Journal of Supramolecular Structure 5:65 (39)–72 (46) (1976)

Cell Shape Changes and Transmembrane Receptor Uncoupling Induced by Tertiary Amine Local Anesthetics

Garth L. Nicolson

Department of Developmental and Cell Biology, University of California, Irvine, California 92717 and Department of Cancer Biology, The Salk Institute for Biological Studies, San Diego, California 92112

George Poste

Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, New York 14263

Tertiary amine local anesthetics (dibucaine, tetracaine, procaine, etc.) modify cell morphology, concanavalin A (Con A)-mediated agglutinability and redistribution of Con A receptors. Con A agglutination of untransformed mouse 3T3 cells was enhanced at low concentrations of local anesthetics, and the dynamics of fluorescent-Con A indicated that ligand-induced clustering was increased in the presence of the drugs. In contast, these drugs inhibited Con A-induced receptor capping on mouse spleen cells. These effects can be duplicated by combinations of vinblastine (or colchicine) and cytochalasin B suggesting that local anesthetics act on microtubule and microfilament assemblies which are involved in the trans-membrane control of cell surface receptor mobility and distribution. It is proposed that tertiary amine local anesthetics displace plasma membrane-bound Ca^{2+}, resulting in disengagement of microfilament systems from the plasma membrane and increased cellular Ca^{2+} concentration to levels which disrupt microtubular organization. The possible involvement of cellular Ca^{2+} in cytoskeletal destruction by local anesthetics was investigated utilizing Ca^{2+}-specific ionophores A23187 and X537A. In media containing Ca^{2+} and cytochalasin B these ionophores caused effects similar to tertiary amine local anesthetics.

Key words: cell morphology; Con A receptors; cytochalasin B; ionophore; lectin agglutination. ligand-induced clustering; local anesthetics; microfilament; microtubule, receptor capping; vinblastine

INTRODUCTION

Due to their amphipathic structures, tertiary amine local anesthetics partition into and act on cellular membranes (1–4). Although their anesthetic site of action has not been determined, local anesthetics interact with membrane lipids (3–10), particularly anionic phospholipids (1, 7, 8, 10), and they probably interfere with ATP-dependent transport and bind to other membrane protein components (11–13). An important property of local anesthetics is their ability to displace Ca^{2+} from membranes (2, 8, 9, 14) and produce molecular disordering in lipid bilayers (10, 15).

Local anesthetics are known to affect a variety of cellular processes, in addition to their anesthetic properties on excitable cell membranes (1–3, 5). At low concentrations where anesthetics have little direct effect on membrane lipid fluidity (10, 15), these drugs alter cell volume (2, 16), osmotic fragility (16), spreading (17), locomotion (18), adhesion (19, 20), axonal transport (21), endocytosis (17), exocytosis (22, 23) and fusion (23, 24). More recently low concentrations of tertiary amine local anesthetics have been found to modify lectin-mediated cell agglutination (25–27) and the dynamics of cell surface receptors (26–28). In particular, local anesthetics enhanced the susceptibility of untransformed BALB/3T3 cells to agglutination by concanavalin A (Con A), and facilitated lectin-induced redistribution of Con A receptors on these cells (26, 27). In contrast, local anesthetics at similar concentrations inhibited ligand-induced capping of immunoglobulin receptors on mouse lymphocytes (27, 28) suggesting that their actions might involve disruption of the cytoplasmic structural elements associated with the cell plasma membrane such as microtubules and microfilaments. These membrane-associated assemblies, collectively called the cytoskeletal system, have been proposed to be involved in certain energy-dependent processes such as capping where clustered ligand-receptor complexes are swept to and held at one pole of a cell prior to endocytosis or shedding (29–32). Microtubules are sensitive to disruptive drugs such as colchicine and the Vinca alkaloids (33), and they are thought to act as "anchors" which restrict the mobility of certain cell surface receptors (34–37), because disruption of microtubular systems in many cases facilitates receptor redistribution by multivalent ligands (25, 27, 34–36, 38, 39). The other important class of membrane-associated cytoskeletal elements, microfilaments, also affect the mobility and distribution of surface receptors (27, 29, 38, 40). Microfilaments can be disrupted by cytochalasin B (41, 42) and D (43). Importantly, local anesthetics appear to disrupt both microfilament and microtubule systems (26, 27, 44) causing enhancement of ligand-induced redistribution or clustering (25–27), but inhibiting polar redistribution or capping (26–28).

