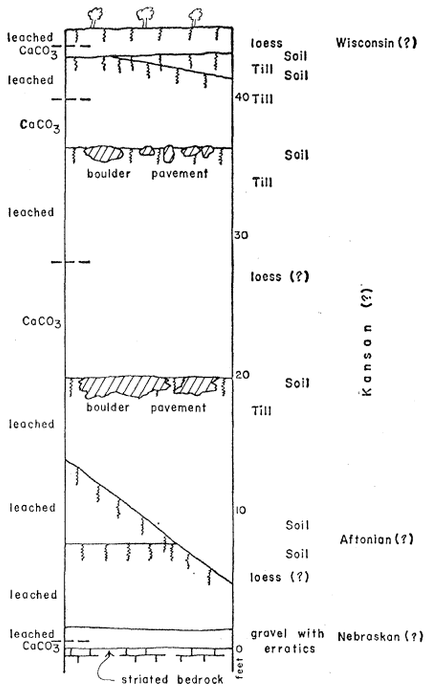

Fig. 1. Diagrammatic columnar section of glacial deposits and weathering zones exposed in a quarry near Doniphan, Kansas. Hatched areas are boulders and masses of shattered, transported rock from nearby sources.

or black in areas that were poorly drained, is the early Kansan (Nickerson) till. This is said to be the most extensive of the till sheets present in Nebraska and Kansas. Its upper surface locally shows development of a thin soil (Fontanelle), which is in turn overlaid in some localities by the medial Kansan (Cedar Bluffs) till, which is deeply oxidized in its upper part. The upper Kansan (Clarkson) till, previously considered to be of early Illinoian age, is present only in northeastern Nebraska.

The record of stadial subdivisions of Early Pleistocene glaciations in Kansas (*2*) is more complex than that now recognized in Nebraska. It is also more precise in that the data come from a single, truly remarkable exposure, thereby escaping some of the inevitable doubts that accompany interpretive correlation of separate parts from several different localities.

In a single vertical section 13.7 m high and 33.5 m wide at one end of an abandoned quarry near Doniphan, 8 km north of Atchison, Kansas (Fig. 1), there is exposed a sequence of four tills plus one gravel that contains erratic rock fragments. Separating these stratigraphic units, and indicating episodes of nonglacial conditions, are six buried soils or weathering zones, readily recognizable by horizonation and color changes, as well as by alternations of carbonate-rich and carbonate-free (leached) zones. At the base of the section the erratic fragment–bearing gravel rests on a striated bedrock surface. Both the excellence of the exposure and clear contrasts in physical and chemical characteristics of the various horizons preclude confusion of slump blocks with true stratigraphic sequence.

Assignment of age designations to the five glacial members of this section is impossible by a numbers system admitting of only two glaciations that affected northeastern Kansas. A relatively thick, dark-gray, well-developed soil below the lowest till is believed to be the Aftonian soil. If this speculative correlation is correct, the Nebraskan glaciation is represented in this locality only by the striated bedrock surface and the very sparse scattering of fragments of igneous and metamorphic rock in the overlying gravel. The four tills and interbedded nonglacial sediments, together with the weathering zones, above the Aftonian(?) horizon would then be the result of four stadial advances of the Kansan glacier.

Of greatest significance in these discoveries is the clear demonstration of complexity in the Early Pleistocene glacial record and the present absence of recognized criteria that indicate with certainty the age of individual Early Pleistocene till bodies. If two tills are present in a single exposure, the upper is not necessarily of Kansan age, the lower not necessarily of Nebraskan. A series of till sheets resulting from stadial fluctuations may occur in unusually well-preserved deposits from either glaciation. Therefore all assignments of Nebraskan or Kansan ages, or of both, to bodies of glacial, proglacial, or periglacial sediments must be reexamined in the context of multiple stades.

A corollary is that any fossils and any geologic or climatic events that have been assigned ages based solely on association with an Early Pleistocene till also must be restudied within the complex framework that current field investigations are revealing. Furthermore, the problem affects not only areas in which Early Pleistocene sediments are exposed at the surface, but also areas of subsurface occurrences beneath younger Pleistocene deposits.

