isolation procedure necessarily modifies the cell membrane, we performed these experiments in order to assess the contribution of the tissue environment to the evocation of the cellular response. The observations indicate that the perturbation produced by the small hydrostatic pressure applied directly to the isolated cells alters the amounts of cyclic nucleotides in cells and that these changes are cell specific and dependent on the matrix (or cell environment).

In this context, we view the tissue as a stimulus-receptor system in which the distortion of the cell membrane produced by a mechanical, electrical (*9*), or chemical (*10*) perturbation initiates specific events in the bone remodeling process through cyclic nucleotide modulation. The meaning of the reduction in cyclic AMP can be inferred from observations in other experimental systems. In fibroblast cultures low levels of cyclic AMP are correlated with growth stimulation, whereas addition of cyclic AMP inhibits growth (*3*). Similarly, the growth-stimulating hormone, somatomedin, inhibits the adenylate cyclase activity of chondrocyte and chick-embryo cartilage preparations in vitro (*11*). The concurrent drop in cyclic GMP observed in the tissue does not fit the hypothesis of reciprocal changes in the two cyclic nucleotides (*12*). In contrast, the response of the isolated cells (from the distal segment) seems to follow this pattern. A deeper understanding of these differences could come from the study of the mechanism by which pressure modulates the cyclic nucleotide levels. Regardless of mechanism, however, our results demonstrate that, under the experimental conditions described, the immediate response of long bone in vitro to a pressure of physiological magnitude includes a change in the levels of cyclic AMP and cyclic GMP. The cyclic nucleotides may play the role of messengers in the conveyance of the mechanical perturbation to the biochemical machinery of the cell, serving as regulatory molecules in the cytodifferentiation required for bone remodeling.

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Membrane Protein Analysis by Two-Dimensional Immunoelectrophoresis

Abstract. *Water-insoluble membrane proteins may be analyzed by a new, rapid technique that combines electrophoresis on high-resolution sodium dodecyl sulfate (SDS) polyacrylamide gels and immunoelectrophoresis. After separation in the first dimension by electrophoresis in SDS, the proteins are subjected to a second electrophoresis at right angles through a two-layered buffered agarose gel. They first pass through a layer containing Lubrol PX which forms complexes with free SDS and then into an antiserum layer where antigen-antibody precipitates form. Precipitin arcs appear at positions corresponding to the antigens separated in the first dimension. The effectiveness of the technique was demonstrated with frog and cattle opsins, human erythrocyte membrane proteins, and their rabbit antiserums and for several water soluble proteins. By this method two fundamental parameters, molecular weight and antigenicity, may be readily used for analysis of membrane proteins.*

Antiserums to cellular membrane constituents are valuable for studying the spatial arrangement of antigens on cell surfaces, their distribution among various cell types, and the effects of antibody binding on membrane-mediated cell functions. However, the water insolubility of many membrane macromolecules has made it difficult to confirm the specificity of the corresponding antiserums or to identify the antigens unequivocally.

Membrane proteins are now routinely separated and analyzed by electrophoresis on sodium dodecyl sulfate (SDS) polyacrylamide gels, a method that furnishes reproducible polypeptide patterns for several membrane systems (*1*–*3*). No other system has comparable advantages of simplicity, sensitivity, and resolution in the analysis of the water-insoluble proteins of biological membranes. We therefore designed a compatible immunochemical assay by combining SDS polyacrylamide gel electrophoresis in the first dimension (*4*) with immunoelectrophoresis into antiserum in the second dimension.

The procedure is an extensive modification of the techniques of "rocket," and

"crossed" immunoelectrophoresis of Laurell and others (*5*) and is described in Fig. 1. The agarose gel for the second dimension is cast in two layers; the first is a narrow zone containing the nonionic detergent Lubrol PX (ICI, Sigma), the second layer contains the antiserum or purified antibody. The nonionic detergent forms a complex with most of the SDS and thereby prevents nonspecific SDS precipitation of antiserum (*6*). As a result, specific precipitin arcs are seen at positions corresponding to the location of the antigen in the first dimension, with negligible interference by nonspecific precipitation (Fig. 2). In order to obtain the best precipitin arcs, optimum concentrations of antigen and antiserum must be used. These vary depending upon the antibody and antigen. For three membrane proteins [opsin (*7*), glycophorin (*8*), and spectrin (*9*)], we used 0.05 to 1.0 μg of protein and 3 to 12 percent of the corresponding antiserum by volume or comparable amounts of a purified immunoglobulin G (IgG) fraction from immune serum in the agarose layer.

