

IN VITRO INCORPORATION OF GLYCINE-1-<sup>14</sup>C IN RETICULOCYTES

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## INTRODUCTION

It is known that the reticulofilamentous substance characteristic of reticulocytes is essentially constituted by ribonucleoproteins (CASPERSSON<sup>1</sup>, THORELL<sup>2</sup>, DUSTIN<sup>3</sup>, DAVIDSON *et al.*<sup>4</sup>, HOLLOWAY AND RIPLEY<sup>5</sup>). It is probably a remainder of the basophilic constituents of the cytoplasm of the erythroblasts, which disappears gradually as the synthesis of hemoglobin proceeds (THORELL<sup>2</sup>).

It has, on the other hand, been established that the synthesis of hemoglobin goes on in the reticulocytes (BORSOOK *et al.*<sup>6</sup>, HOLLOWAY AND RIPLEY<sup>5</sup>, CHANTRENNE AND KORITZ<sup>7</sup>), although these have left the bone marrow and are in the circulating blood, and that they have begun accomplishing their specific function of carrying oxygen from the lungs to the tissues.

That protein synthesis is carried on in these non-nucleated cells is a further argument in favour of the theory that the cytoplasmic constituents containing ribonucleic acid, especially the microsomes are the direct agents of protein synthesis. This is a confirmation of results obtained with enucleated unicellular organisms by BRACHET<sup>8</sup> and BRACHET AND CHANTRENNE<sup>9</sup>.

In particular, BORSOOK *et al.*<sup>6</sup>, CHANTRENNE AND KORITZ<sup>7</sup> have demonstrated that the incorporation of certain amino acids in the proteins of reticulocytes is rather high, whilst it is almost negligible in the mature erythrocytes. This result was obtained by studying the incorporation of the amino acids in blood samples taken from animals before and during a reticulocyte crisis.

We have attempted, in the present paper, to establish by direct radioautographic observation whether the incorporation of a labelled amino acid proceeds in a similar fashion. Furthermore, as the reticulocytes have been classified into different morphological groups according to their degree of maturation, we have attempted to find out whether these different types of cells incorporate the amino acid differently or not.

## EXPERIMENTAL TECHNIQUE

Reticulocytosis in a rabbit was produced by two injections of phenylhydrazine at 24 hours interval (1 ml of a neutralized 2.5% solution in NaCl 0.9%) and the withdrawal of 30 ml of circulating blood 3 days after the phenylhydrazine treatment; 48 hours later the animal was bled and the reticulocyte count was 32%. 1.25 ml of blood were taken and incubated for 4 hours at 38° with 0.4 ml of 0.1 M glucose dissolved in NaCl 0.9% and 1 ml of a physiological solution containing 173  $\gamma$  of glycine having a radioactivity of 5.4  $\mu$ C.

Afterwards the red cell suspension was washed once with a 1 N glycine solution and 9 times with Ringers solution. Immediately after the last centrifugation, Brilliant Cresyl Blue was added to

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the cells for 15' at 37° to stain vitally the reticulofilamentous substance. Smears on gelatinized microscopical slides were then made. These should not be too dense and the cells must be well separated from each other for accurate observation. The slides were fixed for 2' in methyl alcohol, then dried in air. The cells can now be radioautographed with a technique permitting the observation of individual electrons emitted by them<sup>10</sup>.

Photographic emulsion (G<sub>1</sub> Ilford in gel form) diluted with 2 parts of water was poured on to the slides so as to obtain a layer about 20 $\mu$  thick, when dry. (Previous attempts using 40 $\mu$  thick layers were not satisfactory because the erythrocytes could not be adequately stained afterwards.) When the emulsion was dry, the slides were stored for 8 days in an underground room and were surrounded by lead blocks to avoid, as much as possible, contamination due to cosmic rays. Afterwards the slides were treated for 20' at 14° C with Amidol developer (pH 7.8)<sup>11</sup>:

Amidol (Diaminophenol)	1.125 g
Na sulfite	4.5 g
KBr 10% solution	2 ml
Distilled water	250 ml

They were then fixed in 1:3 saturated Na thiosulfate for 30'. After washing in running water, the red cells were stained with Giemsa solution.

