Measurement of Osmotic Fragility of Fibroblasts. (23933)

JACK ROSENBLUTH AND GEORGE D. PAPPAS* (Introduced by L. Thomas)
Departments of Pathology and Anatomy, N. Y. University College of Medicine, New York City

Studies of physical properties of a number of different types of cell membranes have led to a proposed basic structure for the cell membrane, described as consisting of continuous bimolecular film of lipid with a layer of protein adsorbed on both surfaces(1,2,3,7). The "elasticity and great mechanical strength" of the membrane have been attributed to cross-linked polypeptide chains in these protein layers(4). In such a membrane it might be expected that hydrolysis of polypeptide links in the outer protein layer would reduce the mechanical strength of the membrane. Experiments described below attempted to demonstrate this by subjecting samples of fibroblasts to 2 proteolytic enzymes, trypsin and papain, and measuring osmotic fragility of treated and untreated cells. It was assumed that a decrease in mechanical strength of the membrane would be reflected by increase in osmotic fragility of the cells. Trypsin was used because of its well known dispersing effect on fibroblasts and other cells grown *in vitro*, suggesting activity at cell surface. Papain was used because of its reported ability to digest certain living cells(5,6). A comparison is also made between osmotic swelling in fibroblasts and in erythrocytes. The latter swell in a manner suggesting that erythrocyte cell membrane is a nearly inexpansible structure, which permits an increase in cell volume by changing in shape from disc to sphere and not by increasing in surface area(8,9). In contrast to this, observations show (described below) that the fibroblast cell membrane is capable of considerable expansion in area. This, along with the relative rigidity of form of the erythrocyte membrane, even after hemolysis(10) suggests basic structural differences between erythrocyte and fibroblast cell membranes.

Methods. Osmotic fragility measurements on fibroblasts. Cells were obtained from 5-day-old cultures of Earle's strain L mouse fibroblasts grown *in vitro* in medium consisting of 40% horse serum, 40% Hanks solution, and 20% chick embryo extract, plus penicillin and streptomycin. The cells were centrifuged from culture medium for 5 minutes at 1,000 rpm and then rinsed twice in Hanks solution (containing no phenol red or other indicator). The packed cells were then diluted 1-10 by volume in isotonic Hanks solution, and 0.10 ml aliquots of resulting suspension were added to tubes containing 3½ ml each of Hanks solution in dilutions ranging from isotonic strength (100%) to distilled water. After thirty minutes exposure the tubes were centrifuged and supernatant solutions ("A") were saved. The cells in each tube were then resuspended in distilled water for 2 hours, centrifuged out, and supernatants ("B") again saved. The degree of lysis was determined by measuring optical density at 260 mμ of each pair of supernatant solutions, and computing the percent lysis at each dilution as $\frac{[\text{O.D.}] \text{ of "A"} \times 100}{[\text{O.D.}] \text{ of "A" plus } [\text{O.D.}] \text{ of "B"}}$.

Results indicate a small amount of cell lysis at concentrations between 100% and approximately 13% isotonicity. This is probably due to mechanical effects of centrifugation and pipetting plus surface effects of glassware, which was not siliconized. Beginning

TABLE I. Absorption of Supernatant Solutions.

Final % isotonicity (% Hanks)	$\left[\frac{\text{Optical}}{\text{density}}\right]_{\lambda=260}$ Supernatant A	B	% lysis
100	.041	.216	16
57	.046	.258	15
36	.020	.276	7
30	.023	.458	5
23	.028	.473	6
19	.044	.516	8
13	.070	.443	14
11	.127	.375	25
8	.256	.229	53
6	.388	.117	77
3	.422	.063	87

* Present address: Department of Anatomy, College of Physicians and Surgeons, N. Y. City.

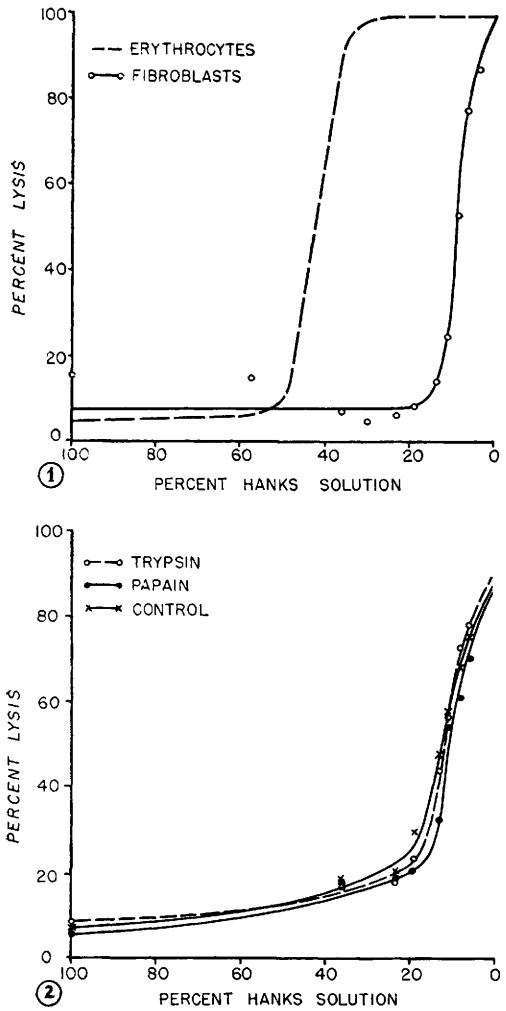

FIG. 1. Comparative osmotic fragility of erythrocytes (from Wintrobe[12]) and strain L fibroblasts.