Several water-soluble proteins and water-insoluble membrane proteins and their antiserums were tested. Two soluble proteins, *Escherichia coli* asparaginase (Merck) and human IgG (fraction 2, Lederle) formed precipitin arcs with their antiserums in this two-dimensional system. The entire IgG molecule was reactive with the serum we tested (Hyland), but arcs were not visible next to the H and L chains separated from the IgG. Rabbit antibodies to frog rod outer segments (ROS) or to purified opsin (*7*) formed precipitin arcs with either frog opsin or cattle opsin (Fig. 2). When glycophorin, the major sialoglycoprotein from the human red cell membrane (*8*), was subjected first to electrophoresis in SDS polyacrylamide gels and then electrophoresed into antiserum to a peptide of glycophorin, precipitin arcs appeared at positions corresponding to sialoglycoprotein bands PAS 1 and PAS 2 (*1*). Red cell membranes (*10*) also formed similar precipitin arcs with the same antiserum (Fig. 2B). Using antiserums to purified spectrin from human red cells (*9*) and longer electrophoresis in the first dimension in a 4 percent polyacrylamide gel, we readily separated the precipitin arcs for the two major spectrin bands I and II (*1*).

The two-dimensional electrophoretic method is quantitative, as would be predicted by analogy with Laurell's one-dimensional system (*5*). First-dimension gels were loaded with varied amounts of ROS protein as determined by amino acid analysis and absorbance at 500 nm. The opsin regions were cut out and electrophoresed in parallel through the Lubrol layer into the same agarose gel containing antiserum to ROS. The peak height, measured from the Lubrol layer, was directly proportional to the amount of antigen (Fig. 3).

As a check on the specificity of immunoprecipitation, opsin labeled biosynthetically with L-[^{14}C]leucine (*7*) was subjected

