Biochimica et Biophysica Acta, 341 (1974) 412–420

BBA 67189

CYCLIC NUCLEOTIDE PHOSPHODIESTERASE OF *CHLAMYDOMONAS REINHARDTII*

ULRICH FISCHER and NIKOLAUS AMRHEIN*

Institute of Plant Physiology, Ruhr-University, D-4630 Bochum (Germany)

(Received October 26th, 1973)

SUMMARY

Cyclic nucleotide phosphodiesterase (EC 3.1.4.17) activity was characterized in crude and $(NH_4)_2SO_4$-fractionated extracts of the green alga *Chlamydomonas reinhardtii*. With cyclic AMP as substrate, the formation of 5′-AMP was optimal at pH 8.5 and required the presence of a sulfhydryl reagent and a divalent cation, Mg^{2+} and Mn^{2+} being the most effective. Methylxanthines, papaverine, inorganic phosphate and pyrophosphate, ATP and Co^{2+} inhibit the enzyme, while imidazole stimulates the activity. At 2 mM substrate concentration, cyclic CMP is hydrolyzed at three times the rate for cyclic AMP, while the cyclic 3′,5′-derivatives of GMP, IMP, TMP and UMP were hydrolyzed at lower rates. No hydrolysis of N^6, $O^{2'}$-dibutyryl cyclic AMP was observed. The *Chlamydomonas reinhardtii* enzyme closely resembles cyclic nucleotide phosphodiesterases from other micro-organisms and animal tissues, and is distinctly different from cyclic nucleotide phosphodiesterases that have been found in higher plants.

INTRODUCTION

Since Sutherland and Rall [1] described the presence of an enzyme in extracts of mammalian heart, brain and liver that catalyses the hydrolysis of cyclic 3′,5′-AMP (cyclic AMP) to 5′-AMP, the enzyme has been found in many animal tissues [2, 3], bacteria [4, 5], slime molds [6, 7], lower and higher fungi [8–10], and protozoans [11–13] as well. Since phosphodiesterase (EC 3.1.4.17) is considered to play an important role in the regulation of intracellular concentrations of cyclic AMP, many efforts have been made to characterize the enzymes from these various sources, and the finding that the administration of various drugs, which are known inhibitors of phosphodiesterase in vitro, can be used in vivo to increase the intracellular level of cyclic AMP in animal cells, has been of great, especially pharmacological, interest [14]. As Butcher and Sutherland [15] have shown, methylxanthines represent one group of potent inhibitors of phosphodiesterase. In a recent study Rubin and Filner [16] demonstrated the inhibitory effects of the methylxanthines caffeine and aminophylline

* To whom correspondence should be addressed.

(a dimer of theophylline) on flagellar function and regeneration of the biflagellate unicellular green alga *Chlamydomonas reinhardtii*. Subsequently Amrhein and Filner [17] established the occurrence of cyclic AMP in *C. reinhardtii* and showed that administration of aminophylline brings about a drastic increase in the concentration of the cyclic nucleotide in the cells. It was further shown by these authors that crude extracts of *C. reinhardtii* hydrolyze cyclic AMP to 5'-AMP in a theophylline-sensitive reaction. It seemed promising to us to study the cyclic nucleotide phosphodiesterase activity of *C. reinhardtii* in more detail, since it appeared to differ markedly in its properties from cyclic nucleotide phosphodiesterases described for higher green plants [18–20].

EXPERIMENTAL PROCEDURES

Materials

The clone derived from wild-type *C. reinhardtii*, (+) mating type, used in our previous work [17], was used throughout this investigation. Nucleotide and nucleoside derivatives and dithiothreitol were obtained from Boehringer, Mannheim; papaverine from Boehringer, Ingelheim; imidazole and caffeine from Fluka AG, Buchs; theophylline from Schuchardt, Munich; aminophylline from K. and K. Laboratories, Plainview, N.Y.; snake venom (*Crotalus atrox*) from Sigma, St. Louis, Mo. Cyclic [8-^{3}H]AMP with a specific activity of 27.5 Ci/mmole was purchased from the Radiochemical Centre, Amersham. All standard chemical reagents in analytical grade were obtained from Merck, Darmstadt.

