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Properties of a toxin from the sea anemone *Stoichactis helianthus*, including specific binding to sphingomyelin

(cytolytic protein/hemolysis/coelenterate/membranes)

ALAN W. BERNHEIMER AND LOIS S. AVIGAD

Department of Microbiology, New York University School of Medicine, New York, N.Y. 10016

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ABSTRACT **_Stoichactis helianthus_ toxin, a protein derived presumably from the nematocysts, was purified to homogeneity. It has a molecular weight of about 16,000, an isoelectric pH of 9.8, and it contains approximately 3.7% carbohydrate. It is powerfully hemolytic for erythrocytes derived from a variety of animal species, those of the cat being the most sensitive and those of the guinea pig the most resistant. The toxin is lytic also for rabbit blood platelets, and it destroys cultured fibroblasts but is inactive for several kinds of bacterial protoplasts and spheroplasts. The hemolytic activity is specifically inhibited by sphingomyelin, and it is proposed that this phospholipid is the constituent of the membrane which functions as receptor for the toxin. Supporting evidence includes the findings that enzymes known to destroy sphingomyelin (*a*) prevent erythrocyte membranes from inhibiting hemolysis, and (*b*) render erythrocytes resistant to lysis by the toxin. The mechanism underlying hemolysis may involve translocation of membrane sphingomyelin by virtue of a specific affinity of the coelenterate protein for this phospholipid.**

A toxin, presumably derived from the nematocysts, of *Stoichactis helianthus* was recently purified and studied by Devlin (1). Some of the properties of this toxin suggest a superficial resemblance to a group of cytolytic toxins of bacterial origin that have been under study in our and other laboratories (2, 3). In order better to evaluate the apparent similarity between the coelenterate toxin and those of bacteria we undertook to extend the findings of Devlin.

MATERIALS AND METHODS

Toxin. A sample (F-4) of purified *S. helianthus* toxin was generously provided by Dr. John P. Devlin. It had been purified by a slight modification of the procedure published earlier (1).

Estimation of Hemolytic Activity and Inhibition of Hemolysis. Test solutions were assayed as in ref. 4 but without gelatin. A solution of toxin was assayed with erythrocyte suspensions prepared from six rabbits. Five of the titers were within the range of variation expected from the possible error of the assay, but the sixth was about twice as great. The assays were not significantly affected by inclusion in the system of 5 mM $MgCl_2$, $CaCl_2$, ethylene diamine tetraacetate, or 0.1% gelatin. With either sheep or rabbit erythrocytes, the toxin did not show a "hot-cold" effect demonstrable with certain hemolytic phospholipases (3).

Capacity of lipids and other substances to inhibit hemolysis was estimated as in ref. 4 with 3 hemolytic units (HU) of *Stoichactis* toxin F-4 and omission of gelatin. Lipid suspensions were prepared as in ref. 4.

Reagents. Phospholipids were purchased from Sylvana Chemical Co., Supelco, Inc., and Applied Science Labs, Inc. Chloroform-methanol extract of brain was prepared as in ref. 5, and partially purified brain ganglioside as in ref. 6. Phosphatidylglycerol from *Micrococcus lysodeikticus* was a gift from Dr. Peter Owen. A sample of ceramide (preparation A) was kindly provided by Dr. Meir Lev. A second sample (preparation B), derived from bovine brain sphingomyelin, was purchased from Sigma Chemical Co.

Staphylococcal sphingomyelinase (EC 3.1.4.12; sphingomyelin cholinephosphohydrolase) was prepared as in ref. 7. Phospholipase C (EC 3.1.4.3; phosphatidylcholine cholinephosphohydrolase) from *Clostridium perfringens* was purified from culture filtrates by isoelectric focusing followed by gel filtration in Sephadex G-100 (Pharmacia), and was preserved in 50% glycerol at 4°. Phospholipase D (EC 3.1.4.4; phosphatidylcholine phosphatidohydrolase) from *Corynebacterium ovis* (8) was concentrated from culture filtrates by precipitation with ammonium sulfate and purified by fractionation on Sephadex G-100 followed by electrofocusing. *Corynebacterium ovis* antitoxin was immune sheep serum generously supplied by Dr. H. R. Carne.

Amino-Acid Analysis. Purified toxin was hydrolyzed in 6 M HCl at 110° for 21 hr. After centrifugation and removal of HCl, analyses were done with a Beckman model 120C automatic amino-acid analyzer using a recorder equipped with an expanded range bridge.

