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# Mechanisms of Hydrolysis of Adenosine 5′-Triphosphate, Adenosine 5′-Diphosphate, and Inorganic Pyrophosphate in Aqueous Perchloric Acid†

Graham J. Hutchings, Barbara E. C. Banks,* Margaret Mruzek, John H. Ridd, and Charles A. Vernon

ABSTRACT: The acid-catalyzed hydrolysis of adenosine 5′-triphosphate (ATP) has been found to give rise both to adenosine 5′-diphosphate (ADP) and inorganic phosphate and to adenosine 5′-phosphate (AMP) and inorganic pyrophosphate. Kinetic and isotope studies on the mechanism of hydrolysis of ATP therefore depend on a knowledge of the mechanism of hydrolysis of the polyphosphate products, ADP and inorganic pyrophosphate. The latter reactions have been studied over the acidity range 1–5 M perchloric acid at 25 °C while the more complex problem of the hydrolysis of ATP has been followed at a single acidity (3 M perchloric acid). The positions of bond fission have been determined for both ATP and ADP.

It has been known for a long time that monoesters of phosphoric acid are extremely resistant to nonenzymic hydrolysis. This is, of course, consistent with their common function as intermediary metabolites. The monoesters of the polycondensed phosphoric acids, although still relatively stable chemically, are more labile and undergo hydrolysis under conditions where the products (the parent monoesters of phosphoric acid) are further hydrolyzed only very slowly. For example, it has been shown that ADP,[1] ATP, and adenosine 5′-tetraphosphate are hydrolyzed rapidly in 1 M sulfuric at 100 °C whereas the product, AMP, is not (Liebecq, 1957).

From about 1950 onward determined efforts were made by a number of groups to establish the mechanisms involved in

† From the Christopher Ingold Laboratories (G.J.H., M.M., J.H.R., and C.A.V.) and the Department of Physiology (B.E.C.B.), University College London, London WC1E 6BT, England. *Received January 5, 1981; revised manuscript received May 18, 1981.* The Science Research Council (United Kingdom) provided financial assistance (to G.J.H.).

[1] Abbreviations used: AMP, adenosine 5′-phosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; $P_i$ and $PP_i$, inorganic phosphate and pyrophosphate.

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the nonenzymic hydrolyses of monoesters of phosphoric acid (and to a lesser extent of diesters and of triesters). A powerful tool proved to be the use of $^{18}O$ tracer. This not only provides a definitive method for the determination of the position of bond fission but can also reveal whether oxygen exchange into the substrate is a concomitant of the hydrolytic reaction. Although considerable progress was made, the fundamental question about the mechanism of nucleophilic attack on phosphorus in phosphate esters remained unanswered since no simple criterion of molecularity was available (all solvolytic reactions show first-order kinetics irrespective of mechanism).

In contrast, very little mechanistic work has been done with the monoesters of the condensed polyphosphoric acids. We now report a study, using kinetic and tracer techniques, of the acid-catalyzed hydrolysis of ADP and of ATP. Our most important finding is that we can detect all the four possible ways in which AMP can be produced from ATP.

Apart from its intrinsic chemical interest, we feel that this work may be of use to biochemists studying the synthesis of ATP (as it occurs in mitochondria, for example) by using $^{18}O$ tracer techniques. Obviously the total incorporation of tracer can be established by complete hydrolysis of the synthesized ATP to AMP and inorganic phosphate—given that there is no oxygen exchange into unchanged substrate during hydrolysis. The distribution of tracer, however, can only be established if the positions of bond fission in the hydrolytic reaction are known.

## Materials and Methods

*Materials.* Adenine nucleotides were purchased from Sigma Chemical Co. Other chemical agents were of AR grade or of the highest purity available. Other materials were as follows: Bio-Rad Ag50 WX4 200–400 mesh cation-exchange resin (hydrogen form); inorganic pyrophosphatase (EC 3.6.1.1) from Merck; $^{18}O$-enriched water, Prochem, from BOC Ltd. (1.29 atom % $^{18}O$ excess); active charcoal, Norit GSX.

*Kinetic Studies.* (*a*) *Estimation of Inorganic Phosphate.* The colorimetric method of Fiske & Subbarrow (1925) as modified by King (1932) was used. Solutions of reducing agent (1-naphthol-2-amino-4sulfonic acid, sodium sulfite, and sodium metabisulfite) were calibrated daily and discarded after 1 week.

(*b*) *Estimation of Inorganic Pyrophosphate.* The enzyme inorganic pyrophosphatase specifically catalyzes the hydrolysis of pyrophosphate to inorganic phosphate at pH 7 and 25 °C. In order to determine inorganic pyrophosphate in the presence of inorganic phosphate, we used the following procedure: Aliquots (1 mL) of the solution to be analyzed were pipetted into two 10-mL volumetric flasks. To one flask was added inorganic pyrophosphatase (10 μL, 1 unit of activity) while Tris buffer (1 mL, pH 7.0, 1 M Tris containing 0.001 M $MgCl_2$) was added to both aliquots, and the flasks were incubated at 25 °C for 15 min before analysis for inorganic phosphate as in (a). The concentration of inorganic pyrophosphate is found from the difference in the amounts of inorganic phosphate in the two aliquots. By this method, 0.1 mmol of inorganic pyrophosphate can be assayed in the presence of 5 mmol of inorganic phosphate.

(*c*) *Kinetics of Formation of Inorganic Phosphate.* ADP, ATP, or inorganic pyrophosphate were dissolved in perchloric acid solutions of the required strength (10 mL) and the solutions maintained at the required temperature, ±0.02 °C, by using a Gallenkamp thermostirrer. All kinetic runs were carried out by using initial substrate concentrations of ~10 mM. Duplicate aliquots (100 μL) were removed at suitable intervals by using an Eppendorf sampler and diluted in 5 mL of water at 0 °C in 10-mL volumetric flasks. Samples were stored at 0 °C prior to assay.

(*d*) *Kinetics of Formation of Inorganic Pyrophosphate.* ATP was dissolved in perchloric acid of the required strength and held at 25 °C as in (c). Duplicate aliquots (100 μL) were removed at suitable intervals and diluted in 5 mL of water at 0 °C in 10-mL flasks. One of each pair of aliquots was neutralized with potassium hydroxide (10 M) and stored at 0 °C. Potassium perchlorate was removed from the neutralized solution by centrifugation, and aliquots (1 mL) were analyzed for inorganic pyrophosphate as in (b). The second of each pair of aliquots was analyzed directly for inorganic phosphate.