WAKEFIELD DORT, JR.
Department of Geology,
University of Kansas, Lawrence

References and Notes

1. E. C. Reed and V. H. Dreezen, *Nebraska Geol. Surv. Bull. 23* (1965); E. C. Reed *et al.*, in *The Quaternary of the United States*, H. E. Wright and D. Frey, Eds. (Princeton Univ. Press, Princeton, N.J., 1965), pp. 187–202.
2. W. Dort, Jr., *Abstr. Geol. Soc. Amer. Ann. Mtg. 1965*, p. 46; in preparation.
3. Work aided by the State Geological Survey of Kansas and by the University of Kansas General Research Fund.

2 September 1966

Staphylococcal Alpha-Toxin: Effects on Artificial Lipid Spherules

Abstract. *Staphylococcal alpha-toxin induces the release of previously sequestered anions or glucose from artificial phospholipid spherules, an effect abolished by specific antitoxin. Alpha-toxin resembles streptolysin S in releasing anions or glucose from spherules prepared without cholesterol, and can be distinguished from the membrane-active polyene amphotericin B, which preferentially disrupts spherules containing cholesterol. It may affect biological structures by a similiar interaction with membrane phospholipids.*

Staphylococcal α-toxin, a protein of approximately 44,000 molecular weight, can be isolated in relative purity from culture filtrates; it induces hemolysis in vitro, causes necrosis of skin in vivo, and is lethal to small laboratory animals (*1*). It also disrupts platelets and lysosomes in vitro and is cytotoxic for cultured mammalian cells (*2*).

These effects have been attributed to a primary action of the toxin upon membranes which bound cells, their organelles, or both (*1, 3*). However, the identity of the component of the membranes of cells or their organelles with which the toxin reacts has not been established. Streptolysin S, an analogous bacterial exotoxin, destroys lysosomes and erythrocytes by virtue of its capacity to disrupt their limiting membranes, and it promotes changes in the gross permeability to ions, glucose, and glycine of artificial phospholipid-cholesterol spherules by disrupting their lamellar substructure (*4*). Streptolysin S produces these effects by an interaction with lecithin, or with long-chain polar lipids, rather than with membrane sterols. Its behavior was opposite to that of amphotericin B (a polyene antibiotic), which preferentially disrupted natural or artificial lipid structures containing sterols (*5*).

In order to clarify the mechanism of action of α-toxin, its effect was tested on artificial lipid spherules that are reasonable models of natural membranes (*4*). We varied the ratio of phospholipid to cholesterol in the target spherules in order to determine whether α-toxin resembled streptolysin S in not requiring sterol to disrupt lipid structures, or whether it resembled amphotericin B in being cholesterol-dependent.

The staphylococcal α-toxin (*6*) was used in the form of stock solutions containing 10,000 hemolytic units per milliliter (0.6 mg of protein per milliliter of approximately 70-percent purity) in 0.03*M* borate buffer, *p*H 8.2. Artificial phospholipid spherules (*4*) of varying lipid composition were prepared as follows. Ovolecithin, cholesterol, dicetyl phosphate, or stearylamine (in molar ratios described below) were dissolved in chloroform, dried in a vacuum to remove solvent, and permitted to "swell" in 0.145*M* or 0.290*M* solutions of K_2CrO_4, KH_2PO_4, or glucose for 6 hours at 23°C. Anions or glucose not trapped within the spherules (15 μmole of lipid per milliliter) were removed by dialysis against 12 changes (900 ml each) of an isotonic mixture of NaCl and KCl. Dialysis was facilitated by rotating the spherule-filled dialysis sacs (6 ml of suspension to 900 ml dialyzate) from the bar of an automatic tissue-processing unit for 18 hours at 23°C. The dialyzed spherules (1-ml portions), now containing only sequestered ions or glucose, were dispensed into smaller dialysis sacs to which 0.05 ml of

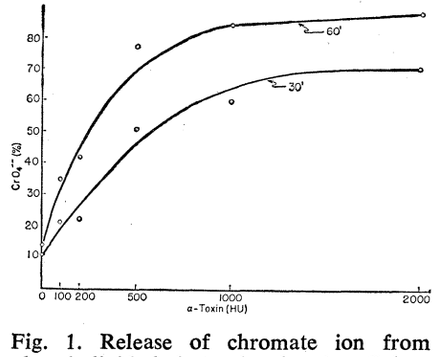

Fig. 1. Release of chromate ion from phospholipid-cholesterol spherules. Spherules (a mixture of ovolecithin, dicetyl phosphate, and cholesterol in molar ratios of 70 : 20 : 10) were incubated for 30 and 60 minutes with increasing amounts of staphylococcal α-toxin (37°C). Release of divalent anion is expressed as percentage of total anion (CrO_4^{--}) trapped by the lipid spherules (15 μmole of lipid per milliliter).

