ARTICLE:

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Thermodynamic Parameters for the Hydrolysis of Inorganic Pyrophosphate at pH 7.4 as a Function of $[Mg^{2+}]$, $[K^+]$, and Ionic Strength Determined from Equilibrium Studies of the Reaction

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SUMMARY

The equilibrium between inorganic pyrophosphate and inorganic orthophosphate was studied by direct measurement of PP_i. The reaction was catalyzed by yeast pyrophosphatase in the presence of Mg^{2+} ions or by alkaline phosphatase in the absence of metal ions. The concentration of PP_i was determined by an isotope derivative method. Identical concentrations of PP_i were obtained whether the reaction proceeded from orthophosphate or pyrophosphate provided other conditions were identical. The equilibrium concentrations were measured at varying Mg^{2+} and K^+ ion concentrations and at different ionic strengths. All measurements were performed at pH 7.4. Equilibrium was established at different temperatures and the enthalpy changes were determined.

The experimental data were used to compute an equilibrium constant (K_{ionic}) based on the ionic species

$$HPP^{3-} \rightleftarrows 2HP^{2-} + H^+$$

K_{ionic} was found to $(2.41 \pm 0.20) \times 10^{-4}$ M² (zero ionic strength) corresponding to a $\Delta G_{ionic} = (4.98 \pm 0.05)$ Cal × mole^{-1} (20.84 kJ × mole^{-1}). It was constant over the variety of conditions studied. From the experiments carried out in absence of metal ions and low ionic strength $\Delta G'^{0}_{obs}$ was found to (-5.63 ± 0.02) Cal × mole^{-1} (-23.56 kJ × mole^{-1}) calculated for zero ionic strength making due allowances for dissociation at pH 7.4. $\Delta H'^{0}_{obs}$ was found to (-1.90 ± 0.37) Cal × mole^{-1} (-7.95 kJ × mole^{-1}) and $T\Delta S'^{0}_{obs}$ to 3.87 Cal × mole^{-1} (16.19 kJ × mole^{-1}) (the latter two results given for ionic strength 0.127). Increasing the Mg^{2+} ion concentration makes the reaction more unfavorable, thus at 26 mM free $[Mg^{2+}]$ and 18 mM free $[K^+]$ at ionic strength 0.1 $\Delta G'^{0}_{obs}$ was found to (-2.73 ± 0.07) Cal × mole^{-1} (-11.42 kJ × mole^{-1}).

The stoichiometry of the participation of H^+ and metal ions in the composite reaction was calculated. The contribution of the observed enthalpy and entropy terms to the variation in $\Delta G'^{0}_{obs}$ is discussed in relation to the changes in production or consumption of H^+ and metal ions. At free $[Mg^{2+}]$ above 0.3 mM and low free $[K^+]$ (17 to 43 mM) at ionic strengths around 0.05 the reaction was found to consume acid. The $T\Delta S'^{0}_{obs}$ term which is negative was found to pass through a maximum negative value of -4.17 Cal × mole^{-1} (-17.53 kJ × mole^{-1}) at 12.2 mM free $[Mg^{2+}]$. The entropy term is numerically considerably larger than the enthalpy term, resulting in a less favorable reaction. $\Delta H'^{0}_{obs}$ values were calculated on the basis of ΔH^0 for dissociation and association reactions and agreement with the experimental results was obtained in the cases where ΔH^0 values reported in the literature were measured at experimental conditions similar to ours.

From $\Delta G'^{0}_{obs}$ measured at the ionic strength and $[K^+]$ and $[Mg^{2+}]$ prevailing in the cell, ΔG for the PP_i hydrolysis in the cell was calculated to -4.0 Cal × mole^{-1} (-16.74 kJ × mole^{-1}) on the basis of 1 mM free $[Mg^{2+}]$, 150 mM free $[K^+]$, 2.42 mM free $[P_i]$, and concentrations of PP_i measured in freeze biopsies from rat liver to (6.2 ± 0.3 (S.E. n = 6)) nmoles per g wet weight. The implication of this result for the utilization of the free energy of PP_i hydrolysis in the cell is discussed.

In an appendix a set of equations were introduced, which permit computation of equilibrium data also in a general case.

Inorganic pyrophosphate plays a central role in the chemistry of living cells. As discussed by Lehninger (1) and by Stetten (2) synthetic reactions which produce pyrophosphate are subject to an additional "thermodynamic pull" due to the subsequent hydrolysis of pyrophosphate by widely distributed pyrophosphatases. Consideration of inorganic pyrophosphate as an energy source arises also from the investigations of Arion and Nordlie (3) and of Stetten and Taft (4), who have extended observations previously reported by Rafter (5), Segal (6), and Hass and Byrne (7). They have shown that inorganic pyrophosphate can be used to phosphorylate glucose *in vitro*.

In attempts to understand the role of inorganic pyrophosphate it is important to have accurate thermodynamic data for its hydrolysis, particularly at pH values and ionic strengths prevailing in the cell. However, great uncertainty exists about these parameters. Direct measurements of the equilibrium

of the pyrophosphatase reaction have been carried out by Stiller *et al.* (8). Their preliminary experiments gave values for $\Delta G^{0'}_{obs}$ of -5.8 to -7.4 Cal $\times$ mole^{-1} at 25°, pH 7.5, and ionic strength 0.25.[1] Calculations of the free energy change of hydrolysis of inorganic pyrophosphate have been made by Alberty (9) and by Wood *et al.* (10) by adding the free energy changes of hydrolysis of several different reactions. However, their results differ considerably due to insufficient data concerning the equilibrium constants for many of the reactions used to arrive at the pyrophosphatase reaction. Furthermore, uncertainties about the stability constants for Mg^{2+} complexes contribute in a similar way to the discrepancy between their results.

The values for the free energy change of hydrolysis of inorganic pyrophosphate so far reported can therefore not be regarded as reliable.

Similar uncertainty has hitherto also existed concerning the enthalpy change and the contribution of the entropy change to the free energy change of hydrolysis of inorganic pyrophosphate. The lack of agreement is striking when one considers the value of -2.1 Cal $\times$ mole^{-1} for the free energy change of the hydrolysis of pyrophosphate estimated (11) from a value for ΔH calculated from the heats of formation of pyrophosphate and orthophosphate. Although the same authors in a note together with Stiller *et al.* (8) corrected this high value caused by their unawareness of the anomalous low partial molal entropies of the pyrophosphate species, calorimetric measurements carried out later (12) do not fully elucidate the thermodynamic quantities underlying the hydrolysis of inorganic pyrophosphate, especially not with respect to the entropy contribution.

In the present paper an isotope derivative method for determination of microquantities of inorganic pyrophosphate (13) has been used to measure final concentrations of inorganic pyrophosphate after equilibrium has been established from both directions in the reaction:

$$PP_i \rightleftarrows P_i + P_i$$

under well defined conditions. The temperature dependence of the equilibrium has been used to calculate the enthalpy change and the entropy change contribution. In addition, digital computer calculations have been carried out with the aim of converting the experimental results into an equilibrium constant based on selected ionic species not comprising metal complexes.

Finally standard enthalpy change for the metal association and acid dissociation reactions involved have been used to calculate the enthalpy change for the composite reaction.

EXPERIMENTAL PROCEDURE

Isotope Assay for Determination of Inorganic Pyrophosphate—The assay previously described (13) was based upon the formation of [5-^{3}H]UTP during a reaction between [5-^{3}H]UDP-glucose and PP_i catalyzed by UDP-glucose pyrophosphorylase (EC 2.7.7.9) under conditions where formation of [5-^{3}H]UTP was favored by addition of phosphoglucomutase (EC 2.7.5.1) $NADP^+$, and glucose 6-phosphate dehydrogenase (EC 1.1.1.49). From the specific activity of the [5-^{3}H]UTP isolated with excess of carrier the amount of PP_i was calculated by comparison with standard assays. Although the principle in the assay used in the present work is the same, several refinements have been introduced. Preparation of [5-^{3}H]UDP-glucose with a specific activity of 22 Ci per mM by conversion of commercially available [5-^{3}H]UTP via the UDP-glucose pyrophosphorylase reaction was carried out as described earlier (13). Reaction mixtures, usually in portions of 3 ml, containing 0.1 M triethanolamine buffer, pH 7.6, 4 mM $MgCl_2$, 6 mM mercaptoethanol, 1 mM $NADP^+$, and 0.2 mM [5-^{3}H]UDP-glucose diluted to a specific activity of 500 mCi per mM were incubated for 1 hour at 25° with 3 units of inorganic pyrophosphatase (EC 3.6.1.1) in order to remove endogenous pyrophosphate. The enzyme was removed by ultrafiltration overnight in the cold using the filter technique shown in Fig. 2. The ultrafiltrate was frozen to $-70°$ in portions of 450 μl until used. To 450 μl of the reaction mixture were then added 8 units of phosphoglucomutase and 3.5 units of glucose 6-phosphate dehydrogenase in a volume of 50 μl of 0.01 M triethanolamine buffer, pH 7.6. The enzymes were freshly dialyzed against the same buffer. To 50-μl samples were added 50 μl of reaction mixture supplemented with the enzymes, and preincubation in small disposable plastic tubes was carried out for 15 min at 25°. The reaction was then initiated by addition of 1 unit of UDP-glucose pyrophosphorylase to each sample and the incubation was continued for another 15 min. The reaction was stopped by boiling for 5 min. The samples were cooled under tap water, and 1 ml of water containing 200 nmoles of UDP-glucose, and 100 nmoles of UTP were added to each sample. The [5-^{3}H]UTP formed, now diluted with the carrier, was isolated for determination of the specific activity using the thin layer chromatography technique previously described (13).

