increases their effectiveness appreciably in inhibiting free-radical oxidative processes in the vitreous body.

LITERATURE CITED

1. Yu. A. Vladimirov and A. I. Archakov, Lipid Peroxidation in Biological Membranes [in Russian], Moscow (1972), pp. 172-196.
2. R. A. Gundorova and G. A. Petropavlovskaya, Penetrating Wounds and Contusions of the Eye [in Russian], Moscow (1975).
3. R. A. Gundorova, A. A. Malaev, and A. M. Yuzhakov, Trauma of the Eye [in Russian], Moscow (1986), pp. 273-286.
4. N. A. Plokhinskii, Biometrics [in Russian], Moscow (1970), p. 18.
5. N. M. Éfendiev, Vest. Oftal'mol., No. 1, 43 (1983).
6. N. M. Éfendiev, N. K. Neiman-Zade, É. M. Kulieva, and A. I. Dzhafarov, Byull. Éksp. Biol. Med., No. 5, 569 (1987).

ENDOGENOUS PROTEOLYSIS IN HUMAN ERYTHROCYTE MEMBRANE PREPARATIONS

P. S. Gromov, S. F. Zakharov,
M. Gaczyńska, M. Soszyński,
G. Bartosz, V. Leyko, and
S. S. Shishkin

UDC 612.111.1.015.1:577.152.344

KEY WORDS: erythrocyte membrane; proteolysis; two-dimensional electrophoresis.

The problem of endogenous proteolytic activity in erythrocyte membranes has attracted the attention of many investigators engaged in the study of the structural and functional organization of cell membranes. This is because of the important role of various membrane-bound enzymes in the processes coordinating the formation of families of structurally interconnected membrane proteins and also in cell aging [8, 13]. Usually in the course of such investigations the need arises to determine the nature of activity discovered. This is a difficult task because of the high degree of contamination of erythrocyte membrane preparations by leukocytes, which may give rise to undesirable artefacts. The study of the protein components of human erythrocyte membranes also has a different aspect. In recent years extensive research has begun in a new field of biochemical genetics, known as "molecular anatomy," with the object of compiling a full catalog of erythrocyte membrane proteins on the basis of their mapping by two-dimensional electrophoresis [1, 9]. For and adequate and correct solution to many of the problems in this direction, the purity of the erythrocyte membrane preparations obtained and assessment of the degree of proteolytic degradation of the membrane proteins are of fundamental importance.

It was accordingly decided to study the effect of the method used to isolate human erythrocyte membranes on the endogenous proteolytic activity discovered in them and, at the same time, to study the degree of contamination of the preparations of erythrocyte "ghosts" by other forms of blood cells, primarily leukocytes. For this purpose, proteolytic degradation of erythrocyte membrane proteins under the influence of enzymes of both endogenous and leukocytic origin was investigated by electrophoretic methods, including the modified method of two-dimensional electrophoresis described in [11].

EXPERIMENTAL METHOD

Erythrocytes were obtained from 20 ml of heparinized blood by two methods. Method A: erythrocytes were separated from leukocytes and plasma by centrifugation 3 or 4 times at 1000g for 15 min. The white film of leukocytes was removed from the surface of the erythro-

Institute of Medical Genetics, Academy of Medical Sciences of the USSR, Moscow. Department of Biophysics, Lodz University, Poland.

(Presented by Academician of the Academy of Medical Sciences of the USSR N. P. Bochkov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 106, No. 9, pp. 292-297, September, 1988. Original article submitted April 27, 1987.