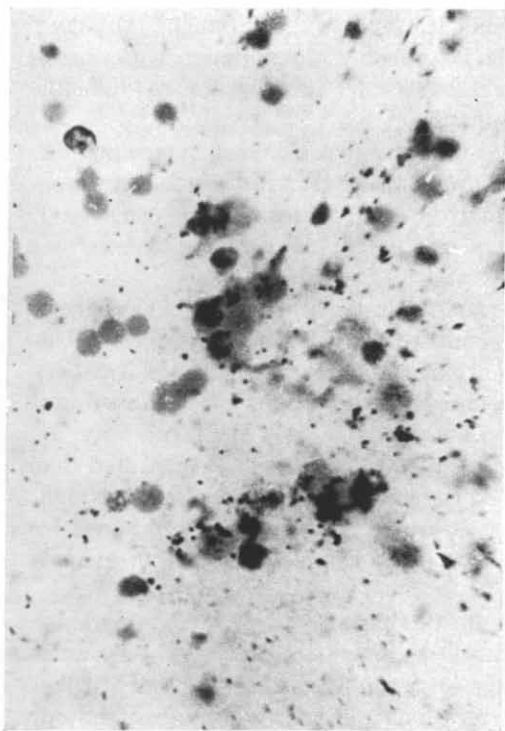


Fig. 1

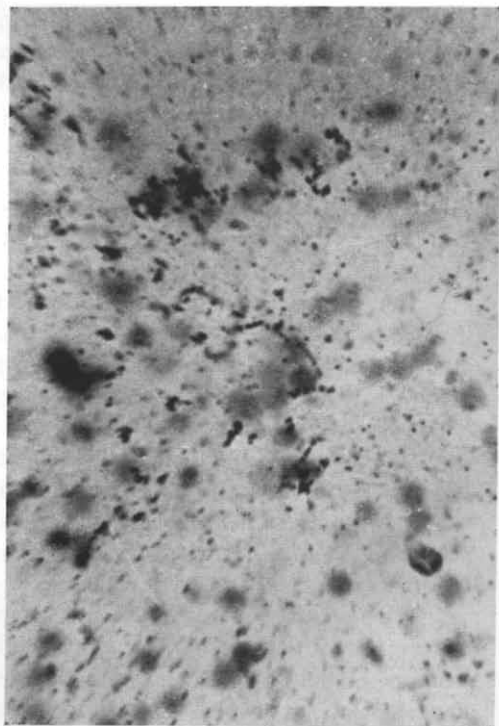


Fig. 2

Groups of erythrocytes and reticulocytes incubated in the presence of glycine-1-<sup>14</sup>C.

Fig. 1. Focussed on the cells.

Fig. 2. Focussed on the electron tracks. These originate mostly from the reticulocytes.

## RESULTS

A. Incorporation of the amino acid was estimated by counting the tracks having their origin in reticulocytes and erythrocytes. The tracks can still be satisfactorily identified in spite of the thinness of the emulsion. The calculation can only give a relative

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evaluation of the activity of the reticulocytes compared to the erythrocytes and not an absolute determination of the activity of each element.

The following values were found:

109 tracks from 1000 erythrocytes.

880 tracks from 1000 reticulocytes.

62 tracks for  $4 \cdot 10^4 \mu^2$  of background corresponding to a surface of 1000 erythrocytes.

In conclusion:

1. The differences between the number of tracks found in the erythrocytes and in a corresponding area of the background is too small to be statistically significant: one can not state with certainty that the mature erythrocytes incorporate glycine.

2. The incorporation of glycine by the reticulocytes is evident.

B. A second count was made during which the reticulocytes were divided into 4 groups according to the classification of HEILMEYER<sup>12</sup>, which has been universally adopted. This classification rests on the quantity and morphology of the reticulofilamentous substance.