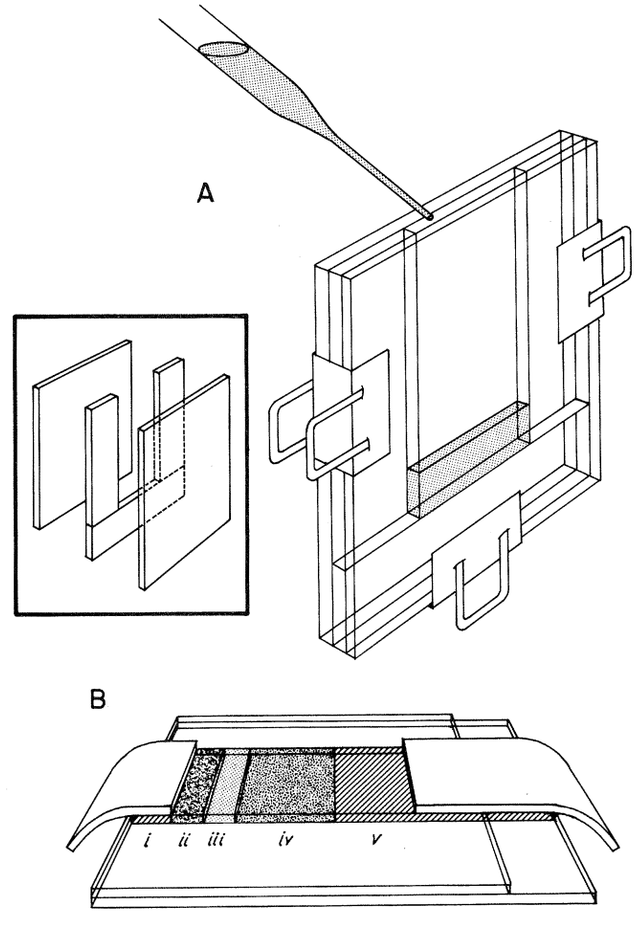

Fig. 1. Preparation of immunoelectrophoretic gels (second dimension). (A) Molds for the agarose gels consisted of two projection slide cover glasses (Kodak) (8.3 by 10.2 cm) separated by three slides (25 by 75 by 1.5 mm) (Clay-Adams) and held together with large binder clips. Molds and pipettes were warmed in a 60°C oven. The buffer for agarose and Lubrol solutions and for electrophoresis was diluted fivefold from a stock solution (*1*) [0.4*M* tris, 0.2*M* sodium acetate, 0.005*M* disodium ethylenediaminetetraacetate (Na_2EDTA), titrated to *p*H 7.4 with glacial acetic acid]. Agarose (electrophoresis grade, Nutritional Biochemicals) was dissolved in buffer (1.1 g/100 ml) and kept molten (79° to 90°C). Pyrex tubes, some containing Lubrol PX (10 percent, weight to volume, in buffer) were warmed to 55° to 60°C in a heating block. To pour the first agarose layer containing Lubrol, 17 volumes of hot agarose were added to 3 volumes of 10 percent Lubrol solution (to a final concentration of 1.5 percent, weight to volume, Lubrol) and the mixture was pipetted into the mold to a depth of 5 mm. After the gel had set (5 minutes), the mold was returned to the 60°C oven. Meanwhile, hot agarose was pipetted into one of the Pyrex tubes and cooled to about 55°C. Serum or immunoglobulin G (IgG) solution (at room temperature) was then added to the agarose, stirred, and quickly pipetted into the warm mold on top of the Lubrol layer to a depth of 1.5 to 3 cm. After this second layer had cooled, the mold was filled with buffered 1.1 percent agarose. When the gel had set, the clips were removed and the top plate was slid off with a piece of window glass (2.3 mm thick) as a stop to keep the gel and spacers from tearing or sliding. The bottom spacer slide was removed, and the SDS polyacrylamide strip was cut from a slab gel (*4*) and abutted against the Lubrol agarose layer. Buffered 1.1 percent agarose was added next to the SDS polyacrylamide gel strip to complete contacts with the wick. Bromphenol blue was added as a marker at the junction between the polyacrylamide and Lubrol-agarose layers, then this junction was sealed with hot agarose. Synthetic sponge wicks (ExaPhor, LKB) were placed over the agarose bridges. (Whatman No. 3 paper, doubled, may also be used; the resistivity is twice that of the sponge.) (B) The completed slab consisted of (i) an agarose bridge, (ii) the SDS polyacrylamide sample strip, (iii) a 5-mm-wide zone of 1.5 percent Lubrol PX in agarose, (iv) a zone, 1.5 to 3.0 cm wide, of agarose containing antiserum, and (v) a final agarose bridge. Electrophoresis was conducted in a horizontal tank at 54 volts over the entire length (5 cm) of the gel, measured at the wick contact point; the current was 4 ma/cm width. (The total voltage was 90 volts over a gel 5 cm long and a 17-cm wick.) Gels were maintained for 1 to 2 hours at constant voltage or until the bromphenol blue marker had traveled 3 to 4 cm. Large-scale immunoelectrophoresis was accomplished by casting the gels in large molds (180 by 180 by 1.5 mm) and subjecting them to electrophoresis on a cooled Savant model FP-22A flat plate; a Savant model HV-3000A power supply was used. The bromphenol blue front was marked with India ink, and the gel was immediately photographed against a black background with side lighting. In order to remove soluble proteins and nonspecific precipitation (which may occur in the red cell ghost system), gels were washed overnight in a stirred fenestrated holder in 0.075*M* NaCl. The gels were covered with Whatman 1 paper, weighted, and dried at 60°C for 2 to 3 hours or at 22°C overnight; they were then stained with 0.11 percent amido black in a mixture of methanol, water, and acetic acid (50 : 50 : 10 by volume) and destained in the same solvent, or if overstained, in a mixture of 95 percent methanol and 5 percent Na_2CO_3 (approximately 4 : 1 by volume). Staining often brings up faint precipitin arcs. They can then be autoradiographed on Kodak RP Royal X-Omat x-ray film (*7*) and stored indefinitely.

to coelectrophoresis with asparaginase (molecular weight, 34,000; Merck), which migrates in nearly the same position as opsin on SDS polyacrylamide gels. The two superimposed proteins were then electrophoresed in the second dimension into agarose containing antibody to asparaginase. Asparaginase and the antibody to asparaginase formed a precipitin arc which contained less than 6 percent of the radioactive [^{14}C]opsin precipitable by antibody to ROS. Furthermore, [^{14}C]opsin was not precipitated in this two-dimensional system by antiserum to IgG, and IgG and its constituent chains and asparaginase were not precipitated by antiserum to ROS.

