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TOPOLOGY OF N-ETHYLMALEIMIDE IN NORMAL HUMAN ERYTHROCYTE MEMBRANE

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ABSTRACT. Normal human, hemoglobin free erythrocyte ghosts were labelled with ^{14}C-N-Ethylmaleimide and incubated at two different temperatures 4^{o}C and 37^{o}C. Gel electrophoresis, autoradiography and nuclear scintillation counting techniques were used in analysing which proteins were labelled. The results of the experiments with incubation at 4^{o}C and 37^{o}C indicated that 35 % of the NEM was associated with spectrin and 50 % was bound to spectrin- actin complex.

1. INTRODUCTION

Electrophoresis of ghost membrane proteins in polyacrylamide gels containing 1 % SDS yields a pattern in which six well resolved bands are observed [1]. These bands are separated according to their molecular weight. Of the total membrane protein, about one-third are contained in two major bands, bands I and bands II, weighing 240,000 and 220,000 respectively. These bands are called spectrin. Bands 5 weighing 43,000-39,000 are called actin. Spectrin, together with actin play a major role in stabilising the membrane and maintaining its discoid shape [2]. To study the protein components of erythrocyte membranes, the intact membranes were labelled N-ethylmaleimide (NEM) [3,4] and maleimide anologue sulfhydryl spin labels [5-9]. However very few studies are available in the literature in mapping the binding sites of NEM and malemide spin labels to erythrocyte membranes [4,10].

In this research, NEM binding sites to normal human erytrocyte membranes have been investigated by labelling the membranes with ^{14}C-NEM. Gel electrophoresis, autoradiography and nuclear scintillation counting tecniques have been used to show which protein components were labelled.

2. MATERIALS AND METHODS

Hemoglobin-free white membrne ghosts were prepared from normal human erythrocytes according to the methods in [11,12]. The red blood cells were washed three times with 150 mM NaCl saline solution buffered with 5 mM sodium phosphate at pH 8 (1 volume of cell + 10 volume of buffer solution).

1 ml of packed cell was lysed by resuspension in 40 ml of cold 5 mM sodium phosphate (pH 8) and was centrifuged at 24,000 g for twenty minutes to form pellets which were washed 3 more times by suspension in the same buffer at the same speed. The resulting pellets were hemoglobin free white unsealed erythrocyte ghosts.

Spectrin-actin was purified according to the method of Bennett and Branton [13]. White membrane ghosts were washed in 0,3 mM Na_2HPO_4, pH 7.6 at 0^oC. The pellets are resuspended in a final volume of 5 ml in 0,3 mM Na_2HPO_4, pH 7.6, and incubated for 25 min. at 37^oC and the suspension centrifuged for 15 min. at 225,000 g. The resulting supernant contains approximately 80 % of the erytrocyte spectrin and almost all bands 5 or erythrocyte actin. The spectrin-actin solution was concentrated to 3 mg/ml by using aquacide (Calbiochem, CA.). Protein concentration was determined by using Lowry method [14]. Hemoglobin free erythrocyte ghosts were labelled with ^{14}C-N-ethylmaleimde with specific activity 23.7 mCi/mmol (New England Nuclear) in hypotonic buffer. Some of the samples were incubated at 37^oC and the others were incubated at 4^oC for one hour. They then were washed three times by suspension in hypotonic buffer, followed by centrifugation (10,000 g-30,000 g). Radioactive labelled spectrin-actin was prepared from labelled erythrocyte ghosts or spectrin-actin complex was purified first then spectrin-actin was labelled with ^{14}C-NEM.