LECTIN-INDUCED AGGLUTINATION AND RECEPTOR REDISTRIBUTION

Local anesthetics at low concentrations enhance lectin-mediated agglutinability and receptor mobility on untransformed 3T3 cells (Table I). Although these effects can be partially attained by treatment with cytochalasin B alone, the combination of cytochalasin B plus vinblastine closely mimics the action of tertiary amine local anesthetics (Table I), consistent with our previous results (26, 27). In other experiments we have shown that drug treatments do not modify lectin binding to the cell surface (26, 27, 49). In addition, local anesthetics failed to produce an additive effect if added together with colchicine and cytochalasin B (27). We interpret these results as evidence that local anesthetics modify both plasma membrane-associated microtubules and microfilaments involved in the transmembrane regulation of cell surface receptor mobility and distribution.

Further evidence for action of local anesthetics in modifying cytoskeletal systems has been obtained using mouse splenic lymphocytes. Anesthetics dramatically inhibit ligand-induced capping of surface immunoglobulin receptors (26–28). In addition, they also block Con A-induced cap formation on spleen cells (Table II) and prevent the maintenance of preformed caps (27) similar to that caused by cytochalasin B (27, 38, 40) or colchicine plus cytochalasin B (27, 38).

TABLE I. Effect of Tertiary Amine Local Anesthetics on Concanavalin A-Induced Cell Agglutination and Receptor Redistribution on Mouse 3T3 Cells

Treatment*	Drug concentration	Concentration of Con A causing half-maximal agglutination†	Redistribution of fluorescent-Con A after drug treatment‡	
			% Uniform	% Clustered
Untreated	–	1100 μg/ml	> 90	< 10
Dibucaine	0.2 mM	160	< 10	> 90
Tetracaine	0.5 mM	200	< 20	> 80
Vinblastine	0.01 mM	1100	> 90	< 10
Cytochalasin B	10 μg/ml	700	> 80	< 20
Vinblastine + cytochalasin B	0.01 mM 10 μg/ml	200	< 20	> 80
Dimethylsulfoxide	0.1%	1100	> 90	< 10

*Cells were incubated in suspension for 30 min at 37°C in serum-free media.
†Agglutination was scored at 20°C as described previously (45, 46).
‡Redistribution of cell-bound fluorescent-Con A was determined as in (27, 47, 48). Cells were observed after 15 min at 20°C. The data represent the results of three different experiments.

TABLE II. The Effects of Local Anesthetics and Cytochalasin B on Capping of Concanavalin A Receptors on Mouse Spleen Cells*

Treatment	Concentration	Incubation time (min)	% Caps†
Untreated	Control	—	39
Dibucaine	2×10^{-4} M	30	< 1
Tetracaine	5×10^{-4} M	30	8
Procaine	1×10^{-2} M	30	14
Cytochalasin B	10 μg/ml	60	< 15
Vinblastine + cytochalasin B	0.01 mM 10 μg/ml	60	< 1
Dimethylsulfoxide	0.25% (v/v)	60	35

*Spleen cells were preincubated in serum-free RPMI 1640 medium with or without the various drug supplements for the indicated times and then incubated with fluorescein-conjugated Con A for 30 min at 37°C after which the percentage of cells displaying caps of Con A receptors was measured as described in Ref. 27. The results represent mean values derived from three separate experiments.
†Percentage of stained cells with caps.