*$^{18}O$ Tracer Studies.* The positions of bond fission were determined by carrying out the reactions in water enriched in $^{18}O$. The $^{18}O$ contents of inorganic phosphate or water were determined by the method of Boyer et al. (1961). The $CO_2$ produced was analyzed for $^{18}O$ by using an AEI MS20 isotope mass ratio spectrometer.

*Isolation of Inorganic Phosphate.* Reaction mixtures were neutralized with potassium hydroxide (10 M) at 0 °C and the precipitated potassium perchlorate was removed by centrifugation. The pH of the supernatant was adjusted to pH 3.5 with HCl (2 M). Active charcoal (0.6 g, Norit GSX) was added, the suspension shaken for 20 min, and the charcoal removed by centrifugation. The procedure was repeated twice more removing more than 99.9% of adenine nucleotides from the solution. The last traces of charcoal were removed from the final supernatant by passage through a millipore filter. The pH was adjusted to 4.5 (HCl, 2 M) and the solution evaporated to dryness in a rotary evaporator. The residual solid was dissolved in distilled water (1 mL), the pH adjusted to 4.5, and absolute ethanol (8 mL) added to precipitate the potassium dihydrogen orthophosphate. The solid was removed by centrifugation and dried at 110 °C. To avoid contamination of the product by potassium perchlorate, we used the method of Welch (Welch, 1960). The solid was weighed and dissolved in the minimum volume of water required for that weight of pure potassium dihydrogen orthophosphate (11.71 g dissolved in 100 mL). The solution was heated to facilitate solution and then cooled in ice for 20 min, whereupon potassium perchlorate is preferentially precipitated. The precipitate was spun off, and pure potassium dihydrogen orthophosphate was precipitated by the addition of ethanol. The solid was removed by centrifugation and dried at 110 °C.

A sample of $^{18}O$-enriched potassium dihydrogen orthophosphate was prepared by reaction between phosphorus pentachloride and an excess of $H_2{}^{18}O$ and isolated by the above method. It was found to have the correct $^{18}O$ atom % excess, within experimental error (1.318, cf. 1.293 abundance in the water from which it was prepared).

*Tracer Incorporation into Adenine Nucleotides.* All tracer experiments were carried out with 50 mM solutions of adenine nucleotides. After suitable periods at 25 °C, reaction was stopped by cooling to 0 °C and the solution neutralized with potassium hydroxide (10 M). Potassium perchlorate was removed by centrifugation and the pH of the supernatant adjusted to 3.5 with HCl (2 M). Active charcoal (0.6 g, Norit GSX) was added and the suspension shaken vigorously for 20 min. The charcoal was spun off and washed 3 times with normal water to remove retained enriched solvents. The washed charcoal was suspended in perchloric acid (3 M) at 100 °C for 5 min to give 100% hydrolysis. The inorganic phosphate released was isolated as described above, after neutralization with potassium hydroxide, and the $^{18}O$ excess

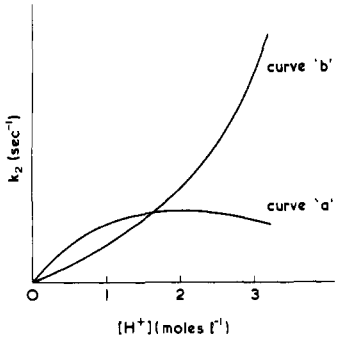


FIGURE 1: Hypothetical dependence on acidity of the rate coefficient for hydrolysis of ADP given that the monoanion (curve a) or the neutral molecule (curve b) is the reactive species.

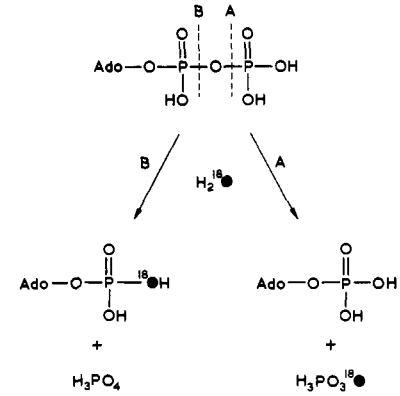


FIGURE 2: Possible labeling resulting from hydrolysis of ADP in water enriched in $H_2^{18}O$.

abundance determined. Negligible exchange of oxygen between the inorganic phosphate and the water occurs under these conditions (Bunton et al., 1961).

*Analysis of Kinetic Data.* (*a*) *Hydrolysis of Pyrophosphoric Acid.* The hydrolysis of pyrophosphoric acid (~0.01 M), catalyzed by strong acid, was found to be first order with respect to the formation of inorganic phosphate. First-order rate constants ($k_4$) were calculated in the usual way.

(*b*) *Hydrolysis of Adenosine Diphosphate, ADP.* The hydrolysis of ADP to AMP and inorganic phosphate is known (Liebecq, 1957) to be much faster than the subsequent hydrolysis of AMP. In the present work, in 3 M perchloric acid at 25 °C, the rate factors have been found to differ by $10^3$ so the reaction

$$\text{AMP} \rightarrow \text{adenosine} + \text{P}_i$$

has been neglected in studies of the hydrolysis of both ADP and of ATP. The hydrolysis of ADP in solutions of perchloric acid between 0.75 and 5 M has been followed at 25 °C and in 3 M acid over the temperature range 25–52.5° C. At the lower acid concentrations, some ADP is present as the monoanion, and it is necessary to correct the rate coefficients to refer only to the amount of the neutral species present.

If the neutral species were the species undergoing hydrolysis, then the dependence of rate on acidity would be as shown in curve a of Figure 1, whereas if a conjugate acid were to be reactive, the dependence of rate on acidity would be as shown in curve b. The experimental data are as shown in curve b, and it is therefore presumed that it is a conjugate acid that is the reactive species. The percentage of the monoanion is calculated from the known p$K_a$ of ADP (Dawson et al., 1969) and the acidity by using the Henderson–Hasselbach equation.

The hydrolysis of ADP (~0.01 M) does not significantly alter the acidity of the medium, and the reaction showed first-order kinetics. The rate coefficients, $k_2$, were calculated as in (a).