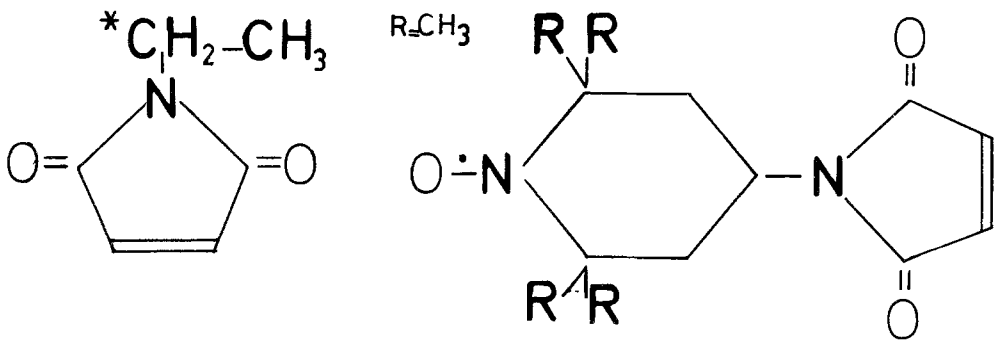
All samples were subjected to 7.5 % polyacrylamide slab gel electrophoresis containing 1 % sodium dodecyl sulfate with similar apparatus in [15] The gels were run at 27 mA until bromophenol blue tracking dye migrated to 1 cm of the bottom of the gel. The gel was nudged into a big crystallization dish and covered wth Coomasie blue stain. It was stained overnight with gentle stirring, and then was destained with destaining solution. The gel of the labelled samples was dried under the vacuum and autoradiography was performed. The bands of the gel were then sliced and were put in different tubes. H_2PO_4 was added and incubated in an oven at 60^oC for 24 hours. Then they were counted by nuclear scintillation counter (Beckman LS-3150 T Model Scintillation Counter).

3. RESULTS AND DISCUSSION

Lenard labelled intact membranes from different animal species with ^{14}C-NEM (see Figure 1. a) and showed that 50 % of the total NEM was associated with the spectrin-actin complex [4]. In this work membranes were incubated with ^{14}C-NEM at 37^oC.

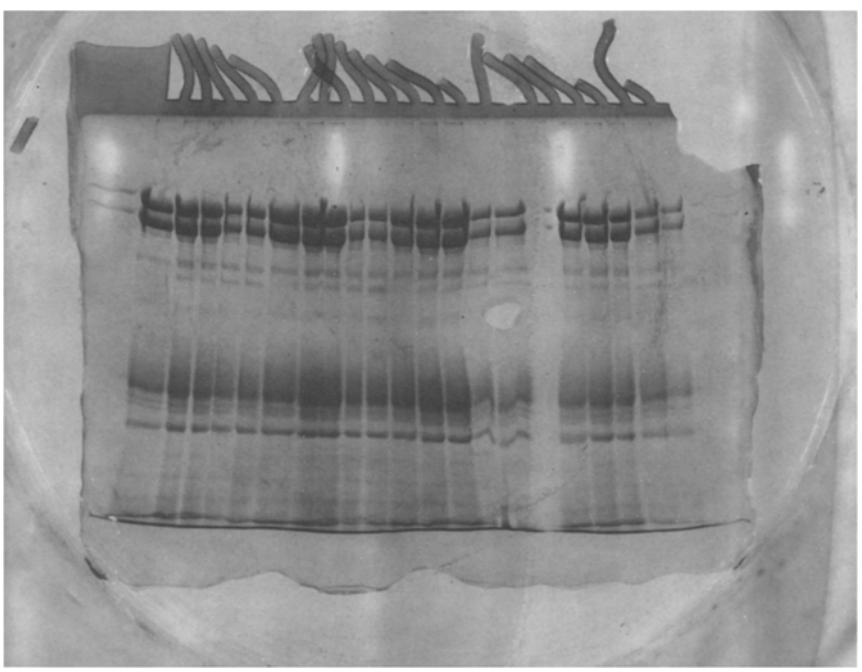
Early EPR studies of normal human erythrocyte membranes labelled with N-(1 oxyl-2,2,6,6- tetramethyl 4-piperidinyl) maleimide (see Figure 1. b) which is a derivative of NEM indicated that 75 % of the total labelled sites belong to the spectrin-actin complex [10]. Membranes were incubated with N-Maleimide at 4^oC. It was also indicated that the differences between the results of [4] and [10] may be due to the difference of the molecules and different incubation temperatures used.

