A STUDY OF THE EFFECT OF X-RAYS UPON THE ULTRASTRUCTURE OF THE ERYTHROCYTE MEMBRANE

by

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INTRODUCTION

Many authors have already studied the effect of X-rays upon erythrocytes. In most cases they tried to discover whether the doses of X-rays generally used in radiotherapy have a harmful effect upon red blood corpuscles and to what extent the erythrocytes *in vivo* irradiated take part in the total irradiative effect in an organism.

Different authors therefore studied the erythrocytes irradiated not only *in vivo* but chiefly *in vitro*.

In the first case, when irradiating a body with the usual healing doses we cannot discover any qualitative changes in erythrocytes in blood slides. The quantitative changes caused by great or repeated doses of X-rays have to be explained not only as the result of the direct effect on nature erythrocytes circulating in blood but first of all as the result of the retarding effect of irradiation upon the erythropoiesis.

The irradiation of blood corpuscles *in vitro* has proved that small doses of X-rays diminish the minimal osmotic resistance against hypotonic solutions; greater doses of irradiation, first of all of soft irradiation, cause a hemolysis (BONIN AND BLEIDORN[1], HAUSMANN[2], HOLTHUSEN[3], LEVIN AND PIFFAULT[4], etc.).

There are different explanations for the mechanism of the hemolytic effect of X-rays. According to HOLTHUSEN[3] the irradiation causes hemolysis in that it attacks the colloids of the blood corpuscle membrane and changes the degree of their dispersion. LIECHTI AND WILBRAND[5] describe the disintegration of erythrocytes caused by X-rays as an entirely osmotic hemolysis dependent on the change of the membrane permeability. Under the effect of irradiation blood corpuscles lose their normal selective impermeability for cations, so that salts from the surrounding medium can penetrate without hindrance and cause osmotic swelling or even hemolysis. The degree of the permeability for cations depends on the dose of X-rays, the rate of hemolysis on the ionic composition of the medium in which the blood corpuscles are situated.

EXPERIMENTAL PART

Starting from the basic fact that X-rays destroy the erythrocyte membrane in that they change its physico-chemical qualities, we posed the question whether the changes caused by irradiation would also show themselves as structural changes that could be observed in the electron microscope.

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METHODS

1. *Preparation of the blood suspension*

Blood drawn off by venal puncture into sterile natrium citrate was supplied with the RINGER solution and centrifuged for 10 minutes at the rate of 2500 r.p.m. The washing fluid was poured off, the sediment was again supplemented with RINGER solution, shaken and centrifuged. This process was repeated five times until we got a pure suspension of washed erythrocytes.

2. *Irradiation with X-rays*

The blood suspension was brought into a paraffin cell (volume 1 ml) and irradiated with X-rays of medium hardness, total dose 24 000 r/80 kV, 4 mA, f: 10 cm, without filter, dose 390 r/min on the surface of the suspension 1 cm^2. The incident dose was measured with a Victoreen dosimeter in the air. After the irradiation no hemolysis was observed in the suspension.

3. *Preparation*

After irradiation the erythrocytes were hemolysed immediately with distilled water, the blood stromata were centrifuged down and again washed thoroughly several times in order to rid them completely of residues of hemoglobin or of the coagulated substances of the blood plasm respectively. One drop of the thin suspension of the blood stromata washed in distilled water was placed on a collodion membrane and left to dry completely at room temperature.

In order to preserve the stromata in a natural form as much as possible we did not use usual fixatives (alcohol, formol, osmic acid, etc.) for preparation because all these substances could considerably change the natural structure of the blood membrane.

The contrast of the object was increased only by shadow technique using chromium (WILLIAMS AND WYCKOFF[6]).

The photographs were made with a 50 kV electron microscope, type EMU 2a RCA-USA.

RESULTS OF THE OBSERVATION

The hemolysed erythrocytes are dispersed in the preparations, either isolated or in groups. Often they are accompanied by a mass of coagulated proteins which we cannot identify with certainty as having originated from the inner contents of the blood corpuscle or in the blood plasm.

A. *Non-irradiated blood corpuscles*

Fig. 1. The hemolysed blood corpuscles appear as double disk with thin walls and circular shape. Their diameter, measured and compared with a normal erythrocyte under physiological conditions, is a little larger; it is comparable with the flattening of a hollow vesicle. The disk is formed of a coherent fine granulated morphological membrane. On its surface masses of coagulated proteins are attached. The homogeneous structure of the membrane is damaged at its right border with a deformation representing an opening in the blood corpuscle vesicle through which the contents of the erythrocyte flowed out into the medium during the hemolysis. Such fissures regularly appear in membranes. They are mostly round or oval and have sharp borders (Fig. 2) formed by the elasticity of the blood corpuscle membrane. Their size differs; it evidently depends on the individual osmotic resistence of each blood corpuscle which, in hypotonic medium, is responsible for the speed of hemolysis and thus for the size and form of the membrane fissure.

