INHIBITION OF CELLULAR DIFFERENTIATION BY PHOSPHOLIPASE C

I. Effects of the Enzyme on Myogenesis and Chondrogenesis In Vitro

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ABSTRACT

In cell culture, a partially purified commercial preparation of phospholipase C (PLC) from *Clostridium welchii* inhibited fusion of myoblasts at concentrations of 12–50 μg per ml. At lower concentrations, PLC-treated cultures were indistinguishable from controls, and at concentrations above 100 μg per ml, PLC-treated cells detached from their substrates. The effect was reversible and fusion resumed approximately one cell cycle time after removal of the enzyme. Neither the percent of cells in the mitotic cycle nor the duration of the different phases of the cycle were altered by PLC at concentrations which inhibited fusion. Cell motility was not reduced by the enzyme. Unfused, PLC-treated myoblasts were virtually indistinguishable in ultrastructure from untreated cells just before fusion. In the presence of PLC, mononucleated myogenic cells did not synthesize thick (150 Å) filaments. Treatment of culture medium with insolubilized commercial PLC did not abolish the capacity of the medium to support myogenesis. Chondrocytes treated with PLC divided repeatedly but failed to synthesize metachromatic matrix and failed to incorporate labeled sulfate into chondroitin sulfate. PLC was further purified by chromatography on Sephadex G-100. The resulting preparation was free of detectable protease, yielded one band on SDS-acrylamide gel electrophoresis, and displayed all of the biological activities of the less pure material.

INTRODUCTION

Through largely indirect evidence, the cell surface has been implicated in the control of cellular differentiation. Many types of cells do not express their specialized properties in isolation from other cells but appear to require surface-to-surface contact in order to carry out their differentiated functions. Myoblasts and chondrocytes are examples of such cell types. In order to form skeletal muscle fibers, myoblasts must contact each other and then fuse to form multinucleated myotubes (14, 16, 27, 34). Fusion occurs only during the G_1 (or G_0) phase of the cell cycle (3, 28), apparently requires the presence of calcium (29), and may require a mitosis to be expressed (3, 25). Chondrocytes, in multiplying cultures, first begin to deposit extracellular cartilage matrix in areas between cells where there is close surface-to-surface contact (35). The molecular basis for surface interaction or "cell recognition" is unknown. It has been proposed that enzymes on the cell periphery are involved in surface interactions (32). In this report we describe initial studies which may lead to an identification

of some of the molecular species involved in the cell recognition phenomena preceding fusion of myoblasts or deposition of matrix by chondrocytes. We have found that phospholipase C (PLC) reversibly inhibits fusion of myoblasts without significantly altering cell surface activities associated with mitosis or cell movement. PLC also suppresses cartilage matrix synthesis by chondrocytes. The antidifferentiation effect of PLC is similar to that of 5-bromodeoxyuridine (1, 4, 36) and may result from the liberation of a class of proteins from the cell surface.

MATERIALS AND METHODS

Cell Culture

Myogenic cells and vertebral chondrocytes were prepared from 11-day chick embryos as described previously (25). The cells were plated at an initial density of 0.33×10^6 per ml of medium (Eagle's Minimal Essential Medium (MEM), 7.8 parts; horse serum, 1.0 part; embryo extract, 1.0 part; penicillin-streptomycin solution, 0.1 part; fungizone, 0.1 part).

In all experiments, 0.5×10^6 cells were grown at 37.5°C on gelatin- or plasma clot-coated 22-mm square cover slips in 35-mm Petri dishes in a water-saturated atmosphere containing 5% CO_2 in air. The medium was changed on the day after initiation of the cultures and every other day thereafter. At appropriate times myogenic cell cultures were washed once with Earle's balanced salt solution (BSS), fixed in ethanol:formalin:acetic acid (20:2:1), and stained with Ehrlich's hematoxylin. Chondrocyte cultures were fixed and stained with toluidine blue according to the procedure of Danes and Bearn (7).

Electron Microscopy

Cultures were rinsed with BSS and fixed for 90 min with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 0.01 M $CaCl_2$. They were then rinsed in 0.1 M sucrose in the same buffer and were postfixed for 60 min in 1% OsO_4, also in cacodylate buffer. Still in the culture dishes, the tissues were passed through graded ethanols and were embedded in Epon. Sections were stained with uranyl acetate and lead citrate and were examined in a Philips 200 microscope.

Autoradiography

Myogenic cells were exposed to [*methyl*-^{3}H]thymidine (New England Nuclear, Boston, Mass., sp act 6.7 Ci per mmol) at a concentration of 1.0 μCi per ml of culture medium. For cell cycle determinations, cultures were exposed to the isotope for 15 min on the second day in vitro. At the end of the 15-min period, several cultures were fixed immediately. The remaining dishes were washed three times with BSS, and fresh medium containing 0.2 mM unlabeled thymidine was added. Several cultures were fixed every 2 h thereafter. PLC at a concentration of 25 μg per ml had been present in the medium of half the cultures for 24 h before the addition of labeled thymidine. This concentration was maintained throughout the determination of the cell cycle time. In another experiment, [*methyl*-^{3}H]thymidine was present during the second 24 h of a 2-day exposure to PLC. PLC and labeled thymidine were then removed and fresh medium containing unlabeled thymidine was added. Cultures were fixed after incubation for 12 or 24 h in unlabeled medium. In all cases, autoradiograms were prepared by dipping slide-mounted cover slips in Kodak NTB3 emulsion diluted 1:1 with water. Exposures were from 3 days to 1 wk. Autoradiograms were developed in Microdol-X and were stained with Ehrlich's hematoxylin.