(*c*) *Hydrolysis of Adenosine Triphosphate.* The relevant equations describing the reactions occurring on acid-catalyzed hydrolysis of ATP are

$$\text{ATP} \xrightarrow{k_1} \text{ADP} + \text{P}_i \quad (1)$$

$$\text{ADP} \xrightarrow{k_2} \text{AMP} + \text{P}_i \quad (2)$$

$$\text{ATP} \xrightarrow{k_3} \text{AMP} + \text{PP}_i \quad (3)$$

$$\text{PP}_i \xrightarrow{k_4} 2\text{P}_i \quad (4)$$

Reactions 2 and 4 were studied separately, and hence $k_2$ and $k_4$ were known. The concentrations of ADP, $PP_i$, and $P_i$ can be expressed in terms of the initial concentration of substrate, $[ATP]_0$, the four rate coefficients, and the time $t$, in seconds (eq 5–7).

$$[\text{ADP}] = \frac{k_1[\text{ATP}]_0}{k_2 - k_1 - k_3}(\exp[-(k_1 + k_3)t] - \exp(-k_2 t)) \quad (5)$$

$$[\text{PP}_i] = \frac{k_3[\text{ATP}]_0}{k_4 - k_1 - k_3}(\exp[-(k_1 + k_3)t] - \exp(-k_4 t)) \quad (6)$$

$$[\text{P}_i] = [\text{ATP}]_0\left(\frac{-1}{k_1 + k_3}\left(k_1 + \frac{2k_4k_3}{k_4 - k_1 - k_3} + \frac{k_1k_2}{k_2 - k_1 - k_3}\right)(\exp[-(k_1 + k_3)t] - 1) + \frac{2k_3}{k_4 - k_1 - k_3}[\exp(-k_4 t) - 1] + \frac{k_1}{k_2 - k_1 - k_3}[\exp(-k_2 t) - 1]\right) \quad (7)$$

If $k_1 \gg k_3$, i.e., when inorganic pyrophosphate is not a product of hydrolysis of ATP, eq 7 reduces to

$$[\text{P}_i] = [\text{ATP}]_0\left[2 - \left(1 + \frac{k_2}{k_2 - k_1}\right)\exp(-k_1 t) + \frac{k_1}{k_2 - k_1}\exp(-k_2 t)\right] \quad (8)$$

The hydrolysis of ATP (~0.01 M) in perchloric acid (3 M) at 25 °C has been followed from the variation both of inorganic phosphate and of inorganic pyrophosphate. The rate coefficients $k_2$ and $k_4$ are known under these conditions. Their values have been substituted into eq 6 and 7, and the best values of the rate coefficients $k_1$ and $k_3$ were then obtained by fitting the experimental data to the equations using the method of least squares. The computer program used is given elsewhere (Hutchings, 1975). In addition, because it has been found that $k_1 \gg k_3$, the simplified form of eq 7, eq 8, has also been used to calculate values of $k_1$ and $k_2$ by a least-squares fit of the experimental variation of $[P_i]$ with time.

*Analysis of $^{18}O$ Tracer Data.* (*a*) *Hydrolysis of ADP.* The hydrolysis of ADP to AMP and $P_i$ can occur by fission at position A or B (Figure 2), giving, in $^{18}O$-enriched water, the products indicated. Both enriched products may be formed, and the percentage fission at the two positions is calculated by

$$\%\ \text{fission at A} = \frac{4p}{s} \times 100$$

where $p$ and $s$ are the excess abundances of $^{18}O$ in the inorganic phosphate and solvent, respectively

$$\%\ \text{fission at B} = (100 - \%\ \text{fission at A})$$

The factor of 4 arises because 100% fission at position A would

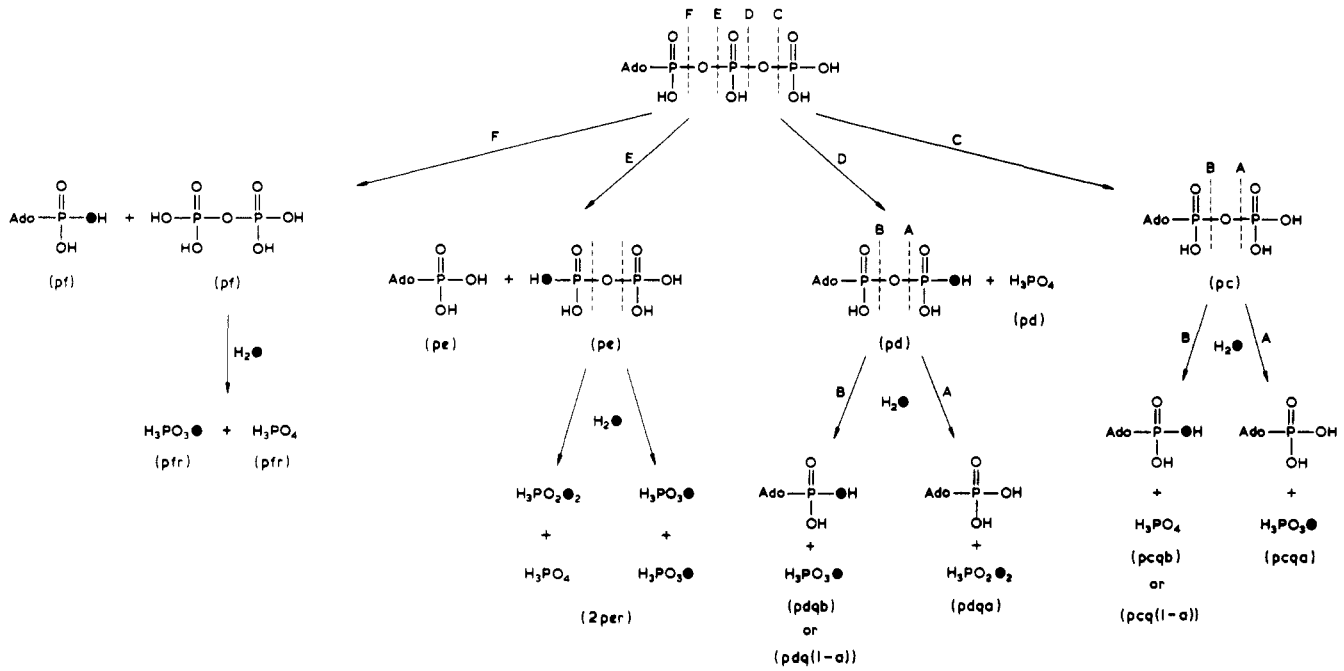

FIGURE 3: Possible labeling resulting from hydrolysis of ATP and subsequently of ADP and inorganic pyrophosphate in water enriched in $H_2{}^{18}O$. ● = $^{18}O$; $p$ = fraction of 1 mole of ATP hydrolyzed; $q$ = fraction of ADP formed that is subsequently hydrolyzed; $r$ = fraction of inorganic pyrophosphate formed that is subsequently hydrolyzed; $c$, $d$, $e$, and $f$ are the ratios of bond fission occurring in ADP in positions C, D, E, and F ($c + d + e + f = 1$); $a$, $b$ are the ratios of bond fission occurring in ADP at positions A and B; ($a + b = 1$).

