masses; the shape of the ridge frequently suggests overriding of one mass by the other. In sequential photographs, short ridges may be seen to fuse into longer ones. Long ridges may undergo uniform lateral displacement, or only a local displacement, producing an arch. Sometimes an arched ridge curves into a whorl, suggesting that the resistance of the two sides of the arch were unequal. From the movement of the 3T3 clumps, epidermal cells at some distance from the center of a developing whorl can be seen to move toward its center. Though the photographs shown were usually taken at intervals of 2 to 3 days, displacements were very appreciable within 1 day.

All the cultures shown in Figs. 1 to 4 were grown in 50-mm dishes, and the whorls are of fairly uniform size; cultures grown to confluence in 33-mm dishes gave rise to whorls similar in other respects but of smaller size. It therefore seems that the size of a whorl is determined by the size of the field available for organization.

In the digits of embryos, the ridges form over epidermal folds that extend down into the dermis. These folds possess an expanded basal layer of proliferating cells which should generate more progeny per unit of skin surface than are produced between the folds, thus sustaining the form of the ridge. This is not possible in the cultures, since the basal cells are held in a flat layer by the surface of the dish. Vertical sections show that the ridges formed in culture consist mostly of differentiated cells resembling the upper layers of skin, while the basal layer of the ridge is very similar to that of the thin regions. Cell growth contributes to pattern formation, and omission of epidermal growth factor slows pattern formation appreciably; but unlike the situation in the digits, there may be no greater rate of proliferation under the ridges than elsewhere. On the other hand, the forces generating curvature of the ridges into whorls in culture should also be acting in the embryonic digits. Possible evolutionary relations between arches, loops, and whorls in the digits have been suggested (*1, 13*), but it seems to have been generally accepted that during embryogenesis the ridges first appear in their definitive pattern (*1, 9*). In view of the behavior of the cultured keratinocytes, this interpretation (or assumption) should be reexamined.

Although in the human, epidermal ridges are formed only on the volar surfaces of the hands and feet, the epidermal cells in our experiments originated from newborn foreskin. Even keratinocytes originating from corneal epithelium and the conjunctiva behave like epidermal cells in culture (*14*), and in the present experiments give rise to whorls very similar to those in Figs. 1 to 4 (*15*). There must therefore be some feature of the culture conditions which permits the keratinocytes to express properties similar to those they express in vivo in certain locations only. In the embryonic digits, the unusual amount of mesenchyme in the pads is probably essential for ridge formation (*9*), and might temporarily permit the ridges an unusual degree of mobility not found elsewhere on the body, but duplicated in the cultures.

On the basis of the idea that cell movement is an important factor in pattern formation in embryonic digits, it is possible to suggest an explanation for a curious influence of chromosome number on the pattern. Increasing numbers of X or Y chromosomes in the human diminish the total epidermal ridge count (*16*), even though the extra chromosomes are known to be largely or completely inactive genetically. It may be postulated that the bulkier nucleus resulting from the presence of the extra chromosomes would inhibit to a slight degree the cell movements and the distortion of cell shape necessary to produce curvature of the ridges, thus reducing the overall complexity of the patterns.

The degree of development of the patterns in culture varies in repeated trials and cannot be expected to equal in precision that of dermatoglyphs; even so, it may be that careful control of culture conditions would make it possible to distinguish some differences between the epidermal cells of individuals whose dermatoglyphs differ for genetic reasons. Any such differences would, of course, support the idea that the cell movements observed in culture are important in embryonic ridge formation.

HOWARD GREEN, JUDITH THOMAS
Department of Biology, Massachusetts Institute of Technology, Cambridge 02139

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15. To our knowledge no cell type other than the keratinocyte gives rise to whorls in culture; however, see Elsdale and Wasoff (*11*). In a few trials, keratinocytes originating from nasopharyngeal epithelium, while resembling in many respects those of skin and conjunctiva, did not give rise to patterns in culture. This may be typical for keratinocytes arising from internal as opposed to external stratified squamous epithelia. This finding is in accord with the idea that though patterns may be influenced by mesenchymal cells, they are formed as a result of intrinsic properties of the keratinocytes.
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Preparation of Sarcolemmal Membrane from Myocardial Tissue Culture Monolayer by High-Velocity Gas Dissection

