ELECTRON MICROSCOPE ANALYSIS OF YOUNG AND OLD RED BLOOD CELLS STAINED WITH COLLOIDAL IRON FOR SURFACE CHARGE EVALUATION

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ABSTRACT

Human and rabbit red blood cells, separated into "young" and "old" age groups by differential flotation on phthalate esters, were fixed with glutaraldehyde and labeled with colloidal ferric oxide. Electron micrographs of thin sections of young cells showed a uniform and dense depostion of positive iron particles. Old cells showed particles deposited irregularly. leaving unlabeled gaps on the membrane surface. Red cells incubated with 10 units/ml receptor-destroying enzyme (RDE) demonstrate a reduced labeling, similar to that of old cells. After neuraminic acid had been removed from red cells by 20 units/ml RDE, no iron particles were found on membrane surfaces. The different labeling of young, old, and RDE-treated human and rabbit red cells was correlated with their electric mobility and agglutinability by poly-L-lysine. The contradiction between the apparent similarity in charge density of human and rabbit red cells as estimated by density of iron particles and the markedly lower electric mobility of rabbit red cells is discussed.

INTRODUCTION

The electric mobility of red blood cells from different mammalian species differ from one species to another (1). Also, separated fractions of "old" red blood cells move more slowly in the electric field than "young" red cells of the same blood sample (2, 3).

Adsorption of myxovirus, followed by elution, results in a marked decrease in the electric mobility of red cells (4); the degree of reduction depends on the myxovirus type (5). Incubation of red blood cells with receptor-destroying enzyme (RDE) derived from *Vibrio cholerae* cultures almost completely removes the negative charge from the cells (5).

The substrate for the receptor-destroying enzyme on the red cell surface is neuraminic acid (6), and treatment with neuraminidase reduces the electric mobility of red cells (7). It has been demonstrated by other methods that the negative charge on the red cell surface can be ascribed almost entirely to the carboxylic group of neuraminic acid (8–10). *n*-Acethyl neuraminic acid (NANA) was also found to be the major component in the reaction of the negatively charged red cell surface with the positively charged poly-L-lysine (11). A correlation was demonstrated between the rate of agglutinability with poly-L-lysine and the electric mobility as well as the absence of agglutinability and reduction of electric mobility in neuraminidase-treated red cells (12, 13).

A recently developed technique for the electron microscopic identification of the site of acid mucopolysacharides on membrane surface by use of

colloidal ferric oxide or thorium hydroxide at low pH (14, 15) permits the location of the site of negative charges on the membrane surface.

The aim of the present work was to determine the location and density of the negative charges on the surface of young and old, human and rabbit red cells and to see whether a correlation can be established between the labeling capacity by positively charged colloidal ferric oxide and the electric mobility and agglutinability differences between old and young cells. The relationship between the reduced electric mobility and agglutinability of RDE-treated red cells and their labeling capacity was also studied.

MATERIALS AND METHODS

Fresh human or rabbit heparinized blood taken by venipuncture was separated into young and old red cell fractions, by differential flotation on phthalate esters[1] after the density distribution of the cells (16) had been determined. Separation was performed by centrifuging the samples in polyethylene test tubes at 10,000 *g* in a Beckman-spinco microfuge at room temperature. The separated red cell fractions were resuspended in 0.9% NaCl solution and washed twice by sedimentation and resuspension.

Treatment with receptor-destroying enzyme (RDE), derived from *Vibrio cholerae* (obtained from Burroughs Wellcome & Co., London, England), was carried out by incubation of 1 volume of washed packed cells with 1 volume of the enzyme at the desired concentration (10, 20, 50, and 100 units/ml) in a thermostatic bath at 37°C for 45–60 min. The incubation was stopped by adding ice-cold saline solution and by washing the treated red cells twice with cold saline. Control samples were treated with heat-inactivated enzyme.