α-toxin, streptolysin S or amphotericin B were added. To control samples, 0.05 ml of appropriate solvent was added as follows: borate buffer for α-toxin, saline for streptolysin S, dimethyl sulfoxide for amphotericin B. To determine the maximum amount of anion or glucose that could be released, one sample of each set was incubated with Triton X-100 (0.2 percent by volume), a detergent which physically disrupts the spherules (*4*). Leakage of anions or glucose from the spherules, through the sacs, into smaller test tubes was measured at 30 and 60 minutes.

Spherules were prepared with molar ratios as follows: ovolecithin, 70; dicetyl phosphate, 20; cholesterol, 10; the release of CrO_4^{--} was determined at 30 and 60 minutes after addition of increasing amounts of α-toxin (Fig. 1). Above 500 hemolytic units per milliliter (0.03 mg/ml), or at 35.4 hemolytic units per micromole of lipid, the release of anion induced by α-toxin approached a plateau. The toxin was so active that at high concentrations it released almost as much chromate ion as Triton X-100 did (Table 1). Incubation of spherules which had been neutralized by antitoxin resulted in abolition of the toxin's capacity to provoke anion release (Table 1). Antitoxin alone, at protein concentrations which equalled or exceeded those of the toxin, had no significant effect upon release of CrO_4^{--}. These experiments were repeated with a monovalent anion of approximately equivalent hydrated radius, $H_2PO_4^-$, as marker (Fig. 2). Increasing amounts of the toxin released progressively more monovalent anion. Its activity approximated that of streptolysin S, when the concentrations of both were expressed as hemolytic units per unit volume. These experiments indicated that α-toxin has the capacity to interact with phospholipid-cholesterol spherules to induce gross changes in their permeability to mono- and divalent anions. Furthermore, the activity of toxin preparations was neutralized by specific antibody.

Spherules were prepared to contain glucose (0.145*M*), and the sign of the charged component within the membranes was varied, while cholesterol and ovolecithin were kept constant. Toxin released comparable amounts of glucose from positively and negatively charged membranes (Table 2, *a* and *b*). Thus, the toxin altered the permeability of spherules to both

Table 1. Effect of staphylococcal α-toxin and its antibody on release of CrO_4^{--} from artificial lipid spherules. Suspensions of spherules containing 15 μmole of lipid per milliliter were prepared with molar ratios of ovolecithin, 70: dicetyl phosphate, 20: cholesterol, 10. These had trapped a "total" of 7.79 μmole of CrO_4^{--} per milliliter of lipid suspension; results are expressed as percentages of this amount of anion released at 30 and 60 minutes.

Agent	CrO_4^{--} released	
	30 min (%)	60 min (%)
Control	18.5	24.5
α-Toxin *	47.2	71.1
Antitoxin †	20.1	26.4
α-Toxin + antitoxin ‡	14.1	24.6
Triton X-100 §	61.7	89.0

* 1000 hemolytic units. † 0.1 ml (100 units). ‡ Antitoxin, 0.1 ml (100 units). § Triton X-100.

charged and uncharged molecular species (anions and glucose), and the effects were independent of the net surface charge on the spherules. Positively charged structures both trapped more and released less glucose than did negatively charged spherules, thereby indicating that they were less permeable to glucose. When glucose was sequestered from isoosmotic solutions rather than from equimolar ones (Table 2, *c*), α-toxin still released glucose in amounts proportional to those released from spherules swollen in 0.145*M* glucose.

