INFECTION AND IMMUNITY, Dec. 1977, p. 688-693

Vol. 18, No. 3
Printed in U.S.A.

Effects of Streptolysin O on Transport of Amino Acids, Nucleosides, and Glucose Analogs in Mammalian Cells

JAMES L. DUNCAN* AND LELA BUCKINGHAM

Department of Microbiology-Immunology, Northwestern University Dental and Medical Schools, Chicago, Illinois 60611

Received for publication 21 June 1977

The transport of several amino acids, nucleosides, and glucose analogs was studied in HeLa cells treated with sublethal concentrations of streptolysin O. A differential effect of toxin on the various solutes tested was observed. The uptake of the neutral amino acids alanine, α-aminoisobutyric acid, leucine, and phenylalanine was reduced by about 60 to 70%. Less inhibition of transport was observed with acidic and basic amino acids, and the uptake of nucleosides and glucose analogs was reduced by 20% or less. The decreased uptake of α-aminoisobutyric acid could be explained by the inability of toxin-treated cells to retain this amino acid. The altered transport of phenylalanine and lysine, however, appeared to be due to decreased initial rates of uptake rather than to the loss of these amino acids from intracellular pools in toxin-treated cells. After treatment with sublethal concentrations of streptolysin O, the cells recovered their ability to transport and accumulate α-aminoisobutyric acid in about 4 h. The data suggest that the alterations in membrane transport observed in toxin-treated cells are due to an effect of streptolysin O on specific transport systems, rather than to the production of holes or pores in the membrane.

The biological properties and mode of action of the cytolytic toxin streptolysin O (SLO) have been studied for many years, primarily with mammalian erythrocytes as the target cells. SLO is thought to act on the plasma membrane of sensitive cells, and considerable evidence from experiments on erythrocytes and erythrocyte membranes suggests that membrane cholesterol is the binding site, and probably the target, of this toxin (4). In addition to erythrocytes, a variety of nucleated mammalian cells have been shown to be susceptible to the lethal effects of SLO (9, 10), and several recent studies have focused on the effects of sublethal concentrations of the toxin on mammalian cells (7). The chemotactic activity of human neutrophils was decreased about 50% by sublethal concentrations of a highly purified SLO preparation (3), and similar effects were observed with the closely related cytolysin produced by *Clostridium perfringens* theta-toxin (20). The effects of low SLO concentrations on human lymphocytes have been studied by Andersen et al. (1, 2). Phytohemagglutinin-induced blast transformation of lymphocytes was suppressed by greater than 80% when the cells were first treated with SLO, and the data suggested that SLO may interfere with the phytohemagglutinin receptor sites on the lymphocyte membrane. In a related study, sheep erythrocyte-lymphocyte rosette (E-rosette) formation was shown to be inhibited by treating the lymphocytes with sublethal concentrations of SLO, and it was suggested that the toxin altered the erythrocyte receptor site on the membranes of T-lymphocytes. The ability of mouse peritoneal macrophages to phagocytize an M protein-negative strain of *Streptococcus pyogenes* was reduced 50% by SLO concentrations that killed only 5% of the cells (11). The results of all these studies probably reflect reversible membrane damage due to alterations in membrane structure or function produced by sublethal concentrations of SLO.

An alternative procedure for studying toxin-induced membrane damage was developed by Thelestam and Möllby (16-18), who examined the effects of *C. perfringens* theta-toxin and other lytic agents on human diploid fibroblasts by measuring the leakage of radioisotopically labeled markers from the cells. Theta-toxin caused a differential release of markers from the cells in the following order: α-aminoisobutyric acid (AIB) > nucleotides > ^{51}Cr = uptake of trypan blue and morphological changes > ribonucleic acid. The release of labeled compounds was thus related to their molecular size, and Thelestam and Möllby concluded that the differential release was due to leakage of the compounds through functional holes produced in the membrane by the toxin. Considerable leakage of AIB and nucleotide label occurred in toxin-treated cells, which, however, showed no

signs of damage as detected by morphological observations and trypan blue exclusion.