0007-4888/88/0009-1255$12.50

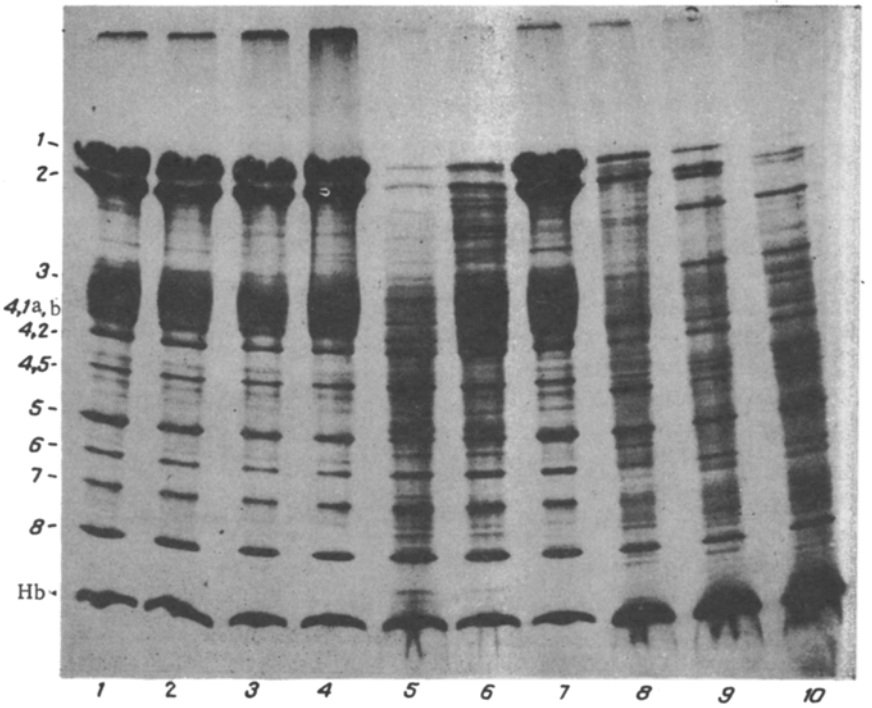

Fig. 1. One-way electrophoresis by the method in [11] of erythrocyte membrane proteins obtained by different methods and incubated for different times in 5 mM Na-phosphate buffer. Nomenclature of proteins according to [6]: pH 7.4 at 37°C. 1-6) Membranes obtained by method B: 1) control, 2) incubation for 24 h, 3) for 48 h, 4) for 72 h, 5) membranes + 100 μg leukocytes (incubation for 24 h), 6) membranes + 10 μg leukocytes (incubation for 24 h); 8-10) membranes obtained by method A: 8) incubation for 24 h, 9) for 48 h, 10) for 72 h; 7) control.

cyte residue by decantation. This is currently the most widely used method of obtaining erythrocytes [3, 7]. Method B: a combination of the two stages of purification described previously [1] with a change in the order of the stages. To begin with the heparinized blood was allowed to stand in 1.5% dextran 500 T solution in 0.15 M NaCl, after which the erythrocytes were further purified by passage through a column with HBS-cellulose. The membranes were obtained by the traditional method [3]. A suspension of membranes in 5 mM Na-phosphate buffer (pH 7.4) was incubated at 37°C for different lengths of time, lyophilized, and analyzed electrophoretically. One-way electrophoresis was carried out by the method in [10]. In the modification of two-way electrophoresis which was used the nonpolar detergent Triton X-100 was replaced by its analog Triton X-305, which has a longer hydrophobic chain, thus improving its lytic ability. This substitution necessitated an increase of the temperature at which separation in the first direction took place, namely electrofocusing to 30-35°C. This modification, described in detail by the writers previously [2] yielded gels with improved reproducibility and resolution by two-way electrophoresis.

EXPERIMENTAL RESULTS

Proteolytic degradation in erythrocyte membrane preparations was characterized by comparing gels of membrane proteins obtained by electrophoresis before and after incubation of the membrane suspension in 5 mM Na-phosphate buffer at 37°C. Membrane preparations obtained by the traditional method A were clearly subjected to endoproteolytic degradation during incubation, as shown by gradual disappearance of the principal high-molecular-weight proteins (spectrin, protein 3) and by the appearance of a large number of additional fractions (Fig. 1). At the same time, the preparations obtained by method B retain a typical distribution of fractions of the main proteins even for a very long incubation in the indicated conditions (up to 72 h), and only addition of a leucocyte suspension to the membrane preparations caused intense degradation of the membrane proteins.

