DETERMINATION OF THE EQUILIBRIUM OF THE HEXOKINASE REACTION AND THE FREE ENERGY OF HYDROLYSIS OF ADENOSINE TRIPHOSPHATE*

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The proper evaluation of the energetics of the reactions in which ATP[1] participates requires knowledge of the free energy of hydrolysis of ATP. Previous estimations (1–8) have depended upon the calculation of the free energies of formation of one or more of the components of the summation reactions used, except those of Levintow and Meister (7) and of Morales *et al.* (8) which are based on reasonable estimations of the free energy of hydrolysis of glutamine and asparagine. Values for the free energy of hydrolysis of ATP have varied from Meyerhof's estimate of −12 kilocalories (1) at pH 7.8 to the value of −7.0 kilocalories at pH 7.0 as calculated by Morales *et al.* (8) from the data of Levintow and Meister (7).

A relatively direct estimation of the free energy of hydrolysis of ATP is afforded by use of the coupled reactions

(1) $$\text{ATP} + \text{glucose} \rightleftarrows \text{G6P} + \text{ADP} \quad (\Delta F_1)$$

(2) $$\text{G6P} + \text{H}_2\text{O} \rightleftarrows \text{P}_i + \text{glucose} \quad (\Delta F_2)$$

the summation of which gives

(3) $$\text{ATP} + \text{H}_2\text{O} \rightleftarrows \text{ADP} + \text{P}_i \quad (\Delta F_3)$$

An experimentally determined value of ΔF_2 has been previously reported (9); thus determination of the free energy, ΔF_1, of the hexokinase reaction (Equation 1) would allow the calculation of the free energy of hydrolysis of ATP (ΔF_3). Information about the equilibrium of the hexokinase re-

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[1] Abbreviations used in this paper are adenosine triphosphate, ATP; adenosine diphosphate, ADP; glucose-6-phosphate, G6P; fructose-6-phosphate, F6P; hexose-6-phosphate (mixture of G6P and F6P), H6P; inorganic orthophosphate, P_i; perchloric acid, PCA; ethylenediaminetetraacetic acid, EDTA.

action is also of value for assessment of expected concentrations of the reaction participants in living systems.

The energetics of the hexokinase reaction are unfavorable for the determination of its equilibrium constant at pH 7 and by ordinary chemical means. Most of the studies were thus conducted at pH 6 with use of radioisotopic techniques for measurement of the equilibrium concentration of glucose arising from G6P-C^{14} and ADP as initial reactants. With these reactants, formation of free glucose was readily demonstrated in harmony with other reports (10, 11), which appeared while these studies were in progress, showing reversibility of the hexokinase reaction. Methods were developed for the isolation and determination of the equilibrium glucose by isotopic dilution and ion exchange techniques. The effects of time, carrier composition, magnesium and potassium chloride concentration, G6P to nucleotide ratio, and pH on the equilibrium were investigated.

EXPERIMENTAL

Preparation and Assay of Reagents—Hexokinase was prepared and crystallized from Fraction 5 of Berger *et al.* (12). To Fraction 5 were added 1 M phosphate buffer, pH 7.0, 0.0215 M EDTA, pH 7.0, and saturated ammonium sulfate, pH 7.0, to final concentrations of 0.1 M, 0.0001 M, and 0.9 saturation, respectively. The precipitated protein was redissolved in 0.1 M phosphate buffer, pH 7.0, containing 0.002 M EDTA. The solution was brought to incipient turbidity with saturated ammonium sulfate, pH 7.0. On standing, crystals containing hexokinase appeared. After 2 weeks, the suspension was centrifuged and the residue was twice washed with 3 ml. portions of 0.1 M phosphate buffer, pH 7.0, containing 0.8–0.9 saturated ammonium sulfate. The residue was redissolved in 0.1 M phosphate, pH 7.0, again treated with the saturated ammonium sulfate to incipient turbidity, and allowed to crystallize. All steps in the crystallization procedure were conducted with cold solutions in a refrigerated room at 1–4°.

Hexokinase was assayed and units were expressed according to Berger *et al.* (12), except that 0.3 to 0.4 mg. of serum albumin was added per flask to stabilize the enzyme. The enzyme was stored at 3° in its precipitation medium. The recrystallized hexokinase was shown to be free of detectable adenosinetriphosphatase, adenylate kinase, phosphoglucomutase, G6P dehydrogenase, phosphofructokinase, aldolase, and phosphatases. Although the crystals appeared to be microscopically homogeneous, the preparation contained a highly active phosphoglucoisomerase.