Abstract. *A high-velocity stream of nitrogen is used to simultaneously disrupt myocardial cells in monolayer culture and fractionate their sarcolemmal membranes. The membranes show a high degree of ultrastructural and enzymatic purity, with less than 1 percent intracellular residuum. They are produced in less than 1 second and remain as tightly adherent sheets on the surface on which the cells were grown. The cells are exposed to no agent other than nitrogen gas during the preparative procedure.*

A technique was previously developed that allows continuous, on-line measurement of isotopic exchange in neonatal rat heart cells in culture (*1*). The basis of the technique is growth of the cells on the surface of disks composed of scintillator plastic in such a way that the monolayer is attached directly to the isotopic detector. The approach has been used to evaluate the characteristics of calcium and potassium exchange in whole cultured cells with particular attention to the role of the sarcolemma and the surface coat–external lamina complex in these exchanges (*1–3*). It became clear that study of the isolated sarcolemmal complex

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Table 1. Enzyme activities and protein contents of sarcolemmal membranes. Activities of Mg^{2+}-adenosinetriphosphatase (Mg^{2+}-ATPase), Na^+,K^+-adenosinetriphosphatase (Na^+,K^+-ATPase), and 5′-adenosinemonophosphatase (5′-AMPase) are expressed as micromoles of inorganic phosphate (P_i) per milligram of protein per hour. The activity of succinic dehydrogenase (SDH) is given as micromoles of *p*-iodonitrotetrazolium violet (INT) per milligram of protein per hour. Protein values are micrograms per dish. The number of experiments is shown in parentheses.

Preparation	Activity (μmole P_i/mg-hour)			Activity of SDH (μmole INT/ mg-hour)	Protein (μg per dish)
	Mg^{2+}-ATPase	Na^+,K^+-ATPase	5′-AMPase		
Cell homogenate	1.7 ± 0.3 (4)	1.0 ± 0.3 (4)	2.2 ± 1.2 (3)	0.9 ± 0.2 (3)	797 ± 103 (3)
Membrane	11.6 ± 2.3 (5)	15.2 ± 3.8 (5)	24.6 ± 4.0 (9)	N.D.*	6.3 ± 1.3 (14)

*N.D., not detectable.

from the cells would be valuable, and this led to the development of a new purely physical method for the preparation of membranes directly from the cellular monolayer on the scintillator disks or culture plates. In the new technique the intracellular material is removed and the membranes are left attached to the disk. The technique has been used with myocardial cell and fibroblast cell cultures and should be applicable to any monolayer tissue culture.

The cells are obtained from neonatal rats by an enzymatic digestion process with trypsin (catalog No. 70730, Gibco, Grand Island, N.Y.) and grown in a monolayer on the surface of a standard 50-mm culture dish (*4*) or on the surface of a 48 by 1.5 mm disk composed of polystyrene combined with scintillation material (*5*). The proportion of myoblasts is increased by incubation of the freshly dissociated cells in a petri dish for 2 to 3 hours. During this time the mesenchymal cells settle and attach, while those in suspension—that is, the myoblast-enriched ones—can be poured over the culture dish or disks (*6*). At 3 days the culture is 80 to 90 percent myoblastic as defined by electron microscopic examination and is beating synchronously.

The basis of the procedure is the direction of a stream of N_2 gas at high velocity across the surface of the cellular monolayer. When the flow has the proper velocity and orientation, the uppermost cell membranes are torn open and the intracellular contents are blown out and removed. The residual membranes are rolled and flattened on the disk or dish surface. It is desirable to restrict the technique to monolayer cultures. Multilayer cultures produce a greater intracellular residuum since the N_2 stream is not optimally oriented for clean dissection of all layers.

