Vol. 97, No. 2, 1980
November 28, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 649-653

TOXINS EXTRACTED FROM AN ALASKAN ISOLATE OF *PROTOGONYAULAX* SP.

S. Hall, P. B. Reichardt, and R. A. Neve'

Institute of Marine Science and Department of Chemistry
University of Alaska, Fairbanks, Alaska 99701

Received October 15,1980

SUMMARY: Analysis of an aqueous acetic acid extract of *Protogonyaulax* sp. clone P107 from Porpoise Islands, Alaska, revealed four substances herein designated B1, B2, C1, and C2. These have relatively low toxicity until they are converted, on exposure to low pH, to saxitoxin, neosaxitoxin, gonyautoxin 2, and gonyautoxin 3, respectively which increases their toxicities by factors of about 10, 6, 20, and 5. Neosaxitoxin, gonyautoxin 1, and gonyautoxin 4 were found, but in smaller quantities. Saxitoxin, gonyautoxin 2, and gonyautoxin 3, if present, were below the levels that could be detected with confidence.

INTRODUCTION: Shellfish in Alaska are occasionally toxic (1-5). Past studies have left in doubt both the origin (6-9) and chemical nature (10-13) of this toxicity. We have begun to re-examine the problem by culturing dinoflagellates from the Pacific coast to study their growth and toxin production and have completed, to date, over 30 analyses of 11 isolates from 6 locations, ranging from San Francisco to the Aleutian Islands. We report here the toxin analysis of an extract of one isolate. It is of particular interest that four of the component toxins undergo facile conversion under relatively mild conditions to yield previously described toxins, and that the toxicity of these components prior to conversion is greatly suppressed.

EXPERIMENTAL: *Protogonyaulax* sp. (14) clone PI07 from Porpoise Islands, Alaska (58° 19.32'N, 135° 26.6'W) was grown in a 500L batch of "f/2" enriched seawater medium (15), under continuous fluorescent illumination. During early log phase growth, at a density of 2000 cells/mL, the cells were filtered from a 100L aliquot. The resulting slurry was centrifuged briefly and the packed cells extracted with 170mL 1M aqueous acetic acid in 5 portions. The combined supernatants were freeze-dried to 0.59 g solids and rehydrated with 3.0mL water to an opaque, reddish, slightly viscous suspension, pH 4.5. A 1.0mL aliquot of this suspension was applied to a glass column which had been packed with Bio-Gel P-2 (Bio-Rad Laboratories) and equilibrated with 0.1M acetic acid to give a bed 1.76 cm × 118.6cm. The column was eluted with 0.1M acetic acid at 12.9mL/h and fractions collected over 12.0min intervals, starting before the void volume eluted.

Aliquots of all fractions were applied (16) to a silica gel thin layer chromatography (TLC) plate (E. Merck), sprayed with 1% hydrogen peroxide, and heated for 15min at 120°C. Fractions giving fluorescent spots under 366nm UV were spotted along with standards of saxitoxin, neosaxitoxin, gonyautoxin 2, and gonyautoxin 3 onto aluminum-backed silica gel TLC plates (E. Merck). These were developed (13) in pyridine:ethyl acetate:acetic acid:water (15:5:3:4) for 3h, dried, checked for fluorescence, and visualized as above.

0006-291X/80/220649-05$01.00/0

Following this, similar fractions were pooled and two sets of 1.0mL aliquots removed for toxicity determinations by mouse assay (17), with minor variations from the standard method. The first set of aliquots was diluted with 2.2mL water. The second set was acidified with 0.1mL 4M HCl, heated for 5min in a 100°C bath, cooled, and diluted with 2.1mL water in the case of toxic fractions or, in the case of those fractions known from previous runs to contain little or no toxin, 2.0mL water and 0.1mL NaOH of a concentration adjusted to give a pH of 2.3 in the final 3.2mL solution. To determine what changes resulted from this treatment, about 2mL from each of the ten toxic groups from each set were freeze-dried and taken up in 0.2mL water. These, accompanied by spots from the groups without freeze-drying, were applied to TLC plates and run as above.

Aliquots of the original acetic acid extract and of the concentrated suspension applied to the column were similarly acidified, heated, and assayed, indicating at least 80% recovery from extraction to the start of chromatography.

RESULTS and DISCUSSION: Our results (Fig. 1) show four components we designate B1, B2, C1, and C2, accompanied by lesser amounts of neosaxitoxin (10), gonyautoxin 1 (18), and gonyautoxin 4 (10). Photography of the TLC plates under UV revealed faint spots, not detectable when the plates were viewed directly, corresponding to gonyautoxins 2 and 3 (19). Since trace conversion of C1 and C2 (see below) would account for the observed intensity, the presence of gonyautoxins 2 and 3 in the extract is uncertain. No saxitoxin (20) was detected in the extract. C1 and C2 may correspond to the "new toxins" encountered by Oshima (21) in a Japanese isolate of *Protogonyaulax*.

