Furthermore, a theory that specifically accounted for any one of the reactions involving ionic species would leave unexplained the very similar standard free energy change for the reaction between un-ionized species, *i.e.*, equation (13). Likewise no theory would be complete

$$atpH_4 + H_2O \rightleftharpoons adpH_3 + H_3PO_4 \qquad (13)$$

that explained specifically this particular reaction. The missing element in all instances is a consideration of the ionization processes.

The standpoint thus reached is that no single reaction such as (18) or (13) is sufficient for an adequate theoretical treatment of the reactivity of ATP: the reactions involving both ionized and un-ionized species have to be accounted for, and hence the associated ionization equilibria assume a theoretical significance equal to that of the hydrolysis reactions themselves.

References

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

Ashby, J. H., Clarke, H. B., Crook, E. M., and Datta, S. P. (1955), *Biochem. J. 59*, 203.

Ashby, J. H., Crook, E. M., and Datta, S. P. (1954), *Biochem. J. 56*, 198.

Bates, R. G., and Acree, S. F. (1943), *J. Res. Nat. Bur. Std. 30*, 129.

Bates, R. G., and Pinching, G. (1949), *J. Am. Chem. Soc. 71*, 1274.

Benzinger, T., Hems, R., Burton, K., and Kitzinger, C. (1959), *Biochem. J. 71*, 400.

Benzinger, T., and Hems, R. (1956), *Proc. Nat. Acad. Sci. U. S. 42*, 896.

Burton, K. (1959), *Biochem. J. 71*, 388.

Datta, S. P., and Grzybowski, A. K. (1961), Biochemist's Handbook, Long, C., editor, Princeton, N. J., Van Nostrand, Table 8, p. 45.

Elliot, J. S., Sharp, R. F. and Lewis, L. (1958) *J. Phys. Chem. 62*, 686.

George, P., and Rutman, R. J. (1960), *Progr. Biophys. Biophys. Chem. 10*, 1.

Izatt, R. M., and Christensen, J. J. (1962), *J. Phys. Chem. 66*, 359.

Kumler, W. D. (1938), *J. Am. Chem. Soc. 60*, 859.

Levintow, L., and Meister, A. (1954), *J. Biol. Chem. 209*, 265.

Mattock, G. (1961), pH Measurement and Titration, New York, Macmillan Co., Chap. 13.

Melchior, N. C. (1954), *J. Biol. Chem. 208*, 615.

Rutman, R. J., and George, P. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1094.

Scatchard, G., and Breckenridge, P. C. (1954), *J. Phys. Chem. 58*, 596.

Smith, R. M., and Alberty, R. A. (1956), *J. Am. Chem. Soc. 78*, 2376.

Snell, F. M. (1955) Electrochemistry in Biology and Medicine, Shedlorsky, T., editor, New York, John Wiley, Chap. 15, p. 284.

Stokes, J. M. (1945), *Trans. Faraday Soc. 41*, 685.

Wingfield, B., and Acree, S. F. (1937), *J. Res. Nat. Bur. Std. 19*, 163.

Estimates of Thermodynamic Data for the Formation of the Mg^{2+} Complexes of ATP and ADP at Zero Ionic Strength*

PHILIP GEORGE, ROGER C. PHILLIPS, S.J., AND ROBERT J. RUTMAN

From the John Harrison Laboratory of Chemistry, University of Pennsylvania, Philadelphia

Received December 4, 1962

In order to calculate thermodynamic data for the formation of the Mg^{2+} complexes of ATP and ADP at zero ionic strength from stability constant measurements at finite ionic strengths, an empirical extrapolation procedure is proposed, based on the assumption that the activity coefficient ratios for nucleotide species of the same charge type are identical at the same ionic strength. Calculations of the standard free energy, enthalpy, and entropy changes have been carried out with data in the literature, and quantitative aspects of the influence of complex formation on the hydrolysis of ATP yielding ADP and orthophosphate are discussed.