Treatment of the reaction mixture with inorganic pyrophosphatase prior to use causes a decrease in the endogenous PP_i in the assay down to 5×10^{-9} M as tested from control experiments using distilled water as sample (the blank value in Fig. 1). By use of acid-treated utensils throughout, together with disposable plastic equipment where possible, contamination with PP_i was avoided. PP_i concentrations down to 10^{-8} M could be measured with an intra-assay analytical error (S.D.) of 5%. The interassay S.D. was about 10%. The favorable equilibrium position of the reaction due to the auxiliary enzyme system added causes a 100% incorporation of PP_i into [5-^{3}H]UDP-glucose present in excess in the assay thus forming stoichiometric amounts of [5-^{3}H]UTP. Linear standard curves were always obtained within the concentration range investigated (Fig. 1). The reliability of the PP_i measurements in the different equilibrium mixtures used have been tested by recovery experiments described under "Results."

Preparation of Tetra-n-propyl Ammonium Phosphate Buffers—Commercial tetra-*n*-propyl ammonium chloride was converted to its hydroxide by ion exchange chromatography on Dowex 1, 200 to 400 mesh on hydroxide form. Orthophosphoric acid was titrated with the tetra-*n*-propyl ammonium hydroxide to pH 7.4 and adjusted with water to the desired molarity.

Materials—[5-^{3}H]UTP (as tetrasodium salt) with a specific activity around 20 Ci per mM was purchased from NEN Chemicals, GmbH, Germany. UDP-glucose, UTP, $NADP^+$, UDP-glucose pyrophosphorylase, phosphoglucomutase, glucose 6-phosphate dehydrogenase, inorganic pyrophosphatase from yeast, and alkaline phosphatase (EC 3.1.3.1) from calf intestine were purchased

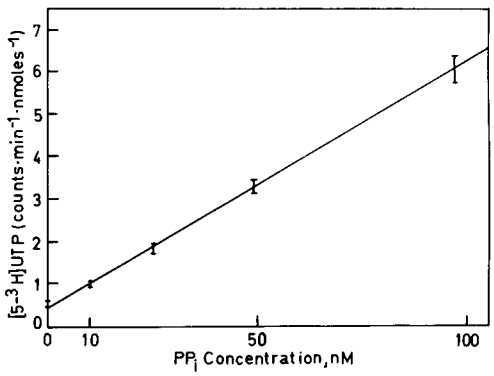

FIG. 1. Standard curve obtained with the isotope derivative method for determination of inorganic pyrophosphate (PP_i). Freshly prepared standard solutions of PP_i in the range from 10^{-8} M to 10^{-7} M in twice distilled water were used. PP_i was measured as described in the experimental section. The *vertical lines* indicate S.D.

[1] The prime superscript denotes that the quantity is evaluated with the concentration of H^+ of the experiment as its standard state instead of the 1 molal standard state. The "obs" subscript denotes what actually has been measured (observed) at the particular experimental condition.

from Boehringer and Soehne, Mannheim, Germany. Potassium orthophosphate (both primary and secondary), orthophosphoric acid, tetrasodium pyrophosphate and $MgCl_2$ were purchased from E. Merck, Darmstadt, Germany, all the chemicals being of analytical grade. Triethanolamine was bought from Boehringer and Soehne, Mannheim, Germany. Tetra-*n*-propyl ammonium chloride was purchased from Eastman Kodak Co., Rochester.

Procedure—The composition of the phosphate buffers used is given in Table II. Because of the limited solubility of magnesium phosphate, addition of $MgCl_2$ in concentrations higher than 10 mM was only carried out with 10 mM buffers. When an ionic strength higher than that of the buffer substances and the metal ions themselves was desired, addition of KCl and tetra-*n*-propyl ammonium chloride, respectively, was employed (Table II). The use of the latter salt to increase the ionic strength makes it possible to study the reaction at different ionic strengths without affecting the free metal ion concentrations to any larger extent.

Pyrophosphatase equilibrium was established in the various buffers by incubation with inorganic pyrophosphatase in the presence of Mg^{2+} ion and with alkaline phosphatase in the absence of this metal ion using the method described in Fig. 2.

RESULTS

Recovery Experiments—In Fig. 3 standard curves are shown obtained by preparing standard solutions of PP_i in 50 mM potassium phosphate buffer, pH 7.4, and in the same buffer with addition of 5 mM $MgCl_2$. Both buffers were brought to pyrophosphatase equilibrium and freed from the enzymes as described in Fig. 2. The intercepts of the two straight lines with the ordinate are not different from the equilibrium concentrations of PP_i measured before addition of known amounts of PP_i, indicating that recovery within the analytical error was achieved over a broad range of PP_i concentrations in the presence of high orthophosphate concentrations. From Table I is seen that PP_i added to various of the tetra-*n*-propyl ammonium phosphate buffers was recovered within the experimental error, indicating that no interference from this cation was introduced.

Determination of K'_{obs}—Equilibrium was reached after 30 to 60 min of incubation with inorganic pyrophosphatase (0.2 units per ml) at 25° and free Mg^{2+} at a concentration approximately 1 mM (for details see Fig. 2). However, equilibrium was in addition established over a temperature range from 5°–40° for determination of $\Delta H^{0'}_{obs}$ (Fig. 4). Therefore, incubation times from 12 to 48 hours were used as routine, the longest time was

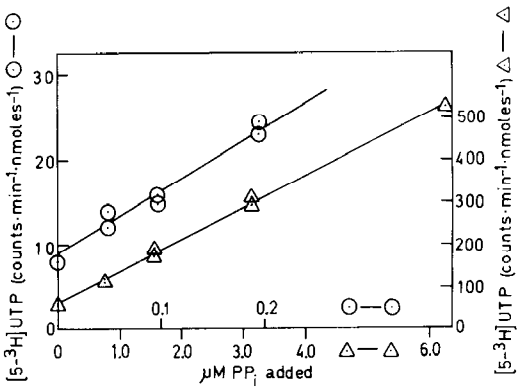

FIG. 3. Recovery of inorganic pyrophosphate (PP_i) in the presence of high orthophosphate concentrations. Orthophosphate buffers, pH 7.4, 50 mM (○) and identical buffer containing in addition 5 mM $MgCl_2$ (△) were brought to pyrophosphatase equilibrium using the technique described in Fig. 2. The equilibrium concentrations of PP_i were found to be 7.89 ± 0.01 and 60.10 ± 9.29 cpm × $nmole^{-1}$, respectively. (The results can be converted to μM PP_i from standard curves similar to Fig. 1.) The intercepts on the ordinate of the two recovery curves (8.67 ± 0.01 and 62.43 ± 7.99 cpm × $nmole^{-1}$) are not significantly different from the equilibrium concentrations of PP_i measured prior to addition of known amounts of PP_i ($0.6 < p < 0.7$ and $0.8 < p < 0.9$, respectively).

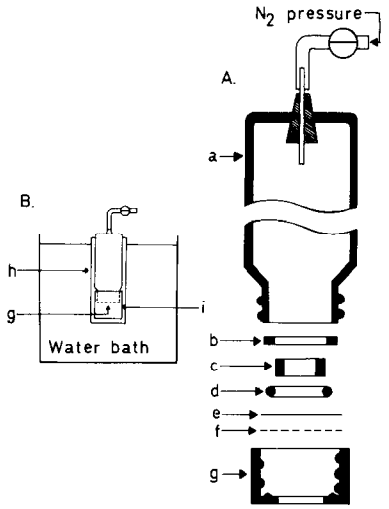

FIG. 2. Equipment used to incubate the orthophosphate buffers with pyrophosphatase to equilibrium and to free the mixtures for the enzyme. *a*, 30-ml polyethylene vial (vial for liquid scintillation counting); *b*, plastic washer; *c*, plastic ring which fit into the O-ring (*d*); *e*, dialysis membrane (cellophane, Union Carbide); *f*, perlon sieve (Monodur 90 S, Vereinigte Seidenwebereien AG Krefeld, Germany); *g*, screw cap to the vial; *h*, sealing rubber jacket; *i*, reservoir. To the various mixtures to be equilibrated (Table II) was added inorganic pyrophosphatase when Mg^{2+} was present, otherwise alkaline phosphatase, to enzyme concentrations of 0.2 unit per ml and 3 units per ml, respectively. The enzymes were freshly dialyzed against excess of the same mixtures to be equilibrated in order to remove $(NH_4)_2SO_4$. Portions between 1 and 5 ml of the mixtures were placed in the vials (*a*), tightened with the dialysis membrane (*e*) by means of the equipment shown (*A*). The filters sealed with the rubber jacket (*h*) were incubated at the desired temperatures in waterbath (*B*). After 12 to 48 hours incubation (see discussion under "Results"), 4 atm of N_2 pressure were applied as shown in *A* and the incubation was continued for approximately 4 hours until enough mixture was collected in the reservoir (*i*) for determination of PP_i.

