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20 December 1963.

## Alpha-Chain of Human Hemoglobin: Occurrence in vivo

Abstract. *A minor hemoglobin component, apparently representing uncombined α-chains has been detected in the hemolysates of persons with inherited β-chain deficiency of moderate or severe degree. This finding supports the hypothesis that there is independent control of the synthesis of α-chains.*

Each type of peptide chain ($\alpha$, $\beta$, $\gamma$, $\delta$) of the normal human hemoglobin is under the control of a separate structural gene (*1–3*). Inherited deficiencies in the production of $\alpha$-chains, known as $\alpha$-thalassemias (*4*), result in an excess of the complementary chains $\beta$, $\gamma$ or $\delta$; such excess can be recognized by the presence of the tetramer molecules $\beta_4$ or $\gamma_4$, or both, and possibly also $\delta_4$ (*5*). In the inherited deficiencies of $\beta$-chain production, the $\beta$-thalassemias, no surplus of $\alpha$-chains has been recognized, even in homozygous individuals. This failure to observe a fraction corresponding to $\alpha$-chain alone or $\alpha$-chain polymers—even though $\alpha$-chains are capable of separate existence in vitro (*6*)—has been variously interpreted. It has been suggested (i) that the presence of $\beta$- or of its equivalent $\gamma$- or $\delta$-chains is necessary for the release of $\alpha$-chains in order to permit formation of complete hemoglobin molecules (*2, 7*), and (ii) that the $\alpha$-chain gene may have a nonautonomous regulation, the amount of $\alpha$-chains produced being dependent on the amount of $\beta$- or $\gamma$-chains available (*3*). According to a different interpretation, uncombined $\alpha$-chains may be precipitated and form visible water-insoluble inclusion bodies within red cell precursors and young red cells, such cells dying prematurely (*8*); thus, no $\alpha$-chains are found in the hemolysate. The latter view is strengthened by the observation that artificially separated $\alpha$-chains precipitate within 24 hours when stored in dilute solution at 37°C (*7*). The possibility that a small amount of the unprecipitated $\alpha$-chains was still present in suitable cells had yet to be tested.

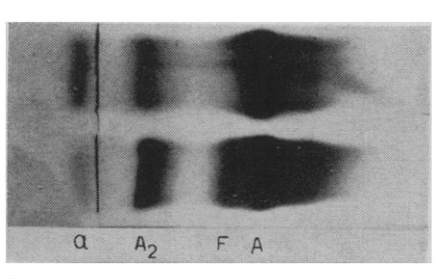


Fig. 1. Comparison of hemoglobin solution containing artificially separated $\alpha$-chains (upper sample), with hemolysate from homozygous $\beta$-thalassemia. Starch-gel electrophoresis, benzidine stain; anode to the right.

Blood specimens were processed immediately after withdrawal and handled thereafter at 4° to 6°C. The cells were washed repeatedly with cold saline solution (0.9 NaCl) buffered to *p*H 7.2 containing 0.005*M* KCN; they were lysed with a very dilute buffer. Stromal material was removed by centrifugation at 15,000*g* for 15 minutes at 4°C. Horizontal starch-gel electrophoresis of approximately 40 to 60 $\mu$l of the hemolysate was performed in a discontinuous barbital-TEB buffer system at *p*H 8.2 for 6 to 7 hours in a water-cooled apparatus. The gels were then stained with a very sensitive benzidine stain (*9*). The whole procedure was completed within 12 hours.

