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FURTHER OBSERVATIONS ON THE ADENOSINE PHOSPHATASES OF COBRA VENOM

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SUMMARY

Dried cobra venom has been dialysed under various conditions. The effect on the $\alpha\beta$-ATP-ase, 5'-nucleotidase and DPN-*pyro*phosphatase, also that of chelating agents and of certain metal ions, is reported. The effect of specific antiserum has been investigated. The venom hydrolyses inosine triphosphate and coenzyme A in a manner analogous to the breakdown of ATP and DPN respectively. Flavine mononucleotide, ribose-5-phosphate and nicotinamide mononucleotide are hydrolysed at only 0.5, 1 and 2 % of the rate of adenosine monophosphate hydrolysis. Thiamin *pyro*phosphate is not hydrolysed.

INTRODUCTION

In a previous publication[1] an enzyme system of cobra venom was described which breaks down ATP by means of $\alpha\beta$-ATP-ase and 5'-nucleotidase[2] liberating *pyro*-P, *ortho*-P and adenosine. This system occurs in other snake venoms[3,4] and in semen[5], from which sources both enzymes have been studied in the partially purified state. The dried venom available to the author, however, contained practically no active non-specific phosphatase or $\beta\gamma$-ATP-ase, thus facilitating a direct study of the venom enzymes mentioned above.

MATERIALS AND METHODS

ATP, ADP and AMP were those described in an earlier publication[1]. ITP and IMP were prepared by Mr. D. H. WILLIAMSON according to the method of KLEINZELLER[6]. The IMP was almost 100 % pure. The ITP analysed as follows: ITP, 48.4: IDP, 42.3: IMP, 4.7: *ortho*-P, 3.7: *pyro*-P, 1.0 %. DPN was obtained from yeast by the method of LEPAGE[7]. (See Table VII, col. 2 for analysis.) FMN, reputed to be 90 % pure, was kindly supplied by the Sigma Chemical Company. Chromatography showed two fluorescent spots with R_F values of 0.095 and 0.129. The lower spot contained 14 % of the phosphorus and 5.5 % of the fluorescence. TPP solution (20 mM) was kindly supplied by Dr. W. BARTLEY. It contained a trace of *ortho*-P. R-5-P was prepared by Mr. D. H. WILLIAMSON by acid hydrolysis of NMN and isolated as the Ba salt. Aqueous solutions of the Na salt were prepared as required. Coenzyme A was a 50 % pure sample made by the method of KAPLAN AND LIPMANN[8] and given by Mrs. M. HOKIN. Run in *n*-butanol–acetic acid–water mixture, it showed under u.v. light one spot R_F 0.013 and one in the AMP area, both containing P. All stock chemicals were of A.R. purity.

Cobra (Naia Naia) *venom* was the dried material described previously[1]. Stock solutions containing 5.4 mg/ml were made up in water or in 0.08 M veronal buffer, pH 8.4. Between pH 7.0 and 9.0 in veronal buffer the venom activity towards mono- and diphenylphosphates was about 1.0 and 0.2 % respectively of that towards AMP and DPN. The natural, non-specific phosphatases[9] were presumably inactivated during drying and storage of the venom. No $\beta\gamma$-ATP-ase was present. No phosphates or u.v. light-absorbing materials were detected in the venom (*cf.* DOERY[10]). Venom in veronal buffer, pH 8.4, was used in some experiments after a preliminary dialysis at 20°, overnight against similar buffer. This is termed "buffer-dialysed" venom solution. Venom dialysed otherwise is described where necessary. All proportions by weight, and percentages refer to the weight of original dried (not dialysed) venom.

Antiserum was the dried material described previously[1]. It was weighed as required and dissolved in the buffer solution. No contaminating phosphates or u.v. light-absorbing materials were detected.

All solutions were stored at —20° without deterioration.

The following abbreviations are used in the text: ATP, ADP, AMP, adenosine-5'-tri-, di-, and monophosphate; ITP, IDP, IMP, inosine-5'-tri-, di-, and monophosphate; DPN, diphosphopyridine nucleotide; TPP, thiamin *pyro*phosphate; *ortho*-P and *pyro*-P, inorganic *ortho*- and *pyro*-phosphate; FMN, flavine mononucleotide; NMN, nicotinamide mononucleotide; R-5-P, ribose-5-phosphate; EDTA, ethylenediaminetetraacetic acid; tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid.