Enzymes

Phospholipase C (Sigma Chemical Co., St. Louis, Mo.; from *Clostridium welchii*; sp act 2.0 U per mg) was a partially purified enzyme from bacterial culture medium prepared according to Ellner (9). Further purification was carried out either by the procedure of Zwaal et al. (38) or by our own method. In our procedure, 10 mg of PLC were dissolved in 0.1 M phosphate buffer, pH 6.5, and applied to a 1.6×90 cm column of Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) previously equilibrated with the same buffer. Fractions of 4.0 ml were eluted with the phosphate buffer at rates of 20 or 55 ml per h. There was no significant difference in fractionation with these two rates. Optical density at 280 nm was continuously monitored with a double-beam recording spectrophotometer. Samples (10 or 100 μl) of each fraction were assayed for PLC activity and for effect on myogenic cells or chondrocytes which had been in culture for 24 h. Enzymatic activity was assayed by a procedure similar to that reported by Mavis et al. (23). The assay was based on the fact that PLC releases water-soluble phosphorylcholine from lecithin. Using phosphatidylcholine labeled only in the choline moiety, enzymatic activity can be measured by extracting the water-soluble counts released by PLC from an ether solution of lecithin. For each sample to be assayed, 10 μl (10,000 cpm) of [1,2-^{14}C]-phosphatidylcholine chloride (International Chemical and Nuclear Corporation, Burbank Calif., sp act 117 mCi per mmol) were dissolved in 2.0 ml of ether containing 1.0 mg of unlabeled L-α-lecithin (Sigma Chemical Co.) as a carrier. The ether solution was placed in a screw-capped tube containing 0.4 ml of 0.1 M phosphate buffer, pH 7.1 with 1.0 mM $CaCl_2$.

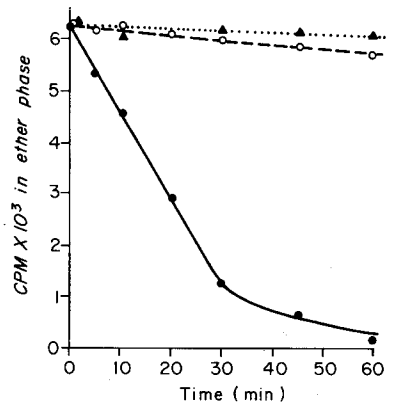

FIGURE 1 Kinetics of enzymatic degradation of [^{14}C]lecithin. Ordinate represents PLC activity measured as counts per minute remaining in ether phase. Ether was counted since it was the top phase and a sample could be collected cleanly without contamination from the water phase which contained labeled phosphorylcholine and emulsified undegraded lecithin. Depicted are the complete reaction mixture with 0.1 mg of commercial PLC (-●—●-), reaction mixture without PLC (-○---○--), and complete reaction mixture containing 1.0 mg lysolecithin, an inhibitor of PLC (··△···△··). Boiled PLC yielded a curve similar to that with no enzyme.

To start the reaction, 0.1 ml of a column fraction was added and the tube was shaken vigorously at room temperature for 30 min. The phases were then permitted to separate and 0.5 ml of the ether phase was counted in 10 ml of scintillation fluid. Preliminary experiments (Fig. 1) demonstrated the usefulness of this procedure as an assay.

A purified preparation of PLC from *B. cereus* was a gift of Dr. L. L. M. van Deenen.

Commercial and purified PLC preparations were subjected to electrophoresis in 5% polyacrylamide gels in 1% sodium dodecyl sulfate according to the procedure of Maizel (20). PLC preparations were run on gels 10 cm long at 3 V/cm for 7 h. The gels were then stained with amido black.

Collagenase (Worthington Biochemical Corp., Freehold, N. J.), testicular hyaluronidase (Calbiochem, San Diego, Calif.), chondroitinase ABC (Miles Research Div., Miles Laboratories Inc., Elkhart, Ind.), pronase (Calbiochem), phospholipase A (Sigma Chemical Co.; from bee venom, 1180 U per mg), and phospholipase D (Sigma Chemical Co.; from cabbage, 22 U per mg) were dissolved in BSS and sterilized by Millipore filtration. These preparations were diluted into tissue culture medium with no further purification.

Agarose Coupling of PLC

PLC was attached to agarose beads (Sepharose 4B, Pharmacia Fine Chemicals, Inc.) according to the procedure of Cuatrecasas (5). 50 mg of PLC were dissolved in 5.0 ml of 0.1 M phosphate buffer, pH 7.1, made 50% (vol/vol) with glycerol. This solution was then added to CNBr-activated agarose beads and was stirred overnight at 4°C. The beads were then washed with the same buffer until no enzymatic activity could be detected in the washings. Settled, coupled beads (10 μl) were assayed for PLC activity as described above and were found to release as much label from [^{14}C]lecithin as 20–25 μg of commercial enzyme.

Coupled beads (0.1 ml) were incubated for 16 h at 37.5°C with 10.0 ml of complete tissue culture medium. The beads were then removed by centrifugation and the medium was filtered. Myogenic cells were cultured in this medium. Control media were either treated with agarose beads which had been subjected to all steps of the coupling procedure but without PLC or contained crude PLC but no beads.

Extracellular Matrix Synthesis

Incorporation of [^{35}S]sulfate ($Na_2{}^{35}SO_4$, New England Nuclear, sp act 738 mCi per mmol), into polysaccharide secreted by chondrocytes was measured according to previously described procedures (24, 26). Labeled sulfate, at a concentration of 5.0 μCi per ml of medium, was added to untreated 6-day-old cultures; to cultures which had been exposed to 12.5 or 25.0 μg of PLC per ml for 6 days; to PLC-treated cultures 48 h after removal of PLC; and to PLC-treated cultures which were trypsinized and permitted to grow to confluence in the absence of enzyme. The label was present in the medium for a 24-h period before the cultures were frozen on a dry-ice acetone mixture. Media and cells with associated matrix were separately digested with pronase and precipitated with cold trichloroacetic acid. The supernatants were exhaustively dialyzed against 0.01 M sodium sulfate and finally against distilled water. Samples were counted in a scintillation counter and were characterized by electrophoresis on cellulose acetate strips in pyridinium formate buffer (0.1 M, pH 3.0, 10 V/cm, 60 min, 25°C).

