In contrast, the MST of 15.2 days for 23 male B6 grafts contralateral to B6.*H-2*$^k$ male grafts was significantly lower ($P < .005$).

If the immunogenicity of H-Y varies on different *H-2* backgrounds, as the above results suggest, then it should be possible to demonstrate this difference in single (unilateral) grafts. Accordingly, we used the congenic strains B10 and B10.BR (*11*) for the following experiment. Three groups of (B10 × B10.BR)$F_1$ hybrid females (*H-2*$^b$/*H-2*$^k$) received individual skin grafts from either B10 males, B10.BR males, or (B10 × B10.BR)$F_1$ males (Table 1). The B10.BR (*H-2*$^k$) male grafts (MST, 14.5 days) were rejected significantly faster than were B10 (*H-2*$^b$) male grafts (MST, 20.0 days) ($P < .005$). The MST of 17.5 days for male grafts of $F_1$ hybrid origin was intermediate between the values for male grafts from the two parental strains; this suggests a gene dosage effect.

The only histoincompatibility between donor and host in this experiment involves the H-Y antigen. Inasmuch as the origin of H-Y is identical in the congenic donors that we employed, and because the only difference between the grafts was their *H-2* genotype, *H-2* was in some way responsible for the disparate survival of B10 and B10.BR male grafts on the $F_1$ hybrid females. We conclude, therefore, that the immunogenicity of H-Y is influenced by the *H-2* locus; specifically, that H-Y antigens are more immunogenic on *H-2*$^k$ cells than they are on *H-2*$^b$ cells. On one hand, there could be a functional relation between the two loci such that the ontogeny of the H-Y antigen is in some way dependent on *H-2* (*12*). Alternatively, these differences might result from steric interference between *H-2* cell surface components and the binding of neighboring H-Y sites to anti-Y receptors. In any case, the effectiveness of rejection of incompatible grafts may depend not only on the ability of the host to respond to graft antigens, but also on the genetic background of the transplant donor.

STEPHEN S. WACHTEL
*Division of Immunology, Memorial Sloan-Kettering Cancer Center, New York 10021*
DAVID L. GASSER
WILLYS K. SILVERS
*Departments of Human Genetics and Pathology, University of Pennsylvania School of Medicine, Philadelphia 19174*

**References and Notes**

1. E. J. Eichwald and C. R. Silmser, *Transplant. Bull.* **2**, 148 (1955); T. S. Hauschka, *ibid.*, pp. 154.
2. D. L. Gasser and W. K. Silvers, *Advan. Immunol.* **15**, 215 (1972).
3. R. E. Billingham and W. K. Silvers, *J. Immunol.* **85**, 14 (1960).
4. D. L. Gasser and W. K. Silvers, *ibid.* **106**, 875 (1971); D. W. Bailey and J. Hoste, *Transplantation* **11**, 404 (1971).
5. Females bearing *H-2*$^k$ frequently reject second skin grafts from males of the same strain [(*2*); E. Goldberg, E. A. Boyse, M. Scheid, D. Bennett, *Nature* **238**, 55 (1972)].
6. W. K. Silvers and R. E. Billingham, *Science* **158**, 118 (1967).
7. There is one report of possible allelic differences at the *H-Y* locus in mice [W. H. Hildemann and E. L. Cooper, *Transplantation* **5**, 707 (1967)]. In two independent experiments, one by D.L.G. in Philadelphia and the other by S.S.W. in New York, we attempted to confirm the findings of Hildemann and Cooper but were unable to do so.
8. S. S. Wachtel, D. L. Gasser, W. K. Silvers, *Transplant. Proc.* **5**, 295 (1973).
9. R. E. Billingham, in *Transplantation of Tissues and Cells*, R. E. Billingham and W. K. Silvers, Eds. (Wistar Institute, Philadelphia, 1961), pp. 1–26.
10. J. T. Litchfield, *J. Pharmacol. Exp. Ther.* **97**, 399 (1949).
11. Females of strain B10.BR (*H-2*$^k$) reject B10.BR male skin grafts slowly or not at all. In contrast, B10 females (*H-2*$^b$) rapidly destroy B10 male skin grafts (MST, 19 days). This disparity is related to the *H-2* genotype of the responding female [D. L. Gasser and W. K. Silvers, *Transplantation* **12**, 412 (1971)].
12. One possible mechanism for the *H-2* dependence of the H-Y antigen could be that this antigen is coded for by an *H-2* linked gene that is turned on only in a male environment (D. C. Shreffler, personal communication). Until allelic differences at the *H-Y* locus are conclusively demonstrated so that it is possible to study linkage relationships of this locus, this hypothesis is probably not testable.
13. Supported by PHS grant AI-09275 and, in part, by NCI grant CA-08748.