MORPHOLOGY OF ANESTHETIC-TREATED CELLS

Local anesthetics produce dramatic morphological changes in confluent BALB/c 3T3 cells (44). These cells are normally flat, polygonal, endothelial-like cells which are density-inhibited at about $5–12 \times 10^4$ cells/cm^2. At confluency 3T3 cells contain an extensive network of microfilaments and microtubules (44, 50). Addition of dibucaine (2×10^{-4} M), tetracaine (5×10^{-4} M) or procaine (10×10^{-4} M) produced cell rounding at anesthetic doses; the differing potencies of these drugs corresponded well with their octanol-water partition coefficients (17). Examination by electron microscopy revealed that the local anesthetic-treated cells were devoid of identifiable microfilaments or microtubules (44). Within 5 min of exposure to local anesthetics, the plasma membranes on the flat 3T3 cells lifted in many places forming surface "blebs" resembling the surface zeiotic

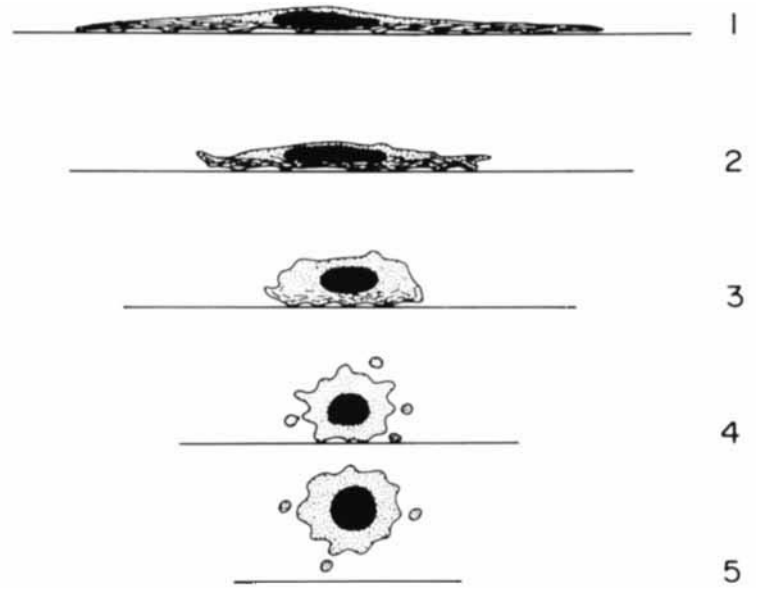

Fig. 1. Diagrammatic sequence of morphological changes induced in BALB/c 3T3 cells by treatment with tetracaine for 30 min at 37°C. At low concentrations of anesthetic (~ 0.5–0.8 mM) the normally flat, extended 3T3 cells (1) retract and round-up (2–3). Higher tetracaine concentrations (0.8 mM–1.0 mM) result in complete cell rounding and loss of membrane-associated cytoskeletal assemblies (4). Concentrations of 1 mM tetracaine or above result in cell detachment from substrate (5) (see Ref. 44 for details and original data).

blebs produced by cytochalasin B (42) or cytochalasin D (43). A few minutes later many of these surface blebs broke off from the cell as vesicles containing cell cytoplasm, but no viable arrays of cytoskeletal components (44). These changes are documented in Ref. 44 and illustrated in Fig. 1.

Other reports have indicated that general (51) and local (52, 53) anesthetics cause ultrastructural alterations in cell cytoskeletal systems. The local anesthetic lidocaine has been shown to impair microtubule assembly in vitro (52), and halothane, a gaseous inhalation general anesthetic, is known to cause microfilament breakdown in mouse neuroblastoma cells (53).