Estimation of Mg^{++} and Zn^{++}: The colorimetric method of GARNER[11] was used to determine Mg^{++} in the venom and in a TCA extract, both after incineration in a platinum crucible at dull red heat. Potassium cyanide (5 %, 0.1 ml per 0.5 ml of solution) was used to overcome the inhibitory effect of Zn^{++} on colour-formation. The values obtained were about 2.0 mg and 1.3 mg/g dried venom respectively. Zn^{++} was determined with dithizone[12], 2.2 mg/g dried venom was found. These values correspond to a concentration in the natural venom of 29 m*M* Mg^{++} and 12 m*M* Zn^{++}, assuming a 33 % solid content and a specific gravity of 1.058 for the natural venom[13]. The dried venom left 2.14 % ash, most of which was removed on water dialysis. It contained no detectable manganese or cobalt.

Nicotinamide determination: AMP and NMN, the initial breakdown products of DPN, run together when paper-chromatographed in *n*-butanol–acetic acid–water (see Table I). For the determination of the NMN in the eluted spot the nicotinic acid produced by alkaline hydrolysis was estimated by the method of BANDIER AND HALD[14] with certain modifications introduced to avoid the formation of dark colours: viz. addition of extra CNBr after the heating process, to stabilise the metol; preparation of CNBr, ice cold, immediately before use; its addition to the solution before heating; use of metol within 1 h and decolorisation with charcoal if necessary. A reproducible straight-line graph was obtained giving 3 % accuracy. The necessary chromatogram areas were eluted with water. AMP did not affect the result.

TABLE I

R_F VALUES OF SOME SUBSTANCES IN *n*-BUTANOL–ACETIC ACID–WATER

The substances were run in the solvent upper layer by descending chromatography at about 20° for varying periods on No. 4 Whatman paper. The values were obtained with mixtures 2 to 7 days old.

Substance	R_F	*Substance*	R_F
DPN	0.013	AMP	0.12
ITP	0.017	R-5-P	0.12
ATP	0.020	*Ortho*-P	0.22
ADP	0.033	Guanine	0.23
TPP	0.035	Inosine	0.29
IDP	0.039	Hypoxanthine	0.37
Pyro-P	0.062	Adenosine	0.42
FMN	0.095	Adenine	0.50
IMP	0.10	Phenylphosphate	0.73
NMN	0.11	Diphenylphosphate	0.93

Incubation and sampling were carried out essentially as described previously[1], with modifications where necessary. Venom concentrations of 0.11–0.14 % were normally obtained giving about 85–111 μM magnesium and 35–45 μM zinc. Natural cobra venom is about 300 times more concentrated than the final incubation mixtures used in these experiments (see[13]).

Chromatographic separation and analysis: Separation of most substances was effected as described previously[1]. When only the *pyro*-P was required from ATP or ITP breakdown the formic acid:isopropyl ether solvent mixture of HANES AND ISHERWOOD[15] was used. Mixtures of adenosine phosphates and DPN were resolved by the method of KREBS AND HEMS[16]. No. 4 Whatman paper was used throughout.

Enzymic "activities" are compared using an arbitrary unit, being μmoles/ml of substrate hydrolysed in 30 min at 30° by a 0.1 % cobra venom solution, or by the enzyme equivalent to this amount of venom. Approximate optimal substrate concentrations were determined for ATP, ADP, AMP and DPN. They are, respectively 1.4–1.7; 12–13; 2.6–3.0; 12–20. The corresponding enzyme activities are 1.4–1.5; approx. 1.5; 3.4–3.6; 8.9–11. Apparent Michaelis constants are 0.4–0.5; 3.0; 0.11; 5.0.

RESULTS

Hydrolysis of adenosine phosphates

Effect of pH change on 5'-nucleotidase and ATP-ase: The optimum pH of the venom 5'-nucleotidase was 6.8 to 7.0 (phosphate buffer). The "activity" in veronal buffer was similar at this pH but decreased more rapidly on the alkaline side (see Fig. 1). This may represent an inhibition by the barbiturate ion, the concentration of which is very small at pH 7.0 (where the free acid crystallises readily). In tris buffer activities were increased at all pH values. The ATP-ase optimum was pH 8.6–8.8 (veronal buffer) (*cf.* HEPPEL AND HILMOE[4]). At pH 8.2 tris buffer effected 163 % enhancement. At pH 8.5 borate buffer (0.1 *M*) inhibited 5'-nucleotidase 40 % and ATP-ase 15 % compared with veronal buffer. Similar borate inhibition and/or tris activation has been reported for these enzymes from semen[3,4] and retina[17]. *Pyro*-P (30 m*M*) inhibited 5'-nucleotidase 67 % and ATP-ase 38 % in veronal buffer, pH 8.4. One tenth of this *pyro*-P concentration had no significant effect upon either enzyme.

