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ELECTROPHORETIC, ENZYME, AND PRELIMINARY TOXICITY STUDIES OF THE VENOM OF THE SMALL-SCALED SNAKE, *PARADEMANSIA MICROLEPIDOTA* (SERPENTES: ELAPIDAE), WITH ADDITIONAL DATA ON ITS DISTRIBUTION

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ABSTRACT

Parademansia microlepidota, a species recently resurrected from the synonomy of *Oxyuranus scutellatus*, has been collected live for the first time. The species occurs only in the Lake Eyre Drainage Basin and is confined to 'ashy downs' areas of the channel systems of Cooper Creek and the Diamantina River.

Electrophoretic and enzyme studies of its venom confirm that *P. microlepidota* is a distinct species. Two directional polyacrylamide gel electrophoresis patterns from *P. microlepidota* venom are clearly distinct from those of *Oxyuranus scutellatus*, *Pseudonaja textilis*, *Pseudechis australis*, and *Notechis scutatus*. The venom of *P. microlepidota* contains more hyaluronidase activity than venoms of other Australian Elapids examined.

Toxicity studies suggest that the venom of *P. microlepidota* is the most toxic terrestial snake venom known.

arademansia microlepidota (McCoy) (the all-scaled or 'Fierce' Snake) was described in 1879 from two specimens collected in north-western Victoria. A third specimen from 'Fort Bourke' (30°02'S, 145°49'E) was described in error by Macleay (1882) as a new species, *Diemenia ferox*. This specimen has since been lost, and only the two type specimens were known. *P. microlepidota* was recognised as a distinct but apparently 'lost' species for many years (Kinghorn 1923, 1929, 1955, 1956); it resembles *Oxyuranus scutellatus* (Peters), the Taipan, superficially and Worrell (1963a, b) treated *P. microlepidota* and *O. scutellatus* as conspecific. After freshly preserved specimens of *P. microlepidota* were donated to the Queensland Museum from southwestern Queensland, and additional specimens were collected by J. Wombey of the C.S.I.R.O., Darwin from mid-western Queensland, a search of state museum reference collections revealed more specimens, all in the South Australian Museum. Examination of all these specimens (fourteen) and the types of *Diemenia microlepidota* enabled confirmation of the specific status of *P. microlepidota* (Covacevich and Wombey 1976).

A serious case of snakebite in southwestern Queensland was attributed to *O. scutellatus* (Trinca 1969) but when the specimen concerned was examined and compared with both *O. scutellatus* and *P. microlepidota* (Covacevich and Wombey 1976) it proved to be *P. microlepidota*. At the time of the bite the specimen was regarded as one of the Brown Snakes, *Pseudonaja* spp., and *Pseudonaja textilis* antivenene was administered, but the patient's survival was attributed to readily available intensive medical care rather than a satisfactory response to *Pseudonaja textilis* antivenene.

P. microlepidota and *O. scutellatus* are both very large Elapids and, as such, are potentially dangerous to humans. The latter has been responsible for many fatalities and *P. mi-*

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(1) How so? If nobody else has examined it then how can they be sure of an error??
(2) Where did Wombey get them??

crolepidota has been responsible for one known near fatality, despite the fact that it occurs in only sparsely settled areas. The largest *P. microlepidota* encountered to date measures 2·0 m and most preserved museum specimens and live specimens collected recently measure between 1·5 m and 1·8 m. *O. scutellatus* is alleged to attain a length of close to 3·5 m. The largest specimen in the Queensland Museum reference collection (J26935) measures 2·5 m and specimens of 1·5 m to 2·0 m are commonly encountered.

Live specimens of *P. microlepidota* were collected in late 1975 by two of us (C.T. and J.C.) and have been maintained successfully in captivity since. One specimen is illustrated in Plate 1. Collection of live specimens has permitted examination of the venom of *P. microlepidota* for the first time. Kinghorn (1956) regarded this species as 'deadly venomous' presumably because of its size and apparent resemblance to *O. scutellatus*, a known dangerous snake.

