LXXVII. PHOSPHOESTERASES OF BONE AND SNAKE VENOMS

BY JOHN MASSON GULLAND AND
ELISABETH MAUDE JACKSON

From the Chemistry Department, University College, Nottingham

(Received 12 January 1938)

IN the course of experiments on the constitution of substances containing organically combined phosphorus in the form of phosphoric esters, it became necessary to obtain preparations of some of the different types of phosphoesterases [Kay, 1936] free from each other. Although many investigators have described the phosphoesterases obtained from a large and varied number of animal and vegetable tissues, it is believed that the present communication records hitherto untried sources which should be of value in this field.

SECTION A

ALKALINE PHOSPHOMONOESTERASE

One of the most readily accessible sources of the "alkaline" type (Class A 1, liberating phosphoric acid from phenylphosphate, glycerophosphate, nucleotides [Kay, 1936]) of phosphomonoesterase is bone, and Martland & Robison [1929] have described a simple method of isolating the enzyme from the leg bones of young rabbits. It is, however, frequently difficult or costly to obtain such animals, and it is now shown that the metatarsal bones of sheep forelegs, which are slaughter-house offal, are suitable material for large-scale preparations. The material obtained from these bones by the Martland-Robison method contains both phosphomonoesterase and phosphodiesterase, and these may be separated by adsorption on norite charcoal, which adsorbs both enzymes. Elution with borate buffer at pH 8.6 liberates the phosphomonoesterase, but leaves the phosphodiesterase on the norite, from which we have as yet been unable to elute it. The same procedure has been used to purify the phosphomonoesterase of guinea-pig femurs. Arising out of these observations, a method is now described for the preparation of phosphomonoesterase from beef or sheep bones and the simultaneous elimination of phosphodiesterase by direct adsorption of the enzymes on norite charcoal from the crude autolysate without the alcohol-ether precipitation of the Martland-Robison method, which is costly and troublesome if carried out on a large scale.

EXPERIMENTAL

Estimations of phosphate and phenol. All estimations of inorganic phosphate in this paper were made by Briggs's colorimetric method, and of phenol by the method of Folin & Denis [1915]. Every experiment was accompanied by a control without enzyme which was negative as regards liberation of phosphate or phenol respectively.

Preparation. The metatarsal bones of sheep forelegs or of guinea-pig femurs, obtained as soon as possible after death, were skinned, scraped free from tissue, sawn lengthwise or split, freed from marrow, crushed and allowed to autolyse

for 7–10 days in five times their weight of water containing chloroform. Methcol (2 vol.) and ether (3 vol.) were added to the filtered extract, and the resulting precipitate (A) was centrifuged and dried over phosphoric oxide. Less successful methods of precipitating active material were (i) adjustment of the reaction to pH 4.5, and (ii) adjustment to pH 4.5 followed by saturation with ammonium sulphate.

Table I records the activities of precipitate A from sheep bones: similar results were obtained with guinea-pig bones.

Table I

(a) Sodium β -glycerophosphate 3.8 mg.; precipitate A 20 mg.; pH 8.6 Clark and Lubs borate buffer 5 ml.; $M/10$ magnesium sulphate 0.5 ml. Diluted to 10 ml. with water. pH 8.6. 37°. 2 ml. samples.

(b) Yeast adenylic acid, or muscle adenylic acid, 5.1 mg., in dilute sodium carbonate, or sodium diphenylphosphate 6.0 mg.; precipitate A 30 mg.; pH 8.6 borate buffer 5 ml.; $M/10$ magnesium sulphate 0.5 ml. Diluted to 10 ml. with water. pH 8.6. 2 ml. samples.

Time hr.	Glycero- phosphate hydrol. %	Yeast adenylic hydrol. %	Muscle adenylic hydrol. %	Diphenyl- phosphate hydrol. %
0	0	0	0	0
2	49	44	49	15
4	—	60	76	—
7	49	—	—	21
8	—	73	77	—
16	—	—	—	23

Dialysis of precipitate A. An aqueous suspension was dialysed through No. 400 cellophane against distilled water until free from inorganic phosphate. The enzyme was then precipitated by addition of methcol-ether (2 : 3), centrifuged and dried over phosphoric oxide. This treatment resulted in a considerable increase in hydrolytic activity as shown by Table II.