Methods

Culture conditions. Stock cultures of *C. reinhardtii* were maintained on slants of 2% agar and Tris–acetate–phosphate [17] medium. Liquid cultures were grown either in 1 l Fernbach flasks at 28 °C on a reciprocal shaker under continuous illumination with 2000 lux of white fluorescent light, or in a light thermostat as devised by Lorenzen [21] (Kniese, Marburg) under aeration with CO_2–air (1.5 : 98.5, v/v) and the same light and temperature conditions as above.

Cell concentration determination. Cells were fixed with 2.5% glutaraldehyde to render them immotile, and titers were determined on a counting slide.

Sterility tests. Cultures were routinely examined under the phase-contrast microscope, and aliquots were plated on various nutrient agars to check for microbial contamination.

Enzyme extraction. Cells were harvested at the end of the logarithmic growth phase (about 10^7 cells/ml, when grown in Fernbach flasks or $3 \cdot 10^7$ cells/ml, when grown in the light thermostat) by centrifugation in a Christ-Junior continuous-flow centrifuge at 10 000 rev./min for 10 min. About 0.5 ml of packed cells was sonicated (Branson Sonifier S 125) for four 15-s intervals in 3 ml of 40 mM Tris–HCl, pH 8.5, containing 2 mM $MgSO_4$ and 10 mM L-cysteine. The homogenate was centrifuged for 20 min at 20 000 $\times$ *g* and 0 °C and the pellet was discarded. The supernatant will be referred to as the crude extract. Solid $(NH_4)_2SO_4$ was added to the crude extract to 20% saturation with constant stirring. After 30 min in an ice-bath the precipitated material was pelleted by centrifugation at 10 000 $\times$ *g* for 10 min and discarded. The supernatant was brought to 40% $(NH_4)_2SO_4$ saturation and after centrifugation the precipitate

was redissolved in 5–10 ml of the extraction buffer and dialyzed overnight against 3 l of the same buffer at 4 °C. The dialysate was centrifuged at 20 000 × *g* for 15 min, and the pellet was discarded. The supernatant will be referred to as the $(NH_4)_2SO_4$-fractionated extract. The specific activity of phosphodiesterase in this fraction was about three times higher than that of the initial 20 000 × *g* supernatant. It was found later that lyophilized algae can be used as a convenient source for phosphodiesterase as well.

Assays of cyclic nucleotide phosphodiesterase. Two methods were employed to determine the activity of the enzyme. For the routine assay (Method I) cyclic [8-^{3}H]-AMP was used as substrate. The reaction mixture contained 40 mM Tris–HCl, pH 8.5, 2 mM $MgSO_4$, 10 mM L-cysteine, 0.2 mM cyclic [8-^{3}H]AMP (spec. act. 2.72 Ci/mole) and cell extract (0.3–0.5 mg protein) in a total volume of 0.5 ml. After a 5-min preincubation at 30 °C the reaction was started by the addition of the extract. After various lengths of time (usually after 0, 10 and 30 min) the reaction was stopped by the addition of 1 ml 10% trichloroacetic acid solution, and after 1 h in an icebath precipitated material was removed by centrifugation in a clinical centrifuge at top speed for 10 min. The supernatant was extracted four times with 5 ml diethylether, and after addition of carrier amounts of cyclic AMP, 5′-AMP, and adenosine (250 nmoles each) the sample was evaporated in an air stream. The material, redissolved by the addition of 0.2 ml water and heating in a boiling water bath, was then chromatographed on silica gel GF_{254} thin-layer plates in the solvent system *n*-butanol–methanol–ethylacetate–conc. NH_3 (7 : 3 : 4 : 4, by vol.). Bands of 5′-AMP, cyclic AMP and adenosine were identified by their ultraviolet absorption, scratched off the plate and transferred to scintillation vials. 5 ml of scintillation fluid (40 ml Scintol 3, Koch-Light Laboratories, in 1 l toluene) were added, and the vials were left in the dark for at least 5 h to let the gel phosphorescence subside before the radioactivity was determined in a Berthold–Frieseke Betaszint 5000 liquid scintillation counter. After subtraction of the counts in the 5′-AMP and adenosine bands of the zero-time control the percentage of remaining cyclic AMP was calculated. It was ensured in a series of control experiments that there was a linear relationship between the ^{3}H-activity applied to the silica gel and the ^{3}H-activity measured which was absorbed onto the gel.