RESULTS

Analysis and Molecular Weight Estimation of Toxin by Electrophoresis in Sodium Dodecyl Sulfate-Polyacrylamide Gel. Fifty micrograms of toxin (F-4) were subjected to electrophoresis in sodium dodecyl sulfate-polyacrylamide gel according to Weber and Osborn (9). The gels showed a single, heavily stained major band and up to three faint minor bands. The major band migrated the same distance whether or not the toxin was preincubated for 2 hr in 0.01 M sodium phosphate, pH 7.0, containing one percent sodium dodecyl sulfate and one percent 2-mercaptoethanol. The molecular weight of the major band was estimated to be 16,000, using the conditions of ref. 9 and the reference proteins indicated in Fig. 1.

Isoelectric Focusing. Isoelectric focusing (10) was carried out in a linear density gradient prepared from (*a*) a less dense solution consisting of 5 mg of toxin (F-4), 4 ml of 8% (wt/vol) ampholine (pH 3.5–10; obtained from LKB Instruments), and sufficient water to bring the volume to 55 ml, and (*b*) a more dense solution of 32 ml of water, 8.5 ml of 8% (wt/vol) ampholine of the same pH range, and 25 g of sucrose. Focusing was done at about 4° for 42 hr, in a 110-ml electrophoresis column (LKB Instruments) with a final potential difference of 600 V. Fractions having a volume of 4 ml each were examined for absorbance at 280 nm, for pH,

Abbreviations: Tris-saline, 0.145 M NaCl–0.01 M Tris pH 7.2; HU, hemolytic unit(s).

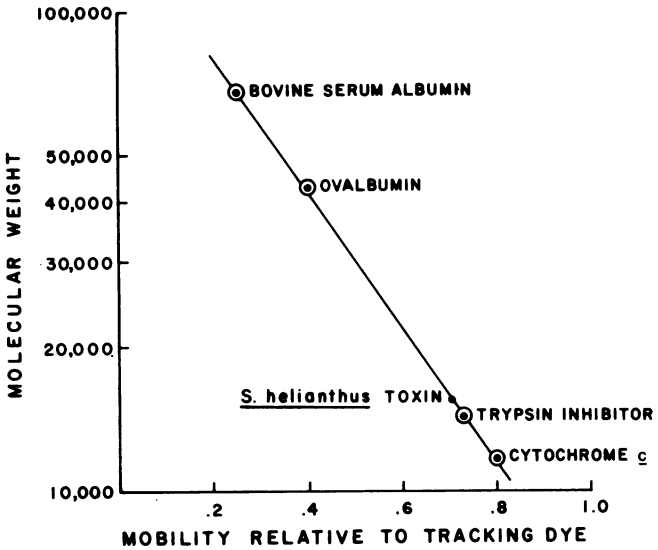

FIG. 1. Estimation of molecular weight of *S. helianthus* toxin from electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gel.

and for hemolytic activity. All the recovered hemolytic activity coincides with a major protein peak, and the toxin, as detected by hemolytic activity, is isoelectric at about pH 9.8 (Fig. 2). For preparative purposes five times as much toxin was subjected to focusing as described. Appropriate fractions were precipitated with, and stored in, saturated ammonium sulfate. The product had a 280 nm absorbance of 2.85 and a specific activity of 70,000 HU/unit of 280 nm absorbance as compared to 55,000 HU/unit of 280 nm absorbance of the starting material (F-4). The total hemolytic activity of the product was about 40% that initially subjected to electrofocusing.

Analysis of the Electrophoretically Purified Product. Four-tenths milliliter of the product was centrifuged, the supernatant discarded, and the pellet dissolved in a minimal volume of 0.01 M Tris buffer, pH 7.4. This was dialyzed against 800 ml of cold 0.01 M Tris buffer, pH 7.4. The resulting solution was clarified at 12,000 × *g* for 10 min, and a portion of the supernatant containing about 40 μg of protein was subjected to electrophoresis in sodium dodecyl sulfate-polyacrylamide gel as before. The stained gel revealed a single heavy band and no detectable protein impurities. The diluted product showed an ultraviolet absorption spectrum typical of a protein. The amino-acid analysis is given in Table 1. Carbohydrate was estimated by the phenol-sulfuric acid method (11). Assuming that a 0.1% solution of toxin gives a 280 nm absorbance of 0.8, F-4 toxin contained 4.6% carbohydrate as glucose and the electrofocused product contained 3.7%.