The determination of stability constants for the formation of the Mg^{2+} complexes of ATP and ADP[1] presents considerable experimental problems, and precise values are particularly difficult to obtain in the low ionic strength region which would permit extrapolation to zero ionic strength and hence the evaluation of the true thermodynamic constants. Considering the change in charge in the two reactions shown in equations (1) and (2), a very pronounced ionic strength effect is to be

$$atp^{4-} + Mg^{2+} \longrightarrow atpMg^{2-} \qquad (1)$$

$$adp^{3-} + Mg^{2+} \longrightarrow adpMg^{1-} \qquad (2)$$

* This work was undertaken as part of a research program on the thermodynamics of phosphate reactions supported by USPHS grant AM-04764.

[1] The usual abbreviations AMP, ADP, and ATP for adenosine mono-, di-, and triphosphate, respectively, are used when there is no need to specify the ionic species precisely. When this is essential amp^{2-}, adp^{3-}, and atp^{4-} are used to designate the nucleotide species in which the phosphate groups are fully ionized; the corresponding conjugate acids are thus $ampH^{1-}$, $adpH^{2-}$, $atpH^{3-}$, *etc.*

anticipated, with the stability constant increasing as the ionic strength is decreased throughout the Debye-Hückel range.

In the absence of the desired experimental data at low ionic strengths, we propose an empirical extrapolation procedure which appears to give reasonable estimates for the thermodynamic values. It is based upon the assumption that the activity coefficient ratios for nucleotide species of the same charge type are identical at the same ionic strength. In the calculations which are presented below, we have obtained the required activity coefficient ratios from our previous studies of the secondary ionization of the terminal phosphate groups in AMP, ADP, and ATP (Phillips *et al.*, 1963).

Equilibrium Constants upon Which the Calculations Are Based

A survey of the literature shows that a variety of values have been obtained for the apparent stability constants of $atpMg^{2-}$ and $adpMg^{1-}$. These have been collected in Table I. Although the empirical extrapo-

TABLE I
VALUES REPORTED FOR THE STABILITY CONSTANTS OF $atpMg^{-2}$ AND $adpMg^{-1}$

Worker	Method	Supporting Electrolyte[a]	Temp.	$atpMg^{1-}$, log K_c	$adpMg^{1-}$, log K_c
Martell and Schwarzenbach (1956)	*p*H titration	0.1 M KCl	20°	4.00 ± 0.04	3.11 ±0.05
Smith and Alberty (1956)	*p*H titration	0.2 M tpaBr	25°	3.47 ± 0.03	3.00 ± 0.05
Nanninga (1957)	Resin competition	0.15 M NaCl	10°	3.06	
			23°	3.34	
			43°	3.50	
		0.1 M NaCl	25°	3.61 ± 0.03	3.03 ± 0.03
Walaas (1958)	Resin competition	0.1 M NaCl	23°	4.04	3.15
Burton (1959)	Competition in solution with 8-hydroxyquinoline	0.1 M tbeaBr	25°	4.58	3.34
		0.1 M tbeaBr	35°	—	3.48
		0.1 M tbeaBr	64°	4.99	3.84
		0.22 M tbeaBr	25°	4.35	3.23
O'Sullivan and Perrin (1961)	Competition in solution with 8-hydroxyquinoline	0.1 M Tris buffer	30°	4.30 ± 0.04	—
		0.1 M Triethanolamine buffer	30°	4.89 ± 0.04	—
		0.1 M *N*-ethylmorpholine buffer	30°	4.93 ± 0.04	—
	*p*H titration	0.1 M teaBr	30°	5.02 ± 0.06	—
Nanninga (1961)	Resin competition	0.1 M teaBr	25°	4.37 ± 0.03	—
	*p*H titration	0.1 M teaBr	25°	4.43 ± 0.03	—
Taqui Kahn and Martell (1962)	*p*H titration	0.1 M KNO_3	25°	—	3.19 ± 0.01

[a] The abbreviations tea, tpa, and tbea stand for the tetraethyl, tetrapropyl, and tributyl-ethyl ammonium ion respectively.

lation procedure could be applied to any of the tabulated constants, the data of Burton (1959) have been selected for several reasons: (1) values at two ionic strengths allow a check to be made on the calculated K_0s; (2) the temperature variation allows calculation of thermodynamic values; (3) the supporting electrolyte, tributylethylammonium bromide, has an electrostatically weak cation which eliminates the complication of the cation of the medium competing with the Mg^{2+} ion for the phosphate; and (4) the values are about midway between the extreme values reported.

