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THE TOXIC PROTEINS OF COBRA VENOM

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Abstract—Carboxymethyl cellulose chromatography permits separation of five distinct toxic proteins from the venom of the Indian cobra (*Naja naja*). These account for 90 per cent of the toxicity against mice. After gel filtration on Sephadex G-50 columns, three of the toxic fractions are more potent than the crude venom by factors of 6–9. They differ from one another in their sensitivities to heat and trypsin inactivation. Phospholipase does not appear to contribute significantly to the lethal effect. The two most abundant fractions, cobramines A and B, are less toxic than the crude venom and account for its cardiotoxic activity.

OF THE MANY proteins of cobra venom, several are lethal in a variety of laboratory animals. They fall into the general classes of neurotoxins and cardiotoxins.[1] One of these which has been extensively purified[2] is obtained from the Formosan cobra (*Naja naja atra*). It is called cobrotoxin and has seven times the toxicity of the crude material. Since it constitutes only 6 per cent of the crude venom protein, it can account for ~40 per cent of the total toxic effect of the whole venom. Although two to three toxic activities have been identified in the venom of *Naja naja* (Indian cobra) and *Hemachatus hemachates* (Ringhals cobra) by electrophoretic or chromatographic methods,[3,4] the potency of these preparations was considerably less than that of the crude venom.

During the isolation and purification of a membrane-active basic protein from *N. naja* venom,[5] at least five proteins which are lethal to mice have been separated. These account for more than 90 per cent of the toxic activity of the crude venom. Two of these, cobramines A and B, have previously been found to have marked effects on a number of transport systems, probably through effects on the cell membrane.[6,7] The other three proteins are present in lesser amounts, although they are considerably more toxic than the original venom or cobramines A and B.

METHODS AND MATERIALS

Chromatography. The initial fractionation of the lyophilized *N. naja* venom was carried out by using an ionic strength gradient elution on carboxymethyl cellulose as described previously.[5] Peak fractions were pooled and lyophilized. Gel filtration with Sephadex G-50 on a 1·5 × 56 cm column at 24° was performed after pre-equilibration with 0·05 M ammonium acetate buffer, pH 5·0. Five- to ten-mg aliquots of lyophilized fractions from the carboxymethyl cellulose column were applied in 0·1 ml of the ammonium acetate buffer and 1-ml fractions were collected at a flow rate of 20 ml/hr.

The protein content of column fractions was estimated by the absorption at 280 mμ in the Beckman DU spectrophotometer. The protein content of the pooled fractions

used in toxicity and enzyme assays was determined by the method of Lowry *et al.* with bovine albumin as standard.[8]

Assays for phospholipase A (EC 3.1.1.4), phosphodiesterase (EC 3.1.4.1), alkaline monophosphatase (EC 3.1.3.1) and I^- accumulation inhibition were performed as previously described.[5]

Paper electrophoresis was done for 22 hr in 0·15 M sodium phosphate buffer (pH 6·0) at 3 V/cm on Whatman No. 2 paper with 150–250 μg of protein per strip. Protein bands were identified with Amido Schwartz stain. After location of the components, protein was eluted over 12–24 hr in 0·154 M sodium chloride.

Toxicity was assayed by i.p. injection of serial 2-fold dilutions of protein (in 0·2 ml of 0·154 M NaCl) into groups of 6 adult male Swiss Albino mice 25–30 g. With fractions more toxic than the crude venom (fractions 5–8, 11), the LD_{50} was determined by injection of groups of 6 mice; the dose to each group was increased by increments of 1 μg protein until 50 per cent mortality was observed. The LD_{50} of these fractions was ascertained within 1 μg and was consistent from sample to sample. Mortality was assessed at 24 hr, although this rarely occurred later than 6–8 hr after injection.

Lyophilized *N. naja* venom was obtained from the Sigma Chemical Co.; carboxymethyl cellulose (CM-52 Whatman) from H. Reeve Angel & Co.; trypsin (2 × recrystallized) and lysozyme (4 × recrystallized) from the Worthington Biochemical Corp. All other chemicals were of reagent grade and triply distilled water was used throughout.