P. microlepidota is apparently confined to the Lake Eyre Drainage Basin of far southwestern Queensland and northeastern South Australia. No specimens have been collected from Victoria or New South Wales since the types of *P. microlepidota* and its synonym *D. ferox* were described late in the nineteenth century. It seems reasonable to assume therefore that the species no longer occurs in this area or, more likely in the light of recent field work, that it has never occurred there and that the museum records may be erroneous. The broad occurrence of *P. microlepidota* (and of its nearest relative *O. scutellatus)* has been described and mapped by Covacevich and Wombey 1976. The species is known from 120 km southeast of Boulia, western Queensland to just south of the Queensland-South Australia border and seems to be confined to the channel systems of Cooper Creek and the Diamantina River. In this area *P. microlepidota* favours the soft loams of the 'ashy downs' which are also favoured in the southwest by *Rattus villosissimus* (the Plague Rat). Populations of this latter species fluctuate dramatically (Covacevich and Easton 1974) and, when present in large numbers, the rats live in extensive interconnected burrow systems which are utilized by *P. microlepidota* as shelters and which also house their prime food source. When rat populations are very low, even appearing absent altogether, the burrows remain, and present evidence suggests that *P. microlepidota* continues to utilize the burrows but virtually stops feeding. The 'ashy downs' also crack extensively during dry periods and this provides additional shelter, enabling *P. microlepidota* to survive high temperatures apparently without food or water for long periods. The species does not occur continuously throughout its range but is found in isolated 'pockets', a characteristic shared with *O. scutellatus*. A population of *P. microlepidota* in the Windorah area is being studied by two of us (C.T. and J.C.) in an effort to obtain data on the biology of this highly specialised snake.

P. microlepidota has variously been referred to *Diemenia*, the genus which included the Brown Snakes (now *Pseudonaja* spp.); *Pseudechis* (the Black Snakes) and *Oxyuranus scutellatus* (the Taipan). It is easily distinguished from *Pseudonaja* spp. and *Pseudechis* spp. by external features such as head shape, head and body scales, and colour but is externally similar to *O. scutellatus*. The presence of 23 midbody scales, an unusually high count for Australian elapid snakes, in both *P. microlepidota* and *O. scuttellatus*, and other similarities, have apparently led to some of the confusion between these two species. The external and skull differences between *P. microlepidota* and *O. scutellatus* are summarised by Covacevich and Wombey (1976). Detailed analysis of the generic relationships between *P. microlepidota, O. scutellatus, Pseudonaja* spp., and *Pseudechis* spp. has not been completed but external and skull features suggest that *Parademansia* most resembles *Oxyuranus*, shares some characteristics with *Pseudonaja* spp., and least resembles *Pseudechis* spp. Examination of the venom of *Parademansia, Oxyuranus scutellatus, Pseudonaja* spp., and *Pseudechis australis* (the Mulga Snake) confirms this preliminary observation.

THE VENOM OF *P. MICROLEPIDOTA*

A two directional polyacrylamide gel electrophoresis (P.A.G.E.) system for characterising venom was developed by two of us (A.B. and S.S.) as an aid to the detection and identification of snake venom in clothing and biological fluids. The method was applied, in fact, only as an analytical procedure following the development of a more sensitive radioimmunoassay by Coulter et al (1974). It was considered that this improved P.A.G.E. system together with selected enzyme and toxicity studies would allow critical comparison of elapid venoms.

MATERIALS AND METHODS

VENOM COLLECTION

All snakes were housed in laboratory cages, one snake to a cage, and fed on freshly killed mice and day-old chickens. Individual snakes refusing dead food were fed minced whole animals through a catheter. The *Parademansia* were fed on mice only. The snakes, with one exception, were in excellent condition when the programme was started and remained so. The exception (No. 16) had been in captivity for some time and was very thin. This snake, the largest in the group, gave low venom yields.