With all the proteins studied, including opsin, red cell membranes, asparaginase, and IgG, we observed specific immunoprecipitates between SDS-solubilized antigens and antibodies. It appears, therefore, that many proteins, and all those tested, can recover antigenicity upon electrophoresis from SDS gels.

In one system, the rabbits were immunized with SDS-solubilized proteins (SDS-opsin) isolated from polyacrylamide gels (*7*). The extent of the contribution of SDS and denaturation to the formation of the immunogenic complex in this case is not known. However, antiserums prepared against SDS-opsin react in Ouchterlony double diffusion gels with ROS dissolved in Emulphogene (General Aniline and Film), a detergent that retains the "native" spectral properties of rhodopsin. Thus, antiserums to proteins isolated from SDS polyacrylamide gels do not require SDS-solubilized antigens for reaction by double diffusion.

Clearest results were obtained with an agarose layer containing Lubrol PX inserted between the SDS polyacrylamide gel and the antibody-agarose layer in the second dimension (Fig. 1). A single agarose gel rather than a two-layered gel was used for the second dimension in earlier experiments. This gel contained only antiserum or antiserum mixed with 1.5 percent Lubrol (Fig. 2A). In these simpler single-layered gels, larger amounts of antigen and antiserum were necessary to distinguish the precipitin arc from the nonspecific background line caused by SDS precipitation of serum proteins. Despite these limitations, the simpler gels were useful when the antigen was labeled with ^{14}C since they could be autoradiographed to localize the immunoprecipitated antigen (*7*). The nonspecific background line contained negligible radioactivity.

In the two-layered gels, SDS and Lubrol may form mixed micelles in the Lubrol layer (*11, 12*). Mixed micelles of a similar anionic-nonionic detergent pair have decreased electrophoretic mobility compared to the pure anionic detergent (*13*). Using pinacryptol yellow to stain SDS (*14*) after the second dimension of electrophoresis, we found that SDS from the polyacrylamide strip had passed through the Lubrol layer and entered the antiserum layer with slower electrophoretic mobility than free SDS. It appears that these mixed SDS-Lubrol micelles do not nonspecifically precipitate antiserum as readily as does SDS alone and thus specific precipitates may be more readily visualized. Triton X-100 (Rohm and Haas; Sigma) appears to form similar micelles with SDS in this system.

Other two-dimensional immunoelectrophoretic studies of red cell membrane antigens and ROS have been reported (*15*). These procedures either did not utilize a highly resolving system for the initial separation or required elution and resolubilization in nonionic detergents before the second stage of immunoelectrophoresis.

Our procedure may be useful in membrane structural analysis (i) to verify the reactivity of antiserums with the membrane proteins from which they were prepared; (ii) to test for the existence of related proteins in a membrane fraction, such as subunits, multimers, cross-linked molecules, and peptide fragments; (iii) to monitor the isolation of proteins; (iv) to locate and identify radioactively labeled proteins by autoradiography (*7*); and (v) to analyze the synthesis and transport of a membrane protein such as opsin (*7*). Some