In blood samples from patients with homozygous $\beta$-thalassemia—that is, with severe $\beta$-chain deficiency—a weak, benzidine-positive band, moving more slowly than hemoglobin $A_2$, was detected upon electrophoresis; its position was 2 to 3 mm cathodic to the slit of insertion of the sample. No such band was seen in hematologically normal subjects or in patients with anemias, or those with pronounced reticulocytosis due to acquired conditions, or those with $\alpha$-thalassemias; this band was also absent in cord blood. The band was more prominent in splenectomized patients including six $\beta$-thalassemia homozygotes, one $\beta$-thalassemia–hemoglobin E and one $\beta$-thalassemia–hemoglobin Pylos combination. In exceptional instances the zone was visible, prior to staining, as a faint pinkish area and was estimated to represent 0.5 percent of the hemoglobin content of the hemolysate. In samples from nonsplenectomized cases of homozygous $\beta$-thalassemia the band was weaker; from the intensity of staining it was estimated to be approximately 0.1 to 0.3 percent. Even smaller amounts were found in all six instances of hemoglobin S–$\beta$-thalassemia disease. No such band has been detected so far in a few cases of simple $\beta$-thalassemia trait; we also failed to observe it in samples from one splenectomized and two out of seven nonsplenectomized cases of homozygous $\beta$-thalassemia.

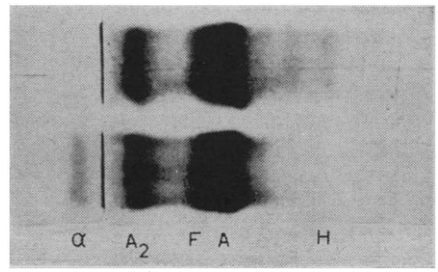


Fig. 2. The addition of pure hemoglobin H (upper sample) has caused disappearance of the cathodic minor component present in the lower sample. Hemolysate from a splenectomized case of homozygous $\beta$-thalassemia.

The electrophoretic position of this minor component corresponds exactly to that of $\alpha$-chains separated in vitro (*6, 7*) as demonstrated by direct comparison (Fig. 1). No catalase activity is present in this area of the electrophoretic strip. Adding, just prior to electrophoresis, either pure $\beta_4$-component (hemoglobin H) or hemolysates containing $\beta_4$-component to samples exhibiting this cathodic fraction always resulted in its complete disappearance (Fig. 2). Conventional treatment of the hemolysate with toluol caused complete disappearance of the band (*10*). Storage of the sample at 4° to 6°C led to progressive weakening of the zone, but in the samples where it had been prominent it could still be detected after 3 to 4 days of storage. When the red cells were fractionated by differential centrifugation the bottom layer contained either a smaller amount of the cathodic band than the top layer or none at all. Chemical identification of the new component has been delayed because of its extremely low concentration.

The electrophoretic position of this minor component and its ready combination with $\beta$-chains of hemoglobin as well as its occurrence in $\beta$-chain deficiencies suggest that it represents uncombined $\alpha$-chains. The failure to observe this band, contrary to expectation, in a few cases of severe $\beta$-chain

deficiency may be explained by accepting the hypothesis of either a complete precipitation of all $\alpha$-chains in vivo, or their combination with free $\gamma$-chains after lysis of the cells. Indeed, a small excess of $\gamma$-chains has been demonstrated in hemolysates of $\beta$-thalassemia homozygotes (*11*). This excess is probably present in only a portion of the total number of red cells. Rapid formation of complete hemoglobin molecules has been observed after the addition in vitro of $\beta_4$- or $\gamma_4$-hemoglobin to hemoglobin-containing $\alpha$-chains (*12*). Therefore the release of any free $\gamma$-chains after lysis is expected to lead to the formation of $\alpha_2\gamma_2$ molecules, hemoglobin F, resulting in reduction or complete disappearance of the $\alpha$-chain zone. In spite of these exceptions, there appears to be a gradation in the number of $\alpha$-chains detectable by electrophoresis, samples from splenectomized homozygous $\beta$-thalassemias exhibiting usually the largest amounts, from nonsplenectomized cases smaller quantities, and from hemoglobin S–$\beta$-thalassemias only traces. A similar gradation occurs in the number of cells carrying inclusion bodies (*8*). In simple $\beta$-thalassemia trait the excess of $\alpha$-chains may be below the limits of sensitivity of our methods, but other interpretations could also apply.