It was originally noted by Proctor (22) that cell-free extracts of *Protogonyaulax* increased in apparent toxicity after brief heating at low pH. We have found this observation essential to understanding the chemistry and manipulation of the toxins, and refer to the effect as "Proctor enhancement". Our preliminary experiments (23) indicated that the toxicity of cell extracts increased to an approximately constant value after heating at 100°C in 0.1M HCl for periods from 2.5 to 25min, or after 5min heating with HCl concentrations ranging from 0.1 to 1.0M. Lower concentrations of HCl did not suffice for complete conversion in 5min at 100°C, although the conversion occurs to some extent in HCl at room temperature over longer periods.

Assay of grouped fractions from this run showed that this treatment increased the toxicity of those containing primarily B1, B2, C1, and C2 by factors of about 10, 6, 20, and 5, respectively, while those containing primarily neosaxitosin and gonyautoxin 4 did not change significantly. The rather large increases in toxicity raise the interesting possibility that, for instance, C1 itself is not toxic, but that the observed toxicity is due to trace conversion once the solution has been diluted for assay.

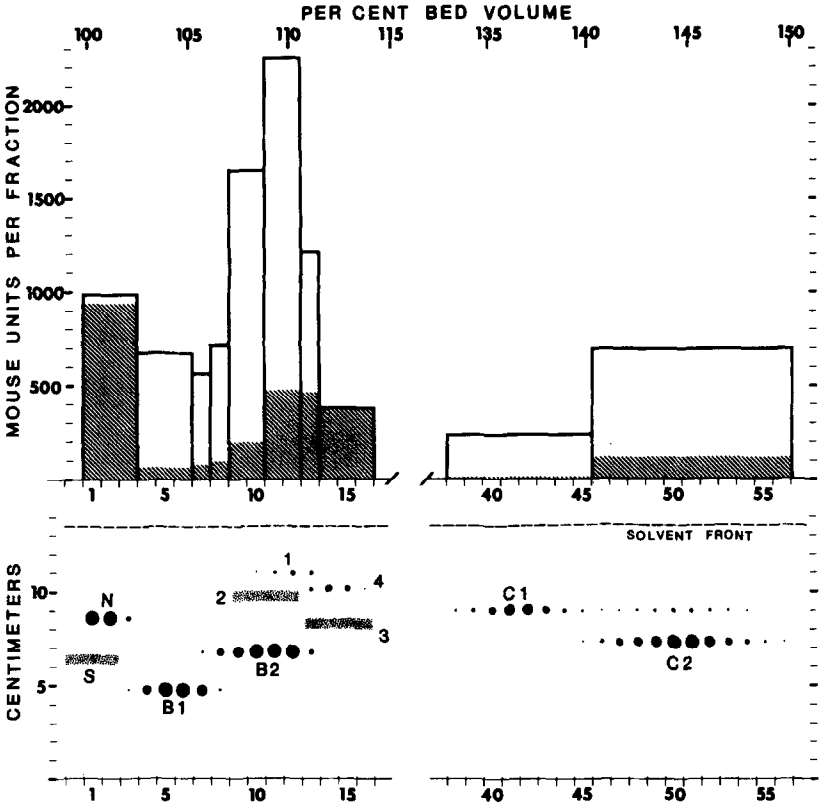

Fig. 1a (upper). Mouse assays of toxicity in grouped fractions before (hatched area) and after (outline) Proctor enhancement. The top axis shows elution volume expressed as per cent of the 228mL bed volume, from the start of sample application. This parameter appears to be highly reproducible in this system despite alterations in column geometry and is thus useful in characterizing the toxins. Fraction numbers along the bottom axis start with the first toxic fraction, at about 100% bed volume. Toxicity, the vertical axis, is in mouse units per fraction. These assays account for 95% of the 27,000 mouse units applied. The toxicity of fractions eluting before 100%, between 114 and 133%, and after 150% bed volume totalled less than 5% of that applied.

Fig. 1b (lower). TLC of individual fractions, positioned to correspond with the toxicity chromatogram above. Symbols: S = saxitoxin; N = neosaxitoxin; 1, 2, 3, 4 = gonyautoxins 1 through 4. The elution positions of S, 2, and 3, indicated by the stippled areas, were established in other runs. Spot size in this diagram was chosen to indicate relative fluorescent intensity under 366nm UV, rather than the size and shape seen on the TLC. The standards, interspersed with the fractions when the plates were run, are not shown. Units along the vertical axis are centimeters of travel after 3h development.

TLC indicated that the increases in toxicity correspond primarily to the conversion of B1 to saxitoxin, B2 to neosaxitoxin, C1 to gonyautoxin 2, and C2 to gonyautoxin 3, with slight interconversion between the resulting gonyautoxins 2 and 3. TLC revealed no change in neosaxitoxin or gonyautoxins 1 and 4. There is the possibility that other minor products result from these conversions, among them traces of what may be gonyautoxins 1 and 4, which

have been observed following the treatment of larger quantities of C1 or C2. It should be noted that the sequence followed does not exclude the possibility of some alteration during the freeze-drying of the HCl solutions, although this has been evaluated and appears to be merely partial conversion to the same products. There was little change due to the freeze-drying of the acetic acid solutions except in the case of B2, where some transformation to neosaxitoxin was apparent. It is thus likely that a portion of the neosaxitoxin observed in the analysis of P107 results from alteration of B2 during handling of the extract. Similarly, interconversion between the three pairs (gonyautoxins 1 and 4, gonyautoxins 2 and 3, C1 and C2) occurs to a small extent during handling, so the appearance of both does not necessarily indicate that each was present in the organism. Gonyautoxins 2 and 3 are known to be epimeric. From their behavior, such a relationship appears likely for the other two pairs.