From these studies it would seem that to understand the effects of cytolytic toxins on sensitive cells, it will be necessary to evaluate not only the lytic effects, but also the subtle and reversible membrane changes produced by these toxins. This report describes the effects of sublethal concentrations of SLO on HeLa cells. The transport of amino acids, nucleosides, and glucose analogs was used to assess alterations in membrane function produced by the toxin.

MATERIALS AND METHODS

SLO. SLO was partially purified by gel filtration on Sephadex G-100 and by ion-exchange chromatography on diethylaminoethyl (DEAE)-Sephadex as previously described (8). The toxin had a specific activity of approximately 200,000 hemolytic units per mg of protein. Cholesterol completely inactivated the hemolytic and lethal effects of the toxin preparation on erythrocytes and HeLa cells, respectively. The toxin was concentrated by lyophilization, quick-frozen, and stored at −80°C in 0.5-ml portions. Before use, SLO was activated by the addition of 5 μl of 10 mM cysteine or 200 mM dithiothreitol. The toxin was assayed as previously described (6), by using a 1.7% suspension of rabbit erythrocytes. One hemolytic unit of toxin is defined as the greatest dilution of SLO that produces 50% hemolysis when incubated with an equal volume of the erythrocyte suspension for 30 min at 37°C.

Cell cultures. Stock cultures of HeLa cells were grown as monolayers in glass prescription bottles (32 ounces [ca. 0.95 liter]) in Eagle minimal essential medium (MEM) containing Hanks salts (Grand Island Biological Co., Grand Island, N.Y.), supplemented with 5% calf serum (ISI, Inc., Cary, Ill.) and an antibiotic solution containing penicillin, streptomycin, and amphotericin B (ISI). The cultures were propagated at 37°C in an atmosphere of air plus CO_2 (95:5) in humidified incubators. The cells used in the experiments were transferred from stock cultures and grown in plastic dishes (60 by 15 mm or 35 by 10 mm).

Transport experiments. (i) Uptake. Cell monolayers were washed with phosphate-buffered saline (PBS: NaCl, 137 mM; KH_2PO_4, 2 mM; Na_2HPO_4, 8 mM [pH 7.4]) and then exposed to SLO in Hanks balanced salt solution that contained no Ca^{2+} or Mg^{2+}. Preliminary experiments suggested that SLO activity was slightly greater in this solution than in ordinary Hanks solution or MEM. In a few experiments, the cells were exposed to toxin in PBS. After incubation at 37°C for 20 min, the monolayers were washed with PBS and then incubated in MEM containing 0.5 μCi of the solute being tested. After incubation at 37°C for 20 min, the uptake was terminated by removing the labeled solution by suction and quickly washing the cells one time in ice-cold PBS.

The cells were removed from the dishes by adding 1 ml of water plus 1 drop of 1 N NaOH to the monolayers. An equal volume of 5% trichloroacetic acid was added, and the material was heated for 10 min at 90°C. After the solution was centrifuged, acid-soluble counts were determined by adding 0.2 ml of the supernatant to 3a70B liquid scintillation cocktail (Research Products International, Elk Grove Village, Ill.). The acid-precipitable pellet was washed with 5% trichloroacetic acid and then dissolved in 1 ml of water containing 1 drop of 1 N NaOH. A 0.1-ml sample was added to the scintillation cocktail for determining precipitable counts. The radioactivity was counted in a Packard model 3385 liquid scintillation spectrometer.

The initial uptake rates of some amino acids were studied by exposing the cultures to toxin and then floating the culture dishes in a 37°C water bath. Different concentrations of amino acid in Hanks balanced salt solution were added to the cells and, after a 90-s incubation period, the total amount of amino acid accumulated was determined as described above.