Sensitivity of Erythrocytes to Lysis. Suspensions of washed erythrocytes [in 0.145 M NaCl–0.01 M Tris, pH 7.2 (Tris-saline)] from various species of animals were prepared to contain the same amount of hemoglobin as in the standard rabbit erythrocyte suspensions used for routine titrations of hemolytic activity. A solution containing 1 mg of toxin (F-4) per ml gave the following results in HU/ml: cat, 100,000; rabbit, 43,000; sheep, 33,000; dog, 30,000; rat, 12,000; man, 8,800; horse, 5,600; and guinea pig, 4,200. The data indicate that erythrocytes from different species differ rather widely in sensitivity to lysis by the toxin, erythrocytes from the cat being about 25 times as sensitive as those from the guinea pig.

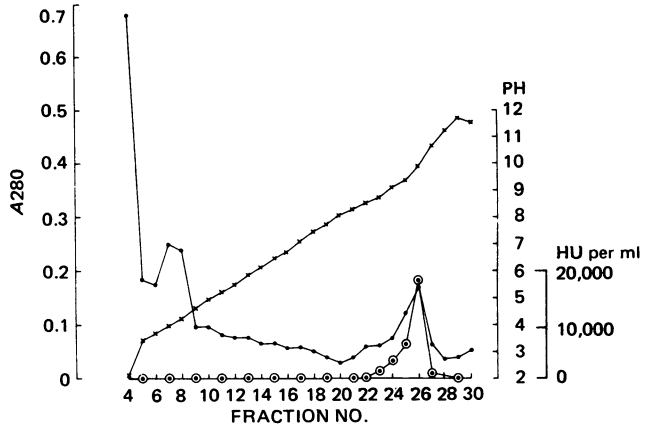

FIG. 2. Isoelectric focusing of toxin. Solid circles: protein as 280 nm absorbance; open circles: toxin as hemolytic activity; crosses: pH.

Inhibition of Lysis. Lipids and other materials were tested for capacity to inhibit lysis of rabbit erythrocytes by toxin (F-4). The results, shown in Table 2, indicate that among the phospholipids tested inhibition was obtained only with sphingomyelin. Ceramide, part of the sphingomyelin molecule, inhibited at a concentration more than 10 times that of sphingomyelin. Inhibition was obtained with whole serum as well as with lipoprotein-containing fractions of serum. Presumably, inhibition by serum, lipoproteins, and crude brain lipids is due to contained sphingomyelin.

Inhibition by Erythrocyte Membranes of Toxin-Induced Hemolysis. Decreasing amounts of osmotically prepared rabbit erythrocyte membranes (method A, ref. 12) were added to a series of tubes, and the volume was brought to 0.5 ml with Tris-saline. To each was added 0.5 ml of electrofocused toxin diluted in Tris-saline to contain about 3 μg

Table 1. Amino-acid composition

Amino acid	Mol per 100 g*	Mol per mol Phe	Presumed residues per molecular weight 16,000 ± 1000
Asp	0.0701	3.4	17
Thr	0.0374	1.8	9
Ser	0.0463	2.3	12
Glu	0.0469	2.3	12
Pro	0.0218	1.1	6
Gly	0.0775	3.8	19
Ala	0.0415	2.0	10
Half-Cys†	0.0092	0.5	3?
Val	0.0381	1.9	10
Met	0.0177	0.9	5
Ile	0.0319	1.6	8
Leu	0.0401	2.0	10
Tyr	0.0374	1.8	9
Phe	0.0204	1.0	5
His	0.0072	0.35	2
Lys	0.0377	1.8	9
Arg	0.0245	1.2	6
Trp			?
Total			152

* Average of two determinations.
† Cysteic acid.