Fig. 3. In single cases, numerous openings of quite different forms appeared in the membranes of control blood corpuscles; their unsymmetric and varied form gives evidence that the elasticity of the membrane did not take part in breaking the blood corpuscle vesicle. Since the whole character of the surface also differs from the granulated

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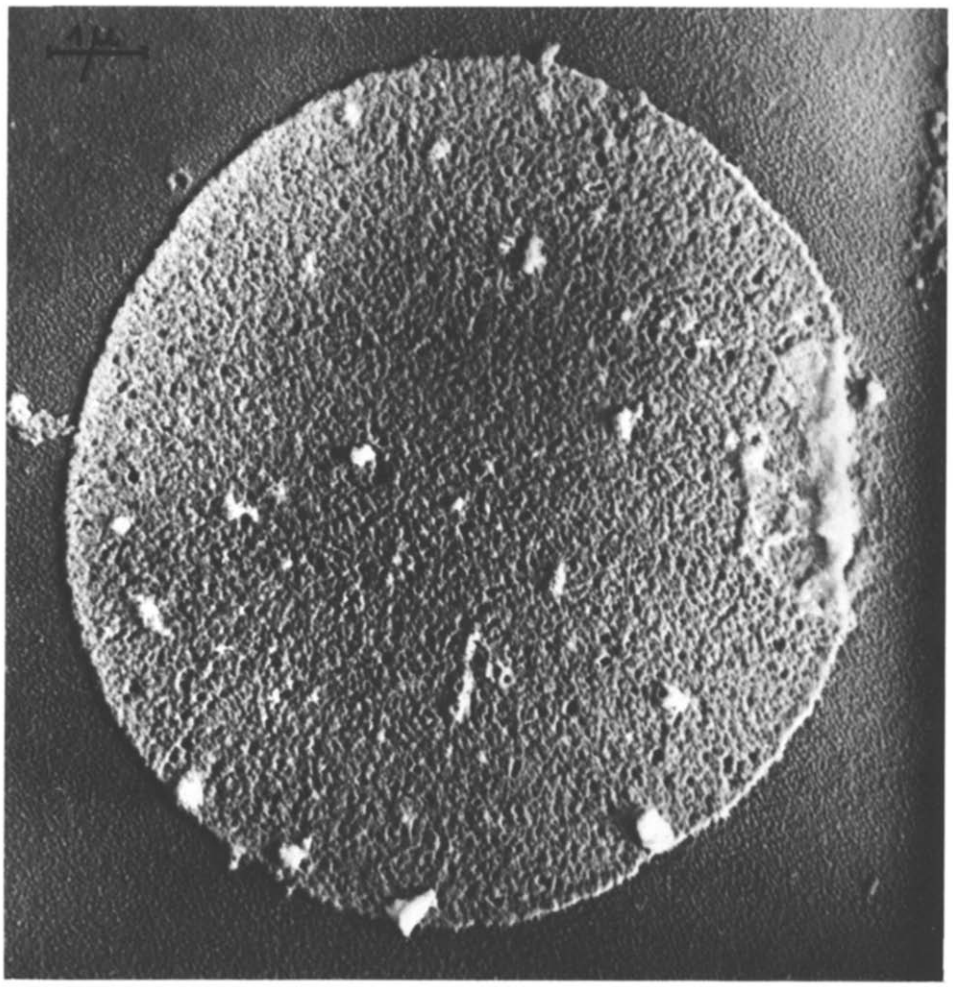

Fig. 1. Discussion see p. 316

structure of a normal erythrocyte we presume that here we have *in vivo* functionally inferior blood corpuscles, which are probably too old.