RESULTS

Fractionation on carboxymethyl cellulose

Crude *N. naja* venom was separated into 15 fractions by chromatography on carboxymethyl cellulose (Fig. 1). Three major areas show a significant increase in toxic potency: fractions 4–5, 6–8, and 11. Aliquots of the column eluate *between* these three areas had little toxicity per milligram of protein, suggesting that they were different toxins.

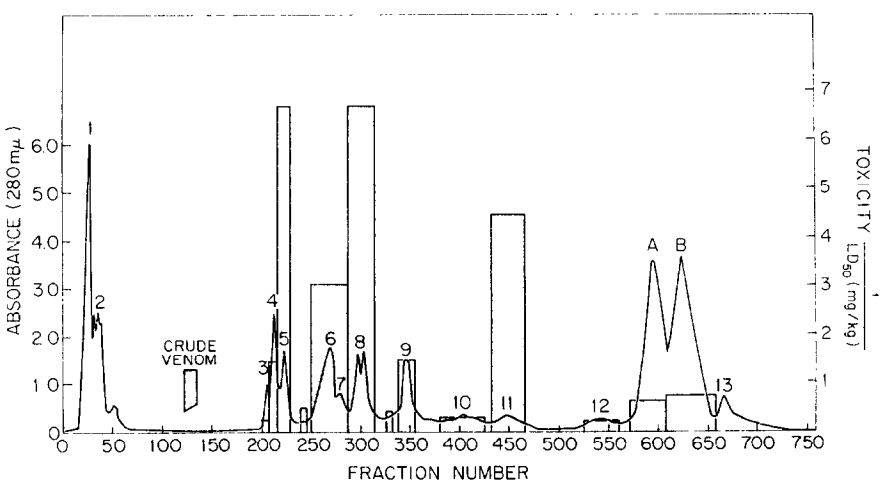
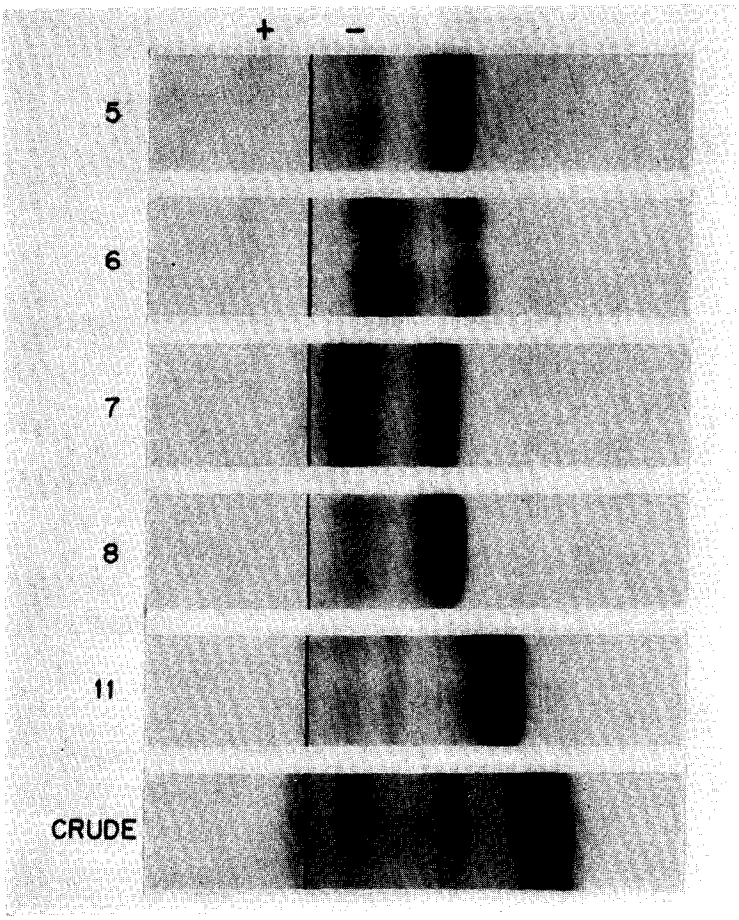