TABLE I

Recovery of inorganic pyrophosphate (PP_i) added to some equilibrated tetra-n-propyl ammonium phosphate buffers used in experiments

The numbers in parentheses refer to the experiments in Table II.

Composition of buffers, pH 7.4	Equilibrium concentration of PP_i at the temperature indicated, measured before addition of PP_i		PP_i added	PP_i found	Recovery
		μM	μM		%
50 mM tetra-*n*-propyl ammonium phosphate buffer (3)	18.5°	0.099	0.070	0.180	107
	25.0°	0.106	0.140	0.255	104
0.010 M tetra-*n*-propyl ammonium phosphate buffer + 0.020 M $MgCl_2$ (20)	11.2°	0.491	0.563	1.220	116
	19.0°	0.608	0.563	1.265	108
	25.0°	0.833	0.563	1.420	102
0.010 M tetra-*n*-propyl ammonium phosphate buffer + 5 mM $MgCl_2$ (19)	38.0°	0.459	0.140	0.626	96
0.010 M tetra-*n*-propyl ammonium phosphate buffer + 5 mM $MgCl_2$ + 0.255 M tetra-*n*-propyl ammonium chloride (21)	25.0°	0.210	0.140	0.368	105

used in the experiments with excess of free Mg^{2+} which inhibits the inorganic pyrophosphatase, and also in some experiments with alkaline phosphatase where high orthophosphate concentrations were present as these are known to inhibit the pyrophosphatase activity of this enzyme (14). The data obtained are presented in Table II and K'_{obs} calculated as shown.

Evidence for Equilibrium—Incubation of the reaction mixture up to 1 week did not alter the results obtained using incubation times up to 48 hours. The same value for K'_{obs} was obtained when equilibria were approached from either direction (Table II). The content of inorganic pyrophosphate in commercial orthophosphate preparations (0.01 to 0.04 mole %) of analytical grade found by us and by others (15) was always far above the equilibrium concentrations of pyrophosphate obtained. Therefore, removal of pyrophosphate was necessary in order to carry out the reaction from orthophosphate. Removal of pyrophosphate was impossible using anion exchange chromatography and recrystallization. The desired buffers were therefore diluted 10 times with water and treated with inorganic pyrophosphatase for 2 days. The enzyme was removed by ultrafiltration using the technique shown in Fig. 2 and the ultrafiltrate subsequently freeze-dried in plastic equipment and redissolved to the original volume. Concentrations of inorganic pyrophosphate not different from the blank value in the isotope assay used for measuring the pyrophosphate (10^{-9} M) were found in the buffers treated in this way. Since the pyrophosphatase reaction is exothermic, reincubation at higher temperatures of the buffers brought to equilibrium at the low temperatures results in formation of pyrophosphate from orthophosphate to equilibrium concentrations not different from those obtained by proceeding from the opposite side at identical high temperatures (Table III). The equilibrium constant of the pyrophosphatase reaction is very sensitive to dilution due to the square term in the numerator. From Experiments 1 and 2 in Table II is seen that the same value for K'_{obs} was obtained using 25 and 50 mM buffers in the absence of Mg^{2+} apart from

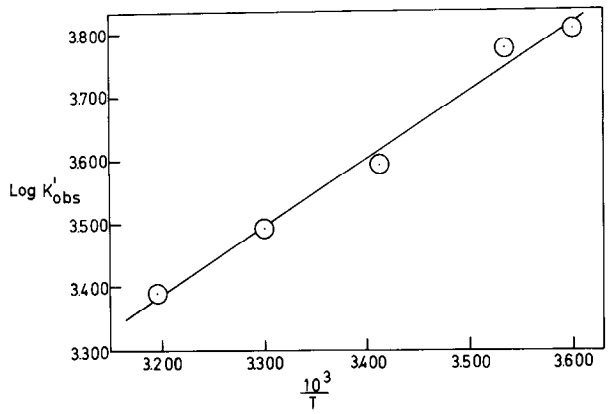

FIG. 4. Plot of log K'_{obs} *versus* 1/T for determination of $\Delta H^{0'}_{obs}$. The composition of the phosphate buffer is given in Experiment 12 in Table II. The equilibrium was established at the temperatures indicated using the technique described in Fig. 2. The slope of the straight line equal to $-\Delta H^{0'}_{obs}/(2.3R)$ gives (-4.93 ± 0.81) Cal × mole^{-1} (Experiment 12 in Table V). The result is given as mean ±S.E. calculated from the standard deviation of the slope. Similar plots give the other results presented in Table V.

TABLE II

Determination of equilibrium constant (K'_{obs}) of pyrophosphatase reaction $PP_i \rightleftarrows 2\ P_i$ at pH 7.4 and 25°

Phosphate buffers, pH 7.4, containing K^+ and tetra-*n*-propyl ammonium as supporting cations, respectively, were brought to pyrophosphatase equilibrium at 25° by treatment with alkaline phosphatase using the technique shown in Fig. 2. Similar buffers containing $MgCl_2$ or in addition KCl and tetra-*n*-propyl ammonium chloride, respectively, were treated in the same way with inorganic pyrophosphatase. The equilibrium was established by reaction from the left because the orthophosphate contains endogenous PP_i (0.01 to 0.04 mole %), whereby the initial PP_i concentration was far above the equilibrium concentrations obtained. Conducting of the reaction from the right as indicated was brought about in the buffers freed from PP_i prior to incubation. K'_{obs} was calculated from the P_i molarity of the buffers, and the equilibrium concentrations of PP_i measured by the isotope derivative method described under "Experimental Procedure." Figures are given as mean ± S.E. Number of experiments, *n*, are given in parentheses.

Experiment No.	Molarity of the buffers $[P_i]$	Equilibrium concentration $[PP_i]$	Total concentrations				$\frac{[P_i]^2}{[PP_i]}$ $K'_{obs} \times 10^{-2}$	Reaction conducted from right $K'_{obs} \times 10^{-2}$
			$[Mg^{2+}]$	$[K^+]$	tetra-n-propyl	$[Cl^-]$		
	mM	μM	mM	mM	mM	mM	M	M
1	25	0.052	0	45	0	0	120 ± 5 (n = 4)	
2	50	0.159	0	90	0	0	157 ± 3 (n = 9)	
3	50	0.147	0	0	90	0	170 ± 7 (n = 8)	
4	2.5	0.003	0.1	4.5	0	0.2	25 (n = 1)	
5	5.0	0.010	0.1	9.0	0	0.2	26.3 (n = 1)	
6	20	0.338	5.0	36	0	10	11.8 ± 0.1 (n =16)	
7	10	0.203	5.0	18	0	10	4.93 ± 0.34 (n = 4)	
8	10	0.465	10	18	0	20	2.15 ± 0.19 (n = 4)	
9	10	0.870	20	18	0	40	1.15 ± 0.11 (n = 4)	
10	10	0.995	40	18	0	80	1.01 ± 0.12 (n = 3)	
11	20	0.106	0.5	44.5	0	14.6	37.7 (n = 1)	
12	25	0.177	1.0	45	0	20	35.3 ± 2.10 (n = 4)	
13	50	0.357	1.0	207	0	120	70.0 ± 1.20 (n = 4)	
14	10	0.180	5.0	44	0	36	5.56 (n = 1)	
15	25	0.725	5.0	215	0	180	8.62 ± 2.10 (n = 2)	7.87 ± 0.34 (n = 3)[a]
16	50	1.85	5.0	195	0	115	13.5 ± 1.06 (n = 7)	10.50 ± 1.12 (n = 4)[a]
17	75	3.00	5.0	53.5	0	50	18.5 ± 3.51 (n = 3)	21.50 ± 3.24 (n = 2)[a]
18	50	1.85	10.0	180	0	110	13.5 ± 0.40 (n = 8)	13.30 ± 1.20 (n = 2)[a]
19	10	0.259	5.0	0	18	10	3.87 ± 0.34 (n = 4)	
20	10	0.903	20.0	0	18	40	1.11 ± 0.06 (n = 8)	
21	10	0.213	5.0	0	220	230	4.69 ± 0.51 (n = 3)	
22	10	0.733	20.0	0	220	260	1.36 ± 0.13 (n = 3)	

[a] K'_{obs} values not different from the K'_{obs} values obtained by reaction from left $0.8 < P < 0.1$

TABLE III

Equilibrium concentrations of PP_i obtained after reincubation at higher temperatures of some of the mixtures brought to equilibrium at lower temperatures

The Experiment numbers refer to the experiments in Table II. The incubation method is described in Fig. 2.

