from degenerating retina and other tissues, as suggested by the authors. However, if one accepts this latter concept, one must also accept that the degree of retinal degeneration is somehow related to the degree of vascular permeability. Such a relationship is plausible.[5]

The present consensus regarding SRF proteins is that many originate from plasma (probably via leakage through an abnormally permeable choriocapillaris Bruch's membrane and retinal pigment epithelium[5]), some from degenerating retina, and some from other sources.[2-5, 7, 8] Most probably, SRF lipids have a similar dual or multicompartmental origin.

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To the Editor:

We did realize that there is some correlation between lipid and protein concentration. Our statement "... no *specific* correlation ...based an *visual* inspection ..." referred to the scattering appearance of the data. For example, a sample showing a high lipid concentration of 2.06 has a protein concentration of only 11.6 mg. per milliliter. The 20 cases selected in our study are far from a complete representation of the complicated clinical variations. If more data were collected, one might see a nonlinear correlation, or other phenomena not indicated in our data. That is the reason we did not attempt to discuss the statistical analyses.

We also agree that part of the lipid is derived from the blood. The portion derived from the blood could be more significant in samples having high protein concentration. The statement "... lipid in subretinal fluid does not result from a *nonspecific* leakage ..." referred to simple diffusion of original serum constituents, where molecules equilibrate reversibly on both sides of the blood vessel. We have not excluded the unknown mechanism where large molecular weight lipoproteins are actively transported into the subretinal space. We have not been able to demonstrate any lipase activity in the subretinal fluid. It seems to us that the degradative processes occur inside the macrophages and tissue cells.

The subretinal esterases described in our previous paper (Lam et al., INVESTIGATIVE OPHTHALMOLOGY, 1972) are not concerned with degradation of lipids.

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Clean Start For The Retinal Pigment Epithelium

To the Editor:

One of the chief purposes of publication of research results is to provide the research community with data that can be used as a basis for the next logical attack on the unknown. Ideally, experiments performed in one laboratory should be reproducible in another one, thereby facilitating progress toward solving problems of common interest. It is an axiom of both science and philosophy, however, that ones conclusions are only as valid as ones assumptions, and therein lies many problems. The assumptions of one laboratory may not be the same as those of another; data cannot be shared or transferred, and experiments must be repeated.

The recent National Eye Institute Symposium* on "The Pigment Epithelium: Its Relationship to

*Oct. 15 through 17, 1975, Bethesda, Md.

the Retina in Health and Disease" brought into focus not only the importance of this cell layer in retinal function but also—perhaps more importantly—how little we actually know about these cells. Several laboratories are now engaged in analyzing the chemical composition of isolated pigment epithelial cells from a variety of animal species. It became clear during the October Symposium that very few investigators were aware of the pitfalls inherent in isolating these cells free of contaminating tissue. It was therefore felt that for those who may in the future be interested in studying retinal pigment epithelial (RPE) cells, whether it be for biochemistry, cell biology, physiology, or any other basic discipline, that certain procedures be followed to ensure against "clean experiments on dirty preparations."

We have invested considerable time and effort in just this problem, and although the details of the preparative procedures have been published,[1, 2] it was felt that certain of the steps used required further comment. The following then are what we have found necessary to isolate RPE cells in good yield, and in a high state of purity from large eyes such as cattle, dogs, cats.

Isolation and decontamination of cells. The neural retina is gently folded inward with a tweezers and snipped off at the optic disc. Sucrose solution (see below) is placed in the posterior eye cup and the pigment cells are then brushed free with a camel's hair brush; the released cells are aspirated with a Pasteur pipette. The crude suspension is poured through cheese cloth to remove large tissue fragments which are invariably present no matter how much care is taken. Much (about 50 per cent) of the material brushed out of the eye cup, and still remaining after filtering through cheese cloth, is contamination (red blood cells, rod outer segments [ROS] and retinal fragments).