The chamber and valvular arrangement used are illustrated in Fig. 1. The chamber is machined from stainless steel. The walls are at least 1.0 cm thick to support pressures of 1800 to 2200 pounds per square inch (psi). The purpose of the chamber is to provide a stable support for the culture and to prevent drying of the membranes after their isolation. Compressed N_2 at 1800 to 2200 psi is released through the valve positioned in the center of the chamber. The valve is 8 mm in diameter and is brought into firm direct contact with the surface of the disk or dish. This is accomplished by elevating the chamber floor by means of the turnscrew shown. The disk or dish is held in position and prevented from moving laterally by three pins equidistant about the outer edge.

The dimensions and configuration of the valve outlet are of critical importance. The internal valve cylinder and valve are beveled (radius of curvature, 1.55 mm or 0.062 inch), as illustrated in Fig. 1, so that the N_2 exits from the valve head in a stream that is essentially parallel to the surface of the culture. If the gas exits downward the cells are swept from the surface, and if it exits even marginally upward the cells remain intact. The velocity of the N_2 stream is also important. If less than 1800 psi is used to generate the stream, the cellular residuum is increased. The valve opening is also critical. It is set at 0.30 mm (0.012 inch) for optimal results. A larger opening leaves a cellular residuum and a smaller opening tends to sweep the cells from the surface.

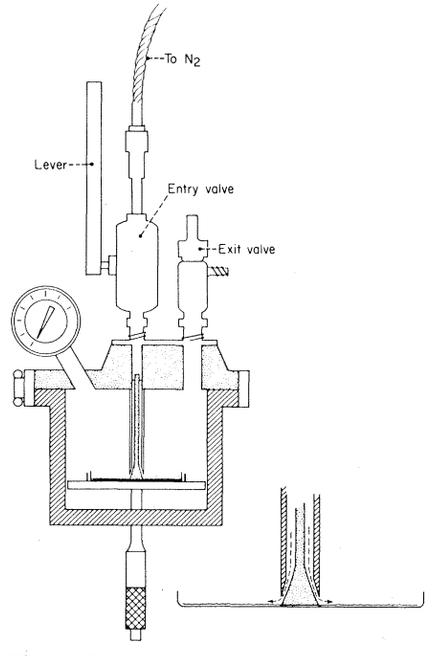


Fig. 1. Section of chamber for membrane preparation. The high-pressure line from the N_2 tank is opened to the entry valve. With the chamber closed and sealed and the monolayer culture oriented as shown and as described in the text, the entry valve is rapidly opened by means of the attached lever. The gas is directed through the beveled valve mounted on the culture, as shown in the inset. With N_2 tank pressures of 1800 to 2200 psi, the optimal valve opening is 0.30 mm. The tank and entry valves are then closed and the chamber decompressed via the exit valve. The chamber is then opened and the dish or disk is removed with the membranes adherent.

The procedure for membrane preparation is simple. The disk or culture dish is mounted on the floor of the chamber with the cellular monolayer directed upward. Before it is mounted, the culture medium is poured off and the disk or dish shaken a few times to remove excess adherent fluid. The top of the chamber is closed and sealed with the two stainless steel semicircular rings, as shown in Fig. 1. A circular steel band is fitted and secured over the rings to prevent them from detaching laterally. The floor of the chamber is then elevated by rotating the turnscrew to bring the center of the culture disk or dish into firm contact with the surface of the valve. The culture is now oriented to receive the N_2 stream. The entry of the gas to the valve applied to the culture is controlled by a valve located in the high-pressure line attached to a large (H size) N_2 tank. A minimum pressure of 1800 psi is used. The valve is opened rapidly (within 1 second) with the aid of the attached lever. The high-velocity N_2 stream exits through the circumferential 0.30-mm opening and travels radially over the culture surface, tearing open the upper surface of the cells and blowing the cellular material to the sides of the chamber. The pressure in the sealed chamber rapidly equilibrates (< 1 second) to that in the tank, and flow ceases. The entry valve is closed and the chamber is decompressed over the

course of 30 to 60 seconds by opening the exit valve (Fig. 1). It should be emphasized that the technique does not depend on decompression to rupture the cells. The membranes are produced by the shearing force of the N_2 stream tearing the cells open and expelling the intracellular contents. The chamber is opened and the disk or dish is removed, placed in culture medium or a physiological salt solution, and examined under the phase microscope. The expansion of the gas in the chamber produces some drying of the membranes but not enough to produce denaturation of the enzymes (see Table 1). The total time required for mounting the dish, fractionating the membranes, and removing the dish is less than 2 minutes. An acceptable preparation has sheets of flat or partially rolled membranous material with virtually no debris. Ultrastructural examination confirms the morphological purity of the preparation.