RESULTS

Effect of Commercial PLC on Myogenic Cells

A series of myogenic cell cultures were set up at an initial density of 0.5 × 10^6 cells in 1.5 ml of medium in 35-mm dishes. The cells were permitted to attach to the substrate (plasma clot or gelatin)

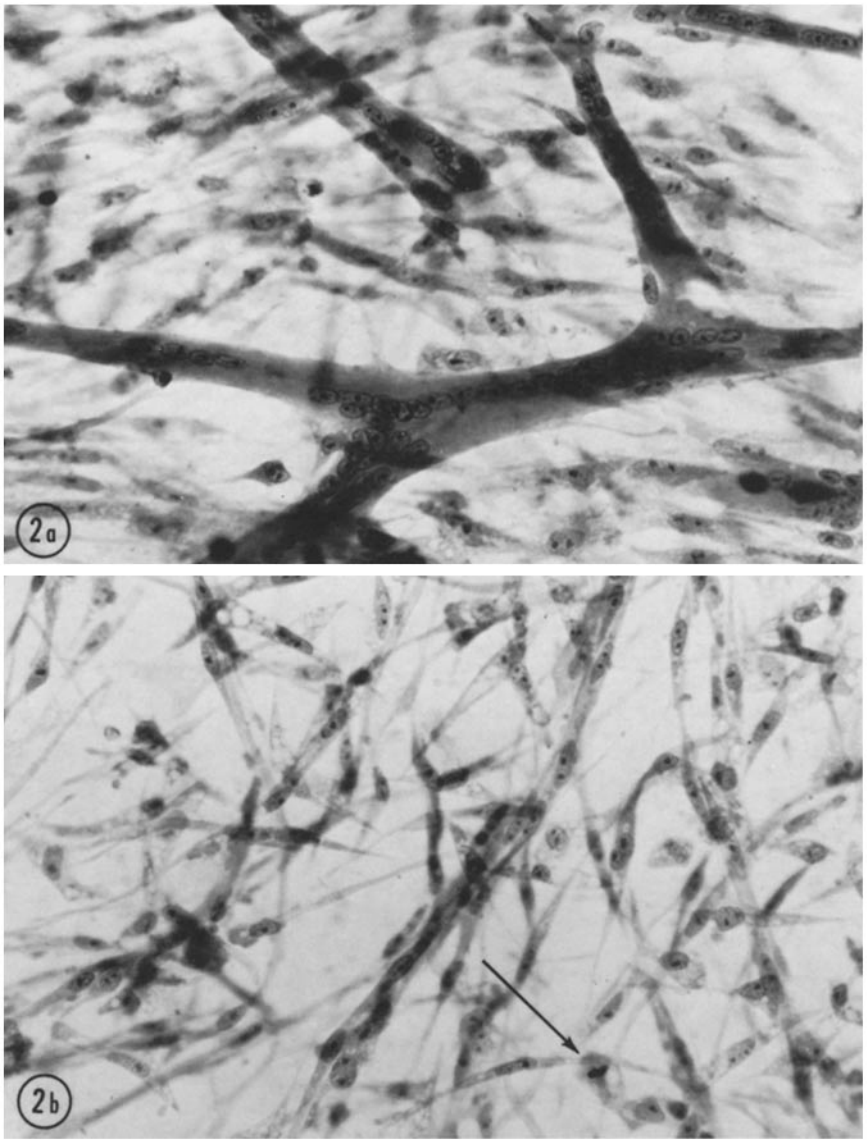

FIGURE 2 Multinucleated myotubes in a control culture (Fig. 2 a). Unfused myogenic cells in PLC (25 μg per ml) (Fig. 2 b). Mitotic figure can be seen in PLC culture (arrow). Both cultures were fixed on day 4. × 240.

overnight. The medium was then removed and fresh medium containing 0, 3, 6, 12.5, 25, or 50 μg of commercial PLC was added. Control dishes received boiled PLC or PLC which had been dialyzed against BSS. The concentrations of PLC were kept constant when ever the medium was changed. By the 3rd day, multinucleated myotubes had formed in cultures containing 0, 3, or 6 μg of PLC and in cultures containing boiled enzyme (Fig. 2 a). These cultures were indistinguishable from each other in that there were no differences in size, number, or degree of multinuclearity of myotubes in any of the concentration of PLC. In contrast, cultures containing dialyzed or undialyzed PLC at concentrations of 12, 25, or 50 μg per ml had no myotubes (Fig. 2 b). To determine if a contaminating protease might have prevented fusion, myogenic cells were cultured in medium containing trypsin, pronase, or collagenase at concentrations of 10 and 20 μg per ml.

Myotubes formed in these cultures and were indistinguishable from controls. Similarly, neither hyaluronidase nor chondroitinase ABC prevented fusion at concentrations of 10–20 μg per ml. Neither commercial PLC nor more purified PLC (see below) had detectable proteolytic activity in an azoalbumin assay (37) which could detect trypsin- or pronase-induced proteolysis at enzyme concentrations of 0.2 μg per ml. Phospholipase A, at concentrations of 5–50 μg per ml, caused vacuolization and death of cells. Phospholipase D had no effect on fusion up to concentrations of 20–30 μg per ml. At higher concentrations, cells detached or died.

Mitotic figures were found in all concentrations of PLC. Cell density (cells per unit area) was identical in all concentrations of PLC in which no fusion occurred. PLC-treated cells showed no tendency to pile up locally, indicating that motility was not markedly impaired. However, cells in PLC were, on the average, more spindle shaped than untreated cells. Multicellular strings were observed in PLC-treated cultures. Similar strings are commonly seen in control dishes as an apparent prelude to fusion. However, since there is no certain method of identifying nonstriated myoblasts, it is possible that none of the cells in the strings were engaged in any prefusion activities. In spite of the close contacts between cells in such strings, no fusion was observed with the electron microscope (see below). PLC-treated cells continued to multiply in the presence of the enzyme and, by the 4th–5th day, reached confluence and formed multilayers. No myotubes were observed up to the 12th day in the presence of PLC. Hence, the effect of the enzyme was not merely to delay fusion.

With the light microscope it is often difficult to be certain that cells are fused. Therefore, control and PLC-treated cultures were fixed with 2.5% glutaraldehyde and examined in the electron microscope (Fig. 3). Surface membranes of mononucleated cells were closely apposed in PLC-treated cultures, but no fusion was observed. Membranes and cytoplasmic organelles in enzyme-treated cells were indistinguishable from those of control cells. Many cells in PLC contained microtubules and intermediate-sized filaments (11). No thick (150 Å) and few thin (60 Å) filaments were found upon measuring filament diameters on micrographs. Controls for measurements were thick and thin filaments found in 6-day myotubes in untreated cultures.