B. *The irradiated blood corpuscles*

Fig. 4. Even after irradiation the blood corpuscle membrane keeps its round shape and size; the structure of the membrane is more coarsely granulated in comparison with the non-irradiated erythrocytes. The radiation, however, caused further changes visible especially on the border parts of the membrane which is strewn with round openings. This regular perforation of the membrane surface resembling a perforated target is very striking especially in comparison with the non-irradiated erythrocyte (Fig. 1). There is no larger fissure in this membrane through which the contents of the blood corpuscle usually flow out during hemolysis. Therefore we must suppose that the penetration of

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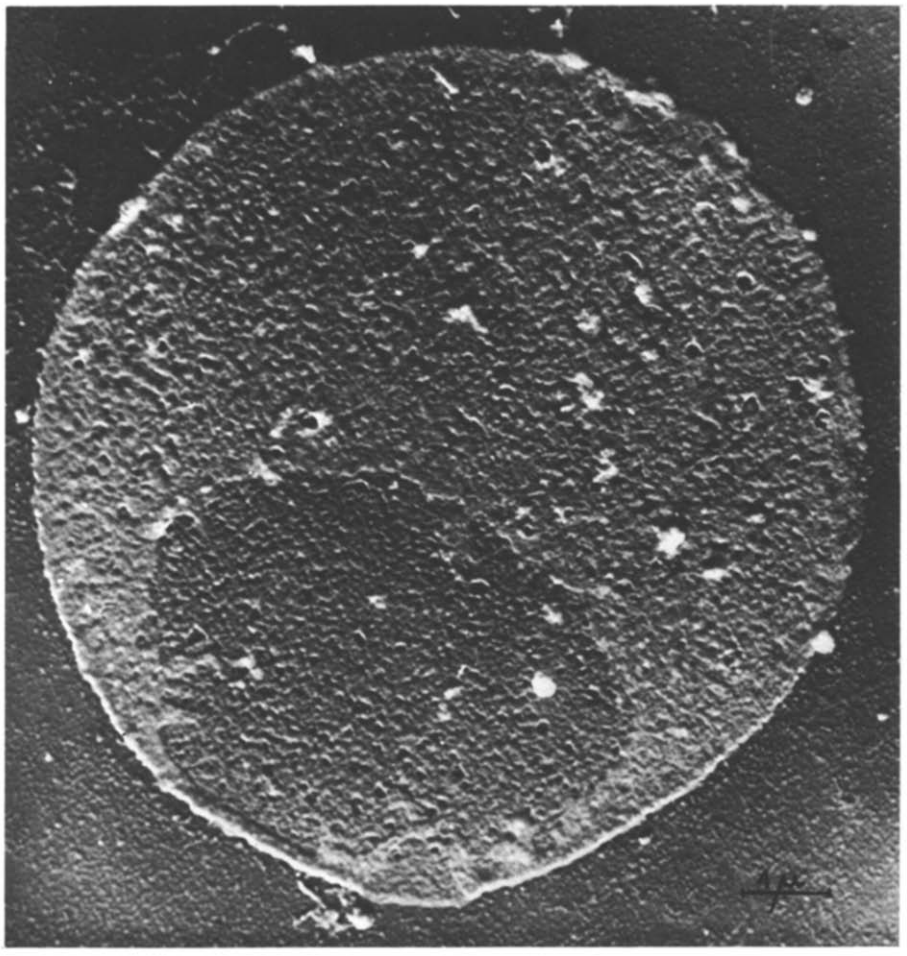

Fig. 2. Discussion see p. 316

hemoglobin into the watery surroundings occurred slowly through these openings. But a slow hemolysis like this is possible only if we have a blood corpuscle of high osmotic resistance, the membrane of which is not torn by the usual large fissure in hypotonic medium. Therefore we believe that the considerable osmotic resistance of the blood corpuscle membrane had its origin in colloido-chemical changes caused by radiation.

Far greater changes which we could not find in the control preparations appeared in further cases:-

Fig. 5. The oval shape of the erythrocyte is caused by the formation of a fold which proceeds from the upper to the lower border of the blood corpuscle and causes a narrowing of the erythrocyte disk in horizontal direction. The basic structure of the membrane remains unchanged. Apart from some greater, craterlike openings in the lower half of the disk further extensive destructions occurred in the blood corpuscle membrane.

