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Enzymic Hydrolysis of Adenosine Phosphates by Cobra Venom

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Zeller (1948, 1950*a*) found that snake venoms liberate one molecule of inorganic orthophosphate (ortho-P) from adenosinetriphosphate (ATP), and concluded that the venoms contain an enzyme which hydrolyses ATP to adenosinediphosphate (ADP) and ortho-P. In an attempt to use the enzyme of cobra venom for the preparation of ADP from ATP Zeller's observation of the formation of one molecule of ortho-P from ATP was confirmed but no ADP was found. A further study of the reaction revealed that the enzyme, in fact, hydrolyses ATP to adenosine, inorganic pyrophosphate (pyro-P) and ortho-P, and that ADP is not an intermediate in this reaction.

EXPERIMENTAL

Materials and methods

Adenosine phosphates. The barium salt of ATP was prepared from rabbit muscle (see LePage, 1949) and converted into the sodium salt as described by Eggleston & Hems (1952). The concentration of ATP in the stock solution was about 6.5 mg./ml. The preparation contained some ADP, ortho-P and pyro-P; the quantities of these impurities are stated for each experiment. A sample of adenosinemonophosphate (AMP) (free acid) was supplied by Roche Products Ltd., through the courtesy of Dr F. Bergel and Dr A. L. Morrison. It showed no other phosphates or

adenosine derivatives when tested by the chromatographic procedure described below. The concentration of the stock solution was 14 mg./ml. AMP, neutralized to pH 8.4. Barium-ADP was obtained from Schwarz Laboratories Inc., New York. It was doubly decomposed with Na_2SO_4 and was also found to be free from phosphates and other adenosine derivatives when tested chromatographically.

Cobra venom. A sample of dried cobra venom (*Naja naja*) was kindly supplied by the late Dr C. H. Kellaway, F.R.S. A stock solution of 5.4 mg./ml. was used in all experiments.

Antiserum. A sample of dried cobra 'antivenine' was also supplied by Dr Kellaway. This is a dried serum prepared by Lamb (1902, 1904) from the blood of rabbits which had received, about every 10 days for 4 or 5 months, subcutaneous injections of cobra venom. This serum forms a precipitate with cobra venom which is maximal when the ratio of venom to antiserum is 1 to 4.

Incubation and sampling. Stock solutions of cobra venom (0.2 ml.), adenosine phosphate (0.2 ml.) and 0.1 M-barbitone buffer (Michaelis, 1930), pH 8.4 (0.4 ml.), were mixed and incubated at 30°. When antiserum was included it was dissolved in the buffer solution. The pH chosen is near the optimum for the venom ATP-ase (Zeller, 1948, 1950*a*) and 5-nucleotidase (Gulland & Jackson, 1938). A sample (0.1 ml.) was taken immediately after mixing and transferred to 30% (w/v) aqueous trichloroacetic acid solution (0.05 ml.). Other samples were collected at intervals and treated in the same way. They were stored in the 'deep freeze' till they were analysed.

Chromatographic analysis. Separation of the adenosine derivatives and phosphates was carried out by chromatography. The method of Eggleston & Hems (1952) was used with certain modifications necessitated by the presence of

Fig. 1. A tracing of a chromatogram of phosphate compounds run in *n*-butanol-acetic acid-water for 60 hr. at 20°. (1) Ortho-P; (2) pyro-P; (3) AMP; (4) ADP (small spot) and ATP; (5) mixture. P content of the pyro-P 'spot', sectioned as shown, was as follows: (2*a*) 1.07; (2*b*) 0.54; (2*c*) 0.12 and (5*a*) 1.51; (5*b*) 0.20 μ g. P.

Table 1. *Recovery of ATP and other phosphates from a mixture*

(For chromatographic procedure, see text. The quantities stated were contained in 5 μ l. of water.)

Material	Amount of P (μ g.) in material	
	Added	Recovered
Adenosinetriphosphate	1.81	1.88
Adenosinediphosphate	0.42	0.37
Adenosinemonophosphate	2.32	2.31
Pyro-P	1.73	1.71
Ortho-P	0.21	0.22

pyro-P, which was found to have about the same R_f value as ADP in the solvents of Eggleston & Hems. When these were replaced by a *n*-butanol-acetic acid-water mixture (40:10:50, v/v), a mixture of ATP, ADP, AMP, adenosine,