In order to investigate whether different temperature used in early studies may cause these discrepancies, in this research, the human white erythrocyte ghosts were labelled with ^{14}C-NEM and were incubated at two different temperatures, 4^oC and 37^oC.



(a) (b)

Figure 1 a) Schematic representation of Ethylmaleimide, N- Ethyl-l-^{14}C and b) N-Maleimido Tempo.

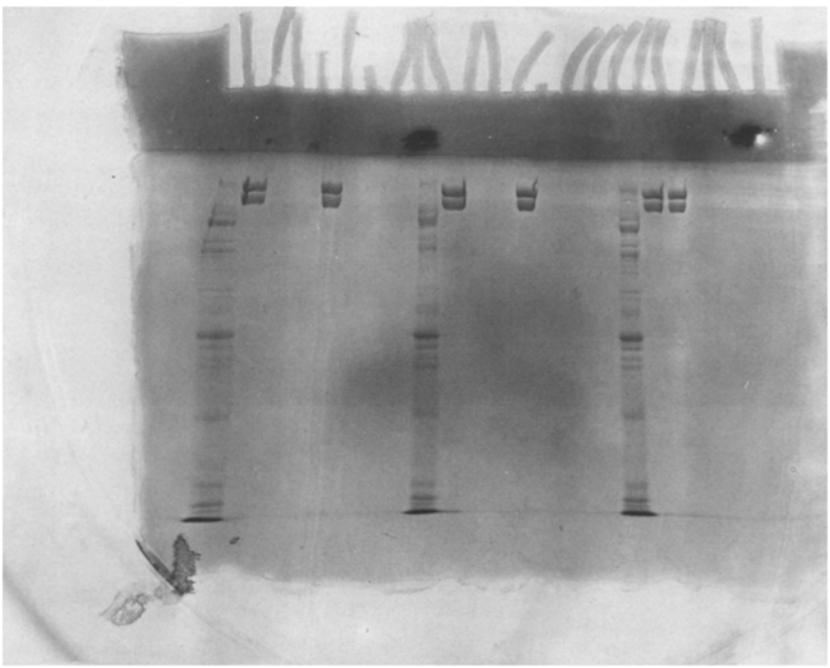


A B C

Figure 2. SDS-Polyacrylamide slab gel electrophoresis of samples A) hemoglobin free erythrocyte ghost. B) and C) hemoglobin free erythrocyte ghost labelled with ^{14}C-NEM and incubated at $4^{o}C$ and $37^{o}C$ respectively.

Figure 2 shows the SDS-polyacrylamide slab gel electrophoresis of radioactive labelled hemoglobin free normal human erythrocyte ghosts incubated at two different temperatures, 4°C and 37°C. More information about bands observed, can be obtained from [1]. No variation in the characteristic pattern of the radioactive labelled ghost membrane have been detected at the two different incubation temperatures.

Figure 3 shows the SDS-polyacrylamide slab gel electrophoresis of labelled spectrin-actin. Spectrin-actin can be extracted from radioactive labelled erythrocyte ghosts as easily as from unlabelled ghosts.



B A A B

Figure 3. SDS-polyacrylamide slab gel electrophoresis of radioactive labelled A) spectrin-actin B) spectrin depleted white erythrocyte ghost.

Figure 4 shows the autoradiogram of the gel of erythrocyte ghosts, labelled with ^{14}C-NEM, incubated at two different temperatures, 4°C and 37°C respectively. Empty columns belong to unlabelled ghosts. This picture represents the radioactivity distribution in the gels.