The venom used for studies at the Commonwealth Serum Laboratories and for assessing yields was collected over a period of three months. Venom samples were obtained by inducing the snakes to bite a tightly stretched latex membrane over a glass container. The venom from each snake was frozen immediately and later dried under an ultimate vacuum of 5×10^{-3} torr, weighed, and then stored under vacuum.

The pooled venom used for toxicity and cross-protection studies was mixed in the liquid state after extraction, and then processed as above.

TWO DIRECTIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS (P.A.G.E.)

The method used was based on that of Davis (1964) and Kochwa (1964), with modifications so that venom components can move to either anode or cathode according to the net charge of the individual components. To achieve this a separation gel was polymerised both above and below the venom sample.

REAGENTS: The following special reagents were required for the P.A.G.E.: Acrylamide, N,N'-methylenebisacrylamide (BIS), Riboflavin, Tris (hydroxymethyl) methylamine (TRIS), and Bromophenol blue from British Drug Houses Ltd.; Xylene Brilliant Cyanin G (Michrome No. 1224) from Edward Gurr Ltd.; N,N,N',N',-tetramethylethylene-diamine (TEMED) from Eastman Organic Chemicals; and 'Photo-flo 200' from Kodak.

The following stock solutions were prepared:

(a) 0·28% Ammonium persulphate (prepared weekly).
(b) 3·02 M TRIS pH 8·9, 0·46% TEMED. The pH was adjusted with HCl.
(c) 60% acrylamide. Before use the acrylamide was recrystallized from chloroform.
(d) 1·2% BIS. Before use the BIS was recrystallized from acetone.
(e) 0·988 M TRIS pH 6·7, 0·92% TEMED. The pH was adjusted with HCl.
(f) 3·6% BIS.
(g) 8 mg/ml Riboflavin. Insoluble material was removed by filtration with Whatman No. 41 filter paper.
(h) 64% sucrose.
(i) The electrode buffer consisted of 0·6 g/litre TRIS and 2·88 g/litre glycine.

PROCEDURE: Twelve clean glass tubes 140 mm long x 5 mm were rinsed in 1/200 dilution of 'Photo-flo 200' and without drying were set vertically in a stand with the lower ends closed. The lower small pore separation (anodal) gel (7%) was formed by mixing the following stock solutions at room temperature:
(a) 4 ml: (b) 2 ml: (c) 1·87 ml: (d) 2·45 ml: (H_2O) 5·68 ml

The tubes were filled to a height of 65 mm with this gel solution. Water was carefully layered on top of each 'liquid' gel to ensure a horizontal gel surface. Polymerization of the gel took approximately 20 min. The large pore spacer gel was made by mixing the following volumes of stock solutions:
(e) 0·5 ml: (c) 0·5 ml: (f) 2 ml: (g) 0·5 ml: (h) 2·5 ml: (H_2O) 2 ml

Aliquots of 200 μ l were added to each tube and allowed to polymerize under a water layer which was then removed. The sample gel mixture was prepared as follows:
(e) 0·5 ml: (c) 0·5 ml: (f) 2 ml: (g) 0·5 ml: (h) 2·5 ml

For analysis of snake venoms, 200 μ g of venom was dissolved in 190 μ l of the sample gel mixture which was made up to 250 μ l total volume with water. A total of 200 μ l of this sample gel mixture was allowed to polymerize in the appropriate tube under water. The water was then removed and the upper cathodal small pore separation gel added to a height of 135 mm. This gel was of the same composition as the lower anodal gel.

Electrophoresis was carried out in 'LABQUIP' electrophoresis apparatus filled with the electrode buffer. Bromphenol blue (1 ml of 0·001% per 200 ml of buffer) was added to the upper or cathodal compartment of the apparatus as a marker. Tubes were electrophoresed at 3 ma/tube until the marker was 5 mm above the bottom of the tubes. Time of electrophoresis was 2·5 hours.

