- Hughes, W. L., Saroff, H. A. & Carney, A. L. (1949). *J. Amer. chem. Soc.* **71**, 2479.
- Kauzmann, W. & Simpson, R. B. (1953). *J. Amer. chem. Soc.* **75**, 5154.
- Kegeles, G. & Gutter, F. J. (1951). *J. Amer. chem. Soc.* **73**, 3770.
- Kharush, F. (1950). *J. Amer. chem. Soc.* **72**, 2705.
- Koenig, V. L. (1950). *Arch. Biochem.* **25**, 241.
- Koenig, V. L. & Pedersen, K. O. (1950). *Arch. Biochem.* **25**, 97.
- Lewis, G. N. & Kasha, M. (1945). *J. Amer. chem. Soc.* **67**, 994.
- Linderström-Lang, K. & Schellman, J. (1954). *Biochim. biophys. Acta*, **15**, 156.
- Marinesco, N. (1927). *J. Chim. phys.* **24**, 593.
- Miller, G. L. & Price, W. C. (1946). *Arch. Biochem.* **10**, 467.
- Ogston, A. G. (1953). *Trans. Faraday Soc.* **49**, 1481.
- Oncley, J. L. (1942). *Chem. Rev.* **30**, 433.
- Pedersen, K. O. (1945). *Ultracentrifugal Studies on Serum and Serum Fractions*. Uppsala: Almqvist and Wiksells.
- Pedersen, K. O. (1953). *Disc. Faraday Soc.* **13**, 49.
- Perlmann, G. E. & Longworth, L. G. (1948). *J. Amer. chem. Soc.* **70**, 2719.
- Perrin, F. (1926). *J. Phys. Radium*, **7**, 390.
- Perrin, F. (1929). *Ann. Phys., Paris*, **12**, 169.
- Rau, K. L. (1949). *Optik, Stuttgart*, **5**, 277.
- Reichmann, M. E. & Charlwood, P. A. (1954). *Canad. J. Chem.* **32**, 1092.
- Schachman, H. K. & Kauzmann, W. J. (1949). *J. phys. Chem.* **53**, 150.
- Schachman, H. K. & Lauffer, M. A. (1949). *J. Amer. chem. Soc.* **71**, 536.
- Schellman, J. (1955). *C.R. Lab. Carlsberg, (Sér. chim.)*. (In the Press.)
- Scheraga, H. A. & Mandelkern, L. (1953). *J. Amer. chem. Soc.* **75**, 179.
- Simpson, R. B. & Kauzmann, W. (1953). *J. Amer. chem. Soc.* **75**, 5139.
- Singleton, C. R. & Weinberger, L. A. (1951). *J. Amer. chem. Soc.* **73**, 4574.
- Svedberg, T. & Sjögren, B. (1930). *J. Amer. chem. Soc.* **52**, 2855.
- Tanford, C. & Buzzell, J. G. (1954). *J. Amer. chem. Soc.* **76**, 3356.
- Tsao, T. C. (1953). *Biochim. biophys. Acta*, **11**, 227, 236.
- Waugh, D. F. & Yphantis, D. A. (1952). *Rev. sci. Instrum.* **23**, 609.
- Weber, G. (1952a). *Biochem. J.* **51**, 155.
- Weber, G. (1952b). *Biochem. J.* **51**, 145.
- Weber, G. (1953a). *Disc. Faraday Soc.* **13**, 33.
- Weber, G. (1953b). *Advanc. Protein Chem.* **8**, 447.
- Williams, R. C., Backus, R. C. & Steere, R. L. (1951). *J. Amer. chem. Soc.* **73**, 2062.
- Wright, F. E. (1934). *J. opt. Soc. Amer.* **24**, 206.
- Yang, J. T. & Foster, J. F. (1954). *J. Amer. chem. Soc.* **76**, 1593.

Cholinesterase Inactivation by Snake Venoms

BY C. Y. LEE, C. C. CHANG AND K. KAMIJO

The Pharmacological Institute, College of Medicine, National Taiwan University, Taipei, Formosa, China, and the Department of Physiology and Pharmacology, Graduate School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, U.S.A.