(ii) Intracellular amino acid pools. Monolayer cultures were washed with PBS and then incubated for 20 to 30 min at 37°C in MEM containing 0.5 μCi of the labeled compound being tested per ml. The label was then removed and the cells were washed once in ice-cold PBS. SLO or control solution in Hanks balanced salt solution containing no Ca^{2+} or Mg^{2+} was added to the dishes, and the cultures were incubated at 37°C. At various times, duplicate cultures of toxin-treated and control cells were removed from the incubator, the incubation solution was removed by suction, and the cells were washed once with ice-cold PBS. The acid-soluble material remaining in the cells was determined as described above. Duplicate cultures that had been exposed to toxin or control solutions for 10 s served as the zero-time exposure in these experiments.

(iii) Viable counts. In each experiment, viable counts were determined by trypan blue exclusion for duplicate cultures exposed to toxin or control solutions for 20 min. After correction for the number of cells killed by the toxin, the radioactive counts taken up by or remaining in the cells were expressed as counts per minute per 10^6 viable cells. No correction was made for variation between individual cultures of the duplicate samples. The control solutions consisted of heat-inactivated or cholesterol-inactivated SLO.

Radioisotopes. ^{3}H-labeled leucine (50 Ci/mmol), AIB (10 Ci/mmol), 3-*O*-methyl-D-glucose (3.6 Ci/mmol), 2-deoxy-D-glucose (10 Ci/mmol), and thymidine (40 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. ^{3}H-labeled alanine (41 Ci/mmol), aspartic acid (0.3 Ci/mmol), phenylalanine (12 Ci/mmol), glutamic acid (2 Ci/mmol), lysine (15 Ci/mmol), and uridine (51 Ci/mmol) were purchased from Amersham/Searle, Arlington Heights, Ill.

RESULTS

The strategy in these experiments was to treat HeLa cells with SLO concentrations that would kill only a small number of cells (≤10%) as detected by trypan blue exclusion and cell counts. Several preliminary experiments indicated that approximately 5 hemolytic units of

SLO achieved this effect. It was also observed that cell killing was reflected primarily as a decrease in total cells in toxin-treated cultures, rather than as an increase of trypan blue-stained cells. In initial experiments, the uptake of the non-metabolizable amino acid AIB was examined over a 20-min period in toxin-treated and control cells. The results of a typical experiment (Fig. 1) demonstrate that AIB accumulation was greatly reduced during this time period in cells treated with sublethal concentrations of SLO.

Based on this initial experiment, the uptake of a number of amino acids, nucleosides (thymidine and uridine), and sugars (2-deoxyglucose and 3-*O*-methyl-glucose) by toxin-treated and control cultures was studied by measuring the incorporation of radioactive label into total acid-soluble and -insoluble cellular material during a 20-min incubation period. The results in Fig. 2 show a wide range of effects depending upon the substrate tested. In general, the uptake of nucleosides and glucose analogs was not greatly affected in cells receiving a sublethal dose of toxin, whereas the uptake of certain amino acids (alanine, AIB, leucine, and phenylalanine) was inhibited by approximately 60 to 70%. Other amino acids were only partially affected.

The reduced accumulation of amino acids could have been the result of decreased influx, increased efflux, or a combination of both effects. Therefore, the possibility that a rapid efflux of amino acids was occurring in toxin-treated cells was examined by preincubating the cells in MEM containing a labeled amino acid and then exposing the cultures to sublethal concentrations of SLO and monitoring the label remaining in acid-soluble pools during a 30-min incubation period. The results (Fig. 3A) show that a rapid loss of AIB occurred between 5 and 10 min after toxin addition. The loss of AIB from control cells occurred at a much slower rate. A rapid decrease in the soluble phenylalanine pools was seen between 10 and 20 min (Fig. 3B), but, in contrast to AIB, the loss of phenylalanine was similar in both toxin-treated and control cells. In other experiments, the loss of soluble phenylalanine pools was more gradual, but again there was very little difference seen between toxin and control cultures. When alanine was examined, there appeared to be a somewhat greater decrease in the soluble pools of this amino acid in toxin-treated cultures than in control cells (Fig. 3C). Toxin had no effect on the soluble radioactive pools of lysine, the other amino acid examined in these experiments.