FIG. 1. Fractionation of *N. naja* venom on carboxymethyl cellulose. Protein was eluted with an ascending ionic strength gradient of ammonium acetate buffer, 0·005 to 0·5 M. The width of the bars showing toxicity indicates the fractions pooled for each peak. The crude venom toxicity is included for reference. Toxicity <10% of the crude venom was considered negligible.



i. 2. Paper electrophoresis of the carboxymethyl cellulose fractions (Whatman No. 2 paper, 3 V/cm, 22 hr; 0·15 M sodium phosphate buffer, pH 6·0).

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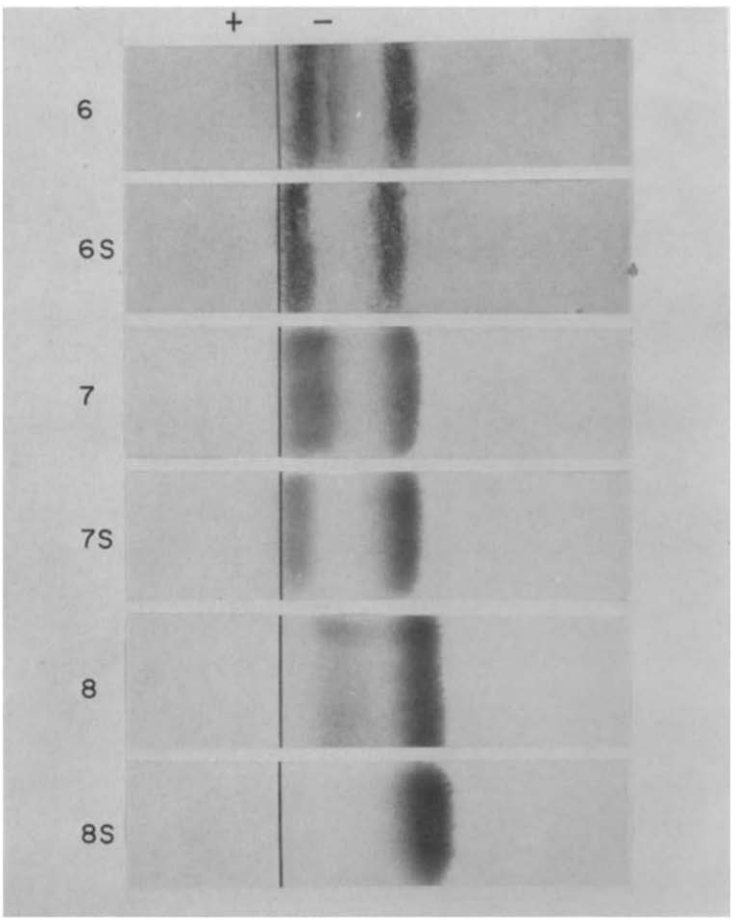

FIG. 5. Comparison of the electrophoretic patterns of fractions 6–8 before and after Sephadex chromatography. Electropherograms labeled with an S are the fractions after Sephadex treatment.

The relative amount of each fraction in the crude venom was determined by planimetry of the chromatogram (Table 1). Although cobramines A and B (A and B of Fig. 1) were incompletely separated, they were purified by subsequent rechromatography[5] on carboxymethyl cellulose and separate values for toxicity were obtained by using the purified proteins. Of the 7000 units of toxicity applied to the column (total area/LD_{50} of crude), 6670 units or 95 per cent were recovered in these fractions.

