References

Abrams, A. (1958), *J. Biol. Chem. 230*, 949.
Brumfitt, W. (1959), *Brit. J. Exptl. Pathol. 40*, 441.
Brumfitt, W., Wardlaw, A. C., and Park, J. T. (1958), *Nature 181*, 1783.
Cummins, C. S., and Harris, H. (1956), *J. Gen. Microbiol. 14*, 583.
Feiner, R. R., Meyer, K., and Steinberg, A. (1946), *J. Bacteriol. 52*, 375.
Fleming, A. (1922), *Proc. Roy. Soc. (London) B93*, 306.
Fleming, A. and Allison, V. D. (1927), *Brit. J. Exptl. Pathol. 8*, 214.
Ghuysen, J. M., and Strominger, J. L. (1963), *Biochemistry 2*, 1119.
Hallauer, C. (1929), *Zentr. Bakteriol. Parasitenk., Abt. I Ref. Orig. 114*, 519.
Hancock, R. (1960), *Biochim. Biophys. Acta 37*, 42.
Hestrin, S. (1949), *J. Biol. Chem., 180*, 249.
Jeanes, A., Wise, C. S., and Dimmler, R. J. (1951), *Anal. Chem. 23*, 415.
Jeanloz, R. W., Sharon, N., and Flowers, H. M. (1963), *Biochem. Biophys. Res. Commun. 13*, 20.
Litwack, G. (1958), *Nature 181*, 1348.
Litwack, G., and Prasad, A. L. N. (1962), *Nature 196*, 543.
Lowry, O. H., and Lopez, J. A. (1946), *J. Biol. Chem. 162*, 421.
McDonald, I. J. (1963), *Can. J. Microbiol. 9*, 415.
Mandelstam, J., and Rogers, H. J. (1959), *Biochem. J. 72*, 654.
Perkins, H. R. (1960), *Biochem. J. 74*, 186.
Prasad, A. L. N., and Litwack, G. (1961), *Biochim. Biophys. Acta 46*, 452.
Prasad, A. L. N., and Litwack, G. (1963), *Anal. Biochem. 6*, 328.
Primosigh, J., Pelzer, H., Maass, D., and Weidel, W. (1961), *Biochim. Biophys. Acta 46*, 68.
Salton, M. R. J., and Ghuysen, J. M. (1959), *Biochim. Biophys. Acta 36*, 552.
Smith, I. (1960), Chromatographic and Electrophoretic Techniques, Vol. I, New York, Interscience.
Stechschulte, M. A. L. (1961), Ph.D. dissertation, Catholic University of America.
Thatcher, F. S., and Simon, W. (1957), International Bulletin of Bacteriological Nomenclature and Taxonomy, Vol. VII, p. 21.
Work, E. (1964), *Nature 201*, 1107.

Comparison of the Levels of Phosphodiesterase, Endonuclease, and Monophosphatases in Several Snake Venoms*

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ABSTRACT: The venoms of five species of snake have been analyzed for phosphodiesterase, 5′-nucleotidase, endonuclease, and alkaline nonspecific phosphatase. Using very dilute solutions of venom, it has been possible to determine endonuclease directly in crude venom by the spectrophotometric method. The venoms of *Agkistrodon piscivorus*, *Bothrops atrox*, *Crotalus adamanteus*, and *Crotalus atrox* resemble each other approximately in contents and relative proportions of the four enzymes.

The venom of *Naja nigricollis* contains an unusually large amount of the nonspecific phosphatase, making it unsuitable as a starting material for the purification of phosphodiesterase.

Snake venoms are known to contain at least four enzymes involved in the hydrolysis of phosphate bonds (Georgatsos and Laskowski, 1962; Sulkowski *et al.*, 1963). For the purpose of isolating a particular enzyme, it would be desirable to find a venom with a high concentration of that enzyme and a low concentration of the others. The problem of selecting the most desirable venom for the isolation of phosphodiesterase has become acute, since the *Bothrops atrox* venom formerly used for this purpose is no longer available in this country. The venoms of five species of snake have been assayed for endonuclease, phosphodiesterase, 5′-nucleotidase, and alkaline nonspecific phosphatase. The results indicate that with one exception the venoms have rather similar complements of enzymes.

In previous work on venom endonuclease, Georgatsos

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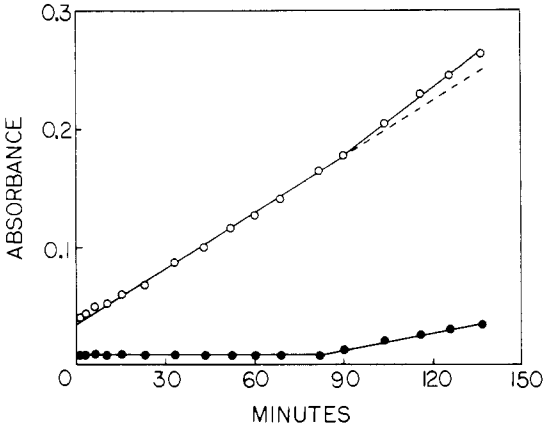

FIGURE 1: Determination of endonuclease in *Crotalus adamanteus* venom. The procedure followed was that of Kunitz (1950), modified as described in the experimental section. The solution assayed contained 1.85×10^{-2} mg of dry venom per ml. O—O, A_{260}; ●—●, A_{330}.