Since Burton's measurements were made at *p*H values well on the alkaline side of the secondary phosphate ionization of ATP and ADP, *i.e.*, at *p*H 8.8 and 7.9, respectively, the reactions studied approximated very closely the reactions between the single ionic species shown in (1) and (2). The concentration equilibrium constants could thus be written specifically as equation (3) for the ATP reaction and as equation (4) for the

$$K_c = \frac{[atpMg^{2-}]}{[atp^{4-}] \times [Mg^{2+}]} \quad (3)$$

$$K_c = \frac{[adpMg^{1-}]}{[adp^{3-}] \times [Mg^{2+}]} \quad (4)$$

ADP reaction. The values Burton obtained under various experimental conditions are listed in Table I.

ESTIMATION OF STABILITY CONSTANTS FOR THE Mg^{2+} COMPLEXES OF ATP AND ADP AT ZERO IONIC STRENGTH

Taking the ATP reaction first, the stability constant at zero ionic strength is related to the concentration constant by equation (5). Now it has been found that

$$K_0 = K_c \times \frac{\gamma_{atpMg^{2-}}}{\gamma_{atp^{4-}} \times \gamma_{Mg^{2+}}} \quad (5)$$

in the presence of an electrostatically weak cation the secondary phosphate ionizations of ATP, ADP, and AMP obey the Debye-Hückel limiting law at low ionic strength (Phillips *et al.*, 1963). Furthermore, the common adenosine moiety makes a big contribution to the over-all size of the molecules. Hence it is a reasonable assumption that the salting-out term in the simple empirical equation (6) is also quite similar for species of

$$\log \gamma = -a\sqrt{\mu} + b\mu \quad (6)$$

like charge. As a first approximation, therefore, certain substitutions may be made for the activity coefficients of the nucleotide species. Multiplying numerator and denominator of the activity coefficient term in equation (5) by $\gamma_{adp^{3-}}$, it can be rewritten,

$$\frac{\gamma_{atpMg^{2-}}}{\gamma_{atp^{4-}} \times \gamma_{Mg^{2+}}} = \frac{\gamma_{atpMg^{2-}}}{\gamma_{adp^{3-}}} \times \frac{\gamma_{adp^{3-}}}{\gamma_{atp^{4-}}} \times \frac{1}{\gamma_{Mg^{2+}}}$$

and, substituting $\gamma_{adpH^{2-}}$ for $\gamma_{atpMg^{2-}}$ and $\gamma_{atpH^{3-}}$ for $\gamma_{adp^{3-}}$, it can be written as equation (7). Since the

$$\frac{\gamma_{atpMg^{2-}}}{\gamma_{atp^{4-}} \times \gamma_{Mg^{2+}}} \approx \frac{\gamma_{adpH^{2-}}}{\gamma_{adp^{3-}}} \times \frac{\gamma_{atpH^{3-}}}{\gamma_{atp^{4-}}} \times \frac{1}{\gamma_{Mg^{2+}}} \quad (7)$$

concentration stability constants were determined in the presence of an electrostatically weak cation, appropriate values for the activity coefficient ratios $\gamma_{adpH^{2-}}/\gamma_{adp^{3-}}$ and $\gamma_{atpH^{3-}}/\gamma_{atp^{4-}}$ can be obtained from the variation of pK' with $\sqrt{\mu}$ for the secondary phosphate ionization of ADP and ATP established with tetra *n*-propylammonium salts (Phillips *et al.*, 1963). The values for the ratios at 25° are 2.50 and 3.88, respectively, at $\mu = 0.11$, and 2.57 and 4.26, respectively, at $\mu = 0.22$.