The second method (Method II) was employed for substrates, for which radioactive compounds were not commercially available. The reaction mixture was identical with the mixture used for the routine assay, except that the substrate concentration was 2 mM and a 4- to 5-times higher protein concentration was used. The mixture was incubated at 30 °C for various lengths of time, and the reaction terminated by heating in a boiling water bath for 5 min. The reaction tubes were placed in an icebath for 15 min and then centrifuged for 15 min at 48 000 × *g*. 50 µl *C. atrox* venom (0.5 mg/ml 40 mM Tris–HCl, pH 7.5) were added to the supernatant, which was incubated for 20 min at 30 °C. The reaction was terminated by the addition of 50 µl 70% trichloroacetic acid solution. After standing in an icebath for 15 min the precipitate formed was removed by centrifugation, and the resulting supernatant was analysed for P_i by the method of Fiske and SubbaRow [22]. Since there was considerable release of P_i in the absence of any cyclic nucleotide added, a blank was run for each experiment containing no substrate. Assays by both methods were done under conditions such that the rate of cyclic AMP hydrolysis was linear with respect to the time of incubation and the concentration of protein.

Determination of protein content. Protein concentrations were determined according to the method of Lowry et al. [23], as modified by Heimer and Filner [24], using bovine serum albumin as a standard. The specific activity of the enzyme is defined as nmoles cyclic AMP hydrolyzed per min and per mg of protein.

EXPERIMENTAL RESULTS

Crude extracts of *C. reinhardtii* are able to hydrolyze the 3'-ester bond of cyclic 3',5'-AMP to form 5'-AMP [17]. Fig. 1 shows the time-course of disappearance of cyclic AMP from the reaction mixture and the concomitant formation of 5'-AMP. Due to the presence of 5'-nucleotidase activity in the crude extract 5'-AMP is further hydrolyzed to adenosine, which does not seem to be converted into further products

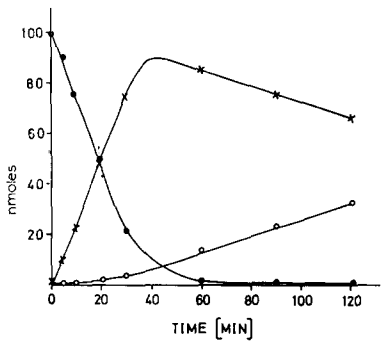

Fig. 1. Hydrolysis of cyclic AMP by a crude extract from *C. reinhardtii*. Time-course of disappearance of cyclic AMP (●—●) and appearance of 5'-AMP (×—×) and adenosine (○—○) in the reaction mixture under standard conditions (Method I). At the higher cyclic AMP concentrations used in this study as compared to our previous work [17], relatively little conversion of 5'-AMP to adenosine is observed during the incubation period.

to any major extent. Since cyclic nucleotide phosphodiesterases from higher green plants are known to preferentially hydrolyze the 5'-ester bond of cyclic AMP [18, 20], the earlier finding that the product of hydrolysis of cyclic AMP by *C. reinhardtii* extracts is 5'-AMP [17], was reinvestigated. AMP was eluted from the silica gel with hot water and rechromatographed on silica gel in the solvent system isopropanol–conc. NH_3–0.1 M H_3BO_3 (7 : 1 : 2, by vol.) [25] after addition of 3'-AMP and 5'-AMP carrier material. More than 99% of the radioactivity were found in the 5'-AMP band thus clearly establishing that 5'-AMP is the sole product of *C. reinhardtii* cyclic nucleotide phosphodiesterase.

Effect of pH and temperature on enzyme activity and stability

The optimal pH for cyclic AMP hydrolysis was found to be 8.5 in Tris–HCl buffer. This value compares favorably with the pH optima reported for cyclic nucleotide phosphodiesterases from other microorganisms [4–13, 20] and animal systems [2, 3], but differs markedly from the acid pH optimum of higher plant phosphodiesterases [18–20]. The optimal temperature for the enzymic reaction was 30 °C.

