[CONTRIBUTION FROM THE NAVAL MEDICAL RESEARCH INSTITUTE]

Rates and Energetics of Activation of the Acid-catalyzed Hydrolysis of Adenosine Triphosphate[1a,b]

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The activation energetics of hydrolysis of the biologically important adenosine triphosphoric acid (ATP) molecule have been studied over the temperature range 40–50°, for comparison with corresponding quantities for the hydrolysis of the simpler polyphosphates and with the myosin-catalyzed ATP dephosphorylation process. At *p*H 1.33 and in the presence of 0.30 *M* added sodium chloride, the following quantities of activation (at 40°) were observed for ATP hydrolysis: E^{A}, 21.2 ± 0.4 kcal./mole; $\Delta F^{\ddagger}$, 26.0 ± 0.5 kcal./mole; $\Delta H^{\ddagger}$ 20.6 ± 0.4 kcal./mole; $\Delta S^{\ddagger}$ −17.4 ± 0.4 e.u. These quantities are uniformly higher than the corresponding values associated with hydrolysis of the enzyme–substrate complex for the myosin-catalyzed reaction (Ouellet, Laidler and Morales), but nearly identical with the activation quantities for simple di- and triphosphate hydrolysis under the same conditions.

The rates of acid-catalyzed ATP hydrolysis at 50° are intermediate in value between those rates previously observed for triphosphoric and pyrophosphoric acid hydrolysis, are directly proportional to the acidity of the medium in the *p*H range 1–1.5, and are dependent in a complex way on total salt concentration.

An important element of recent theories of the chemistry of muscle action[2] involves the myosin-catalyzed dephosphorylation of the adenosine triphosphate (ATP) ion at some stage of the fiber expansion–contraction cycle. Since the energy requirements and work involved in muscular function would seem to be intimately associated with this enzymatic process, a knowledge of the energetics[3] of this terminal phosphate cleavage under catalysis by muscle adenosine triphosphatase is of considerable significance. However, a fuller understanding of any specialized role of ATP in the energy transfer process would also require some knowledge of the intrinsic ease and energetics of hydrolysis of the terminal phosphate linkage itself, in the absence of the enzyme system.

Accordingly, the present paper deals with a limited study of the kinetics and energetics of activation of the purely chemical analog of the enzymatic cleavage process, *i.e.*, the hydrolytic fission of the terminal phosphate group from the free acid ATP under relatively mild acid catalysis.

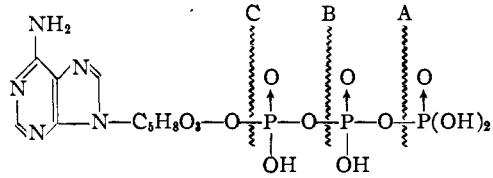
$$ATP + H_2O \xrightarrow{H^+} ADP + H_3PO_4$$

Acidic conditions were employed to obtain rates of reaction of the same order of magnitude as those observed in the enzymatic work.[3]

Information as to the activation energetics of this acid-catalyzed process can be compared with corresponding data[3] on phosphate cleavage from the enzyme–substrate complex in the myosin-catalyzed reaction, and the entire picture examined for any kinetic evidence of peculiarity in the terminal phosphate linkage, such as the localized "high-energy phosphate bond" character[4,5] commonly attributed to this linkage. Further contrast can be made with the available equilibrium data[6] on hydrolysis of compounds of the ATP type.

Discussion

In studying the acid-catalyzed hydrolysis of the terminal phosphate group in the ATP molecule, nucleophilic attack of water resulting in cleavage



at the terminal position A could well be accompanied by fission of a pyrophosphate residue as shown at B, or cleavage of the sugar–phosphorus linkage at C. That these three processes occur at rates different enough to permit isolation of the terminal cleavage reaction at A can be inferred from previous studies[7] on certain related model compounds. It has been shown[7a] that under acidic conditions nearly equivalent to those employed in this work, the single-stage cleavage of triphosphoric acid to pyro- and orthophosphate is at least six times faster than the corresponding hydrolysis of pyrophosphoric acid to orthophosphate. On this basis it would be expected that cleavage at linkage A should be intrinsically faster than that at B, and that the orthophosphate concentration observed as a function of time would result almost entirely from hydrolysis at A, in the first stages of reaction. This conclusion is substantially upheld by the reproducible linearity of first-order rate plots over the first 25–50% of each ATP hydrolysis run, since an appreciable amount of hydrolysis at B in this interval would lead to excessive amounts of phosphate production, through the intermediate pyrophosphate cleavage, and ultimately to deviation from the observed first-order kinetics at fixed *p*H.

