a range from 0 to 400 μg of protein (Fig. 3). There was a profound diminution of activity of the sphingomyelin-cleaving enzyme in preparations from patients with Niemann-Pick disease compared with those obtained from normal human beings and patients with various other disorders (Table 2). Although we studied only three patients with Niemann-Pick disease the mean value of sphingomyelin-cleaving enzyme in the control series was 4.4 units per milligram of protein, whereas it was 0.36 unit per milligram of protein for patients with Niemann-Pick disease. This difference in mean is highly significant (*8*). The specificity of this assay is indicated by the fact that enzyme activity in the leukocyte preparations obtained from patients with other sphingolipodystrophic conditions, such as Gaucher's disease and Fabry's disease, was normal. Thus, our techniques should be useful in the diagnosis of Gaucher's disease and Niemann-Pick disease. Whether these procedures can be used to detect heterozygous carriers of these diseases remains to be seen.

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References and Notes

1. R. O. Brady, J. N. Kanfer, D. Shapiro, *Biochem. Biophys. Research Commun.* **18**, 221 (1965); R. O. Brady, J. N. Kanfer, R. M. Bradley, D. Shapiro, *J. Clin. Investigation* **45**, 1112 (1966).
2. R. O. Brady, J. N. Kanfer, M. B. Mock, D. S. Fredrickson, *Proc. Nat. Acad. Sci. U.S.* **55**, 366 (1966).
3. R. O. Brady, A. E. Gal, J. N. Kanfer, R. M. Bradley, *J. Biol. Chem.* **240**, 3766 (1965); J. N. Kanfer, O. M. Young, D. Shapiro, R. O. Brady, *ibid.* **241**, 1081 (1966).
4. R. O. Brady and R. M. Bradley, unpublished observations.
5. Z. A. Cohn and J. G. Hirsch, *J. Exp. Med.* **112**, 983 (1960).
6. R. O. Brady, J. N. Kanfer, D. Shapiro, *J. Biol. Chem.* **240**, 39 (1965).
7. J. P. Kampine, R. O. Brady, R. A. Yankee, J. N. Kanfer, D. Shapiro, A. E. Gal, in preparation.
8. R. A. Fisher, *Statistical Methods for Research Workers* (Hafner, New York, ed. 13, 1958).
9. A portion of this work was performed under Section 104(K) of Public Law 480, 83rd Congress, Agreement No. 42515. We thank Dr. D. S. Fredrickson of NHI for his interest and assistance in obtaining some of the blood samples.

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D-Gluconic Acid: Isolation from the Defensive Secretion of the Cockroach Eurycotis decipiens

Abstract. *The major water-soluble constituent of the defensive secretion of* Eurycotis decipiens *was identified as gluconic acid, isolated in the form of calcium* D-*gluconate. The acid, in equilibrium with its lactones, is present in unusually high concentration.*

Adults of *Eurycotis decipiens* (Kirby) have a large defensive gland which opens through the intersegmental membrane between the sixth and seventh sternites. Except for its white color, the large secretion-filled reservoir is similar in appearance and position to the gland which is found in *Eurycotis floridana* (Walker) (*1*) and which produces and stores 2-hexenal (*2*) for defensive purposes.

The aqueous, acidic, milky fluid (0.58 ml) from 72 specimens of *E. decipiens* (*3*) was dissolved in methanol. Volatiles which included 2-hexenal (about 5 percent) (identified by infrared spectra and gas-liquid chromatography) were removed in a vacuum. After extraction with methylene chloride, the residue (39.6 mg) was dissolved in methanol (1 ml). The infrared spectrum of a disc obtained from a lyophilized aqueous potassium bromide solution of the methanol-soluble fraction was similar (essentially identical from 1900 to 500 cm^{-1}) to that derived from an equilibrated aqueous solution of δ-D-gluconolactone. A chromatogram of the methanol-soluble fraction was developed with a mixture of butanol, acetic acid, and water (4:1:5) and sprayed with silver nitrate in acetone and then alcoholic sodium hydroxide. By comparison with chromatograms of known materials the pattern of spots could be interpreted in order of increasing mobility as an *O*-gluconylgluconic acid (*4*), δ-gluconolactone and gluconic acid, methyl gluconate, and γ-gluconolactone. Methyl gluconate was an artifact of storage in methanol. Gas-liquid chromatography (*5*) of material subjected to trimethylsilylation indicated the presence of a gluconolactone but not of glucose.