Table 2. Results of inhibition tests

	Amount required to inhibit 2/3 the test amount* of toxin (μg)
Phosphatidylcholine	>500
Phosphatidylethanolamine	>500
Phosphatidylserine	>500
Phosphatidylinositol	>500
Phosphatidylglycerol	>500
Diphosphatidylglycerol	>500
Sphingomyelin (prep. A)	1.5
Sphingomyelin (prep. B)	2.0
Ceramide (prep. A)	70
Ceramide (prep. B)	35
Sphingosine	>500
Cholesterol	>500
Cerebrosides	>500
Partially purified brain gangliosides†	>500
Chloroform-methanol extracted brain lipids	2.5
Human serum	0.008‡
α-Globulin fraction of human serum	5
β-Lipoprotein fraction of human serum	10
Human serum albumin	>500
Concanavalin A	>500

* Three hemolytic units.
† Two additional specimens from commercial sources gave the same result.
‡ Measured in μl.

of toxin per ml. After 60 min at 20° the mixtures were diluted with 19 volumes of Tris-saline and titrated for hemolytic activity. About 1 μl of membrane preparation inhibited most of the hemolytic activity. Similar results were obtained (*a*) when the membranes and toxin were allowed to stand in an ice bath, and (*b*) when membranes prepared from horse or guinea pig erythrocytes were used. Membranes from cat erythrocytes were about three times as effective in inhibiting lysis as those from rabbit.

Effect of Pretreatment of Membranes with Staphylococcal Sphingomyelinase on Ability of Membranes to Inhibit Hemolysis. One-tenth milliliter amounts of rabbit erythrocyte membranes were mixed with 0.1 ml of each of a series of 10-fold dilutions of staphylococcal sphingomyelinase in the presence of 0.01 M $MgCl_2$ in Tris-saline. After 60 min at 37° the mixtures were diluted 50-fold with Tris-saline, and 0.25 ml of each was tested for its effect on *S. helianthus* toxin under the conditions of the preceding experiment. As little as 0.5 μg of sphingomyelinase per ml completely abolished inhibition of toxin by erythrocyte membranes, and 0.05 μg gave partial inhibition. In an additional experiment, samples of a solution containing 2 μg of sphingomyelinase per ml of Tris-saline were heated for 15 min at 60° and 100°. Heating at 60° and 100° reduced the effect of the sphingomyelinase by about 25% and >67%, respectively.

Effect of Pretreatment of Membranes with Phospholipases C and D. A similar experiment was carried out with *Clostridium perfringens* phospholipase C and with 0.01 M $CaCl_2$ instead of 0.01 M $MgCl_2$. To neutralize the hemolytic activity of the clostridial enzyme, 10 μl of gas gangrene antitoxin was added to each mixture immediately after the membranes had been incubated with the enzyme. About 5 μg of clostridial enzyme per ml were needed to reduce by 50% the toxin-inhibiting effect of the membranes, that is, about 100 times as much clostridial enzyme was needed to produce the same effect as the staphylococcal sphingomyelinase. Erythrocyte membranes were also pretreated with phospholipase D from *Corynebacterium ovis*. Again, about 5 μg/ml, or about 100 times the concentration, was required for an effect comparable to that of staphylococcal sphingomyelinase. Addition of 5 μl of *C. ovis* antitoxin largely prevented the phospholipase D from abolishing membrane inhibition of toxin-induced hemolysis.

Effect of Pretreatment of Erythrocytes with Phospholipases C and D. Washed 1.4% (vol/vol) suspensions of sheep and rabbit erythrocytes in Tris-saline were used. To 5-ml portions were added 5 ml of Tris-saline containing 0.01 M $MgCl_2$, 0.2% gelatin, and various quantities of corynebacterial phospholipase D. After 30 min at 37° the mixtures were centrifuged at 1100 × *g* for 5 min. The supernatants were discarded, and each erythrocyte pellet was suspended in 10 ml of Tris-saline. Hemolytic titrations were carried out with electrofocused toxin and the erythrocyte suspensions. Phospholipase D rendered both sheep and rabbit erythrocytes insensitive to lysis (Table 3). Exposure of these erythrocytes to the enzyme at concentrations of 2 μg/ml and 0.2 μg/ml reduced their sensitivity by more than 100-fold. A similar experiment in which rabbit erythrocytes were treated with staphylococcal sphingomyelinase C showed that exposure of erythrocytes to the enzyme at a concentration of about 0.2 μg/ml reduced their sensitivity to toxin about 140-fold (Table 3).