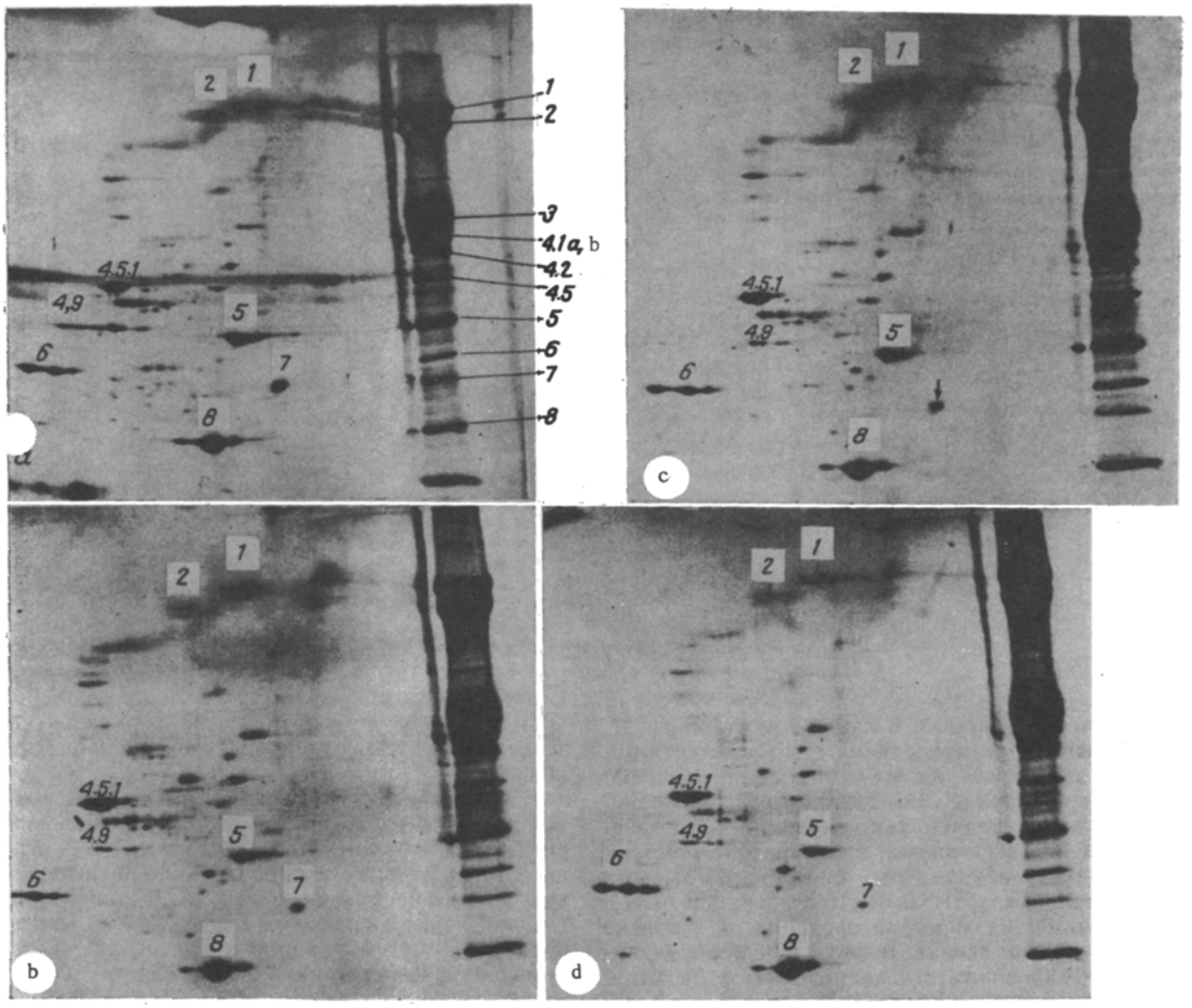

Fig. 2. Two-way electrophoresis of erythrocyte membrane proteins obtained by method B. a) Control; b-d) incubation at 37°C in 5 mM Na-phosphate buffer (pH 7.4); b) incubation for 24 h, c) for 48 h, d) for 72 h. Nomenclature of proteins as in [6]. One-way lane of corresponding specimen shown alongside two-way gel. Arrows indicate membrane proteins whose quantitative proportion falls during incubation. Circle indicates peptides whose content increases during endoproteolysis.

Two-way electrophoresis of the membrane proteins led to a sharp increase in the number of fractions to be analyzed and more detailed results of endoproteolysis of erythrocyte membrane preparations obtained by the method B to be given. Long-term incubation under these same conditions did not lead to the appearance of additional peptide spots on the gels as degradation products of membrane proteins of higher molecular weight as a result of proteolysis (Fig. 2). From a qualitative aspect, all the gels obtained by two-way electrophoresis, shown in Fig. 2, appear identical. Unfortunately we were unable to undertake an instrumental qualitative analysis of the two-way gels, but it could be concluded from visual assessment that the relative proportion of some principal membrane proteins decrease somewhat during incubation: spectrins, protein 4.9, actin(5), and protein 7. Since no additional spots with mol. wt. of over 15 kilodaltons (kD) (mol. wt. of globin) were present on the gels, this suggests that proteolysis of the above-mentioned proteins (Fig. 2, arrows) is characterized by the formation of peptides with mol. wt. of under 15 kD. Only one spot with mol. wt. of about 40 kD can be seen on the gels illustrated, and its intensity increased progressively during incubation (Fig. 2, circles). This peptide may perhaps act as a marker of functional proteolysis of membrane proteins, and from this point of view it is of great interest during the study of membrane-bound proteases. Besides the characteristic features of protein degradation mentioned above (a decrease in the relative proportion of several of the principal membrane proteins), observed during incubation under the same conditions, the