Table 2. *Decomposition of ATP and ADP by cobra venom*

Material	Amount of material (μ moles) at											
	0.5 hr.			1.5 hr.			4 hr.					
	Found	Observed change	Calculated change	Found	Observed change	Calculated change	Found	Observed change	Calculated change	Found	Observed change	Calculated change
Adenosinetriphosphate	1.44	—	—	0.19	—1.25	—	0	—1.44	—	0	—	—
Adenosinediphosphate	0.60	—	—	0.24	—0.35	—	0	—0.60	—	0	—	—
Adenosinemonophosphate	0	0	—	0	0	—	0	0	—	0	0	—
Pyro-P	0.04	0.35	0.29	1.10	1.06	1.25	1.35	1.31	1.44	1.35	1.31	1.44
Ortho-P	0.74	0.53	0.61	2.56	1.82	1.97	3.48	2.74	2.64	3.48	2.74	2.64
Adenosine	0	0.63	0.45	1.77	1.77	1.61	2.12	2.12	2.04	2.12	2.12	2.04

(Incubation at 30° in 0.05M-barbitone buffer (pH 8.4). Venom, 0.14% (w/v). Vol. of mixture, 0.8 ml. 'Observed change' refers to the increase above the zero value. 'Calculated change' is based on the assumption that ATP yields equimolar amounts of adenosine, ortho-P and pyro-P, and that ADP yields one equivalent of adenosine and two of ortho-P.)

ortho-P and pyro-P separated when run in the downward direction for 50 hr. None of the substances decomposed under these conditions. After about 30 hr. the adenosine spots approached the bottom end of the paper where they were located by ultraviolet light. To obtain the adenosine values the section of the paper containing the substance was cut off whilst the strip was still wet with solvent. The 'run' was continued, and after 50–60 hr. the ortho-P reached the bottom of the shortened paper. The excised section was air-dried, the adenosine was eluted with 3 ml. 0.1 M-phosphate buffer, pH 7.0, at 20° for about 30 min. and the absorption of the eluate at 280 m μ . was measured spectrophotometrically. When P analyses were not required the chromatogram was run for 28 hr. and the adenosine compounds removed and estimated as described above for adenosine. It was sufficiently accurate to estimate ATP and ADP together as ATP, since the P content affects the molar extinction coefficients of such compounds only slightly (see Kalekar, 1947), and the proportion of ADP was small.

Table 3. *Effect of antiserum on the decomposition of ADP by cobra venom*

(Incubation at 30° in 0.02 M-barbitone buffer (pH 8.4). Venom, 0.10 % (w/v). Antiserum, 2.0 mg. Vol. of mixture, 0.5 ml.)

Material	Amount of material (μ moles) at		
	0 hr.	4 hr.	21 hr.
(a) Without antiserum			
Adenosinediphosphate	2.76	0.77	0.15
Adenosinemonophosphate	0	0	0
Pyro-P	0	0	0
Ortho-P	0	4.24	5.52
(b) With antiserum			
Adenosinediphosphate	2.75	1.47	0.18
Adenosinemonophosphate	0	1.11	0.42
Pyro-P	0	0	0
Ortho-P	0	1.47	4.75

A tracing of a 60 hr. chromatogram of a mixture of adenosine derivatives and phosphates is shown in Fig. 1. Table 1 shows the satisfactory recovery of the added substances. It is noteworthy that pyro-P tails badly when run alone but tailing is so much decreased when a mixture of phosphates is present that separation offers no difficulty.

Table 4. *Effect of antiserum on the decomposition of AMP by cobra venom*

(Incubation at 30° in 0.05 M-barbitone buffer (pH 8.4). Venom, 0.14 % (w/v). Antiserum, 4.4 mg. Vol. of mixture, 0.8 ml.)

Material	Amount of material (μ moles) at					
	0 hr.	0.5 hr.	1.5 hr.	4 hr.	7 hr.	24 hr.
(a) Without antiserum						
Adenosinemonophosphate	7.90	2.10	> 0.05	0	—	—
Ortho-P	0	6.15	8.18	8.24	—	—
Adenosine	0	5.77	6.93	7.55	—	—
(b) With antiserum						
Adenosinemonophosphate	8.32	—	7.42	6.67	5.35	2.01
Ortho-P	0	—	1.04	1.38	2.91	6.17
Adenosine	0	—	0.44	1.09	2.02	5.78

40.2

Nomenclature. According to a suggestion of Dr W. Klyne, Dr R. S. Cahn and Dr R. K. Callow the three P-atoms of ATP are distinguished as follows:

RESULTS

Hydrolysis of adenosine phosphates by cobra venom.

The time curves of the effect of cobra venom on ATP and ADP are shown in Tables 2 and 3. The column '0 hr.' gives the composition of the starting material. All ATP and such ADP as was present were completely dephosphorylated after 4 hr. in the experiment recorded in Table 2. As the concentrations of ADP and ATP fell those of adenosine, pyro-P and

Table 5. *Decomposition of inorganic pyrophosphate by cobra venom*

(For conditions, see Table 2.)

