The Enthalpy of Hydrolysis of Various 3',5'- and 2',3'-Cyclic Nucleotides*

(Received for publication, August 18, 1970)

STEPHEN A. RUDOLPH, EDWARD M. JOHNSON,† AND PAUL GREENGARD‡

From the Departments of Chemistry and Pharmacology, Yale University, New Haven, Connecticut 06520

SUMMARY

The enthalpy of hydrolysis of the 3'-bond of several cyclic 3',5'-nucleotides and of the 2'-bond of several cyclic 2',3'-nucleotides has been measured calorimetrically. In each case, the enthalpy was indicative of a high energy bond, although values for different compounds varied from -7.7 to -14.1 kcal mole⁻¹. The enthalpy of hydrolysis of the cyclic 3',5'-nucleotides, but not that of the cyclic 2',3'-nucleotides, was markedly affected by the nature of the base.

Materials and Methods

Enthalpies of hydrolysis were determined by measuring the heat evolved upon enzymic conversion of the cyclic 3',5'-nucleotides to the 5'-nucleotides and of the cyclic 2',3'-nucleotides to the 3'-nucleotides. A tabulation of the enzymes and experimental conditions employed is shown in Table I. Experiments which employed ribonuclease T₁ were performed at pH 6.5 because this enzyme has a sharp optimum at that pH (9). The enthalpy values should be independent of the pH of the reaction mixture between pH 6.5 and pH 7.3. It was found experimentally that the enthalpy of hydrolysis of cyclic 2',3'-UMP was the same, within experimental error, whether the hydrolysis reaction was carried out in the presence of (a) 36 mM potassium phosphate, pH 7.3; (b) 36 mM potassium phosphate, pH 6.5; or (c) 36 mM potassium phosphate, 1 mM imidazole, 1 mM MgCl₂, pH 7.3. Moreover, as reported earlier (6), the enthalphy of hydrolysis of cyclic 3',5'-AMP is independent of the concentration of Mg¹¹⁺, at least up to a Mg¹¹⁺ concentration of 1 mM.

Both batch and flow calorimetric methods were employed. Batch calorimetry was carried out in a Beckman model 190 B microcalorimeter by means of methods described previously (6). Flow calorimetry was performed in a similar instrument with flow modifications as described by Sturtevant and Lyons (10). As in previous experiments (6), heats of dilution of substrates and enzymes were found to be negligible. Concentrations of cyclic nucleotides were measured by ultraviolet spectroscopy using a Cary model 14 spectrophotometer. Absorbance measurements were made at neutral pH at the respective maxima in 1-cm cuvettes. Wave lengths and extinction coefficients are shown in Table II.

Cyclic 3',5'-AMP and cyclic 3',5'-UMP were obtained as the sodium salts from Boehringer Mannheim. Cyclic 2',3'-AMP, cyclic 2',3'-CMP, and cyclic 2',3'-UMP were obtained as the sodium salts from Sigma, and cyclic 2',3'-GMP was obtained as the t-butyl ammonium salt from Sigma. Cyclic 3',5'-dAMP was made available through the courtesy of Dr. P. K. Chang, of the Pharmacology Department at Yale University. It was not possible to measure the enthalpies of hydrolysis of cyclic 3',5'-CMP, cyclic 3',5'-dTMP, and dibutyryl cyclic 3',5'-AMP in the present investigation because enzymes could not be found which would hydrolyze these nucleotides rapidly enough to carry out accurate calorimetric measurements.

Identity and purity of the cyclic nucleotides were established by paper chromatography. All nucleotides were found to be chromatographically pure except cyclic 2',3'-GMP and cyclic

Cyclic 3',5'-AMP has been recognized in the past several years (1, 2) as a key substance in the regulation of many metabolic processes. A variety of evidence, recently summarized (3), suggests that cyclic guanosine 3',5'-monophosphate also plays a significant role in metabolism. Cyclic 3',5'-AMP, cyclic 3',5'-GMP, and several other cyclic 3',5'-nucleotides have been found to activate a number of protein kinases, although there is considerable variation in the efficacy with which the various cyclic nucleotides activate any given enzyme (4, 5). Because the cyclic 3',5'-phosphate moiety is essential for the biological activity of these compounds, an understanding of the nature of this moiety is of considerable importance. In particular, measuring the thermodynamic parameters associated with breaking the ester linkage at position 3' to yield the ordinary 5'-nucleotides has been measured calorimetrically. In each case, the enthalpy was indicative of a high energy bond, although values for different compounds varied from -7.7 to -14.1 kcal mole⁻¹. The enthalpy of hydrolysis of the cyclic 3',5'-nucleotides, but not that of the cyclic 2',3'-nucleotides, was markedly affected by the nature of the base.