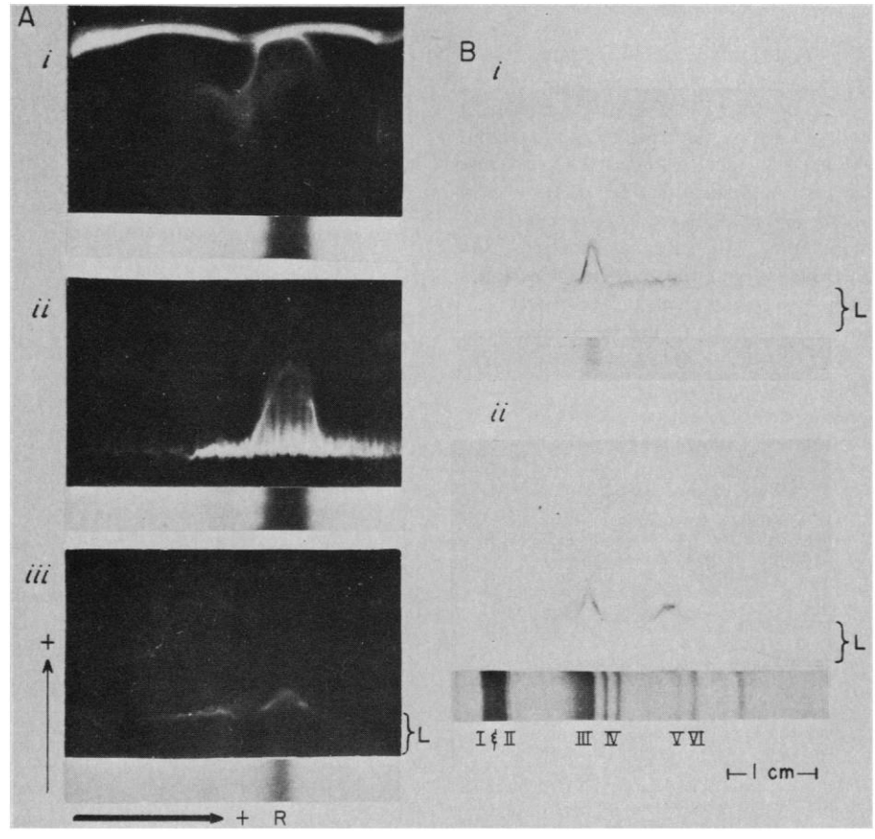

Fig. 2. Immunoelectrophoresis of ROS and red cell ghost membrane proteins. (A) Comparison of different types of agarose gel (second dimension). The antigen is frog retina ROS membranes (*3*). Antiserums were produced as described (*7*). The IgG fraction of antiserum to ROS (*16*) was concentrated to 14 mg/ml (Amicon UM-10). (i) The agarose gel contained 12 percent (by volume) IgG fraction of antiserum to ROS. The polyacrylamide strip contained 8.5 μg of frog ROS protein. A stained reference gel is shown. Excess SDS causes nonspecific precipitation at the top. (ii) The agarose gel (a single layer) contained both 8 percent IgG from antiserum to ROS, IgG fraction, and 1.5 percent Lubrol PX. The antigen was 8.5 μg of frog ROS protein. The zone of nonspecific precipitation has moved below the precipitin arcs. (iii) The agarose gel was in two layers, as described in Fig. 1. The lower layer contained 1.5 percent Lubrol PX and the upper contained 5 percent IgG from antiserum to ROS. Antigen consisted of 0.2 μg of frog ROS protein. (The stained strip used as reference contains 0.6 μg of opsin.) Since nonspecific precipitation was eliminated, the sensitivity of antigen detection was enhanced about 40-fold and less antibody was required. (B) Precipitin arcs produced by electrophoresis of glycophorin or red cell membranes through Lubrol PX into agarose containing antiserum (12 percent by volume) to the carboxyterminal peptide (C-2) of glycophorin (*8*). (i) The polyacrylamide strip contained 1.5 μg of human red cell glycophorin (0.6 μg of protein). (ii) The polyacrylamide contained human red cell membranes (12 μg of protein and an estimated 1.2 μg of glycophorin, or 0.5 μg of glycophorin protein). Major protein bands are assigned according to (*1*).

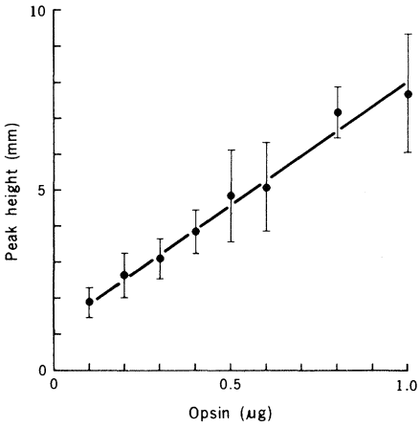

Fig. 3. Peak height as a function of the amount of opsin migrating during electrophoresis in the first dimension and subsequently into antiserum to ROS. One standard deviation (four measurements) is shown for each point.

membrane components may not be able to regenerate antigenic determinants after the native conformation or supramolecular interactions are disturbed by SDS solubilization. For such systems this procedure may not be applicable. However, the simplicity of the technique and the ease of immunization with antigens isolated from SDS polyacrylamide gels should permit a large variety of systems to be explored.