Reversibility of Fusion Inhibition

A series of cultures were set up in commercial PLC at a concentration of 25 μg per ml of medium. On the 3rd, 4th, and 5th days, the PLC medium was removed and the cells were washed *in situ* with BSS. Fresh medium containing no PLC was then added. At 2-h intervals several cultures were fixed. Multinucleated myotubes began to appear 8–10 h after removal of enzyme (Fig. 4). Spontaneous contractions were observed in these myotubes by the 12th–18th h. This experiment demonstrated that the effect of PLC was reversible and was not a result of selective killing of muscle precursor cells in the population. Although the 8–10-h period corresponds to the time required for one traverse of the cell cycle (see below, and reference 3), it may represent the time required for repair or resynthesis of cell surface constituents released or damaged by PLC and may only be fortuitously equal to the cycle time.

PLC and the Cell Cycle

Although mitotic figures were observed in PLC-treated cultures, it seemed possible that the enzyme was preferentially blocking cell division in muscle precursors but not in the "contaminating" connective tissue cells in the cultures. If PLC were acting in this manner, fusion would be inhibited because of the failure of myoblasts to enter the G_1 phase of the cell cycle or to acquire the capability to fuse through a critical or quantal mitosis (3). The following experiments indicate that PLC does not act in this way.

[*Methyl*-^{3}H]thymidine at a concentration of 1.0 μCi per ml was added to 2-day-old cultures which had been in commercial PLC (25 μg per ml) for 1 day. After a further incubation for 24 h, the enzyme and labeled thymidine were removed and the cells were permitted to recover in the presence of 0.2 mM unlabeled thymidine. After myotubes had formed, autoradiograms were prepared and showed that almost all nuclei in myotubes were labeled (Fig. 5). In a second experiment, cell cycle times were determined according to conventional techniques (3, 31). Two series of cultures were set up. One had 25 μg of PLC per ml; the other had no PLC. The labeling index (percent of nuclei incorporating labeled thymidine at zero

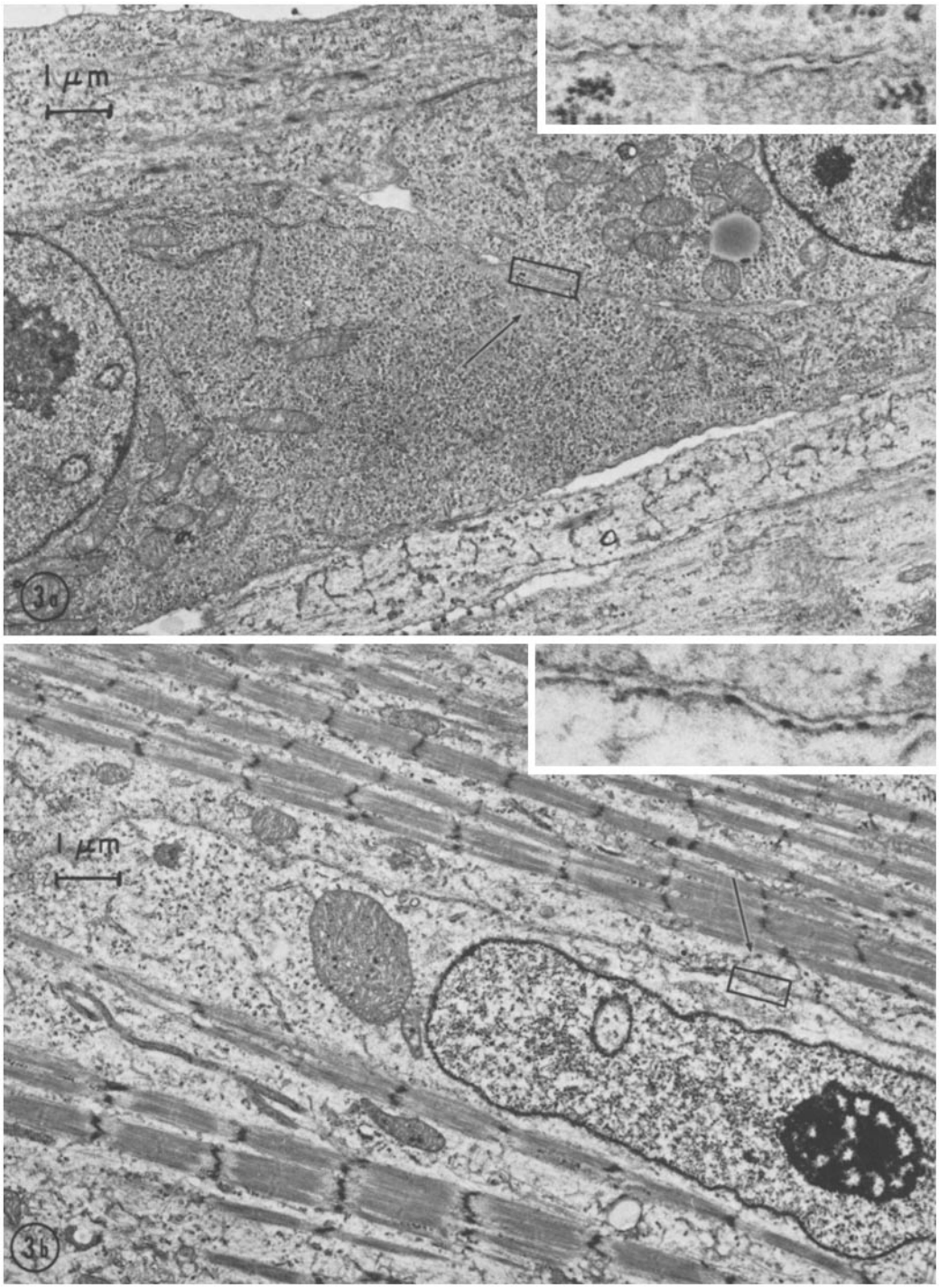

FIGURE 3 Electron micrographs of PLC-treated (Fig. 3 a) and control myogenic cells (Fig. 3 b) on day 6. Cells in PLC are in close proximity but surface membranes are intact. Control cells are also in close proximity but contain myofibrils. Insets are higher power views of membrane regions outlined by rectangles (arrows). Electron-opaque plaques of unknown significance are visible on the surfaces of both control and PLC-treated cells. × 9,000. Insets, × 60,000.