Table II

Yeast adenylic acid or muscle adenylic acid 5.6 mg.; dialysed enzyme preparation 30 mg.; pH 8.6 borate buffer 5 ml.; $M/10$ magnesium sulphate 0.5 ml. Diluted to 10 ml. with water, pH 8.6. 37°. 2 ml. samples.

Time hr.	Yeast adenylic hydrol. %	Muscle adenylic hydrol. %
0	—	—
2	78	78
4	110	—
6	110	106

*Purification by adsorption of monoesterase in precipitate A
from sheep or guinea-pig bones*

The norite charcoal used in these experiments had been extracted successively with the following hot solvents—20 % HCl, alcohol and glacial acetic acid—being washed thoroughly with much distilled water between each extraction, and had then been dried at 110°. A mixture of charcoal (800 mg.) and a finely ground suspension of precipitate A (300 mg.) in water (10 ml.) was shaken occasionally for 1 hr. at 0°, and the charcoal was collected, washed with water and eluted overnight with pH 8.6 borate buffer (12 ml.). This solution contained phosphomonoesterase, but no phosphodiesterase, as shown in the

case of sheep bone preparations in Table III. It showed no differentiation in the rates of hydrolysis of purine and pyrimidine nucleotides or of adenosine-3- and 5-phosphates. Similar results were obtained with guinea-pig bone preparations.

Table III

(a) Sodium β -glycerophosphate 3.76 mg. or sodium diphenylphosphate 5 mg.; enzyme solution 2 ml.; pH 8.6 borate buffer 5 ml.; $M/10$ magnesium sulphate 0.5 ml. Diluted to 10 ml. with water. pH 8.6. 37°. 2 ml. samples.

(b) Yeast adenylic acid, muscle adenylic acid or cytidylic acid, 14.5 mg. in dilute sodium carbonate; enzyme solution 2 ml.; pH 8.6 borate buffer 10 ml.; $M/10$ magnesium sulphate 1.0 ml. Diluted to 20 ml. with water. pH 8.6. 37°. 2 ml. samples.

Time hr.	Glycero- phosphate hydrol. %	Diphenyl- phosphate hydrol. %	Yeast adenylic hydrol. %	Muscle adenylic hydrol. %	Cytidylic hydrol. %
0	0	0	0	0	0
2	41	0	14	10	10
4	57	0	25	25	13
6	—	0	37	29	23
24	62	0	64	64	60

The pH optimum of the purified phosphomonoesterase of sheep bone, using β -glycerophosphate as substrate, was at pH 8.8 (Fig. 1).

Fig. 1. Sodium β -glycerophosphate 3.03 mg. in water 2 ml.; enzyme solution 2 ml.; borate or veronal buffer 5 ml.; $M/10$ magnesium sulphate 0.5 ml. Diluted to 10 ml. with water. 3 hr. 37°. 4 ml. samples.

Attempts to obtain the phosphodiesterase of sheep bone. The following procedures were unsuccessful:

(i) Preservation of suspension of precipitate A at pH 4.5 for 2 days in the hope of inactivating the monoesterase. No appreciable inactivation of either enzyme occurred.

(ii) After the monoesterase had been eluted from the charcoal, elution with dilute acetic acid yielded a solution which had no diesterase activity towards diphenylphosphate at pH 8.6.

(iii) After elution of the monoesterase, elution with pH 7 phosphate buffer or $M/5$ potassium dihydrogen phosphate, followed by dialysis to remove phosphate, failed to liberate diesterase.

Simultaneous preparation and purification of bone phosphomonoesterase by adsorption without alcohol-ether precipitation

Beef leg-bones, not necessarily obtained immediately after the death of the animal, were freed from marrow, sawn and hashed in pieces of about the size of a walnut or smaller, and autolysed for 7 days with four times their weight of water containing chloroform. The phosphoesterases in this autolysate (400 ml.) were adsorbed on purified norite charcoal (3 g.) for 1–1.5 hr. at 0°, and the charcoal was collected, washed with water and eluted with pH 8.6 borate buffer (20 ml.).

A further adsorption of the filtrate (200 ml.) from the first adsorption was carried out by means of norite (1 g.) for 1 hr. at 0°, followed by washing with water and elution of the norite with pH 8.6 borate buffer (10 ml.).

The above elutions contained monoesterase but not diesterase, and analogous experiments with the autolysates from sheep metatarsals had similar results (Table IV). The procedure described above has the additional advantage that inorganic phosphate is automatically removed from the enzyme preparation, since it remains in the filtrates.