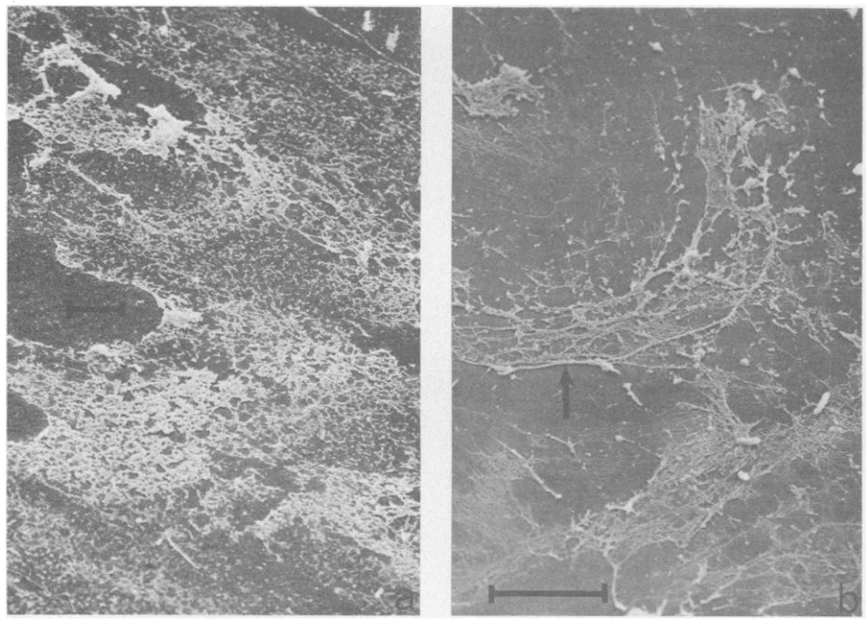
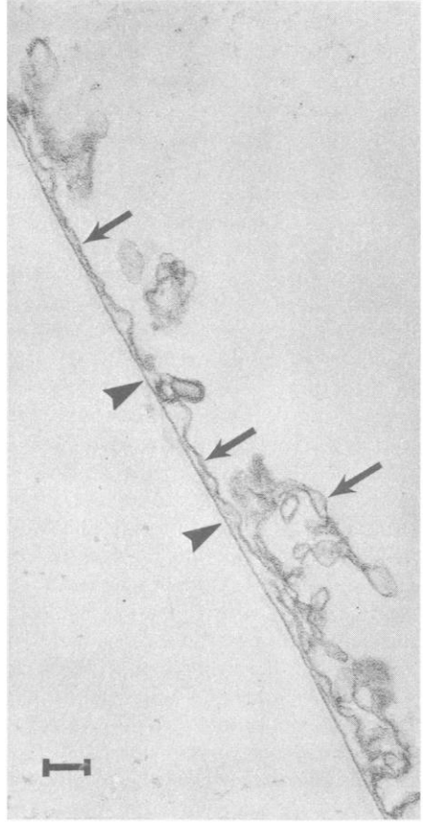


Fig. 2. Scanning electron micrographs of membranes from myoblasts grown in tissue culture. Membranes (a) cover a large portion of the petri dish and (b) appear in sheets or in some cases are rolled (arrow). Scale bars, 10 μm.



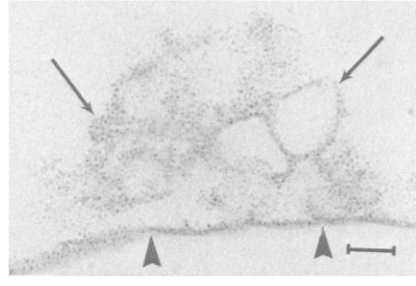


Fig. 3 (left). Transmission electron micrograph of isolated membranes from myoblasts. The straight dark line (arrowheads) running the length of the picture represents a layer of the Falcon dish (Bioquest Corp.) removed in the processing. The membranes are indicated by arrows. Scale bar, 0.1 μm. Fig. 4 (above). Transmission electron micrograph of isolated membranes exposed to cationic ferritin (0.33 mg/ml) (Miles-Yeda Ltd.). The membranes are stained with granules of cationic ferritin (arrows). The staining layer at the bottom (arrowheads) is not cellular in origin but is a charged styrene layer from the Falcon plastic dish (*14*). Scale bar, 0.1 μm.