Apparent Absence of Sphingomyelinase Activity of Toxin. A mixture of about 60 μg of electrofocused toxin and 1 mg of sphingomyelin in 0.4 ml of Tris-saline containing 0.01 M $MgCl_2$ was allowed to stand at 37° for 60 min. The

Table 3. Effect of pretreatment of erythrocytes with corynebacterial phospholipase D and with staphylococcal sphingomyelinase C on their susceptibility to lysis by toxin

Approximate concentration of enzyme to which erythrocytes were exposed (μg/ml)	Hemolytic activity (HU/ml) of *S. helianthus* toxin using: Sheep erythrocytes treated with phospholipase D	Rabbit erythrocytes treated with phospholipase D	Rabbit erythrocytes treated with sphingomyelinase C
2	<200	<200	—
0.2	6,000	<200	1,300
0.02	130,000	60,000	160,000
0.002	170,000	120,000	230,000
0	215,000	270,000	185,000

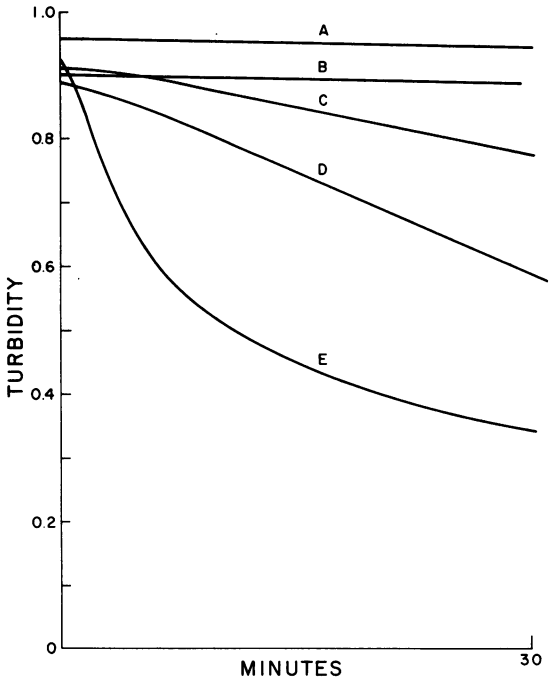

FIG. 3. Lysis of rabbit blood platelets by toxin. A, no toxin; B, 0.1 HU/ml; C, 1 HU/ml; D, 2 HU/ml; E, 10 HU/ml.

solution was extracted with two volumes of chloroform-methanol 2:1 (vol/vol) and the extract was subjected to thin-layer chromatography according to Broekhuyse (13, 14), but using only the first solvent system. Staining with iodine vapor revealed a single spot having the R_F of sphingomyelin, whereas in a control reaction mixture containing staphylococcal sphingomyelinase the sphingomyelin spot largely disappeared and a new spot appeared near the solvent front. Rabbit erythrocyte membranes were also exposed to toxin and centrifuged, and the lipids extracted from the pellet by the method of Reed *et al.* (15). The extracted lipids were analyzed by two-dimensional thin-layer chromatography (13, 14) and stained with iodine vapor. Visual inspection of test and control plates indicated that the toxin had not caused a significant alteration of the sphingomyelin or of other major membrane phospholipids.

***In Vitro* Destruction of Platelets and Fibroblasts.** Since the only known biological effects of *S. helianthus* toxin are erythrocyte lysis and lethality (1), it was of interest to test the toxin on other mammalian cells. Rabbit platelets prepared as in ref. 16 were suspended in Tris-saline containing 0.1% bovine serum albumin. After addition of toxin F-4, turbidity was followed in a model 14 Cary recording spectrophotometer at a wave length of 520 nm. Ten HU of toxin per ml produced gross lysis, and as little as 1 HU/ml had a detectable lytic effect (Fig. 3). Hence, rabbit platelets showed the same order of sensitivity as rabbit erythrocytes.

Human foreskin fibroblasts (FS4) were cultivated according to Vilcek and Havell (17) without serum. A solution containing 1 mg of toxin (F-4) per ml in serum-free medium was sterilized by filtration through a Millipore Millex disposable filter (0.45 μm). One-half milliliter of each of a series of 5-fold dilutions, prepared in serum-free medium, was added to 4.5 ml of fibroblast culture. Table 4 shows that about 8 HU damaged the fibroblasts.

Lack of Reactivity Between Toxin and Bacterial Membranes, Protoplasts, and Spheroplasts. One percent (vol/vol) suspensions of membranes from either *Micrococcus lysodeikticus* or *Neisseria gonorrhoeae*, tested under the same conditions as erythrocyte membranes, failed to inhibit lysis. Toxin at 1000 HU/ml caused no reduction in turbidity of suspensions of protoplasts of *M. lysodeikticus* or *Bacillus megaterium* or of spheroplasts of *Escherichia coli* in 30 min at 37°.