The preparations of uniformly labeled glucose-C^{14} (Research Specialties Company) were purified by ascending paper chromatography in *n*-propanol-water (776:226, v/v). A preparation of glucose-1-C^{14} (National

Bureau of Standards) was used without further purification. The non-hexokinase-reactive materials in the glucose preparations were determined by reaction with excess ATP at pH 7. The results are presented in Table I. Under the conditions used, all but 0.003 per cent of pure glucose would be expected to be converted to H6P. The method thus makes a sensitive test for impurities and shows that considerable radioactive material other than D-glucose was present in some preparations.

H6P-C^{14} was prepared from the uniformly labeled glucose-C^{14} by use of crystalline hexokinase and ATP. The H6P-C^{14} was precipitated as the barium salt, washed free of glucose, and converted to the sodium salt with

TABLE I

Measurement of Impurities in Glucose-C^{14} Preparations

The substances indicated were incubated for 30 minutes at 30° with 300 units of crystalline hexokinase, 100 μmoles of ATP, 0.4 mg. of serum albumin, and 200 μmoles of $MgCl_2$ in a total volume of 2 ml. at pH 7.0. The solution was deproteinized with 0.8 ml. of 70 per cent PCA, and 7.5 mg. of fructose and 17.5 mg. of glucose were added as carrier. The hexose-6-phosphate and other ions were removed on ion exchange columns and the residual radioactivity of the solution was determined as described in the text.

Glucose-C^{14} preparation No.	Glucose used	Observed radioactivity of aliquot plated	Per cent impurity*
	μmoles	*c.p.m.*	
14, uniformly labeled.....	6.4	68	0.17
15, " "	2.6	767	5.0
16, C-1-labeled.....	7.8	42	0.13

* Calculated as the per cent of the total radioactivity of the glucose preparation not removed by the experimental procedure.

sodium sulfate. All of the radioactivity in freshly prepared solutions of H6P-C^{14} could be removed by ion exchange resins. After 10 weeks storage at −6°, about 1 per cent of the radioactivity was not removed by the resins. This apparent rate of decomposition is greater than would be anticipated for non-radioactive H6P.

Because of the phosphoglucoisomerase activity of the hexokinase, both F6P and G6P were present in the H6P-C^{14} preparation. Their combined concentrations were determined by measurement of total ester phosphate. Fortunately, yeast hexokinase will rapidly phosphorylate either glucose or fructose. Also F6P and G6P are known to have close to the same free energy of hydrolysis (5, 9) and are acid-stable esters. Therefore, the presence of phosphoglucoisomerase in the hexokinase did not interfere appreciably in the equilibrium determinations.

Unlabeled G6P was purchased as the crystalline barium salt (Sigma Chemical Company). Analysis for neutral sugars after removal of G6P on ion exchange resins was made by the phenol-sulfuric acid method (13); the results showed the equivalent of 0.2 per cent glucose in the G6P.

ATP was the crystalline disodium salt (Sigma). The monosodium salt of ADP (Pabst) was treated with an excess of glucose and crystalline hexokinase to remove any contaminating ATP. Complete absence of ATP in the ADP preparation was an experimental requisite. The concentrations of ATP and ADP were measured by the absorption at 260 mμ and by the P_i liberated upon heating in 1 N HCl for 10 minutes in a boiling water bath.

Separation of Glucose-C^{14} from H6P-C^{14}—Preliminary experiments demonstrated that satisfactory separation of small amounts of glucose-C^{14} from relatively large excesses of H6P-C^{14} could be achieved with a mixture of equal parts of heated Dowex 50-(H), 200 to 400 mesh, and IRA-410-(OH). When necessary, the flow rate was reduced by the addition of powdered IRA-410-(OH). The separation was facilitated by the observation that heating of the resins for 6 hours at 90° markedly reduced their retention of glucose.

Deproteinized filtrates containing added carrier glucose and fructose were passed through a resin column 1 cm. in diameter and 10 cm. high, followed by washing with about 12 ml. of water. To the combined filtrate and wash were added about 50 μmoles of unlabeled H6P to dilute the remaining H6P-C^{14}, and the solution was passed through another column of the mixed resins, 1 cm. by 7 cm., followed by a wash of about 12 ml. of water. The completeness of separation attainable is illustrated by an experiment in which the final filtrate from a preparation of H6P equivalent to 5.47×10^5 c.p.m. upon evaporation to dryness gave only 1.5 ± 1.2 c.p.m. above background.

Radioactivity Determinations—Aliquots containing about 1 mg. of hexose were transferred to planchets of 2.4 cm. diameter, dried uniformly, and counted in an internal gas flow counter. The activity was calculated to the 1 mg. level by use of a predetermined glucose self-absorption curve. Replicate determinations usually agreed within ±2 per cent. To allow calculation of the specific activity, the concentration of glucose plus fructose in the solutions was determined by the dinitrosalicylic acid procedure (14); glucose and fructose give equal color in this method.