Electron Microscopy

Treated or untreated red cells were fixed with 2% glutaraldehyde in phosphate buffer, pH 7.3, at room temperature for 30 min. The fixed cells were washed twice with distilled water and resuspended in positive or negative colloidal iron solution for 30 min. The preparation of the stock solution and the staining procedure were carried out according to the method of Gasic et al. (14). After staining, the suspension was washed twice in 12% acetic acid solution, once in distilled water, and postfixed with 1% aqueous OsO_4 at room temperature (14). The samples were dehydrated in acetone and embedded in Vestopal W. Thin grey sections of approximately 600 A thickness were cut with a Danon-Yeda ultramicrotome and mounted on Formvar carbon-coated copper grids. Micrographs were taken with a JEM-7 or JEM-T7 electron microscope at 80 kv and 60 kv, respectively.

In comparing the density of attached colloidal particles on the surfaces of different cell membranes, a curvimeter was used to measure the length of membrane on which the number of black dots was counted from micrographs enlarged to a magnification of 20,000. Colloidal particles were not counted on parts of the membrane that were tangentically sectioned.

Rate of Agglutination Measurements

1 volume of washed, packed red blood cells from RDE-treated and untreated blood samples separated into young and old age groups was resuspended in 500 volumes of 0.9% NaCl solution buffered to pH 7.3 by Veronal-acetate. Rate of agglutination measurements were performed with the Fragiligraph Model D-2 (Elron Electronic Industries, Ltd., Haifa, Israel), according to the method described elsewhere (13). Positively charged poly-L-lysine n = 100 was employed as the agglutination agent for human blood, and poly-L-lysine n[2] = 1000 was used for rabbit blood.

Electric mobilities of 100 cells from each sample, 50 cells in each direction, were measured in a Zeiss Cytopherometer at a current of 5 ma at a thermostatically controlled temperature of 24°C. The diluted working suspension of the red cells was made up in an isotonic phosphate-buffered saline at pH 7.4.

RESULTS

The surface of the red cell membrane is labeled with positively charged colloidal iron particles at pH 1.8.

Young human red blood cells, from a separated fraction labeled with a nondialyzed positive colloidal iron suspension, showed a uniformly distributed deposition of colloidal particles along the membrane surface (Fig. 1). When a dialyzed suspension was used for labeling, there was a tendency toward the formation of an aggregated and coarse particle deposition on the membrane surface as described by Gasic et al. (14).

Old human red cells from a separated fraction showed particles deposited irregularly, leaving unlabeled gaps on the membrane surface (Fig. 2).

Treatment of human red blood cells with RDE at increasing concentrations of the enzyme resulted in a progressive reduction in the labeling capacity with positive particles. Incubation of 0.1 ml washed, packed whole population human red

[1] This was purchased from Miles-Yeda Ltd., Rehovot, Israel.

[2] n = degree of polymerization (number of lysine residues).

