than that of $H_2PO_4^-$ (−38) is also qualitatively in accord with this view, though it is probable that additional sources of entropy differences, over and above the statistical and resonance sources, are important.

Values of the heat of reaction 2, calculated according to equation 7, are given in the last column of Table I. These values are constant (average deviation from the mean = ±80 cal. per mole) over the entire pH range, which indicates that the ionization corrections have been correctly estimated. The precision uncertainty interval of 200 cal. per mole given in Table I includes an allowance for the uncertainty of the calorimetric calibrations.

In previous work, the heat of the reaction

$$ATP^{4-} + RNH_2 + H_2O = ADP^{3-} + HPO_4^{=} + RNH_3^+ \quad (8)$$

has been found[2] to be $\Delta H = -15{,}900$ cal. per mole. Here RNH_2 represents tris-(hydroxymethyl)-aminomethane (THAM), and ADP is adenosine diphosphate. Since THAM has a heat of ionization[8] $\Delta H = -10{,}900$ cal. per mole, the reaction

$$ATP^{4-} + H_2O = ADP^{3-} + HPO_4^{=} + H^+; \quad \Delta H = -5000 \text{ cal./mole} \quad (9)$$

has a heat of −5,000 cal. per mole.[9] Alberty, Smith and Bock[10] have shown that the heats of the third ionization of ADP and of the fourth ionization of ATP are negligible (for ADP, $pK' = 6.26$ and 6.27 at 25 and 38°, respectively, and for ATP 6.48 and 6.50) so that

$$ATP^{3-} + H_2O = ADP^{2-} + H_2PO_4^-; \quad \Delta H = -5{,}800 \text{ cal./mole} \quad (10)$$

The heat of hydrolysis of pyrophosphate at pH 7.3 and ionic strength 0.6–1.0 M has been reported[3] as −5810 cal./mole. This value was determined under conditions such that it applies to a reaction taking place with no pH change in the absence of a buffer. According to Schwartzenbach and Zurc,[11] the third and fourth ionizations of pyrophosphoric acid have $pK' = 6.08$ and 8.45 in 0.1 N KCl at 20°. If we assume these values to apply at 25° and a higher ionic strength, we conclude that more than 90% of the pyrophosphate is in the form $HP_2O_7^{3-}$ at pH 7.3. We then have to a good approximation

$$HP_2O_7^{3-} + H_2O = H_2PO_4^- + HPO_4^{=}; \quad \Delta H = -5{,}800 \text{ cal./mole} \quad (11)$$

If we restrict our attention to reactions in which there is no net liberation or absorption of hydrogen ions, data are available for three types of reaction

$$XOPO_3H^- + H_2O = XOH + H_2PO_4^- \quad (12)$$

$$YOPO_3^{=} + H_2O = YOH + HPO_4^{=} \quad (13)$$

$$ZOPO_3^{=} + H_2O = ZO^- + H_2PO_4^- \quad (14)$$

The enthalpy changes for these reactions are summarized in Table III. It is immediately evident that comparison of the enthalpy for the ester hydrolysis with the enthalpies for the hydrolyses of the "high energy" phosphate bonds depends on an arbitary choice of a particular reaction type. Thus, from reactions 12 one would conclude that the hydrolysis of the "low energy" ester bond is even more exothermic than the hydrolyses of the anhydride bonds, whereas reactions 14 lead to a comparison more in line with what might have been expected.

An equally arbitrary basis upon which to make a comparison is the actual reaction which would take place in each case at pH 7 in the absence of a buffer. For the "high energy" compounds, the figures given in Table III would still be approximately correct, and for the ester the hydrolysis heat would be closely the mean of the last two figures for this substance given in Table III, $\Delta H =$ −400 cal. per mole.

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[7] W. D. Kumler and J. J. Eiler, THIS JOURNAL, **65**, 2355 (1943).

[8] J. M. Sturtevant, unpublished result.

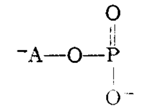
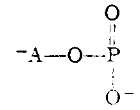
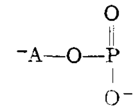
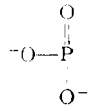
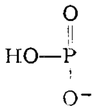
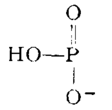
[9] This value is in good agreement with that reported by Podolsky, *et al.*, (ref. 2) at the Atlantic City meeting of the Federation of American Societies for Experimental Biology, April, 1954. A complete account of the basis for Podolsky's result will be given in a forthcoming publication.

[10] R. A. Alberty, R. M. Smith and R. M. Bock, *J. Biol. Chem.*, **193**, 425 (1951).

[11] G. Schwarzenbach and J. Zurc, *Monatsh.*, **81**, 202 (1950).