(Received 23 July 1955)

Although the instability of the cholinesterase of cobra venom in aqueous solution has been pointed out by previous authors (Mounter, 1951; Augustinsson & Grahn, 1952), its cause is unknown. During studies of the cholinesterase of Formosan snake venoms, a high potency of cholinesterase inactivation (anticholinesterase) was found in the cholinesterase-containing venom of the Formosan cobra (*Naja naja atra*) (Chang & Lee, 1955). The existence of such anticholinesterase activity was proved by adding the cobra venom to the venom of *Bungarus multicinctus*, a very potent source of specific (true, acetyl-, aceto-) cholinesterase. Furthermore, in the presence of MgCl_2 (0.04M) its anticholinesterase activity was completely inhibited.

The present work was undertaken to throw more light on the nature of the anticholinesterase activity of the cobra venom and to ascertain

whether it can affect cholinesterase preparations from other sources. The results were presented in abstract at the meeting of the American Association for the Advancement of Science, Berkeley, California, in December, 1954.

EXPERIMENTAL

Measurement of cholinesterase activity. The electrometric titration method described by Alles & Hawes (1940) was adopted in most experiments for the measurement of cholinesterase activity with the aid of a Beckman pH meter, model G, unless otherwise stated. To 14 ml. of 0.0032M acetylcholine chloride in 0.04M- MgCl_2 , 1 ml. of test solution was added. The reaction mixture was kept constantly at 37° by circulating water from a water bath. The pH was kept at 7.4, and the mixture was titrated with 0.02N-NaOH to neutralize the liberated acetic acid. Cholinesterase activity is expressed in ml. of 0.02N-NaOH consumed during the first 5 min. of enzymic hydrolysis of substrate.

In some experiments, rates of hydrolysis were measured by the Warburg manometric technique (Ammon, 1933). Each flask contained a total volume of 3 ml., all solutions being made up in 0.03M-NaHCO₃, and after attachment to the manometers the flasks were gassed for 5 min. with N₂+CO₂ (95:5, v/v). No other salt was added to the system, unless otherwise indicated. The cholinesterase activity is expressed in b_{50} values, i.e. the amount of CO₂ in μ l. evolved during 30 min. minus the corresponding value for non-enzymic hydrolysis.

Venoms. Formosan cobra (*Naja naja atra*) venom was used for the study of the anticholinesterase activity, and the venom of *Bungarus multicinctus* was selected as the source of cholinesterase in most experiments. Venoms of other genera, which displayed no cholinesterase activity, were also tested for comparison in some experiments. The dried venoms were dissolved in cold distilled water before each experiment and stored in the ice box until used.

Cholinesterase preparations from tissues. Cholinesterase preparations from erythrocytes, serum, heart muscle and brain of rabbits were prepared in the manner described by Augustinsson (1948).

RESULTS

Effect of cations on *Naja*-venom anticholinesterase activity. It has been demonstrated previously (Chang & Lee, 1955) that MgCl₂ (0.04M) inhibits the anticholinesterase activity so completely that a typical bell-shaped pS-activity curve can be obtained for the *Naja*-venom cholinesterase in the

presence of the salt. In order to determine how other cations affect the anticholinesterase activity of *Naja* venom, the venom solutions (*Naja* venom alone or *Naja* venom and *Bungarus* venom) were incubated with various salt solutions of different concentrations for 20 min. at 37° and the remaining cholinesterase was measured (Tables 1 and 2). It must be pointed out that the MgCl₂ added to the enzyme solution (1 ml.) did not significantly change the concentration of this cation in the substrate solution (14 ml.) during the measurement of cholinesterase. It can readily be seen that all the cations tested inhibited the *Naja*-venom anticholinesterase, although the degrees of inhibition varied considerably. In general, bivalent ions were more active than univalent ions. Thus, 0.02M-MgCl₂ or -CaCl₂ was sufficient for complete inhibition of the *Naja*-venom anticholinesterase, whereas with NaCl, KCl, or NH₄Cl 0.1M solutions were needed. Among the univalent ions NH₄⁺ was more active than Na⁺ or K⁺. Higher concentrations of either of the cations not only inhibited the *Naja*-venom anticholinesterase completely but also increased the cholinesterase activity beyond the original values. A possible explanation of these results is given in the Discussion.