19 March 1973; revised 30 April 1973 ■

# Plasma Membranes: Isolation from Naturally Fused and Lysolecithin-Treated Muscle Cells

Abstract. *A medium containing bicarbonate and calcium was used to isolate plasma membranes of cultured muscle cells. Membranes from differentiated myotubes, as well as the labile, largely unfused, lysolecithin-treated cells from the same culture could be isolated by identical manipulations. Adenylate cyclase of high specific activity was assayed in plasma membranes from both types of cells. Lysolecithin treatment apparently interferes with the metabolism and turnover of membrane phospholipids and thus prevents the natural fusion of muscle cells.*

Skeletal muscle cells differentiating in vitro present a useful model for studying the role of the plasma membrane (*1*) in cellular growth and differentiation. In this system, single muscle cells (myoblasts) naturally fuse into myotubes, and the multinucleate skeletal muscle fibers can eventually be formed by subsequent fusion of myoblasts and myotubes (*2*). The plasma membrane of these muscle fibers can be further studied during synapse formation with dissociated cells of neural origin, also grown in culture (*3*). The isolation of plasma membrane from cultured muscle cells at various stages of differentiation offers advantages in studying specialized membrane structures since replicate plates from muscle cell cultures can also be used for electrophysiological and cytological measurements (*4*).

We now report a method of isolating plasma membranes from rat muscle cells cultured in regular medium and exhibiting extensive fusion, as well as muscle cells cultured in lysolecithin-containing medium and exhibiting confluent but unfused cellular growth. Selected biochemical comparisons between the two preparations are also presented.

The characteristic growths of rat muscle cells in a mixture of common culture media (R) or media treated with lysolecithin (LL) (at a concentration of 150 $\mu$g per milliliter of medium) have been described (*5*). After the LL mixture was replaced with regular medium (LLR) at any time during culture, the fusion inhibition, imposed by lysolecithin, was completely reversible and occurred over a 6- to 8-hour interval (*5*). The muscle cells were sampled after 7 days in culture—when cell confluency and myotube formation were well established (between days 5 to 6)—in petri plates containing regular medium (R). Half the culture plates were treated with lysolecithin starting from day 3 of culture until day 7. Culture medium was changed every other day.

After repeated washing in Hanks solution, the cells were collected by gently scraping the culture plates (*5*). This method was preferable to the use of enzymes which could have caused loss of membrane components. After being scraped in ice-cold medium, the cells were centrifuged at 1000 rev/min for 10 minutes, and the collection medium, consisting of 0.3*M* sucrose, 5 m*M* Tricine [*N*-tris (hydroxymethyl)-methylglycine], *p*H 7.5, 1 m*M* $CaCl_2$, and 5 m*M* KCN, was discarded. The cells were homogenized with 12 to 15 strokes in a 5-ml-capacity Dounce

Table 1. Radioactivity from [$^3$H]glycerol in phospholipids (PL) of isolated membranes from muscle cells grown in regular or lysolecithin medium. Muscle cells (7-day-old) were grown (*5*) in regular medium (R) or in lysolecithin medium (LL); the egg lysolecithin (General Biochemicals) was added from the day 3 of culture (150 $\mu$g per milliliter of medium). Glycerol (New England Nuclear) labeled in the $\beta$ position was used to avoid complications from the normal extensive turnover when the glycerol skeleton is labeled in the $\alpha$ position. The specific activity of the original compound was 200 mc/mmole, and 1.25 mc per milliliter of medium was used for a 3-hour labeling period. After this period, the cells were collected after washing first with ice-cold complete medium and then with Hanks solution. The membrane fractions were separated and examined for lipid incorporation (*16–18*). Abbreviations: PM, plasma membrane; Ms, microsome; Mt, mitochondria; Lys-r, lysosome-rich fractions.