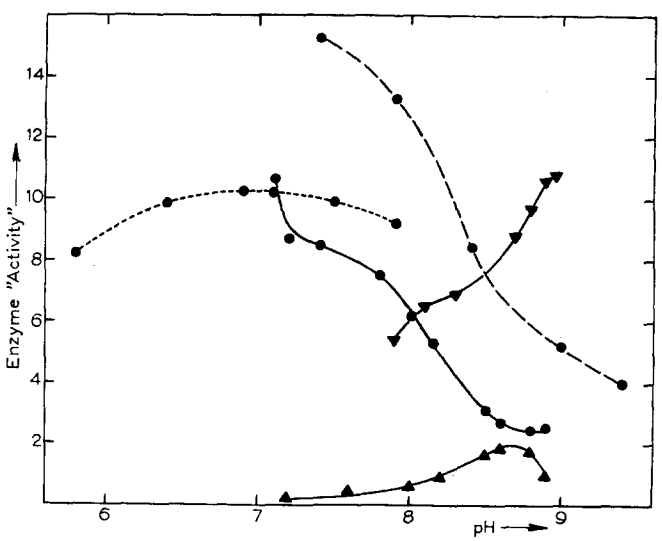

Fig. 1. Effect of pH change on $\alpha\beta$-ATP-ase (▲), 5'-nucleotidase (●), and DPN-pyrophosphatase (▼) in various buffers: 0.1 *M* phosphate – – – –; 0.1 *M* tris — — —; 0.08 *M* veronal ———.

The pH stability (7 days at room temperature) of 5'-nucleotidase was examined at the following pH values: 2.9, 4.3, 5.8 (acetate buffer); 7.0, 8.4 (veronal buffer). The activities (determined in veronal buffer pH 7.0) were respectively 0.03, 4.7, 17.2, 11.9, 15.6.

Dialysis of cobra venom and addition of metal ions: $ZnSO_4$ and $MgCl_2$ added to the crude venom solution up to 1.15 m*M* had no effect on either enzyme.

Dialysis of the venom at 20° against water effected a reduction of both enzymic activities and ultimately removed most of the Mg^{++} and Zn^{++}. Table II shows these results, and the effect of Mg^{++} and Ca^{++} on the dialysed enzymes. During the dialysis a precipitate formed, which contained 25 % of the 5'-nucleotidase activity. Dialysis against distilled water at 0° for 14 days reduced the 5'-nucleotidase activity (veronal buffer, pH 8.4) to 70 % of its former value while dialysis against 0.9 % KCl at 0° reduced it to 96 %.

TABLE II

EFFECT OF WATER-DIALYSIS ON ATPASE AND 5'-NUCLEOTIDASE

Venom (0.54 %) dialysed against distilled water at 20°, then used at 0.14 %. Incubation at 30° in 0.025 *M* veronal buffer, pH 8.4.

Time of dialysis (days)	*Total zinc/g venom (μg)*	*Mg^{++}*/g venom (μg)*	*Enzyme "activity"*	
			ATP-ase	*5'-nucleotidase*
0	2,200	1,300	1.4	3.4
2	1,150	525	1.5	0.2
4	314	490	1.0	—
6	< 20	< 50	0.4	—
2 (+ 0.015 *M* Mg^{++})			1.9	0.3
2 (+ 0.015 *M* Ca^{++})			0.8	0.2

* Measured on TCA extract.

Dialysis of venom against 0.08 *M* veronal buffer, pH 8.4 effected 79 % enhancement of the 5'-nucleotidase. Zinc sulphate (47 μ*M*) neutralised this effect. Mn^{++}, Co^{++} and Mg^{++}, in decreasing order, raised the activity further. Ca^{++} inhibited slightly, confirming a previous report[3] (see Table III). Such dialysis hardly affected the ATP-ase (Table IV). Dialysis at the same pH against 0.008 *M* buffer increased the 5'-nucleotidase activity (in 0.08 *M* buffer) to 9.3 (at pH 8.4) and 16.0 (at pH 7.1)

TABLE III

EFFECT OF CERTAIN METAL IONS ON 5'-NUCLEOTIDASE

Conditions as for "buffer-dialysed" venom, Table IV.