Table IV

(a) Sodium β -glycerophosphate 3.62 mg.; enzyme solution from 1st or 2nd adsorption 4 ml.; pH 8.6 borate buffer 5 ml.; $M/10$ magnesium sulphate solution 0.5 ml. Diluted to 12 ml. with water. 2 ml. samples.

(b) Sodium diphenylphosphate 6.7 mg.; enzyme solution 4 ml.; pH 8.6 borate buffer 5 ml.; $M/10$ magnesium sulphate 0.5 ml. Diluted to 20 ml. with water. 37°. 4 ml. samples.

Time hr. from zero	Beef		Glycero- phosphate hydrol. %	Sheep	
	1st adsorption			1st adsorption	
	Glycero- phosphate hydrol. %	Diphenyl- phosphate hydrol. %		Glycero- phosphate hydrol. %	Diphenyl- phosphate hydrol. %
4	70	0	32	40	0
24	72	0	—	67	0

SECTION B

PHOSPHODIESTERASES

Many animal and vegetable tissues contain both phosphomonoesterases and phosphodiesterases (liberating phenol, but not phosphoric acid, from diphenylphosphate), but despite the efforts of various investigators there is no recorded instance in which a diesterase preparation, free from monoesterase, has been obtained from such sources by removal of the monoesterase. In the present work, for example, all attempts failed to obtain the diesterase of sheep bone from the charcoal adsorbate of this enzyme and phosphomonoesterase. These results find parallels in the lack of success of Uzawa [1932] in eluting from adsorbents the diesterases of a variety of animal and vegetable sources. The same lack of success is apparent if one assumes—as seems to be justified—that the polynucleotidases, which split nucleic acids into their constituent nucleotides, are wholly or in part diesterases. Thus, for example, Levene & Dillon [1932] and Makino [1934] obtained from intestinal mucosa preparations of mixed polynucleotidase and nucleotidase, the latter enzyme being a phosphomonoesterase in the terms of this classification. Levene & Dillon were unable to separate these enzymes, but Makino was apparently able to use the mixture to prepare both nucleotides and nucleosides.

The nearest approach to the production of an enzyme preparation having diesterase, but no monoesterase, activity is to be found in the work of Klein [1933], who obtained from calf's intestinal mucosa a mixture of thymonucleinase, a polynucleotidase specific for thymus nucleic acid, and of a phosphomonoesterase which eliminated phosphoric acid from the resulting nucleotides. He was able to suppress the action of the phosphomonoesterase by means of sodium arsenate without interfering with the activity of the thymonucleinase.

Uzawa [1932], however, found in the venom of a Japanese snake known as the Habu (*Trimeresurus flavoviridis*) a diesterase entirely free from monoesterase, and he also stated that the venom of the Mamushi (*Agkistrodon blomhoffi*) also contains phosphodiesterase, without referring to the presence, or absence, of monoesterase. Klein & Rossi [1935], on the other hand, found both diesterase and monoesterase in a specimen of Habu venom obtained from Japan, and were moreover unable to separate these enzymes.

Table V

Monophenylphosphate or diphenylphosphate, 13 mg., in dilute sodium carbonate; venom 20 mg., pH 8.6 borate buffer 10 ml. Diluted to 20 ml. with water and incubated at 37°. 2 ml. samples. In the case of monoesterase the degree of hydrolysis was calculated from estimations of inorganic phosphate, in the case of diesterase from estimations of phenol. When a monoesterase accompanied the diesterase, theory demanded the liberation of two phenol molecules from one of diphenylphosphate, but when no monoesterase was present only one molecule of phenol should be liberated. The figures for percentage hydrolysis are calculated on this basis.