The results described above suggested that the inhibitory effects on phenylalanine and lysine accumulation could be accounted for primarily by an effect of toxin on uptake. Further support for this interpretation was provided by experiments in which phenylalanine influx as a function of extracellular phenylalanine concentration was examined in toxin-treated and control cultures during a 90-s incubation period. The results (Fig. 4) demonstrate that the initial rates of phenylalanine uptake are decreased in toxin-treated cultures even at very low substrate concentrations.

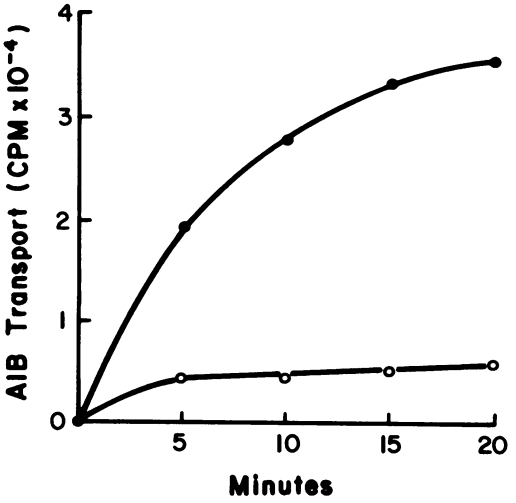

FIG. 1. *AIB transport in control (●) and SLO-treated (○) HeLa cells. The cultures were exposed to toxin or control solutions for 20 min and then incubated in [³H]AIB. At various times, the amount of AIB present in the cells was determined.*

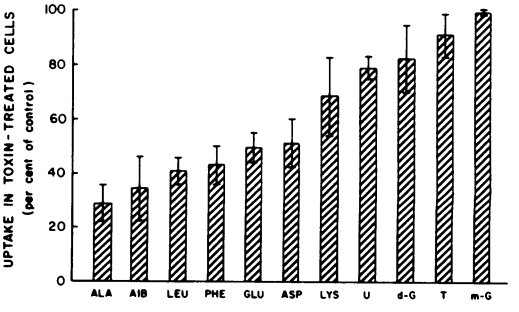

FIG. 2. *Transport of amino acids, nucleosides, and glucose analogs in SLO-treated cells. HeLa cell cultures were treated with SLO or control solutions for 20 min and then exposed to 0.5 μCi of each compound per ml. The amount of label present in acid-soluble plus acid-precipitable materials after a 20-min incubation period was determined. Vertical bars represent the standard error of the mean. ALA, Alanine; LEU, leucine; PHE, phenylalanine; GLU, glutamic acid; ASP, aspartic acid; LYS, lysine; U, uridine; d-G, 2-deoxy-D-glucose; T, thymidine; m-G, 3-O-methyl-D-glucose.*