| Fraction | Lipid P per mg of protein ($\mu$mole) | | Phospholipid (% total counts) | | Phospholipid specific activity: P ($10^4$ count min$^{-1}$ $\mu$mole$^{-1}$) | | Phospholipid specific activity: Protein ($10^4$ count min$^{-1}$ mg$^{-1}$) | |
|---|---|---|---|---|---|---|---|---|
| | R | LL | R | LL | R | LL | R | LL |
| PM | 0.97 | 0.47 | 31.3 | 58.8 | 11.1 | 4.7 | 10.8 | 0.22 |
| Ms | 0.44 | 1.08 | 48.0 | 29.7 | 20.1 | 3.1 | 8.8 | 3.2 |
| Mt | 0.73 | 1.67 | 59.5 | 15.2 | 10.5 | 2.0 | 7.8 | 3.3 |
| Lys-r | 0.84 | 1.08 | 62.0 | 39.5 | 15.4 | 13.3 | 13.0 | 14.4 |

homogenizer with an estimated clearance of 0.013 mm. The homogenate was centrifuged at 1500*g* for 10 minutes in a Sorvall SS-34 rotor. The supernatant resulting from this homogenization was used for separation of cell organelles and intracellular membranes. The portion sedimenting at 1500*g* was used for preparation of plasma membranes.

The plasma membranes were prepared from the 1500*g* pellet in a hypotonic medium with 1 m*M* $NaHCO_3$ and 2 m*M* $CaCl_2$. The pellet was diluted in the hypotonic medium to 9 ml and homogenized again in a larger Dounce homogenizer (six strokes) with an estimated clearance of 0.009 mm, recentrifuged, and redispersed twice in decreasing volumes of the hypotonic medium (2500 rev/min for 10 minutes each at 4°C). The pellet was then dispersed with the small Dounce homogenizer in hypotonic medium and mixed with 70 percent sucrose to give 50 percent sucrose (density of 1.24 g/cm$^3$). The muscle cell plasma membranes were obtained after modification of the methods used for separation of rat liver membranes (*6*). Discontinuous gradients were prepared with bicarbonate–calcium chloride medium in 5-ml Spinco tubes. The following sucrose densities were used—dispersed pellet: 1.200, 1.188, 1.177, 1.167, and 1.155. The tubes were centrifuged in a Spinco SW-39 rotor at 30,000 rev/min for 105 minutes. The plasma membrane fractions were then collected by aspiration of the interface material between the layers with densities of 1.155 and 1.167, of the 1.167 layer itself, and of the interface between 1.167 and 1.177. Care was given to avoid the lipid in the topmost layer (where the density was 1.155). The aspirated material was diluted in Tricine-calcium medium and collected after 20 minutes centrifugation at 18,000 rev/min in the Sorvall RC2B centrifuge.

Striking differences in the profiles of fixed, stained, and sectioned material from R- and LL-type cells were noted in electron micrographs (Fig. 1) (*7*). The plasma membranes of fused cells from a 7-day-old muscle culture showed dense, well-defined contiguous profiles of highly ordered arrays and long stretches of membranes in cross section (Fig. 1A). Replicate plates of this same culture exposed to LL from days 3 to 9 of the culture period showed plasma membranes of unfused cells lightly stained and with diffused but separable profiles. Interconnections between adjacent cells were not easily distinguished (Fig. 1B). The LL cell membranes were especially fragile when examined with negative contrast staining (not shown) and lacked the extensive structure of normally fused cells.

Neither succinate dehydrogenase nor oxidation of NADH (*8*) could be detected in the isolated plasma membranes. Glucose-6-phosphatase (*p*H 6.2), an enzyme characteristic of "microsomal" fractions, was detected in the plasma membrane fractions at 18 to 30 percent of the specific activity found in the microsomal fractions (*8*). The specific activity of glucose-6-phosphatase in the "microsomal" fractions from normal 7-day cultures (R) varied between 10 to 12 $\mu$mole per milligram of protein per hour, with comparable preparations from LL cells also yielding similar specific activities.