give rise to inorganic phosphate with 25% of the $^{18}O$ enrichment of the water. Neither ADP nor inorganic phosphate undergoes oxygen isotope exchange with the solvent under the conditions of reaction investigated in the present work.

(*b*) *Hydrolysis of ATP.* The four possible positions of bond fission in ATP are positions C, D, E, and F as given in Figure 3. Cleavage at C or D will give rise to ADP which will then be hydrolyzed at positions A and B as already described. If a fraction $p$ of 1 mol of ATP undergoes hydrolysis in solvent enriched in $^{18}O$ and this involves fission at positions C, D, E, and F in the ratio $c/d/e/f$ ($c + d + e + f = 1$), then the amounts of labeled and unlabeled products are given in the first four lines of Table I. If fractions $q$ and $r$ of the products ADP and $PP_i$, respectively, then undergo further hydrolysis, the amounts of labeled and unlabeled inorganic phosphate produced are shown in the last six lines of Table I. The percentage excess $^{18}O$ in the inorganic phosphate, $E_p$, is related to the percentage excess in the solvent, $E_s$, by

$$E_p = \frac{E_s p}{4n}[c + q(ac + ad + d) + r(2e + f)] \qquad (9)$$

where $n$ is the number of moles of inorganic phosphate formed per mole of ATP hydrolyzed and $a$ is the fraction of ADP undergoing fission at position A of Figure 2. The term in brackets is simply the sum of all terms in Table I giving labeled inorganic phosphate. The fractions $p$, $q$, and $r$ are calculated as follows. (a) The rate of disappearance of ATP is given by

$$\frac{-\mathrm{d}[\mathrm{ATP}]}{\mathrm{d}t} = [\mathrm{ATP}](k_1 + k_3) \qquad (10)$$

When $t = 0$, $[\mathrm{ATP}] = [\mathrm{ATP}]_0$ and therefore

$$[\mathrm{ATP}] = [\mathrm{ATP}]_0 e^{-(k_1+k_3)t} \qquad (11)$$

Given values of $k_1$ and $k_3$, [ATP] can be found for any given value of $n$ and hence of $t$. The magnitude of $p$ is simply 1 − [ATP]. (b) The amount of inorganic pyrophosphate unhydrolyzed is given by eq 6. From the ratio of $k_3/k_1$ and the proportion, $p$, of ATP hydrolyzed for a given value of $n$, the proportion of $PP_i$ formed can be calculated as $k_3p/k_1$, and hence the amount of $PP_i$ hydrolyzed is given by the difference between the amount formed and the amount unhydrolyzed. The proportion, $r$, is the ratio of the amount of $PP_i$ hydrolyzed to the amount of $PP_i$ formed. (c) The amount of $P_i$ formed from ATP was given by $p(1 - k_3/k_1)$ and that from $PP_i$ has been calculated in (b), allowing for the fact that $PP_i$ forms 2 mol of inorganic phosphate. The difference between $n$ and the sum of these two quantities is the amount of $P_i$ formed from ADP. The proportion, $q$, of ADP hydrolyzed can then be calculated.

Table I: Moles of Labeled and Unlabeled Products Produced by the Concurrent and Consecutive Reactions in the Hydrolysis of 1 mol of ATP in a Solvent Labeled with $H_2{}^{18}O$[a]

| | | products | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $PP_i$ | | ADP | | $P_i$ | | | |
| substrate | position of fission | $^{18}O$[b] | $O$[c] | $^{18}O$[b] | $O$[c] | $^{18}O$[b] | | $O$[c] | |
| ATP | C | | | | | *pc* | *pc* | | |
| ATP | D | | | *pd* | | | | *pd* | |
| ATP | E | *pe* | | | | | | | |
| ATP | F | | *pf* | | | | | | |
| ADP[b] | A | | | | | *qapd*[d] | | | |
| ADP[b] | B | | | | | *q*(1 − *a*)*pd* | | | |
| ADP[c] | A | | | | | *qapc* | | | |
| ADP[c] | B | | | | | | | *q*(1 − *a*)*pc* | |
| $PP_i$[b] | | | | | | *2rpe* | | | |
| $PP_i$[c] | | | | | | *rpf* | | *rpf* | |

[a] The fractions of ATP, ADP, and $PP_i$ hydrolyzed are taken as $p$, $q$, and $r$, respectively, and the letters $a$–$f$ refer to the fractions of reaction involving fission at bonds A–F in Figures 2 and 3. [b] $^{18}O$ Labeled. [c] Unlabeled. [d] Doubly labeled.

All quantities in eq 9 are either known or measurable with the exception of $c$, $d$, $e$, and $f$. Given that $(e + f)/(c + d) = k_3/k_1$, i.e., the ratio of the rates of formation of $PP_i$ and ADP, respectively, and that $c + d + e + f = 1$, the values of $c + d$ and $e + f$ can be found.

## Results

(*a*) *Acid-Catalyzed Hydrolysis of Pyrophosphoric Acid.* Table II gives the first-order rate coefficients ($k_4$) for the

Table II: First-Order Rate Coefficients for Hydrolysis of Pyrophosphoric Acid in Aqueous Perchloric Acid

| temp (°C) | $[HClO_4]$ (M) | $10^5 \times k_4$ ($s^{-1}$) |
|---|---|---|
| 25.0 | 1.0 | 1.64[a] |
| 25.0 | 2.0 | 3.77 |
| 25.0 | 3.0 | 7.07 |
| 25.0 | 4.0 | 12.5 |
| 25.0 | 5.0 | 17.0 |
| 28.8 | 3.0 | 10.07 |
| 37.3 | 3.0 | 20.6 |
| 40.1 | 3.0 | 26.4 |
| 100.0 | 3.0 | (1656)[b] |

[a] Corrected to allow for the presence of monoanion. [b] Calculated from the Arrhenius parameters.