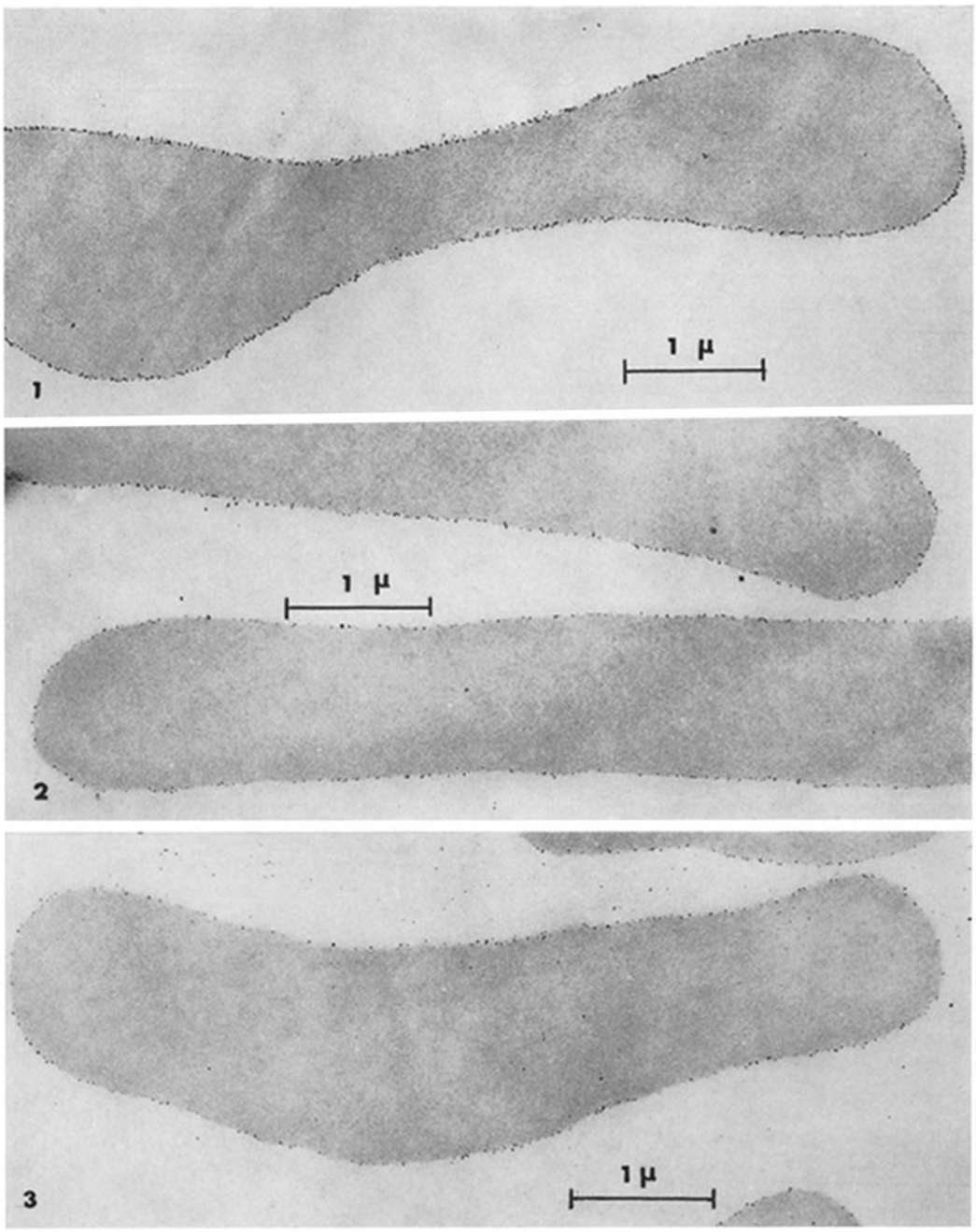

FIGURE 1 Human red blood cells from a young fraction separated by differential flotation, fixed in glutaraldehyde, and labeled with a nondialyzed positive colloidal iron suspension. Colloidal particles are uniformly distributed along the membrane surface. × 20,000.

FIGURE 2 Human red blood cells from an old fraction separated by differential flotation, fixed in glutaraldehyde, and labeled with a nondialyzed positive colloidal iron suspension. Iron particles are deposited irregularly, leaving unlabeled gaps on the membrane surface. × 20,000.

FIGURE 3 Unseparated human red blood cells treated with RDE (10 units/ml) and labeled with nondialyzed positive colloidal suspension. Deposition of colloidal iron particles is similar in amount and disposition to that of an old human red blood cell (see Fig. 2). × 20,000.