Methods of Enzyme Inactivation—In the experiments to be reported, a large amount of hexokinase was used to insure attainment of equilibrium. The method of reaction termination must completely and rapidly inactivate the enzyme without causing a shift in equilibrium or a measurable hydrolysis of H6P. Experiments showed that a final concentration of 10

per cent PCA did not result in any interfering hydrolysis of G6P. Inactivation of the hexokinase by heat (100°, 4 minutes) gave approximately the same measured equilibrium concentration of glucose as when PCA was used, after correction for the hydrolysis of H6P caused by the heating. This result and the obtaining of the anticipated shift in equilibrium with change in pH demonstrate that addition of PCA did not result in a serious shift in the equilibrium reactant concentrations before enzyme inactivation. The use of PCA had the added advantage that most of the perchlorate ion could be removed as $KClO_4$ before resin treatment.

Equilibrium Measurements Starting with H6P-C^{14} and ADP—Appropriate concentrations (usually of the order of 0.005 M) of H6P-C^{14} and of ADP, together with magnesium chloride and approximately 1800 units of recrystallized hexokinase per ml., were incubated at 30°. No added buffer ions were used in order to facilitate subsequent separation of the G6P from free glucose and to avoid possible interference by interaction of buffer components with reactants. The pH was determined by electrodes inserted directly into the reaction mixture and the initial pH of the reactants was adjusted to obtain the final pH as required. When desired, a 1 or 2 ml. aliquot was transferred into sufficient cold PCA solution to give a final 10 per cent PCA concentration, and the mixture was cooled for 20 minutes in an ice bath. Unless otherwise noted, 0.5 ml. of a carrier solution containing 7.5 mg. of fructose and 17.5 mg. of glucose was added. After 20 additional minutes the protein was removed by centrifugation, the solution was neutralized to about pH 7 with 4 M KOH, then chilled for 20 more minutes, and the potassium perchlorate was removed by centrifugation. The hexose-C^{14} was separated from the H6P-C^{14} and the specific activity of the free hexose was determined as previously described. From this value and the previously determined specific activity of the H6P, the equilibrium concentrations of the various reactants were calculated. The values for various experiments are summarized in Table II, where the primary purpose of the experiments is indicated in the footnote.

That equilibrium between the hexose-C^{14} and H6P-C^{14} was reached in the 20 minute incubation period commonly used is shown by the following observations: The amount of hexokinase used was sufficient to cause measurable net reaction (as measured by change in pH of the medium) to reach completion within 2 minutes, no further change in the concentration of reactants occurred when the incubation time was doubled to 40 minutes, separate tests showed that the hexokinase was fully active catalytically at the end of the incubation period, variation of pH or Mg concentration at the end of 20 minute incubation periods caused shifts in reactant concentration of the expected direction and magnitude, and, as shown later, approximately the same equilibrium constant was obtained

by measurement starting either with ADP and H6P or with ATP and glucose, although the latter measurements, for experimental reasons, are less accurate.

TABLE II

Initial and Final Concentrations from Equilibria of Reverse Reaction

Experiment No.*	Initial molarity × 10^3		Final total molarity × 10^3				pH
	ADP	H6P	Hexose = ATP	H6P	ADP	Mg	
1 (a)	4.46	7.42	0.316	7.10	4.15	2.14	6.07
(b)	4.46	7.42	0.312	7.10	4.11	2.14	6.07
(c)	4.46	7.42	0.310	7.08	4.11	2.14	6.07
2 (a)	7.86	7.14	0.406	6.74	7.46	3.95	6.00
(b)	7.55	6.84	0.432	6.41	7.12	7.56	6.03
(c)	7.25	6.57	0.453	6.12	6.80	10.84	6.02
(d)	6.99	6.34	0.455	5.89	6.54	14.00	6.03
(e)	6.37	5.79	0.417	5.38	5.96	90.3	6.04
3 (a)	5.00	3.88	0.203	3.68	4.80	2.50	6.00
(b)	4.88	3.78	0.234	3.55	4.65	4.85	6.01
(c)	4.40	3.41	0.261	3.15	4.15	85.5	6.00
4 (a)	4.44	6.91	0.341	6.57	4.10	2.17	5.94
(b)	4.44	6.91	0.348	6.57	4.09	2.17	5.94
(c)	4.42	6.90	0.328	6.57	4.09	2.16	6.02
(d)	4.18	6.55	0.297	6.26	3.89	2.05	6.12
5 (a)	3.57	7.35	0.398	6.96	2.74	80	6.01
(b)	6.09	5.86	0.446	5.42	5.65	119	6.04
(c)	7.62	4.44	0.379	4.05	7.24	177	6.03
(d)	7.62	4.44	0.371	4.07	7.25	177	6.03
6 (a)	7.08	8.38	0.207	8.17	6.88	155	7.00
(b)	7.00	8.35	0.315	8.04	6.69	153	6.67
(c)	6.94	8.25	0.422	7.83	6.52	151	6.34
(d)	6.84	8.14	0.584	7.56	6.26	149	5.99
(e)	6.70	8.10	0.710	7.29	6.00	146	5.70