The gels are stained essentially by the method of Diezel et al (1972) but with the dye being prepared in 10% trichloracetic acid.

ASSAY OF HYALURONIDASE ACTIVITY

Hyaluronidase activity was determined by the method of Dorfmann (1955). Hyaluronic acid was obtained from Worthington Biochemicals, and the source of hyaluronidase was 'Rondase' as marketed by Evans Medical Ltd. Bovine Serum Albumin was purchased from Armour Laboratories. The results are expressed as units of hyaluronidase activity per mg venom.

TOXICITY STUDIES, LD_{50} DETERMINATION*

Stock venom solutions of 1 mg/ml were prepared in 0·85% saline in appropriate aliquots and stored at -20°C. Samples of these stock solutions were thawed out and diluted with 0·85% saline as necessary prior to each toxicity determination, the dilution interval was 1:1·25. C.S.L. white Swiss mice in the weight range of 17 to 20 g were used in the assay. Four mice were used at each level which was given subcutaneously (S.C.) in a volume of 0·2 ml. Results were read as mice dead or alive at 48 hours.

The LD_{50} of each venom was calculated using the method of Spearman-Karber as adapted by Baxter and Gallichio (1976).

The formula employed was as follows:

$$\log_{10} LD_{50} = {}^{x}100 \pm \frac{d}{n}\left[(\Sigma r) - \frac{n}{2}\right]$$

where LD_{50} is the 50% lethal dose end point; $^{x}100$ is the $\log_{10}$ dose giving 100% deaths at that dose and all higher doses; d is the $\log_{10}$ dose interval; n is the number of mice at each dose interval; and Σ r is the total number of mice dying between and including the 0% and 100% response levels to increasing doses of venom.

RESULTS

The venom yields obtained by regularly 'milking' individual specimens of *P. microlepidota* are shown in Table 1. The significance of these yields will be considered later in the light of the toxicity studies.

The P.A.G.E. patterns or 'finger prints' of a number of important elapid venoms are shown in Plate 2, fig. 1. The venoms studied were from a pool collected by milking many individual snakes. Venoms of different species have distinct patterns due to a variable number of components of differing mobilities and concentrations. The cathodal gel demonstrates the presence of positively charged proteins which further characterise the venoms. In the case of *N. scutatus* venom the cathodal moving proteins are known to be neurotoxins. Broad and Coulter at C.S.L. have also shown that the major cathodal band in the case of *P. textilis* venom is an important neurotoxin.

Plate 2, figs. 2 and 3 examine more closely the electrophoresed components of various *O. scutellatus* and *P. microlepidota* venom samples. Far greater uniformity can be seen amongst the individual single milkings of the latter species than in *O. scutellatus*. Because the sample B shown in Plate 2, fig. 2 was so different from other P.A.G.E. patterns of *O. scutellatus*, a further milking, sample H, was obtained from this particular specimen. As can be seen the same result was obtained. Toxicity studies on this particular venom showed it to be far less toxic than the other *O. scutellatus* venom samples.

Hyaluronidase activity of the venom of seven specimens of *P. microlepidota* ranging between 12·0 and 15·0 units/mg venom is shown in Table 1. For eight specimens of *O. scutellatus* it was considerably lower — 3·0, 3·0, 4·7, 5·0, 6·1, 8·2, 8·6, and 8·7. Hyaluronidase activity for large samples of pooled venom from *P. microlepidota* and *O. scutellatus* was 11·8 and 7·5 respectively.

The results of LD_{50} determinations on venom pools from the four most toxic Australian snakes were: 0·43 *(Parademansia microlepidota)*, 0·90 *(Pseudonaja textilis)*, 1·52 *(Oxyuranus scutellatus)*, and 3·82 *(Notechis scutatus)*. LD_{50} determinations for individual specimens of *P. microlepidota* are listed in Table 1 and confirm its extremely high toxicity in mice. Preliminary studies in guinea pigs suggest similar relative toxicities to those obtained in mice.