No.	*Additions to reaction (Ultimate concn. mM)*	*"Activity"*
1	None	6.1
2	Mg^{++} (0.005)	6.1
3	Mg^{++} (0.047)	6.6
4	Zn^{++} (0.047)	3.6
5	Mn^{++} (0.005)	9.6
6	Co^{++} (0.005)	8.5
7	Ca^{++} (0.047)	5.8

Zn^{++} (47 μ*M*) reduced these values to 6.6 and 12 respectively. Mn^{++} (5 μ*M*) had no additional effect. Dialysis against tris buffer (0.01 *M*) at pH 8.5 resulted in a reduced

5′-nucleotidase activity (measured in 0.1 *M* tris) (10.4 at pH 7.4 and 3.9 at pH 9.0) compared with the undialysed venom. Mn^{++} (5 μM) had little effect on this dialysed product. Zn^{++} (47 μM) inhibited about 25 % at both pH values.

Both enzymes were unaffected by NaF (50 m*M*) but were completely inhibited by Cu^{++} probably indicating the involvement of amino groups (*cf.* [18] for alkaline phosphatase). ATP-ase was inhibited 50 % by sodium iodoacetate (45 m*M*).

Effect of chelating agents upon ATP-ase and 5′-nucleotidase: A preliminary experiment showed that dithizone increased the 5′-nucleotidase activity of the crude venom, while the ATP-ase was unaffected. 8-Hydroxy quinoline effected complete inhibition of both enzymes. The effect of various EDTA concentrations (8.4, 42, 420 and 4200 μM) on the enzymes of crude and "buffer-dialysed" venom was determined (Table IV). After dialysis less EDTA was required for complete inhibition of both enzymes. This inhibition was reversed by $MgCl_2$ with considerable enhancement in the case of 5′-nucleotidase. Mg^{++} had a greater effect upon the ATP-ase after the "buffer-dialysed" venom:EDTA mixtures had been dialysed against equimolar veronal buffer (pH 8.4) overnight, even though it was found that such treatment would remove only about 50 % of the EDTA:metal complexes and less of EDTA itself (measured spectrophotometrically). (Table IV). The high Mg^{++} concentrations seemed essential for the re-activation of ATP-ase since dialysis overnight of "buffer-dialysed" venom:EDTA:magnesium ("280 m*M*") mixtures (Table IV, No. 15) against equimolar veronal buffer (pH 8.4) gave a low ATP-ase activity (0.35).

Mn^{++} had greater and Be^{++} less effect than Mg^{++} in overcoming EDTA-inactivation of 5′-nucleotidase. Ca^{++} had no effect with either enzyme (*cf.* [19]). Excess Mg^{++} and Mn^{++} inhibit 5′-nucleotidase. Zn^{++} inhibits the Mg^{++}-reactivated enzyme (Tables IV and V).

TABLE IV

EFFECT OF EDTA AND MAGNESIUM UPON ATP-ASE AND 5′-NUCLEOTIDASE

Incubation at 30° in 0.024 *M* veronal buffer, pH 8.4. Venom and "buffer-dialysed" venom, 0.11 %. AMP and ATP concentrations 5.6 m*M*.

No.	*Additions to reaction (Ultimate concn. mM)*	*5′-nucleotidase "activity"*		*ATP-ase "activity"*	
		Crude venom	*Dialysed venom*	*Crude venom*	*Dialysed venom*
1	None	3.4	6.1	1.4	1.3
8	EDTA (0.008)	—	7.3	—	1.9
9	EDTA (0.042)	3.7	6.5 (3.3*)	1.1	1.6
10	EDTA (0.42)	5.3	**0.3 (0*)	0.6	0
11	EDTA (4.2)	0	—	< 0.1	—
12	No. 10 + Mg^{++} (0.56)	—	2.2	—	0 (0.7***)
13	No. 10 + Mg^{++} (5.6)	—	14.0 (0*)	—	0.1 (0.9***)
14	No. 10 + Mg^{++} (28)	—	15.8	—	—
15	No. 10 + Mg^{++} (280)	—	4.1	—	1.4 (1.8***)
16	No. 11 + Mg^{++} (280)	12.2	—	1.0	—
17	No. 11 + Mg^{++} (2,800)	4.8	—	0.9	—

* After storage of EDTA-treated venom at — 20° for 21 days.
** Non-specific phosphatase activity < 0.05.
*** After dialysis of No. 10 overnight against equimolar veronal buffer, pH 8.4 at 20°.