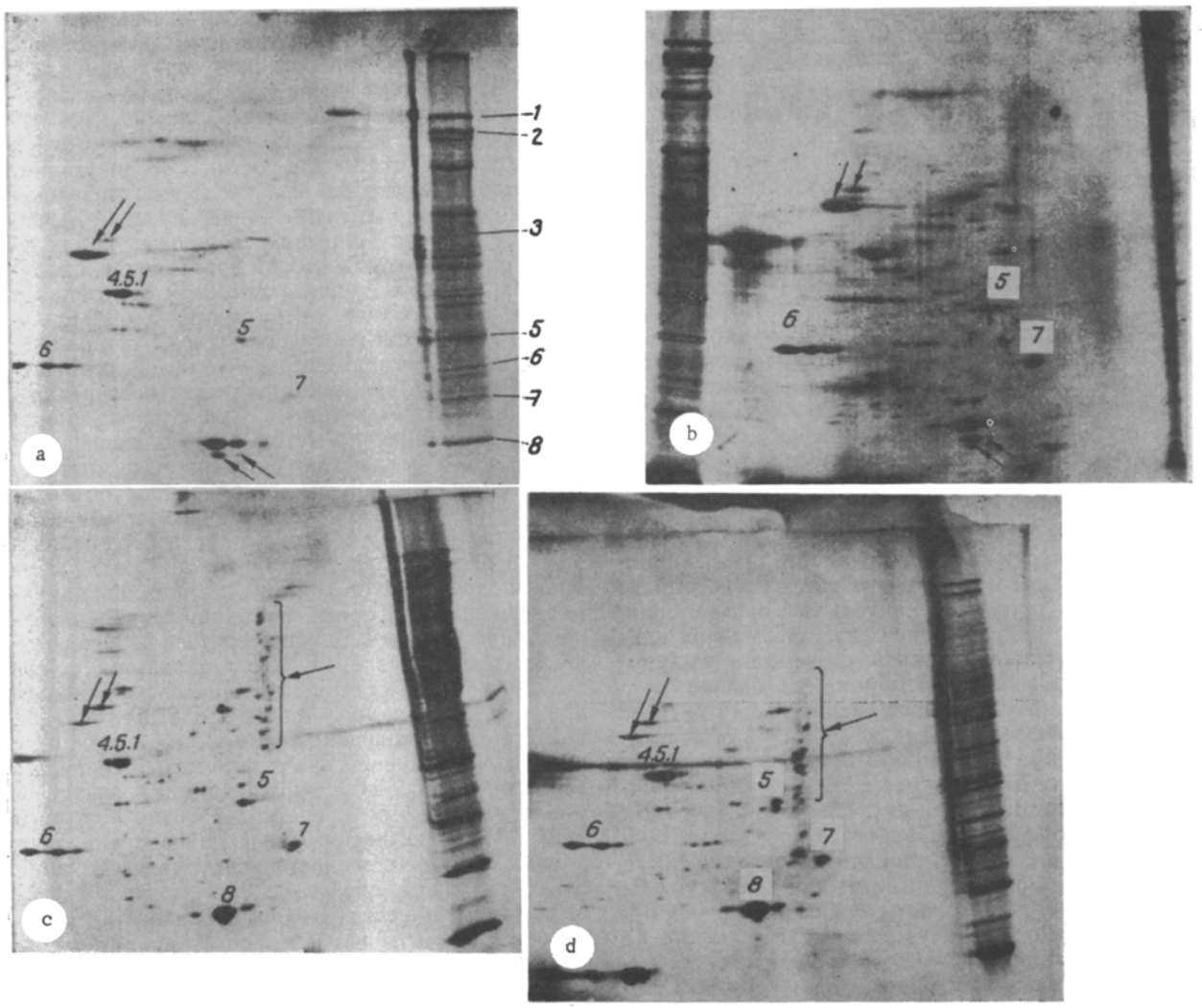

Fig. 3. Two-way electrophoresis of human erythrocyte membrane proteins obtained by method A (a, c) and method B (b, d). a) Incubation for 24 h, c) incubation for 48 h, b) membranes + 10 μg leukocytes to 1 mg membranes (incubation for 24 h); d) membranes + 100 μg leukocytes to 1 mg membranes (incubation for 24 h). Nomenclature as in [6]. Arrows indicate additional identical peptides.