EFFECTS OF CALCIUM IONOPHORES

If membrane-associated microtubules are involved in trans-membrane control of cell surface receptors, ligand-induced redistribution and lectin-mediated agglutination should be affected by cytoplasmic Ca^{2+} which is known to regulate the polymerization of tubulin subunits (54, 55) and depolarization of microtubules (56, 57). To examine the role of cytoplasmic Ca^{2+} in regulating cytoskeletal trans-membrane receptor control, we have utilized Ca^{2+}-specific ionophores such as A23187 and X537A (49). Calcium ionophores (in the presence of 4×10^{-4} to 1×10^{-3} M Ca^{2+}) reduced the agglutinability of SV40-transformed 3T3 cells by Con A (49) similar to the action of colchicine on these cells (27, 58). Further, the inhibition of SV3T3 cell agglutination caused by the calcium ionophores could not be augmented by the addition of colchicine (49). However, Con A agglutination of untransformed 3T3 cells was not appreciably altered by A23187 or X537A, unless cytochalasin B was additionally present (49). In these latter experiments the concentra-

tion of Con A required for maximum cell agglutination of 3T3 cells was ~1,400 μg/ml in the controls, ~1,200 μg/ml with A23187 (5 μg/ml) plus Ca^{2+}, ~1,100 μg/ml with cytochalasin B (15 μg/ml), and 250 μg/ml with A23187 plus Ca^{2+} in the presence of cytochalasin B (49). The binding of ^{3}H-Con A to SV3T3 or 3T3 cells was not affected by these treatments (49).

Lectins such as Con A under certain conditions can alternatively promote capping of their surface receptors on mouse lymphoid cells or inhibit the capping of other receptors such as surface-immunoglobulin. Low Con A concentrations (usually ≤ 5 μg/ml) or incubation of cells at 0–4°C prior to addition of Con A generally results in Con A-induced Con A receptor capping (34–36), but addition of higher Con A concentrations (usually > 25 μg/ml at 37°C inhibits capping of surface-immunoglobulin receptors on spleen cells. Loor et al. (30), Yahara and Edelman (35, 36, 59) and de Petris (38, 40) have shown that colchicine blocks the Con A inhibition of anti-immunoglobulin-induced capping, suggesting that Con A binding to its receptors could modulate the mobility and distribution of other lymphocyte cells surface receptors through a microtubular system. We reasoned that if a microtubular system was involved in "anchoring" certain lymphocyte surface receptors as proposed by Yahara and Edelman (35, 36, 59), then calcium ionophores in culture medium containing Ca^{2+} might well mimic the effects of colchicine or vinblastine (49). A23187 or X537A were found to block the Con A inhibition of anti-immunoglobulin-induced capping, but these drugs alone had little effect on cap formation (49), while Valinomycin, a K^{+}-specific ionophore, had no effect on either capping or Con A inhibition of capping (49). Schreiner and Unanue (60) have recently reported that treatment of mouse spleen lymphocytes with A23187 in the presence of extracellular Ca^{2+} stopped capping without addition of Con A. The possible reasons for this discrepancy in the action of calcium ionophores is not immediately apparent, but they could reflect different experimental conditions, drug lots, etc. The important point here, however, is that experimental manipulation of cellular Ca^{2+} can result in alterations in cytoskeletal elements.

LOCAL ANESTHETICS AND CELLULAR CALCIUM

The mechanism of local anesthetic action on cytoskeletal systems is unknown, but the experiments described here and elsewhere (26, 27, 49, 60) suggest that Ca^{2+}-sensitive functions are involved. Local anesthetics possess high affinity for Ca^{2+}-binding sites and are able to displace Ca^{2+} from cellular membranes (2, 8, 9). Anesthetic-induced alterations in Ca^{2+} binding to the plasma membrane and cell membrane-associated cytoskeletal structures could perturb the linkage of integral membrane receptors to these cytoplasmic elements at the inner surface of the plasma membrane (26, 27, 44, 49). An example of this possibility can be found in the human erythrocyte membrane where Ca^{2+} is involved in stabilizing a peripheral membrane spectrin-actin network at the inner membrane surface (61–63) which is involved in the trans-membrane control of at least two surface glycoproteins (64, 65). Unbound spectrin will only reassociate with membranes in the presence of Ca^{2+} (66), and similar forces could be involved in stabilizing cytoskeletal systems to the inner membrane surfaces of other cell types (67). Local anesthetics cause dramatic changes in erythrocyte morphology (68), and these effects might be due, in part, to modifications in Ca^{2+} sensitive components at the inner surface (44).