Table III: First-Order Rate Coefficients ($k_2$) for Hydrolysis of Adenosine Diphosphate in Aqueous Perchloric Acid

| temp (°C) | $[HClO_4]$ (M) | $10^5 \times k_2$ ($s^{-1}$) |
|---|---|---|
| 25.0 | 0.75 | 0.535[a] |
| 25.0 | 1.0 | 0.795[a] |
| 25.0 | 2.0 | 2.43 |
| 25.0 | 3.0 | 5.20 |
| 25.0 | 4.0 | 10.7 |
| 25.0 | 5.0 | 20.7 |
| 25.0 | 3.0 | 7.33[b] |
| 25.0 | 3.0 | 7.73[c] |
| 33.5 | 3.0 | 12.1 |
| 42.0 | 3.0 | 26.3 |
| 52.5 | 3.0 | 66.0 |
| 100.0 | 3.0 | (2190)[d] |

[a] Corrected to allow for the presence of unreactive monoanion ($pK_{a_1} = 1$). [b] Containing also sodium chloride (0.5 M). [c] Containing also sodium perchlorate (0.5 M). [d] Calculated from the Arrhenius parameters.

hydrolysis of pyrophosphoric acid in aqueous perchloric acid (1–5 M) at 25 °C and in 3 M perchloric acid over the temperature range 25–40 °C. The Arrhenius parameters were calculated to be (3 M $HClO_4$) $E_A$ = 64.8 kJ $mol^{-1}$, $A = 5.6 \times 10^7$ $s^{-1}$, and $\Delta S^*(25\ °C) = -107.2$ J $K^{-1}$ $mol^{-1}$. The conventional Hammett plot (log $k_4$ against $-H_0$) gives a curve with the slope decreasing with increasing acidity (0.67–0.27) and leads to no useful information. Analysis of the data by the method advocated by Bunnett & Olsen (1966) (plotting log $k_4 + H_0$ against $H_0$ + log $[H^+]$) gives an approximately linear plot with a slope ($\phi$) of 0.742 (correlation coefficient = 0.998). This value of $\phi$ is consistent with a mechanism in which water is involved in the rate-determining step.

(*b*) *Acid-Catalyzed Hydrolysis of Adenosine Diphosphate.* Table III gives the first-order rate coefficients ($k_2$) for the hydrolysis of ADP to AMP and inorganic phosphate in aqueous perchloric acid (0.75–5 M) at 25 °C and in 3 M perchloric acid over the temperature range 25–52.2 °C. The Arrhenius parameters were calculated to be (3 M $HClO_4$) $E_A$ = 72 kJ $mol^{-1}$, $A = 5.5 \times 10^8$ $s^{-1}$, and $\Delta S^*(25\ °C) = -85.5$ J $K^{-1}$ $mol^{-1}$.

As with pyrophosphoric acid the conventional Hammett plot gave a curve with a slope decreasing with increasing acidity (0.93–0.59). Analysis of the data by the Bunnett–Olsen method, however, gave an approximately linear plot with a slope ($\phi$) of 0.46 (correlation coefficient = 0.995). This value is also consistent with a mechanism in which water is involved in the rate-determining step.

Addition of sodium chloride (apart from an ionic strength effect) did not significantly increase the rate. As previously found for the acid-catalyzed hydrolysis of methyl phosphate proceeding by phosphorus–oxygen fission (Bunton et al., 1958), chloride ion is not an effective nucleophile toward a phosphorus center.

Table IV: Analysis of the Isotope Content of Inorganic Phosphate ($P_i$) during Hydrolysis of ADP in Aqueous Perchloric Acid Enriched in $H_2{}^{18}O$

| temp (°C) | $[HClO_4]$ (M) | % reaction | % excess $^{18}O$ | | % fission at position A |
|---|---|---|---|---|---|
| | | | solvent | $P_i$ | |
| 25 | 1 | 75 | 1.240 | 0.2244 | 72.4 |
| 25 | 3 | 75 | 1.119 | 0.1888 | 67.5 |
| 25 | 3 | 100 | 1.119 | 0.1867 | 66.7 |
| 25 | 5 | 75 | 0.973 | 0.1093 | 44.2 |
| 100 | 3 | 87.5 | 1.119 | 0.1620 | 58.0 |

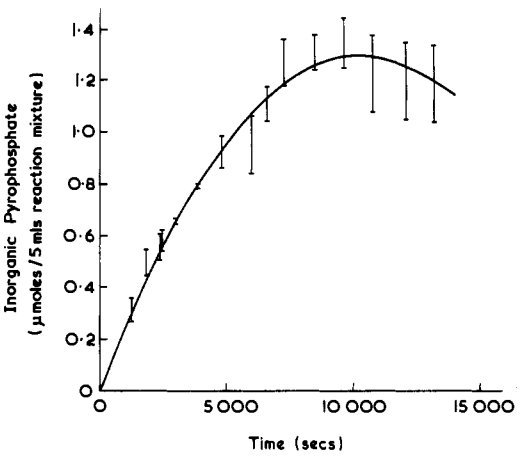


FIGURE 4: Production of inorganic pyrophosphate during the hydrolysis of ATP at 25 °C in 3 M perchloric acid.

*Positions of Bond Fission.* The hydrolysis of ADP in aqueous perchloric acid solutions enriched with $H_2{}^{18}O$ gave a considerably lower incorporation of label into inorganic phosphate than would have been the case for complete fission at position A of Figure 2. The data are given in Table IV. The percentage of bond fission at A is seen to decrease with increasing acidity of the medium and, to a lesser extent, with increasing temperature. The relatively small change of percentage fission at position A with change in temperature indicates that the temperature dependence is roughly the same for fission in the two positions A and B.