appearance of several additional protein spots, completely absent on the corresponding gels of erythrocyte membrane proteins obtained by method B (Fig. 2), was observed in this case (Fig. 3a, b). It can be tentatively suggested that these products are the results of non-specific proteolytic degradation of high-molecular-weight membrane proteins, due to incomplete removal of leukocytes from the packed red cells. To test this hypothesis, leukocytes were added in the form of a suspension in 5 mM Na-phosphate buffer, in a dose of 10 and 100 μg/mg membranes to erythrocyte membrane preparations obtained by method B before incubation. Comparison of the corresponding two-way gels (Fig. 3a, c; b, d) shows that the addition of leukocytes to membrane preparations caused the appearance of analogous proteolysis products on the gels after electrophoresis. The most typical protein spots, identical with respect to the parameters studied, are indicated by arrows in Fig. 3.

Two main conclusions may be drawn from the results. First, preparation of membranes by the traditional method does not rule out their contamination with leukocytic enzymes. Second, the use of sedimentation in dextran 500 T followed by purification of the erythrocytes on HBS cellulose to remove leukocytes enables erythrocyte membranes with a low level of endogenous proteolytic activity to be obtained. This suggests that these preparations are virtually free from contaminating enzymes of leukocytic origin. According to our own observations, preparations of erythrocyte membranes obtained by method A, after hemolysis of the erythrocytes, always contain a small dense residue, evidently consisting of contaminating

leukocytes, in the diffuse membrane residue. This residue is usually removed mechanically by the investigator [6]. It must be pointed out that the procedure used for removal (method A) is difficult to monitor and, as our results show, it does not always ensure complete and reliable removal of leukocytes from the erythrocyte membrane preparations. The protein composition of erythrocyte membranes obtained by method A was analyzed in [2] by two-way electrophoresis, and the protease activity which was found was characterized by the authors cited as being on a relatively low level. However, it was evidently much higher than in our own preparations, for even after incubation for 30 min under conditions analogous with those described above considerable changes were found on the gels, reflecting in particular an increase in the representation of the majority of protein spots. According to our own observations, this fact is the result of nonspecific proteolysis, arising on account of the presence of traces of leukocytes in the membrane preparations.

The very low level of endoproteolytic activity which we found in erythrocyte membrane preparations obtained by method B may perhaps reflect the situation found in vivo. Meanwhile the nature of this activity may be different and it requires further study. For example, it is not impossible that it may be due to enzymes adsorbed from the cytosol of the erythrocytes. Erythrocyte cytosol proteinases are currently being actively studied [9]. Some erythrocytic proteolytic enzymes may perhaps have a mixed localization between the cytosol and erythrocyte membrane. Analysis of the strength of association of proteases with the membrane and characterization of their specific properties are essential for a final solution to the problem of the nature of proteolytic activity found in human erythrocyte membrane preparations. At the same time, the low level of proteolytic activity in preparations obtained by method B makes it possible to obtain erythrocyte membranes with stable protein composition, suitable for construction of two-dimensional protein maps and for the compiling of a catalog of erythrocyte membrane proteins on this basis.

LITERATURE CITED

1. P. S. Gromov, A. M. Shandala, L. I. Kovalev, and S. S. Shishkin, Byull. Éksp. Biol. Med., No. 7, 28 (1986).
2. B. R. Copeland, S. A. Todd, and C. E. Furlong, Am. J. Hum. Genet., 34, 15 (1982).
3. J. T. Dodge, C. Mitchell, and D. J. Hanahan, Arch. Biochem., 100, 119 (1963).
4. J. M. Fagan, L. Waxman, and A. L. Goldberg, J. Biol. Chem., 261, 5700 (1986).
5. G. Fairbanks, T. L. Steck, and D. F. H. Wallach, Biochemistry (Washington), 10, 2606 (1971).
6. M. Gaczynska, G. Bartosz, J. Rosin, and M. Soszynski, Int. J. Biochem., 17, 1237 (1985).
7. M. Gaczynska and G. Bartosz, Zagadn. Biophiz. Wspolsz., 11, 97 (1986).
8. D. Harrel and M. Morrison, Arch. Biochem., 193, 158 (1979).
9. R. J. Kirschner and A. L. Goldberg, J. Biol. Chem., 258, 967 (1983).
10. U. K. Laemmli, Nature, 227, 680 (1970).
11. P. H. O'Farrell, J. Biol. Chem., 250, 4007 (1975).
12. K. Sorensen and G. Bartosz, Zagadn. Biophiz. Wspolsz., 11, 135 (1986).