Techniques were designed to study the ultrastructural configuration of the membranes with scanning and transmission electron microscopy over wide areas of the petri dishes. In addition, polycationic ferritin, a histochemical marker for negatively charged surfaces (*7*), is applied to the membranes to demonstrate the intactness of the surface coat.

The membranes adherent to the petri dish are easily prepared for scanning electron microscopy. Small disks (10 mm) are mechanically punched out of the bottom of the petri dish containing the adherent membranes. They are fixed in buffered glutaraldehyde, critical point-dried, fixed to specimen holders and coated with platinum, and viewed in the ETEC AutoScan electron microscope. All areas selected, except the center of the dish directly beneath the inlet valve, show patches of adherent membrane in a sheetlike configuration or as cylindrical rolls (Fig. 2, a and b).

For studies with the transmission electron microscope (Siemens 1A) methods were devised to ensure (i) that the membranes were processed while still attached to the petri dish, (ii) that the membranes were cut in cross section, and (iii) that the membranes could be easily located in the microscope. Fixation and dehydration are done by pouring solutions into a petri dish containing adherent membranes, using the procedure previously described for intact myoblasts (*1*). For embedding, a thin layer (< 2 mm) of Epon is placed in the petri dish and allowed to remain overnight at room temperature, and then after 1 day at 60°C the disks of cured Epon containing the membranes are separated from the dish. Rectangles of Epon (8 by 5 mm) are cut out, the sides of the rectangles are painted with Pelican ink, and each rectangle is reembedded between two layers of fresh Epon. The rectangle outlined in ink is then easily located in the Epon block for sectioning. Figure 3 illustrates a typical section obtained with this technique. The membranes lie in sheets or are thrown into folds and are free of any identifiable intracellular contaminants. In some experiments the membranes are exposed to cationic ferritin (0.33 mg/ml, *p*H 7.1) for 1 minute and

then rinsed and processed for electron microscopy. Cationic ferritin is seen to bind to the membranes (Fig. 4), indicating the intactness of the negatively charged surface coat. The surface coat is an integral part of the membrane and has been recognized as important in the determination of specific functional properties of the cell (*3, 8*).

A number of enzymatic markers were measured in the cell homogenate and in the membrane preparation. The cell homogenate is prepared by washing two culture dishes twice with homogenizing solutions [sucrose, 250 m*M*; histidine, 30 m*M*; EDTA, 5 m*M*; sodium deoxycholate, 0.01 percent (weight to volume); and tris buffer, 5 m*M* (*p*H 6.8)]. The cells are scraped and homogenized in 2.0 ml of homogenizing solution with ten strokes of a Teflon-glass Elvehjem-Potter homogenizer. Protein is analyzed by the method of Lowry *et al.* (*9*), Na^+,K^+-adenosinetriphosphatase according to Philipson and Edelman (*10*), and succinic dehydrogenase (SDH) and 5′-nucleotidase according to Wong and Zull (*11*).

Membrane enzymes are assayed by adding the reagents directly to the culture dishes on which the membranes were prepared and incubating at 37°C for 30 minutes. Reagents are then poured from the plates and assayed for reaction products. To determine Na^+,K^+-adenosinetriphosphatase activity this procedure is first done with 20 m*M* KCl in the reaction medium; it is then repeated with the same dish but in the absence of KCl, and the difference between the results of the two assays is taken as the enzyme activity.

To determine protein, the membranes on the plates are washed with distilled H_2O; 6 ml of H_2O is then added and the H_2O is frozen. The ice is allowed to thaw slightly and the disk of ice is rubbed vigorously against the bottom of the dish. This leaves the membranes floating as the ice melts. The H_2O and membranes are poured into a centrifuge tube, the membranes are precipitated with 10 percent trichloracetic acid and spun down, and protein is determined (*3, 8*). Protein and enzyme activities are shown in Table 1.