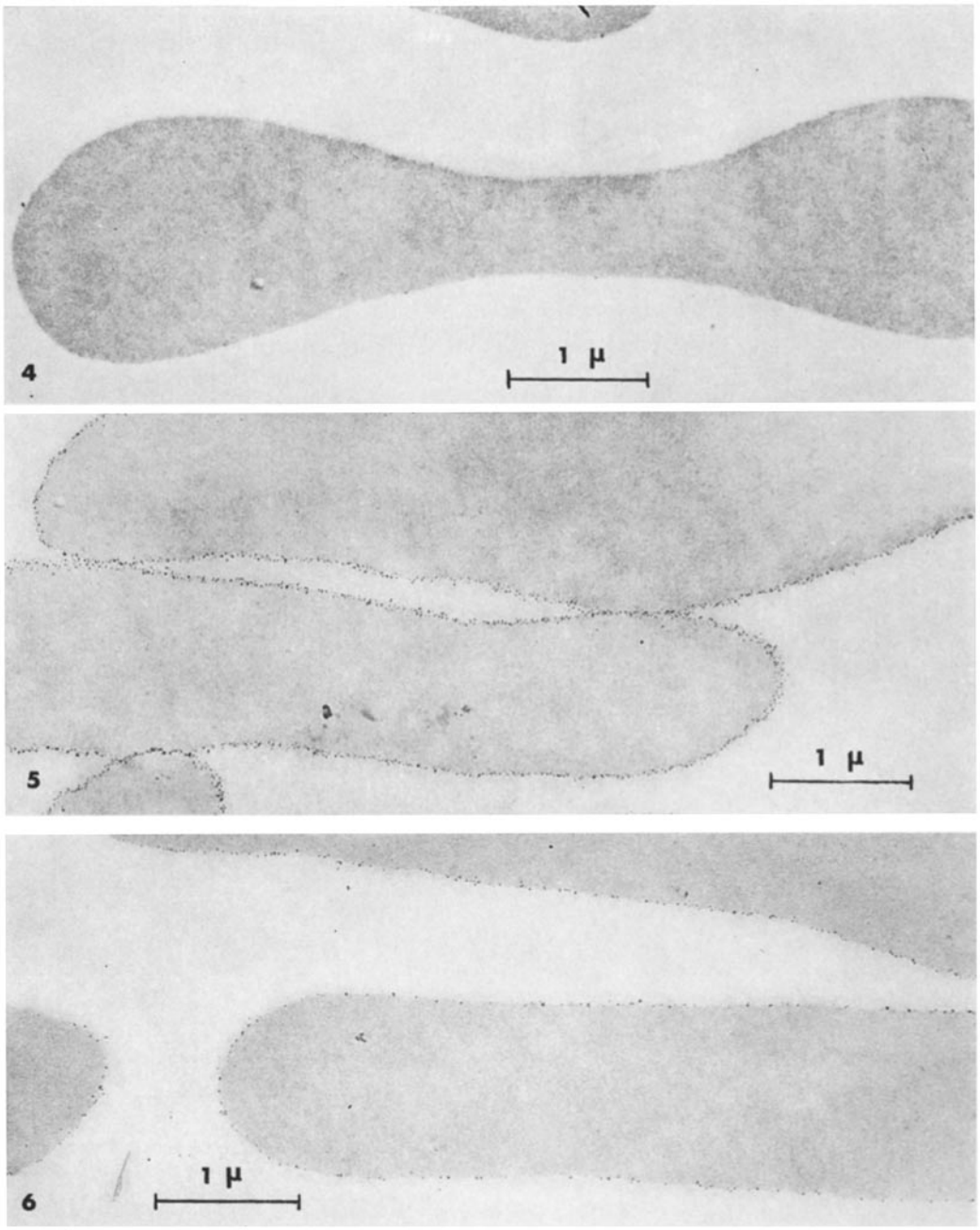

FIGURE 4 Unseparated human red blood cells treated with RDE (20 units/ml) and labeled with nondialyzed positive colloidal iron suspension. There is practically no deposition of colloidal particles on the membrane surface. × 20,000.

FIGURE 5 Young rabbit red blood cells, labeled with a nondialyzed positive colloidal iron suspension, showing a deposition similar to that of young human red blood cells. × 20,000.

FIGURE 6 Old rabbit red blood cells, labeled with a nondialyzed positive colloidal iron suspension, showing particles deposited irregularly, leaving unlabeled gaps on the membrane surface. × 20,000.

TABLE I

Correlation Between Electric Mobility of Red Blood Cells and the Density of Colloidal Iron Oxide Particles Counted on Electron Micrographs, for Comparative Evaluation of Surface Charge*

Blood sample	No. of particles per micron length of membrane		Electric mobility: μ/sec/v/cm	
	Decrease		Decrease	
		%		%
Human red blood cells				
Young	20.4		1.32	
Old	12.6	35.0	1.03	24
10U/ml RDE	11.8	38.0	0.96	28
20 U/ml RDE	0.0		0.27	80
Rabbit red blood cells				
Young	17.8		0.70	
Old	12.0	32.5	0.60	14
10 U/ml RDE	10.8	39.0	0.57	18
20 U/ml RDE	0.0		0.21	70