The inactivation of cholinesterase by the *Naja*-venom anticholinesterase was irreversible: MgCl₂ (0.04M) did not restore the cholinesterase activity

Table 1. *Effect of cations on anticholinesterase activity of Naja venom*

Naja venom (200 μ g./ml. final concentration in the incubation mixture) was incubated with various salts at 37° for 20 min. The remaining cholinesterase (ChE) from 1 ml. of the incubation mixture was measured and the anticholinesterase (antiChE) activity (%) was computed against the original *Naja*-venom ChE. The original ChE before incubation was 0.23 and after incubation in the absence of any salt it decreased to nil.

Salt concn. (M)	MgCl ₂		CaCl ₂		NaCl		KCl		NH ₄ Cl	
	ChE	AntiChE (%)	ChE	AntiChE (%)	ChE	AntiChE (%)	ChE	AntiChE (%)	ChE	AntiChE (%)
1.0	—	—	—	—	0.27	-17	0.27	-17	—	—
0.1	0.32	-36	0.27	-17	0.22	4.5	0.21	9	0.27	-17
0.02	0.23	0	0.23	0	0.006	97	0.01	96	0.095	59
0.002	0	100	0	100	—	—	—	—	—	—

Table 2. *Effect of cations on anticholinesterase activity of Naja venom*

In 1 ml. of incubation mixture, 30 μ g. of *Bungarus* venom and 30 μ g. of *Naja* venom (which was pre-incubated at 37° for 10 min. to destroy its own cholinesterase) were contained. Original cholinesterase (ChE) activity of 30 μ g. *Bungarus* venom before incubation was 0.36, which decreased to 0.27 when incubated alone without *Naja* venom in the absence of any salt. The latter value was taken as control and used for the calculation of *Naja*-venom anticholinesterase (antiChE) activity (%). It was found to be 85% when no cations were added.

Salt concn. (M)	MgCl ₂		CaCl ₂		NaCl		KCl		NH ₄ Cl	
	ChE	AntiChE (%)	ChE	AntiChE (%)	ChE	AntiChE (%)	ChE	AntiChE (%)	ChE	AntiChE (%)
1.0	—	—	—	—	0.43	-59	0.46	-70	—	—
0.1	0.44	-63	0.42	-56	0.39	-44	0.42	-56	0.45	-67
0.02	0.37	-37	0.35	-30	0.04	85	0.05	81	0.28	-4
0.002	0.04	85	0.04	85	—	—	—	—	—	—

after the enzyme had been inactivated by the *Naja*-venom anticholinesterase.

On the other hand, inhibition of the *Naja*-venom anticholinesterase by $MgCl_2$ (0.02M) or KCl (0.1M) was not irreversible, since the anticholinesterase activity could be restored by diluting the mixture of the *Naja* venom and metal ions to the degree that no inhibition occurred (Table 3).

Effects of organic substances on the Naja-venom anticholinesterase activity. There seemed to exist

Table 3. *Reversibility of Naja-venom anticholinesterase, inhibited by cations, after dilution*

600 $\mu g./ml.$ of *Naja* venom (after destroying its cholinesterase (ChE) by heating at 37° for 10 min.) was pre-incubated with $MgCl_2$ (final concn. 0.02M) or KCl (final concn. 0.1M) for 20 min. Thereafter, the mixture was diluted 10 times and its anticholinesterase (antiChE) activity against *Bungarus* venom was measured under the same conditions as in Table 2. Original ChE of 30 $\mu g.$ of *Bungarus* venom before incubation was 0.30, which decreased to 0.25 after incubation.