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6. Proteins having a molecular weight of less than about 150,000 migrate readily from the standard 5.8 percent gel (*1*) and may be detected by fixing and staining after electrophoresis into agarose lacking antiserum. Gels with 4 percent or less are satisfactory as the first dimension for studying larger molecules. It appears that some SDS is still attached to the protein after migration through the Lubrol layer. This was shown by the migration, under electrophoresis, of opsin, IgG, and H and L chains into the SDS polyacrylamide gel, then in the second dimension through the Lubrol layer into buffered agarose lacking antiserum. All of the proteins migrated into the agarose approximately the same distance; if all SDS has been removed, the IgG and the H and L chains should have been retarded or should have reversed their direction of migration and not been seen at all. At *p*H 7.4, IgG in the agarose layer migrates slightly toward the cathode (that is, toward the entering antigens). Gels run at *p*H 7.4 and *p*H 8.6 are not significantly different.
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Enteropancreatic Circulation of Digestive Enzymes

Abstract. *Intact digestive enzymes can be absorbed by the intestine and resecreted by the pancreas. The pancreas, therefore, appears to be able to recycle proteins much as the liver recycles bile salts, although the magnitude of this process remains uncertain.*

At least some digestive enzyme undergoes an enteropancreatic circulation, analogous to the enterohepatic circulation of bile salts; that is, intact digestive enzyme molecules are absorbed by the intestine and subsequently resecreted by the pancreas. The existence of this cycle was shown directly with radioactive enzyme and inferred from the following observations: (i) The baso-lateral cell membranes of pancreatic tissue are permeable to at least some digestive enzymes; (ii) there is the potential for transpancreatic movement of these enzymes from blood to duct lumen through the secretory cells; and (iii) the small intestine is permeable to several digestive enzymes.

In studies on the kinetics of [^{3}H]chymotrypsinogen (bovine) uptake by pancreatic tissue in vitro (*1*) we found that at least a portion of the cell membrane of the acinar cell, the pancreatic secretory cell, was permeable to this and presumably other digestive enzymes that were added to the suspending medium. This exogenous enzyme equilibrated with endogenous secretory protein of the same enzyme species in the cytoplasm and zymogen granules, crossing both zymogen granule (*1, 2*) and cell membrane (*1*) in the process. Since in this study the investigators used slices of pancreatic tissue no more than 0.1 mm thick that expose both apical and baso-lateral cell membranes to the bathing medium (*3*), it was not clear which part of the cell membrane was permeable to the enzyme. As a control, the uptake of exogenous enzyme was monitored in the same manner (*1*) by using strips of pancreatic tissue, that is, unsliced segments of rabbit pancreas in which the apical membrane is not exposed to the bathing medium (*3*). In these strips, after correction for intercellular space (with albumin) and a substantial wash (sufficient to remove 90 percent of the albumin) with a medium containing a high concentration of unlabeled chymotrypsinogen (40 μ*M*), a considerable amount of [^{3}H]chymotrypsinogen was still associated with the tissue. Since the magnitude of uptake was substantial [unsliced uptake was about 20 percent less than the previously reported values for tissue (*1*)], in all likelihood uptake across the baso-lateral surface of the cell was at least partially involved in the attainment of equilibrium between exogenous enzyme and intracellular digestive enzyme pools.

If secretory protein can enter the cell from the "blood" side, and if once these molecules are in the cell they mix with secretory pools, then this protein should, of course, eventually be secreted. In order to test for this, an in vitro preparation of whole rabbit pancreas was used (*4*). After excision, the whole organ was suspended by means of attached intestinal tissue on a Lucite frame that was in turn placed in a bath containing a physiological salt solution and oxygenated with 95 percent O_2 and 5 percent CO_2 without recourse to vascular perfusion. The pancreatic duct was cannulated and secretion collected directly from it without contamination by bath water, thereby maintaining the polarity of the natural secretory process. [^{3}H]Chymotrypsinogen (bovine) and [^{131}I]albumin (human) (to control for the leakiness of the system) were added and