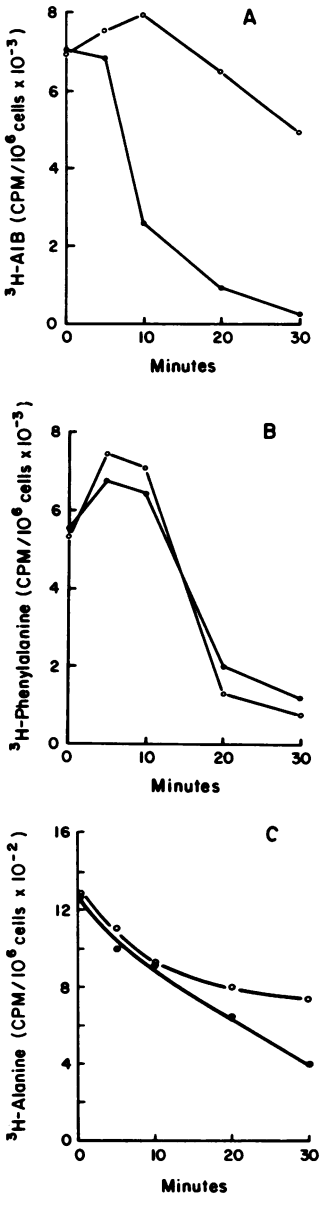

FIG. 3. *Effect of SLO on amino acid pools in HeLa cells. The cultures were incubated in the presence of labeled AIB (A), phenylalanine (B), or alanine (C) for 20 to 30 min and then exposed to toxin (●) or control (○) solutions. At various times, the amount of each amino acid remaining in acid-soluble pools was determined.*

Since the cells in all the experiments described above had been exposed to sublethal concentrations of toxin for short periods of time, the possibility that the cultures could recover their transport properties was investigated. Cells were treated with SLO or control solutions for 20 min and then incubated in complete medium at 37°C. At various times, the cells were washed and then tested for their ability to accumulate AIB. The results from three such experiments are shown in Fig. 5. The ability to accumulate AIB gradually increased, and within 4 h the cells appeared to have completely recovered.

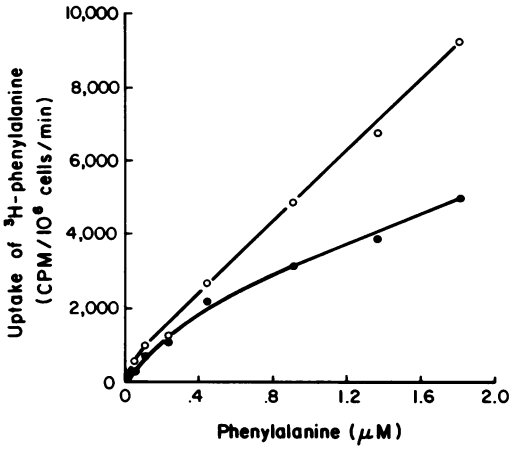

FIG. 4. *Uptake of phenylalanine in SLO-treated (●) and control (○) HeLa cells. After exposure to toxin or control solutions for 20 min, the cultures were incubated in various concentrations of phenylalanine for 90 s. The incorporation of label into acid-soluble plus acid-precipitable material was determined.*

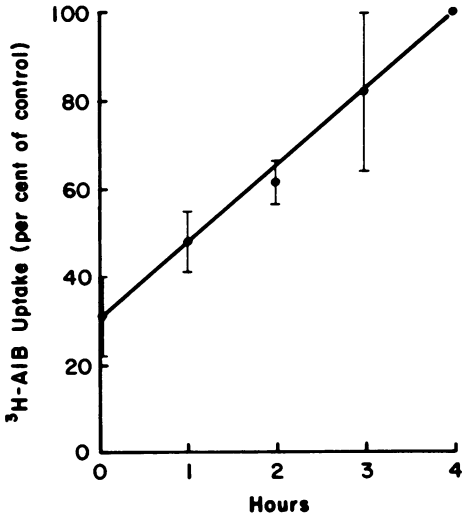

FIG. 5. *Recovery of AIB transport in SLO-treated cells. HeLa cells were exposed to toxin for 20 min and then incubated in complete MEM. At various times, the ability of the cultures to accumulate AIB was determined by incubating them for 20 min in the presence of [^{3}H]AIB. Vertical bars represent the standard error of the mean.*

DISCUSSION

The results described here suggest that sublethal concentrations of SLO can reversibly alter membrane function in HeLa cells. The ability of the cells to transport (and/or maintain) certain amino acids was significantly decreased by SLO; in contrast, the transport of at least one amino acid, lysine, as well as of nucleosides and glucose analogs, was not greatly affected. The amino acids were affected in the following order: small neutrals > large neutrals > acidic > large basic.