Bearing in mind the principle that for dilute solutions the activity coefficient of a strong electrolyte is the same in all solutions of the same ionic strength, values for $\gamma_{Mg^{2+}}$ can be obtained by interpolation from the mean activity coefficients for $MgBr_2$ and $Mg(NO_3)_2$, tabulated by Robinson and Stokes (1940), Stokes (1948), and Latimer (1952). Values of 0.64 at $\mu = 0.11$ and 0.58 at $\mu = 0.22$ are adopted for 25°.

Substituting these values for $\gamma_{adpH^{2-}}/\gamma_{adp^{3-}}$, $\gamma_{atpH^{3-}}/\gamma_{atp^{4-}}$, and $\gamma_{Mg^{2+}}$, and the values of K_c from Table I in

TABLE II

EXPERIMENTAL VALUES OF K_c (BURTON, 1959) AND CALCULATED VALUES OF K_0 FOR THE FORMATION OF THE Mg^{2+} COMPLEXES OF ATP AND ADP AT 25°

Stability Constant (M^{-1})	ATP		ADP	
	$\mu = 0.11$	$\mu = 0.22$	$\mu = 0.11$	$\mu = 0.22$
K_c, exptl.	3.8×10^4	2.25×10^4	2.2×10^3	1.7×10^3
K_0, calcd.	5.8×10^5	4.2×10^5	1.4×10^4	1.1×10^4
Mean K_0	5.0×10^5		1.25×10^4	

TABLE III

VALUES FOR THE STANDARD FREE ENERGY, ENTHALPY, AND ENTROPY OF FORMATION OF THE Mg^{2+} COMPLEXES OF ATP AND ADP AT 25°, BASED UPON ESTIMATES OF THE STABILITY CONSTANTS AT ZERO IONIC STRENGTH

All values are in kcal/mole, except ΔS^0, which is in e.u.

Reaction	ΔF^0	ΔH	$T\Delta S^0$	ΔS^0
$atp^{4-} + Mg^{2+} \rightleftharpoons atpMg^{2-}$	−7.8	+4.8 ± 1.0	+12.6 ± 1.0	+42 ± 3
$adp^{3-} + Mg^{2+} \rightleftharpoons adpMg^{1-}$	−5.6	+5.8 ± 1.0	+11.4 ± 1.0	+38 ± 3

equations (5) and (7), K_0 is found to be 5.8×10^5 and 4.2×10^5 M^{-1} from the data at $\mu = 0.11$ and 0.22, respectively, at 25°, giving a mean value of 5.0×10^5 M^{-1}.

The corresponding equation relating K_0 and K_c for the ADP reaction is given by equation (8). Multiplying

$$K_0 = K_c \times \frac{\gamma_{adpMg^{1-}}}{\gamma_{adp^{3-}} \times \gamma_{Mg^{2+}}} \qquad (8)$$

numerator and denominator of the activity coefficient term by $\gamma_{amp^{2-}}$,

$$\frac{\gamma_{adpMg^{1-}}}{\gamma_{adp^{3-}} \times \gamma_{Mg^{2+}}} = \frac{\gamma_{adpMg^{1-}}}{\gamma_{amp^{2-}}} \times \frac{\gamma_{amp^{2-}}}{\gamma_{adp^{3-}}} \times \frac{1}{\gamma_{Mg^{2+}}}$$

and, substituting $\gamma_{ampH^{1-}}$ for $\gamma_{adpMg^{1-}}$ and $\gamma_{adpH^{2-}}$ for $\gamma_{amp^{2-}}$, it follows that

$$\frac{\gamma_{adpMg^{1-}}}{\gamma_{adp^{3-}} \times \gamma_{Mg^{2+}}} \approx \frac{\gamma_{ampH^{1-}}}{\gamma_{amp^{2-}}} \times \frac{\gamma_{adpH^{2-}}}{\gamma_{adp^{3-}}} \times \frac{1}{\gamma_{Mg^{2+}}} \qquad (9)$$

From the plot of pK' against $\sqrt{\mu}$ for the secondary phosphate ionization of AMP (Phillips *et al.*, 1963) the values 1.62 and 1.48 are obtained for $\gamma_{ampH^{1-}}/\gamma_{amp^{2-}}$ at $\mu = 0.11$ and 0.22 respectively at 25°. Together with the previous values for $\gamma_{adpH^{2-}}/\gamma_{adp^{3-}}$ and $\gamma_{Mg^{2+}}$, and those for K_c from Table I, substitution in equations (8) and (9) gives the values of K_0, 1.4×10^4 and 1.1×10^4 M^{-1} for the data at $\mu = 0.11$ and 0.22, respectively, at 25°, with a mean value of 1.25×10^4 M^{-1}.