DISCUSSION

Two-directional electrophoresis of the principal Australian elapid venoms establishes their individual patterns or characteristic 'finger prints'. The innovation of displaying the cathodal moving components as well as those seeking the anode allows the comparison of the basic proteins which probably have neurotoxic activity.

Considerable uniformity is seen in P.A.G.E. patterns of the milkings from individual specimens of *P. microlepidota*. Initially we thought this was because the specimens were all collected from a limited area. It was not seen with individual milkings from specimens of *O. scutellatus* which come from diverse areas. Overseas experience has been quite different. Johnson et al (1967) found that milkings from individual specimens of

* LD_{50} is the quantity of venom which kills 50% of a group of a specified animal receiving that amount of venom by a specified route.

Crotalus atrox could be clearly distinguished from each other by the use of one directional P.A.G.E. Taborska (1971) discovered similar variations amongst individual venom milkings from *Echis carinatus*. More recently Glenn and Straight (1977) reported similar studies on the venom of *Crotalus viridis* concolor. Not only were the electrophoretic patterns of the venoms collected from the 'same area' considerably different but they varied in lethality.

Further work is proceeding upon individual milkings from other species of Australian snakes from geographically remote areas as well as those found in close proximity to each other. It will be interesting to see if other species show the remarkable electrophoretic homogeneity seen with *P. microlepidota*. If they do not, then some explanation will have to be found for the lack of intraspecific variation in *P. microlepidota*.

It was interesting to find a specimen of *O. scutellatus* which yielded on two separate occasions a venom with only one major component and greatly reduced *in vivo* toxicity. This venom is being studied further to determine the properties of this major component. The specimen has a normal healthy appearance and its venom output is normal.

A further distinction between *P. microlepidota* venom and venom from *O. scutellatus* is seen by the much higher level of hyaluronidase activity or spreading factor seen in the former. Such activity will accelerate the onset of systemic toxic effects of the venom by facilitating its movement in the tissue.

The studies of *P. microlepidota* venom toxicity in mice are very significant as they suggest this snake is potentially the most toxic terrestrial snake. The average yield of 44·2 mg is enough venom to kill over 50,000 mice. The maximum yield of 110 mg would kill 125,000 mice. *Oxyuranus scutellatus* with an average yield on milkings of 120 mg (Garnet 1969) could account for some 47,500 mice. The average quantity of venom collected from *Notechis scutatus* is 35 mg, which could kill some 4,500 mice.

Ophiophagus hannah (the King Cobra) yields more venom than any other terrestrial snake — an average of 420 mg (Canthavorn 1969), with a subcutaneous LD_{50} for 20 g mice of 34·5 μ g (Minton 1974). This quantity would kill some 6,000 mice. Brown (1973) considered *O. scutellatus* (the Taipan) was the most 'deadly' snake in the world, presumably because of the combination of its high venom yield (average 120 mg) and high venom toxicity (LD_{50} of 1·52 μ g). In the light of the information above and in Table 1 this position appears to be challenged by *P. microlepidota*.

Neutralization of Venom with Antivenene

We have found that *O. scutellatus* antivenene quite satisfactorily neutralized this venom *in vivo*. The Australian Polyvalent Antivenene made by C.S.L. is also quite satisfactory if the former antivenene is not available. *Pseudonaja textilis* or *Notechis scutatus* antivenenes are ineffective.