Although the leakage of amino acids from the cells was not measured directly, the results of experiments on the fate of acid-soluble pools in toxin-treated and control cells allow us to assess the importance of leakage in the uptake effects. The decreased ability of the cells to accumulate AIB seems to be due, in large part, to the inability of the cells to maintain intracellular pools of this compound. Since AIB is not incorporated into acid-precipitable material, its rapid loss from the pools can only be accounted for by leakage out of the cells, an observation that agrees with the results of Thelestam and Möllby (16–18). The failure of the toxin-treated cells to accumulate phenylalanine, lysine, and, to a lesser extent, alanine, on the other hand, appears to reflect primarily a decrease in the rate of uptake of these amino acids, rather than leakage. There was little or no difference in the soluble pools of these amino acids in toxin-treated versus control cells, and it follows, therefore, that leakage of the amino acids could not account for the dramatic inhibition in uptake which was observed. Whether toxin acts primarily on uptake or on efflux mechanisms might be related to the transport systems involved for each amino acid. AIB and alanine have been shown to be transported by the Na^+-dependent A and ASC systems in several types of cells, whereas phenylalanine, at least in part, and leucine are transported by the L system, generally considered to be Na^+ independent (5, 12, 13). The differential transport effects, therefore, may depend on the sensitivity of various transport systems to toxin-induced membrane alterations.

The mechanism by which SLO could exert differential effects on transport systems in the cell membrane is not known, but the recent finding of hydrophobic complex formation between SLO and inhibitory sterols by Prigent and Alouf (15), as well as the proposal that SLO and other SH-activatable cytolytic toxins may "extract" or sequester cholesterol from its normal position in the erythrocyte membrane bilayer (8; C. J. Smyth and J. L. Duncan, *in* J. Jeljaszewicz and T. Wadstrom [ed.], *Bacterial Toxins and Cell Membranes*, in press), may offer an explanation for these results. It has been suggested that localized areas of different lipid composition associated with transport functions exist within the cell membrane (14), and it is known that the activity of some membrane proteins, for example adenosine triphosphatase (19), is dependent on the presence of phospholipids or cholesterol in their immediate environment. Thus, the sequestration or removal of cholesterol might change the lipid composition in the immediate environment of certain transport systems, resulting in an alteration of transport properties.

Based on leakage studies, Thelestam and Möllby concluded that the related SH-activatable cytolysin theta-toxin produced functional holes of a constant size in the membrane of human diploid fibroblasts (18). Furthermore, the differential release of AIB and uridine was attributed to a more rapid diffusion of AIB through the holes. Although some of the results reported here might be compatible with such a proposal, we tend to interpret our data as supporting a more specific effect of SLO on certain transport systems. The phenylalanine experiments, which show that the rate of uptake is decreased in toxin-treated cells, but that intracellular pools of this amino acid are unaffected, are difficult to explain in terms of holes present in the membrane. Furthermore, there was no obvious relationship between the molecular weights of the solutes studied here and their transport in toxin-treated cells. Thus, the differential effects on amino acid accumulation and the finding that the transport (and presumably leakage) of nucleosides and glucose analogs are only slightly affected also suggest an effect (or lack of effect) on specific transport processes.

These results provide further evidence that SLO may alter physiological processes in sensitive cells at concentrations that are not lethal for the cells. All of the effects that have been described thus far appear to be related to membrane changes produced by the toxin, presumably through its effect on membrane cholesterol. The possibility that this toxin plays a role in the pathogenesis of streptococcal infections, therefore, should be considered in terms of its more subtle, and perhaps reversible, effects on tissue cells.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI-11644 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Andersen, B. R., and J. J. Amirault.** 1976. Decreased E-rosette formation following streptolysin-O treatment.