TABLE 1. TOXICITIES OF THE FRACTIONS OF *n. naja* VENOM OBTAINED BY CARBOXYMETHYL CELLULOSE CHROMATOGRAPHY

Fraction	Toxicity (LD_{50}, mg/kg)	Area of chromatogram (mm^2)	Toxicity per fraction (area/LD_{50})	Fraction recovered toxicity (%)
Crude	0·76	5310*	7000	
1	>7·6	630	0	0
2	>7·6	140	0	0
3	5·5	70	10	0
4	0·73	170	230	3
5	0·15	140	930	14
6	0·33	170	520	8
7	0·33	270	820	12
8	0·15	310	2170	32
9	0·69	200	290	4
10	3·6	140	40	1
11	0·22	40	180	3
12	4·5	60	10	0
13	1·6	220	140	2
Combramine A & B		2200	1400	21
Combramine A	1·6			
Cobramine B	1·5			
Total		4750†	6670	100

* Area of entire chromatogram.
† Area of the collected peaks.

Therefore, significant antagonism or synergism among the various proteins is unlikely.

Ninety per cent of the toxicity of the crude venom is localized to four groups of fractions. Three of these (5, 6–8, 11) were considerably more toxic than the crude venom and two (cobramines A and B) had only one-half the toxicity. In spite of this lower toxicity, since cobramines A and B constitute about 40 per cent of the venom protein, they contribute one-fifth of the toxic activity as assayed in the mouse. It is of interest that fractions 1 and 3, which have been shown to have 12 and 7 times the phospholipase A activity of the crude venom,[5] had little or no toxic effect. This finding is consistent with previous reports in which these two activities were separated.[1, 9, 10]

All fractions produced similar effects in the mice. There was increasing cyanosis with a decrease in rate and amplitude of respiration. Convulsions sometimes occurred just before death. In addition, the behavior of the animals immediately after i.p. injection of even nonlethal amounts of cobramine A or B suggested a local irritant effect of these basic proteins. A similar response occurred after injection of crude venom.

Paper electrophoresis of fractions 5–8 and 11 at pH 6·0 showed that two or more proteins were present in each fraction (Fig. 2). The most cathodal component contained all the toxic activity in each case.

Gel filtration

The toxic fractions were purified on Sephadex G-50 (Figs. 3 and 4). An unretarded and one or more retarded components were present in fractions 5–8 and 11, indicating a mixture of both low (<30,000) and high molecular weight proteins. The toxic activity was limited to the major retarded protein in all. Cobramines A (not shown) and B have a single peak with identical peak elution volumes.

Further purification of four of the fractions was obtained by this technique as indicated by the decrease in the LD_{50} (Table 2). These values were obtained by pooling the 3–4 peak tubes of the major retarded protein in each case.

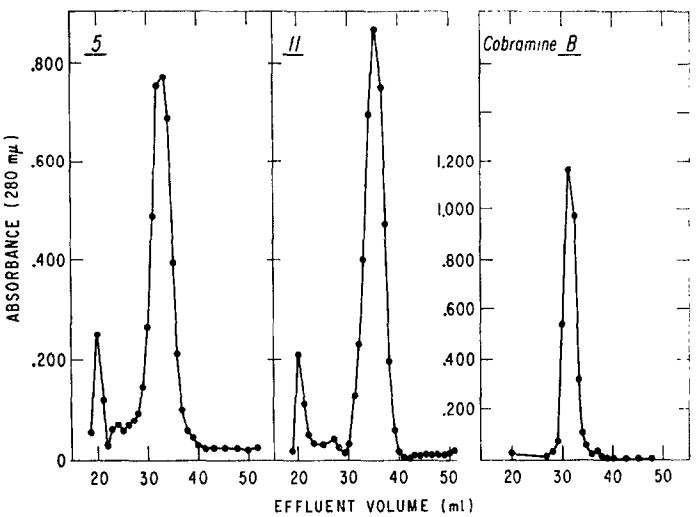

FIG. 3. Elution patterns of fractions 5 and 11 and of cobramine B on Sephadex G-50 (ammonium acetate buffer, 0·05 M, at pH 5·0).

