ENZYMATIC DEAMINATION OF ADENOSINE 2',3'-CYCLIC PHOSPHATE IN *DROSOPHILA MELANOGASTER*

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Summary

Four nucleoside cyclic phosphates were isolated from the hot ethanol extracts of the third instar larvae of *Drosophila melanogaster* and identified as follows: cytidine 2',3'-cyclic phosphate(Cp!), uridine 2',3'-cyclic phosphate(Up!), guanosine 2',3'-cyclic phosphate(Gp!) and inosine 2',3'-cyclic phosphate(Ip!).

The occurrence of Ip! instead of Ap! in the larvae suggests the presence of a deaminase which catalyzes the conversion of Ap! to Ip!. Such an enzyme has indeed been shown to be present in *Drosophila* larvae.

Pyrimidine nucleoside 2',3'-cyclic phosphates were first isolated as intermediates of RNA degradation by RNase *in vitro*(Markam and Smith, 1952). It is generally accepted that nucleoside 2',3'-cyclic phosphates are formed as intermediary or final products during the enzymatic degradation of RNA (Pierpoint, 1956; Asano, 1959; Shiio et al., 1966; Barker et al., 1967). They were also detected in the acid soluble fraction from *E.coli* strain B in phosphorus deficiency(Maruyama and Mizuno, 1966) and from hen's oviduct (Nakanishi et al., 1967).

The abbreviations used are: Cp!, cytidine 2',3'-cyclic phosphate; Up!, uridine 2',3'-cyclic phosphate; Gp!, guanosine 2',3'-cyclic phosphate; Ip!, inosine 2',3'-cyclic phosphate; Ap!, adenosine 2',3'-cyclic phosphate.

During the investigation on the purine and pyrimidine derivatives in Drosophila, it was found that the nucleoside cyclic phosphates were detected in the hot aqueous ethanol extract of the third instar larvae of D. melanogaster. An unusual feature of the present investigation is the fact that Ip!, but not Ap! was detected in the cyclic nucleotide fraction. This suggests the occurrence of an enzyme capable of converting Ap! to Ip! in Drosophila larvae. The present paper deals with the isolation and structure of these nucleoside cyclic phosphates and the enzymatic deamination of Ap!.

Experimental

Chemicals - Ap! and Cp! were purchased from Sigma Chemical Company. Other nucleotides and nucleosides were obtained from Kokoku Rayon and Pulp Co. Ltd. RNase T_1 was purchased from Sankyo Co. Ltd. and pancreatic RNase from Worthington Biochemical Company.

Materials - The third inster larvae of Drosophila melanogaster(Oregon -R, wild type strain) reared on the usual corn meal-molasses medium at 25°, were collected and used for the analyses. The starvation of larvae was conducted in deep petri dishes containing moist cellulose powder to avoid contamination of the nucleotides from feeding yeast.

Paper chromatography and paper electrophoresis - Paper chromatography was carried out by the descending technique in the following solvent systems: (A) n-butanol-ethanol-water(52:32:16) (B) ethanol-1 M ammonium acetate, pH 7.5(7.5:3) (C) ethanol-1 M ammonium acetate saturated with sodium borate(7:3) (D) isopropanol-water(7:3) with ammonia in the vapor phase. Paper electrophoresis was conducted in 0.05 M ammonium acetate, pH 5.0 and 0.05 M ammonium formate, pH 3.6.

Analytical procedures - The procedure for measurements of phosphate (Lowry et al., 1954) was modified to a micro scale. UV absorption spectra between 210-310 mμ were measured in a Cary recording spectrophotometer Model 14.

Partial purification of adenosine deaminase from Drosophila - The enzyme was purified about 30-fold by the ammonium sulfate fractionation (50-70% saturation), followed by chromatography on a Bio-Gel P-150 column. The partially purified enzyme catalyzed the conversion of adenosine and Ap! to inosine and Ip!, respectively. A relative substrate specificity (ratio of activity with Ap! to that with adenosine) was 0.13. This fraction also has shown low activities of phosphodiesterase and phosphatase.

Conditions of enzymatic deamination - Reaction mixture contains 0.75 μ mole of Ap!, 40 mM Tris-HCl, pH 7.5 and 0.1 mg(as protein) of the enzyme, in a total volume of 0.15 ml.

Results

Isolation and structure of nucleoside cyclic phosphate - Nucleotides were extracted with a hot aqueous ethanol(70% v/v) from Drosophila larvae. After concentrated with a rotatory evaporater, the nucleotides were absorbed on charcoal and eluated with aqueous ammoniacal ethanol solution(0.2% ammonia in 50% ethanol). The effluent was evaporated to a small volume and was applied as a zone on Toyo No.527 filter paper for descending paper chromatography in solvent(A), in which nucleoside cyclic phosphates were separated from other nucleotides. The nucleoside cyclic phosphate fraction was then separated from nucleosides and free bases with paper electrophoresis in 0.05 M ammonium acetate, pH 5.0. The fraction was subjected to the paper chromatography in solvent(B) to ensure that it was free from nucleoside 2'(3')-phosphates(Table I). After desalting in solvent(A), nucleoside cyclic phosphates were separated each other with paper electrophoresis in 0.05 M ammonium formate, pH 3.6(Table I). Four spots were recognized and identified as cytidine cyclic phosphate, uridine cyclic phosphate, guanosine cyclic phosphate and inosine cyclic phosphate by their Rf values on paper chromatography, mobilities on paper electrophoresis and UV absorption spectra. All the samples eluated from the spots gave molar ratio of base to phosphate as 1. The following results led to the conclu-