Group I corresponds to the younger reticulocytes, and group IV to the more mature ones (HEILMEYER<sup>12</sup>, PAOLINO AND PINNA PINTOR<sup>13</sup>).

The ratio of the number of tracks of each class of cells to the corresponding number of those cells is the following:

Class	I	II	III	IV
No. of tracks				
No. of reticulocytes:	1.33	1.21	0.83	0.62

It is obvious that the incorporation is highest in the younger reticulocytes and decreases as their maturation proceeds.

#### DISCUSSION AND CONCLUSION

During the maturation of the erythroblastic cells in bone marrow, it has been established that the cytoplasmic basophilia disappears progressively during the simultaneous increase of hemoglobin in the cytoplasm. The nucleus becomes picnotic and caryolytic in the polychromatophils and orthochromatic erythroblasts and is eliminated in further stages.

The presence of a nucleus seems to be necessary for maintaining the integrity of the microsomes (BRACHET<sup>8</sup>): in the present case, the degeneration and disappearance of the nucleus are concomitant with the disappearance of the cytoplasmic basophilia. The reticulofilamentous substance of the reticulocytes is probably the last remnant of this basophilic material; this substance disappears rapidly and the life of the reticulocytes lasts but a few days. The reticulofilamentous substance, in this short time, changes its shape and its localization in the cell which makes it possible to distinguish the younger and older forms of the different classes. In these, however, this basophilic residue appears to be still capable of maintaining a synthetic activity, as the forementioned authors have demonstrated.

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In our own experiments, we have attempted to give a radioautographic evidence of this remaining activity. Our results, as has been seen, have confirmed these findings, even though, owing to the present limitations of the technique, it has not been possible to obtain quantitative values of the amounts of amino acid incorporated.

Furthermore, in the younger reticulocytes (class I), the incorporation is considerably higher (approximatively double) than that in the more mature elements; in other words, as maturation of the reticulocytes to the stable form of the erythrocytes proceeds, incorporation of glycine is diminished and the synthetic activity disappears almost completely with the disappearance of cytoplasmic basophilia.

#### SUMMARY

A  $\beta$ -ray track radioautographic study of the *in vitro* incorporation of glycine- $^{14}\text{C}$  in rabbit blood containing a high percentage of reticulocytes has been accomplished. Whereas the reticulocytes incorporate appreciable amounts of the amino acid, the mature erythrocytes have an activity not significantly above that of the background. If the reticulocytes are divided into the 4 Heilmeyer classes according to their degree of maturation, we have observed that the youngest have a much greater activity than the more mature ones.

#### RÉSUMÉ

Une étude de l'incorporation *in vitro* de la glycine- $^{14}\text{C}$  dans du sang de lapin contenant un taux élevé de réticulocytes a été faite à l'aide d'une technique autoradiographique permettant l'observation des traces individuelles des électrons émis.

Alors que les réticulocytes incorporent des quantités appréciables de l'acide aminé, les érythrocytes mûrs ont une activité qui n'excède pas de façon significative celle du "background". Si les réticulocytes sont classés d'après leur degré de maturation suivant les types de Heilmeyer, nous avons observé que les plus jeunes ont une activité beaucoup plus considérable que les réticulocytes ayant atteint un stade plus avancé de leur maturation.

#### ZUSAMMENFASSUNG

Der *in vitro*-Einbau von Glycin- $^{14}\text{C}$  in einen hohen Prozentsatz Reticulocyten enthaltenden Kaninchenblut wurde mit Hilfe der  $\beta$ -Strahlen-radioautographischen Spurenuntersuchung ausgeführt. Während die Reticulocyten beträchtliche Mengen Aminosäuren einbauen ergab sich bei den reifen Erythrocyten eine gegen den "Hintergrund" nicht bedeutend erhöhte Aktivität. Bei der Einteilung der Reticulocyten nach ihrer Reife in die vier Heilmeyrklassen konnte beobachtet werden, dass die jüngsten Reticulocyten eine weitaus grössere Aktivität besitzen als die reiferen.

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