The further problem of cleavage at the sugar hydroxyl–phosphorus linkage C does not appear to

(1) (a) The opinions in this paper are those of the author, and do not necessarily reflect the views of the Navy Department. (b) Presented in part before the Second International Congress of Biochemistry, Paris, France, July 21–27, 1952.

(2) For recent discussions and leading references see: (a) A. Szent-Györgyi, "Chemistry of Muscular Contraction," Second Ed., Academic Press, Inc., New York, N. Y., 1951; (b) M. F. Morales and J. Botts, *Arch. Biochem.*, **37**, 283 (1952).

(3) L. Ouellet, K. Laidler and M. F. Morales, *ibid.*, **39**, 37 (1952).

(4) (a) F. Lipmann, *Advances in Enzymol.*, **1**, 99 (1941); (b) H. M. Kalckar, *Chem. Revs.*, **28**, 71 (1941).

(5) For a discussion of the non-localized, electrostatic "high-energy" reactant effect in ATP leading to thermodynamic instability, see T. L. Hill and M. F. Morales, THIS JOURNAL, **73**, 1656 (1951).

(6) See for example the compilation of data by P. Oesper, "A Symposium on Phosphorus Metabolism," The Johns Hopkins Press, Baltimore, Md., 1951, p. 525.

(7) (a) S. L. Friess, THIS JOURNAL, **74**, 4027 (1952); (b) **74**, 5521 (1952).

complicate the kinetics, since previous work[7b] on the model primary hydroxyl–phosphate bond in fructose-6-phosphoric acid reveals a very marked stability toward hydrolysis under the conditions of the present study, leading to rate constants smaller than those of the A type by a factor of at least 10^2.

Hydrolysis Rates, Temperature Dependence.—A first series of kinetic runs was designed to yield temperature coefficient data for the ATP hydrolysis under the conditions previously used,[7a] in order to facilitate study of the terminal solvolysis reaction (A). In these runs sufficient hydrochloric acid was used in each solution to set its initial *p*H value (25°) at 1.33, and all solutions were prepared 0.300 *M* in added sodium chloride. Data from a typical first-order rate determination in this series are shown plotted in Fig. 1. Results of runs made in

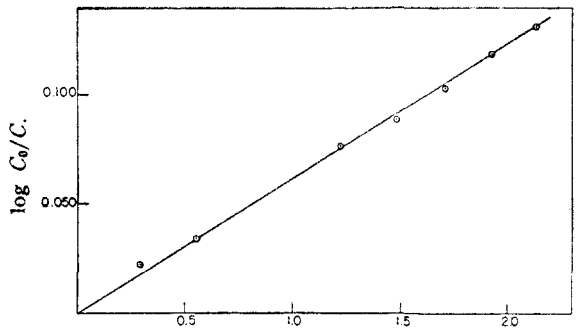


Fig. 1.—Acid-catalyzed ATP hydrolysis: $(ATP)_0$, 0.0198 *M*; temperature, 50.24°; *p*H 1.33.

duplicate are given in Table I, with the indicated accuracy for first-order rate constant (k_1) values that were obtained analytically from the kinetic data.

TABLE I

TEMPERATURE COEFFICIENT DATA FOR ATP HYDROLYSIS RATES

$(ATP)_0$, 0.0198*M*; (NaCl added), 0.300*M*; *p*H 1.33

Temperature,[a] °C.	k_1 (sec.$^{-1}$ × 10^6)
39.94	4.67 ± 0.16
43.82	7.22 ± 0.48
47.06	10.0 ± 0.3
50.24	13.9 ± 0.5

[a] Temperatures held constant to ±0.02°.