Salt added	ChE activity	AntiChE activity (%)
None (control)	0.03	88
$MgCl_2$	0.02	92
KCl	0.01	96

some parallelism between inhibition of the anticholinesterase activity of *Naja* venom and stabilization or activation of cholinesterase by various cations, so it was of interest to ascertain whether or not certain organic substances which have been proved to act as stabilizers (Mounter, 1951) or activators of cholinesterase (Nachmansohn & Lederer, 1939; Chaudhuri, 1950a, b) could inhibit the *Naja*-venom anticholinesterase. The results of such experiments are shown in Tables 4 and 5.

It will be seen that cysteine, reduced glutathione, sodium citrate and hydrocyanic acid at a concentration of 0.02M, and gelatin, bovine serum albumin and gum acacia at a concentration of 0.05% not only inhibited the *Naja*-venom anticholinesterase completely but also increased the cholinesterase activity beyond the original values.

The cholinesterase of *Naja* or *Bungarus* venoms, once inactivated by the *Naja* venom anticholinesterase after 20 min. of incubation, could not be reactivated by the addition of cysteine, reduced glutathione, citrate or gelatin.

Effect of hydrogen-ion concentration on the anticholinesterase activity of Naja venom. *Bungarus*-venom cholinesterase (100 $\mu g./ml.$) was pre-incubated with *Naja* venom (20 $\mu g./ml.$) at various pH values for 40 min. at 37° and the remaining cholinesterase was measured at pH 7.4.

Table 4. *Effect of various organic substances on Naja-venom anticholinesterase*

Experimental conditions were the same as in Table 1. ChE, cholinesterase.

Reagent	Concn.	ChE (original)	ChE (after incubation)	AntiChE (%)
None	—	0.23	0	100
Cysteine	0.02M	0.23	0.25	-9
Glutathione	0.02M	0.23	0.30	-30
Citrate	0.02M	0.23	0.30	-30
Hydrocyanic acid	0.02M	0.20	0.20	0
Gelatin	0.05% (w/v)	0.22	0.25	-14
Bovine serum albumin	0.05% (w/v)	0.22	0.25	-14
Gum acacia	0.05% (w/v)	0.22	0.25	-14

Table 5. *Effect of various organic substances on Naja-venom anticholinesterase*

Experimental conditions were the same as in Table 2.

Reagent	Concn.	ChE control	ChE after incubation with <i>Naja</i> venom	AntiChE %
None	—	0.28*	0.03	89
Cysteine	0.02M	0.28	0.39	-39
Glutathione	0.02M	0.28	0.44	-57
Citrate	0.02M	0.28	0.43	-54
None	—	0.24†	0.03	88
Hydrocyanic acid	0.02M	0.24	0.32	-33
Gelatin	0.05% (w/v)	0.24	0.40	-67
Bovine serum albumin	0.05% (w/v)	0.24	0.40	-67
Gum acacia	0.05% (w/v)	0.24	0.35	-46

* Original value of *Bungarus*-venom cholinesterase (ChE) before incubation, 0.36.

† Original value of *Bungarus*-venom ChE before incubation, 0.29.

As shown in Fig. 1, the *Naja*-venom anticholinesterase had a broad range of optimum activity, between pH 6.0 and 8.5. It started to decline abruptly at pH 5.5 and lost its activity completely below pH 5.0; on the alkaline side it started to decline more gradually at pH 9.0 and lost its activity completely above pH 10.

The pH range for the anticholinesterase activity of *Naja* venom was similar to that for specific cholinesterase (Wilson & Bergmann, 1950; Chang & Lee, 1955).

Effect of temperature on the Naja-venom anticholinesterase. As shown in Table 6, the *Naja*-venom anticholinesterase was stable below 50° but was completely destroyed by heating at 60° for 10 min. The heat-treated *Naja* venom seemed to stabilize the *Bungarus*-venom cholinesterase slightly during incubation.

Effect of dialysis on the Naja-venom anticholinesterase. As 0.2% (w/v) solution of *Naja* venom was dialysed at 5° for 2 days against distilled water, in a cellophan membrane. It will be seen from Table 7 that more than 80% of the *Naja*-venom anticholinesterase was lost after dialysis, since the anticholinesterase of 100 µg. of the dialysed venom

was found to be much less than that of 20 µg. of the undialysed venom.