Snake	Habitat and Family	Venom collected	Monophenyl-phosphate		Diphenyl-phosphate	
			Time hr.	Hydrol. % of theory	Time hr.	Hydrol. % of theory
<i>Notechis scutatus</i> Tiger snake	Australia Colubridae	1931-34 mixed	6	9.5	24	9.1
<i>Bungarus fasciatus</i> Banded krait	South-east Asia Colubridae	May 1937	24	0	24	41.6
<i>Naja haje</i> Egyptian cobra	North and Central Africa Colubridae	Jan. 1936	6	59.0	24	29.0
<i>Naja Naja</i> Black spitting var. of Indian cobra	Malaya Colubridae	1936	3	64.0	24	33.0
<i>Sepedon haemachata</i> Ringhals	South Africa Colubridae	Oct. 1935	6	22.9	5	21.6
<i>Vipera russellii</i> Russell's viper	India, etc. Viperidae	Aug. 1937	24	0	24	41.8
<i>Bitis arietans</i> Puff-adder	South Africa Viperidae	Nov. 1936	24	58.0	24	14.9
<i>Agkistrodon piscivorus</i> Water moccasin	Southern U.S.A. Crotalidae	May 1937	24	0	24	20.4
<i>Agkistrodon mokasin</i> Copperhead	Eastern U.S.A. Crotalidae	1935	24	0	24	13.9
<i>Trimeresurus Wagleri</i> Wagler's pit viper	Malaya Crotalidae	May 1935	2	64.3	24	17.5
<i>Crotalus oreganus</i> Oregon rattlesnake	Western U.S.A. Crotalidae	May 1935	24	0	24	24.6
<i>Crotalus adamanteus</i> Diamond rattlesnake	Southern U.S.A. Crotalidae	July 1936	24	0	24	45.0

These discrepancies, the need of finding a comparatively accessible source of monoesterase-free diesterase, and a desire to enquire whether the enzyme content varies with the snake family, prompted us to examine the venoms of snakes as regards their "alkaline" monoesterase and their diesterase activities, and we have been able to do this through the generosity of Dr Burgess Barnett, to whom we acknowledge with pleasure our indebtedness and gratitude for advice and for supplying the venoms. The venoms were extracted from living snakes and were dried *in vacuo* at 35° immediately after extraction. They were then hermetically sealed and stored in the dark. The results are tabulated in Table V.

The substrates examined were monophenylphosphate (monoesterase) and diphenylphosphate (diesterase); the actions of the venoms on other simple substrates such as α - and β -glycerophosphates have not yet been studied. It was necessary to use the same substrates throughout so as to obtain comparative results, and this restriction was also enforced because the amount of venom available in several cases was small and the material difficult to procure.

Several venoms contained diesterase but not monoesterase, whereas none contained monoesterase but no diesterase. In connexion with the work of Uzawa and of Klein & Rossi, mentioned above, it may be observed that *Trimersurus wagleri* venom contained both mono- and di-esterases and that the venoms of *Agkistrodon piscivorus* and *A. mokasin* contained diesterase but not monoesterase.

Apparently the snake family—Crotalidae, Colubridae, Viperidae—is not a deciding factor in determining which of the phosphoesterases are present in the venom.

The pH-activity curve was determined for the diesterase in the venom of Russell's viper (Fig. 2).

Fig. 2. Sodium diphenylphosphate 3.08 mg. in water 2 ml.; Russell's viper venom 7 mg.; borate or veronal buffer 5 ml.; diluted to 10 ml. with water and incubated at 37° for 3 hr. 4 ml. samples.

SUMMARY

1. The phosphomonoesterase (optimum pH 8.8, sheep) and the phosphodiesterases of sheep, beef and guinea-pig bones are adsorbed by norite charcoal from aqueous solution. Since the diesterase is not eluted, the monoesterase, which can be eluted, is purified from diesterase and inorganic phosphate.

2. Such adsorptions may be carried out with the solution of an enzyme preparation obtained by the customary precipitation with alcohol and ether, or

may be used with a crude bone autolysate, thus obviating this precipitation completely.

3. Of the venoms of twelve different snakes of the viperidae, crotalidae and colubridae, seven contained both phosphomonoesterase and phosphodiesterase, whereas five contained phosphodiesterase only, optimum at pH 8.3 in the case of Russell's viper venom.

4. Apparently the snake family is not a deciding factor in determining which phosphoesterases are present in the venom.

We wish to express our gratitude to the Government Grant Committee of the Royal Society for a research grant.

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ERRATA

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page 152, Table IIB. *Insert headings to columns as follows:*

mg. ascorbic acid which should be oxidised provided the
ratio be 1:1 (col. 5), 1:2 (col. 6)

Align col. 7 with col. 8 above.

page 180, line 13 *for* second theory, mentioned in the introduction
read the Warburg-Wieland theory

line 25 *for* 527 $m\mu$ *read* 555 $m\mu$

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page 596, line 13 *for* Kay *read* Folley and Kay, etc.