First-order rate constants were evaluated in terms of orthophosphate production, assuming one liberated phosphate ion to correspond to one hydrolyzed ATP molecule. This over-all k_1 calculation does not separate the individual contributions of the various ionic ATP species to the observed values of the rate constants, but only presents their summation, for comparison with similarly obtained constants for hydrolysis of the polyphosphates.[7a] Under the conditions used in this phase of the work (*p*H 1.33), the reactant form of ATP would consist largely[8] of the first ionization product, H_3ATP^-. The over-all k_1 values obtained were, in general, reproducible within about 5% for duplicate runs. Comparison of these rate constants with those observed[7a] for polyphosphate hydrolysis reveals that k_1 for ATP hydrolysis at a fixed temperature and acidity is intermediate in magnitude between the values found for tri- and pyrophosphoric acids under the same conditions.

The rate data of Table I were plotted according to the Arrhenius equation, with the resulting straight line shown in Fig. 2. Application of the

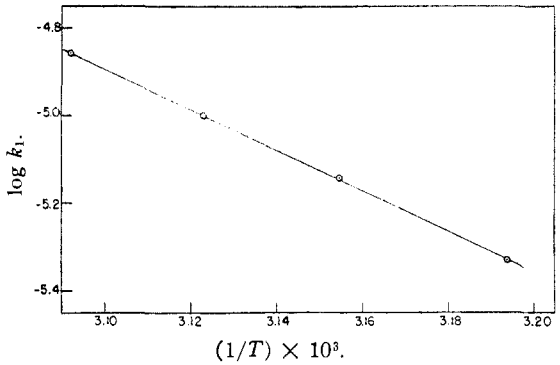


Fig. 2.—Activation energy plot for ATP hydrolysis: $(ATP)_0$, 0.0198 *M*; (added NaCl), 0.300 *M*; *p*H 1.33.

method of least squares to the linear log k_1 *vs.* $1/T$ data gave the value of the energy of activation, E^A, shown in Table II. Also included in Table II are values for the thermodynamic quantities of activation $\Delta F^{\ddagger}$, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ calculated for the reference temperature 40.0°. For comparison purposes, the corresponding data for the equivalent hydrolyses of pyro- and triphosphoric acids[7a] are also tabulated.

TABLE II

ENERGETICS OF ACTIVATION FOR POLYPHOSPHATE HYDROLYSIS

Temperature, 40.0°, (added NaCl) 0.300 *M*

	E^A, kcal./mole	$\Delta F^{\ddagger}$ kcal./mole	$\Delta H^{\ddagger}$ kcal./mole	$\Delta S^{\ddagger}$, e.u.
ATP	21.2 ± 0.4	26.0 ± 0.5	20.6 ± 0.4	−17.4 ± 0.4
Triphosphoric acid[7a]	22.9	25.5	22.3	−10.3
Pyrophosphoric acid[7a]	22.8	26.7	22.2	−14.5

Several interesting points are to be noted from the results in Table II. First, with respect to E^A values, the "tail" of the ATP species is by no means unique in the magnitude of the activation energy associated with its dephosphorylation, as compared with the very nearly equal values found for hydrolysis of the other polyphosphates. That the adenosine residue of ATP does not confer a special, localized activity on the extremity of the triphosphate tail is also compatible with the observation that the 21 kcal. value of the energy hump for ATP hydrolysis is within the 15–23 kcal./mole range usually found[9] for the mechanistically similar process of acid-catalyzed hydrolysis of esters of organic acids.

Quite as striking as the equivalence of E^A values

(8) From an apparent pK'_a value in 0.15 *M* NaCl solution, Alberty and co-workers have estimated that at *p*H 2 the average ionic charge of ATP is about −1.7; R. A. Alberty, R. M. Smith and R. M. Bock, *J. Biol. Chem.*, **193**, 425 (1951).