MATERIALS AND METHODS

Batch calorimetry was carried out in a Beckman model 190 B microcalorimeter by means of methods described previously (6). Flow calorimetry was performed in a similar instrument with flow modifications as described by Sturtevant and Lyons (10). As in previous experiments (6), heats of dilution of substrates and enzymes were found to be negligible. Concentrations of cyclic nucleotides were measured by ultraviolet spectroscopy using a Cary model 14 spectrophotometer. Absorbance measurements were made at neutral pH at the respective maxima in 1-cm cuvettes. Wave lengths and extinction coefficients are shown in Table II.

Cyclic 3',5'-IMP and cyclic 3',5'-UMP were obtained as the sodium salts from Boehringer Mannheim. Cyclic 2',3'-AMP, cyclic 2',3'-CMP, and cyclic 2',3'-UMP were obtained as the sodium salts from Sigma, and cyclic 2',3'-GMP was obtained as the t-butyl ammonium salt from Sigma. Cyclic 3',5'-dAMP was made available through the courtesy of Dr. P. K. Chang, of the Pharmacology Department at Yale University. It was not possible to measure the enthalpies of hydrolysis of cyclic 3',5'-CMP, cyclic 3',5'-dTMP, and dibutyryl cyclic 3',5'-AMP in the present investigation because enzymes could not be found which would hydrolyze these nucleotides rapidly enough to carry out accurate calorimetric measurements.

Identity and purity of the cyclic nucleotides were established by paper chromatography. All nucleotides were found to be chromatographically pure except cyclic 2',3'-GMP and cyclic

* This investigation was supported by Grants GB 06033X1 and GB 8391 from the National Science Foundation and by Grants GM 04725, NS 08440, GM 12589, and MH 17387 from the United States Public Health Service.

† Predoctoral Trainee of the United States Public Health Service.

‡ To whom correspondence should be addressed.
Experimental conditions for calorimetric experiments

<table>
<thead>
<tr>
<th>Cyclic nucleotide</th>
<th>pH</th>
<th>Buffer system</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclic 3',5'-IMP</td>
<td>7.3</td>
<td>Potassium phosphate, 36 mM; imidazole, 1 mM; MgCl₂, 1 mM</td>
<td>Rovina heart phosphodiesterase</td>
</tr>
<tr>
<td>Cyclic 3',5'-dAMP</td>
<td>7.3</td>
<td>Potassium phosphate, 36 mM; imidazole, 1 mM; MgCl₂, 1 mM</td>
<td>Canine heart phosphodiesterase</td>
</tr>
<tr>
<td>Cyclic 3',5'-UMP</td>
<td>6.5</td>
<td>Tris-HCl, 0.20 mM</td>
<td>Ribonuclease T₄</td>
</tr>
<tr>
<td>Cyclic 2',3'-AMP</td>
<td>6.5</td>
<td>Potassium phosphate, 36 mM</td>
<td>Ribonuclease A*</td>
</tr>
<tr>
<td>Cyclic 2',3'-GMP</td>
<td>7.3</td>
<td>Potassium phosphate, 36 mM</td>
<td>Ribonuclease A*</td>
</tr>
<tr>
<td>Cyclic 2',3'-CMP</td>
<td>7.3</td>
<td>Potassium phosphate, 36 mM</td>
<td>Ribonuclease A*</td>
</tr>
<tr>
<td>Cyclic 2',3'-UMP</td>
<td>7.3</td>
<td>Potassium phosphate, 36 mM</td>
<td>Ribonuclease A*</td>
</tr>
</tbody>
</table>

a All at 25°C.

b Prepared according to the method of Butcher and Sutherland (7), with minor modifications.

c Prepared according to the method of Nair (8).

d From Calbiochem (prepared by Sankyo Chemical Company, Tokyo, Japan).

TABLE II

Wave lengths of maximum absorbance and extinction coefficients

<table>
<thead>
<tr>
<th>Cyclic nucleotide</th>
<th>Wave length</th>
<th>Extinction coefficient</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μm</td>
<td>ε</td>
<td></td>
</tr>
<tr>
<td>Cyclic 3',5'-IMP</td>
<td>248.5</td>
<td>12,200</td>
<td>Roethinger Mannheim</td>
</tr>
<tr>
<td>Cyclic 3',5'-dAMP</td>
<td>259</td>
<td>14,500</td>
<td>Drummond et al. (11)</td>
</tr>
<tr>
<td>Cyclic 3',5'-UMP</td>
<td>261</td>
<td>9,940</td>
<td>Smith, Drummond, and Khorana (12)</td>
</tr>
<tr>
<td>Cyclic 2',3'-AMP</td>
<td>259</td>
<td>14,850</td>
<td>Sigma</td>
</tr>
<tr>
<td>Cyclic 2',3'-GMP</td>
<td>254</td>
<td>13,300</td>
<td>Sigma</td>
</tr>
<tr>
<td>Cyclic 2',3'-CMP</td>
<td>268</td>
<td>8,900</td>
<td>Hearn (13)</td>
</tr>
<tr>
<td>Cyclic 2',3'-UMP</td>
<td>259</td>
<td>9,570</td>
<td>Brown, Magrath, and Todd (14)</td>
</tr>
</tbody>
</table>

3',5'-dAMP. These contained small amounts of 3'-GMP and 5'-dAMP, respectively. Concentrations of these impurities were determined chromatographically, and suitable corrections were applied. These impurities could have no effect on the reaction enthalpies measured because they are the reaction products.