Table 4. Cytopathic effect of toxin on cultured human fibroblasts

Final concentration of toxin (HU/ml)	Toxicity* at: 1.25 hr	4 hr	22 hr
5000	2.5	3	3.5
1000	2	3	3.5
200	1.5	2	2.5
40	1	1	1
8	±	1	1
1.6	±	±	±
0	0	0	0

* Toxicity for human foreskin fibroblasts was scored visually on a 0 to 4 basis, as determined by degree of sloughing of cells from the culture dish. Complete sloughing was scored as 4; no morphological change as 0.

DISCUSSION

The foregoing data indicate that the hemolytic toxin of *S. helianthus* is a protein of molecular weight about 16,000. It does not appear to be dissociable into subunits, nor is there evidence for aggregate formation. Since an aim of this study was to compare this toxin with bacterial toxins exhibiting similar biological activities, it is notable that with the exception of streptolysin S virtually all the latter proteins fall within the molecular weight range of 30,000 to 70,000 (2, 3). Another difference between the bacterial cytolytic toxins and the coelenterate toxin is the presence of carbohydrate in the latter, as first demonstrated by Devlin (1) and confirmed here. *S. helianthus* toxin is a basic protein, and it may be significant that this feature is shared by a number of bacterial cytolytic toxins that are known or thought to react with membrane phospholipids, notably the major component of staphylococcal α-toxin, staphylococcal β-toxin, and corynebacterial phospholipase D. In common with several cytolytic proteins of bacterial origin, the hemolytic activity of the coelenterate toxin depends upon the erythrocyte species tested. As with most of the systems involving bacterial toxins, the basis for the differences in susceptibility is unknown. The order of sensitivity of erythrocytes to the toxin of the Indo-Pacific sea wasp *Chironex fleckeri*, said to be the most dangerous venomous marine animal known (18), has been reported (19) to be rat > human > guinea pig. This is the same order as for *S. helianthus* toxin, but additional data for the former toxin would be desirable.

Of interest is the finding that sphingomyelin is unique among the phospholipids tested in inhibiting hemolysis. We interpret this to mean that sphingomyelin is the specific membrane receptor for the toxin, and that this phospholipid is intimately involved in the events that result in lysis of the cell. Additional support for the role of sphingomyelin as receptor for the toxin is provided by the finding that (*a*) erythrocyte membranes, but not bacterial membranes (which lack sphingomyelin), inhibit toxin-induced hemolysis; (*b*) the inhibition is abolished by pretreatment of erythrocyte mem-

branes with staphylococcal sphingomyelinase C (which is specific for sphingomyelin and lysophosphatidylcholine), with clostridial phospholipase C (known to hydrolyze sphingomyelin as well as other phospholipids), or with corynebacterial phospholipase D [which has been shown (8) to hydrolyze membrane sphingomyelin]; and (*c*) whole erythrocytes are rendered resistant to lysis by the toxin upon pretreatment with either staphylococcal sphingomyelinase C or corynebacterial phospholipase D. Consistent also with these results is the finding that bacterial protoplasts and spheroplasts are insusceptible to the action of the toxin.

No evidence could be adduced for the possibility that *S. helianthus* toxin might itself be a sphingomyelinase. We, therefore, postulate that the toxin is a protein having a specific affinity for sphingomyelin, and that translocation of this particular membrane constituent causes changes in permeability leading to lysis. In this connection it is of interest that *C. fleckeri* toxin has been shown (20) to be relatively specifically inhibited by gangliosides, and its lytic action is thought to be probably not enzymic but associated with a nonenzymic interaction between gangliosides and the hemolytic protein. Perhaps the best precedent for this kind of hemolytic mechanism is to be found among the SH-activated, or oxygen-labile, bacterial cytolysins which have a specific affinity for membrane cholesterol (2, 21). A different mechanism has been proposed (22) to explain hemolysis produced by the nematocyst toxin of the sea anemone *Aiptasia pallida*, namely, that lysis is caused by enzymic action of venom phospholipase A on membrane phospholipids.

It is possible that *S. helianthus* toxin and an antitumor agent isolated from *Stoichactis kenti* (23) are similar substances. Among the coelenterate toxins that have been characterized biochemically *S. helianthus* toxin appears to resemble more closely than others the nematocyst toxin of the sea anemone *Actinia equina* of the northern Adriatic shore. This was found (24) to be a hemolytic and lethal protein of molecular weight about 20,000, and isoelectric at pH 12.4. There is also some resemblance in the amino-acid compositions of the two proteins.

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