The activity of adenylate cyclase was also determined. Adenylate cyclase was of some interest because the membrane-bound form is influenced by detergents (*9*) and the hormonal response of this enzyme is dependent on the stage of muscle cell differentiation (*10*). The specific activity of the plasma membrane fractions was 6 to 12 times higher than the microsomal or mitochondrial fractions (Fig. 2, legend). In contrast to

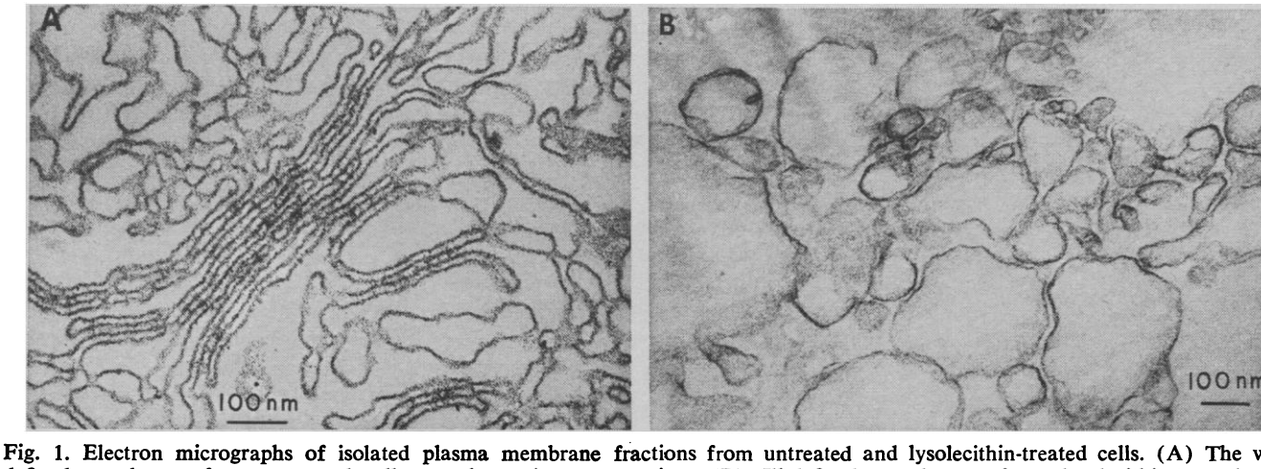


Fig. 1. Electron micrographs of isolated plasma membrane fractions from untreated and lysolecithin-treated cells. (A) The well-defined membranes from untreated cells are shown in cross section. (B) Ill-defined membranes from lysolecithin-treated cells in cross section. The differences in size of the vesicles between the two membrane preparations are obvious.

the unchanged glucose-6-phosphatase specific activity of both the plasma membrane and microsomal fractions, the cyclase activity of the LL treated plasma membranes was 30 percent higher than that from plasma membranes of untreated cells. The importance of the cyclase in plasma membranes is also attested by the fact that increases of the cyclase in the other two fractions of LL cells was less than 13 percent as compared to the comparable R cell fractions. Seven-day cultures of R and LL cells were confluent (*5*) but exhibited lowered concentration of intracellular cyclic adenosine monophosphate (AMP), in contrast to other cells in culture (*11, 12*). Media were replaced every other day (4 ml per 60-mm dish) during muscle cell cultures, and the low cyclic AMP concentrations arising from depletion of serum growth factors seem unlikely, as has been indicated in the tissue culture experiments of Seifert and Paul (*13*).

The effect of LL treatment on muscle cell plasma membranes was interpreted to stem primarily from interference with metabolism and turnover of new phospholipids. The turnover of phospholipids increases in such plasma membrane–mediated phenomena as phagocytosis, secretion, and serum-stimulated relief of contact inhibition (*14, 15*), and increases in phospholipid turnover have been noted to parallel increases in membrane movement (*15*).