Comparing the values of K_c and K_0, listed in Table II, two points may be noted. First, it can be seen that the values of K_0 are more nearly equal than those of K_c in both cases, as indeed they should be if the estimates of the activity coefficients are well based: they should, of course, be identical. In part the discrepancy can be attributed to uncertainty in the values adopted for $\gamma_{Mg^{2+}}$: 0.66 instead of 0.64 at $\mu = 0.11$ and 0.56 instead of 0.58 at $\mu = 0.22$ would be acceptable in view of the interpolation errors, and on this basis the values K_0 for the ATP reaction would be closer still, 5.6 and 4.4×10^5 M^{-1}. In addition, part of the discrepancy undoubtedly arises from the assumption made regarding the ratios of activity coefficients for the nucleotide species. But since the values calculated for K_0 are almost within the experimental error of the measurements, namely, about 10%, the extrapolation procedure would appear to be justified as a reasonable approximation, and any further refinement of the present data is uncalled for.

Secondly, it can be seen that the activity coefficient term as a whole is large in magnitude, as anticipated for reactions of this type. For the formation of the ATP complex, K_0 is about 17 times K_c; and for the ADP complex, K_0 is about 6.5 times K_c. The standard free energy changes for complex formation calculated from the values of K_0 are thus appreciably more favorable than those based upon the values of K_c at $\mu = 0.11$, namely, −7.8 instead of −6.3 kcal/mole for the ATP reaction, and −5.6 instead of −4.6 kcal/mole for the ADP reaction.

ESTIMATES OF THE STANDARD FREE ENERGY, ENTHALPY, AND ENTROPY OF FORMATION OF THE Mg^{2+} COMPLEXES OF ATP AND ADP

On the basis of the K_c values in Table I, Burton (1959) calculated the enthalpy changes to be about +5 and +6 kcal/mole for the ATP and ADP reactions, respectively, and the entropy change to be about +36 e.u. for both reactions. The more reliable values for the standard free energy changes calculated from the estimates of K_0 will, however, be reflected in the values for the entropy changes, and new calculations have therefore been made. For this purpose rather more precise values of ΔH have been obtained from the data at $\mu = 0.11$ in Table I, taking into account the experimental error of ±10% in K_c, *viz.*, +4.8 ± 1.0 and 5.8 ± 1.0 kcal/mole for the ATP and ADP reactions respectively. Substituting the values of ΔF^0 and ΔH in the thermodynamic equation (equation 10), $T\Delta S^0$ is found

$$\Delta F^0 = \Delta H - T\Delta S^0 \qquad (10)$$

to be +12.6 and +11.4 kcal/mole, respectively, and the corresponding entropy values are +42 and +38 e.u. These thermodynamic data are brought together in Table III.

It can be seen that the entropy changes are more positive than those calculated from the K_c data, especially in the case of the ATP reaction. But the values are still quite similar in magnitude for both reactions, and this feature merits closer examination because a substantially more favorable entropy change might have been expected to accompany the partial cancellation of the higher charge of atp^{4-}.

The data for the secondary phosphate ionization of ATP, ADP, and AMP are very relevant to this question. Written in reverse as the formation of "proton complexes" to correspond to the formation of the metal ion

TABLE IV

A COMPARISON OF THERMODYNAMIC DATA FOR THE HYDROLYSIS OF ATP AT 25° AND *p*H 10 0 IN THE ABSENCE AND PRESENCE OF Mg^{2+} IONS, BASED UPON ESTIMATES OF THE EQUILIBRIUM CONSTANTS AT ZERO IONIC STRENGTH

All values are in kcal/mole, except $\Delta S'$, which is in e.u.