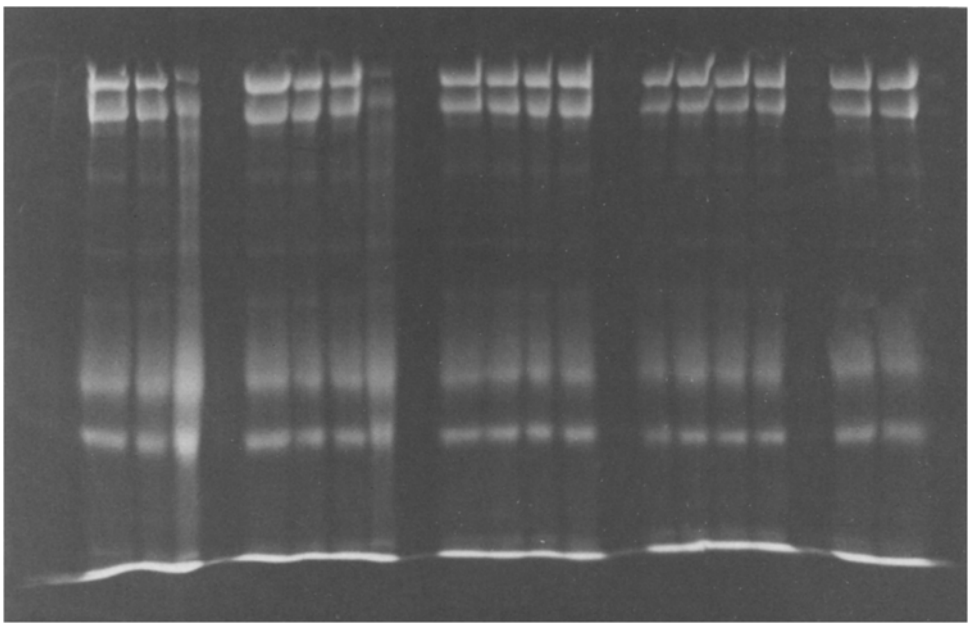


Figure 4. Autoradiogram of erythrocyte ghosts. Empty columns belong to unlabelled ghosts.

Results of nuclear scintillation counting for samples incubated at $4^{O}C$ are shown in Figure 5. It was observed that 35 % of the total NEM was bound to spectrin and 50 % of the NEM was bound to spectrin-actin complex.

Results of radioactivity counting for samples incubated at $37^{O}C$ (see Figure 6) also indicated that 35 % of the NEM was associated with spectrin and 50 % of the NEM was bound to spectrin-actin. The results are in agreement with [4] and we can say that different incubation temperatures may not cause any variation .

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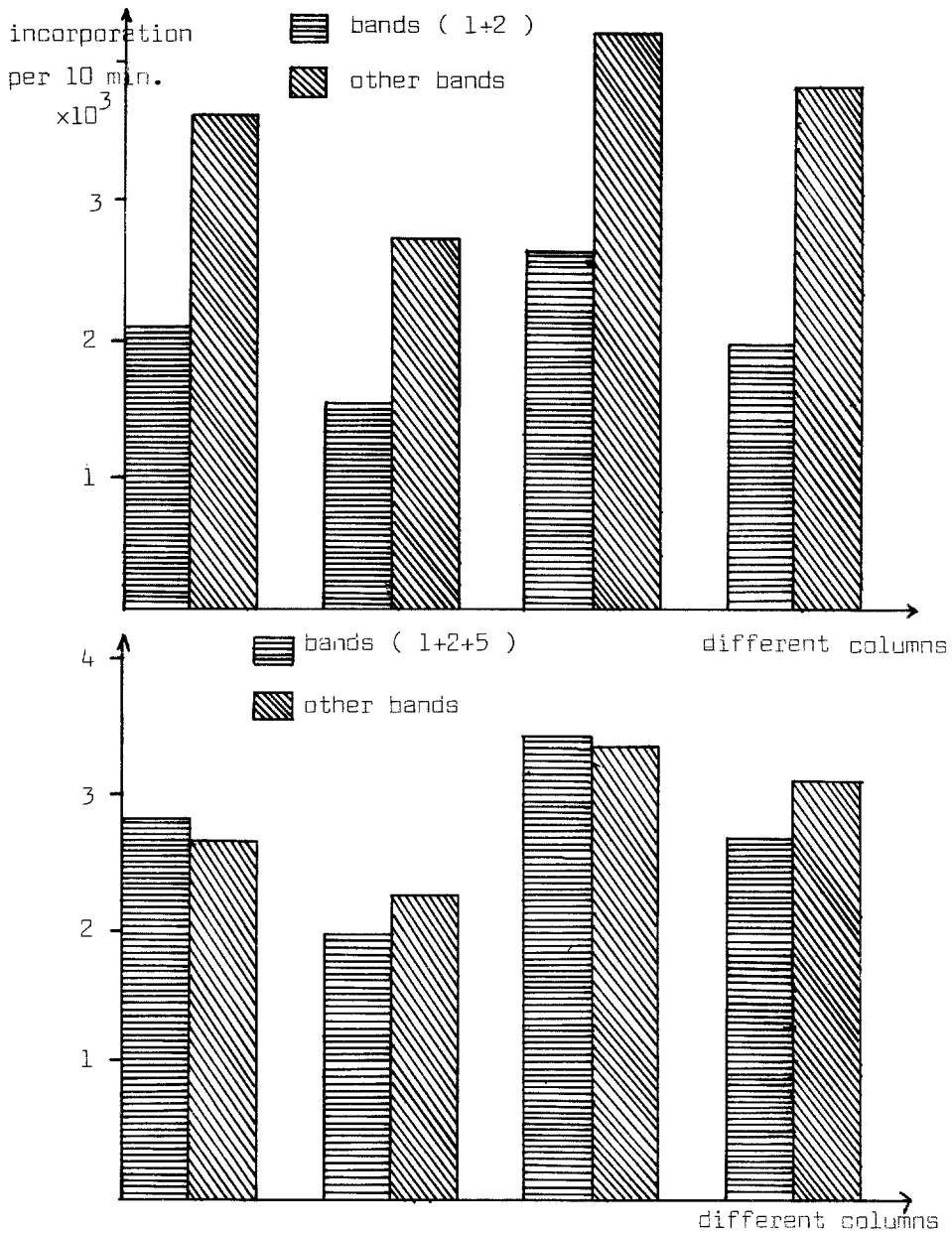