TABLE III

HEATS OF CONSTANT pH, UNBUFFERED HYDROLYSES OF PHOSPHATE COMPOUNDS AT 25°[a]

	Reaction 12		Reaction 13		Reaction 14	
Substrate	x	$-\Delta H$, cal./mole	y	$-\Delta H$, cal./mole	z	$-\Delta H$, cal./mole
p-Nitrophenol	$O_2N\cdot C_6H_4$	6280	$O_2N\cdot C_6H_4$	2760	$O_2N\cdot C_6H_4$	−1900
ATP	 $^{-}A{-}O{-}P(=O)(O^{-})$	5800	 $^{-}A{-}O{-}P(=O)(O^{-})$	5000	 $^{-}A{-}O{-}P(=O)(O^{-})$	5000
Pyrophosphate	 $^{-}O{-}P(=O)(O^{-})$	5800	 $HO{-}P(=O)(O^{-})$	5800	 $HO{-}P(=O)(O^{-})$	5800

[a] A represents the adenylate residue.

The primary factors in making the above comparisons so sensitive to the reaction type selected are the high heats of ionization of p-nitrophenol and p-nitrophenylphosphate. An additional factor which would have to be included in any complete discussion of the various heat values is the electrostatic interactions between the ions of various charge types. For example, reaction 13 for the ester involves no separation of charges, whereas in the case of ATP this reaction results in the separation of two doubly charged negative ions. A considerable *positive* contribution of electrostatic origin would be expected[12] in the ΔH of the ATP reaction if the effective dielectric constant for this case has a temperature coefficient similar to that of pure water.

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NEW HAVEN, CONN.

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[12] The electrostatic contributions to enthalpies of reaction, and their variation with ionic strength, will be discussed in a future communication.

[CONTRIBUTION NO. 1243 FROM THE DEPARTMENT OF CHEMISTRY OF YALE UNIVERSITY]

The Hydration of Desoxyribonucleic Acid

BY JUI H. WANG

RECEIVED AUGUST 6, 1954

The self-diffusion coefficient of water in aqueous sodium desoxyribonucleate solution at 25° was determined as a function of concentration The hydration of sodium desoxyribonucleate computed from the measured results is about 0.35 g. of water per g. of the dry desoxyribonucleate. The effect of added salt on the hydration and shape of the nucleic acid molecule is examined, and the broadening of the proton magnetic resonance lines in aqueous sodium desoxyribonucleate solutions is discussed.

The hydration of nucleic acids has been the subject of speculation of many investigators.[1,2] Recently Jacobson, Anderson and Arnold[3] have made some interesting measurements on the proton magnetic resonance in aqueous sodium desoxyribonucleate solutions. They concluded that desoxyribonucleic acid is much more highly hydrated than hemocyanin, egg albumin, etc., and that on adding sodium chloride to the nucleic acid solutions considerable amounts of water previously bound are released to the normal water state. Since some quantitative knowledge of this hydration is of importance both to the size and shape studies by dynamic method and to the development of the theory of line-breadth of proton magnetic resonance in aqueous solutions, the subject is further examined by means of self-diffusion measurements in the present work. The method of computing the hydration of the macromolecule from the measured self-diffusion coefficient of water in solution has been described in an earlier article.[4] The experimental procedure for self-diffusion measurements is essentially the same as that used by Wang, Anfinsen and Polestra.[5]

Experimental

Diffusion Measurements.—H_2O^{18} was used as tracer to determine the self-diffusion coefficients of water in sodium desoxyribonucleate solutions. This was supplied by Stuart Oxygen Company, and obtained on allocation from the Isotopes Division, U. S. Atomic Energy Commission. The experimental method has already been described.[5] The diffusion samples were analyzed directly by means of a Consolidated-401 mass-spectrometer.

Preparation of Bath Solutions.—Desoxyribonucleic acid prepared from thymus by Delta Chemical Works, New York, was used. This was dissolved in distilled water by stirring overnight at 1°. The solution was then kept in an ice-bath and slowly neutralized with dilute sodium hydroxide solution until pH 7.8. Some sodium chloride was now added to the solution. The mixture was then alternately dialyzed and concentrated (by evaporation through the cellulose bag at 1°) for 7 days. The resulting solution was

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[1] B. Jacobson, *Nature*, **172**, 666 (1953).

[2] M. E. Reichmann, S. A. Rice, C. A. Thomas and P. Doty, THIS JOURNAL, **76**, 3047 (1954).

[3] B. Jacobson, W. A. Anderson and J. T. Arnold, *Nature*, **173**, 772 (1954).

[4] J. H. Wang, THIS JOURNAL, **76**, 4755 (1954).

[5] J. H. Wang, C. B. Anfinsen and F. M. Polestra, *ibid.*, **76**, 4763 (1954).