Influence of divalent cations, EDTA and sulfhydryl reagents

Mg^{2+} or Mn^{2+} are required for maximal activity of cyclic nucleotide phosphodiesterase from animal sources, and chelating agents like EDTA are inhibitors of these enzymes [2, 3]. The enzyme from *Saccharomyces carlsbergensis* is stimulated by Mn^{2+} only [8], and the phosphodiesterase of *Serratia marcescens* requires Fe^{2+}, Ca^{2+} or Ba^{2+} for maximum activity, while the activity of higher plant phosphodiesterases is not influenced by metal ions [18–20]. The *C. reinhardtii* enzyme requires both Mg^{2+} and cysteine for maximal activity, and EDTA acts as an inhibitor (Table I). Of the

TABLE I

EFFECT OF OMISSION OF Mg^{2+} AND CYSTEINE FROM THE STANDARD REACTION MIXTURE ON PHOSPHODIESTERASE ACTIVITY

Cells were homogenized in 40 mM Tris–HCl (pH 8.5), and phosphodiesterase activity was determined by Method I. The complete reaction mixture contained 40 mM Tris–HCl (pH 8.5), 2 mM $MgSO_4$, and 10 mM L-cysteine.

Reaction mixture	Relative activity
Complete	100
Omit Mg^{2+}	31
Omit Mg^{2+} Add 1 mM EDTA	18.5
Omit cysteine	36.2
Omit cysteine and Mg^{2+}	16.9

various metal ions tested, both Mg^{2+} and Mn^{2+} effectively stimulated the hydrolysis of cyclic AMP, a 2.5-fold stimulation of enzyme activity being obtained with 2 mM Mg^{2+} or 0.2 mM Mn^{2+}, respectively. Ca^{2+} and Ba^{2+} are slightly stimulatory at high concentrations (20 mM), while Zn^{2+} and especially Co^{2+} are strongly inhibitory at 20 mM. Of the three commonly used sulfhydryl reagents, 2-mercaptoethanol, dithiothreitol (Cleland's Reagent) and cysteine, cysteine was most effective in stimulating phosphodiesterase activity (a 3-fold increase in activity at 10 mM).

K_m value for cyclic AMP and the relative rates of hydrolysis of cyclic nucleoside monophosphates

The determinations of cyclic AMP hydrolysis at various substrate concentrations showed Michaelis–Menten kinetics, and the K_m was calculated to be $8.5 \cdot 10^{-5}$ M from a Lineweaver–Burk plot. Identical K_m values were found when crude or $(NH_4)_2SO_4$-fractionated extracts were used. In an attempt to obtain information on the substrate specificity of the *C. reinhardtii* phosphodiesterase, various cyclic nucleoside monophosphates were tested as substrates using the release of P_i from the corresponding nucleoside monophosphates by *C. atrox* venom 5′-nucleotidase as measure for enzymatic activity as described under the Experimental Procedures.

With 2 mM substrate, the enzyme showed the following relative rates (%) of hydrolysis of substrates: cyclic AMP (100%), cyclic CMP (300%), cyclic GMP (50%), cyclic IMP (45%), cyclic TMP (35%), cyclic UMP (30%), N^6,$O^{2'}$-dibutyryl cyclic AMP (0%). The preferential hydrolysis of cyclic CMP at 3 times the rate for cyclic

AMP is an astonishing characteristic of the *C. reinhardtii* enzyme and seems to be a unique property among other known phosphodiesterases. The only other phosphodiesterase known to hydrolyze cyclic CMP to any major extent is that of *S. marcescens* [5], but with the enzyme of this organism the rate of cyclic CMP hydrolysis was only one-sixth of the rate for cyclic AMP. To ensure that cyclic CMP was in fact hydrolyzed and did not stimulate the release of P_i from other sources in the crude extract, the reaction mixtures containing cyclic CMP were chromatographed on silica gel, before and after the incubation period. Spectrophotometric measurements of the absorbance at 260 nm of the eluates of the cyclic CMP and 5′-CMP bands gave results identical to results obtained with the P_i measurements. Since these experiments were carried out with barely purified extracts, it remains to be shown, however, that cyclic AMP and cyclic CMP are hydrolyzed by the same enzyme. Failure to observe the hydrolysis of N^6, $O^{2'}$-dibutyryl cyclic AMP is in accordance with reports for mammalian and bacterial phosphodiesterases [2, 5], but differs from the pea phosphodiesterase [18].