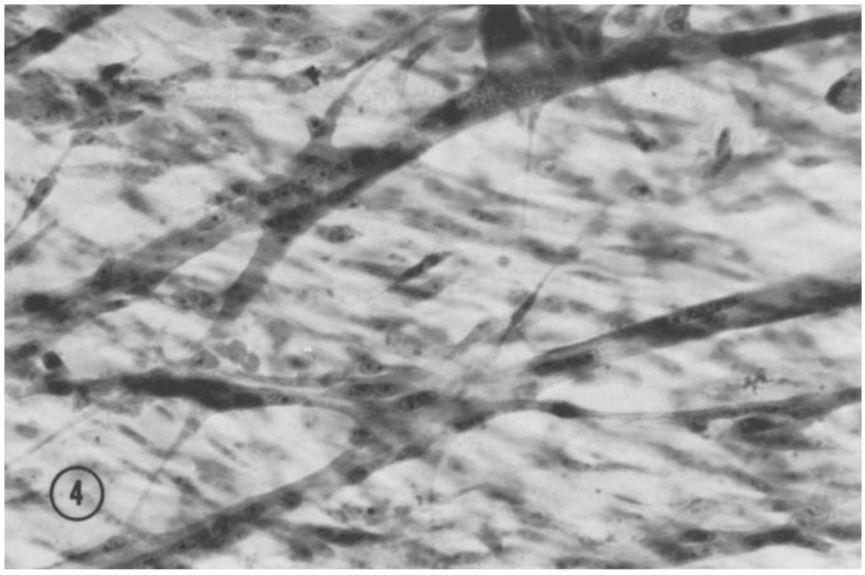

FIGURE 4 Myotubes forming in a culture 10 h after recovery from PLC treatment. Day 4. × 240.

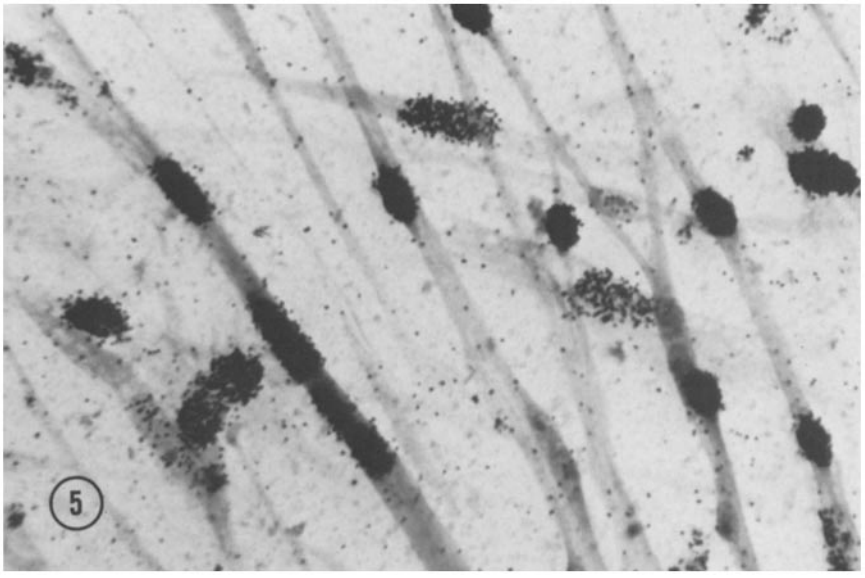

FIGURE 5 Labeled nuclei in myotubes recovering from PLC-induced fusion block. Cells were fixed 12 h after removal of PLC and [^{3}H]thymidine. × 400.

time) was determined for both control and PLC-treated cultures by counting 1,000 nuclei in each of four cultures. 32% of control nuclei and 33% of PLC-treated nuclei were labeled at the end of a 15-min exposure to radioactive thymidine. Therefore, the same number of cells were in cycle in PLC-treated cultures as in controls. It is apparent from Fig. 6 that the duration of the cell cycle and its component phases was unaffected by concentrations of PLC which inhibited fusion. These experiments implied that the inhibition of fusion was not a secondary result of alteration of the cell cycle and that, if a mitosis were required before fusion would occur, PLC did not act by stopping that mitosis.

Direct Effect of PLC on Cells

In the experiments described above, PLC was used in medium together with cells. It was pos-

sible that the enzyme did not act directly on the cells but rather on some component of the tissue culture medium. Degradation of a factor required for fusion (15) or release of an inhibitor from a component of the medium could lead to the observed failure of myotube formation. Commercial PLC was therefore coupled to a removable substrate (agarose beads) and was incubated with complete medium for 16 h. The substrate and attached, active, enzyme were then removed by centrifugation. Myogenic cells formed myotubes when cultured in this medium (Fig. 7). It therefore appeared that PLC was acting directly on the myogenic cells and presumably on the cell surface.

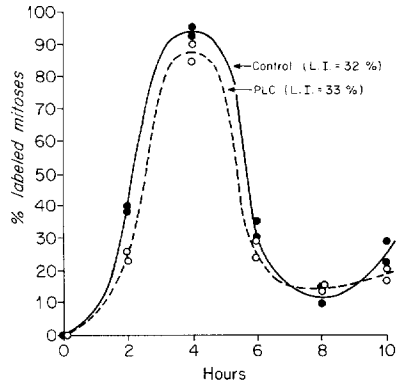

FIGURE 6 Cell cycle determinations for untreated myogenic cells (-●—●-) and myogenic cells in PLC (25 μg per ml) (--○--○--). Curves are identical and abeling indices (L.I.) were approximately equal.

