# Glycoprotein Biosynthesis in Human Reticulocytes: A Lesion in Thalassemia

Abstract. *The rate of incorporation of glucosamine into the glycoprotein of reticulocyte stroma is greatly increased in thalassemia. This increase suggests a major enzymic disorder in the buildup of cellular membrane and reconciles the accumulation of material showing a positive reaction with periodic acid Schiff reagent with the clinical picture of oligocythemia and microcytosis.*

Like many anemias, thalassemia syndromes are characterized by erythropoietic cells with a low hemoglobin concentration. In particular the concentration of hemoglobin A is low whereas those of hemoglobins F and $A_2$ are generally elevated. Theories (*1, 2*) that the low concentration of hemoglobin A was due to the production of a structurally abnormal $\beta$-chain can be discounted on the basis of Guidotti's analysis (*3*). Recent experimental evidence (*4, 5*) directly supports the proposal (*2*) that the rate of $\beta$-chain synthesis is greatly retarded. In some cases of thalassemia, however, the lesser synthesis of hemoglobin A appears to be somewhat counterbalanced by an elevated synthesis of hemoglobin F (*5*). In any event, the decreased synthesis of hemoglobin A does not in itself account for such clinical expressions of thalassemia as oligocythemia and microcytosis. Indeed, both in fetal blood and in that of patients with familial fetal hemoglobinemia, where the total hemoglobin is substantially represented by hemoglobin F, the erythropoietic cells behave normally (*6*).

Thalassemia syndromes are highly complex and the clinical picture probably results from the combined effects of several genetic defects. Our work concerns abnormalities in the synthesis of the glycoprotein of the cellular membrane in human reticulocytes. The glycoprotein of the erythrocyte membrane is important because it includes the M and N antigens (*7*) and virus receptor material, and it strongly influences the electrophoretic mobility by virtue of its sialic acid (*8*). The glycoprotein contains mannose and *N*-acetylgalactosamine in addition to the sialic acid. In studies with rabbit reticulocytes (*9*), active synthesis of glycoprotein was indicated by incorporation of $C^{14}$-glucosamine. Nearly all the glycoprotein of the erythrocyte appears to be located at the outer periphery of the plasma membrane (*8*).

Blood from donors with severe thalassemia as evaluated clinically and chemically (85 to 95 percent hemoglobin F and high content of hemoglobin $A_2$) was used. The blood cells were washed three times in 20 volumes of cold 0.145*M* NaCl, and the buffy coat was removed. The cell solution contained 2 to 8 percent reticulocytes (*10*). The washed cells (2 volumes) were incubated (*10*) with modified Krebs-Ringer solution (1 volume) containing $C^{14}$-glucosamine (120 $\mu$c/$\mu$mole, 1 $\mu$c/ml). Portions were removed at intervals, and the cells were washed in cold saline and lysed in $H_2O$. The *p*H was adjusted to 5.8, and the stromata were collected by centrifugation, successively washed four times in 0.145*M* NaCl, three times in trichloroacetic acid, once in a mixture of ethanol and ether (1 : 1), and twice in 0.5 percent HCl in acetone, and finally dissolved in 1*N* NaOH; the radioactivity was determined (*10*). The percentage of hemoglobin F was determined by alkali denaturation (*11*).