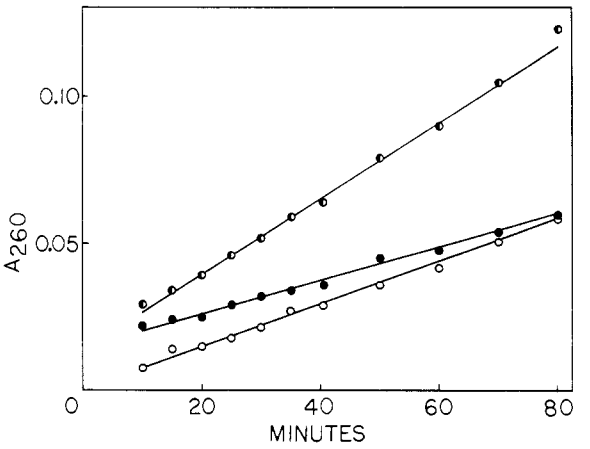

FIGURE 2: Effect of adding purified endonuclease to crude venom. Endonuclease activities were determined by the method of Kunitz (1950), modified as described in the experimental section. O—O, purified endonuclease, prepared according to Georgatsos and Laskowski (1962), 7.2×10^{-4} units/ml; ●—●, *Crotalus adamanteus* venom solution, 5.8×10^{-4} units/ml; ◐—◐, endonuclease plus venom, concentration of each component as before.

and Laskowski (1962) were unable to determine the level of this enzyme in crude venom by the spectrophotometric method of Kunitz (1950), because the addition of venom to a DNA solution at *p*H 5.0 caused a precipitate to form. It has been found that by reducing the amount of venom used in the assay, formation of a precipitate can be delayed beyond the time required for the determination of activity. This procedure for the assay of endonuclease is described in detail.

Experimental Procedure

DNA was prepared from calf thymus by the method of Kay *et al.* (1952). Dried or lyophilized samples of snake venom were obtained from the following sources: *Crotalus adamanteus* from Ross Allen's Reptile Institute, Silver Springs, Fla.; *Crotalus atrox* from Shellie Downs, Jr., Micanopy, Fla.; and *Agkistrodon piscivorus* from Miami Serpentarium Laboratories, Miami, Fla. *Bothrops atrox* venom had been obtained previously from Brazil through the courtesy of Dr. K. H. Slotta of Miami. *Naja nigricollis* venom was a gift from Dr. Paul Boquet, Institut Pasteur, Paris. Suspensions of each venom in water were prepared at a concentration of 14 mg/ml. After having been allowed to stand for 2–3 hours at 0°, the suspensions were centrifuged to remove undissolved material. The supernatant solutions were stored at 0° and were diluted with water as required for individual assays.

Phosphodiesterase, nonspecific phosphatase, and 5′-nucleotidase were determined essentially as described by Sulkowski *et al.* (1963). To determine phosphodiesterase, a reaction mixture containing 1.0 ml of 0.1 M Tris-HCl buffer, *p*H 8.9, 1.2 ml of 0.001 M calcium bis-*p*-nitrophenyl phosphate, diluted venom, and enough water to make a total of 3.0 ml was incubated in the spectrophotometer at 37°. The increase in A_{400} with time was read over a period of up to 30 minutes against a blank containing all components except venom.

The nonspecific phosphatase was determined in the same manner as phosphodiesterase, except that the reaction mixture contained 1.0 ml of 0.1 M glycine-NaOH buffer, *p*H 8.9, 1.2 ml of 0.001 M sodium *p*-nitrophenyl phosphate, 0.3 ml of 0.01 M $MgCl_2$, diluted venom, and water to make a total of 3.0 ml.

To determine 5′-nucleotidase, a reaction mixture containing 0.1 ml of 1.0 M glycine-NaOH buffer, *p*H 9.0, 0.1 ml of 0.01 M $MgCl_2$, 0.3 ml of 0.01 M AMP, diluted venom, and water to make a total of 1.0 ml was incubated at 37° for 20 minutes. The reaction was stopped by adding 4 ml of 10% trichloroacetic acid, and the liberated phosphate was determined by the method of Fiske and Subbarow (1925).

The dilutions and amounts of standard venom solution used in each assay were such that a maximum change of 0.02 A_{400} units per minute was obtained in the assays for phosphodiesterase and the nonspecific phosphatase, and a maximum A_{860} of 0.85 was obtained in the determination of 5′-nucleotidase. In each case, assays were also run on smaller amounts of venom to demonstrate that the proportional region of the assay methods had not been exceeded. The results obtained at 37° were converted to 25°, using factors of 0.44 for phosphodiesterase, 0.70 for the nonspecific phosphatase, and 0.25 for 5′-nucleotidase. Enzyme activities were expressed as μmoles of substrate transformed per minute at 25°, in accordance with the recommendations of the Commission on Enzymes (Report of the Commission, 1961). For all four enzymes (including endonuclease,

TABLE I: Levels of Enzymes in Five Snake Venoms.