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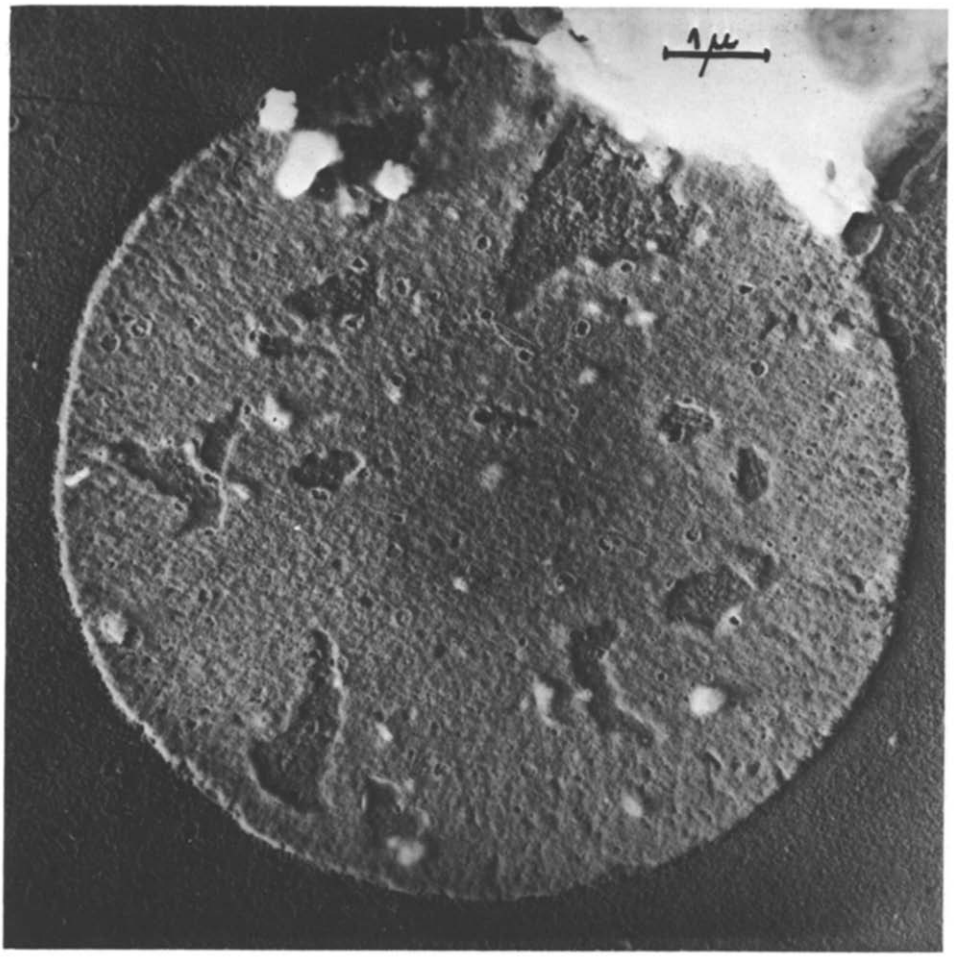

Fig. 3. Discussion see p. 316

The integrity of the membrane is destroyed in the upper parts of the membrane by the falling out of whole areas of the blood corpuscle wall. In the defective parts only a fine fibrillar structure remained, probably of protein character.

Fig. 6, 7. The next micrographs show us that the fibrillar structure is evidently not an occasional phenomenon. The distribution and the course of the fibrils show a certain regularity, but not of such a degree that it would correspond to a symmetrical circular or radial texture. The density of fibrils varies and so does the thickness, fluctuating between 20–50 mμ. The fibrils of the network interweave irregularly and as they do not lie on the same level it seems probable that the fibrillar structure has several layers.

Small spherical structures adhere to some of these fibrils. We believe that they are aggregates of lipoid molecules which not only gather on the fibrils but also fill up the space between individual fibrils. The coherent residues of the preserved membrane wall