The results, expressed in counts per minute in stroma per milliliter of reticulocytes, are shown in Fig. 1. The difference between the two sets of curves is striking and shows that, in thalassemia, glycoprotein biosynthesis in stroma is greatly elevated. After 90 minutes the incorporation of glucosamine in the thalassemia cell (curve *A*) is more than ten times greater than in that of the control (curves *D* and *E*). This result probably cannot be explained on the basis of nonspecific factors such as the age of the cells or environmental conditions *in situ*, since similar conditions exist in the peripheral blood of patients with different anemias. In thalassemia, maturation of erythroid cells is retarded (*12*), and circulating reticulocytes therefore would not be expected to show increased biosynthesis. Further evidence for normal or slower rate of maturation of erythroblasts in thalassemia comes from the work of Stohlman (*13*) and others: the finding of normal or elevated levels of metabolic enzymes (*14*) and of normal concentrations of ribosomes and polysomes (*15*). If thalassemic reticulocytes were less mature than the normal, the concentrations of the ribosomes and polysomes should have been different. Although there is no evidence that thalassemic reticulocytes arise from early erythroid forms, this interpretation has been offered to explain the elevation of hemoglobin F in thalassemia (*15*). The remote possibility exists therefore that the results of Fig. 1 may be explained on the basis of reticulocyte age. But the results of Fig. 1 appear to correlate with the histochemical observation (*16*) of material showing a positive reaction with the periodic acid Schiff reagent (PAS-positive) in the erythroblasts and erythrocytes of these patients. Conceivably, PAS-positive material represents an intermediate state or abnormal by-product of the membrane glycoprotein. Glycoproteins containing sialic acid give an intense reaction in the PAS test (*17*); this suggests disorder or deficiency in thalassemia along the enzymic pathway of buildup of the cellular membrane. Apparently the high incorporation of glucosamine into the stroma fraction reflects the synthesis of the PAS-positive material rather than the synthesis of normal glycoprotein in the cell membrane; this material may be attached to the membrane, since the activity was located in the washed stroma. Further, the attachment of the PAS-material to

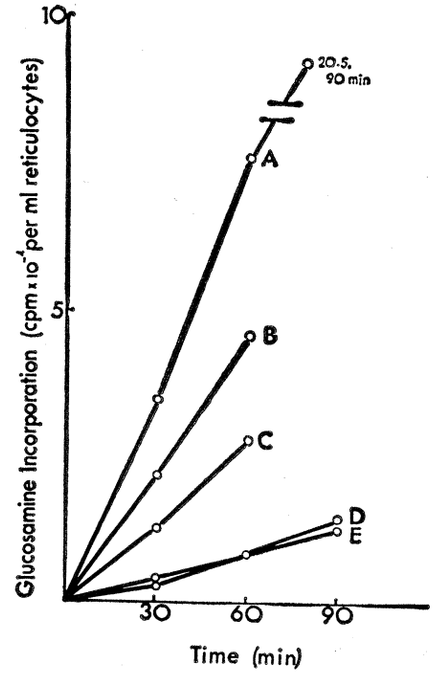


Fig. 1. The rate of incorporation of $C^{14}$-glucosamine into stroma of human reticulocytes. *A, B,* and *C* are for cells with thalassemia major. *D* is a case of mild favism, and *E* is a case of acute bleeding.

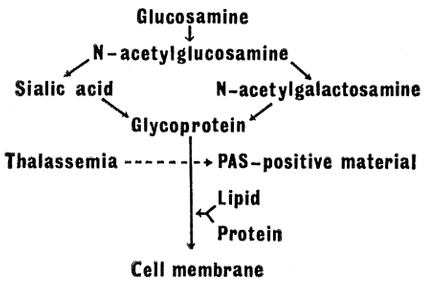


Fig. 2. The biosynthetic pathway of the carbohydrate of the cellular membrane and the proposed block in thalassemia.

the stroma is very likely on the basis of results (*18*) which show an elevation (50 to 80 percent) in the sialic acid content of stroma isolated from the thalassemic erythrocytes. Neither the PAS-positive material nor the radioactivity is identifiable with glycogen, since the former is not destroyed by salivary amylase, and the latter is not in the fraction soluble in trichloroacetic acid. Sometimes PAS-positive material is found in erythroblasts in other anemic conditions.