Relationship between anticholinesterase and proteolytic activity of various snake venoms. In order to ascertain whether or not the anticholinesterase activity of *Naja* venom is due to a proteolytic enzyme in the venom, the two types of activity of various snake venoms were compared. As shown in Table 8, the proteolytic activity of the *Naja* venom was the least, whereas its anticholinesterase activity was the most potent among the venoms

Table 6. *Effect of temperature on stability of Naja-venom anticholinesterase*

1 mg./ml. of *Naja* venom was pre-incubated at the given temperature for 10 min. Thereafter, 30 µg. of the treated venom was incubated with 30 µg. of *Bungarus* venom in 1 ml. at 37° for 20 min. Cholinesterase (ChE) activity of 30 µg. of *Bungarus* venom alone after incubation for the same period was 0.23.

Temperature (°)	Residual ChE	AntiChE activity (%)
0	0.04	83
37	0.04	83
50	0.04	83
60	0.25	- 8
70	0.25	- 8
80	0.24	- 4
100	0.24	- 4

Fig. 1. Effect of hydrogen-ion concentration on the *Naja*-venom anticholinesterase activity.

Table 7. *Effect of dialysis on Naja-venom anticholinesterase*

After dialysis, *Naja* venom was diluted and the anticholinesterase activities of 20 and 100 µg. were measured by incubation with 50 µg. of *Bungarus* venom in 1 ml. at 37° for 20 min.

Dose of <i>Naja</i> venom (µg./ml.)	Anticholinesterase activity (%)	
	Without dialysis	After dialysis
20	75	10
100	90	52

Table 8. *Relationship between anticholinesterase and proteolytic activities of various snake venoms*

Source of venom	Proteolytic activity*		Dose (mg.)	Anticholinesterase†	
	MgCl ₂ absent	MgCl ₂ (0.04 M) present		MgCl ₂ absent	MgCl ₂ (0.04 M) present
<i>Naja naja atra</i>	0.07	0.10	0.03	90	- 55
<i>Trimeresurus mucrosquamatus</i>	0.42	0.36	1.0	60	- 64
<i>Trimeresurus gramineus</i>	0.33	—	1.0	40	- 64
<i>Agkistrodon acutus</i>	0.33	—	1.0	5	- 60
<i>Vipera russellii formosensis</i>	0.11	—	1.0	- 20	- 64

* A 3.6% solution of casein was digested by venoms (2 mg. dry wt.; 1.0 mg./ml.) at pH 8.0, 40° for 1 hr. Activity is expressed as the difference in amounts (ml.) of 0.05N ethanolic KOH needed to titrate 2.0 ml. of digestion mixture, which was brought to 90% ethanolic solution, before and after incubation.

† Venoms were incubated with 30 µg. of *Bungarus* venom in 1 ml. at 37° for 20 min. The pH of the incubation mixture was adjusted to 8.0-8.2. Anticholinesterase activity is expressed as a percentage of inhibition of the *Bungarus*-venom ChE.

tested. In general, no parallelism could be found between the two types of activity. Moreover, $MgCl_2$ (0.04M) inhibited the anticholinesterase activity completely but scarcely affected the proteolytic activity.

From these experiments, it may be concluded that the anticholinesterase activity of *Naja* venom seems not to be due to a proteolytic enzyme.

Inhibition of Naja-venom anticholinesterase by cholinesterase preparations from various tissues of rabbits. *Naja*-venom anticholinesterase was easily inhibited by metal ions and other substances as mentioned above, and it remained to be determined whether cholinesterase preparations from mammalian tissues could be affected by the *Naja*-venom anticholinesterase.

As shown in Table 9, all the cholinesterase preparations tested (erythrocyte, serum, heart muscle and brain) not only failed to be inactivated by the

at 100° for 10 min., whereas the cholinesterase of brain homogenate was destroyed by heating at 60° for the same period (Table 10).