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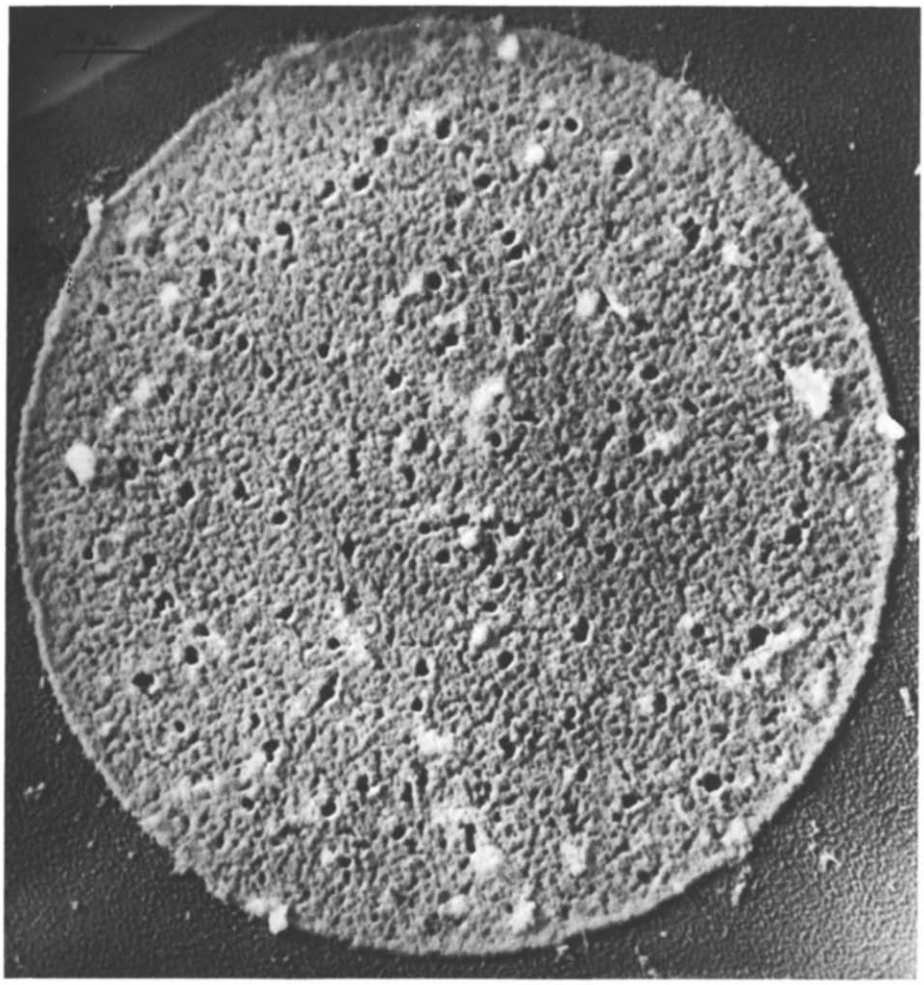

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indicate that the wall is formed by these aggregates of globular formations which are so densely squeezed together that they cover the basic fibrillar structure.

DISCUSSION

The submicroscopic structure of the blood corpuscle membrane was the object of numerous investigations not only for its relation to the structure of the erythrocyte itself but chiefly from the biophysical point of view. The blood corpuscle membrane is an ideal natural model of the surface membranes in general which are of such great importance for the living cell.

According to chemical analyses the basis of the membrane is formed of lipoproteid,

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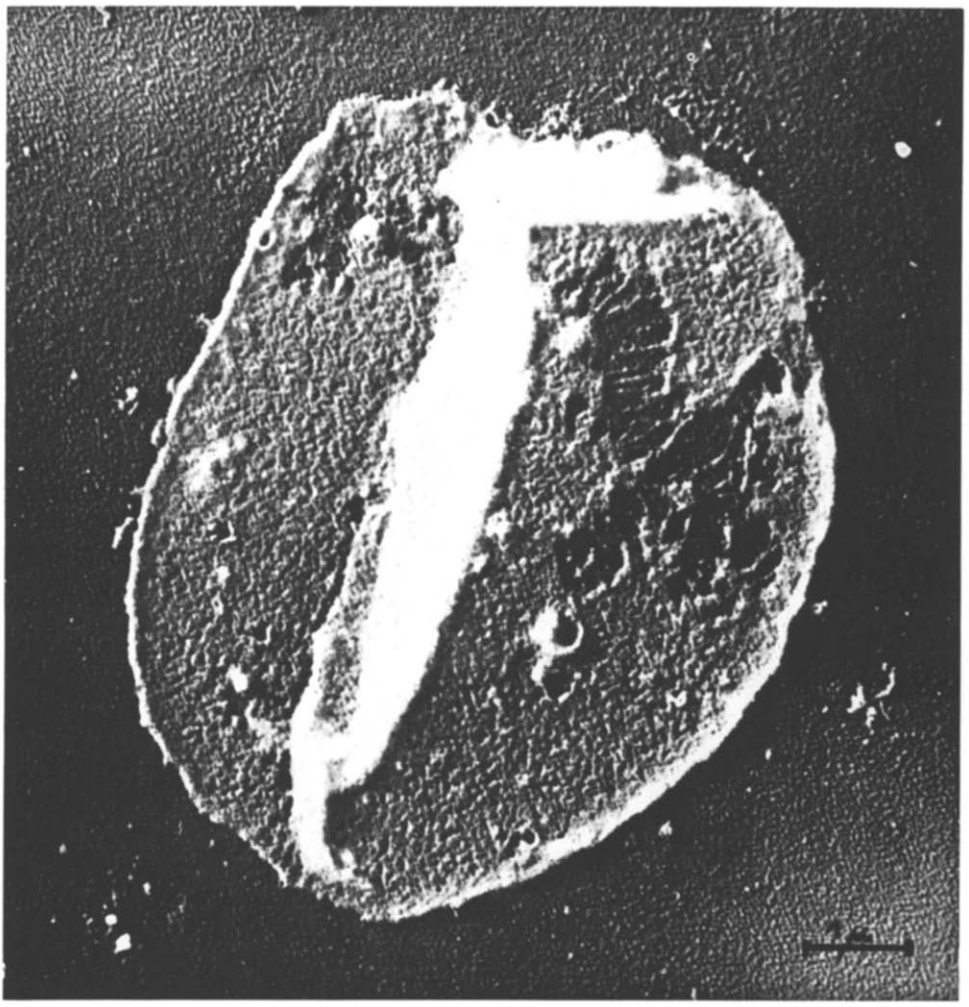