The gluconic acid fraction was passed through a cation exchange resin (H^+ form). After the effluent was treated with calcium carbonate, calcium D-gluconate monohydrate ($[\alpha]_D^{28} = +5°$) was isolated by crystallization from water and identified by comparison of infrared spectra (Fig. 1). The D-gluconic acid, in equilibrium with its lactones, is a major constituent (6.8 to 8.2 percent, weight to volume) of the secretion. After *Eurycotis decipiens* ejects its secretion, the volatiles, 2-hexenal and water, evaporate and leave a mixture of gluconic acid and lactones as a residue. The secretion effectively repelled the fire ant (*6*), probably because of the presence of 2-hexenal.

The compound 2-hexenal has previously been identified in the defensive secretions of Hemiptera, cockroaches, and an ant (*7*).

Aldonic acids have not been found previously in plants or animals in appreciable amounts. L-Arabonic acid has been isolated from gum arabic (*8*) and from *Austrocedrus chilensis* (D. Don), Florin and Boutelje (*9*). Gluconic acid (about 0.18 percent by weight) occurs in pasteurized honey (*10*) where it originates in a honey

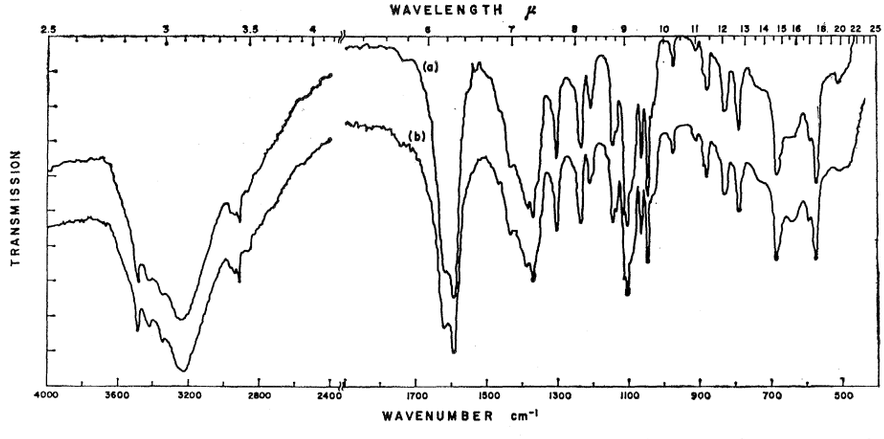

Fig. 1. Infrared spectra of calcium D-gluconate monohydrate (KBr disc). Curve *a* is the spectrum of the salt obtained from the methanol-soluble fraction of *E. decipiens* secretion. Curve *b* is that of an analytically pure sample from the recrystallization of commercial material.

glucose-oxidase system (*11*). The occurrence of D-gluconic acid in *E. decipiens* is another example of a well-known substance that occurs in unusually high concentration in an arthropod defensive secretion.

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References and Notes

1. B. Stay, *Ann. Entomol. Soc. Amer.* **50**, 514 (1957).
2. L. M. Roth, W. D. Niegisch, W. H. Stahl, *Science* **123**, 670 (1956).
3. The secretion was collected from each adult in a centrifuge tube fashioned from a graduated, thick-walled, 4-mm capillary fused to 22-mm or larger tubing. The insect was placed with its abdomen toward the bottom of the tube and was then anesthetized with CO_2. As the cockroach became anesthetized it ejected its defensive secretion into the tube which was then centrifuged prior to the next "milking."
4. A tentative identification of a slow-moving trace component also found in aged, concentrated aqueous solutions of authentic δ-gluconolactone [see H. S. Isbell and H. L. Frush, *Methods Carbohydrate Chem.* **2**, 16 (1963)].
5. C. C. Sweeley, R. Bentley, M. Makita, W. W. Wells, *J. Amer. Chem. Soc.* **85**, 2497 (1963).
6. We thank E. O. Wilson for the use of his fire ant colony.
7. L. M. Roth and T. Eisner, *Ann. Rev. Entomol.* **7**, 107 (1962); T. Eisner and J. Meinwald, *Science* **153**, 1341 (1966).
8. R. H. Schleif, T. Higuchi, L. W. Busse, *J. Amer. Pharm. Assoc., Sci. Ed.* **40**, 98 (1961).
9. A. Assarsson, B. Lindberg, H. Vorbrüggen, *Acta Chem. Scand.* **13**, 1395 (1959).
10. E. E. Stinson, M. H. Subers, J. Petty, J. W. White, Jr., *Arch. Biochem. Biophys.* **89**, 6 (1960).
11. J. W. White, M. H. Subers, A. I. Schepartz, *Biochim. Biophys. Acta* **173**, 57 (1963).