(9) For a compilation of data on E^A values for the acid-catalyzed hydrolysis of aliphatic and aromatic esters see (a) A. E. Remick, "Electronic Interpretations of Organic Chemistry," Second Ed., John Wiley and Sons, Inc., New York, N. Y., 1949, p. 422; (b) P. N. Rylander and D. S. Tarbell, THIS JOURNAL, **72**, 3021 (1950).

is the close correspondence between $\Delta F^{\pm}$ and $\Delta H^{\pm}$ values for ATP and for the two polyphosphate species in Table II, pointing to the similarity in the activation processes for all the members of the group. Indeed, the greatest differentiation in activation ease for the three polyphosphates is to be found in the values for their entropies of activation, with $\Delta S^{\pm}$ for the ATP dephosphorylation turning out more negative than the corresponding pyro- and triphosphate values. This could well be a function of the greater complexity of structure of the ATP molecule as compared to pyro- and triphosphoric acids, requiring more extensive solvation and precise orientation in its hydrolysis transition state, relative to its ground state as a reactant, than that found in the simpler polyphosphate hydrolyses. It is of interest in this connection that the difference in $\Delta S^{\pm}$ values between ATP and triphosphoric acid reactions (*ca.* 7 e.u.) is far greater than the approximately 1 e.u. that might be expected if ATP were considered to be a simple derivative of triphosphate with one of the two equivalent end phosphorus positions hindered or completely blocked to nucleophilic attack because of the adenosine substitution.

Another curious point concerns the kinetically-determined energetics of activation of the ATP hydrolysis reaction, as contrasted to the over-all free energy change calculated for the equilibrium process. Lipmann[4a] and others[6] have pointed to the abnormally large $-\Delta F^0$ value (*ca.* 10.5 kcal./mole) for the hydrolysis of ATP, setting it apart from normal polyphosphates and endowing it with the quality of a localized, terminal "high-energy phosphate bond," but this differentiation does not show up in the energetics of the acid-catalyzed *activation* process.

Acid-catalysis of Rates.—To obtain some estimate of the relative magnitude of the *p*H effect on the ATP hydrolysis, runs were made in which, at a constant added salt level, small variations in HCl content were allowed to change initial *p*H values (glass electrode) by small increments. A representative set of data is reproduced in Table III below.

TABLE III

EFFECT OF *p*H ON RATE CONSTANTS FOR ATP HYDROLYSIS

Temperature, 49.53 ± 0.02°; (added NaCl), 0.300 *M*; $(ATP)_0$, 0.0198 *M*

*p*H	k_1 (sec.$^{-1}$ × 10^5)
1.13	1.42 ± 0.07
1.20	1.37 ± .03
1.35	1.26 ± .08

These data indicate a virtually linear dependence of rate constant on *p*H in this limited region of acidity, with increasing acidity producing an increase in the observed k_1 value.

Dependence of Rates on Total Salt Concentration.—All of the preceding measurements were made at approximately constant initial concentrations of ATP and of added salt, to conform to conditions used in the previous model work. Since the hydrolysis involves a large, negatively charged ion, it was of some interest to observe what effect variation of the total ionic strength has on the reaction rate. This variation was carried out by the addition of successively larger increments of sodium chloride to a series of otherwise identical rate runs, with the results indicated in Table IV.

TABLE IV

EFFECT OF ADDED SALT ON THE HYDROLYSIS RATE

Temperature, 49.53 ± 0.02°; *p*H 1.35; $(ATP)_0$, 0.0198 *M*

(Total NaCl),[a] *M*	Ionic strength,[b] μ	k_1, sec.$^{-1}$ × 10^5
0.079	0.147	1.70 ± 0.15
.154	.222	2.05 ± .06
.229	.297	1.23 ± .17
.379	.447	1.27 ± .10

[a] NaCl added, plus the NaCl resulting from the Na_2SO_4 precipitation and subsequent HCl acidification procedures. [b] Total ionic strength, assuming all ATP as H_3ATP^-.

It is seen that the rate constant is quite dependent on small changes in ionic strength at values of μ much below 0.30, but that this dependence is much less marked (and indeed k_1 is almost constant, within experimental error) for values of μ lying between 0.30 and 0.40. This condition on μ held for the bulk of the measurements in this study.