Effects of Purified PLC

All of the experiments described above were repeated with purified PLC and the results were identical to those obtained with commercial PLC. Both enzymatic activity and fusion inhibition were followed at each stage of purification. An example is shown in Fig. 8 which depicts optical density, PLC activity, and fusion inhibition as functions of fraction number from the chromatography of partially purified PLC on Sephadex G-100. It can be seen that PLC activity and fusion inhibition ran together on the column. The enzyme apparently represented approximately 5% of the total protein in the commercial preparation as measured by the procedure of Lowry et al. (19). Approximately 0.5 μg of the purified material were sufficient to inhibit fusion in cultures containing 1.5 ml of medium. Upon SDS-polyacrylamide electrophoresis, the purified material displayed one band (Fig. 9). In the absence of serum in the tissue culture medium, cells in PLC began to leak intracellular protein after about 1–2 h. Transfer to serum-containing medium led to recovery but only if transfer was accomplished in less than 4 h. Apparently there is some factor in serum which either protects the cells from the lytic

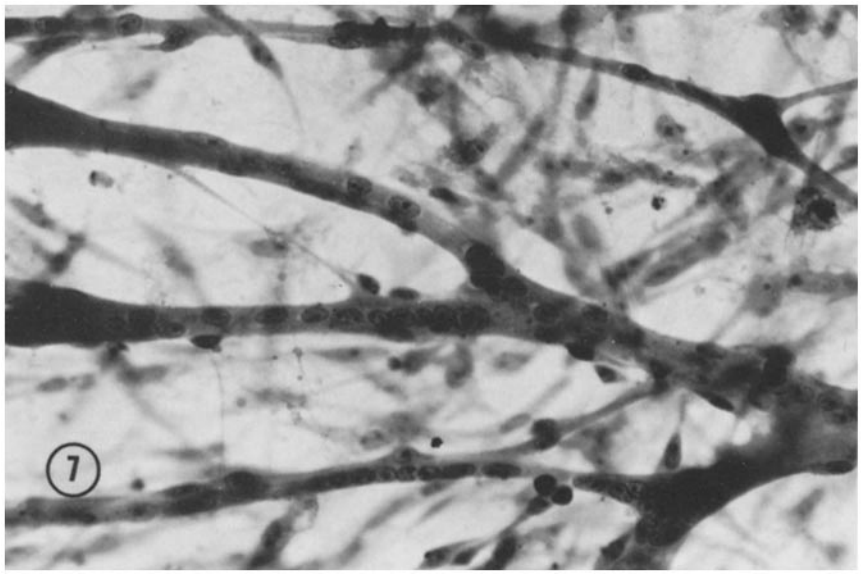

FIGURE 7 Myotubes which formed in medium treated with PLC coupled to agarose beads. × 240.

effects of PLC or is, perhaps, a substrate for the enzyme so that the effective dose which the cells "see" may be much lower than the actual amount of material placed into the medium. PLC from *B. cereus* which was active as a lipase in our assay had no effect on fusion over a range of concentrations from 0.1 to 10 μg per ml of medium.

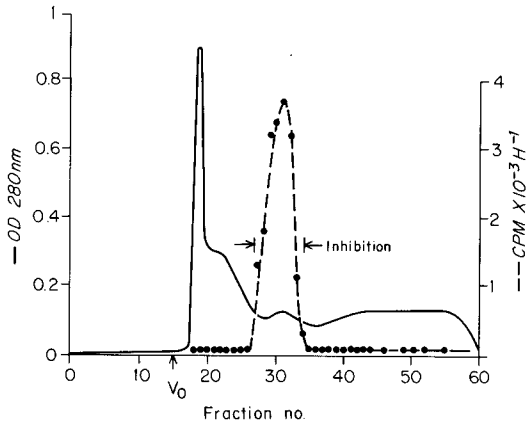

FIGURE 8 Chromatography of 10 mg of commercial PLC on Sephadex G-100. Represented are optical density at 280 nm (solid line) and PLC activity (dashed line). Inhibition of fusion of myoblasts and inhibition of matrix synthesis by chondrocytes were observed only in fractions with PLC activity. In all column runs a small peak of optical density was observed in the region with enzymatic activity.

Effect of PLC on Chondrocytes

Chondrocytes were grown in 12.5 or 25 μg of commercial PLC per ml. As with myogenic cells, chondrocytes multiplied and migrated in the presence of the enzyme. When both control and PLC-treated cultures had reached confluence and controls had deposited extracellular matrix (Fig. 10 a), cultures were fixed and stained with toluidine blue. PLC-treated chondrocytes failed to deposit metachromatic matrix material (Fig. 10 b). PLC was removed from the medium and the cells were permitted to remain in culture for up to 6 days more. Again no metachromatic matrix was observed. Confluent PLC-treated chondrocytes were subcultured and allowed to grow to confluence again, but no cartilage matrix was detected. Incorporation of [^{35}S]sulfate into polysaccharide was markedly inhibited in PLC-treated and recovered cells (Table I). Electrophoresis of sulfated polysaccharide from enzyme-treated cultures showed little if any synthesis of chondroitin sulfate (Fig. 11). Both cells and medium were assayed to rule out the possibility that cells which did not stain metachromatically might have synthesized matrix materials but were unable to deposit them in an insoluble extracellular structure.

DISCUSSION

It is apparent that PLC selectively and reversibly inhibits fusion of myoblasts. PLC also inhibits

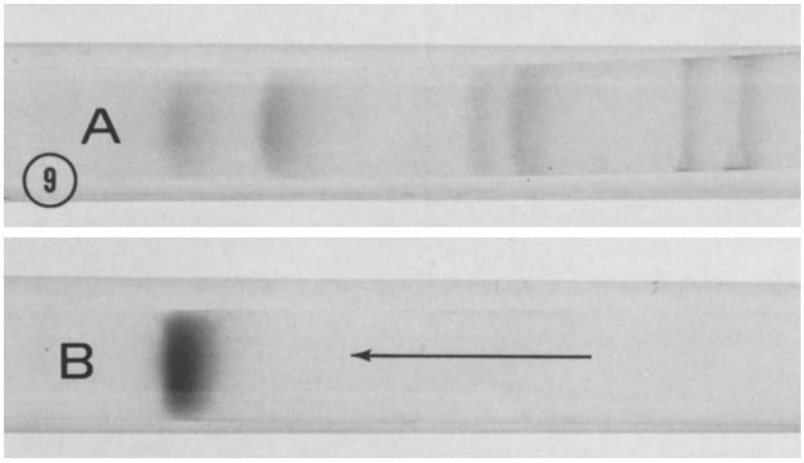

FIGURE 9 Polyacrylamide gels stained with amido black and cleared in 7% acetic acid. Gel *A* contained crude PLC and showed at least six bands. Gel *B* contained PLC fraction from G-100 column illustrated in Fig. 8. Only one band was seen. This band corresponded to the farthest migrating (lowest molecular weight) band of the crude enzyme. Arrow indicates direction of migration.