Fig. 5. Discussion see p. 318

i.e., the lipoid and protein components in mutual chemical linkage. In the lipoid part the hydrophylic phosphatides, chiefly lecithin predominate; the rest is formed by cholesterol, some fats and fatty acids. The precise chemical structure of the protein called stromatin has not yet been made clear.

The different opinions of authors regarding the structure of the blood corpuscle membrane and the mutual arrangement of both components in space are due to the use of different indirect physico-chemical methods of investigation (GORTER AND GRENDEL[7], MOND AND HOFFMANN[8], SCHMITT, BEAR, AND PONDER[9], BUNGENBERG DE JONG AND WINKLER[10], etc.).

Similar investigations were carried out by WOLPERS[11], who studied the erythrocyte membranes in an electron microscope and found that the basis of the membrane is formed by a micellar network structure of a long protein in which the lipoids are

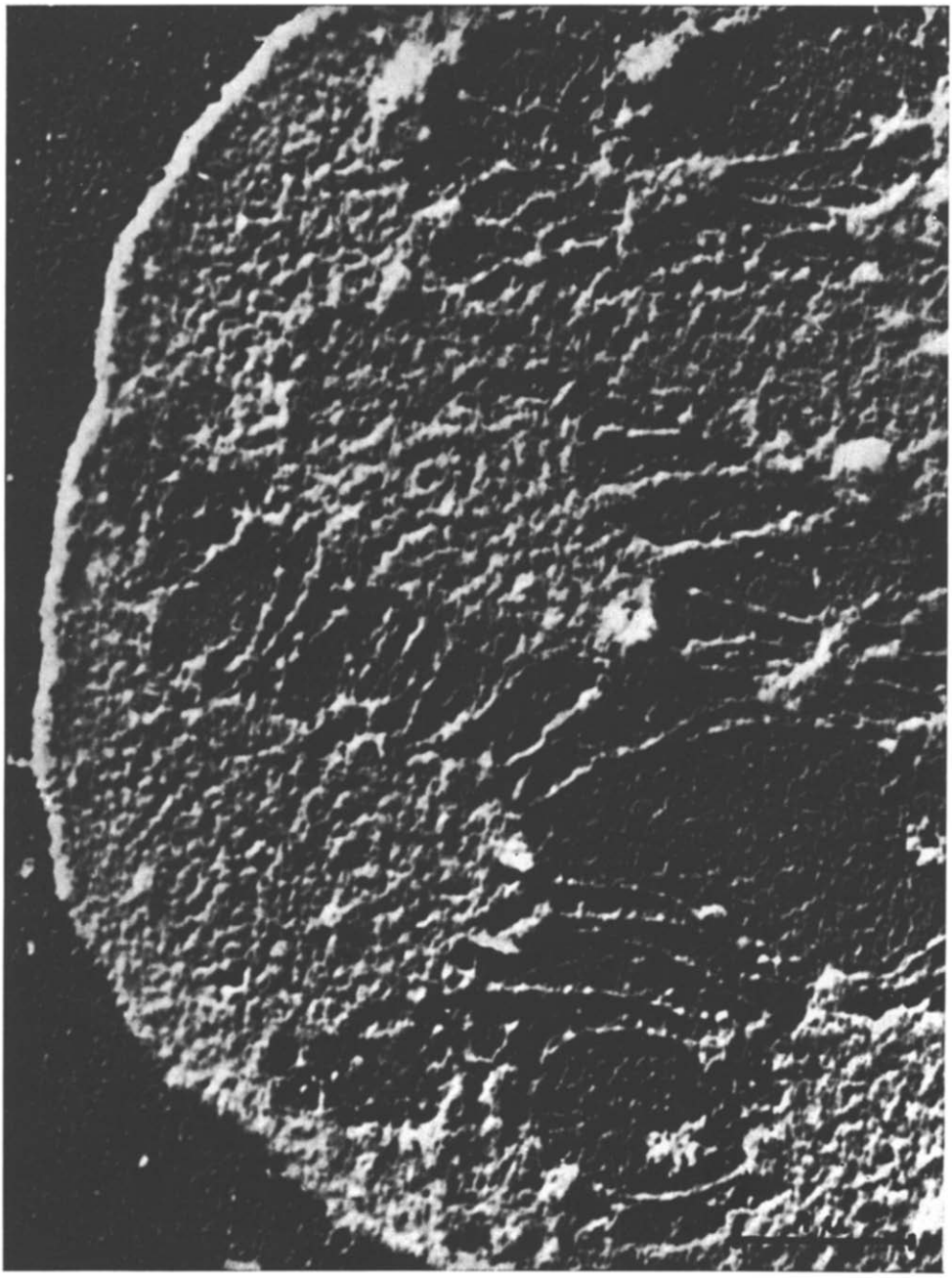

Fig. 6. Discussion see p. 319

Fig. 7. Discussion see p. 319

interlaid as intersubstances. For the preparation of membranes he used, however, either osmic acid as a fixative, or he extracted lipoids with a fatty solvent. So he used agents which themselves can severely destroy the original membrane structure. Therefore FREY-WYSSLING[12] believes that the above mentioned discoveries are not natural structures but artefacts.

It is remarkable that our discoveries agree strikingly with WOLPERS' results although the experimental method was quite different. The control tests show that our method of preparing the blood corpuscle membrane is really very careful as we did not use any fixative which could influence the membrane structure. Therefore the changes in the blood corpuscle membrane originated under the influence of X-rays and they proceed, in our opinion, as follows (ŽÁČEK AND ŽÁČKOVÁ[13]):

Lipoproteid with a protein molecule is the main component in the structure of the erythrocyte membrane as the basic structural unit. The protein has an electric charge protected by lipids. The absorbed ionizing radiation decomposes part of the protective lipids (lecithin?) and makes it possible for water and crystalloids to penetrate to the protein. Then ions of crystalloids change the electric charge of the protein. The consequence of this is a further liberation of ionogenically linked lipids and a gradual disintegration of the whole molecule.

The lipoproteid ceases to exist as a whole; the protein denaturates, and loose lipids fall out partially from the membrane during the hemolysis and washing, so that the coherent basic protein ultrastructure is laid bare.