In cells containing membrane-associated microtubule-microfilament systems, local anesthetics probably cause displacement of Ca^{2+} from cellular membranes, and could produce competitive inhibition of Ca^{2+}-sensitive functions required for microtubule integ-

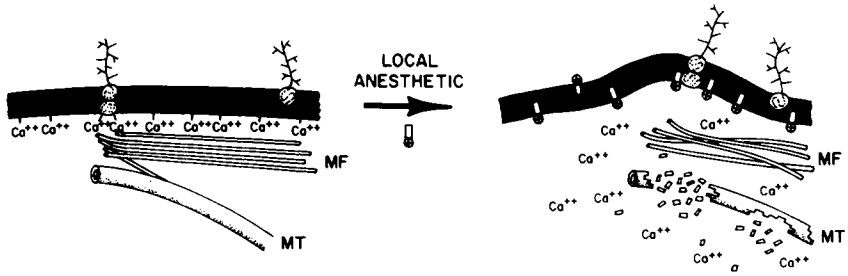

Fig. 2. Proposal to explain the action of tertiary amine local anesthetics on cell membranes and membrane-associated cytoskeletal assemblies. The partitioning of drug molecules into cellular membranes results in displacement of membrane-bound Ca^{2+} which could be involved in stabilizing microfilaments (MF) to the plasma membrane inner surface. In addition, the resulting increase in free cellular Ca^{2+} could be responsible for microtubule (MT) depolymerization. (Not drawn to scale.)

rity. The structural integrity of microtubules is particularly sensitive to Ca^{2+} concentrations in vitro, and cytoplasmic Ca^{2+} concentrations have been proposed to control the state of cellular microtubule polymerization (55, 56). An anesthetic-induced release of membrane-bound Ca^{2+} might raise intracellular Ca^{2+} concentrations to levels ($> 10^{-5}$ M) capable of microtubule depolymerization (Fig. 2). Although the nature of the effects of local anesthetics on microfilament systems is not known, these cytoskeletal elements could also possess Ca^{2+} sensitive functions. Alternatively, membrane-bound Ca^{2+} could stabilize and link actin-containing microfilament systems to the plasma membrane similar to actin-spectrin complexes in human erythrocyte membranes. In this case local anesthetics would cause an uncoupling of microfilaments from the plasma membrane, an event that appears to occur within minutes after tetracaine treatment of 3T3 cells (44). Of course, a combination of these mechanisms and others not yet identified could be involved in the membrane association or attachment and activities of the cytoskeletal elements (67).

Although previous proposals for the mechanism of action of local anesthetics in producing alterations in cell morphology and other functional changes have been based primarily on the ability of these drugs to interact with membrane lipids (2, 8, 9, 68), we suggest that the effects of these agents on membrane-associated cytoskeletal assemblies must also be considered. Indeed, in certain instances anesthetic-induced alterations in cytoskeletal elements may represent the primary mechanism underlying the observed changes in cell function. This is particularly apparent in our experiments where combinations of cytoskeletal disrupting drugs such as colchicine (or vinblastine) plus cytochalasin B produced similar effects to local anesthetics on cell agglutination and receptor redistribution and mobility.

ACKNOWLEDGMENTS

We thank J. Smith, P. Newhouse and A. Brodginski for valuable technical assistance. This work was supported by NCI contract CB-33879 from the Tumor Immunology Program (to G. L. Nicolson), NSF grant BMS-72-02107 from the Human Cell Biology Program (to G. L. Nicolson) and NIH grant CA-13393 (to G. Poste).