Comparison of Acid-catalyzed with Enzymatically-catalyzed ATP Hydrolysis.—A final point of contrast can be made between the kinetics of acid-catalyzed ATP hydrolysis noted above and those of the myosin-catalyzed dephosphorylation as observed by Ouellet, Laidler and Morales.[3] It might be observed that the reaction conditions employed in each case are by no means equivalent, since the enzymatic work was carried out at *p*H 7.0 in a solution 0.6 *M* in KCl and containing 10^{-3} *M* Ca^{++} ion, in which solution the predominant ATP species present were polyvalent anions. However, from the standpoint that catalysis by hydronium ion at low *p*H produces rates of ATP dephosphorylation of the same order of magnitude as those found for myosin catalysis in neutral solution, the energetics of these processes occurring at nearly equivalent catalytic levels can be compared.

In the enzymatic process, the standard Michaelis–Menten treatment presupposes an enzyme–ATP complex as the species undergoing hydrolysis, and it is for this reaction that the following activation data[3] are given: $\Delta F^{\pm}$, 14.8 kcal./mole; $\Delta H^{\pm}$, 12.4 kcal./mole; $\Delta S^{\pm}$, −8.0 e.u. Comparison of these data with those for ATP in Table II reveals that the activation quantities for the enzymatic process are of the same sign as, and uniformly lower in absolute value than, those for the acid-catalyzed dephosphorylation reaction. Although it is not feasible to compare $\Delta S^{\pm}$ quantities for the two processes because of the nebulous character of both the ground and transition states of the enzyme–substrate complex reacting with water, it is quite reasonable that the free energy and the enthalpy of activation should be lower for the reaction employing the more efficient and highly-specific enzyme catalyst.

Experimental

Materials.—Samples of the dibarium salt of ATP available from the Sigma Chemical Co. and from Nutritional Biochemicals Corporation were used throughout this study. Each sample was analyzed by the ion-exchange technique

of Cohn and Carter,[10] with the chief impurity in each case consisting of adenosine diphosphate (and an equivalent quantity of inorganic phosphate). The Sigma sample analyzed for 71% of dibarium adenosine triphosphate by dry weight, while that from Nutritional Biochemicals Corp. gave an 86% analytical figure. Kinetic dephosphorylation runs on these two samples under the same conditions (49.53°, *p*H 1.35) gave values of the respective rate constants (1.26 ± 0.08, 1.27 ± 0.10 × 10^{-5} sec.$^{-1}$) identical within experimental error, pointing to the relative hydrolytic inertness of the varying amounts of adenosine diphosphate found in these materials.

Triply distilled water was used in all the work, and the other reagents employed were of analytical grade.

Procedure for Kinetic Runs.—In a representative rate determination, a weighed sample of the dry barium salt was treated in a 50-ml. beaker with 20 ml. of water and the desired volume of a standard 1.3 *M* solution of hydrochloric acid. After stirring to effect solution of the salt, the barium ion was precipitated by the slow addition, with continual stirring, of the required amount of sodium sulfate. The mixture was allowed to settle for about 10 minutes at 5°, and then filtered through a prewashed analytical filter paper directly into the 50-ml. volumetric flask to be used as the reaction vessel. In a run employing added sodium chloride, a preweighed quantity of salt had already been transferred to the reaction flask before the filtration procedure. About 15 ml. of water was used to aid the filtration and in washing the precipitated barium sulfate. The solution was then made to about 48 ml., placed in a water thermostat held constant to ± 0.02° or better (as measured using Beckmann thermometers calibrated with an N.B.S. certified thermometer), and allowed to come to temperature during an approximately 15-minute interval. It was finally made to volume with water already at the bath temperature, and the first 2-ml. aliquot taken for analysis after about 20 minutes.

Aliquots of reaction mixture were quenched by addition to a large volume of water containing sufficient sodium acetate to neutralize all free acid, and the resulting solutions made to the proper volume for colorimetric estimation of orthophosphate present, using the method of Lowry and Lopez.[11] ATP concentrations at times *t* were evaluated from the initial weights of samples of known purity and the inorganic phosphate concentrations at *t*, assuming one orthophosphate ion to be derived from one hydrolyzed ATP molecule. The observed phosphate ion concentrations in the reaction mixture aliquots were corrected for the blank phosphate content of the initial samples.