(*c*) *Acid-Catalyzed Hydrolysis of Adenosine Triphosphate.* The hydrolysis of ATP in 3 M perchloric acid at 25 °C has been followed from the production of pyrophosphoric acid ($PP_i$) and of inorganic phosphate ($P_i$). Under these conditions, the values of $k_2$ and $k_4$ are known (Tables II and III, respectively). The concentration of pyrophosphoric acid rises to a maximum at ~10 000 s and then falls (eq 6, Figure 4) whereas that of inorganic phosphate increases steadily with time. The best values of $k_1$ and $k_3$ have been found by substituting $k_2$ and $k_4$ and carrying out a least-squares fit of the experimental data to eq 6 and 7. The values are $k_1 = 11.5 \times 10^{-5}$ $s^{-1}$ and $k_3 = 6.4 \times 10^{-6}$ $s^{-1}$.

Because $k_1 \gg k_3$, eq 8 has been used to calculate $k_1$ and $k_2$ under different conditions of acidity by using only the experimentally determined variation of $[P_i]$ with time. The values of $k_1$ and $k_2$ so calculated are given in Table V. The calculated values of $k_2$ agree well with the corresponding measured values given in Table III, and, in the case of 3 M perchloric acid, the value of $k_1$ calculated by the simplified eq 8 ($12.0 \times 10^{-5}$ $s^{-1}$) agrees with the value calculated from the full eq 7 ($11.5 \times 10^{-5}$ $s^{-1}$).

*Positions of Bond Fission.* The hydrolysis of ATP was carried out at 25 °C in 3 M perchloric acid enriched in $H_2{}^{18}O$

Table V: First-Order Rate Coefficients for Hydrolysis of ATP to ADP ($k_1$) and for Subsequent Hydrolysis of ADP ($k_2$) Calculated from the Rate of Formation of Inorganic Phosphate according to the Approximation Represented by Equation 8[a]

| $[HClO_4]$ (M) | $10^5 \times k_1$ ($s^{-1}$) | $10^5 \times k_2$ ($s^{-1}$) |
|---|---|---|
| 0.75 | 2.63[b] | 0.63[b] |
| 1.0 | 2.70[b] | 0.65[b] |
| 2.0 | 6.74 | 2.57 |
| 3.0 | 12.0 | 4.74 |
| 4.0 | 20.3 | 11.4 |
| 5.0 | 31.0 | 19.3 |

[a] Temperature, 25 °C. [b] Corrected to allow for the presence of monoanion.

($E_s$ = 1.119%). Reaction was stopped when 1.5 mol of inorganic phosphate had been formed from 1 mol of substrate ($n$ = 1.5 in eq 9, $t$ = 25 200 s, $k_1 = 11.5 \times 10^{-5}$ $s^{-1}$, and $k_3 = 6.4 \times 10^{-6}$ $s^{-1}$ in equation 11. The enrichment of $^{18}O$ in the inorganic phosphate was found to be 0.2204 atom %. The values of $p$, $q$, and $r$ were calculated to be 0.953, 0.582, and 0.717, respectively. The appropriate value of $a$ from Table IV is 0.67. From the relative rates of formation of ADP (i.e., $k_1$) and inorganic pyrophosphate (i.e., $k_3$), it follows that $c + d = 0.9473$ and $e + f = 0.0527$) ($c + d + e + f = 1$). Insufficient information is available for calculation of the individual values of $c$, $d$, $e$, and $f$ from eq 9, but the values of $c$ and $d$ are insensitive to the relative values of $e$ and $f$: Assuming $f = 0$ and $e = 0.0527$, $c$ can be calculated to be 0.59, and $d$ can be calculated to be 0.36 whereas for $f = 0.0527$ and $e = 0$, $c$ is 0.67 and $d$ is 0.28. It follows that the percentage of bond fission at position C lies between 59 and 67% whereas at position D the limits are 36% and 28%, respectively. The respective means are 63% and 32%.

The possibility that under these reaction conditions, the rate of exchange directly into the substrate was comparable with the rate of hydrolysis was checked by stopping the hydrolysis after 5% reaction and determining the $^{18}O$ enrichment in inorganic phosphate formed by hydrolysis of the unreacted ATP in unlabeled solvent. The result ($E_p$ = 0.009%) was within normal limits.

## Discussion

The rate of hydrolysis of ADP in solutions of perchloric acid at 25 °C is, at some particular concentration of acid, not very different from that of inorganic pyrophosphate (Tables II and III). Not unexpectedly, the adenosine moiety has little effect on the kinetic behavior of the diphosphate chain.

We did not detect a reaction involving oxygen exchange into the substrate without hydrolysis occurring. This finding is mechanistically important, and we shall return to the point later. It also makes the interpretation of the results obtained on hydrolyzing ADP in water containing an excess abundance of $^{18}O$ particularly easy: the inorganic phosphate produced by hyrolysis does not itself undergo oxygen exchange with solvent under the relevant experimental conditions (Bunton et al., 1961), and therefore isotopic analysis of this product immediately gives the ratio in which bond fission occurs in the two possible positions A and B in Figure 2. The data are given in Table IV. It can be seen that fission must occur both at position A, entailing labeling of the inorganic phosphate, and at position B, giving an unlabeled product. At low acidity, bond fission occurs preferentially at position A (A/B, 0.72:0.38), but this preference decreases as the acidity increases and also, at some fixed acidity, as the temperature is raised. The important point is that bond fission does occur in both positions, and, although there is a small variation with acidity and temperature, the ratio is never far removed from what it necessarily is in inorganic pyrophosphate, that is, 50:50. The presence of the adenosine moiety therefore hardly affects either the rate of hydrolysis to form inorganic phosphate or the position of nucleophilic substitution.

Table VI: Rate Coefficients ($k_A$ and $k_B$) for Hydrolysis Reactions of ADP Leading to Fission at Positions A and B (Figure 2) and Corresponding Rate Coefficients ($k_p$) for Hydrolysis of Pyrophosphoric Acid

| temp (°C) | $[HClO_4]$ (M) | $H_0$[a] | $10^5 \times k_A$ ($s^{-1}$) | $10^5 \times k_B$ ($s^{-1}$) | $10^5 \times k_p$ ($s^{-1}$) |
|---|---|---|---|---|---|
| 25 | 1.0 | −0.22 | 0.58 | 0.22 | 0.82 |
| 25 | 2.0 | −0.78 | 1.71 | 0.72 | 1.88 |
| 25 | 3.0 | −1.23 | 3.49 | 1.71 | 3.53 |
| 25 | 4.0 | −1.72 | 6.43 | 4.22 | 6.25 |
| 25 | 5.0 | −2.23 | 9.15 | 11.5 | 8.50 |
| 100 | 3.0 | | 1270 | 920 | 828 |

[a] Paul & Long (1957).