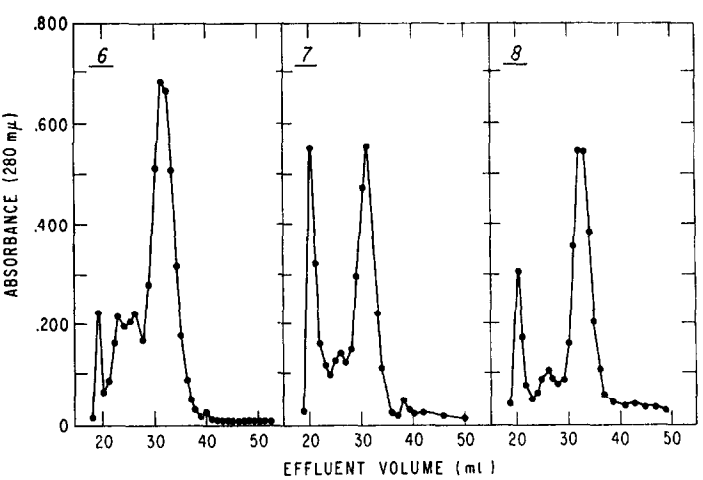

FIG. 4. Elution patterns of fractions 6–8 on Sephadex G-50 (ammonium acetate buffer, 0·05 M, at pH 5·0).

Enzyme assays

Some of the crude fractions contained detectable levels of enzyme activity, but these enzymes could not be causally implicated in the toxicity (Table 3). Obviously no conclusions can be made regarding possible other enzymatic activities.

TABLE 2. TOXICITY AFTER PURIFICATION BY GEL FILTRATION

Fraction	Toxicity (LD_{50}, mg/kg)		Final purification
	Carboxymethyl cellulose peak	Sephadex G-50 peak	(LD_{50} crude)/(LD_{50} toxin)
5	0·33	0·12	6·3
6	0·33	0·34	2·2
7	0·33	0·24	3·2
8	0·15	0·12	6·3
11	0·22	0·08	9·5

TABLE 3. ENZYME ACTIVITIES OF TOXIC FRACTIONS AFTER CARBOXYMETHYL CELLULOSE CHROMATOGRAPHY

Enzyme activity	Crude	5	6	7	8	11	A	B
Phosphodiesterase*	8·4	0·20	0·40	13	40	0·94	0	0
Alkaline phosphatase*	0·63	0·020	0·040	0·032	0·14	0·057	0	0
Phospholipase A†	150	180	350	130	5·9	0·67	0·35	0·099

* Expressed as μmoles *p*-nitrophenol released/mg protein/hr.

† Expressed as μmoles NaOH required/mg protein/min.

Phosphodiesterase. Fractions 7 and 8 contained higher phosphodiesterase activity than the crude venom. This was retarded on Sephadex G-50 with the toxic activity. Unlike the latter, it was completely destroyed by exposure to 100° for 10 min at pH 5·1.

Alkaline monophosphatase. Assay of the gel filtration fractions demonstrated that the low alkaline phosphatase activities in fractions 5–8 were not retarded during gel filtration.

Phospholipase A. The phospholipase A activity of fractions 5–7 was retarded with the toxin. However, if the toxic peaks of the Sephadex column of fraction 6 and 7 are subjected to paper electrophoresis at pH 6·0, two major bands are noted (Fig. 5). The most cathodal band contained only the toxic activity, while the slower band had only phospholipase A activity. The toxic activity of fraction 5 was not separated from phospholipase A activity by electrophoresis, but the heat lability of this fraction (*v.i.*) argues against a causal role of this heat-stable enzyme in the lethal effect.

Inhibition of iodide accumulation. Inhibition of iodide accumulation by thyroid slices has been used as an assay for the effects of cobramine A and B.[5] Brief preincubation of thyroid slices with 12–24 μg of cobramine A or B causes 50 per cent inhibition of subsequent iodide accumulation. Fractions 5, 7 and 8 were ineffective at concentrations of 200 μg/ml. At the same concentration, fraction 6 caused 21 per cent inhibition and fraction 11, 18 per cent. The activity of these two fractions was less than 5 per cent of that of the cobramines.