The analysis reported here has been found reproducible in several runs under the same or slightly altered conditions and the toxins described, each giving a fluorescent spot on oxidation, appear to account for the bulk of the toxicity recoverable from the cells. However, the present data do not exclude the possibility of additional toxins with different properties. Conversely, we find at least one component that oxidizes to give a fluorescent spot, but has no detectable toxicity. The toxins B1, B2, C1, and C2 appear not to be artifacts of the procedure because similar analyses of other isolates give substantially different results (23).

While it remains to be demonstrated how close the array of toxins described here is to that actually produced by cells, the consistency of analyses within one clone, and the difference between clones, indicate that the method provides a useful representation of the natural assembly.

ACKNOWLEDGMENTS: This work was supported by the U.S. Food and Drug Administration and the Alaska Sea Grant Program, cooperatively supported by NOAA Office of Sea Grant, U.S. Department of Commerce, under Grant 04-8-MO1-187 and by the University of Alaska with funds appropriated by the State of Alaska. The authors wish to thank G. J. Mueller for many useful discussions, G. L. Boyer for supplying toxin standards, and F. J. R. Taylor for comments on taxonomy.

REFERENCES

1. Anon. (1976) Morbidity and Mortality Weekly Report **25**, 383-384.
2. Fortuine, R. (1975) Alaska Med. **17**, 71-76.
3. Meyers, H. F., and Hilliard, D. K. (1955) Public Health Reports **70**, 419-420.
4. Orth, F. L., Smelcer, C., Feder, H. M., and Williams, J. (1975) Alaska Sea Grant Program Report No. 75-5, University of Alaska, Fairbanks.
5. Zimmerman, S. T., and McMahon, R. S. (1976) Fishery Bull., Fish Wildl. Serv. U.S. **74**, 679-680.

6. Chang, J. C.-C. (1971) M.S. Thesis, University of Alaska, Fairbanks.
7. Neal, R. A. (1967) Ph.D. Thesis, University of Washington, Seattle.
8. Schantz, E. J. (1960) Ann. N.Y. Acad. Sci. **90**, 843-855.
9. Schantz, E. J., and Magnussen, H. W. (1964) J. Protozool. **11**, 239-242.
10. Oshima, Y., Buckley, L. J., Alam, M., and Shimizu, Y. (1977) Comp. Biochem. Physiol. **57C**, 31-34.
11. Schantz, E. J., Lynch, J. M., Vayvada, G., Matsumoto, K., and Rapoport, H. (1966) Biochemistry **5**, 1191-1195.
12. Schantz, E. J., Mold, J. D., Stanger, D. W., Shavel, J., Riel, F. J., Bowden, J. P., Lynch, J. M., Wyler, R. S., Riegel, B., and Sommer, H. (1957) J. Am. Chem. Soc. **79**, 5230-5235.
13. Shimizu, Y., Fallon, W. E., Wekell, J. C., Gerber, D., Jr., and Gauglitz, E. J., Jr., (1978) J. Agr. Food Chem. **26**, 878-881.
14. Taylor, F. J. R. (1979) *in* Devel. Mar. Biol. **1**, Toxic Dinoflagellate Blooms, Taylor, D. L., and Seliger, H. H., Eds., Elsevier North Holland, New York, pp. 47-56.
15. McLachlan, J. (1973) *in* Handbook of Phycological Methods- Culture Methods and Growth Measurements, Stein, J. R., Ed., Cambridge, New York, pp. 25-51.
16. Buckley, L. J., Ikawa, M., and Sasner, J. J., Jr. (1976) J. Agr. Food Chem. **24**, 107-111.
17. Anon. (1975) Official Methods of Analysis, 12th ed., revised, A.O.A.C., Washington, D.C., pp. 319-321.
18. Shimizu, Y., Alam, M., Oshima, Y., and Fallon, W. E. (1975) Biochem. Biophys. Res. Commun. **66**, 731-737.
19. Boyer, G. L., Schantz, E. J., and Schnoes, H. K. (1978) Chem. Commun. 889-890.
20. Schantz, E. J., Ghazarrossian, V. E., Schnoes, H. K., Strong, F. M., Springer, J. P., Pezzanite, J. O., and Clardy, J. (1975) J. Am. Chem. Soc. **97**, 1238-1239.
21. Oshima, Y., and Yasumoto, T. (1979) *in* Devel. Mar. Biol. **1**, Toxic Dinoflagellate Blooms, Taylor, D. L., and Seliger, H. H., Eds., Elsevier North Holland, New York, pp. 377-380.
22. Proctor, N. H. (1973) Ph.D. Thesis, University of California, San Francisco.
23. Hall, S., unpublished data.