REFERENCES

1. Ritchie, J. M., and Greengard, P., Ann. Rev. Pharmacol. 6:405–430 (1966).
2. Seeman, P., Pharmacol. Rev. 24:583–655 (1972).
3. Eyring, H., Woodbury, J. W., and D'Arrigo, J. S., Anesthesiology 38:415–424 (1973).
4. Feinstein, M. B., J. Gen. Physiol. 48:357–374 (1964).
5. Blaustein, M. P., and Goldman, D. E., Science 153:429–432 (1966).
6. Hauser, H., Penkett, S. A., and Chapman, D., Biochim. Biophys. Acta 183:466–475 (1969).
7. Giotta, G. J., Chan, D. S., and Wang, H. H., Arch. Biochem. Biophys. 163:453–458 (1974).
8. Papahadjopoulos, D., Biochim. Biophys. Acta 211:467–477 (1970).
9. Papahadjopoulos, D., Biochim. Biophys. Acta 265:169–186 (1972).
10. Papahadjopoulos, D., Jacobson, K., Poste, G., and Sheperd, G., Biochim. Biophys. Acta 394:504–519 (1975).
11. Wang, H. H., and Koblin, D. D., Biophys. J. 16:105a (1976).
12. Balzer, H., Naunyn-Schmiedeberg's Arch. Exp. Path. Pharmak. 274:256–272 (1972).
13. Feinstein, M. B., and Paimre, M., Fed. Proc. 28:1643–1648 (1969).
14. Chen, S. S., J. Physiol. 238:313–328 (1974).
15. Hubbell, W. L., Metcalfe, J. C., Metcalfe, S. M., and McConnell, H. M., Biochim. Biophys. Acta 219:415–427 (1970).
16. Roth, S., and Seeman, P., Nature New Biol. 231:284–285 (1971).
17. Rabinovitch, M., and De Stefano, M. J., Exp. Cell Res. 88:153–162 (1974).
18. Gail, M. H., and Boone, C. W., Exp. Cell Res. 73:252–255 (1972).
19. O'Brien, J. R., J. Clin. Pathol. 15:446–455 (1962).
20. Rabinovitch, M., and De Stefano, M. J., J. Cell Biol. 59:165–176 (1973).
21. Byers, M. R., Fink, B. R., Kish, S. J., and Aaseim, G. M., in "Progress in Anesthesiology," B. R. Fink (Ed.), Raven Press, New York, pp. 25–28 (1975).
22. Douglas, W. W., in "Secretory Mechanisms of Exocrine Glands," N. A. Thorn and O. H. Petersen (Eds.), Academic Press, New York, pp. 116–136 (1975).
23. Poste, G., and Allison, A. C., Biochim. Biophys. Acta 300:421–465 (1973).
24. Poste, G., and Reeve, P., Exp. Cell. Res. 72, 556–560 (1972).
25. Poste, G., Papahadjopoulos, D., Jacobson, K., and Vail, W. J., Nature 253:552–554 (1975).
26. Poste, G., Papahadjopoulos, D., Jacobson, K., and Vail, W. J., Biochim. Biophys. Acta 394:520–539 (1975).
27. Poste, G., Papahadjopoulos, D., and Nicolson, G. L., Proc. Natl. Acad. Sci. USA 72:4430–4434 (1975).
28. Ryan, G. B., Unanue, E. R., and Karnovsky, M. J., Nature 250:56–57 (1974).
29. Taylor, R. B., Duffus, P. H., Raff, M. C., and de Petris, S., Nature New Biol. 233:225–229 (1971).
30. Loor, F., Forni, L., and Pernis, B., Eur. J. Immunol. 2:203–212 (1972).
31. Unanue, E. R., and Karnovsky, M. J., Transplant. Rev. 14:184–210 (1973).
32. de Petris, S., and Raff, M. C., in "Locomotion of Tissue Cells," R. Porter and D. N. Fitzsimons (Eds.), Ciba Fdn. Symp. 14, Elsevier-Excerpta Medica-North Holland, Amsterdam, pp. 27–52 (1973).
33. Wilson, L., and Bryan, J., in "Advances in Cell and Molecular Biology," Vol. 3, E. J. Du Praw (Ed.), Academic Press, New York, pp. 21–72 (1974).
34. Edelman, G. M., Yahara, I., and Wang, J. L., Proc. Natl. Acad. Sci. USA 70:1442–1446 (1973).
35. Yahara, I., and Edelman, G. M., Nature 236:152–155 (1973).
36. Yahara, I., and Edelman, G. M., Exp. Cell. Res. 81:143–155 (1973).
37. Berlin, R. D., Oliver, J. M., Ukena, T. E., and Yin, H. H., Nature 247:45–46 (1974).
38. de Petris, S., Nature 250:54–56 (1974).
39. Ukena, T. E., Borysenko, J. Z., Karnovksy, M. J., and Berlin, R. D., J. Cell. Biol. 61:70–82 (1974).
40. de Petris, S., J. Cell Biol. 65:123–146 (1975).
41. Wessells, N. K., Spooner, B. S., Ash, J. F., Bradley, M. O., Luduena, M. A., Taylor, E. L., Wrenn, J. T., and Yamada, K. M., Science 171:135–143 (1971).
42. Carter, S. B., Nature 225:858–859 (1970).
43. Godman, G. C., Miranda, A. F., Deitch, A. D., and Tanenbaum, S. W., J. Cell Biol. 64:644–667 (1975).
44. Nicolson, G. L., Smith, J. R., and Poste, G., J. Cell Biol. 68:395–402 (1976).