DISCUSSION

Chang & Lee (1955) have already suggested that the instability of the cholinesterase of cobra venom could be due to the coexistence of anticholinesterase activity, although the venom studied by them was a Formosan variety. Additional evidence for such a possibility is presented in the present work. Thus substances which stabilize the cholinesterase of cobra venom, such as metal ions (Augustinsson & Grahn, 1952), gelatin, gum acacia and albumin (Mounter, 1951), were shown to protect the cholinesterase from destruction by the *Naja*-venom anticholinesterase. Moreover, the order of the potency for the inhibition of anticholinesterase

Table 9. *Effect of Naja-venom anticholinesterase on cholinesterase from various tissues of rabbits*

Cholinesterase (ChE) activity was measured by the manometric method.

Source of ChE	Cholinesterase activity (b_{30})				
	Control (A)	+ venom (100 μ g.) (B)	Difference (B - A)	+ venom (200 μ g.) (C)	Difference (C - A)
Erythrocyte	9	82	73	146	137
Serum	26	102	76	156	130
Heart muscle	38	143	105	200	162
Brain	86	171	85	215	129
Brain (dialysed)	74	107	33	140	66

Table 10. *Thermostability of the factors inhibiting Naja-venom anticholinesterase in rabbit-brain homogenate*

Cholinesterase activity was measured by the manometric method.

Temperature	Cholinesterase activity (b_{30})				
	Control (A)	+ venom (100 μ g.) (B)	Difference (B - A)	+ venom (200 μ g.) (C)	Difference (C - A)
Control (untreated)	108	161	53	201	93
50°	95	154	59	193	98
60°	6	92	86	151	145
100°	3	86	83	144	141

Naja-venom anticholinesterase, but on the contrary seemed to inhibit the latter activity. Consequently, the *Naja*-venom cholinesterase contributed its activity to that of the cholinesterase of the tissue preparations. It is apparent that cholinesterase preparations from various tissues of the rabbit contain some factor, or factors, which inhibit the *Naja*-venom anticholinesterase completely. Experiments with dialysed brain preparation (30 hr. against distilled water at 5°) indicated that the inhibiting factors were at least partially dialysable (Table 9). These factors were unaffected by heating

activity of various cations was found to be similar to their cholinesterase-stabilizing effects described by Augustinsson & Grahn. Some reducing agents, such as cysteine, reduced glutathione, hydrocyanic acid and citrate, which are activators of cholinesterase (Nachmansohn & Lederer, 1939; Chaudhuri, 1950a, b), were also found to be good inhibitors of the *Naja*-venom anticholinesterase. In addition to inhibiting the *Naja*-venom anticholinesterase, most of these compounds increased the cholinesterase activity beyond its original value. However, experiments with cations, especi-

ally with MgCl_2 , showed that it is unlikely that their effect was due simply to activation of the cholinesterase, since there was sufficient MgCl_2 (0.04M) in the substrate solution during the measurement of cholinesterase. Therefore the increase of cholinesterase activity by pre-incubation with higher concentrations of cations must be due to some unknown mechanism other than simple activation of the enzyme. Since pre-incubation with the cations does not appear to be necessary for full activation of cholinesterase, it is assumed that the venoms contain some inert cholinesterase, which is loosely and reversibly bound to the anticholinesterase, and that the cations (possibly also the other inhibitors of the *Naja*-venom anticholinesterase) can reactivate the inert cholinesterase, but that reactivation is inhibited in the presence of substrate. On the basis of these assumptions, the experimental results can be readily explained. However, once the cholinesterase had been inactivated by incubation with the *Naja*-venom anticholinesterase, none of the inhibitors of the anticholinesterase could restore the cholinesterase activity.

On the other hand, the dilution experiments show that inhibition of the *Naja*-venom anticholinesterase by metal ions is not irreversible. In other words, metal ions do not destroy the *Naja*-venom anticholinesterase but only interfere with its interaction with cholinesterase.

The potent protection of cholinesterase, by the reducing agents tested, from destruction by the *Naja*-venom anticholinesterase might lead to an assumption that the anticholinesterase contains active disulphide groups and produces its effect by oxidizing the thiol groups of cholinesterase. However, this is improbable since the cholinesterase, once inactivated by the *Naja*-venom anticholinesterase, cannot be reactivated by these reducing agents. Moreover, the thiol group in cholinesterase was recently regarded not to be a significant group in the active surface of the enzyme (Mounter & Whittaker, 1953; Markwardt, 1953).