Table I

Properties of nucleoside cyclic phosphates isolated from the larvae of D.melanogaster

Compound	μ moles Pi[a] / μ moles base	Paper chromatography[b] R_{AMP} in solvent(B)	R_{AMP} in solvent(D)	Paper electrophoresis[c] $R_{Ap!}$
Ip!	0.98	2.42	1.88	2.20
Gp!	1.13	2.26	1.84	2.62
Cp!	0.97	2.02	1.82	0.67
Up!	0.95	2.38	1.96	3.20

a) Calculated from the molar absorbancies of IMP, GMP, CMP and UMP.
b) Estimated on Toyo filter paper No. 50 in solvent(B) ethanol-1 M ammonium acetate, pH 7.5(7.5:3) and solvent(D) isopropanol-water(7:3) with ammonia in the vapor phase.
R_{AMP}=mobility of sample(cm)/mobility of AMP(cm)
c) Estimated on Toyo filter paper No. 51 A in 0.05 M ammonium formate, pH 3.6 for 90 minutes at 1000 volts.
$R_{Ap!}$=mobility of sample(cm)/mobility of Ap!(cm)

Table II

Identification of nucleotides obtained by chemical and enzymatic hydrolysis of nucleoside cyclic phosphates.

Compound (from D.m.larvae)	$R_{Ap!}$[a]	Authentic Compound	$R_{Ap!}$[a]
Ip!	1.20	5'-AMP	0.06
Gp!	1.16	5'-GMP	0.03
Cp!	0.96	5'-CMP	0.06
Up!	1.05	5'-UMP	0.06
Ip!,digested[b]	0.23	2'(3')-AMP	0.29
Gp!,digested[b]	0.22	2'(3')-GMP	0.19
Cp!,digested[c]	0.38	2'(3')-CMP	0.39
Up!,digested[c]	0.42	2'(3')-UMP	0.44
Ip!,hydrolyzed[d]	0.22	Inosine	0.42
Gp!,hydrolyzed[d]	0.21	Guanosine	0.38
Cp!,hydrolyzed[d]	0.38	Cytidine	0.57
Up!,hydrolyzed[d]	0.42	Uridine	0.70

a) Estimated on Toyo No.51 A filter paper in solvent(C).
$R_{Ap!}$=mobility of sample(cm)/mobility of Ap!(cm)
b) Reaction mixture contained in a total volume of 0.05 ml, 0.1 μ mole of nucleoside cyclic phosphate, 40 mM Tris-HCl, pH 7,5, 2 mM EDTA, and 2 μg of RNase T_1.
c) Reaction mixture contained in a total volume of 0.05 ml, 0.1 μ mole of nucleoside cyclic phosphate, 20 mM Tris-HCl, pH 7.5, and 2 μg of pancreatic RNase.
d) Hydrolyzed with 0.1 N HCl for 6 hours at a room temperature.

sion that the four nucleotides were, in fact, nucleoside 2',3'-cyclic phosphates.

1) Alkaline phosphomonoesterase hydrolyzes nucleotide monophosphates to produce nucleosides, whose R_f are given in Table II. Under the same condition, the enzyme did not attack the samples in question at all.

2) On treating the pyrimidine nucleoside cyclic phosphates with pancreatic RNase and the purine nucleoside cyclic phosphates with RNase T_1, each converted to the compound with the same R_f value as the corresponding nucleoside 3'-phosphate, as judged by paper chromatography in solvent(C) (Table II). Those RNases are known to be active with nucleoside 2',3'-cyclic phosphate but not with nucleoside 3',5'-cyclic phosphate. The above results, therefore, eliminate the possibility that the nucleotides are 3',5'-isomers.

3) Treatment with 0.1 N HCl at room temperature for 6 hours converted the nucleoside cyclic phosphate samples to the corresponding nucleoside 2' or 3'-phosphate, as judged by paper chromatography in solvent(C)(Table II). Nucleoside 3',5'-cyclic phosphates would not be hydrolyzed by 0.1 N HCl.

Evidence for enzymatic deamination of Ap! - Ap! was incubated at 37° with the enzyme preparation for 90 minutes as described in "Experimental" and the solution was applied to Whatman 3 MM filter paper for paper chromatography in solvent(B). After desalting in solvent(A), hypoxanthine nucleotides were separated from adenine nucleotides by paper electrophoresis in 0.05 M ammonium formate buffer, pH 3.6(Fig. 1). UV absorption spectra of the products were measured as described in "Experimental". The composition of the products derived from Ap! were Ap!(33%), Ip!(50%), 2'(3')-AMP(6%), 2'(3')-IMP(6%), and inosine(5%). In the similar experiment with the mixture of 2' and 3'-AMP, the formation of inosine was observed but neither 2' nor 3'-IMP was detected in the reaction mixture. This indicates that AMP deaminase is absent in the enzyme preparation.