* Experiment 1, reproducibility of results and effect of carrier composition. (a), glucose 25 mg.; (b), glucose 17.4 mg. + fructose 7.4 mg.; (c), glucose 12.0 mg. + fructose 13.0 mg.; Experiments 2 and 3, effect of magnesium concentration. Experiment 4, establishment of equilibrium and the effect of KCl. (a) and (b), after 20 and 40 minutes incubation, respectively; (c), final molarity of KCl was 0.0072 M; (d) final molarity of KCl was 0.248 M. Experiment 5, effect of reactant ratio. The last two values are replicates. Experiment 6, effect of pH.

Values for the dissociation constants of the acid and magnesium nucleotides (15) allowed estimation of the equilibrium concentrations of the various ionic species by using approximations from a series of simultaneous equations. The complex formation of ATP^{-4} and of ADP^{-3} with Mg^{++} is considerably greater than that of $HATP^{-3}$, $HADP^{-2}$, or $H6P^{-2}$,

and thus the complex formation of the latter was neglected for the estimations made (16).

Assuming that the components present are ATP^{-4}, $HATP^{-3}$, $MgATP^{-2}$, ADP^{-3}, $HADP^{-2}$, $MgADP^{-1}$, and Mg^{++}, their concentrations can be calculated from seven simultaneous equations.

(1) $$ATP^{-4} + HATP^{-3} + MgATP^{-2} = (ATP)_T$$

(2) $$ADP^{-3} + HADP^{-2} + MgADP^{-} = (ADP)_T$$

(3) $$Mg^{++} + MgADP^{-} + MgATP^{-2} = (Mg)_T$$

(4) $$\frac{(MgATP^{-2})}{(Mg^{++})(ATP^{-4})} = K_1$$

(5) $$\frac{(MgADP^{-})}{(Mg^{++})(ADP^{-3})} = K_2$$

(6) $$\frac{(H^+)(ATP^{-4})}{(HATP^{-3})} = K_3$$

(7) $$\frac{(H^+)(ADP^{-3})}{(HADP^{-2})} = K_4$$

since $(ATP)_T$, $(ADP)_T$, $(Mg)_T$, K_1, K_2, K_3, K_4, and H^+ are known. Substitution of Equation 6 into Equation 1 gives

(8) $$(ATP)_T = (ATP^{-4}) + \frac{(H^+)(ATP^{-4})}{K_3} + (MgATP^{-2})$$

Substitution of Equation 4 into Equation 8 gives

(9) $$(ATP)_T = (ATP^{-4}) + \frac{(H^+)(ATP^{-4})}{K_3} + K_1(Mg^{++})(ATP^{-4})$$

Similar calculations with ADP yield

(10) $$(ADP)_T = (ATP^{-3}) + \frac{(H^+)(ADP^{-3})}{K_4} + K_2(Mg^{++})(ADP^{-3})$$

By substituting Equations 4 and 5 into Equation 3, one obtains

(11) $$(Mg)_T = (Mg^{++}) + K_2(Mg^{++})(ADP^{-3}) + K_1(Mg^{++})(ATP^{-4})$$

By factoring and dividing, one obtains

(12) $$(Mg^{++}) = \frac{(Mg)_T}{1 + K_2(ADP^{-3}) + K_1(ATP^{-4})}$$

By substituting Equation 12 into Equations 9 and 10, one obtains

(13) $$(ATP)_T = (ATP^{-4})\left(1 + \frac{(H^+)}{K_3} + K_1 \times \frac{(Mg)_T}{1 + K_2(ADP^{-3}) + K_1(ATP^{-4})}\right)$$

and

$$(14)\quad (ADP)_T = (ADP^{-3})\left(1 + \frac{(H^+)}{K_4} + K_2 \times \frac{(Mg)_T}{1 + K_2(ADP^{-3}) + K_1(ATP^{-4})}\right)$$

Equations 13 and 14 include a mutual term. However, further manipulation of the equations so that each would contain only one unknown leads