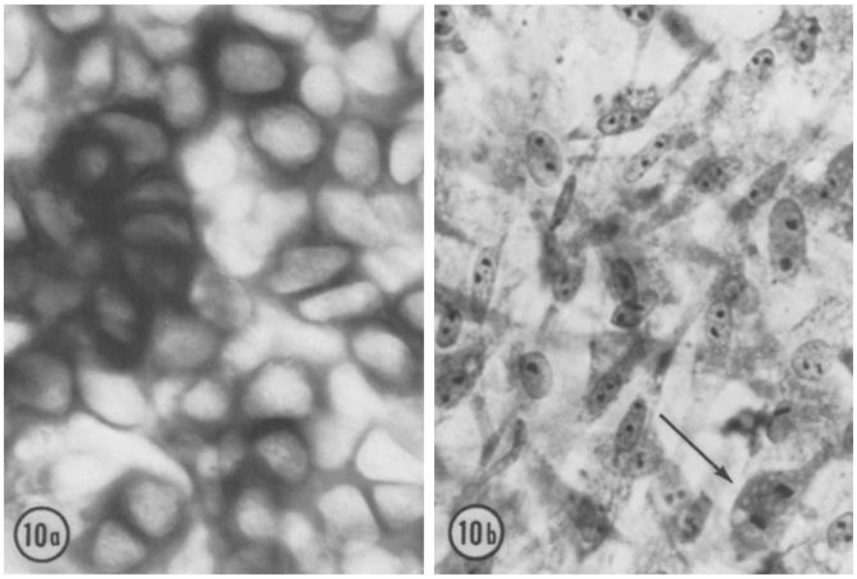

FIGURE 10 Control chondrocytes on day 7 in culture, stained with toluidine blue, (Fig. 10 a). Most cells are surrounded by metachromatic capsules. In PLC, (Fig. 10 b), chondrocytes showed no metachromatic capsules and were stellate or "fibroblastic" in morphology. Mitotic figures were observed in treated cultures (arrow). × 400.

cartilage matrix synthesis by chondrocytes. In both instances the biological effect of PLC is specific in the sense that neither cell division nor cell motility is significantly impaired while the differentiated functions of the two cell types are suppressed. Obviously, then, the cells can make new surface membrane in the presence of the enzyme and can engage in whatever surface activities are required for movement. The mechanism by which PLC exerts its specific antidifferentiation effects is not known. To rationalize our experimental observations we are considering the following hypothesis: PLC alters the surfaces of myoblasts and chondrocytes in such a way that cell recognition phenomena fail to occur. Given a failure in recognition, the processes leading to fusion and contractile protein synthesis by myoblasts or to extracellular matrix synthesis by chondrocytes do not occur in spite of close surface-to-surface apposition.

Other hypotheses concerning the mechanism of differentiation inhibition could, of course, be constructed. We have assumed that PLC does not enter cells (at least in an active form) but we are exploring the alternative possibility. PLC might act to stimulate cell division and keep cells from withdrawing from the mitotic cycle. Under such circumstances myoblasts might not fuse (3) and chondrocytes might not synthesize matrix components (24). The enzyme could be exerting different types of effects on different kinds of cells. Sulfate transport systems in chondrocyte surfaces could be impaired so that chondroitin sulfate synthesis would not occur, while some other myoblast property, unrelated to ion transport, could be altered. PLC might stimulate rapid membrane turnover or repair so that cells divert metabolites normally used in surface differentiations to pathways for basic membrane structures. Since PLC removes phosphorylamines from lecithins, residual diglycerides in the surface membranes could be responsible for the antidifferentiation effects (22). However, no large osmiophilic

TABLE I

Effect of PLC on Incorporation of Labeled Sulfate by Chondrocytes

	cpm ^{35}S in Polysaccharide
Cells	
Control	15,730
PLC-treated	3,860
Medium	
From control cells	14,660
From PLC-treated cells	5,400

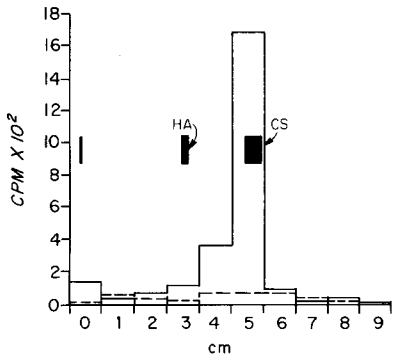

FIGURE 11 Electrophoresis of sulfated polysaccharide extracted from control (solid line) and PLC-treated chondrocytes (dashed line). Polysaccharide from the medium gave similar results. Superimposed is an electrophoresis pattern of a mixture of hyaluronic acid (*HA*) and chondroitin sulfate (*CS*) which served as standards.

droplets were found in electron micrographs of PLC-treated cells. The small, dense, plaques described in Fig. 3 were observed in both control and treated cells. PLC might attach to the surfaces of myoblasts and cover or shield sites which are active in fusion. This possibility is under study. The enzyme could alter membrane lipids such that certain proteins would not function properly (6, 23) or would be released from the membrane (see however, reference 17). We are at present testing this last hypothesis and have found that PLC releases acid-precipitable radioactivity from the surfaces of cells which have been labeled with ^{125}I by the lactoperoxidase procedure (10, 21, 30). We are attempting to characterize the released material both biochemically and biologically. In this regard, it is interesting to note that Lesseps (18) has reported that, of a variety of enzymes, only PLC removed the lanthanum-staining "coat" from embryonic chick cells. It is also possible that the lipase activity of our purified PLC preparation is not responsible for the observed biological effects. In spite of our purification procedure, another activity of the same molecule or of an unseparated contaminant molecule could be the inhibitor of differentiation. With regard to this possibility, purified PLC from *B. cereus* was active as a lipase in our assay but had no effect on fusion or synthesis of cartilage matrix. However, even if the bacillus enzyme is a purer preparation, it is also a different protein with somewhat different substrate specificities (33) compared to clostridial PLC. In the light of these considerations, the results of our experiment with PLC coupled to agarose beads could be explained in another way. A contaminating molecule could have been structurally altered during coupling and hence rendered inactive (or it might not have been coupled). Alternatively, if the antidifferentiation activity is a property of the PLC molecule, then that activity might have been abolished during coupling, while the lipase activity survived.