Inhibition and activation studies

Methylxanthines are known to be competitive inhibitors of animal phosphodiesterases [15], while microbial phosphodiesterases seem to differ from each other in this respect. Whereas the enzymes of *Escherichia coli* [4] and *Dictyostelium discoideum* [6] are not inhibited by theophylline, theophylline inhibition was reported for the enzymes of *S. marcescens* [5], *Physarum polycephalum* [7], *Neurospora crassa* [9], *Coprinus macrorhizus* [10], and *Tetrahymena pyriformis* [12]. As briefly reported previously [17], *C. reinhardtii* belongs to this second group of microorganisms, and as Fig. 2 illustrates, theophylline, aminophylline and caffeine are inhibitors of the

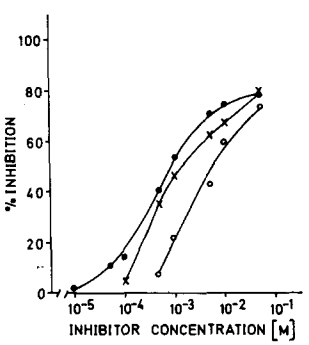

Fig. 2. Inhibition of phosphodiesterase by various methylxanthines. Reaction mixtures containing $(NH_4)_2SO_4$-fractionated enzyme were incubated in the presence of various concentrations of theophylline (●—●), aminophylline (×—×) and caffeine (○—○). The activity of the enzyme was determined by Method I, and the inhibitions are relative to the cyclic AMP hydrolysis in the absence of any methylxanthine. All reaction mixtures contained 40 mM Tris–HCl (pH 8.5), 2 mM $MgSO_4$, 10 mM L-cysteine, and 0.2 mM cyclic AMP.

phosphodiesterase, theophylline being the most effective. Half-maximal inhibition by theophylline occurs at 0.7 mM, while an 8-fold concentration of caffeine was necessary for the same potency of inhibition.

The inhibitory effects of inorganic phosphate (P_i) and pyrophosphate (PP_i) are depicted in Fig. 3. P_i is a relatively weak inhibitor, while PP_i is more potent showing

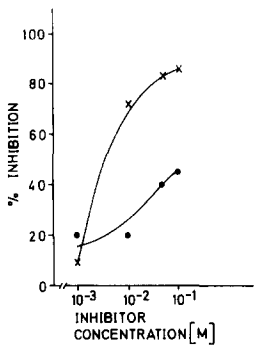

Fig. 3. Inhibition of phosphodiesterase by P_i and PP_i. The phosphodiesterase activity of $(NH_4)_2SO_4$-fractionated extracts was determined by Method I in the presence of various concentrations of P_i (●—●) and PP_i (×—×). The inhibitions are relative to the enzyme activity in the absence of inhibitors. All reaction mixtures contained 40 mM Tris–HCl (pH 8.5), 2 mM $MgSO_4$, 10 mM L-cysteine, and 0.2 mM cyclic AMP.

half-maximal inhibiton at a concentration of 3 mM. P_i inhibits *N. crassa* [9] and trout brain [3] phosphodiesterase activity, while PP_i has been reported to inhibit rat brain [26] and *S. carlsbergensis* [8] phosphodiesterases, possibly acting by virtue of its metal-chelating properties. The effects of various other agents on phosphodiesterase activity are summarized in Table II. The inhibition by cyclic CMP might indicate that one enzyme is responsible for the hydrolysis of both cyclic AMP and cyclic CMP. Papavarine is a potent inhibitor of mammalian phosphodiesterases [27] and of two of the three phosphodiesterases of *Tetrahymena* [12]. Imidazole stimulates the *C. reinhardtii* enzyme. Stimulation by imidazole was reported for phosphodiesterases from mammalian tissues [15], *T. pyriformis* [12] and *N. crassa* [9], but not for phosphodiesterases of *D. discoideum* [6] and *S. marcescens* [5].

Subcellular distribution

Phosphodiesterase occurs in many animal tissues in both soluble and particulate forms [2]. Up to 80% of *N. crassa* phosphodiesterase were found in the 105 000

TABLE II

INFLUENCE OF VARIOUS COMPOUNDS ON PHOSPHODIESTERASE ACTIVITY

Results were obtained by Method I using $(NH_4)_2SO_4$-fractionated extracts. All reaction mixtures contained 40 mM Tris–HCl (pH 8.5), 2 mM $MgSO_4$, 10 mM L-cysteine, 0.2 mM cyclic AMP and additional compounds at concentrations indicated in the table.