Experiment	Equilibrium concentration of PP_i at the temperature indicated		Equilibrium concentration of PP_i obtained by reincubation at the higher temperature		Equilibrium concentration of PP_i obtained by conducting the reaction from the other side, at the high temperature shown	
		μM		μM		μM
20	11.2°	0.491	19.0°	0.543	19.0°	0.608
19	10.8°	0.203	34.6°	0.362	34.6°	0.387
21	10.8°	0.184	34.6°	0.311	34.6°	0.314

the small difference to be expected due to the higher K^+ concentration and the higher ionic strength in Experiment 2. As discussed below the pyrophosphatase reaction is more favorable with increasing ionic strength and free $[K^+]$.

Determination of $\Delta G^{0'}_{obs}$, $\Delta H^{0'}_{obs}$ and $T\Delta S^{0'}_{obs}$ from Observed Equilibrium Constants, K'_{obs}—The formula $\Delta G^{0'}_{obs} = -RT\ln K'_{obs}$ was used to calculate $-\Delta G^{0'}_{obs}$ from the K'_{obs} values in Table II. $\Delta H^{0'}_{obs}$ was calculated from the equilibrium experiments carried out at different temperatures (Fig. 4). Linear plots of log K'_{obs} *versus* $1/T$ were always obtained within the temperature range studied (5°–40°), and $\Delta H^{0'}_{obs}$ was calculated from the slopes equal to $-\Delta H^{0'}_{obs}/(2.3\ R)$. $T\Delta S^{0'}_{obs}$ was calculated from $\Delta G^{0'}_{obs} = \Delta H^{0'}_{obs} - T\Delta S^{0'}_{obs}$. The results obtained are given in Table V.

CALCULATIONS

The observed thermodynamic functions derived from the experimental equilibrium constants, K'_{obs}, are expressed in terms of the total concentrations of all the ionic species of P_i and PP_i (both free and in complex) present at equilibrium at pH 7.4. Because the hydrolysis of PP_i implies that a reactant is converted into a product with much weaker binding properties for metal ions, it is desirable to relate the observed thermodynamic quantities to the thermodynamic quantities for the individual ionic reaction which occurs. Thermodynamic parameters for all the association and dissociation reactions considered were selected from published data (Table IV). No values for ΔH^0 for $KP_2O_7^{3-}$ and $Mg_2P_2O_7$ complex formation were available. ΔH^0 for the first compound was taken to be equal to ΔH^0 for the potassium orthophosphate complex formation (16). The entropy of dissociation of the $Mg_2P_2O_7$ complex was taken to be the same as for the uncharged species $MgHPO_4$ and ΔH^0 calculated with the use of this value for ΔS^0 (9). The media to which the constants apply are indicated in Table IV. Calculations of the free ion concentrations from the total concentrations and calculations of the correct ionic strengths were now possible by use of the appropriate association and dissociation constants in combination with the procedure of Kielland (17) for adjustment of ionic strengths.[2] Calculation of the

[2] Some of the data are presented as a miniprint supplement immediately following this paper. Material published in miniprint form can be easily read with the aid of a large-field reading glass of a type readily available at most opticians. For the convenience of those who prefer to obtain supplementary material in the form of a microfiche or full size photocopies, these same data are available as JBC Document No. 73M-1210. Orders for supplementary material should specify the title, authors, and reference to this paper and the JBC Document Number, the form desired, microfiche or full sized photocopy of 9 pages, and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $2.50 for microfiche or for photocopy.

TABLE IV

Thermodynamic parameters for dissociation and association reactions used in calculations at 25° and pH 7.4

The figures in parentheses are the association constants reported by Schuegraf *et al.* (26).

Acids	K values	ΔH^0
		Cal/mole
$HP_2O_7^{3-} = H^+ + P_2O_7^{4-}$	1.12×10^{-9} (18)[a]	0.40 (19)[b]
$H_2P_2O_7^{2-} = H^+ + HP_2O_7^{3-}$	7.59×10^{-7} (18)[a]	0.11 (19)[a]
$H_2PO_4^- = H^+ + HPO_4^{2-}$	1.66×10^{-7} (20)[c]	0.80 (21)[e]
Complexes		
$Mg^{2+} + P_2O_7^{4-} = MgP_2O_7^{2-}$	2.57×10^5 (22)[f] [1.60×10^6]	3.50 (12)[g]
$Mg^{2+} + HP_2O_7^{3-} = MgHP_2O_7^-$	1.15×10^3 (22)[f] [4.20×10^3]	3.50 (12)[g]
$Mg^{2+} + MgP_2O_7^{2-} = Mg_2P_2O_7$	2.19×10^2 (22)[f] [4.50×10^2]	2.26
$Mg^{2+} + HPO_4^{2-} = MgHPO_4$	76 (20)[c]	2.90 (23)[d]
$Mg^{2+} + Cl^- = MgCl^+$	3.40 (24)	0.00
$K^+ + P_2O_7^{4-} = KP_2O_7^{3-}$	6.30 (25)[f]	6.00
$K^+ + HPO_4^{2-} = KHPO_4^-$	3.10 (16)[h]	6.00 (16)[h]

a. 0.1 M $(CH_3)_4NCl$

b. 0.22 M $(CH_3)_4NCl$

c. 0.2 M $(n\text{-propyl})_4NCl$

d. Zero ionic strength

e. 0.01 ionic strength

f. 1.0 M $(CH_3)_4NCl$

g. 0.05 ionic strength

h. 0.2 ionic strength

stoichiometric mole number, n, for the participation of H^+ and metal ion in the composite reaction was thereafter possible. Finally, on basis of a value of ΔH^0 for the pyrophosphatase reaction taken from the calorimetric experiments of Wu *et al.* (12), it was possible to calculate $\Delta H^{0'}_{obs}$ for the total reaction using the free metal ion concentrations and ΔH^0 for the association and dissociation reactions involved. It becomes thus practicable to compare the $\Delta H^{0'}_{obs}$ values determined from the equilibrium experiments to $\Delta H^{0'}_{obs}$ values calculated from a quite different concept.

Even if the required constants are known, the labor of making all the calculations mentioned above encourages the introduction of approximations and short cuts. Fortunately, the modern digital computer eliminates this problem and so it is possible to take all the individual ionic components reactions into consideration. In order to facilitate the complicated and tedious iterative procedures necessary for the calculation of the free ion concentrations and the correct ionic strengths the general equations worked out in the "Appendix" were used. The concepts used and the details in the calculation are thoroughly treated in this section.

Determination of K_{ionic}. Further Evidence for Equilibrium—For the purpose of comparing the equilibrium data obtained under the different conditions employed the following reference reaction was chosen:

$$HP_2O_7^{3-} \rightleftarrows 2HPO_4^{2-} + H^+$$

The calculation of the equilibrium constant, K_{ionic}, for this

reaction is given in the "Appendix." The calculations are based upon the concept that the observed equilibrium constant K'_{obs} can be expressed as a product of two factors, one which is a true thermodynamic constant (K_{ionic}) reflecting the appropriate quantity for a reference reaction in which each reactant is represented by a particular ionic species not comprising metal complex, and another involving free metal ion concentrations. It follows therefore that the values obtained for K_{ionic}, corrected for activities, must be constant under all conditions provided that the measured quantities are true equilibrium concentrations. From the 22 experiments in Table II an average value ±S.E. of K_{ionic} was found to $(2.41 \pm 0.20) \times 10^{-4}$ M[2] corresponding to a $\Delta G_{ionic} = (4.98 \pm 0.05)$ Cal × mole^{-1} (zero ionic strength).[2] Thus, the constancy of the results obtained strongly indicates that equilibrium has been attained under all the experimental conditions. The agreement between the values calculated from the experiments carried out in the presence and absence of metal ions, respectively, shows that the thermodynamic parameters adopted for association and dissociation reactions involved are valid (Table IV). Moreover, when the larger constants for magnesium binding to PP_i species shown in brackets in Table IV were used no agreement was obtained between the figures from the experiments in the presence and absence of metal ions. In addition the large variations in the values obtained indicate that these binding constants, which have been extrapolated to ionic strength 0.2 by Schuegraf *et al.* (26) from the original measurements of Lambert and Watters (22) at ionic strength 1.0, are not valid.