TABLE III

Concentration of Ionic Species of Nucleotides and Mg^{++} at Equilibrium

Experiment No.	Molarity × 10^5				Molarity × 10^3				
	ATP^{-4}	$HATP^{-3}$	$MgATP^{-2}$	$Mg\text{-}HATP^{-1}$*	ADP^{-3}	$HADP^{-2}$	$MgADP^{-1}$	MgHADP*	Mg^{++}
1 (a)	4.86	12.5	14.4	0.39	1.02	2.15	1.02	0.06	1.02
(b)	4.79	12.4	14.2	0.38	1.01	2.13	1.01	0.06	1.01
(c)	4.76	12.3	14.1	0.38	1.01	2.11	1.00	0.06	1.00
2 (a)	4.82	14.70	21.6	0.69	1.50	3.75	2.25	0.16	1.50
(b)	3.00	8.50	31.6	0.94	1.04	2.44	3.70	0.24	3.55
(c)	2.06	6.00	37.3	1.12	0.72	1.72	4.38	0.29	6.08
(d)	1.50	4.24	39.8	1.17	0.53	1.24	4.75	0.31	8.94
(e)			40.5	1.17			5.60	0.36	83.9
3 (a)	2.68	8.13	9.45	0.30	1.03	2.56	1.22	0.09	1.19
(b)	1.97	5.84	15.50	0.48	0.77	1.88	2.04	0.14	2.64
(c)			25.20	0.08			3.88	0.27	81.8
4 (a)	4.39	15.30	14.20	0.52	0.82	2.36	0.91	0.07	1.11
(b)	4.48	15.68	14.80	0.54	0.82	2.36	0.91	0.07	1.11
(c)	4.63	13.40	14.40	0.44	0.92	2.20	0.97	0.06	1.05
(d)	4.85	11.10	13.70	0.32	1.01	1.92	1.05	0.05	0.95
5 (a)			38.60	1.19			2.56	0.18	76.4
(b)			43.35	1.25			5.31	0.34	113.0
(c)			36.80	1.11			6.67	0.47	169.4
(d)			36.00	1.06			6.67	0.47	169.4
6 (a)			20.6	0.06			6.83	0.05	148
(b)			31.5	0.20			6.59	0.10	146
(c)			41.6	0.60			6.32	0.20	144
(d)			56.6	1.80			5.84	0.42	142
(e)			66.8	4.20			5.25	0.74	139

* Except in the case of considerable excess magnesium, these values are only approximations and are not to be included in a balance computation.

to cumbersome equations. Equations 13 and 14 can be solved by a series of approximations. Once the ATP^{-4} and ADP^{-3} concentrations have been calculated, $HATP^{-3}$ and $HADP^{-2}$ can be readily estimated from Equations 6 and 7, respectively. After the Mg^{++} concentration has been calculated

from Equation 12, the concentration of $MgATP^{-2}$ and $MgADP^{-2}$ can be calculated from Equations 4 and 5, respectively. The balance Equations 1, 2, and 3 serve as a check on the calculations. From the constants for $MgHATP^{-}$ and MgHADP dissociation, and the concentrations of free

TABLE IV

Equilibrium Values Obtained from Reverse Reaction

Experiment No.	K_t		K_{Mg}		K_{Mg-0}	
	At experimental pH	Calculated for pH 6.0	At experimental pH	Calculated for pH 6.0	At experimental pH	Calculated for pH 6.0
1 (a)	294	275	165	155	408	383
(b)	299	279	163	152	406	380
(c)	303	283	162	152	418	391
2 (a)	304	304	173	173	446	446
(b)	245	237	166	161	450	437
(c)	203	199	159	156	408	400
(d)	186	180	154	149	400	383
(e)	184	168	178	161		
3 (a)	430	430	234	234	1830	1830
(b)	300	297	200	199	514	509
(c)	193	193	186	186		
4 (a)	230	245	123	131	310	331
(b)	222	236	116	123	298	317
(c)	249	230	135	132	336	329
(d)	277	248	148	133	361	324
5 (a)	123	120	118	116		
(b)	154	140	148	135		
(c)	205	190	196	183		
(d)	214	198	200	186		
6 (a)	1310	131	1310	131		
(b)	540	115	536	117		
(c)	286	130	282	129		
(d)	139	142	134	137		
(e)	88	175	81	161		

Mg^{++}, $HATP^{-3}$, $HADP^{-2}$, an estimation of the concentration of $MgHATP^{-}$ and MgHADP can be made.

The estimated concentrations of the various ionic species for the experiments outlined in Table II are given in Table III.

Equilibrium constants at the experimental pH were calculated based on the total concentration of reactants, K_t, the concentration of $MgATP^{-2}$ and $MgADP^{-1}$, K_{Mg}, or the concentration of uncomplexed nucleotides, K_{Mg-0}. Equilibrium constants were also extrapolated to pH 6.0 on the

basis of the expected shift of the equilibrium with pH.[2] The respective values are summarized in Table IV. With omission of the value for Experiment 3 (a), the average of twelve values for $K_{Mg=0}$ at pH 6.0 is 386 ± 58.[3] The corresponding K for excess magnesium of 0.076 to 0.17 M is 155 ± 31, an average of eleven values.