First-order rate constants were calculated analytically in the standard manner, and were found to be reproducible within the limits of accuracy of the constants themselves (*i.e.*, about 5% on the average). Occasionally, in scattered runs made at the lower temperatures, some erratic behavior in phosphate production was noted at the start of a given run. In such a case, the rate constant was calculated from the steady portion of the rate curve following cessation of the abnormal initial behavior. Such rate constants were generally verified by duplicate runs of more normal character.

The *p*H values of reaction mixtures were determined on samples withdrawn from solution and cooled to room temperature, using a Beckman *p*H meter.

Acknowledgment.—The technical assistance of Mr. James A. Robinson in some of the kinetic determinations is gratefully acknowledged.

(10) W. E. Cohn and C. E. Carter, THIS JOURNAL, **72**, 4273 (1950).

(11) O. H. Lowry and J. A. Lopez, *J. Biol. Chem.*, **162**, 421 (1946).

BETHESDA, MD.

[CONTRIBUTION FROM THE VENEREAL DISEASE EXPERIMENTAL LABORATORY, U. S. PUBLIC HEALTH SERVICE, UNIVERSITY OF NORTH CAROLINA, SCHOOL OF PUBLIC HEALTH]

A Method for the Preparation of a Stable Brain Fraction Containing Acetylcholinesterase[1]

BY HENRY TAUBER

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A dried fraction containing acetylcholinesterase activity has been prepared from frozen pig brain. The dried material is stable and can be readily redissolved. Although not free of impurities, such preparation represents a convenient source of reproducible enzyme preparations having acetylcholinesterase activity. A few anticholinesterase experiments are presented to indicate the activity and specificity of the enzyme.

Introduction

The preparation of a stable mammalian brain acetylcholinesterase has not been reported previously. Without exception, fresh homogenates or supernatants of homogenates of mammalian brain tissue have been used as the source of this enzyme. Such preparations have obvious limitations as to purity, reproducibility and availability.

There is here reported a rapid, convenient and inexpensive method for the preparation of a stable fraction containing dry, soluble acetylcholinesterase from frozen pig brain. Such a method, while not offering a highly active product, provides a convenient source of the enzyme for *in vitro* studies of anticholinesterases. This is of particular value in the study of new anticholinesterase compounds, which have assumed considerable pharmacological and insecticidal importance. Experiments concerning the enzymic properties of this material are presented to demonstrate its activity as an acetylcholinesterase as opposed to a cholinesterase.

Materials and Solutions

Activator Buffer–Salt Solution.—For full activity the pig brain acetylcholinesterase requires bivalent ions and sodium chloride. The following activator buffer solution was employed: 5.8 g. of NaCl and 0.202 g. of $MgCl_2 \cdot 6H_2O$ were dissolved in 495 ml. of 0.2 *M* phosphate buffer of *p*H 7.0. The solution was readjusted to *p*H 7.0 with 3.5 *N* NaOH, and brought to a final volume of 500 ml. with distilled water.[2a,b] We found the salt concentrations as used in these experiments (final concentration of $MgCl_2$ 0.00027 *M* and NaCl 0.025 *M*) to be optimal for our enzyme preparation.

Substrates.—The acetylcholine bromide and the other substrates were dissolved in 0.1 *M* phosphate buffer at *p*H 7.0. The final substrate concentrations in the 4-ml. digests were 0.004 *M*. The acetylcholine concentration used in these investigations is optimal for acetylcholinesterase of conductive tissue (electric eel, mammalian brain).[2a,b,3]

(1) Presented before the Division of Biological Chemistry of the National Meeting of the American Chemical Society at Atlantic City, N. J., September 14–19, 1952.

(2) (a) I. B. Wilson and F. Bergmann, *J. Biol. Chem.*, **185**, 479 (1950); (b) D. Nachmansohn and M. A. Rothenberg, *ibid.*, **158**, 653 (1945).

(3) K.-B. Augustinsson, *Nature*, **161**, 194 (1948).