Material	Amount of material (μ moles) at		
	0 day	6 days	
	Found	Found	Observed change
Pyro-P	2.8	1.2	-1.6
Ortho-P	0	3.0	3.0

ortho-P rose. No AMP appeared. The data support the assumption that ATP is split into one molecule each of adenosine, pyro-P and ortho-P, and ADP into adenosine and 2 molecules of ortho-P. The pyro-P found was slightly below, and the ortho-P slightly above, the expected values, which can be accounted for by slow hydrolysis of pyro-P (see below). The hydrolysis of ADP (Table 3) is slower than that of ATP. After 21 hr. 95 % of the added ADP had disappeared under the test conditions and the amount of ADP removed and ortho-P formed were approximately in the expected proportion of 1:2. No pyro-P or AMP appeared.

AMP was rapidly hydrolysed to adenosine and ortho-P (Table 4), the initial rate of disappearance of AMP being about 5 times higher than that of

Table 6. *Effect of antiserum on the decomposition of ATP by cobra venom*

(For conditions see Table 4.)

Material	Amount of material (μ moles) at				
	0 hr.	0.5 hr.	1.5 hr.	4 hr.	21 hr.
(a) Without antiserum					
Adenosinetriphosphate	2.06	0.89	0.18	0	—
Adenosinemonophosphate	0.18	0	0	0	—
Adenosine	0	1.28	1.69	2.32	—
(b) With antiserum					
Adenosinetriphosphate	2.24	1.48	0.86	0.03	0
Adenosinemonophosphate	0.21	0.81	1.62	1.85	0
Adenosine	0	0	0	0.50	2.38

Table 7. *Location of ^{32}P before and after decomposition of $[\beta\gamma\text{-}^{32}\text{P}_2]\text{ATP}$ and $[\beta\text{-}^{32}\text{P}]\text{ADP}$ by cobra venom*

(For conditions see Table 2. 'Observed change' refers to the increase above the zero value. 'Calculated change' is based on the assumption that ^{32}P in $[\beta\gamma\text{-}^{32}\text{P}_2]\text{ATP}$ appears as pyro-P and that ^{32}P in $[\beta\text{-}^{32}\text{P}]\text{ADP}$ appears as ortho-P.)

Material	Amount of ^{32}P (counts/min./0.01 ml. of incubated mixture) in material at			
	0 hr. Found	4 hr. Found	Observed change	Calculated change
Adenosinetriphosphate	212	61	-151	—
Adenosinediphosphate	41	11	-30	—
Pyro-P	20	175	155	151
Ortho-P	23	46	23	30

ATP. This confirms the presence of a highly active 5-nucleotidase in cobra venom (Gulland & Jackson, 1938; Zeller, 1950b).

Hydrolysis of inorganic pyrophosphate. The yields of pyro-P and ortho-P from ATP (see Table 2) suggest that the venom contains a weak pyrophosphatase. This is borne out by the experiment recorded in Table 5. Recrystallized $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ (0.2 ml., 4.0 mg./ml.) was incubated under the same conditions as ATP. After 6 days a little above 50 % of the added pyro-P was hydrolysed to ortho-P (Table 5). There was no hydrolysis in the absence of cobra venom under the same conditions. An inorganic pyrophosphatase is thus present in cobra venom but its activity is slight compared with the ATP-ase and 5-nucleotidase.

Action of venom on adenosine. After incubation for several weeks with cobra venom in the presence of chloroform, adenosine remained unchanged and no ribose, adenine or inosine was formed.

Effect of antiserum. Addition of antiserum (4.4 mg. in 0.8 ml. mixture) reduced the rate of disappearance of ATP by about one-third (Table 6). The formation of adenosine was negligible during the first 90 min.; instead AMP accumulated. Antiserum alone did not attack ATP. Dried serum from the normal rabbit had no inhibitory effects on the action of cobra venom.

The observations suggest that two enzymes are concerned with the hydrolysis of ATP, an ATP-ase, which converts ATP into AMP and pyro-P and

a 5-nucleotidase, which hydrolyses AMP to adenosine and ortho-P. The antiserum inhibits the latter enzyme more powerfully than the former (see Table 4 and 6); it also inhibits the decomposition of ADP (Table 3).

Decomposition of isotopic ATP. ATP, prepared according to Hems & Bartley (1953) and in which the β - and γ -P atoms were replaced by ^{32}P , was incubated with cobra venom in the absence and presence of antiserum. The AMP which accumulated during the first 4 hr. in the presence of antiserum showed no radioactivity. In the absence of antiserum practically all of the radioactivity was located in the pyro-P (Table 7). This confirms the conclusions reached in the preceding paragraph.