After keeping the EDTA:venom mixtures at —20° for 21 days, the re-activation of 5′-nucleotidase by Mg^{++} was reduced, *e.g.* see Nos. 9 and 13 of Table IV.

Paper electrophoresis showed that the venom proteins are predominantly basic and the 5′-nucleotidase and ATP-ase activities move towards the cathode at pH 8–8.5. The electrophoretic pattern was not altered by the presence of EDTA.

TABLE V

EFFECT OF CERTAIN METAL IONS ON 5′-NUCLEOTIDASE INACTIVATED WITH EDTA

Conditions as for "buffer-dialysed" venom, Table IV.

No.	*Additions to reaction (Ultimate concn. mM)*	*"Activity"*
10	EDTA (0.42)	0.3
12	No. 10 + Mg^{++} (0.56)	2.2
18	No. 10 + Mn^{++} (0.37)	16.4
19	No. 10 + Mn^{++} (28)	3.4
20	No. 10 + Co^{++} (28)	0
21	No. 10 + Be^{++} (4.2)	8.8
22	No. 12 + Zn^{++} (0.042)	1.2

The inhibitory effect of specific antiserum upon the ATP-ase and 5′-nucleotidase has been reported previously[1]. A correlation was sought between the immunological and the enzymic changes effected under certain conditions.

LAMB[20] reported that after mixing the venom and antiserum, precipitate formation continued for 18–24 h. No parallel decrease in enzyme activity was observed. The reaction, with consequent enzyme inhibition, probably occurs rapidly while "mechanical" coagulation may take longer with little further effect on enzyme activity. CINADER found a decrease of 1–5 % for lecithinase[21] under such conditions but not for ribonuclease[22].

The effect of increasing amounts of antiserum on enzyme activity is shown in Table VI. LAMB[20] found that precipitate formation was maximum at an antiserum/venom ratio of 500/1 (by wt.). The ATP-ase inhibition may parallel the precipitate formation but ratios above 100 were not tried.

TABLE VI

INHIBITION OF ATP-ASE AND 5′-NUCLEOTIDASE BY SPECIFIC ANTISERUM

Incubation at 30° in 0.041 *M* veronal buffer, pH 8.4; 0.14 % venom; 5.0 m*M* AMP or 2.6 m*M* ATP.

Antiserum/g venom (g)	*% Inhibition*	
	5′-nucleotidase	*ATP-ase*
0.25	35	—
0.5	74	—
1	86	20
2	93	—
4	95	49
8	97	—
16	98	—
100	99	79

From an antiserum–venom mixture (4:1 by wt.) left at 20° overnight at pH 8.4 the precipitate was removed by high-speed centrifugation. The supernatant contained 81 % of the ATP-ase and 15 % of the 5′-nucleotidase activity of the original mixture.

Assuming this to be uninhibited enzyme, the inhibition accompanying their incorporation in the precipitate is 96 % for 5'-nucleotidase and 70–75 % for ATP-ase (allowing for inhibition due to AMP accumulation[23]). However, CINADER[21] has reported a loss of activity upon centrifuging a mixture of lecithinase and antiserum, so the calculation may be invalid.

Both enzymes were inactivated by heating at 75° for 30 min. Shorter periods showed the 5'-nucleotidase to be the more heat-stable at pH 8.4. After 1 min it was 91.5 % inhibited—(ATP-ase, 99.6 %); after 5 min, 98.8 %; after 20 min, 99.5 %. Similar results were obtained at pH 7.0. Since LAMB[20] found that the non-heat coagulable proteins of the venom would form an undiminished amount of serological precipitate, it would seem that the two enzymes in question cannot make a large contribution to the precipitate volume.

Hydrolysis of inosinephosphates

The action of the venom on ITP (and IDP) was similar to that on ATP (and ADP). In veronal buffer at pH 8.4, the ITP-ase activity rose above that of the 5'-nucleotidase so that IMP accumulated during the breakdown of ITP. Specific immune serum (× 2 wt. of venom) inhibited these activities to about the same extent as those of the adenosine phosphatases, the 5'-nucleotidase "activity" being reduced to 0.09.