It should follow that muscle cells exhibiting extensive natural fusion should also exhibit increased phospholipid turnover or increased incorporation of radioactive precursors (or both) into plasma membrane phospholipids. When natural fusion is prevented by withdrawal of calcium, the use of large quantities of cyclic AMP, or optimal quantities of lysolecithin (*5*), the turnover or lipid accumulation (or both) in plasma membrane phospholipids should be lower in the treated cells, which adhere strongly to the growth support, than in the untreated cells, which adhere less strongly (*5*).

The percentage of radioactivity in the phospholipid fractions of the membranes is shown in Table 1. Of the radioactivity originally present in the extracted membrane lipids, 85 to 93 percent was recovered from the separated neutral lipids and phospholipids (*16–18*). These neutral lipids were presumably synthesized in mitochondria. To account for this distribution of radioactivity, the neutral lipids were interpreted as being in transport via Golgi apparatus, the reticulum, and the lysosomal vesicles, but not the plasma membrane.

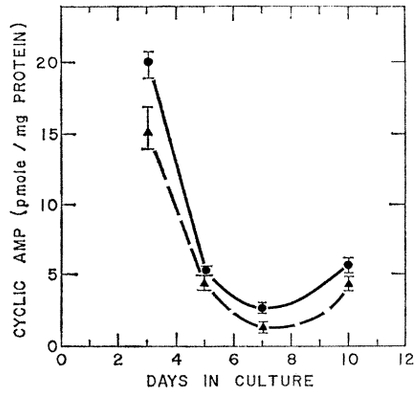


Fig. 2. Plasma membrane adenylate cyclase and cyclic AMP content of muscle cells during culture. The assays for cyclic AMP have been described (*10, 12*). The vertical bars represent range of values obtained from four determinations of cyclic AMP at each averaged point. The specific activities (nanomoles per milligram of protein per minute) of adenylate cyclase of various membrane preparations from 7-day cultures of cells was determined with [8-$^{14}$C]adenosine 5′-triphosphate (*10*). The specific activities in regular medium for plasma membrane, microsome, and mitochondria were 9.6, 0.8, and 1.6 nmole $mg^{-1}$ $min^{-1}$, and the activities in lysolecithin medium were 13.6, 1.1, and 1.9.

Further separation of the plasma membrane phospholipids showed that radioactivity distribution from LL cells was 40, 8, 1, 12, and 2 percent for phosphatidylcholine, serine, ethanolamine, inositol, and cardiolipin, respectively. Similar separation of plasma membrane phospholipids from R cells was 57, 8, 14, 3, and 7 percent for these respective classes. No difference in [$^3$H]lysolecithin radioactivity (1 to 2 percent) was noted between the plasma membranes of the two cell types.

The main aberration of treated cells originated in lipid metabolism (Table 1) and in the expected attrition of some membrane material from the outside of plasma membrane by the surface effects of the lysophosphatide (Fig. 1). Because recovery from LL treatment occurred at any time in culture by replacement with regular medium (LLR) (*5*), the restoration of normal turnover in membrane lipids would be a necessary precedent. Muscle cell fusion involves movement of plasma membrane, and this is probably reflected in increased turnover of membrane phospholipids.

MINOCHER REPORTER
DAN RAVEED
*Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45378*