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Clostridium botulinum Type F: Seasonal Inhibition by Bacillus licheniformis

Abstract. Clostridium botulinum *type F has been identified during the summer months in mud samples from a small stream. Its absence during the period from October to April in these mud samples is attributed to the presence of* Bacillus licheniformis.

During the summer, a survey of sewage lagoons and adjacent streams in eastern North Dakota produced two samples (from a sewage lagoon and a small stream) which yielded cultures producing *Clostridium botulinum* type F toxin. During the fall and winter months the organism could not be identified (as determined by toxicity assays) from mud samples obtained from the small stream. It was determined that *Bacillus licheniformis* was present during these periods and was at least partially responsible for the inhibition of growth and hence toxin production of *Clostridium botulinum* type F.

Previous isolations or identifications of *Cl. botulinum* type F have been few (*1*), and it is altogether possible that seasonal or other variations occur in areas other than reported here. Other workers have noted that certain organisms will inhibit *Cl. botulinum* growth and toxin production, such as *Cl. sporogenes (2), Bacillus sphaericus* (*3*), *Brevibacterium linens* (*4*), and *Escherichia coli* and *Streptococcus faecalis* (*3*).

Mud samples which yielded *Cl. botulinum* type F toxin (or no toxicity) were placed in 1-g amounts into 9 ml of sterile, previously boiled and cooled, brain heart infusion (BHI) broth (Difco). All tubes were incubated in a Brewer anaerobic jar at 30°C for 36 to 40 hours. The supernatant of the culture (4340*g* for 10 minutes) was injected into 6-week-old Swiss mice in 0.4-ml amounts by the intraperitoneal route. The animals were observed for periods up to 5 days; however, all deaths attributable to toxin occurred within 2 days.

The toxin (0.4 ml of the culture supernatant) was identified by injection into mice that had been protected with antitoxin to *Cl. botulinum* types A, B, C, D, E, or F or tetanus (see Table 1). Heated supernatant alone (100°C for 10 minutes) failed to kill mice.

Mud samples obtained in June, July, and August were positive for *Cl. botulinum* type F, whereas samples obtained any time between October and May were negative. These negative samples were inoculated with a toxin-producing strain of *Cl. botulinum* type A (American Type Culture Collection No. 7948) and subsequently cultured as described above. No deaths occurred when culture supernatant was injected into mice, while controls with pure cultures of *Cl. botulinum* type A killed mice and showed type-specific neutralization with antitoxin (Table 2).

Table 1. Neutralization pattern of cultures identified as *Clostridium botulinum* type F. Results are given as the number of mice that died out of the number tested. S, centrifuged supernatant of cultured mud samples; HS, heated supernatant (100°C for 10 minutes).

Source of toxin	Antitoxin	Results
S	None	15/15
S	ABCDE	20/20
S	F	0/10
S	Tetanus	4/4
HS	None	0/8

Serially diluted cultures of these negative mud samples yielded pure cultures of a variety of organisms; of these only *B. licheniformis* inhibited the growth and toxin production of *Cl. botulinum* type A when the two organisms were cultured together in BHI broth. *Bacillus licheniformis* had no effect on the preformed toxin, on the basis of the fact that supernatants of 36-hour cultures of *Cl. botulinum* type A remained toxic after extended incubation in fresh medium with *B. licheniformis*. Mixed cultures of the above organisms (when *Cl. botulinum* type A was initially present in concentrations of 1.6×10^3 organisms or less per milliliter) resulted in nontoxic cultures, provided that *B. licheniformis* was initially present in concentrations above 7.3×10^3 organisms per milliliter (Table 3).

Bacillus licheniformis produces the antibiotic, bacitracin (*5*), which is probably responsible for the growth-inhibiting phenomenon observed here. It is not known why *B. licheniformis* is

Table 2. Inhibitory effect of mud on toxin production by *Cl. botulinum* type A. Results are given as the number of mice that died out of the number tested.

Media inoculated with	Antitoxin	Results
Cl. botulinum type A	None	6/6
Cl. botulinum type A	A	0/6
Cl. botulinum type A + mud	None	0/6
Mud alone	None	0/6

Table 3. Inhibition of toxin production by *B. licheniformis*. Results are given as the number of mice that died out of the number tested. The media was inoculated with 1.6×10^3 *Cl. botulinum* type A per milliliter and various amounts of *B. licheniformis*.

B. licheniformis (cells/ml)	Results
7.3×10^6	0/3
7.3×10^5	0/3
7.3×10^3	4/6
7.3×10^1	3/6
0	6/6