FIG. 2. Effect of trypsin and papain on osmotic fragility of strain L fibroblasts.

at 13% the degree of lysis rises sharply as tonicity approaches zero. Similar results were obtained when tests were run at 5° instead of 37°C, and when cells were suspended in dilutions of sodium chloride solution instead of Hanks solution.

Absorption of supernatant solutions (which was maximal at 260 mμ) was presumed to be due primarily to RNA-protein released from cell cytoplasm(11). This is inferred from microscopic observation that the cell nuclei appeared to remain intact after osmotic disruption of the cytoplasmic membrane. Presence of relatively large amounts of cytoplasmic RNA has been demonstrated in one strain of fibroblasts grown *in vitro*, where the ratio of total cellular RNA to DNA was approximately 5 to 1 in 5-day-old cultures(13, 14). Direct chemical analysis of the supernatant solutions was not done.

Figure 1 shows percent lysis at each dilution of Hanks solution for strain L fibroblasts. For comparison a typical curve of erythrocyte osmotic fragility(12) is included, showing hemolysis beginning at approximately 50% tonicity.

Microscope observations of fibroblast swelling. Wet slide-coverslip preparations of strain L fibroblasts were made and examined at 1,000 X (phase contrast). When distilled water was put under edge of coverslip, each cell swelled rapidly to 3 or 4 times its original diameter. After a variable time, up to about 3 minutes, the cell membrane ruptured abruptly at a point, and the intracellular granules were extruded through the defect as the membrane contracted to its original size. Nuclei showed very little swelling and never burst. Microscopically it appeared that the cell membrane was expansile and that there was tension in the expanded membrane causing it to contract again after its rupture.

Effects of proteolytic enzymes on fibroblast fragility. Washed samples of strain L fibroblasts were suspended in solutions of either trypsin† or papain‡ for 30 minutes at 37°C. The concentration used for both enzymes was 0.5 mg/ml. The cells were then washed twice in Hanks solution and subjected to the fragility test described above. Controls were treated in the same way except that in place of enzymes, plain Hanks solution was substituted. The results are shown in Fig. 2. In enzyme-treated samples and in controls, the curve rises sharply at a tonicity of approximately 13%. The elevated baselines probably represent increased mechanical fragmentation due to extra rinsing, pipetting and centrifugation involved in pretreatment with the enzymes.

† Thrice crystallized trypsin obtained from Worthington Corp., N. J.

‡ Crystalline protease prepared from crude papain by Dr. Emil Smith (Dept. of Biochemistry, University of Utah).

The fact that enzyme treatment produced no increase in cell fragility may be interpreted in one of two ways: 1. There is no substrate for trypsin and papain in the concentrations used at the cell surface of strain L fibroblasts. 2. There is a protein substrate for these enzymes at the cell surface, which was hydrolyzed by the enzymes, but this protein does not play a significant role in imparting strength to the membrane (as measured by osmotic fragility).

Summary. 1. A method is described for measuring osmotic fragility of cells containing no visible pigment and available in limited quantities only. 2. By this method it was found that osmotic fragility of Earle's strain L fibroblasts is considerably less than that of mammalian erythrocytes, fibroblasts lysing at approximately 13% of isotonicity and erythrocytes at approximately 50%. 3. Microscopic observations are described suggesting that the cell membrane of Earle's strain L fibroblasts is an expansile structure. 4. Treatment of strain L fibroblasts with trypsin or papain produced no increase in their osmotic fragility.

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Effect of Vit. E-Deficiency and Dietary Glycine on Tissue Phosphatase Activity.* (23934)

L. C. SMITH AND SAID NEHORAYAN

Department of Biochemistry, School of Medicine, State University of S. Dakota, Vermillion

Carey *et al.*(1) reported that alkaline phosphatase activity of muscle homogenates from dystrophic rabbits was unchanged from that of control animals, whereas a 2-fold increase in muscle acid phosphatase activity from dystrophic rabbits was observed. White and Hess(2) found no alteration in serum phosphatase activity in children and adults afflicted with progressive muscular dystrophy. Tallan(3) showed an increase in level of most free amino acids in muscle extracts of dystrophic rabbits except for the concentration of glycine which was markedly reduced. This was later confirmed by Smith and Nelson(4) who in addition showed that glycine was consistently reduced in 4 other tissues of dystrophic rabbits. It seemed desirable to determine what effect, if any, the dystrophic condition as well as dietary glycine would have on enzymic activity of tissue other than muscle. Our studies describe the acid and alkaline phosphatase activity in liver, kidney, heart, spleen, brain, and muscle of normal and Vit. E-deficient rabbits, and the influence of dietary glycine on phosphatase activity of these tissues.

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Methods. Twelve male rabbits, divided into 4 equal groups, were fed the following diets *ad lib.*: A. Control diet 11 of Goettsch