The value for $K_{Mg=0}$ is based on the concentrations of uncomplexed forms of nucleotides, as given in Table III, and on the total instead of the uncomplexed H6P. Total H6P was used because of the lack of an accurate value for the association constant of Mg^{++} with H6P, the cumbersome nature of the equations if this correction was included, and the

TABLE V

Initial and Final Concentrations from Equilibria of Forward Reaction

Experiment No.*	Initial molarity × 10^3			Final molarity × 10^3 of total species					Final pH
	Glucose	ATP	ADP	Hexose†	ATP	ADP	H6P	Mg^{++}	
7 (a)	3.63	5.08	0	0.0330	1.50	3.58	3.58	117	6.03
(b)	3.29	4.60	2.90	0.0640	1.39	6.11	3.21	105	6.00
(c)	3.18	54.4	2.04	0.0055	51.2	5.04	3.18	102	6.38
8 (a)	3.78	5.00	0	0.0185	1.24	3.76	3.76	2.50	6.00
(b)	3.67	4.85	0	0.0305	1.22	3.64	3.64	4.92	6.08
(c)	3.28	4.25	0	0.0470	1.14	3.24	3.24	99.0	5.94

* Experiment 7, (a), determination of the equilibrium from the forward reaction; (b), effect of addition of ADP to equilibrium reaction mixture of (a); (c), effect of addition of excess ATP to equilibrium reaction mixture of (b). Experiment 8, (a), (b), and (c), effect of increasing magnesium concentration.

† Corrected for non-hexokinase-reactive radioactive impurities.

approximate error introduced in the value of $K_{Mg=0}$ would be less than 10 per cent.

Equilibrium Measurements Starting with Glucose-C^{14} and ATP—These experiments were less extensive than those with the reverse reaction because of experimental difficulties, particularly that any non-hexokinase-

[2] At excess magnesium, nearly all of the reactants form complexes and the production of hydrogen ion is stoichiometric. Then $K_{pH\ 6.0} = K_{pH}(H^+)/10^{-6}$. At zero or catalytic levels of magnesium near pH 6, close to 0.57 mole of hydrogen ion is produced per mole of reaction. Thus near pH 6.0, $K_{pH\ 6.0} = K_{pH}(0.57(H^+))/10^{-6}$. The value of 0.57 mole of H^+ produced was calculated from the generalized equation of Alberty *et al.* (17), together with values given for the acid dissociation constants of $HATP^{-3}$ and $HADP^{-2}$ (15) and of glucose-6-OPO_3H^- (18).

[3] The error is expressed as the standard deviation calculated from the formula $s = \sqrt{\Sigma(x - \bar{x})^2/N - 1}$.

reactive radioactive material in the glucose preparations could introduce serious error into the measurements and the requirement that the initial ATP concentration should be close to the initial glucose concentration. With excess glucose a low, inaccurately measured concentration of ATP would result; with excess ATP the presence of ADP in the ATP prepara-

TABLE VI

Concentration of Ionic Species of Nucleotides and Mg^{++} at Equilibrium

Experiment No.	Molarity × 10^4				Molarity × 10^3				
	ATP^{-4}	$HATP^{-3}$	$MgATP^{-2}$	$MgHATP^{-1}$*	ADP^{-3}	$HADP^{-2}$	Mg-ADP^{-1}	Mg-$HADP$*	Mg^{++}
7 (a)			14.56	0.44			3.37	0.22	112
(b)			13.47	0.43			5.71	0.40	97.5
(c)									
8 (a)	1.72	5.18	5.50	0.17	0.81	2.03	0.88	0.06	1.08
(b)	1.12	2.82	7.60	0.20	0.66	1.36	1.49	0.09	2.28
(c)			11.0	0.40			3.00	0.24	95

* For Experiment 8, these values are approximations and are not to be included in a balance computation.

TABLE VII

Equilibrium Values Obtained from Forward Reaction

Experiment No.	K_t		K_{Mg}		$K_{Mg=0}$	
	At experimental pH	Calculated for pH 6	At experimental pH	Calculated for pH 6	At experimental pH	Calculated for pH 6
7 (a)	260	243	251	233		
(b)	222	222	212	212		
8 (a)	618	618	325	325	828	828
(b)	356	330	236	219	612	567
(c)	172	197	188	215		

tion could introduce serious error. The results of trials with incubation conditions as used previously are summarized in Table V. The calculated amounts of various ionic species are given in Table VI and the equilibrium constants in Table VII. By neglecting the value in Experiment 8 (a), the value for $K_{Mg=0}$ is 567; the value for K with excess Mg^{++} is 221 ± 23.