The precise mechanisms by which the two hydrolytic pathways occur are of some interest since nucleophilic substitution on phosphorus occurs in many enzyme-catalyzed reactions. The relevant questions are, first, is the nucleophile (in this case a water molecule) involved in the rate-determining step and, second, does a five-coordinate species occur as an intermediate?

Since no simple order criterion is available, indirect methods must be used. One such method is to investigate the way in which the rate of hydrolysis depends on acidity. The original version of this method (Zucker & Hammett, 1939) has not proved particularly useful for phosphate esters and, consistently, has led to no mechanistic conclusions in the present study. The Bunnett–Olsen treatment, on the other hand, leads to the conclusion, for both pyrophosphoric acid and ADP, that water is involved in the rate-determining steps.

The hydrolysis of ADP is, however, a composite process, and it is instructive to divide the determined rate coefficients into their component parts ($k_A$ and $k_B$ for fission at positions A and B, respectively). The data are given in Table VI. It is immediately obvious that the two positions of bond fission differ in dependence of rate on acidity (from 1 to 5 M perchloric acid $k_A$ and $k_B$ increase by factors of 16 and 52, respectively). In both cases Bunnett–Olsen plots are approximately linear with slopes of 0.62 (A) (correlation coefficient = 0.988) and 0.231 (B) (correlation coefficient = 0.997). The first value is similar to that observed for pyrophosphoric acid (0.742, correlation coefficient = 0.998), but the second differs significantly and might be taken to indicate a different mechanism. This is, however, extremely unlikely both on general grounds and because all three rate coefficients have similar values. Moreover, the result is predictable: protonation of the proximal phosphoryl oxygen will give a conjugate acid which, because of steric hindrance from the adenosine moiety and because there is one less hydroxyl group, will be less solvated than is the corresponding conjugate acid from the distal phosphoryl atom. Under these circumstances, it is known that, given the same mechanism, the dependence of rate on acidity function will be steeper (Rochester, 1970).

Another criterion of the mechanism for acid-catalyzed hydrolytic reactions is based on the values of the so-called entropies of activation (Schaleger & Long, 1963; Cox & Ramsay, 1964). The criterion is purely empirical and arises because examination of the values for reactions whose mechanisms are known on other grounds shows that there is a dichotomy. Reactions involving the nucleophile in the rate-determining step have values of $\Delta S^{\ddagger}$ that are highly negative whereas for reactions in which the nucleophile is not involved in the

Table VII: Activation Parameters Calculated from the Rate Coefficients for the Hydrolysis of ADP and Pyrophosphoric Acid in 3 M Perchloric Acid

| substrate | rate coeff | $\Delta H^{\ddagger}$ (kJ mol$^{-1}$) | $\Delta S^{\ddagger}$ (J K$^{-1}$ mol$^{-1}$) |
|---|---|---|---|
| ADP | $k_A$ | 70.2 | −94.7 |
| ADP | $k_B$ | 75.1 | −84.5 |
| $PP_i$ | $k_p$ | 64.8 | −112.8 |

rate-determining step, the values of $\Delta S^{\ddagger}$ are close to 0 or slightly positive. Values of the activation parameters are given in Table VII. All three entropies of activation for hydrolysis of ADP at positions A and B and for hydrolysis of inorganic pyrophosphate are large and negative, indicating a common mechanism. Consistently, the values of $\Delta S^{\ddagger}$ for the hydrolysis of monoanions and monophosphate esters are all close to 0 (Cox & Ramsay, 1964), and it has long been thought that this reaction does not involve nucleophilic attack on phosphorus in the rate-determining step (Bunton et al., 1958; Butcher & Westheimer, 1955). We conclude, therefore, that in the acid-catalyzed hydrolysis of ADP (at both positions) and in the acid-catalyzed hydrolysis of pyrophosphoric acid, a water molecule is involved in the rate-determining step. A similar conclusion about the hydrolysis of the latter compound has been reached by Bunton & Chaimovich (1965).

It is generally supposed that nucleophilic attack on phosphorus leads to a trigonal bipyramidal intermediate (Westheimer, 1977). Evidence for such an intermediate is immediately given when oxygen exchange into substrate accompanies hydrolysis. This is found for the acid-catalyzed hydrolysis of methyl phosphate proceeding by phosphorus–oxygen bond fission (Bunton et al., 1958), for the acid-catalyzed hydrolysis of methyl ethylene phosphate (Westheimer, 1970, 1977), and for the acid-catalyzed hydrolysis of some phosphonate esters (Sigal & Westheimer, 1979). It is not observed in the acid-catalyzed hydrolysis of *p*-nitrophenyl phosphate (Barnard et al., 1966) nor in the base-catalyzed hydrolysis of triphenyl phosphate (Barnard et al., 1961), although, in both cases, there are good reasons for supposing that the reactions do proceed via trigonal bipyramidal intermediates. Concomitant oxygen exchange is also not observed in the acid-catalyzed hydrolysis of ADP or of ATP, but this does not exclude the possibility that five-coordinate intermediates exist on the reaction pathways. It is reasonable to suppose that addition of water produces an intermediate of type III (Figure 5), in which a hydroxyl group is not in the apical position, and that pseudorotation to give IV, in which a protonated hydroxyl group is in the apical position, is slower than loss of the phosphate group.