The membrane markers are increased 7- to 15-fold in specific activity. The mitochondrial marker (SDH) is undetectable, and there is more than 100-fold reduction in protein in the course of preparation. To further document the small amount of intracellular residuum in the membrane preparation, cultures are grown on scintillation disks. The cultures are labeled with ^{42}K for 40 to 60 minutes, the disks are inserted into a flow cell, and isotopic washout is continued for 10 minutes, at which time all the remaining ^{42}K is intracellular (*2, 3*). The ^{42}K activity is recorded, the disks are rapidly removed from the flow cell, and membranes are prepared. The disks, with attached membranes, are then reinserted in the flow cell and counted. The residual activity, which represents retained intracellular material, in six preparations was 0.85 ± 0.49 percent—that is 99 percent of the intracellular material labeled by ^{42}K was eliminated by the procedure. The reverse procedure—labeling the membrane of the cells by using a lactoperoxidase iodination technique (*12*)—was not successful because of heavy nonspecific binding of the radioactive iodine to the plastic disks. This obscured the signal from the membranes.

Although by DePierre and Karnovsky's definition (*13*) the technique applies the preparative rather than the analytic approach, it is unique in a number of important respects. The cells are never exposed to any chemical agents that might modify the basic membrane characteristics. Exposure to a critically oriented high-velocity N_2 jet disrupts and fractionates the tissue in a single operation that requires less than 1 second. The membranes remain in a sheetlike configuration and adhere to the surface on which the cells were grown. They are amenable to enzymatic, ionic binding, histochemical, and ultrastructural studies. Such studies can be preceded by a variety of studies of the same cells before isolation of their membranes. The technique should be applicable to any tissue grown in monolayer culture.

G. A. LANGER
J. S. FRANK
K. D. PHILIPSON
Departments of Medicine and Physiology and *Cardiovascular Research Laboratory*, University of California, Los Angeles 90024*

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* Greater Los Angeles Affiliate of the American Heart Association.

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Identification of a *C*-Glucuronide of Δ^6-Tetrahydrocannabinol as a Mouse Liver Conjugate in vivo

Abstract. *Δ^6-Tetrahydrocannabinol-C-4′-glucuronide was found in the livers of mice that had been administered Δ^6-tetrahydrocannabinol. Thus, C-glucuronidation of a compound that contains a free hydroxyl group has been demonstrated in vivo.*

Glucuronidation of foreign compounds is a major metabolic pathway in mammals. Although *O*-, *N*-, and *S*-glucuronidation are well known (*1*), to our knowledge *C*-glucuronidation in vivo has been observed only in a group of pyrazolidine-containing drugs that have a strongly acidic hydrogen on a carbon atom but no hydroxyl, amino, or thiol groups (*2*).

In several chemical reactions, including glucosidation, the C-4′ aromatic position in some cannabinoids is substituted in preference to the free phenolic group (*3*). On this basis we assumed (*4*) that *C*-glucuronidation could take place on that carbon atom. We found that enzymatic conjugation of Δ^6-tetrahydrocannabinol (Δ^6-THC) (**1**) with uridine 5′-diphosphoglucuronic acid in the presence of UDP-glucuronyltransferase led to *C*-glucuronides (*4*). The identification was made by comparison with synthetic substances.

It seemed of importance to determine whether *C*-glucuronidation of Δ^6-THC could also take place in vivo. We report here that Δ^6-THC does undergo *C*-glucuronidation in vivo.

Δ^6-Tetrahydrocannabinol (50 mg) was suspended in 1 ml of a 5 percent solution of Tween 80 in isotonic saline solution. It was administered (100 μl per mouse; 140 mg/kg per dose) intraperitoneally to ten male mice (~ 35 g each) 26 and 2 hours before the animals were killed. The livers (17 g) were removed and were homogenized in a glass homogenizer for 3 minutes in 30 ml of saline solution (*p*H 7). The homogenate was extracted with

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