To remove these contaminants, the cells are gently dispersed on a Vortex mixer and centrifuged at 112 × g for 10 minutes.[1, 2] More recently we have found better morphology of particles using 0.32 M sucrose. In this case, about 140 to 150 × g for 10 minutes is needed for pelleting of the cells. Proper washing, at these recommended centrifugal forces,* is critical, for it is in this step that the intact cells are pelleted, while the contaminants are "floated away" in the supernatant. Increasing the gravitational forces above these values is likely to bring down more contaminants with the cells, while decreasing the centrifugal force would result in poor yields of cells. About six washing cycles are needed to remove most of the red blood cells and to release the ROS from their insertions in the villous processes of the pigment epithelial cells. With patience and careful monitoring of both the pellets and the washes in a light microscope, clean RPE cells are eventually obtained. Exactly how many washes are needed? This requires some experience and judgment. We have found numerous red cells still present after three or four washes,[2] but after a few more cycles, when the supernatant is essentially clear, the pellet is quite free of red blood cells and ROS. Using this method we obtain about four to six milligrams of cells per cattle eye.

Homogenization. Cell suspensions from nearly any source tend to slide around a Teflon pestle during homogenization, in contrast to a solid tissue which is anchored to the wall of the homogenizing tube.[3] Thus, RPE cells, like most other free cells require "pretreatment" before the usual glass-Teflon homogenization. Three methods have been used in the authors' laboratories:

Sonication. This is quick and easy. The washed cells are suspended in saline or buffer. About two bursts of 20 seconds each time (with the sample itself in an ice bath) disintegrates the cells completely. Centrifugation at about 10,000 r.p.m. for five minutes yields: (1) a clear straw-colored* supernatant (or cell-free extract) which usually contains about one-third of the total protein of the cells, and (2) a dark black residue consisting of pigment granules and insoluble membranous material. This method, although completely unsatisfactory for the isolation of morphologically recognizable organelles, is extremely effective for extraction of enzymes (e.g., all of the peroxidase of RPE is solubilized this way) and proteins (e.g., retinol-binding proteins). This extract is *not* the cytosol, which is the specific fractions obtained after high-speed centrifugation of a sucrose homogenate. If the RPE cells are homogenized in sucrose under milder conditions, as described below, cytosol protein accounts for only about 10 per cent of the total cellular protein. Therefore, the cell-free extract obtained by sonication contains proteins and enzymes originating from the cytosol *plus* those solubilized from various cytoplasmic organelles.

French pressure cell. This causes efficient breakage of cell membranes with release of intracellular organelles; however, in our hands the morphology is poor. This instrument usually generates very high pressures (up to 20,000 psi) and probably explains why there is extensive escape of proteins and enzymes from intracellular organelles into the cytosol fraction. Advantages of using a French pressure cell are that it is easy to handle and the instrument is usually readily available.

Nitrogen decompression chamber. This method

*This is best measured with an externally applied tachometer.

*If this solution is pink instead of straw-colored, it means that the red blood cells have not been completely removed during the washing procedure.

also causes efficient breakage of cell membranes, and release of organelles. Its use for disrupting isolated mitochondria under high pressure has been described,[4] but the method may be adapted using relatively lower pressures. Under well-controlled conditions, the organelles from RPE look much better morphologically than do those from French pressure cells. Another advantage of nitrogen cavitation is that the organelles are biochemically intact as well, as shown by fractionation experiments. There is very little leakage of, for example, lysosomal acid hydrolases into the cytosol.

Which method is chosen for cell disruption depends on the type of experiment you want to perform. But in all cases, once the cell membranes have been ruptured by any of the above methods, only one or two up-and-down strokes in a glass-Teflon homogenizer, running at about 1,000 r.p.m., are required to completely disperse the intracellular organelles. Cells prepared by methods two and three are then ready for fractionation, or any of the three preparations may be used as an RPE homogenate.

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