Species of Venom	Units of Enzyme × 10^3 per mg Dry Venom				Ratio of Enzymes: Phosphodiesterase = 1.0		
	Phospho-diesterase	Nonspecific Phosphatase	5′-Nucleo-tidase	Endo-nuclease	Nonspecific Phosphatase	5′-Nucleo-tidase	Endo-nuclease
Agkistrodon piscivorus	7.8	0.55	495	3.3	0.070	64	0.42
Bothrops atrox	36	1.8	1070	3.4	0.050	30	0.095
Crotalus adamanteus	25	1.3	1390	8.8	0.052	56	0.35
Crotalus atrox	8.7	0.39	860	2.8	0.045	99	0.32
Naja nigricollis	17	54	360	1.1	3.2	21	0.065

further on), specific activity was defined as units of activity per mg of dry venom.

Endonuclease was determined as follows: the substrate consisted of DNA, 0.04 mg/ml in 0.2 M Na acetate buffer, *p*H 5.0. Three ml of substrate and 1 ml of enzyme (venom) were mixed and transferred to the spectrophotometer, where they were incubated at 37°. Changes in A_{260} and A_{330} were followed against a blank of substrate and water until A_{330} began to increase at a substantial rate. If this occurred before enough points had been recorded to determine a straight line for A_{260} against time (see Figure 1), the assay was repeated using a more highly diluted venom. Satisfactory results were obtained with dilutions of between 1:75 and 1:110, under which conditions A_{330} remained relatively stable for between 1 and 2 hours. A unit of endonuclease was defined as the amount of enzyme giving an increase in absorbance at 260 mμ of 1.0/minute.

The assay of endonuclease in *Crotalus adamanteus* venom by the procedure just described is illustrated in Figure 1. The onset of the development of turbidity in the solution is clearly indicated by the break in the curve for A_{330}, and a corresponding break in the curve for A_{260} is also visible. Activity was calculated from the portion of the A_{260} curve obtained before A_{330} began to rise. In certain other cases, A_{330} increased very slowly throughout the course of the measurement, with or without showing a break at some point. In such cases, the slope of A_{330} was subtracted from the slope of A_{260} under the assumption that the loss of light by scattering was approximately the same at the two wavelengths.

To test the validity of the method, a solution of purified endonuclease from *Bothrops atrox* venom (Georgatsos and Laskowski, 1962) was assayed by the original method of Kunitz (1950) and was found to have an activity of 1.47×10^{-2} units/ml. After 25-fold dilution, the activity measured by the modified method was 6.8×10^{-4} units/ml, 15% higher than the value expected on the basis of dilution. Figure 2 shows that when 7.2×10^{-4} units of purified endonuclease was added to a solution of *Crotalus adamanteus* venom containing 5.8×10^{-4} units of activity, the resulting mixture had a total activity of 13.2×10^{-4} units, a value closely comparable to the expected 13.0×10^{-4} units. A unit of enzyme in venom, measured as described, is therefore approximately equivalent to a unit of purified enzyme, measured according to Kunitz.

Results and Discussion

The levels and relative amounts of the four enzymes in each of the five venoms are presented in Table I. These data show that the venom of *Naja nigricollis*, a cobra, is unusually high in the nonspecific phosphatase. Since this enzyme is extremely difficult to separate from phosphodiesterase, *Naja* venom can be ruled out as a source for the isolation of the latter enzyme. This venom is also poorest in 5′-nucleotidase and endonuclease. In the four remaining venoms, all obtained from members of the pit viper family, the levels of the different enzymes vary in roughly the same manner from venom to venom. *Bothrops* and *C. adamanteus* are relatively rich in enzymes, while *Agkistrodon* and *C. atrox* are relatively poor, but by factors of less than 4.6. Since the ratios of phosphodiesterase to phosphatase in these four venoms are quite similar, a decision as to which venom represents the most suitable starting material for the preparation of phosphodiesterase depends primarily on the availability of the venom in large quantities. It is possible, however, that a given preparative procedure, such as that described by Björk (1963) for phosphodiesterase in *Bothrops atrox* venom, will give different results when applied to the other venoms.

References

Björk, W. (1963), *J. Biol. Chem. 238*, 2487.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.

Georgatsos, J. G., and Laskowski, M., Sr. (1962), *Biochemistry 1*, 288.

Kay, E. R. M., Simmons, N. S., and Dounce, A. L. (1952), *J. Am. Chem. Soc. 74*, 1724.

Kunitz, M. (1950), *J. Gen. Physiol. 33*, 349.

Report of the Commission on Enzymes of the International Union of Biochemistry (1961), New York, Pergamon, p. 10.

Sulkowski, E., Björk, W., and Laskowski, M., Sr. (1963), *J. Biol. Chem. 238*, 2477.