Proc. Soc. Exp. Biol. Med. **153**:405–407.
2. **Andersen, B. R., and R. Cone.** 1974. Inhibition of human lymphocyte blast transformation by streptolysin O. J. Lab. Clin. Med. **84**:241–248.
3. **Andersen, B. R., and D. E. Van Epps.** 1972. Suppression of chemotactic activity of human neutrophils by streptolysin O. J. Infect. Dis. **125**:353–359.
4. **Bernheimer, A. W.** 1974. Interactions between membranes and cytolytic bacterial toxins. Biochim. Biophys. Acta **344**:27–50.
5. **Christensen, H. N., C. de Cespedes, M. E. Handlogten, and G. Ronquist.** 1973. Energization of amino acid transport, studied for the Ehrlich ascites tumor cell. Biochim. Biophys. Acta **300**:487–522.
6. **Duncan, J. L.** 1974. Characteristics of streptolysin O hemolysis: kinetics of hemoglobin and ^{86}rubidium release. Infect. Immun. **9**:1022–1027.
7. **Duncan, J. L.** 1975. Streptococcal toxins, p. 257–262. *In* D. Schlessinger (ed.), Microbiology—1975. American Society for Microbiology, Washington, D.C.
8. **Duncan, J. L., and R. Schlegel.** 1975. Effect of streptolysin O on erythrocyte membranes, liposomes, and lipid dispersions. A protein-cholesterol interaction. J. Cell Biol. **67**:160–173.
9. **Ginsburg, I.** 1972. Mechanisms of cell and tissue injury induced by group A streptococci: relation to poststreptococcal sequelae. J. Infect. Dis. **126**:294–340.
10. **Halbert, S. P.** 1970. Streptolysin O, p. 69–95. *In* T. C. Montie, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 3. Academic Press Inc., New York.
11. **Ofek, I., S. Bergner-Rabinowitz, and I. Ginsburg.** 1972. Oxygen-stable hemolysins of group A streptococci. VIII. Leukotoxic and antiphagocytic effects of streptolysins S and O. Infect. Immun. **6**:459–464.
12. **Oxender, D. L., and H. N. Christensen.** 1963. Distinct mediating systems for the transport of neutral amino acids by the Ehrlich cell. J. Biol. Chem. **238**:3686–3699.
13. **Oxender, D. S., M. Lee, P. A. Moore, and G. Cecchini.** 1977. Neutral amino acid transport systems of tissue culture cells. J. Biol. Chem. **252**:2675–2679.
14. **Plagemann, P. G. W., and J. Erbe.** 1975. Temperature-dependent changes in activation energies of the transport systems for nucleosides, choline and deoxyglucose of cultures Novikoff rat hepatoma cells and effects of cytochalasin B and lipid solvents. J. Membr. Biol. **25**:381–396.
15. **Prigent, D., and J. E. Alouf.** 1976. Interaction of streptolysin O with sterols. Biochim. Biophys. Acta **443**:288–300.
16. **Thelestam, M., and R. Möllby.** 1975. Determination of toxin-induced leakage of different-size nucleotides through the plasma membrane of human diploid fibroblasts. Infect. Immun. **11**:640–648.
17. **Thelestam, M., and R. Möllby.** 1975. Sensitive assay for detection of toxin-induced damage to the cytoplasmic membrane of human diploid fibroblasts. Infect. Immun. **12**:225–232.
18. **Thelestam, M., and R. Möllby.** 1976. Cytotoxic effects on the plasma membrane of human diploid fibroblasts—a comparative study of leakage tests. Med. Biol. **54**:39–49.
19. **Warren, G. B., M. D. Houslay, J. C. Metcalfe, and N. J. M. Birdsall.** 1975. Cholesterol is excluded from the phospholipid annulus surrounding an active calcium transport protein. Nature (London) **255**:684–687.
20. **Wilkenson, P. C.** 1975. Inhibition of leukocyte locomotion and chemotaxis by lipid-specific bacterial toxins. Nature (London) **255**:485–487.