The dose-response curve relating fusion to concentration of PLC showed an apparent "all-or-none" effect. This result might be due to the difficulty in accurately quantitating fusion with the light microscope. It is also possible that a fine-grained analysis might reveal a graded decrease in number of fused cells between the doses of 6 and 10 μg of commercial PLC (0.3 and 0.5 μg of purified PLC). In view of our ignorance about the mechanism of action of PLC, the all-or-none response remains noteworthy but unexplained.

In many respects the antidifferentiation properties of PLC are similar to those of 5-bromodeoxyuridine (BUdR; 1, 4, 36). PLC and BUdR effects on myoblasts are readily reversible while the effects of these substances on chondrocytes are not easily reversible. PLC, however, does not alter cell morphology to the extent that BUdR does. In PLC, cells are stellate or spindle-shaped but are not as spread out as cells in BUdR. In preliminary experiments we have found that PLC and BUdR release the same types of ^{125}I-labeled proteins from the cell surface. Since BUdR affects the surface properties of chondrocytes (1), it is possible that the antidifferentiation effects of PLC and BUdR result from a common mechanism involving the loss of specific molecules from the cell surface.

In other systems, phospholipases of various types prevent virus-induced cell fusions (2, 12, 13) or may promote fusion of polycation-aggregated cells (8). The specific mechanisms operating to inhibit or induce fusion in those systems is not clear. Study of the mechanism of PLC action in our system may enable us to dissect some of the events both in the differentiation of specific cell surface function and in the control of expression of differentiation which may reside in the surface membrane.

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REFERENCES

1. ABBOTT, J., and H. HOLTZER. 1968. *Proc. Natl. Acad. Sci. U. S. A.* **59**:1144.
2. BARBANTI-BRODANO, G., L. POSSATI, and M. LAPLACA. 1971. *J. Virol.* **8**:796.
3. BISCHOFF, R., and H. HOLTZER. 1969. *J. Cell Biol.* **41**:188.
4. BISCHOFF, R., and H. HOLTZER. 1970. *J. Cell Biol.* **44**:134.
5. CUATRECASAS, P. 1970. *J. Biol. Chem.* **245**:3059.
6. CUATRECASAS, P. 1971. *J. Biol. Chem.* **246**:6532.
7. DANES, B. S., and A. G. BEARN. 1966. *J. Exp. Med.* **123**:1.
8. DE BOER, E., and A. LOYTER. 1971. *FEBS Lett.* **15**:325.
9. ELLNER, P. D. 1961. *J. Bacteriol.* **82**:275.
10. HUBBARD, A. L., and Z. A. COHN. 1972. *J. Cell Biol.* **55**:390.
11. ISHIKAWA, H., R. BISCHOFF, and H. HOLTZER. 1968. *J. Cell Biol.* **38**:538.
12. KOHN, A. 1965. *Virology.* **26**:228.
13. KOHN, A., and C. KLIBANSKY. 1967. *Virology.* **31**:385.
14. KONIGSBERG, I. R., N. MCELVAIN, M. TOOTLE, and H. HERRMANN. 1960. *J. Biophys. Biochem. Cytol.* **8**:333.
15. KONIGSBERG, I. R. 1971. *Dev. Biol.* **26**:133.
16. LASH, J., H. SWIFT, and H. HOLTZER. 1956. *Anat. Rec.* **124**:324.
17. LENARD, J., and S. J. SINGER. 1968. *Science (Wash. D. C.).* **159**:738.
18. LESSEPS, R. J. 1967. *J. Cell Biol.* **34**:173.
19. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. Randall. 1951. *J. Biol. Chem.* **193**:265.
20. MAIZEL, J. V. 1969. *In* Fundamental Techniques in Virology. K. Habel and N. Salzman, editors. Academic Press Inc., New York. 334.
21. MARCHALONIS, J., R. E. CONE, and V. SANTER. 1971. *Biochem. J.* **124**:921.
22. MARTONOSI, A. 1968. *J. Biol. Chem.* **243**:71.
23. MAVIS, R. D., R. M. BELL, and P. R. VAGELOS. 1972. *J. Biol. Chem.* **247**:2835.
24. NAMEROFF, M., and H. HOLTZER. 1967. *Dev. Biol.* **16**:250.
25. NAMEROFF, M., and H. HOLTZER. 1969. *Dev. Biol.* **19**:380.
26. NAMEROFF, M. 1972. *J. Cell Biol.* **54**:166.
27. OKAZAKI, K., and H. HOLTZER. 1966. *Proc. Natl. Acad. Sci. U. S. A.* **56**:1484.
28. O'NEILL, M. C., and F. E. STOCKDALE. 1972. *J. Cell Biol.* **52**:52.
29. PATERSON, B., and R. C. STROHMAN. 1972. *Dev. Biol.* **29**:113.
30. PHILLIPS, D. R., and M. MORRISON. 1971. *Biochemistry.* **10**:1766.
31. QUASTLER, H., and F. G. SHERMAN. 1959. *Exp. Cell Res.* **17**:420.
32. ROTH, S., E. J. MCGUIRE, and S. ROSEMAN. 1971. *J. Cell Biol.* **51**:536.
33. STAHL, W. L. 1973. *Arch. Biochem. Biophys.* **154**: 47.
34. STOCKDALE, F. E., and H. HOLTZER. 1961. *Exp. Cell Res.* **24**:508.
35. STOCKDALE, F. E., J. ABBOTT, S. HOLTZER, and H. HOLTZER. 1963. *Dev. Biol.* **7**:293.
36. STOCKDALE, F. E., K. OKAZAKI, M. NAMEROFF, and H. HOLTZER. 1964. *Science (Wash. D. C.).* **146**:533.
37. TOMARELLI, R. M., J. CHARNEY, and M. L. HARDING. 1949. *J. Lab. Clin. Med.* **34**:428.
38. ZWAAL, R. F. A., B. ROELOFSEN, P. COMFURIUS, and L. L. M. VAN DEENEN. 1971. *Biochim. Biophys. Acta.* **233**:474.