Streptolysin S, Triton X-100, and membrane-active steroids release comparable amounts of anions or glucose from spherules prepared with and without cholesterol (*4*, *5*). In contrast, amphotericin B releases fewer markers from spherules prepared in the absence of cholesterol (*5*). Therefore, whereas streptolysin S, Triton X-100, and the steroids react with phospho-

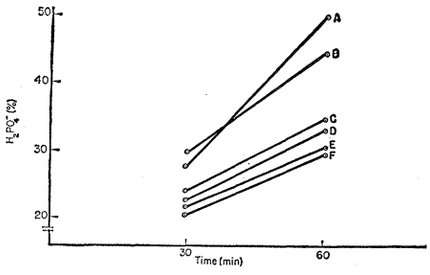

Fig. 2. Release of phosphate ion from phospholipid-cholesterol spherules. Spherules (as in Fig. 1) incubated for 30 and 60 minutes with increasing amounts of staphylococcal α-toxin. SLS, streptolysin S. *A*, 2000 HU of SLS; *B*, 2000 HU of α-toxin; *C*, 1000 HU of α-toxin; *D*, 500 HU of α-toxin; *E*, 200 HU of α-toxin; *F*, control (HU, hemolytic unit).

lipids or long-chain polar lipids, amphotericin preferentially reacts with membrane sterols, as suggested earlier (*7*). To evaluate the role of cholesterol in the action of α-toxin, spherules were prepared in the presence and absence of 10 percent cholesterol. Whereas streptolysin S released equivalent amounts of anion from spherules prepared in either way, amphotericin B released far less CrO_4^{--} from spherules prepared in the absence of cholesterol (Fig. 3). Staphylococcal α-toxin resembled streptolysin S in action and also induced equivalent release of anion, whether or not cholesterol was present. The amounts of anions or glucose trapped did not differ in the two preparations.

Thus staphylococcal α-toxin, in reacting with spherules of purified lipids, produces large changes in their gross permeability to anions or glucose. If the structure of the spherules, which resemble natural lipid membranes in their response to lytic or disruptive agents, consists in aqueous dispersion of multiple, concentric, bimolecular leaflets of lipid, then release of marker ions or glucose may represent exchange diffusion of the small molecules across lipid membranes (*4*, *8*). Anions or glucose are considered to be sequestered in an aqueous phase between lamellae, over and above any *gegen-ion* trapping (*8*). α-Toxin rapidly induced leakage of the previously sequestered marker anions, or of glucose, approaching in its activity that of the nonionic detergent Triton X-100. Such alterations in overall permeability of the spherules cannot be due to nonspecific protein-lipid interactions, since neither antitoxin alone nor toxin-antitoxin mixtures provoked release of markers.

Several globular proteins can be described as possessing relatively hydrophilic or relatively hydrophobic areas (*9*). The principal cohesive forces between polar lipids in aqueous media are the London–van der Waals dispersion forces between adjacent CH_2 groups of adjacent fatty acids (*10*). Insertion of hydrophobic portions of a protein into lipid layers would presumably disrupt the lipid structure in a manner similar to that previously postulated for lysolecithin and streptolysin S (*4*, *8*). Large polyene molecules, such as amphotericin B, which have a macrolide ring with both rigid hydrophobic and more flexible hydrophilic portions might disrupt membranes in similar, but by no means identical, fashion. It is clear from our experiments, and from those reported earlier (*5*), that α-toxin and streptolysin S interact with phospholipids or long-chain polar lipids, whereas amphotericin B preferentially reacts with membranes which contain cholesterol. The direct action of α-toxin upon synthetic lipid membranes is sufficient to explain its effects on erythrocytes, platelets, lysosomes, and cultured cell lines. Indeed, disruption by the toxin of membranes which bound lysosomes, or the cell itself, may explain the intense tissue damage produced by this bacterial product. The elaboration by staphylococci, during natural infection, of a toxin which reacts with lipid components common to many biological systems could augment the microorganism's capacity to form lesions. Since α-toxin interacts with artificial spherules composed of lipids that occur in biological membranes, it is unnecessary to postulate a primary action on membrane protein, polysaccharide, or pathways in cell metabolism in order to explain its effects upon membrane-bound structures that exist in nature.

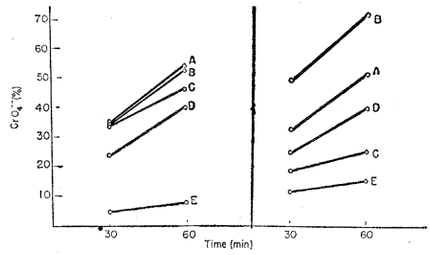

Fig. 3. Release of chromate ion from lipid spherules. Spherules were prepared as a mixture of ovolecithin, dicetyl phosphate, and cholesterol in a molar ratio (left) of 70 : 20 : 10, or as a mixture of ovolecithin and dicetyl phosphate in a molar ratio of 80 : 20. (Right) Leakage of divalent anion was measured 30 and 60 minutes after addition of (*A*) streptolysin S (SLS); (*B*) 0.2 percent (by volume) Triton X-100; (*C*) $10^{-4}M$ amphotericin B; or (*D*) α-toxin in borate buffer; (*E*) control.