Equilibria at Higher pH and Free Energy of Hydrolysis of ATP—The equilibrium constant of the hexokinase reaction as measured by the reverse reaction was calculated for pH values 7 and 8 by means of equations which

consider the ionization of the components (15).[4] The free energy of the hexokinase reaction was calculated from the relationship $\Delta F^0 = -RT \ln K$, which at 30° becomes $\Delta F^0 = -1394 \log_{10} K$. The values are presented in Table VIII.[5] Combined with a free energy of hydrolysis of G6P of close to −3.1 kilocalories,[6] the free energy of hydrolysis of ATP at pH

TABLE VIII

*Equilibrium Constants and Free Energy of Hexokinase Reaction at Given H^+ Concentrations**

pH	Zero Mg^{++}		Excess† Mg^{++}	
	K_t	$-\Delta F$	K_t	$-\Delta F$
		kilocalories		*kilocalories*
6.0	386	3.6	155	
7.0	2,460	4.5	1,550	4.7
8.0	23,500	5.9	15,500	6.2

* See footnote 5.

† Magnesium in a 0.079 to 0.170 M excess.

7.0 and 30° is −7.6 and −7.8 kilocalories at excess and catalytic (zero) concentrations of magnesium, respectively.

[4] By the use of their respective acid dissociation constants, the components of the reaction ATP + hexose ⇌ ADP + G6P can be expressed in terms of their total concentrations at various pH values. Substitution of these terms in the equilibrium expression allows the calculation of $K_{Mg=0}$ at various pH values.

[5] The free energy values calculated from the total concentrations of reactants at equilibrium and at a given pH represent the figure for conversion of 1 mole of the various ionic species of reactants at the given H^+ concentration to 1 mole of the various ionic species of products at the given H^+ concentration. For some purposes, the ΔF^0 for better defined standard states is desirable. At pH 8 and 30°, with catalytic amounts of Mg, the stoichiometry of the reaction is essentially as follows: ATP^{-4} + glucose ⇌ ADP^{-3} + $G6P^{-2}$ + H^+, the corresponding K_{eq} is 2.35×10^{-4}, and the ΔF^0 is 5.05 kilocalories.

[6] A small uncertainty remains in the value for the $-\Delta F$ of G6P hydrolysis. The value of 3.1 kilocalories for the $-\Delta F$ at pH 7 and 30° is calculated from the equilibrium value of 122 (H_2O = 55.5 M) found by Meyerhof and Green (9) at pH 8.5 and 38° with high glucose and unspecified Mg concentration by using the expression $K_{pH} = K_{pH\,8.5} \times ((1 + K_2)/(H^+))/((1 + K_1)/(H^+))$, where K_2 is the acid dissociation constant for H_2PO_4, 1.46×10^{-7} (19), and K_1 is the acid dissociation constant for glucose-6-OPO_3H^-, 9.4×10^{-7} (18). For the temperature correction the van't Hoff expression was used, by taking the ΔH as equal to the ΔF. At pH 8.5, at which the stoichiometry of the hydrolysis reaction is essentially $G6P^{-2}$ + HOH ⇌ glucose + HPO_4^{-2}, the estimated K at 30° is 107 (H_2O = 55.5 M), and the corresponding ΔF^0 = −2.65 kilocalories. This value, together with that for the hexokinase reaction (footnote 5), gives a value of ΔF^0 = 2.40 kilocalories for the stoichiometric reaction ATP^{-4} + HOH ⇌ ATP^{-3} + $HOPO_3^{-2}$ + H^+.

Additional experimental details and other information are given elsewhere (16).

DISCUSSION

The results show that the techniques used give a reasonably close approximation to the equilibrium of the hexokinase reaction. Replication of values was better within a given experimental series than among experimental series made at different times. This probably reflects the comparatively large error in the value of K resulting from small errors in some of the experimental measurements, particularly the pH of the reaction medium. The experimental value of K_t at pH 6.0 of 386 ± 58 corresponds to free energy for the hexokinase reaction of -3610 ± 85 calories. Even if the experimental errors were such as to double the equilibrium constant at pH 7, the estimated free energy of hydrolysis of the ATP would be increased by only about 5 per cent.