SUMMARY

The authors irradiated a suspension of washed erythrocytes with X-rays of total dose 24 000 r and after hemolysis with distilled water they observed changes in the blood corpuscle membrane with an electron microscope. The contrast of the object was increased with the chromium shadow technique; no usual fixatives were used for preparation.

It was found that:

1. The radiation causes considerable destructive changes in the blood corpuscle membrane and these changes are revealed as the falling out of whole areas from the membrane wall. In the destroyed parts only a fine fibrillar network remains, probably of protein character. The fibrils of this network do not lie on the same level and they interweave irregularly. Their thickness fluctuates between 30–50 mμ which corresponds to the dimension of a protein macromolecule.

2. The authors believe that the effect of X-rays on the blood corpuscle membrane lies in the destruction of lipoproteid, the basic structural unit of the membrane. The mutual chemical cohesion of both components is destroyed, the protein component denatures, and lipoid is eliminated and falls out of the blood corpuscle wall during the hemolysis and washing of the suspension. Thus the basic micellar reticular submicroscopic structure of the blood corpuscle membrane is laid bare.

3. The question whether the described ultrastructure exists in the same form *in vivo* or whether it is an artefact caused by the denaturation of protein under the influence of the ionizing radiation still remains unsolved.

RÉSUMÉ

Nous avons irradié par des rayons-X, dose totale 24 000 r, une suspension d'érythrocytes lavés et, après hémolyse par l'eau distillée, nous avons observé au microscope électronique les changements survenus dans les membranes des globules sanguins. Les contrastes de l'objet ont pu être accentués par la technique des ombres au chrome; aucun des fixateurs habituels n'a été employé.

Nous avons constaté:

1. L'irradition produit dans la membrane des globules sanguins des destructions considérables; l'on observe, en effet, que des plaques se détachent de la membrane; il ne subsiste alors dans les

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parties attaquées qu'un enchevêtrement de fibrilles fines, probablement de charactère protéinique. Ces fibrilles ne se trouvent pas au même niveau et sont enchevêtrées de façon irrégulière. Leur épaisseur varie de 30 à 50 mμ ce qui correspond à la dimension d'une macromolécule de protéine.