Reaction	ΔF_{obs}	$\phi RT ln H$	$\Delta F'$	$\Delta H'$	$T\Delta S'$	$\Delta S'$
$atp^{4-} + H_2O \longleftrightarrow adp^{3-} + HPO_4^{2-} + H^+$	−12.7	−13.7	+1.0	−4.4	−5.4	−18
$atpMg^{2-} + H_2O \longleftrightarrow adpMg^{1-} + HPO_4^{2-} + H^+$	−10.5	−13.7	+3.2	−3.4	−6.6	−22
Difference	− 2.2	0	−2.2	−1.0	+1.2	+ 4

complexes, the various entropy changes are as shown below:

$$atp^{4-} + H^+ \longleftrightarrow atpH^{3-} \quad \Delta S^0 = +40.7 \pm 1.0 \text{ e.u.}$$

$$adp^{3-} + H^+ \longleftrightarrow adpH^{2-} \quad \Delta S^0 = +37.4 \pm 1.0 \text{ e.u.}$$

$$amp^{2-} + H^+ \longleftrightarrow ampH^{1-} \quad \Delta S^0 = +33.4 \pm 1.0 \text{ e.u.}$$

(Phillips *et al.*, 1963). Similar data are not available for the next ionization, so the over-all entropy changes accompanying the cancellation of two negative charges by protons, which would afford a more strict comparison with the effect of Mg^{2+}, cannot yet be calculated. Nevertheless there is no reason to suppose from the above values that there would be a *very pronounced* trend toward more positive entropy changes in the series AMP to ADP to ATP. At most the ATP reaction might be expected to be a few entropy units more favorable than the ADP reaction, as indeed the values suggest. Hence, as far as a comparison can be made at present, the data for the ionization reactions and for the formation of the Mg^{2+} complexes appear to be self-consistent.

THE HYDROLYSIS OF ATP INVOLVING Mg^{2+} COMPLEXES OF THE NUCLEOTIDES

For solutions in which the *p*H is greater than the pK' values for the secondary phosphate ionizations of ATP, ADP, and orthophosphate, but less than pK' for the tertiary ionization of orthophosphate, the familiar hydrolysis takes the form of equation (11). However,

$$atp^{4-} + H_2O \longleftrightarrow adp^{3-} + HPO_4^{2-} + H^+ \quad (11)$$

if Mg^{2+} ions are present in concentrations such that the ATP and ADP complexes are fully formed but the orthophosphate remains uncombined, reaction (11) becomes reaction (12). These conditions are readily

$$atpMg^{2-} + H_2O \longleftrightarrow adpMg^{1-} + HPO_4^{2-} + H^+ \quad (12)$$

achieved in practice. In evaluating the standard free energy change for reaction (11) from studies of the glutamine synthetase reaction and the hydrolysis of glutamine, Benzinger *et al.* (1959) have shown that with the experimental conditions employed the data lead first to a value for reaction (12), and hence the difference between the standard free energies for the formation of the Mg^{2+} complexes of ATP and ADP has to be taken into account. From the values of K_c in Table I, Burton (1959) calculated the free energy differences at 25° to be −1.7 kcal/mole.

From a similar assumption with regard to an activity coefficient ratio as those involved in the estimations of K_0, the value for the free energy of hydrolysis of ATP calculated by Benzinger *et al.* (1959) has also been extrapolated to zero ionic strength (Phillips *et al.*, 1963). A comparison can thus be made to show the fundamental influence that complex formation has upon the hydrolysis reaction, free from ionic strength effects. The data for the reactions at *p*H 10 have been chosen for this purpose because at this *p*H both reactions approximate very closely those between the single ionic species represented in reactions (11) and (12). The various thermodynamic terms are listed in Table IV. ΔF_{obs} is the "observed" standard free energy change, evaluated from the equilibrium constant expression,

$$K_{obs} = \frac{[\text{total ADP species at equil.}] \times [\text{total orthophosphate species at equil.}]}{[\text{total ATP species at equilibrium}]}$$