* From each sample, the membranes of 25 cells were measured on the micrograph in the parts where the membrane is perpendicularly sectioned. On these parts the iron particles were counted.

blood cells with 10 units/ml RDE reduced the amount of colloidal iron particles on the membrane surface to approximately that found on labeled old red cells (Fig. 3). Such an alteration of the red cell to simulate an old one is reflected also in changes in the rate of agglutination and mobility (12). Incubation with 20 units/ml RDE removed most of the negative charge of the red cell. No labeling by positively charged iron particles on the membrane surface was seen (Fig. 4), and no agglutinability could be recorded. Measurement of electric mobility (Table I) showed a loss of about 80% of the original charge.

The difference in the labeling capacity of separated young and old rabbit red cells by positive colloidal iron is similar to that for human young and old red cells (Figs. 5 and 6). Similar differences were found in electric mobility (Table I) and agglutinability (Fig. 7). Rabbit red cells treated with 10 units/ml of RDE and afterwards labeled with positive colloidal iron showed a similar reduction in labeling as did human RDE-treated red cells.

When RDE (20 units/ml)-treated rabbit red cells were labeled with negatively charged colloidal iron, a slight and dispersed deposition of colloid particles appeared on the membrane surface. Positive colloidal iron did not label the same sample at all. In a control experiment in which non-RDE–treated rabbit red cells were incubated with the negative colloid, no deposition of colloid particles was apparent. When this technique was applied to RDE–treated human red cells, no labeling with negatively charged particles occurred.

The unit membrane of red cells, usually clearly visible in stained thin sections, was completely absent in all samples labeled with colloidal iron. The low pH of the labeling medium might be the reason for this morphological feature.

DISCUSSION

n-Acethyl neuraminic acid, the main or perhaps the only carrier of the negative charge on the outer surface of the red cell membrane, exerts a strong attractive force to positively charged colloidal particles. Benedetti and Emmelot (17) blocked the acidic receptors on the membrane by the basic poly-peptide poly-L-lysine, thus preventing the association of the positive particles to the membrane surface. The interaction with neuraminic acid was also demonstrated by the complete ab-

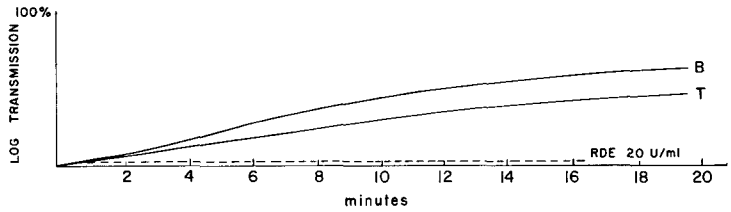

FIGURE 7 The rate of agglutination of human red blood cells by poly-L-lysine n = 100. Agglutination curves of young red cells from a top fraction (*T*), old red cells from a bottom fraction (*B*), and a whole population of red cells treated with 20 units/ml RDE (- - -). Note that at any time after onset of agglutination the old cells are agglutinated in a rate approximately 30% higher than that of the young cells. No agglutination occurs after RDE treatment.

sence of colloidal iron particles on red cell membranes treated with RDE.

Intact young human or rabbit red cells are more negatively charged than are the old cells (2, 3, 13). The present study demonstrated a dense and regular labeling of young red cells and a decrease in labeling capacity and irregular location of the colloid particles on old red cells. The decrease in labeling capacity on the surface of old red cells correlates with their reduction in electric mobility of about 24% in human and 14% in rabbit red cells. Treatment of young red cells with 10 units/ml of RDE results in a reduction of approximately 28% in human and 18% in rabbit red cells in their electric mobility, about 35% in their labeling density, and about 35% increase in the rate of agglutination, making these cells similar to old cells from the electric charge point of view. The removal of neuraminic acid by treatment with higher concentrations (20 units/ml) of RDE results in the absence of sites capable of attaching the iron particles as well as loss of agglutinability and a considerable reduction in electric mobility. The fact that no agglutination by poly-L-lysine occurred and no labeling with colloidal iron particles could be observed, in spite of the remaining charges after treatment with 20 units/ml RDE, may be explained by the sparsity of the charges left. In such a case, even if there is a single charge left, it might not have the capacity to hold the huge molecule or colloidal particle.