Since the pH range for the anticholinesterase activity of *Naja* venom is similar to that for the cholinesterase, it is suggested that the venom anticholinesterase inactivates the cholinesterase by attacking its active centre, just as does tetraethyl pyrophosphate (Wilson, 1954).

The dialysis experiments indicate that the *Naja*-venom anticholinesterase is not a compound of very high molecular weight, such as a protein, although the heating experiments suggested such a possibility. The possibility that the *Naja*-venom anticholinesterase is due to a proteolytic enzyme is unlikely, since no parallelism between two types of activity of various snake venoms could be found.

Moreover, MgCl_2 inhibited the former activity but not the latter.

Finally, since cholinesterase preparations from various tissues contain some factor, or factors, which inhibit the *Naja*-venom anticholinesterase completely, they are unaffected by the venom anticholinesterase. These factors were found to be heat-resistant and at least partially dialysable.

SUMMARY

1. The cholinesterase-inactivating activity (anticholinesterase) of the Formosan cobra venom (*Naja* venom) is reversibly inhibited by various cations. Magnesium is by far the most potent ion tested; sodium and potassium are least active in this respect.

2. Other activators or stabilizers of cholinesterase, such as cysteine, reduced glutathione, citrate, hydrocyanic acid, gelatin, albumin and gum acacia, are also inhibitors of the *Naja*-venom anticholinesterase.

3. The inactivation of cholinesterase by the *Naja*-venom anticholinesterase is irreversible, since, once the cholinesterase has been inactivated by the anticholinesterase, none of the anticholinesterase inhibitors can restore the cholinesterase activity.

4. In addition to the inhibition of the *Naja*-venom anticholinesterase, most of the inhibitors increase the cholinesterase activity beyond its original value. The possible cause is discussed.

5. The optimum pH range for the *Naja*-venom anticholinesterase is between 6.0 and 8.5. It loses its activity completely below 5.0 or above 10.

6. The *Naja*-venom anticholinesterase is completely destroyed by heating at 60° for 10 min. Its activity disappears for the most part after dialysis of a 0.2 % solution.

7. The anticholinesterase activity of *Naja* venom seems not to be due to a proteolytic enzyme, since no parallelism between the two types of activity of various snake venoms could be found.

8. The *Naja*-venom anticholinesterase does not affect the cholinesterase preparations of various tissues of the rabbit such as erythrocyte, serum, heart muscle and brain, since they apparently contain some factors which inhibit the anticholinesterase completely. These factors are heat-resistant (100°, 10 min.) and at least partially dialysable.

The authors wish to thank Dr G. B. Koelle, Graduate School of Medicine, University of Pennsylvania, U.S.A., for his interest and advice in this work. This investigation was supported in part by a research grant [B-282 (C2)] from the National Institutes of Health, U.S. Public Health Service.

REFERENCES

- Alles, G. A. & Hawes, R. C. (1940). *J. biol. Chem.* **133**, 375.
 Ammon, R. (1933). *Pflüg. Arch. ges. Physiol.* **233**, 486.
 Augustinsson, K. B. (1948). *Acta physiol. scand.* **15**, suppl. 52.
 Augustinsson, K. B. & Grahn, M. (1952). *Ark. Kemi*, **4**, 277.
 Chang, C. C. & Lee, C. Y. (1955). *J. med. Ass., Formosa*, **54**, 103.
 Chaudhuri, D. K. (1950a). *Ann. Biochem.* **10**, 65.
 Chaudhuri, D. K. (1950b). *Ann. Biochem.* **10**, 71.
 Markwardt, F. (1953). *Naturwissenschaften*, **40**, 341.
 Mounter, L. A. (1951). *Biochem. J.* **50**, 122.
 Mounter, L. A. & Whittaker, V. P. (1953). *Biochem. J.* **53**, 167.
 Nachmansohn, D. & Lederer, E. (1939). *Bull. Soc. Chim. biol., Paris*, **21**, 797.
 Wilson, I. B. (1954). *The Mechanism of Enzyme Action*, p. 642. Baltimore: The Johns Hopkins Press.
 Wilson, I. B. & Bergmann, F. (1950). *J. biol. Chem.* **186**, 683.