The reticulocytes of curves *A*, *B*, and *C* (Fig. 1) show an interesting variation that can be correlated with the severity of the thalassemia major. The most severe case clinically, a 2-year-old boy with 95 percent hemoglobin F, is illustrated in curve *A*. The donors of cells represented by curves *B* and *C* had less severe clinical symptoms and had 91 and 85 percent of hemoglobin F, respectively. Curve *A* shows the greatest incorporation of glucosamine; curves *B* and *C* show roughly 40 to 60 percent as much at 1 hour. Each of these curves, however, shows incorporation of much more glucosamine than do curves *D* and *E*.

Thus thalassemia may express itself as a function of several biochemical disorders. In particular, synthesis of the glycoprotein of the cellular membrane, or rather the PAS-positive material, is greatly elevated. Possibly a region of a chromosome is damaged, resulting in the malfunction of several genes associated with hemoglobin, ferritin, and glycoprotein biosynthesis. However, a primary genetic defect in only one of these components may produce abnormal behavior of the other components.

A disturbance in enzyme production along the pathway of membrane synthesis, at the point of association of glycoprotein with lipid and protein, could account for the observed accumulation of PAS-positive material (glycoprotein) in thalassemia (Fig. 2). A block, or shunt, at this point would retard membrane synthesis in general. Thus the regulatory mechanisms would be strongly geared to favor synthesis and thereby the incorporation of glucosamine. In theory the clinical symptoms are understandable in the light of impaired synthesis in the membrane, but, since the cell membrane is a macromolecular, heterogeneous structure composed of carbohydrate, lipid, and protein, it is difficult to evaluate the biosynthesis of any component in relation to the whole without additional data. Knowledge of the chemistry and structure of each component, and of how they are assembled to form a complex unit capable of specific biological activity, is almost totally lacking. More detailed characterization of this specific lesion in thalassemia and its relative importance must therefore await further biochemical studies of the membrane.

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## Paraffinic Hydrocarbons in Pasture Plants

Abstract. *The gas chromatographic–mass spectrometric technique recently developed by Ryhage has been applied to the analysis of paraffins extracted from pasture plants, specifically, whole plant and leaves of spotted bur clover* (Medicago arabica). *Normal alkanes from* $C_{18}$ *to* $C_{35}$ *have been found. The* $C_{29}$, $C_{31}$, *and* $C_{33}$ *normal saturated hydrocarbons predominate and* n-$C_{31}$ *is the major component. In the range from* $C_{24}$ *to* $C_{34}$ *the ratio of alkanes with an even number of carbon atoms to those with an odd number is approximately 8 for the whole plant and 5 for the leaves. The distribution of paraffins is similar to that reported for cattle manure and also resembles that of some soils and sediments.*

It has been shown (*1*) by a combined gas chromatographic–mass spectrometric technique (*2*) that certain animal excretion products, such as cattle manure, contain a number of paraffinic hydrocarbons, among which *n*-nonacosane ($C_{29}H_{60}$), *n*-hentriacontane ($C_{31}H_{64}$), and *n*-tritriacontane ($C_{33}H_{68}$) predominate.

In an attempt to elucidate the origin of these hydrocarbons we have considered the following three possibilities, that is, the paraffins may be: (i) end products of bovine metabolism, (ii) metabolic end products of the bacteria living in the bovine digestive tract, and (iii) compounds originally present in the pasture plants eaten by the cattle. Since a number of plants are known to produce waxes which contain paraffins (*3*) the third of these possibilities appeared more likely and was investigated first. Spotted bur clover (*Medicago arabica*) was chosen as the plant to analyze because it constituted the bulk of the cattle's diet at the time our other investigation (*1*) was carried out.

Freshly collected spotted bur clover was allowed to dry at room temperature in the shade for a period of 1 week or longer. Two samples of 2 g each (the first one including the whole plant and the second, leaves only) were each extracted with a benzene-methanol mixture (3 : 1), and the extract was fractionated on a silica gel column into four fractions (*1*). The approximate weights, per gram of extracted material, obtained for each of the four fractions of the second sam-