Determination of Chemical Potential Equation—Completion of the stoichiometry of PP_i hydrolysis by inclusion of the "n" terms for the participation of $n$moles of H^+ and $n$moles of metal ions, respectively, in the reaction gives:

$$PP_i + H_2O = 2P_i + n_H H^+ + n_{Mg}\, Mg^{2+} + n_K K^+$$

The n terms are defined as the number of moles, n, of H^+ and metal ions, respectively, liberated per mole of PP_i hydrolyzed as a result of the differences in the dissociation and association constants, respectively, of the reactants and the products. Where H^+ and also K^+ are consumed, *i.e.* become reactants in the total reaction, the n terms carry a negative sign. The equilibrium constant, K, for the above reaction is:

$$K = \frac{[P_i]^2[H^+]^{n_H}[Mg^{2+}]^{n_{Mg}}[K^+]^{n_K}}{[PP_i]}$$

Combining K with K'_{obs} in Table II, K becomes equal to

$$K = K'_{obs} \times [H^+]^{n_H} \times [Mg^{2+}]^{n_{Mg}} \times [K^+]^{n_K}$$

and rewritten as a free energy relationship the chemical potential equation is:

$$-\Delta G^{0'}_{obs} = -\Delta G^0 - n_H RT \ln(H^+) - n_{Mg} RT \ln(Mg^{2+}) - n_K RT \ln(K^+)$$

where the last three terms according to the suggestion of Phillips (27) can be regarded as hydrogen and metal ion driving or hindering forces that come into play, because the prevailing H^+ and metal ion concentrations in general, *e.g.* in the cell, will have values other than the standard value 1 molal. ΔG^0 applies to reactants and products in their standard state, *i.e.* 1 molal (hypothetical) in a mixture with the properties of an ideal solution.

The individual contributions of these terms to the $\Delta G^{0'}_{obs}$ measured with changing $[Mg^{2+}]$ at fixed pH (7.4) and ionic strengths around 0.05 (Table V) are shown in Fig. 5. It is obvious that even if both ΔG^0 and $n_{Mg} RT \ln(Mg^{2+})$ individually are in favor of forward reaction (Mg^{2+} is produced throughout)

TABLE V

Thermodynamic parameters for hydrolysis of inorganic pyrophosphate (PP_i) at pH 7.4 as a function of Mg^{2+} and K^+ concentrations and ionic strength. Comparison with thermodynamic parameters calculated

The parameters found have been derived from the results in Table II and as shown in Fig. 4. The calculation of the free ion concentrations, the ionic strengths, the enthalpy and entropy changes and the stoichiometric number, n_x, for production (or consumption) of hydrogen and metal ions in the composite reaction has been carried out as described in the calculation section. (n = number of experiments).

Exp. No.	Ionic strength	$[Mg^{2+}]$ free	$[K^+]$ free	Found			Calculated				
				$-\Delta G^{o'}_{obs}$	$-\Delta H^{o'}_{obs}$	$T\Delta S^{o'}_{obs}$	$-\Delta H^{o'}_{obs}$	$T\Delta S^{o'}_{obs}$	n_H	n_{Mg}	n_K
		mM	mM	Cal/mole	Cal/mole	Cal/mole	Cal/mole	Cal/mole	mole	mole	mole
3	0.127	0.00	0.00	5.77 ± 0.02	1.90 ± 0.37 (n = 2)	+ 3.87	3.35	+ 2.42	+ 0.602	0.00	0.00
1	0.058	0.00	42.00	5.56 ± 0.03	2.68 ± 0.25 (n = 1)	+ 2.88	1.97	+ 3.59	+ 0.594	0.00	-0.232
2	0.112	0.00	80.60	5.72 ± 0.01	2.19 ± 0.49 (n = 1)	+ 3.53	1.16	+ 4.56	+ 0.657	0.00	-0.356
4	0.006	0.063	4.45	4.63			5.67	- 1.04	- 0.106	+0.628	-0.038
5	0.011	0.050	8.82	4.66			5.06	- 0.391	+ 0.016	+0.523	-0.070
11	0.058	0.165	42.10	4.88			4.53	+ 0.349	+ 0.014	+0.684	-0.235
12	0.068	0.298	42.10	4.84 ± 0.03	4.93 ± 0.81 (n = 1)	- 0.09	5.00	- 0.162	- 0.074	+0.805	-0.227
6	0.050	1.74	34.30	4.19 ± 0.01	5.49 ± 0.10 (n = 3)	- 1.30	5.98	- 1.79	- 0.253	+1.045	-0.174
7	0.030	2.37	17.50	3.67 ± 0.04	5.66 ± 0.59 (n = 2)	- 1.99	6.32	- 2.65	- 0.290	+1.011	-0.091
14	0.056	2.53	43.00	3.74			5.78	- 2.04	- 0.243	+1.048	-0.198
8	0.038	5.38	17.70	3.18 ± 0.05	6.87 ± 0.74 (n = 2)	- 3.69	5.90	- 2.72	- 0.240	+0.878	-0.070
9	0.061	12.20	17.80	2.82 ± 0.05	6.99 ± 0.79 (n = 1)	- 4.17	5.33	- 2.52	- 0.172	+0.685	-0.048
10	0.110	25.70	17.80	2.73 ± 0.07	4.92 ± 0.52 (n = 1)	- 2.19	4.88	- 2.73	- 0.117	+0.525	-0.032
13	0.221	0.298	191.00	5.24 ± 0.02	1.98 ± 0.18 (n = 3)	+ 3.26	2.19	+ 3.05	+ 0.134	+0.705	-0.616
17	0.139	0.899	45.80	4.46 ± 0.11			5.54	- 1.08	- 0.132	+0.939	-0.203
16	0.213	1.51	181.00	4.27 ± 0.05			3.45	+ 0.814	- 0.084	+1.019	-0.577
15	0.213	1.99	207.00	4.00 ± 0.13			3.27	+ 0.735	- 0.092	+1.061	-0.625
18	0.204	3.06	167.00	4.33 ± 0.04	4.45 ± 0.29 (n = 2)	- 0.12	3.97	+ 0.299	- 0.129	+1.095	-0.518
19	0.029	2.30	0.00	3.53 ± 0.05	7.70 ± 0.96 (n = 1)	- 4.17	6.83	- 3.30	- 0.311	+0.990	0.00
21	0.290	2.56	0.00	3.64 ± 0.06	4.63 ± 0.63 (n = 1)	- 1.99	6.93	- 3.29	- 0.214	+1.012	0.00
20	0.056	12.00	0.00	2.79 ± 0.03	5.73 ± 0.71 (n = 2)	- 2.94	5.52	- 2.74	- 0.178	+0.651	0.00
22	0.265	13.40	0.00	2.91 ± 0.06	6.81 ± 0.80 (n = 1)	- 3.27	5.95	- 3.03	- 0.171	+0.781	0.00

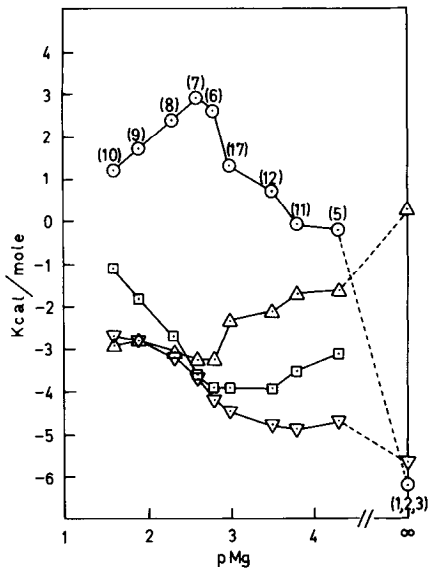

FIG. 5. $\Delta G^{0'}_{obs}$ (▽——▽), ΔG^{0} (△——△), $n_H RT\ln(H^+)$ (○——○) and $n_{Mg} RT\ln(Mg^{2+})$ (□——□) for the hydrolysis of inorganic pyrophosphate as a function of pMg ($-\log [Mg^{2+}]$). The *numbers* in *parentheses* refer to the experiment number in Table V, where the n terms are presented.

with increasing $[Mg^{2+}]$ (decreasing pMg) the $n_H RT\ln(H^+)$ term rapidly switches over from negative to positive values, thus becoming a hindering force due to the considerable H^+ consumption (n_H negative) (Table V). The maximum and minimum for the $n_H RT\ln(H^+)$ and $n_{Mg} RT\ln(Mg^{2+})$ terms, respectively, at the same pMg appear as a consequence of the linkage of H^+ binding and metal ion binding to the equilibrium system. The general theory for this phenomenon has been worked out by Wyman (28).

From the experiments designed to imitate "physiological" conditions, *i.e.* high ionic strength and high $[K^+]$ (Table V), the acid consumption appears to be reduced compared to the experiments at lower $[K^+]$ but identical $[Mg^{2+}]$. This is a result of the fact that the $KHPO_4^{1-}$ complex which is the only potassium complex present in significant amounts occupies 30% of the total orthophosphate concentration under these conditions, and an equivalent amount of acid is liberated as a consequence again of the linkage between H^+ and metal ion binding to the equilibrium system.