Figure 5. Results of incorporation per 10 min. for samples prepared at $4^{o}C$. 35 % of NEM is bound to spectrin, 50 % of the NEM is bound to spectrin-actin.

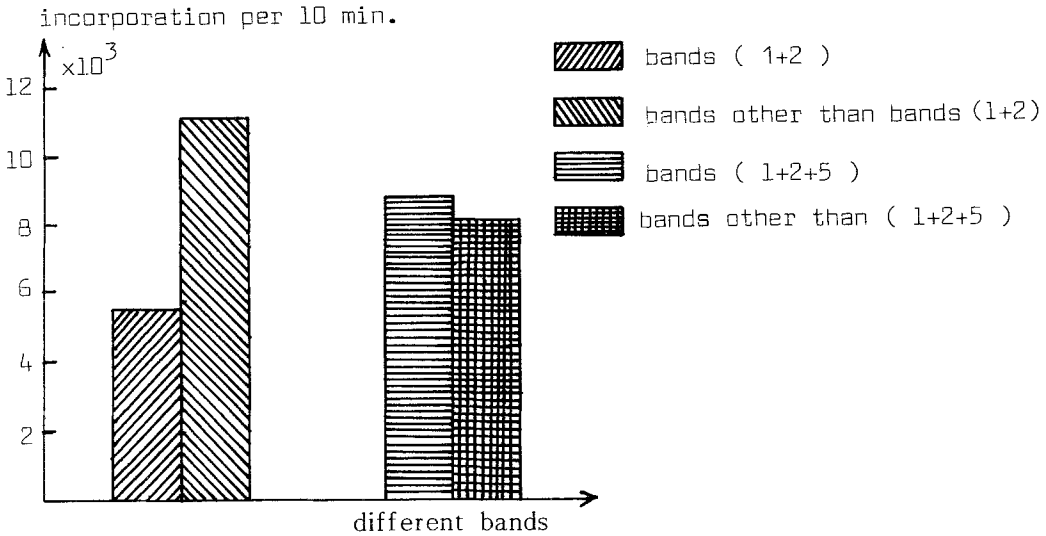

Figure 6. Results of incorporation per 10 min. for samples prepared at 37°C. 35 % of the NEM is associated with spectrin. 50 % of the NEM is bound to spectrin-actin.

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