Characterization of the toxins

On the basis of the above data, it was not possible to determine whether fractions 6, 7 and 8 contained separate toxins or the same toxic protein in different amounts. The mobility of the toxic components of these 3 fractions on paper electrophoresis was sufficiently variable from run to run that differences of the magnitude seen in Figs. 2 and 5 cannot be interpreted. If the same protein does account for all the observed toxicity, then fraction 8 contains the least contamination. Therefore, this fraction was used in subsequent studies.

The toxic proteins of fractions 5, 8 and 11, which can be separated by carboxymethyl cellulose chromatography, are similar in some respects. The molecular weights were all in the same range as estimated by gel filtration, and the potencies (LD_{50} range, 0·08–0·12 mg/kg) were similar. Of greater significance are the different responses to both heat and incubation with trypsin (Table 4).

TABLE 4. PROPERTIES OF THE TOXIC PROTEINS OF *n. naja* VENOM

Conditions	Residual toxicity (%)				
	5	8	11	A	B
Heat*	50	100	67	100	100
Incubation with trypsin†					
After 2 hr	<3	<3	44		
After 24 hr			<3	100	100

* Toxins (50–100 μg/ml) in 0·05 M ammonium acetate buffer, pH 5·1, were incubated in a 100° water bath for 10 min and toxicity was compared before and after heating.

† Protein, 10 mg/ml incubated with 0·2 mg/ml trypsin at 37° in 0·15 M Tris buffer, pH 7·8. Toxicity in trypsin-free controls was unaffected.

Heat stability. After 10 min at 100°, the toxic protein of fraction 5 lost 50 per cent of its activity (Table 4). Fraction 8 lost no activity, while fraction 11 was moderately affected.

Sensitivity to trypsin. Incubation with trypsin for 2 hr reduced the toxic activity of fractions 5 and 8 to less than 3 per cent, while fraction 11 retained 44 per cent activity. Fractions 6 and 7 are similar to fraction 8 in heat resistance and trypsin sensitivity.

The LD_{50} of cobramines A and B was not affected by 24-hr of incubation with trypsin nor by a 10-min exposure to 100° under the stated conditions. We have shown previously that the inhibition of I^- accumulation produced by cobramine B is not affected by similar treatment.

DISCUSSION

Cobra venom produces major toxic changes in the neuromuscular and cardiovascular systems of experimental animals. In the dog and rabbit the primary cause of death is respiratory failure.[11] With artificial respiration, survival is prolonged but death eventually supervenes due to circulatory failure.[12] The cat, which is 2–3 times less sensitive to the crude venom, expires with irreversible hypotension.[13]

It has been suggested that several factors may be involved in the production of these pharmacological effects. One factor, neurotoxin, is thought to cause the curare-like respiratory paralysis in dogs, and electrophysiological studies have verified the

similarity of this component to *d*-tubocurarine.[11] Physicochemical characterization of this protein has not been performed. However, on the basis of the similar potency (seven times that of the original *N. naja atra*), it is probably identical to the low molecular weight, heat-stable protein, cobrotoxin.[2]

The toxic protein of fractions 6–8, like cobrotoxin, accounts for roughly half of the toxic effect of the whole *N. naja* venom. It is also heat-stable at acid pH and sensitive to trypsin, as is the latter. The LD_{50} of both toxins, is about one-seventh of that of their respective crude venoms. It is quite likely that this protein is the *N. naja* equivalent of cobrotoxin or neurotoxin.

Proteins similar to those of fractions 5 and 11 have not been described previously and their physiological mechanism of action is unknown. In terms of absolute potency they are equal to or stronger than the major toxin. These proteins account for only 17 and 3 per cent, respectively, of the effect of the whole venom. In addition, there may be neurotoxic activity in several other chromatographic fractions (Table 1). These have not yet been adequately separated, but would appear to be minor constituents.