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TABLE 1: Yield, Hyaluronidase Activity, and Toxicity of Venom from Seven Specimens of *Parademansia microlepidota*

Specimen	Length (cm)	Sex	Venom yield* (grams dried)	Hyaluronidase activity+ (units/mg venom)	Toxicity× LD_{50} μg
7	155	F	0·03–0·04	13·2	0·31
13	160	F	0·03–0·06	12·8	0·35
14	165	M	0·02–0·04	13·0	0·61
5	170	M	0·02–0·03	15·0	0·35
3	178	F	0·04–0·07	13·0	0·50
11	180	M	0·06–0·11	14·7	0·39
16	193	M	0·02–0·03	12·0	0·28

* six milkings 16.11.1975 — 12.2.1976
\+ 11·8 for large venom pool
× 0·43 for large venom pool

Mr B. Campbell (Queensland Museum) constructively criticized the manuscript, and Mr A. Easton (Queensland Museum) took the photograph of the live specimen of *Parademansia microlepidota*.

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PLATE 1
Specimen of *Parademansia microlepidota* measuring approximately 170 cm, from southwestern Queensland

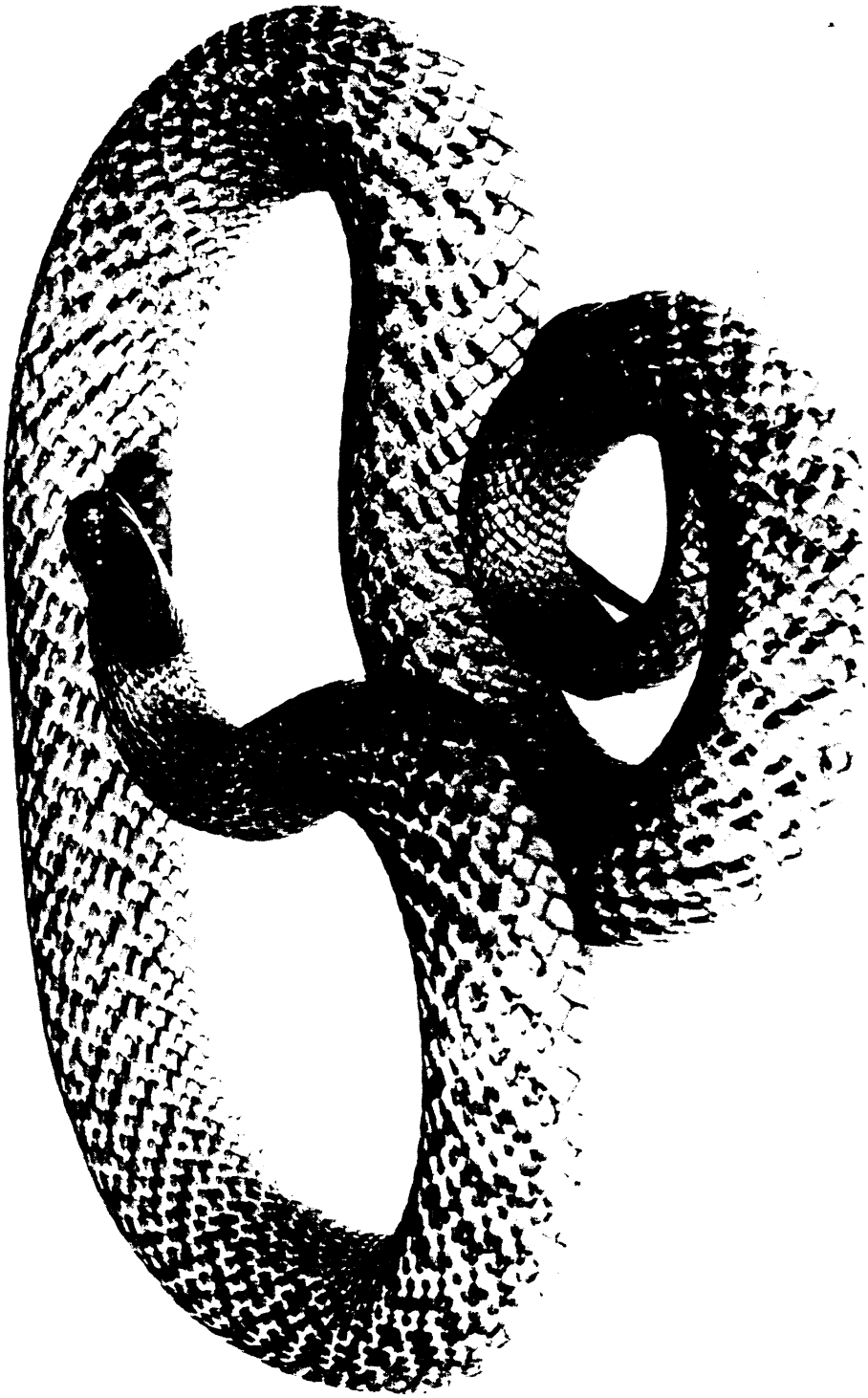