45. Nicolson, G. L., J. Natl. Cancer Inst. 50:1443–1451 (1973).
46. Poste, G., Exp. Cell Res. 73:319–328 (1972).
47. Nicolson, G. L., Nature New Biol. 243:218–220 (1973).
48. Poste, G., and Reeve, P., Nature New Biol. 247:469–471 (1974).
49. Poste, G., and Nicolson, G. L., Biochim. Biophys. Acta 426:148–155 (1976).
50. McNutt, N. S., Culp, L. A., and Black, P. H., J. Cell Biol. 50:691–708 (1971).
51. Allison, A. C., and Nunn, J. F., Lancet 2:1326–1329 (1968).
52. Haschke, R. H., Byers, M. R., and Fink, B. R., J. Neurochem. 22:837–843 (1974).
53. Hinkley, E. R., Jr., and Telse, A. G., J. Cell Biol. 63:531–540 (1974).
54. Weisenberg, R. C., Science 177:1104–1105 (1972).
55. Olmsted, J. B., Marcum, J. M., Johson, K. A., Allen, C., and Borisy, G. G., J. Supramol. Struct. 2:429–450 (1974).
56. Kirschner, M. N., and Williams, R. C., J. Supramol. Struct. 2:412–428 (1974).
57. Kuriyama, R., and Sakai, H., J. Biochem. 75:463–468 (1974).
58. Yin, H. H., Ukena, T. E., and Berlin, R. D., Science 178:867–868 (1972).
59. Yahara, I., and Edelman, G. M., Exp. Cell Res. 91:125–142 (1975).
60. Schreiner, G. F., and Unanue, E. R., J. Exp. Med. 143:15–31 (1976).
61. Marchesi, V. T., Steers, E., Jr., Tillack, T. W., and Marchesi, S. L., in "The Red Cell Membrane, Structure and Function," G. A. Jamieson and T. Greenwalt (Eds.), J. B. Lippincott Co., Philadelphia, pp. 117–130 (1969).
62. Nicolson, G. L., Marchesi, V. T., and Singer, S. J., J. Cell Biol. 51:265–272 (1971).
63. Tilney, L. G., and Detmers, P., J. Cell Biol. 66:508–520 (1975).
64. Nicolson, G. L., and Painter, R. G., J. Cell Biol. 59:395–406 (1973).
65. Ji, T. H., and Nicolson, G. L., Proc. Natl. Acad. Sci. USA 71:2212–2216 (1974).
66. Juliano, R. L., Kimelberg, H. K., and Papahadjopoulos, D., Biochim. Biophys. Acta 241:894–905 (1971).
67. Nicolson, G. L., Biochim. Biophys. Acta Rev. Biomembranes 457:57–108 (1976).
68. Sheetz, M. P., and Singer, S. J., Proc. Natl. Acad. Sci. USA 71:4457–4461 (1974).