The factor causing the delayed cardiovascular effects of crude cobra venom has not been previously isolated, but sufficient evidence is available to attribute these effects to cobramines A and B. In studies kindly performed by Dr. Stephen Hajdu, these enzyme-free, basic proteins can be shown to have a direct cardiotoxic effect on the isometrically contracting frog heart. They can account for all the cardiotoxic activity of the crude *N. naja* venom. Furthermore, the cardiovascular toxin of *N. naja* venom, as assayed in dogs, has been shown to be eluted last from carboxymethyl Sephadex in an ascending pH gradient.[14] Although the present separation technique is not completely comparable, cobramines A and B behave in a similar way on carboxymethyl cellulose (Fig. 1). The primary structure of these proteins is not yet known. However, amino acid analysis of cobramine B has yielded a formula weight of 5840 for the 52 amino acid residues and a molecular weight of 6400 by equilibrium sedimentation.[5] The composition differs substantially from that of neurotoxins in various other venoms.[16–19] It remains to be seen, however, whether these various toxins possess regions of amino acid sequences in common.

In addition to the effects on the heart and, perhaps, on vascular smooth muscle, heat-stable basic proteins of cobra venom cause direct depolarization of skeletal muscle.[11, 15] This effect is quite consistent with the proposed mechanism of action of cobramines A and B based on studies of the iodide accumulation process in the thyroid.[7] Although the initial intent of the thyroid studies had been to investigate the role of membrane phospholipids in iodide transport by the use of phospholipase A, these proteins have profound effects on I^- accumulation in the absence of any phospholipase activity. A marked increase in the efflux rate of preloaded I^-, as well as a decrease in the tissue potassium, is observed after exposure to these proteins, and the inhibition of accumulative transport by intestinal mucosa or kidney[6] is probably due to a similar 'leak'. The same type of mechanism would account for the depolarization or systolic arrest seen in skeletal or cardiac muscle respectively.

REFERENCES

1. B. S. Meldrum, *Pharmac. Rev.* **17**, 393 (1965).
2. C. C. Yang, *J. biol. Chem.* **240**, 1616 (1965).
3. H. I. Bicher, C. Klibansky, J. Shiloah, S. Gitter and A. DeVries, *Biochem. Pharmac.* **14**, 1779 (1965).

4. W. BJÖRK and H. G. BOMAN, *Biochim. biophys. Acta* **34**, 503 (1959).
5. P. R. LARSEN and J. WOLFF, *J. biol. Chem.* in press.
6. P. R. LARSEN and J. WOLFF, *Biochem. Pharmac.*, **16**, 2003 (1967).
7. J. WOLFF, H. SALABÈ, M. AMBROSE and P. R. LARSEN, *J. biol. Chem.* in press.
8. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
9. R. W. P. MASTER and S. S. RAO, *J. biol. Chem.* **236**, 1986 (1961).
10. C. C. YANG, C. J. CHEN and C. C. SU, *J. Biochem., Tokyo* **46**, 1201 (1959).
11. C. C. CHANG and C. Y. LEE, *Br. J. Pharmac. Chemother.* **28**, 172 (1966).
12. J. A. VICK, H. P. CIUCHTA and E. H. POLLEY, *Archs int. Pharmacodyn. Thér.* **153**, 424 (1965).
13. C. Y. LEE and M. T. PENG, *Archs int. Pharmacodyn. Thér.* **133**, 180 (1961).
14. J. A. VICK, H. D. CIUCHTA, C. BROOMFIELD and B. T. CURRIE, *Toxicon* **3**, 237 (1966).
15. B. S. MELDRUM, *Br. J. Pharmac. Chemother.* **25**, 197 (1965).
16. T. SASAKI, *J. pharm. Soc. Japan* **77**, 848 (1957).
17. N. TAMIYA and H. ARAI, *Biochem. J.* **99**, 624 (1966).
18. E. KARLSSON, D. L. EAKER and J. PORATH. *Biochim. biophys. Acta* **127**, 505 (1966).
19. C. ROCHAT, H. ROCHAT, F. MIRINDA and S. LISSITZKY, *Biochemistry, N.Y.* **6**, 578 (1967).