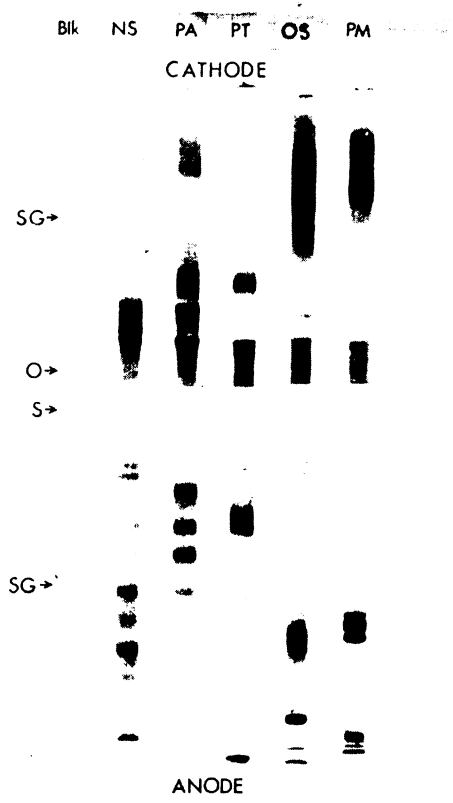
PLATE 2

FIG. 1. Two-directional 'P.A.G.E. patterns' of venom pools from five Australian elapid snakes.
Blk = Blank gel
NS = *Notechis scutatus*
PA = *Pseudechis australis*
PT = *Pseudonaja textilis*
OS = *Oxyuranus scutellatus*
PM = *Parademansia microlepidota*
O = *Application or sample gel*
S = *Spacer gel*
SG = *Separation gel*

FIG. 2. 'P.A.G.E. Patterns' of *Oxyuranus scutellatus*. Samples A to G are from individual snakes from different Queensland localities. Sample H was a requested second milking a month later from snake B. Sample OS is from a major venom pool. Blk is a blank gel.

FIG. 3. 'P.A.G.E. Patterns' of *Parademansia microlepidota*. The numbers indicate the snake (see Table 1) from which the single milking was collected. Sample PM was from a venom pool.

1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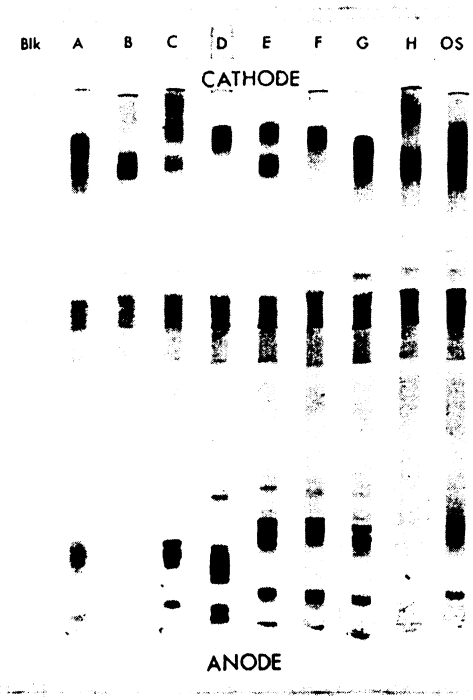
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