Purification and Amino Acid Analysis of Melanophore-expanding Hormone from Hog and Ox Pituitary Glands

BY B. G. BENFEY AND J. L. PURVIS

Departments of Pharmacology and of Biochemistry, McGill University, Montreal, Canada

(Received 18 July 1955)

The preparation of highly purified, if not pure, melanophore-expanding hormone from hog pituitary has recently been reported by Lerner & Lee (1955). The present writers, with the aid of counter-current distribution technique, have succeeded in preparing highly purified melanophore-expanding material, virtually free from adrenocorticotrophic (ACTH) activity, from both hog and ox pituitary. The products from the two sources are not chemically identical. The amino acid composition of the material from hog pituitary differs considerably from that reported by Lerner & Lee (1955). The successful segregation of the melanophore-expanding principle and ACTH along with other evidence indicates the non-identity of the two hormones.

EXPERIMENTAL

Preparation of material for countercurrent distribution

(a) *Hog-pituitary extract.* A quantity (150 g.) of acetone-dried and powdered posterior pituitaries, assaying from 6 to 10 units of melanophore-hormone activity per mg., was divided into 10 g. lots and each lot was extracted with 25 ml. of acetone and 160 ml. of glacial acetic acid at 70°. The material was left in contact with the solvent at this temperature for 10 min. The extracts were centrifuged, the sediment of tissue fragments was removed and washed with 20 ml. each of cold acetone and glacial acetic acid and the washing was added to the previous extract. To the combined supernatant fluids were added 1 ml. of saturated NaCl and a quantity of acetone equal to half the volume of the extract. The resulting precipitate was removed by centrifuging and to the supernatant fluid was added an equal volume of diethyl ether. The precipitate was collected by centrifuging, washed three times with acetone and placed in a desiccator. The total yield of this partially purified material from 150 g. of posterior-pituitary lobes

was 27 g. The melanophore-hormone activity was 30 units/mg., thus representing a recovery of 67.5% of the activity of the original material. Further purification of the product was achieved by dissolving 27 g. of the ether precipitate in 750 ml. of 0.1N acetic acid. Oxycellulose (4.5 g.), previously washed successively with 1N-HCl, water and 0.1N acetic acid, was added and the mixture stirred for 40 hr. at room temperature. The oxycellulose was collected by centrifuging, washed successively with 0.1N acetic acid and water, and then suspended twice in 100 ml. of 0.1N-HCl and stirred for 30 min. to elute the hormone. The oxycellulose was removed by centrifuging and the pH of the combined supernatant fluids was adjusted to 6.5 with aq. N-NH₃. The resulting precipitate was removed by centrifuging and to the supernatant fluid 5 vol. of acetone were added. The resulting precipitate was washed with acetone and stored in an evacuated desiccator. The yield was 660 mg. of a powder which assayed 800 units/mg., representing a recovery of 44% of the activity of the original material. This procedure was based on the methods of Payne, Raben & Astwood (1950), and Astwood, Raben, Payne & Grady (1951), originally developed for the purification of ACTH, first used by Raben, Rosenberg & Astwood (1952) for the purification of melanophore hormone and later modified for this purpose by Landgrebe & Mitchell (1954). The present authors have introduced only slight modifications.

(b) *Ox-pituitary extract.* A quantity of purified powder which had been prepared from ox posterior pituitary lobes by Stehle (1944) was available. Stehle's method involved fractionation of an acetic acid extract of the tissue with organic solvents, and final isolation of the active material by precipitation with picric acid. The powder assayed 150 units/mg.

Countercurrent distribution

For the segregation and purification of the melanophore-expanding substance and ACTH a 100-tube (each 200 ml. capacity) all-glass Craig apparatus was used. For analytical purposes a 24-unit (20 ml.) stainless-steel Craig instrument