Table 2. Effect of staphylococcal α-toxin on release of glucose from artificial lipid spherules of varying composition. Suspensions of spherules containing 15 μmole of lipid per milliliter were prepared with molar ratios indicated as follows, and given either a net negative charge by the incorporation of dicetyl phosphate or a net positive charge by the incorporation of stearylamine. The "total" number of micromoles of glucose trapped by each type of suspension is indicated below, and the results are expressed as the percentages of these amounts released at 30 and 60 minutes. The concentration of Triton (Triton X-100) was 0.2 percent by volume.

Glucose (*M*)	Releasing agent		Glucose released (% total)	
	Identity	Conc.	30 min	60 min
a. *Lecithin, cholesterol, dicetyl phosphate (70 : 10 : 20)*				
0.145	Control		24.7*	39.5
.145	Triton	0.2%	37.9	62.5
.145	α-Toxin	8000 HU	39.4	68.5
	α-Toxin	4000 HU	32.2	58.0
b. *Lecithin, cholesterol, stearylamine (70 : 10 : 20)*				
0.145	Control		7.7†	15.0
.145	Triton	0.2%	27.4	54.0
.145	α-Toxin	8000 HU	40.3	67.7
.145	α-Toxin	4000 HU	23.0	45.8
c. *Lecithin, cholesterol, dicetyl phosphate (70 : 10 : 20)*				
0.290	Control		31.3‡	46.8
.290	Triton	0.2%	41.9	66.2
.290	α-Toxin	2000 HU	37.9	61.6
.290	α-Toxin	1000 HU	34.8	57.0
.290	α-Toxin	500 HU	32.0	52.3

* Total amount trapped was 19.8 μmole per milliliter of lipid suspension. † Total amount trapped was 28.8 μmole per milliliter of lipid suspension. ‡ Total amount trapped was 43.7 μmole per milliliter of lipid suspension.

GERALD WEISSMANN
GRAZIA SESSA
ALAN W. BERNHEIMER
Departments of Medicine and *Microbiology, New York University School of Medicine, New York*

References and Notes

1. A. W. Bernheimer, *Ann. N.Y. Acad. Sci.* **128**, 112 (1965).
2. ——— and L. L. Schwartz, *J. Bacteriol* **87**, 1100 (1964); ———, *J. Pathol. Bacteriol.* **89**, 209 (1965); M. S. Artenstein, M. A. Madoff, L. Weinstein, *Yale J. Biol. Med.* **35**, 373 (1963); M. A. Madoff, M. S. Artenstein, L. Weinstein, *ibid.*, p. 382.
3. A. A. Marucci, *J. Bacteriol.* **86**, 1182, 1189 (1963); M. A. Madoff, L. Z. Cooper, L. Weinstein, *ibid.* **87**, 145 (1964); G. Weissmann, H. Keiser, A. W. Bernheimer, *J. Exp. Med.* **118**, 205 (1963).
4. A. D. Bangham, M. Standish, G. Weissmann, *J. Mol. Biol.* **13**, 253 (1965); G. Weissmann, G. Sessa, S. Weissmann, *Nature* **208**, 649 (1965); ———, *Biochem. Pharmacol.*, in press.
5. G. Weissmann and G. Sessa, *J. Cell Biol.*, in press.
6. A. W. Bernheimer and L. L. Schwartz, *J. Gen. Microbiol.* **30**, 455 (1963).
7. S. C. Kinsky, *Arch. Biochem. Biophys.* **102**, 180 (1963).
8. A. D. Bangham, M. Standish, J. C. Watkins, *J. Mol. Biol.* **13**, 238 (1965).
9. D. T. Warner, in *Mechanisms of Hormone Action*, P. Karlson, Ed. (Academic Press, New York, 1965), p. 83.
10. F. A. Vandenheuvel, *J. Amer. Oil Chem. Soc.* **40**, 455 (1963).

11. Aided by grants from the Life Insurance Medical Research Fund and NIH grants AI-02874 and AM-08363. G.W. is a career investigator of the Health Research Council of the City of New York. A.W.B. holds a PHS research career program award (5K6-AI-14).

19 September 1966