Hydrolysis of pyridine nucleotides

DPN breakdown at the *pyro*-P bridge by snake venoms has been reported[24,25] and has been confirmed for cobra venom by BHATTACHARYA[26] and the present work (Table VII). The NMN produced is hydrolysed at 1.8 % of the rate for AMP which is comparable with the results of HEPPEL AND HILMOE[3] for other snake venoms. Antiserum (× 4 wt. of venom) decreased the DPN-ase activity by 60 %. Part of this inhibition was due to AMP accumulation[23]. (*cf.* ATP-ase). Low EDTA concentrations had little effect upon the DPN-ase activity of dialysed venom but 420 μM EDTA

TABLE VII

HYDROLYSIS OF DPN BY COBRA VENOM

Incubation at 30° in 0.016 *M* veronal buffer, pH 8.4. "Buffer-dialysed" venom, 0.11 %.

Substance	*Concentrations (mM) after various times*			
	0 min	*5 min*	*10 min*	*15 min*
DPN	4.51	3.39	2.71	2.11
(AMP + NMN)*	0.45	2.16	2.86	3.37
NMN**	0	1.10	1.85	2.38
AMP (calc.)	0.45	1.06	1.01	0.99
Ortho-P	0.41	1.01	1.64	2.29
Adenosine	1.40	1.93	2.75	2.98

* From phosphate analysis.
** From nicotinamide analysis.

caused 93 % inhibition. Mg up to 5.6 m*M* did not reverse this inhibition but 280 m*M* Mg effected 100 % enhancement. (*cf.* ATP-ase). The pH optimum of the crude-venom DPN-ase was in the region of pH 9.0 or above. (Fig. 1). For the purified enzyme from

Maine potatoes, KORNBERG AND PRICER[27] found a broad optimum at pH 6.5–8.5 with 50 % reduction at pH 9.0.

The ATP-ase and DPN-ase are inhibited by the other's substrate and there is no summation of rates of hydrolysis. However, the kinetics preclude any possibility of a single enzyme acting on both substrates. With ADP the inhibition is not mutual, ATP-ase only being affected. There is no summation of rates and the kinetics suggest a single enzyme. However, supporting evidence is required especially in view of the discovery of specific nucleoside-5′-diphosphatases[28,29].

Hydrolysis of coenzyme A

Incubation for 18 h (veronal buffer, pH 8.4) with cobra venom effected an almost complete degradation of coenzyme A (as measured microbiologically[30] by Dr. W. S. PIERPOINT) and chromatography showed that the coenzyme had been replaced by two spots, R_F values 0.024 and 0.063. Pantotheine-4-phosphate corresponded with the lower one. *Ortho*-P and adenosine appearing were accounted for by the "AMP" impurity which disappeared after incubation. The upper spot, therefore, which contained twice as much phosphorus as the lower, was probably adenosine-3′,5′-diphosphate suggesting that cobra venom hydrolyses coenzyme A at the *pyro*-P bond without subsequent liberation of *ortho*-P (*cf.* [31]).

No hydrolysis of TPP was apparent after 18 h incubation at pH 8.4.

Hydrolysis at pH 8.4 of FMN and R-5-P was slow ("activities", 0.013 and 0.031 respectively) (*cf.* [3]). The overall effect of EDTA (0.42 m*M*) plus Mg^{++} (5.6 m*M*) on the "buffer-dialysed" venom was 44 % inhibition of FMN hydrolysis and 140 % enhancement of R-5-P breakdown tending to confirm the suggestion[3] that different enzymes are involved.

DISCUSSION

The pH optima found for 5′-nucleotidase fall roughly into two categories: (a) values around 7.0, *viz.* for dialysed extracts[2,32] of nerve tissue (7–7.5), human sperm (6.3), and other human tissues (7.0); (b) values of 8.5–8.9 for the partially purified enzyme of certain snake venoms[9] and bull seminal plasma[3] and for whole bull seminal plasma[23]. The value found for the cobra venom enzyme falls easily into the former category. Sources in this category only contain significant amounts of non-specific monophosphatases. The snake venoms studied by GULLAND AND JACKSON[9] for 5′-nucleotidase were selected for their lack of non-specific enzyme, and in bull seminal plasma the non-specific phosphatase activity is not more than 0.4 % of that of 5′-nucleotidase. The significance, if any, is unknown.