The production of inorganic phosphate from the acid-catalyzed hydrolysis of ATP proceeds at a rate not very different from that of the corresponding process in ADP or in inorganic pyrophosphate. The kinetic form is, however, complicated since the final products (AMP and inorganic phosphate) can arise by two sets of consecutive reactions (eq 1–4). It seems to have been assumed in the past that reaction 1 is the only primary step, but we have shown unequivocally that reaction 3, that is, the production of inorganic pyrophosphate, also occurs. This was established by studying the reaction under conditions where only a small fraction of final product (AMP and inorganic phosphate) had been formed. The pH of aliquots was suitably adjusted, and the increase in inorganic phosphate produced by the addition of the specific enzyme, inorganic pyrophosphatase, was measured. The procedure is necessarily somewhat inaccurate since the solutions have a high background concentration of inorganic phosphate produced

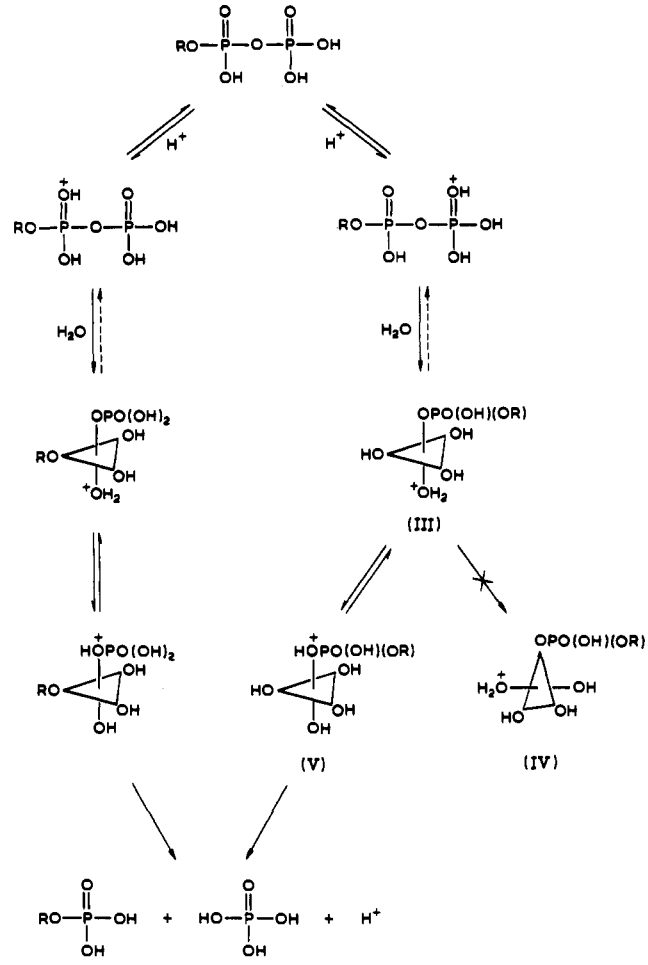


FIGURE 5: Structures of possible intermediates in the hydrolysis of the polyphosphates ATP (R = AMP), ADP (R = Ado), or inorganic pyrophosphate (R = H).

by reactions 1 and 2. Nonetheless, the results are clear enough: as shown in Figure 4, at 25 °C and 3 M perchloric acid, the concentration of pyrophosphoric acid rises to a maximum at ~10 000 s and then falls, due to subsequent hydrolysis by reaction 4.

Obviously, a detailed kinetic analysis of the acid-catalyzed hydrolysis of ATP is necessarily complicated because of the occurrence of four separate reactions. The relevant kinetic equations are given under Materials and Methods. The values of $k_2$ and $k_4$ can be measured independently, but it then remains to discover the values of $k_1$ and, more importantly, $k_3$. We studied the overall production of inorganic phosphate as a function of time. From these data, it is possible, by an iterative procedure based on the least-squares method, to calculate the values of $k_1$ and $k_3$ (computer program supplied on request). Under the stated conditions, the values are $k_1 = 11.5 \times 10^{-5}$ s$^{-1}$ and $k_2 = 6.4 \times 10^{-6}$ s$^{-1}$. The direct production of inorganic phosphate is, therefore, ~20 times faster than the production of pyrophosphoric acid. The difference no doubt arises from slight steric hindrance due to the adenosine moiety.

The consequence of this kinetic complexity on the results of isotopic analysis of inorganic phosphate formed by hydrolysis of ATP in water enriched in $^{18}O$ are given in Figure 3. Our data show that fission must occur at positions C and D and at either E or F, or both, since pyrophosphoric acid is a primary product. To distinguish between positions E and F would require isotopic analysis of the transient pyrophosphate, and this is technically extremely difficult. We may

conclude that the proportions of bond fission in the various positions are at C, 0.59–0.67, at D, 0.36–0.28, and at E + F, 0.053. It seems most likely that bond fission occurs in all four positions.

The importance of these results for biochemists lies partly in the unexpected complexity of the isotope data and also the fact that they provide a firm basis for the use of $^{18}O$ tracer in biosynthetic studies of ADP and ATP. The demonstration that isotope exchange is not a concomitant reaction with hydrolysis shows that the common practice of determining the total incorporation of $^{18}O$ tracer in ATP by complete hydrolysis in acid solution to AMP and inorganic phosphate is justified (Boyer & Bryan, 1966). Any ambiguity about the position of the tracer can, however, only be resolved by using the results presented above.

Acknowledgments

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# Amino Acid Sequence of the Variable Regions of Light Chains from Two Idiotypically Cross-Reactive Human IgM Anti-γ-globulins of the Wa Group†

David W. Andrews‡ and J. Donald Capra*

ABSTRACT: The amino acid sequences of the variable regions of the light chains derived from two idiotypically related human monoclonal rheumatoid factors are reported. The sequences were obtained through automated Edman degradations of the intact light chains, peptides generated from tryptic digests of citraconylated light chains, and peptides obtained from chymotryptic digestions of light chains. Comparison of the sequences suggests that the idiotypic determinant(s) may reside in the framework portions of the two chains or their J segments.

The immune system is designed to recognize and respond to patterns displayed on molecules encountered by the individual. The generation of the set of recognition units and the subsequent regulation of responsiveness are crucial problems in immunology.

Patterns unique to the individual units themselves ("idiotypes") are ideal candidates as the sites used for both recognition (Capra & Kehoe, 1975) and regulation (Jerne, 1974). Whether these sites are structurally identical is open for debate. In order to approach this question, we have attempted in several systems to generate a structural correlate of the serologically defined idiotype.

Structural correlates of idiotypy have been attempted by using both induced antibodies and pathogenetic immunoglobulins. Structural examination of idiotype-bearing induced

† From the Department of Microbiology, The University of Texas Health Science Center at Dallas, Dallas, Texas 75235. *Received April 6, 1981.* Supported by National Institutes of Health Grant AI-12127 and National Science Foundation Grant PCM 7923480.

‡ Recipient of support from a training grant awarded to the Graduate Program in Immunology, The University of Texas Health Science Center, by the National Cancer Institute, Department of Health, Education and Welfare (Grant No. CA 09082). Present address: Harvard University, Biological Laboratories, Cambridge, MA 02138.

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