Mg^{++} has a much greater affinity for ATP^{-4} than for ADP^{-3} and the affinity for the adenine nucleotides is considerably higher than that for other reaction components (15). On this basis increase in the magnesium concentration in the same range as the nucleotide concentration would be expected to shift the equilibrium in the direction of ATP formation. This effect was observed (Experiments 2, 3, and 8 in Tables II and V). Evaluation of the expected quantitative effect of Mg^{++} on the equilibrium is difficult because the association of Mg^{++} with components other than nucleotides may markedly affect the equilibrium when Mg^{++} is present in excess. If it is assumed that the equilibrium is affected only by the combination of Mg^{++} with ATP^{-4} and ADP^{-3}, theoretical values for the ratio of K_t to $K_{Mg=0}$ may be computed. The resulting relationship is indicated in Fig. 1, together with experimental values from Experiment 2 (Table IV). If a combination of Mg^{++} with $H6P^{-2}$ is considered, with an estimated equilibrium constant of 35, the theoretical equilibrium is progressively shifted towards H6P formation (Fig. 1). However, the experimental values of K_t appeared to become constant as Mg^{++} was increased. This independence may arise from the combination of $MgATP^{-2}$ with additional Mg^{++}, or, less likely, from combination of Mg^{++} with hexose in addition to the combination with ATP, ADP, and H6P. While the agreement between the experimental results and theoretical curves of Fig. 1 is fair, the data indicate that all factors influencing the effect of Mg^{++} have not yet been adequately evaluated.

The shift of the equilibrium of the reaction with change in pH was, within experimental error, in agreement with that predicted from the dissociation constants of the reactants. Any changes in the equilibrium with increase in the ionic strength resulting from KCl addition or from

variation in the H6P to nucleotide ratio were less than the experimental variation in the results.

The values at pH 7 of −7.6 and −7.8 kilocalories for the free energy of hydrolysis of ATP in the presence of catalytic or excess quantities of Mg^{++} agree well with the value of Levintow and Meister (7) of −7.9 kilocalories obtained at high Mg^{++} concentration and are slightly higher than the value of −7.0 kilocalories calculated by Morales (8) from the data of Levintow and Meister. The results of experiments given herein, together with those of Levintow and Meister (7), would appear to give a much better evaluation of the free energy of hydrolysis of ATP than less direct previous estimates.

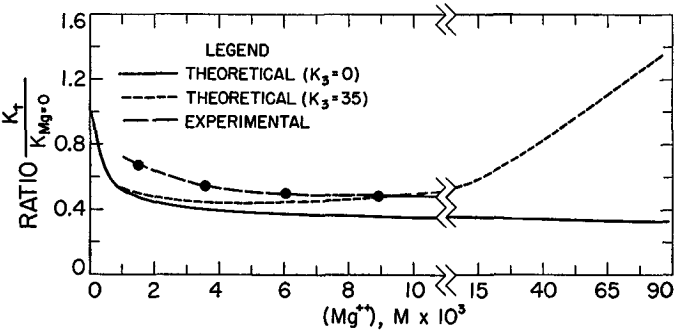

FIG. 1. A comparison of theoretical and experimental effects of Mg^{++} on the hexokinase equilibrium. The theoretical curves were evaluated from the expression $K_t/K_{Mg=0} = ((1 + K_1(Mg^{++}))(1 + K_3(Mg^{++})))/(1 + K_2(Mg^{++}))$ for $K_3 = 0$ and $K_3 = 35$, when K_1, K_2, and K_3 are the association constants for Mg^{++} with ADP^{-3}, ATP^{-4} and $H6P^{-2}$, respectively (16).

SUMMARY

The equilibrium constant of the hexokinase reaction at 30° was determined by use of isotopic dilution techniques for measurement of equilibrium hexose concentrations. The most reliable measurements were made with adenosine diphosphate and hexose-C^{14}-6-phosphate as initial reactants at pH 6.0. The effects of pH, Mg^{++} concentration, ionic strength, and hexose-6-phosphate to nucleotide ratio were evaluated.

The values of K at pH 6.0 for zero (catalytic) Mg^{++} concentration and for 0.079 to 0.017 M excess Mg^{++} are 386 ± 56 and 155 ± 29, respectively. Estimation of K values and calculation of the free energies of the reaction at pH 7.0 give −4.5 and −4.7 kilocalories at pH 7.0 for zero and excess Mg^{++} concentration. Combination with the free energy of hydrolysis of hexose-6-phosphate of −3.1 kilocalories gives −7.6 and −7.8 kilocalories for the free energy of hydrolysis of ATP at zero and excess Mg^{++} concentrations, pH 7.0, and 30°.

Procedures are described for the quantitative removal of hexose-6-phosphate from hexose by use of ion exchange resins, preparation of hexose-C^{14}-6-phosphate, crystallization of hexokinase, and measurement of impurities in glucose-C^{14} preparations.

Addendum—Benzinger and Hems have recently measured the equilibrium of the glutaminase reaction (20). From this measurement and the results of Levintow and Meister (7), they obtained a value of −7.73 kilocalories for the free energy of hydrolysis of ATP at ph 7.0 and 37°. The close agreement between this value, obtained by entirely independent procedures, and that reported herein is gratifying.

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