Enhancement of the 5′-nucleotidase activity after dialysis of the venom against veronal buffer would seem to be due to removal of inhibitory zinc ions, since re-addition of these reduces the activity to its original value. Zinc inhibition of 5′-nucleotidase has been reported previously[33,34], and since it is observed at both pH 7.0 and 8.4 it is likely to be a true inhibitory effect upon the enzyme and not due to a shift of the pH optimum, as reported for acid and alkaline phosphatases[35]. The greater degree of enhancement obtained using more dilute buffer for dialysis is difficult to explain especially in view of the inhibition shown on dialysis in tris buffer. It cannot be due to an increased zinc removal, since re-addition does not effect a

corresponding inhibition. The greater inhibition resulting from dialysis against water at room temperature compared with that at 0° must be ascribed to a combined effect of temperature and ion-removal, since the undialysed enzyme is stable when stored at room temperature at similar pH values. That the enzyme activity is susceptible to ion concentration is shown by the different effects of dialysis against water and 0.9 % KCl at 0°. The partial precipitation of the enzyme and possible denaturation effects during water-dialysis may be partly responsible for this difference.

The influence of manganese on the 5′-nucleotidase of the dialysed venom is contrary to the finding for the bull-semen enzyme[3], where manganese could not replace magnesium, but is in accordance with the recent findings of REIS[34] for the aorta and placenta enzymes (compare also for swine kidney alkaline phosphatase[36]). The effect of cobaltous ion confirms ZELLAR's activation effect[37] and may accord also with REIS's inhibition effect at higher cobalt concentrations[34]. As neither manganese nor cobalt were found in the venom, magnesium remains as a possible naturally-occurring activator. This is supported by the ready reversal of EDTA-inactivation of the enzyme by magnesium ions. However, that this is effected less readily after a prolonged period remains to be explained. Two possibilities arise: (a) denaturation of the enzyme by the EDTA, which may occur without significant change in the mass : charge relationship, and (b) removal of a bound metal ion from the enzyme molecule. There is evidence that venom contains a moiety having bound magnesium since part is not liberated by trichloracetic acid extraction. Furthermore, magnesium was found to accompany buffer-dialysed venom through a buffered, Dowex-50 column under conditions which removed all ionic magnesium[23]. However, denaturation of 5′-nucleotidase by EDTA seems a possibility.

The need for a large excess of magnesium ion for reactivation of EDTA-inactivated ATP-ase and DPN-*pyro*phosphatase suggests that this process is more complex than can be accounted for by mere chelation of EDTA with magnesium. The increased effect of even the highest magnesium concentration after dialysis of the EDTA-venom mixture is difficult to explain unless one assumes a fairly strong combination between enzyme and EDTA. The enzyme may be considerably more sensitive to EDTA than is 5′-nucleotidase. A denaturation by EDTA may be reversible by these high magnesium concentrations. The nature of the magnesium, or other activating influence associated with these enzyme activities warrants further study.

The greater incorporation of the 5′-nucleotidase into the serological precipitate may not mean that it is a more powerful antigen than the ATP-ase but merely that there is more of it in the venom, effecting greater anti-enzyme production. Heat-inactivation is likely to have sufficiently drastic effects on enzyme configuration to make precipitate formation with the anti-enzyme unlikely and since LAMB[20] failed to detect a reduction of serological precipitate after heat-coagulable proteins were removed, it is unlikely that these two enzymes play a major role in its formation. BRAGANCA AND QUASTEL[38] ascribed the toxic principle of cobra venom to a heat-stable enzyme; a lecithinase. This activity would disrupt cell-wall and particulate structures and, alone, would probably be sufficient to be lethal. However, many of the vital cellular cofactors would thus become available to the venom enzymes and would be degraded, *e.g.* ATP, DPN, coenzyme A, uridine-diphosphate-glucuronic acid[39] and probably others. This latter effect may be subsidiary to that of the lecithinase and may not affect significantly the number of experimental animals that

are killed, in which case, heat-inactivation would appear not to alter the toxicity of the venom. The toxicity of snake venoms is now thought to be made up of several "activities", *e.g.* haemolytic, neurotoxic. The separation of these two by electrophoresis has been reported[40]. It would be of interest to know of their respective heat-stability, and enzymic properties. It was reported that the latter "toxin" is a small, dialysable molecule.

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