Another possibility is that the remaining charges after RDE treatment are not due to neuraminic acid (18), and that either by their nature or sparsity they are incapable to interact with either poly-L-lysine or with colloidal iron.

Some sparse dots were seen on the surface of rabbit RDE (20 units/ml)-treated red cells incubated with negatively charged colloidal iron. When this technique was applied to human red cells after RDE-treatment, no labeling with negatively charged iron particles was visible. Whether these sparse dots on rabbit red cells are indicative of a positive charge on the surface of the rabbit red cell, newly appearing after treatment with RDE, is not clear and requires further investigation.

The apparent similarity in charge density between rabbit and human red cells, when estimated by the density of colloidal iron particles (compare Fig. 1 with Fig. 5), is rather surprising in view of the markedly lower electric mobility of rabbit red cells (1). Furthermore, poly-L-lysine n = 1000 was required to agglutinate rabbit red cells, while the shorter polymer of n = 100 agglutinated human red cells (13). However, it was estimated that the iron particle covers 5–16 molecules of sialic acid (19), and even if only 10% of these molecules are ionized, there would be sufficient charge to interact with the iron particles (14). Assuming that these charges are uniformly distributed, the equal density of iron particles on the surface of both, human and rabbit young red blood cells would be understandable. Apparently in spite of the difference in their over-all surface charge both of them contain sufficient charges to saturate the surface with iron particles. The reduction in mobility, label density, and increased rate of agglutination in old cells and in RDE-treated cells should, therefore, be attributed to areas on the red cell surface depleted of negative charges as visualized by the unlabeled gaps on the membrane surface (Figs. 2, 3, and 6).

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REFERENCES

1. EYLAR, E. H., M. A. MADOFF, O. V. BRODY, and J. L. ONCLEY. 1962. *J. Biol. Chem.* **237:** 1992.
2. DANON, D., and Y. MARIKOVSKY. 1961. *C. R. Acad. Sci.* **253:**1271.
3. YEARI, A. 1969. *Blood.* **33:**159.
4. HONIG, M. 1948. *Proc. Soc. Exp. Biol. Med.* **68:** 385.
5. STONE, J. D., and G. L. ADA. 1952. *Brit. J. Exp. Pathol.* **33:**428.
6. GOTTSCHALK, A., and P. E. LIND. 1949. *Nature (London).* **164:**232.
7. COOK, G. M., D. H. HEARD, and G. V. F. SEAMAN. 1961. *Nature (London).* **191:**44.
8. GLAESER, R. M., and H. C. MELL. 1964. *Biochim. Biophys. Acta.* **79:**606.
9. HAYDON, D. A., and G. V. F. SEAMAN. 1967. *Arch. Bioch. Biophys.* **112:**126.
10. SACHTLEBEN, P., and G. RUHENSTROTH-BAUER. 1961. *Nature (London).* **192:**982.
11. DANON, D., C. HOWE, and L. T. LEE. 1965. *Biochim. Biophys. Acta.* **101:**201.
12. MARIKOVSKY, Y., D. DANON, and A. KATCHALSKY. 1966. *Biochim. Biophys. Acta.* **124:**154.

13. DANON, D., Y. MARIKOVSKY, and A. KOHN. 1969. *Experientia (Basel).* **25:**104.
14. GASIC, G. J., L. BERWICK, and M. SORRENTINO. 1968. *Lab. Invest.* **18:**63.
15. REVEL, J. P. 1964. *J. Microsc.* **3:**535.
16. DANON, D., and Y. MARIKOVSKY. 1964. *J. Lab. Clin. Med.* **64:**668.
17. BENEDETTI, E. L., and P. EMMELOT. 1967. *J. Cell Sci.* **2:**499.
18. HAYDON, D. A., and G. V. F. SEAMAN. 1967. *Arch. Biochem. Biophys.* **122:**126.
19. KRAEMER, P. M. 1966. *J. Cell Comp. Physiol.* **67:** 12.