**References and Notes**

1. A. B. Pardee, *In Vitro* **7**, 95 (1971); I. Goldschneider and A. A. Moscona, *J. Cell Biol.* **53**, 435 (1972); W. Ragland, J. L. Pace, R. L. Doak, *Biochem. Biophys. Res. Commun.* **50**, 118 (1973); A. Macieira-Coelho and S. Avrameas, *Proc. Nat. Acad. Sci. U.S.A.* **69**, 2469 (1972).
2. I. R. Konigsberg, *Develop. Biol.* **26**, 133 (1971).
3. G. D. Fischbach, *ibid.* **28**, 407 (1972).
4. H. Criss Hartzell and D. M. Fambrough, *ibid.* **30**, 153 (1973); J. E. Rash and D. M. Fambrough, *ibid.*, p. 188; A. J. Sylkowski, Z. Vogel, M. Nirenberg, *Proc. Nat. Acad. Sci. U.S.A.* **70**, 270 (1973); J. Patrick *et al.*, *ibid.* **69**, 2762 (1972).
5. M. Reporter and G. Norris, *Differentiation* **1**, 83 (1973).
6. T. K. Ray, *Biochim. Biophys. Acta* **196**, 1 (1970); L. Emmelot and C. J. Bos, *J. Membr. Biol.* **9**, 83 (1972).
7. The membrane fractions were fixed for 30 minutes in ice-cold 2 percent glutaraldehyde and 1 percent osmium tetroxide in 0.05*M* each of sucrose and 2,4,6-collidine, *p*H 7.4. Pellets were washed, dehydrated in graded ethanol and acetone, and embedded in epoxy mixture [A. R. Spurr, *J. Ultrastruct. Res.* **26**, 31 (1969)]. The Sorvall microtome-cut silver sections were stained with 1 percent uranyl acetate and lead citrate [E. S. Reynold, *J. Cell Biol.* **17**, 208 (1963)]. Inspection of membrane fractions by negative stain technique utilized three washes in 2 percent methylamine tungstate (R. Oliver and A. E. Faberge, private communication).
8. Succinate dehydrogenase, NADH oxidation, and glucose-6-phosphatase were analyzed after suitable modifications of procedures described in H.-U. Bergmeyer, Ed., *Methods in Enzymatic Analysis* (Academic Press, New York, 1963).
9. C. Gitler, *Annu. Rev. Biophys. Bioenerg.* **1**, 76 (1972); A. Retty, V. Tomas, A. Trevisani, O. Barnabei, *Biochim. Biophys. Acta* **290**, 58 (1972); G. S. Levey, *Biochem. Biophys. Res. Commun.* **43**, 108 (1971); A. B. Schneider and H. Edelhoch, *J. Biol. Chem.* **247**, 4986 (1972).
10. M. Reporter, *Biochem. Biophys. Res. Commun.* **48**, 598 (1972); *J. Gen. Physiol.* **57**, 244 (1971). Adenylate cyclase was assayed according to D. L. Severson, G. I. Drummond, and P. V. Sulakhe [*J. Biol. Chem.* **247**, 2949 (1971)].
11. M. D'Armiento, G. S. Johnson, I. Pastan, *Proc. Nat. Acad. Sci. U.S.A.* **69**, 459 (1972).
12. A. G. Gilman and M. Nirenberg, *Nature* **234**, 356 (1971).
13. W. Seifert and D. Paul, *Nature New Biol.* **240**, 281 (1972).
14. D. G. Gunnigham, *J. Biol. Chem.* **247**, 2464 (1972); J. B. Sanford and M. D. Rosenberg, *Biochim. Biophys. Acta* **288**, 333 (1972).
15. C. A. Pasternak, *J. Cell Biol.* **53**, 231 (1972).
16. Lipids were extracted from these organelles and separated on reagent grade silicic acid columns (Matheson, Coleman & Bell) into neutral and phospholipid fractions (*17–19*). The phospholipids were further separated on preformed, Brinkmann silica gel G glass plates (*19*). The protein content of extracted residues was determined by the Lowry method (*18*), and lipid phosphorus (LP) fractions were determined after "wet ashing" by the method of Bartlett (*19*). Determination of phosphorus (Table 1) was the only chemical analysis made on the lipids. The radioactivity of the separated lipids was monitored by scintillation spectrometry with a Nuclear-Chicago Unilux II machine.
17. V. P. Skipski and M. Barclay, *Methods Enzymol.* **14**, 530 (1969). The phospholipid was separated with a mixture of chloroform, methanol, acetic acid, and water (61:32:5:2); D. B. Weinstein, J. B. Marsh, M. C. Glick, L. Warren, *Biochemistry* **8**, 4103 (1969).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
19. G. R. Bartlett, *ibid.* **234**, 446 (1959).
20. We thank Dr. J. Elovson (University of California, San Diego) for suggesting the use of glycerol, labeled in $\beta$ position, for lipid precursor studies and membrane isolation. Dr. T. Keenan (Purdue) supplied some lipid standards. We thank G. Norris for establishing muscle cultures. This is contribution 495 from the Charles F. Kettering Research Laboratory.

2 April 1973; revised 29 May 1973