2. Nous sommes d'avis que l'effet des rayons-X sur la membrane des globules sanguins consiste en la destruction d'une lipoprotéine constituant l'unité structurale de base de la membrane. La cohésion entre les deux composantes est détruite, la composante protéinique dénaturée et le lipoïde éliminé; il se détache alors de la membrane pendant l'hémolyse et le lavage de la suspension. C'est ainsi que la structure micellaire de base de la membrane des globules sanguins se révèle sous forme d'un réseau à dimensions submicroscopiques.

3. L'ultrastructure ainsi décrite existe-t-elle sous la *même* forme *in vivo* ou bien est-elle au contraire un produit artificiel de la dénaturation des protéines sous l'influence ionisante de la radiation? Cette question attend encore sa solution.

ZUSAMMENFASSUNG

Eine Suspension von gewaschenen Erythrozyten wurde mit X-Strahlen, Gesamtdosis 24 000 r, bestrahlt und nach Hämolyse mit distilliertem Wasser wurden die in der Membrane der Blutkörperchen entstandenen Veränderungen mit dem Elektronenmikroskop untersucht. Die Kontraste des Objektes wurden durch Anwendung der Chrom-Schatten-Technik verstärkt, aber keines der üblichen Fixiermittel angewendet.

Es wurde festgestellt:

1. Die Bestrahlung bewirkt bedeutende Zerstörungen in der Membrane der Blutkörperchen, welche sich durch das Herausfallen ganzer Stücke aus der Membrane zeigen. An den angegriffenen Stellen bleibt nur ein feines Netzwerk zurück, das wahrscheinlich Eiweisscharakter hat. Die Fasern dieses Netzwerks liegen nicht auf dem gleichen Niveau und sind unregelmässig verwoben. Ihre Dicke variiert zwischen 30 und 50 mμ, was den Dimensionen eines Eiweiss-Makromoleküls entspricht.

2. Es wird angenommen, dass die Wirkung der X-Strahlen auf die Membrane der Blutkörperchen in der Zerstörung eines Lipoproteins beruht, welches die Grundstruktureinheit der Membrane darstellt. Die gegenseitige chemische Kohäsion der beiden Komponenten wird zerstört, der Proteinanteil denaturiert; das Lipoid wird entfernt und fällt bei der Hämolyse und dem Auswaschen der Suspension aus der Membrane heraus. So wird die micellare, netzförmige submikroskopische Grundstruktur der Membrane blossgelegt.

3. Die Frage, ob die beschriebene Ultrastruktur in *derselben* Form auch *in vivo* existiert, oder ob sie ein Artefakt aus dem, durch die ionisierende Strahlung denaturierten Eiweiss ist, muss noch unbeantwortet bleiben.

REFERENCES

[1] BONIN AND BLEIDORN, in H. HOLTHUSEN, *Strahlentherapie*, 14 (1923) 561.
[2] HAUSMANN, in H. HOLTHUSEN, *Strahlentherapie*, 14 (1923) 561.
[3] B. HOLTHUSEN, *Strahlentherapie*, 14 (1923) 561.
[4] H. S. LEVIN AND C. PIFFAULT, *Compt. rend. soc. biol.*, 116 (1934) 1324.
[5] A. LIECHTI AND W. WILBRANDT, *Strahlentherapie*, 70 (1941) 541.
[6] R. WILLIAMS AND R. W. G. WYCKOFF, *J. Applied Phys.*, 17 (1946) 23.
[7] E. GORTER AND F. GRENDEL, *J. Exptl Med.*, 41 (1925) 439.
F. GRENDEL, *Biochem. Z.*, 214 (1929) 231.
[8] R. MOND AND F. KOFFMANN, *Pflügers Arch. ges. Physiol.*, 219 (1928) 467.
R. MOND, *Pflügers Arch. ges. Physiol.*, 217 (1927) 618.
[9] P. O. SCHMITT, R. S. BEAR AND E. PONDER, *J. Cellular Comp. Physiol.*, 9 (1936) 89.
[10] K. WINKLER AND H. G. BUNGENBERG DE JONG, *Arch. néerl. physiol.*, 25 (1941) 431.
[11] C. WOLPERS, *Naturwissenschaften*, 29 (1941) 416.
C. WOLPERS, *Klin. Wochenschr.*, 21 (1942) 1049.
[12] A. FREY-WYSSLING, *Submicroscopic morphology of protoplasm and its derivatives*, Elsevier Publ. Comp. New York-Amsterdam, 1948.
[13] J. ŽÁČEK AND V. ŽÁČKOVÁ, *Scripta medica*, 23 (1949) 183, Brno, ČSR.

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