Changes in the concentration of nucleoside cyclic phosphate during

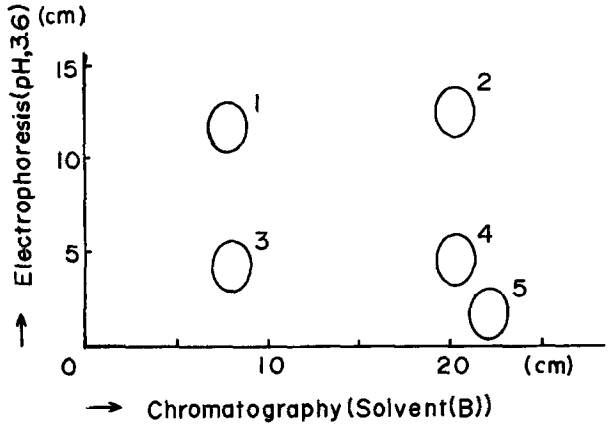

Fig. 1. Two dimentional mapping of the products derived from adenosine 2',3'-cyclic phosphate. The experimental conditions are given in text. The numbered spots are identified as follows: 1 - 2'(3')-IMP, 2 - cyclic 2',3'-IMP, 3 - 2'(3')-AMP, 4 - cyclic 2',3'-AMP, 5 - Inosine.

starvation - Changes in the level of nucleoside cyclic phosphates during starvation were investigated by determining the nucleotide contents in the extracts of larvae at different periods for starvation. As shown in Table III, the nucleoside cyclic phosphate content reached to a maximum point after 2-hours starvation and then declined.

Table III

Changes in the concentration of nucleoside 2',3'-cyclic phosphates of Drosophila during starvation.

Time after starvation(h)	Ip!	Gp!	Up!	Cp!
0	0.010	0.015	0.11	0.12
1	0.015	0.030	0.14	0.13
2	0.040	0.035	0.17	0.19
3	0.025	0.030	0.14	0.12
4	0.015	0.015	0.05	0.06

Concentrations are given in micromoles/g(dry weight) of larvae.

Discussion

According to the hypothesis proposed by Mizuno and Anraku(1967) for the routes of enzymatic degradation of RNA, there are at least two enzyme

systems in E.coli cells, namely, the ribonuclease(RNase I) pathway and the polynucleotide phosphorylase(PNPase)-RNA phosphodiesterase(RNase II) pathway. In the RNase I pathway, RNA is degradated to nucleoside 3'-phosphate through nucleoside 2',3'-cyclic phosphate.

The four nucleoside 2',3'-cyclic phosphates occurred in Drosophila may be intermediary products from RNA by RNase digestion and the RNase I pathway is suggested to be the most probable for the route of the degradation of RNA in the starved larvae.

Although little is known of the biochemical aspects of RNA metabolism during metamorphosis of insects, it is likely that the larval RNA is reutilized for the formation of the adult. However, the form of degradation products(i.e., oligonucleotides, nucleotides or nucleosides) for the reutilization is not known. Our results suggest that the RNase I pathway is working during metamorphosis and the nucleoside cyclic phosphates are key compounds for the reutilization of RNA.

Although Ap! is deaminated at a rate approximately one-eighth that of adenosine, the presence of Ip! in the larvae suggests that Ap! is a substrate for the deaminase in vivo. This also suggests the occurrence of a new metabolic pathway(pathway II) in the adenine nucleotide metabolism of Drosophila(Fig. 2). However,5'-AMP and 2'(3')-AMP were less deaminated at a rate only approximately one-thirthieth that of adenosine. These deaminations seem to be due to prior removal of phosphate by the phosphatase present in the enzyme preparation. On the other hand,it was observed in

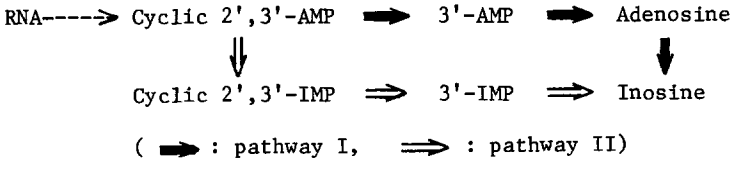

Fig. 2. Pathways of deamination and dephosphorylation of adenosine 2',3'-cyclic phosphate in Drosophila

this laboratory that Ap! was deaminated with the commercial calf intestinal adenosine deaminase(Boehringer Mannheim GmbH, West Germany), but at a rate less than one-hundredth that of adenosine. These results show that the deaminase of Drosophila is distinguished from the calf intestinal adenosine deaminase and the non-specific Taka-Diastase adenosine deaminase(Minato et al., 1966; Sharpless and Wolfenden, 1967). The further purification and characterization of the deaminase in Drosophila are being carried out.

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