As shown elsewhere (George and Rutman, 1960), ΔF_{obs} is made up of a term $\phi RT ln H$, the driving force due to the prevailing low hydrogen ion concentration, and $\Delta F'$, the standard free energy change for the reaction with respect to the 1 molal standard state for the hydrogen ion. ϕ is the number of moles of H^+ required to complete the stoichiometry of the reaction, and in equation (13), relating ΔF_{obs} and $\Delta F'$, ϕ carries a positive sign if H^+ is a product, and a negative sign if a reactant. Since for both reaction (11) and reaction (12), $\phi = +1$, $\phi RT ln H$ has the identical value of −13.7 kcal/mole at *p*H 10.

$$\Delta F_{obs} = \Delta F' + \phi RT ln H \quad (13)$$

At this *p*H the more favorable value of ΔF_{obs} for the ATP hydrolysis in the absence of Mg^{2+} ions thus originates entirely in the difference between the $\Delta F'$ values, *i.e.*, −2.2 kcal/mole. This is, of course, the difference between the standard free energy changes for the formation of the complexes. If the experimental uncertainty attached to the values for ΔH and ΔS^c is set aside (see Table III), it would appear that neither the enthalpy term, $\Delta H'$, nor the entropy term, $T\Delta S'$, is alone responsible for the more favorable value of $\Delta F'$: the former contributes −1.0 kcal/mole and the latter an almost equal amount, −1.2 kcal/mole, as a consequence of the entropy change being more favorable by 4 e.u.

In conclusion, the comparison of the thermodynamic quantities for the two reactions has an interesting bearing on cell economy. Mg^{2+} ions are an essential component of the enzyme systems that catalyze the reactions of ATP. However, a small but significant part of the driving force inherent in the hydrolysis is lost when the reaction involves Mg^{2+} complexes of the nucleotides. The necessary speed is thus achieved at the expense of an equally necessary thermodynamic property.

REFERENCES

Benzinger, T., Kitzinger, C., Hems, R., and Burton, K. (1959), *Biochem. J. 71*, 400.

Burton, K. (1959), *Biochem. J. 71*, 388.

George P., and Rutman, R. J. (1960), *Prog. Biophys. Biophys. Chem. 10*, 1.

Latimer, W. M. (1952), The Oxidation States of the Elements and Their Potentials in Aqueous Solution, ed. 2, New York, Prentice-Hall, Table 86, p. 355.

Martell, A., and Schwarzenbach, G. (1956), *Helv. Chim. Acta 39*, 653.
Nanninga, L. B. (1957), *J. Phys. Chem. 61*, 1144.
Nanninga, L. B. (1961), *Biochim. Biophys. Acta 54*, 330.
O'Sullivan, W., and Perrin, D. (1961), *Biochim. Biophys. Acta 52*, 612.
Phillips, R. C., S.J., George, P., and Rutman, R. J. (1963), *Biochemistry 2*, 501 (this issue).
Robinson, R. A.. and Stokes, R. H. (1940), *Trans. Faraday Soc. 36*, 733.
Smith, R., and Alberty, R. (1956), *J. Am. Chem. Soc. 78*, 2376.
Stokes, R. H. (1948), *Trans. Faraday Soc. 44*, 295.
Taqui Kahn, M., and Martell, A. E. (1962), *J. Am. Chem. Soc. 84*, 3037.
Walaas, E. (1958), *Acta Chem. Scand. 12*, 528.

Separation of Oxidative from Phosphorylative Activity by Proteolysis of Glyceraldehyde-3-Phosphate Dehydrogenase*

I. KRIMSKY AND E. RACKER

From the Department of Biochemistry, The Public Health Research Institute of The City of New York New York 9, New York