The concentrations of the cyclic nucleotides present in the calorimeter immediately after mixing enzyme and substrate ranged from 0.1 to 1.0 mM. Enthalpies of hydrolysis were independent of nucleotide concentration. Amounts of enzyme used were sufficient in most cases to take the reaction to completion (>99%) within 10 min. In the batch calorimetric experiments, the reaction vessel was removed from the calorimeter approximately 10 min after heat evolution was no longer detectable. Enzyme activity in the reaction mixture was then destroyed either by boiling for 5 min or by addition of trichloroacetic acid to a final concentration of 10%. Precipitated protein was removed by centrifugation, and the trichloroacetic acid was extracted with ethyl ether. An aqueous solution of the reaction product was then run in at least two different paper chromatographic solvent systems; in all cases, the reaction mixture gave a single spot in the same position as a sample of authentic reaction product. The presence of unreacted cyclic nucleotides or the products of further breakdown would have been detected at a level of less than 1% of the total ultraviolet absorbing material. Similar procedures were carried out on the effluent from the flow calorimeter. It is thus concluded that all reactions went to at least 99% completion to the expected product.

Determinations of the number of protons liberated during the course of the hydrolysis reaction were carried out under conditions similar to those of the calorimetric experiments, except that the buffer was omitted and the ionic strength was made up with KCl. A Radiometer model TTT-1a automatic titrator (pH-stat) was used for these measurements.

RESULTS AND DISCUSSION

The results of all calorimetric experiments are shown in Table III. The data of Greengard et al. (6) on cyclic 3',5'-AMP and cyclic 3',5'-GMP are included for the purpose of comparison. The present data show that the enthalpy of hydrolysis of each cyclic 3',5'-nucleotide is greater than that of the corresponding cyclic 2',3'-nucleotide.

Changing the nature of the base of the cyclic 2',3'-nucleotides had only a slight effect on the measured enthalpies of hydrolysis. In contrast, the enthalpies of hydrolysis of the cyclic 3',5'-
nucleotides were markedly affected by the nature of the purine or pyrimidine base. Examination of the data shows that the heat of hydrolysis of cyclic 3',5'-GMP is from 2.5 to 3.6 kcal mole\(^{-1}\) lower than the heats observed for the other cyclic 3',5'-purines. It seems significant in this connection that of the purine derivatives used in this study, only guanosine was substituted at position 2. The building of scale models of the cyclic nucleotides used in this study shows that the 2-amino group of the guanine ring may be within 2 Å of one of the unesterified oxygen atoms of the cyclic 3',5'-phosphate group. This would allow the possibility of hydrogen bonding or some other weak interaction which could result in charge delocalization and stabilization of the cyclic phosphate group of cyclic 3',5'-GMP. There is essentially no difference between the enthalpies of hydrolysis of cyclic 2',3'-AMP and cyclic 2',3'-GMP, and model building shows little possibility for interaction between the cyclic 2',3'-phosphate group and the purine ring.

Cyclic 3',5'-AMP has the highest enthalpy of hydrolysis of any of the cyclic nucleotides investigated to date. In addition, the free energy of hydrolysis of the 3'-bond of cyclic 3',5'-AMP has been shown to be \(-11.9\) kcal mole\(^{-1}\) (15). It is thus possible that the energetic properties of the cyclic phosphate moiety of cyclic 3',5'-AMP were an important aspect of the central role which this compound came to assume in the course of the evolution of mechanisms for the regulation of metabolism. Experiments are in progress to determine whether the energy stored in the cyclic phosphate bond of cyclic 3',5'-AMP is actually utilized in the action of this biologically unique substance.

Acknowledgment—We thank Professor J. M. Sturtevant for valuable discussions.

REFERENCES

The Enthalpy of Hydrolysis of Various 3',5'- and 2',3'-Cyclic Nucleotides
Stephen A. Rudolph, Edward M. Johnson and Paul Greengard


Access the most updated version of this article at [http://www.jbc.org/content/246/5/1271](http://www.jbc.org/content/246/5/1271)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/246/5/1271.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/246/5/1271.full.html#ref-list-1](http://www.jbc.org/content/246/5/1271.full.html#ref-list-1)