Relative Contributions of $T\Delta S^{0'}_{obs}$ *and* $-\Delta H^{0'}_{obs}$ *to* $\Delta G^{0'}_{obs}$—From Table V it is apparent that the variation in the $T\Delta S^{0'}_{obs}$ term closely parallels the trend in n_H. For a composite reaction producing or consuming acid, the entropy change plays a larger role in determining $\Delta G^{0'}_{obs}$ than $\Delta H^{0'}_{obs}$ does at reasonably high pH values (29), because the $\Delta S^{0'}_{obs}$ contains an entropy of dilution (or concentration) term, $2.3\ R(n_H pH + n_{Mg} pMg + n_K pK)$. Therefore, the very pronounced shift in $T\Delta S^{0'}_{obs}$ toward negative values shows up clearly when Mg^{2+} ion is introduced and acids start to be consumed (Table V). Likewise at the high $[K^+]$ and $[Mg^{2+}]$ around 2 mM the $T\Delta S^{0'}_{obs}$ term is slightly favorable for the forward reaction as a consequence of the reduced acid consumption and probably by an additional effect from the decreased organization of water structure around the ions produced due to the diminished charge of the $KHPO_4^{1-}$ complex present in high concentration.

From the data in Table V it appears that $\Delta H^{0'}_{obs}$ is negative throughout. Because the ΔH^0 for complex formations is positive (Table IV) the $\Delta H^{0'}_{obs}$ values pass through a minimum (maximum negative) as a consequence of the formation of the Mg^{2+} complex of PP_i first as Mg^{2+} ion is introduced into the system, thus following the trend in n_{Mg} (and n_H in opposite direction). At the high $[K^+]$ the $\Delta H^{0'}_{obs}$ value appears to be less favorable as a consequence of the pronounced formation of the $KHPO_4^{1-}$ complex having a ΔH^0 of 6 Cal × mole^{-1} (Table IV).

ΔH^0_{obs} *Calculated*—The calculations ("Appendix") were performed on the basis of a ΔH^0_{obs} of -3.8 Cal × mole^{-1} obtained by Wu *et al.* (12) in their calorimetric studies of the reaction $HP_2O_7^{3-}$ (pH 8.5) + H_2O = HPO_4^{2-} + $H_2PO_4^{1-}$ (pH 6.9) in the absence of metal ions at ionic strength 0.07. Conversion of this result by adding and subtracting the enthalpy changes (Table IV) for the individual ionic reactions corresponding to the appropriate proportions of ionic species calculated to make up the total equilibrium concentrations in each particular experiment (Table II) gives the figures shown in Table V. The value of (-1.90 ± 0.37) Cal × mole^{-1} found from the equilibrium experiments in the absence of metal ions (Table V, Experiment 3) may be compared to the -3.35 Cal × mole^{-1} calculated. Apart from the possibility of small errors in the calculation arising from the ΔH^0 values for dissociation reactions, the small discrepancy can be explained by use of higher ionic strength and different supporting electrolytes in the equilibrium experiment. Experiments 19 and 21 in Table V clearly indicate that $\Delta H^{0'}_{obs}$ is markedly increased with increasing ionic strength. The phenomenon is, however, not at all reflected in the calculation due to the fact that the ΔH^0 used for magnesium complexing with PP_i species only applies to low ionic strength (Table IV). Values at higher ionic strength are not available and no theoretical guidance exists for correction of ΔH^0 values to other conditions. However, as the ΔH^0 selected in Table IV applies fairly well to many of the experimental conditions employed, good agreements between the experimental and calculated results are obtained (Table V). It is especially interesting to note that at physiological $[K^+]$ the increase in $\Delta H^{0'}_{obs}$ toward less favorable values due to the larger formation of the $KHPO_4^{1-}$ complex mentioned above is excellently reflected in the calculation by use of the ΔH^0 of 6 Cal × mole^{-1} for the formation of this complex determined by Smith *et al.* (16) at ionic strength 0.2 thus comparable to the experimental ionic strengths.

Determination of ΔG *for Hydrolysis of* PP_i *in Cell*—From Experiments 15 and 16 in Table V, which are the best imitation of physiological conditions, $\Delta G^{0'}_{obs}$ for 1 mM $[Mg^{2+}]$, which has been reported to be present in rat liver (30), 150 mM $[K^+]$, and 0.25 ionic strength, pH 7.4, is calculated to -4.0 Cal × mole^{-1}. The concentration of inorganic pyrophosphate in freeze biopsies from rat liver was (6.2 ± 0.3 (S.E., $n = 6$)) nmoles per g wet weight.[3] The P_i concentration has been reported to be 2.42 ± 0.12 mM in rat liver (31). Thus, if these concentrations and the value of -4.0 Cal × mole^{-1} for $-\Delta G^{0'}_{obs}$ is inserted in the formula

$$\Delta G = \Delta G^{0'}_{obs} + RT\ln'' K''$$

ΔG is calculated to -4.0 Cal × mole^{-1}.

[3] The rather high inorganic pyrophosphate concentrations in rat liver reported earlier by us (13) are too high, the reason being that we were unaware of ATP-pyrophosphohydrolase contamination in the preparations of UDP-glucose pyrophosphorylase used in the isotope derivative method. The method is now worked out to be absolutely specific for inorganic pyrophosphate measurements in extracts from biological tissues, and a detailed study of the pyrophosphate concentrations in mammalian tissues is undertaken in our laboratory.

DISCUSSION

The results obtained indicate that the standard free energy change, $\Delta G^{0'}_{obs}$, for the hydrolysis of inorganic pyrophosphate at fixed pH (7.4) is not very favorable in the presence of Mg^{2+} ion. Thus, at 26 mM [Mg^{2+}], 18 mM [K^+], and ionic strength 0.1 (Table V), only a decrease in free energy change of 2.73 Cal × $mole^{-1}$ was found. Moreover, the −4.0 Cal × $mole^{-1}$ found for $\Delta G^{0'}_{obs}$ at physiological conditions, *i.e.* 1 mM [Mg^{2+}], 150 mM [K^+], and ionic strength 0.25 is not particularly different from the standard free energy change of an ordinary ester bound.

The ΔG calculated to −4.0 Cal × $mole^{-1}$ by introducing the actual concentrations of PP_i and P_i found in rat liver may be even less negative. A considerable part of the very low pyrophosphate concentration in the cell (6 μM) is probably bound to proteins and membrane structures. This causes, other things being equal, the $\Delta G^{0'}_{obs}$ to become less negative. The poor affinity demonstrated for this reaction under physiological conditions makes direct chemical coupling to, *e.g.* phosphorylation reactions unlikely. However, the energy in inorganic pyrophosphate may be utilized by chemical coupling to biosynthetic reactions in the following way. The synthetic reaction

$$MgATP + X = XAMP + Mg + 2P_i \quad (1)$$

may be considered the sum of the following reactions

$$AMP + X = XAMP \quad (2)$$

$$ATP = AMP + PP_i \quad (3)$$

$$MgATP = ATP + Mg \quad (4)$$

$$Mg + PP_i = MgPP_i \quad (5)$$

$$MgPP_i = Mg + 2P_i \quad (6)$$

Reaction 2 is the thermodynamically unfavorable reaction which is directed toward synthesis by chemical coupling to Reactions 3 to 5. Due to the higher stability of the magnesium pyrophosphate complex compared to the corresponding ATP complex the pyrophosphate-forming reaction of ATP cleavage is the more favorable as discussed by Schuegraf *et al.* (26). They calculated this reaction to be 2.6 Cal × $mole^{-1}$ more negative than the orthophosphate forming reaction of ATP cleavage at pH 7.5, 37° in the presence of excess Mg^{2+}. Reaction 6 proceeds with ΔG near zero. A mechanism whereby the cell gains the free energy from the pyrophosphate forming reaction of ATP cleavage by means of chemical coupling to the favorable magnesium pyrophosphate association reaction seems clear. The low affinity found for the hydrolysis of the magnesium pyrophosphate complex, the true substrate for the inorganic pyrophosphatase, gives a mechanism whereby the PP_i can be converted to orthophosphate without loss of the energy as heat.

Comparison with Other Values from Literature—The only direct measurements of the pyrophosphatase equilibrium found in the literature are those reported by Stiller *et al.* (8). They determined final concentrations of inorganic pyrophosphate by paper chromatography in experiments in which the reaction was catalyzed by yeast inorganic pyrophosphatase in the presence of Mg^{2+} ion. Their provisional values calculated for zero Mg^{2+} at pH 7.5 and 0.25 ionic strength gave −5.8 to −7.4 Cal × $mole^{-1}$ when they used 5 × 10^5 M^{-1} and 10^7 × M^{-1} as lower and upper limits for the thermodynamic stability constant of $MgP_2O_7^{2-}$, respectively. However, as a consequence of the argumentation for the lower binding constants (Table IV) given under "Calculations," "Determination of K_{ionic}," only the less negative value equal to −5.8 Cal × $mole^{-1}$ should be considered. This result is in excellent agreement with the direct experiment (Table V, Experiment 3) carried out with the ideal cation tetra-*n*-propyl ammonium in the absence of metal ions.