Received December 5, 1962

Controlled digestion of glyceraldehyde-3-phosphate dehydrogenase with chymotrypsin resulted in marked changes in molecular size and enzymic properties of the protein. After digestion, the enzyme no longer catalyzed the over-all reaction of oxidative phosphorylation of glyceraldehyde-3-phosphate but retained the ability to oxidize glyceraldehyde in the absence of phosphate at an undiminished or even accelerated rate. Glyceraldehyde-3-phosphate was oxidized, in the presence of monothioglycerol as acyl acceptor, at a somewhat slower rate, whereas with arsenate as acceptor over 95% of the activity was lost after digestion. With enzyme acting stoichiometrically as acyl acceptor, the rate of oxidation of glyceraldehyde-3-phosphate by digested enzyme was unimpaired or accelerated. Threose-2,4-diphosphate, a potent inhibitor of glyceraldehyde or glyceraldehyde-3-phosphate oxidation by native enzyme, had little or no effect on digested enzyme. Acylation of digested enzyme by oxidation of aldehyde not only was more rapid but was more extensive as compared with native enzyme. This phenomenon was shown to be due to a pronounced inhibition of aldehyde oxidation by reduced diphosphopyridine nucleotide in the case of native enzyme. These findings are discussed in reference to the mode of action of glyceraldehyde-3-phosphate dehydrogenase and to problems of oxidative phosphorylation linked to electron transport.

The enzyme glyceraldehyde-3-phosphate dehydrogenase catalyzes the oxidative phosphorylation of glyceraldehyde-3-P to 1,3-diphosphoglycerate. Studies on the mechanism of action of this enzyme in several laboratories (*cf.* Racker, 1955) revealed that the reaction proceeds in two steps:

$$E-DPN + \text{glyceraldehyde-3-P} \rightleftharpoons \text{3-P-glyceryl}-E + DPNH + H^+ \quad (1)$$

$$\text{3-P-glyceryl}-E + P_i \rightleftharpoons \text{1,3-di-P-glycerate} + E \quad (2)$$

The enzyme-substrate intermediate formed in the first step has been isolated in crystalline form and has been shown to undergo either reduction in the presence of DPNH or cleavage in the presence of arsenate (Krimsky and Racker, 1955).

It is the purpose of this paper to show that the oxidative activity of the enzyme can be separated from the phosphorolytic activity by controlled proteolytic digestion. The properties of the resulting altered enzyme will be described.

Materials and Methods

Preparation of Glyceraldehyde-3-P Dehydrogenase.—Glyceraldehyde-3-P dehydrogenase was prepared from rabbit muscle (Cori *et al.*, 1948), with the modification that 0.005 M EDTA was present in the extracting fluid and was used in this concentration throughout subsequent procedures. The enzyme was recrystallized three times and then further purified by washing the crystals obtained from one rabbit (about 2 g) three times with 800 ml of 3 M $(NH_4)_2SO_4$ solution, *p*H 7.9. The washed crystals were dissolved in 0.005 M EDTA, *p*H 7.8, to give a protein concentration of about 7%, and stored at 2°. The enzyme was stable in this form for several months.

Removal of DPN from Glyceraldehyde-3-P Dehydrogenase.—In order to permit digestion by proteolytic enzymes (Racker and Krimsky, 1958), enzyme-bound DPN was removed with charcoal. The charcoal (Norit A, neutral, Fisher Scientific Co.) was prepared by washing 110 g with 2 liters of 1 N HCl twice by decantation. It was then washed on a Buchner funnel with water until the filtrate was essentially free of acid, then with 0.005 M EDTA, *p*H 7.8 (about 16 liters), until the *p*H of the filtrate was 7.8, and finally with 8 liters of H_2O, and then dried. This exhaustive washing of the charcoal was necessary for the preparation of relatively stable digested glyceraldehyde-3-P dehydrogenase. The charcoal was suspended in 0.005 M EDTA, *p*H 7.2, poured into a column of 1-cm diameter, and allowed to settle. A height of 1.5 cm of wet packed charcoal was used for each 100 mg of enzyme, applied as a 7% solution. The enzyme solution was followed by a volume of 0.005 M EDTA, *p*H 7.2, somewhat in excess of the hold-up volume of the column. Air pressure was used to give a flow rate of about 0.4 ml per minute. About 80% of the protein applied to the column was recovered in the effluent. Use of a larger ratio of charcoal to protein gave rise to greater loss of protein. The 280/260 mμ absorbancy ratio of

* This research was supported in part by U. S. Public Health Service Grant No. C-3463.