DISCUSSION

The observations on the effect of cobra venom on adenosine derivatives are all accounted for by the assumption that the following reactions occur in the presence of the venom.

- (1) $\text{ATP} \rightarrow \text{AMP} + \text{pyrophosphate}$ (rapid).
- (2) $\text{AMP} \rightarrow \text{adenosine} + \text{orthophosphate}$ (very rapid).
- (3) $\text{pyrophosphate} \rightarrow 2 \text{ orthophosphate}$ (very slow, rate about 1 % of that of (1)).
- (4) $\text{ADP} \rightarrow \text{adenosine} + 2 \text{ orthophosphate}$ (slow, rate about 10 % of that of (1)).

As (2) is much more rapid than (1) AMP does not accumulate normally, but when (2) is selectively inhibited by antiserum AMP accumulates.

The enzyme-catalysing reaction (1) hydrolyses the $\alpha\beta$ -pyrophosphate bond of ATP and may therefore be described as $\alpha\beta$ -ATP-ase, in contrast to the common ATP-ases of animal tissues and other materials, which are $\beta\gamma$ -ATP-ases. The enzyme of reaction (2) is the 5-nucleotidase of Gulland & Jackson, which removes phosphate from AMP and inosine 5-phosphate but not from 3-phosphate nucleotides. Reaction (4) is presumably the result of a combined action of an ADP-ase and 5-nucleotidase.

The fission of ATP into equimolar quantities of adenosine, ortho-P and pyro-P has already been recorded, though without experimental details, by Hitchings & Fuller (1939). Zeller (1950*b*) believed that this occurred only 'under certain conditions, e.g. low substrate concentration'. The venom investigated in the present work did not, as Zeller (1951) assumed, transform ATP into ADP or hydrolyse the latter to adenosine and pyrophosphate.

The observation of Bhattacharya & Ghosh (1951) that one equivalent of acid is formed when ADP is decomposed by cobra venom is explained by reaction (1). The known data for the ionization constants of the reactants (Alberty, Smith & Bock, 1951) indicate that the hydrolysis of one pyrophosphate bond yields about one equivalent of acid at pH 7.4.

As Zeller (1951) has pointed out, it is very probable that the $\alpha\beta$ -ATP-ase and 5-nucleotidase of cobra venom are, like the nucleotide pyrophosphatase (Chain, 1938, 1939), components of the toxic principle of the venom. Cobra venom is known to depress the blood pressure, and Kellaway & Trethewie (1940) have demonstrated the release by cobra venom *in vivo* of 'adenyl compounds' (i.e. substances depressing the blood pressure in the atropinized guinea-pig heart preparation). Two of the products of the decomposition of ATP, namely adenosine and pyrophosphate, have such depressor

effects (Green & Stoner, 1950; Bielschowsky & Green, 1944). Thus by two enzyme-catalysed reactions the key metabolite of animal tissues, ATP, is converted into two toxic substances. Moreover, unlike other breakdown products of ATP, adenosine and pyrophosphate cannot be readily resynthesized to ATP by the enzyme systems responsible for oxidative phosphorylation. It remains to be investigated whether the same or different enzymes cause the destruction of nucleotide coenzymes by snake venoms (Chain, 1939; Fleckenstein & Gayer, 1950).

SUMMARY

1. The effect of cobra venom on adenosine phosphates has been studied. The products formed were separated and estimated chromatographically.

2. In the presence of cobra venom, adenosinetriphosphate is almost quantitatively converted into adenosine, orthophosphate and pyrophosphate; adenosinemonophosphate is converted into adenosine and orthophosphate.

3. Antiserum inhibits the hydrolysis of adenosinetriphosphate. In the presence of antiserum, no adenosine is formed in the earlier stages of incubation; instead adenosinemonophosphate appears.

4. Adenosinetriphosphate labelled with ^{32}P in the β and γ positions (for nomenclature see p. 627) yields radioactive pyrophosphate and inactive orthophosphate when incubated with cobra venom.

5. The findings indicate that the venom contains an adenosinetriphosphatase which hydrolyses the $\alpha\beta$ -pyrophosphate bond, differing from the common tissue adenosinetriphosphatases which hydrolyse the $\beta\gamma$ -bond. This enzyme together with the 5-nucleotidase described by Gulland & Jackson (1938) account for the hydrolysis of adenosinetriphosphate.

6. Adenosinediphosphate is slowly hydrolysed by the venom to adenosine and two molecules of orthophosphate. No pyrophosphate is formed.

7. The role of the above enzymes in the toxic action of cobra venom is discussed.

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