Otherwise the results obtained for $\Delta G^{0'}_{obs}$ are considerably less negative than previous reported (9, 10, 26). However, these authors have calculated the equilibrium constant for the pyrophosphatase reaction by algebraic combination of several different reactions. Great uncertainties exist about the equilibrium constants for many of the reactions used, and different values for the stability constants of magnesium complexes reported in the literature have been used. The data for the ATP phosphohydrolase reaction, used as component reaction by all the authors mentioned, can in part be traced back to the studies of the glutaminase reaction by Benzinger *et al.* (32, 33). These experiments were carried out at concentrations of ammonia and glutamate made up to 0.9 M, which made introduction of mean ion activity coefficients necessary to correct the results. It is therefore not surprising that the values obtained by different authors differ as much as they do.

The data reported in the literature for the $\Delta G^{0'}_{obs}$ for the hydrolysis of ATP covers a range from −10 to −5.6 Cal × $mole^{-1}$ (27, 34–36). The less negative value of −5.6 Cal × $mole^{-1}$ obtained by Russian workers (36) may be the best estimate on the basis of the fact that these workers used low substrate concentrations and an isotope dilution method in their studies of the hexokinase reaction, which they combined with the data for the free energy change of the hydrolysis of glucose 6-phosphate obtained by Guinodman (37), who also used an isotope dilution method. Substitution of this result into the calculations of Alberty (9) and Wood *et al.* (10) who obtained a ΔG_{ionic} of 1.56 and 2.40 Cal × $mole^{-1}$, respectively, applying to the same reference reaction as used in the present work, would bring their results into agreement with the 4.70 Cal × $mole^{-1}$ for ΔG_{ionic} calculated from the present equilibrium experiments for the same ionic strength as used by these authors. Moreover, from the direct experiments carried out in the absence of metal ions and at substrate concentrations not too far from ideality (Table V, Experiment 3) calculation of $\Delta G^{0'}_{obs}$ for the hydrolysis of PP_i making due allowance for the dissociation at this pH gives (−5.63 ± 0.02) Cal × $mole^{-1}$ at zero ionic strength. Although this result cannot be compared directly with the more favored values between −7 and −8 Cal × $mole^{-1}$ for the ATP hydrolysis under the same conditions, a discrepancy as large as 1.5 to 2 Cal × $mole^{-1}$ should not be expected. There is still good reason to assume that the more negative values reported are too negative. Further need for investigation of the ATP hydrolysis by direct measurements seems evident.

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Supplemental Material
to
"Thermodynamic Parameters for the Hydrolysis of Inorganic Pyrophosphate (PP_i) at pH 7.4 as a Function of $[Mg^{2+}]_f$, $[K^+]$ and Ionic Strength Determined from Equilibrium Studies of the Reaction"
by
Hans Flodgaard and Peter Fleron

Relation between equilibrium constants and related thermodynamic functions for total and reference reactions.

1. Notation.

Suppose the reactants to be $R_1, R_2, \ldots\ldots, R_n$ participating in the total reaction:

1) $$\sum_{i=1}^{n} \nu_i R_i = 0$$

where ν_i is the stoichiometric mole number, positive for products and negative for reactants whereas it is zero for other species. R_{ij} are the dissociated species of R_i, $j = 1, 2, \ldots\ldots, n_i$, where R_{i1} is chosen as the most acid form, the charge of which is zr_i.

Next, suppose the metal ions to be $S_1, S_2, \ldots\ldots, S_m$, and let a_k be the maximum number of molecules of S_k in a complex, and zs_k be the charge of S_k.

The dissociation and association constants are introduced in the following way:

K_{ij} is the dissociation constant divided by the hydrogen ion concentration for the reaction carried out isohydrically:

$$R_{ij} \rightleftharpoons R_{ij-1} + H^+$$

K_{ijkl} is the association constant for the reaction:

$$S_k + R_{ij}(S_k)_{l-1} \rightleftharpoons R_{ij}(S_k)_l$$

The last definition implies that neither mixed complexes nor complexes involving more than one reactant molecule are considered. However, the formulae given below can easily be extended to include such cases too.

The reference reaction is:

2) $$\nu H^+ + \sum_{i=1}^{n} \nu_i R_{i,ref_i} = 0$$

where R_{i,ref_i} is a particular species chosen among R_{ij}. The constant ν is given by:

$$\nu + \sum_{i=1}^{n} \nu_i (zr_i - ref_i + 1) = 0$$

The concentration of the various species is as follows:

tr_i is the total equilibrium concentration of R_i, while r_{ij} is the equilibrium concentration of free R_{ij}, especially r_i being that of the reference species R_{i,ref_i}. In a similar way, ts_k is the total concentration of S_k, while s_k is the equilibrium concentration of the free S_k.

II. Determination of equilibrium concentrations.

The relations between the total and the free concentrations are expressed by:

$$tr_i = \sum_{j=1}^{n_i} \left(r_{ij} + \sum_{k=1}^{m} \sum_{l=1}^{a_k} (R_{ij}(S_k)_l) \right)$$

$$ts_k = s_k + \sum_{i=1}^{n} \sum_{j=1}^{n_i} \sum_{l=1}^{a_k} l[R_{ij}(S_k)_l]$$

Here all concentrations except r_i and s_k are eliminated by means of K_{ij} and K_{ijkl}.

By definition

$$r_{ij} \begin{cases} r_i \prod_{p=j+1}^{j} K_{ip} & \text{, if } j<ref_i \\ r_i \prod_{p=j+1}^{ref_i} K_{ip}^{-1} & \text{, if } j>ref_i \end{cases}$$

which is equivalent to:

3) $$r_{ij} = r_i \prod_{p=1}^{j} K_{ip} \prod_{p=1}^{ref_i} K_{ip}^{-1}$$

where $K_{i,1} = 1$.

Likewise:

$$[R_{ij}(S_k)_l] = s_k^l r_{ij} \prod_{p=1}^{j} K_{ijkp} = r_i s_k^l \prod_{p=1}^{j} K_{ip} \prod_{p=1}^{ref_i} K_{ip}^{-1} \prod_{p=1}^{l} K_{ijkp}$$

This expression is reduced to an identity when i or k is equal to zero, if

$$r_0 = s_0 = n_0 = n_0 - ref_0 = K_{0l} = K_{i0l} = K_{ij0} = 1.$$

Hence

3) $$[R_{ij}(S_k)_l] = r_i s_k^l B_{ijkl}$$

where

$$B_{ijkl} = \prod_{p=1}^{j} K_{ip} \prod_{p=1}^{ref_i} K_{ip}^{-1} \prod_{p=1}^{l} K_{ijkp}$$

is inserted in the equations for the total concentrations

$$tr_i = \sum_{j=1}^{n_i} \sum_{k=0}^{m} \sum_{l=1}^{a_k} r_i s_k^l B_{ijkl}$$

$$ts_k = \sum_{i=0}^{n} \sum_{j=1}^{n_i} \sum_{l=1}^{a_k} l\, r_i s_k^l B_{ijkl}$$

If A_{ikl} is defined by

$$A_{ikl} = \sum_{j=1}^{n_i} B_{ijkl}$$

then

$$tr_i = \sum_{k=0}^{m} \sum_{l=1}^{a_k} r_i s_k^l A_{ikl}$$

$$ts_k = \sum_{i=0}^{n} \sum_{l=1}^{a_k} l\, r_i s_k^l A_{ikl}$$

which are rearranged to give:

4) $$r_i = \frac{tr_i}{\sum_{k=0}^{m} \sum_{l=1}^{a_k} s_k^l A_{ikl}}$$

5) $$s_k = \frac{ts_k}{\sum_{i=0}^{n} \sum_{l=1}^{a_k} l\, r_i s_k^{l-1} A_{ikl}}$$

This is (n+m) equations containing (n+m) unknowns: r_i and s_k (i and k>0). They are solved in an iterative manner as follows: First, all r_i and s_k (i and k>0) are initialized to zero. Then all r_i (i>0) are found from the first equation 4) and subsequently all s_k (k>0) are found from the second equation 5). This procedure is repeated until the maximum relative difference between two consecutive r_i or s_k is below a certain limit.

The equilibrium concentration of all other species is then given by equation 3).

Determination of the ionic strength.

When the concentration of all the species are known, the ionic strength of the solution can be calculated.

However, since the constants K_{ij} and K_{ijkl} depend on this value, a further iterative procedure is necessary. This is carried out as follows:

First a value of the ionic strength is assumed, and the dissociation and association constants are adjusted to this ionic strength (cf. 17), after which the concentration of all ionic species is determined as described above. The new ionic strength is then given by:

$$\mu = \mu_0 + \frac{1}{2} \sum_{i,j,k,l} (r_i s_k^l B_{ijkl}) (zr_i - j + l)^2 zs_k)^2$$

where μ_0 is the contribution to the ionic strength from ions which are neither a R_i nor a S_k. (In this equation and in the following expressions derived below, the summation limits are omitted when the range includes all defined values).

Now, if the ionic strength calculated deviates more than a certain amount from the preceeding value, the iteration is repeated; adjust the constant etc.