Our findings lend support to the hypothesis that the $\alpha$-chain may be synthesized or released (or both) independently of the presence of $\beta$-, $\gamma$- or $\delta$-chains. However, the degree of this independence cannot be estimated unless the total excess of uncombined $\alpha$-chains, precipitated and nonprecipitated, is accurately measured. The observations also suggest that under certain conditions the hemoglobin pattern of hemolysates may be altered by the combination of hemoglobin fractions occurring in different lines of cells; they further emphasize the importance of alterations that arise during the process of obtaining hemoglobin solutions or during storage.

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9. Benzidine base (100 mg) in 80 ml of distilled water, 10 ml of 1.5*M* acetic acid, 10 ml of 1.5*M* sodium acetate, and 0.5 ml of 30 percent hydrogen peroxide were added just prior to use.
10. We have observed an analogous deleterious effect when chloroform, instead of toluol, is used for the clarification of hemolysates; it causes complete precipitation of hemoglobin $\beta_4$ (hemoglobin H) as well as of the $\alpha$-chain component.
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11 November 1963

## Somatic Mitoses in Cells of Picea glauca Cultivated in vitro

Abstract. *The cytology of one strain of tumor tissue from the spruce tree,* Picea glauca, *grown in vitro for more than a year was examined. The cells of this strain are characterized by a uniform chromosome number of 24, and the strain appears to be quite stable. The implications of the results of this study and previous studies on similar material are discussed.*

In 1960 de Torok and White (*1*) reported that cells of a tumor of *Picea glauca* showed an extreme cytological instability; the chromosome numbers varied between 4 and more than 70 without showing clear modes. Cells of corresponding normal tissues, on the other hand, were quite stable, with most mitoses showing 22 chromosomes, a few tetraploids, and no more aneuploids than one might expect from unavoidable counting errors.

This result appeared to be consistent with the evidence of nutritional and morphological instability reported earlier by Reinert and White (*2*) for a corresponding tumor strain. The chromosome number of 22 differed from the number 24 reported for *Picea glauca, P. pungens,* and *P. abies* by Sax and Sax (*3*). And the strain of tumor tissue currently grown in this laboratory (1961–1963) has been quite stable both morphologically and nutritionally (*4*). In view of these discrepancies it has seemed desirable to reexamine the question.

The materials and methods used by de Torok and White differed somewhat from those used here. Their chromosome counts were made on primary explants, that is, on cells emerging as callus from the cambium of bits of wood transferred directly from the tree to a nutrient substratum while still remaining partially dependent on nutrients drawn from the explants. None of the explants had been out of the tree for more than 2 months; they might reasonably be expected to represent the conditions in the tree itself. They were, however, treated with dichlorobenzene before squashing and were subjected to mild hydrolysis with HCl before staining. What cytological effects this treatment may have had, other than the presumed arrest of mitoses at the metaphase, is not known. The material described in this report was drawn from stock cultures of tumor cells of an isolation which had been cultivated for 1½ to 2 years (40 to 50 passages) and was thus far removed from the parent tree and stabilized on a completely defined nutrient (*4*). The cells were treated briefly with colchicine, without hydrolysis, and were compared with untreated controls. The strain, unlike other strains studied previously, appeared to be morphologically and nutritionally stable.

*Picea* chromosomes are long, and are difficult to examine unless they are shortened and spread by the initial treatment. After testing several fixatives that would shorten the chromosomes before the cells were killed, under a variety of conditions, colchicine was chosen as most satisfactory. The procedure described here consistently yielded good results. The tumor tissue was placed in 1 percent colchicine and kept in the dark for 6 hours; it was transferred to acetic alcohol, 3:1, at room temperature for 24 hours, and then hydrolyzed in 1*N* HCl for 20 minutes at 60°C. It was stored in 50 percent alcohol and stained by the squash method in acetocarmine.

The tumor tissue used had been in cultivation for a minimum of 40 passages or approximately 1½ years. The individual cultures for counting were chosen at random from stocks which were routinely subcultured at 2-week intervals. Preliminary work indicated that the greatest percentage of cells were dividing during the hours of 4:00 A.M. to 8:00 A.M. during the first 6 days of the 14-day passage. For optimum counting efficiency most of the cultures examined were taken from the medium