Addition	Concentration (mM)	Activity (%)
None	—	100
Cyclic CMP	10	71
Papaverine	0.05	67
	0.10	58
Imidazole	1	100
	50	127
	100	144

$\times$ g pellet [9], while 75% of the *Acanthamoeba palestinensis* phosphodiesterase activity were recovered in the postmicrosomal fraction [13]. The *C. reinhardtii* phosphodiesterase is largely, if not entirely, soluble. 87% of the total activity of the crude homogenate were found in the postmicrosomal supernatant (obtained by centrifuging the 20 000 $\times$ g supernatant for 90 min at 100 000 $\times$ g).

DISCUSSION

The presence of a cyclic nucleotide phosphodiesterase, which is inhibited by methylxanthines, in *C. reinhardtii* had been postulated, when it was found that methylxanthines are effective inhibitors of flagellar function and regeneration in this organism [16]. The present study fully confirms this assumption. Even though properties of the enzyme have been studied using crude extracts of the alga, it is evident from the results that the enzyme of *C. reinhardtii* resembles in many respects phosphodiesterases, which have been described for animals, bacteria, slime molds, fungi and protozoans: e.g. hydrolysis of cyclic AMP occurs at the 3′-phosphate ester bond thus giving rise to 5′-AMP as the reaction product. Maximum activity in vitro is exhibited in the alkaline pH range and requires the presence of Mg^{2+} or Mn^{2+}. Inhibition by methylxanthines and stimulation by imidazole are also common features of phosphodiesterases from many sources. An outstanding and hitherto unique characteristic of the enzyme from *C. reinhardtii* is its ability to hydrolyze cyclic CMP at three times the rate for cyclic AMP. While cyclic CMP is resistant to mammalian phosphodiesterases [2], only the enzyme from *S. marcescens* has been reported to hydrolyze this cyclic nucleotide to some extent [5]. We have no information on the possible occurrence of cyclic CMP in *C. reinhardtii*, and it remains to be seen, if the hydrolysis of cyclic CMP has any physiological significance in vivo. A similar case was reported by Hardman and Sutherland [28], who described a phosphodiesterase from dog heart, which hydrolyzed cyclic UMP at 3- to 4-times the rate for cyclic AMP. As stated earlier, it remains to be shown that cyclic AMP and cyclic CMP are hydrolyzed by the same enzyme in extracts of *C. reinhardtii*. Attempts to purify the enzyme to any major extent, which might help to solve this question, have failed so far.

While many parallels exist between properties of the phosphodiesterase of *C. reinhardtii* and phosphodiesterases of other microorganisms and animals, it appears that enzymes from higher plants able to hydrolyze cyclic nucleotides have quite different properties [18–20]. The most thoroughly studied enzymes of this type, from pea seedlings [18] and soybean callus [19], have pH optima in the acid pH range (5.4–6.0 for pea seedlings, 4.2 for soybean callus), do not require divalent metal ions for maximum activity and are insensitive to methylxanthines and imidazole. Furthermore, it was shown for the purified pea enzyme [18], that the products of cyclic AMP hydrolysis are 3′-AMP and 5′-AMP in a ratio of about 7 : 1. The pea enzyme readily hydrolyses 2′,3′-cyclic nucleotides, and it is suggested by the authors, that its physiological function is the hydrolysis of these nucleotides, which are products of RNA degradation by higher plant ribonuclease. This would be in agreement with the apparent lack of convincing evidence for the occurrence of cyclic AMP in higher plants [29, 30]. In a survey of the plant kingdom, to be published elsewhere [20], we found cyclic nucleotide phosphodiesterases with pH optimum in the alkaline region and with 5′-AMP as sole product of cyclic AMP hydrolysis only in various algae, while

extracts from higher plants hydrolyzed cyclic AMP preferentially at an acid pH, and if AMP could be trapped as an intermediate in the formation of adenosine from cyclic AMP, it was either 3′-AMP or a mixture of 3′-AMP and 5′-AMP. We conclude, therefore, that the green alga *C. reinhardtii*, and possibly other algae as well, with respect to the occurrence of cyclic AMP and cyclic AMP metabolizing enzymes is more closely related to protozoans and other heterotrophic organisms than to higher green plants.

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