III. Determination of equilibrium constants and related functions.

Let K'_{obs} and K_{ionic} be the equilibrium constants corresponding to reaction 1) and 2), respectively. Then:

$$K'_{obs} = \prod_{i=1}^{n} tr_i^{\nu_i} \quad , \quad K_{ionic} = [H^+]^{\nu} \prod_{i=1}^{n} r_i^{\nu_i}$$

If the fractions

$$F_i = \frac{r_i}{tr_i}$$

are introduced, then

$$K'_{obs} = K_{ionic} [H^+]^{-\nu} \prod_{i=1}^{n} F_i^{-\nu_i}$$

or from the equation 4)

$$K'_{obs} = K_{ionic} [H^+]^{-\nu} \prod_{i=1}^{n} \left(\sum_{k,l} s_k^l A_{ikl} \right)^{\nu_i}$$

which expresses K'_{obs} as a product of K_{ionic} and a factor containing the free metal ion concentrations and the hydrogen concentration.

(Notice that A_{ikl} depends on $[H^+]$).

The relation between $\Delta H^{o\prime}_{obs}$ and ΔH^o_{ionic} is derived from the formula

6) $$\Delta H^o = RT^2 \frac{\partial \ln K}{\partial T}$$

giving

$$\Delta H^{o\prime}_{obs} = \Delta H^o_{ionic} + RT^2 \sum_{i=1}^{n} \nu_i F_i \sum_{j,k,l} s_k^l \frac{\partial B_{ijkl}}{\partial T}$$

If

$$B = \prod_i K_i^{a_i} \text{, implying that } \ln B = \sum_i a_i \ln K_i$$

then

$$\frac{\partial B}{\partial T} = B \sum_i a_i \frac{\partial \ln K_i}{\partial T}$$

and hence

$$\frac{\partial B_{ijkl}}{\partial T} = B_{ijkl} \left(\sum_{p=1}^{j} \frac{\partial \ln K_{ip}}{\partial T} - \sum_{p=1}^{ref_i} \frac{\partial \ln K_{ip}}{\partial T} + \sum_{p=1}^{l} \frac{\partial \ln K_{ijkp}}{\partial T} \right)$$

where the terms are readily identified from equation 6) as $\frac{\Delta H^o}{RT^2}$

corresponding to the various dissociation and association reactions. When k is zero, ΔH^o is zero. From this follows:

$$\Delta H^{o\prime}_{obs} = \Delta H^o_{ionic} + \sum_{i=1}^{n} \nu_i F_i \sum_{k,l} s_k^l \sum_j B_{ijkl}$$

$$\times \left(\sum_{p=1}^{j} \Delta H^o_{ip} - \sum_{p=1}^{ref_i} \Delta H^o_{ip} + \sum_{p=1}^{l} \Delta H^o_{ijkp} \right)$$

The number of moles produced or consumed of the ion Q ($Q = H^+$ or S_k) during the reaction according to equation 1) is given by (cf. 9):

$$n_Q = \frac{\partial \log K'_{obs}}{\partial pQ}$$

which yields, in a way analogous to the above mentioned

$$n_H = -\sum_{i=1}^{n} \nu_i F_i \sum_{k,l} s_k^l \sum_j (ref_i - j) B_{ijkl}$$

$$n_{S_k} = -\sum_{i=1}^{n} \nu_i F_i \sum_l l\, s_k^l A_{ikl}$$

Table VI. Proportion of species (F_i) calculated to be present in the experiments summarized in Table II.

Calculation of the fractions on basis of the figures from Table II and IV is outlined in the "[illegible]".

The underlined fractions are used to calculate K_{ionic} in Table VII.

Exp.No.	$H_2P_2^{2-}$	HP_2^{3-}	$MgHP_2^-$	KHP_2^-	$H_2PP_i^{2-}$	HPP_i^{3-}	PP_i^{4-}	$MgHPP_i^-$	$MgPP_i^{2-}$	Mg_2PP_i	KPP_i^{3-}	Cl^-	$MgCl^+$
1	0.222	0.567	0.000	0.121	0.0659	0.906	0.0189	0.000	0.000	0.000	0.0095	0.000	0.000
2	0.179	0.674	0.000	0.187	0.0552	0.904	0.0222	0.000	0.000	0.000	0.0183	0.000	0.000
3	0.214	0.784	0.000	0.000	0.0545	0.922	0.0233	0.000	0.000	0.000	0.0000	0.000	0.000
4	0.335	0.651	0.0149	0.0193	0.0400	0.928	0.0040	0.156	0.442	0.0300	0.0004	1.00	0.0005
5	0.310	0.645	0.0099	0.0355	0.0454	0.922	0.0059	0.123	0.395	0.0171	0.0009	1.00	0.0003
6	0.196	0.552	0.159	0.0869	0.0018	0.0739	0.0005	0.110	0.475	0.388	0.0002	0.991	0.0086
7	0.200	0.504	0.250	0.0455	0.0010	0.0114	0.0002	0.0966	0.367	0.524	0.0001	0.987	0.0139
8	0.152	0.407	0.407	0.0348	0.0003	0.0036	0.0001	0.0598	0.243	0.695	0.0000	0.972	0.0278
9	0.104	0.311	0.561	0.0239	0.0001	0.0012	0.0000	0.0344	0.155	0.809	0.0000	0.945	0.0547
10	0.0691	0.243	0.672	0.0160	0.0000	0.0005	0.0000	0.0206	0.109	0.870	0.0000	0.906	0.0942
11	0.218	0.646	0.0162	0.119	0.0212	0.291	0.0061	0.117	0.523	0.0377	0.0051	0.999	0.0008
12	0.210	0.648	0.0270	0.115	0.0134	0.192	0.0042	0.127	0.591	0.0708	0.0020	0.999	0.0014
13	0.177	0.547	0.0117	0.115	0.0139	0.272	0.0079	0.0838	0.570	0.0351	0.0131	0.999	0.0010
14	0.177	0.523	0.003	0.0998	0.0011	0.0150	0.0003	0.0945	0.419	0.470	0.0002	0.998	0.0121
15	0.117	0.497	0.0726	0.113	0.0022	0.0426	0.0012	0.0940	0.604	0.254	0.0022	0.993	0.0066
16	0.124	0.526	0.0583	0.790	0.0030	0.0580	0.0017	0.0986	0.653	0.202	0.0027	0.995	0.0050
17	0.172	0.671	0.0525	0.102	0.0051	0.0922	0.0025	0.109	0.645	0.145	0.0011	0.997	0.0033
18	0.120	0.504	0.116	0.260	0.0013	0.0242	0.0007	0.0845	0.534	0.355	0.0010	0.990	0.0103
19	0.212	0.531	0.256	0.000	0.0010	0.0118	0.0003	0.0980	0.371	0.518	0.0000	0.987	0.0128
20	0.107	0.313	0.580	0.000	0.0003	0.0052	0.0001	0.0564	0.159	0.817	0.0000	0.945	0.0549
21	0.166	0.693	0.139	0.000	0.0016	0.0295	0.0008	0.0897	0.557	0.322	0.0000	0.991	0.0088
22	0.106	0.416	0.476	0.000	0.0001	0.0022	0.0001	0.0376	0.224	0.736	0.0000	0.954	0.0460

Table VII. $K_{ionic} = \frac{[HP^{2-}]^2 [H^+]}{[PPP^{3-}]}$ and ΔG_{ionic} calculated from the proportion of species in Table V and adjusted to zero ionic strength according to the method described in the "[illegible]".

The values of K_{ionic} shown in the fourth column are calculated using the binding constants for magnesiumpyrophosphate complex formation shown in brackets in Table [illegible]. The free $[Mg^{2+}]$ are calculated from the figures in Table [illegible] and [illegible] as described in the calculation section. Mean ± S.E. given below.

Exp. No.	$K_{ionic} \times 10^4$ $\mu=0$	ΔG_{ionic} $\mu=0$	Calculated with higher binding constants for Mg^{2+}: $K_{ionic} \times 10^3$ $\mu=0$	$[Mg^{2+}]$ free
	M^2	Cal/mole	M^2	mM
1	1.72	5.13		0.00
2	1.84	5.09		0.00
3	2.90	4.82		0.00
4	1.15	5.37	0.467	0.063
5	0.946	5.49	0.335	0.050
6	4.77	4.93	3.98	1.74
7	5.66	4.89	5.42	2.37
8	5.18	4.77	5.38	5.38
9	2.79	4.85	3.19	12.20
10	3.25	4.76		25.70
11	1.63	5.17	0.731	0.165
12	2.26	4.97	1.19	0.298
13	1.69	5.14	0.783	0.298
14	3.03	4.80	2.73	2.53
15	1.11	5.39	0.824	1.99
16	1.42	5.25	0.986	1.51
17	2.22	4.98	1.41	0.899
18	5.20	4.77	2.62	3.06
19	3.08	4.79		2.30
20	2.85	4.85		12.00
21	1.75	5.12		2.56
22	2.62	4.88		13.40
	Mean: (2.41±0.20)x 10^{-4} M^2	Mean: (4.90±0.05)x Cal x $mole^{-1}$	Mean: (1.86 ± 0.34)x 10^{-5} M^2	