to overestimate a when synaptic sensitivity to ACh was decreased. Of our experiments, six were in innervated preparations and five were in preparations denervated for 8 to 11 days. The a values in the innervated preparations were 60 ± 10 percent of control values, and those in the denervated preparations were 38 ± 2 percent of controls. We have no explanation for the larger effect in the second group. When DTNB (1 mM) was tested in four of the denervated preparations, it produced a recovery of a to 92 ± 17 percent of the control value (that is, value before DTT) within 25 to 80 minutes. The effects of DTT and DTNB were also confirmed in the innervated cutaneous pectoris preparation (*10*).

The shot effect derived from intracellular voltage measurements offers only a distorted view of the underlying permeability change. Its time constant of decay reflects primarily the surrounding membrane characteristics rather than the relaxation time of the elementary permeability event. Both a diminution in amplitude and shortening of this event would appear as a reduction of a (*1*). In order to decide which of these two mechanisms was responsible for the effect of DTT on a, we resorted to extracellular recording of end plate potentials (EPP's). The time course of decay of these potentials, when measured in the absence of Prostigmine, does not differ greatly from the relaxation time of the elementary permeability change produced by ACh (*1, 11*). Extracellular EPP's were recorded at 12 different junctions, and the effects of DTT (1 mM) were examined at various times between 10 and 65 minutes. In all of these experiments, DTT produced a strong reduction of the EPP amplitude which was fully reversed by DTNB. In the first 30 minutes, the effect of DTT on the duration of the EPP was small, and the decay half-time ($t_{1/2}$) declined on the average to 93 ± 10 percent ($N = 12$) of control values. However, with longer periods of treatment (35 to 65 minutes), a marked shortening of the EPP duration was evident, and the average $t_{1/2}$ declined to 59 ± 9 percent ($N = 7$) of control values. This effect was also fully reversed by DTNB. This result, which indicates an effect of DTT on the duration of the permeability event, may partly account for the observed reduction of a. However, in contrast to the change in $t_{1/2}$, most of the DTT-produced decrease in a had already appeared after 30 minutes of treatment [after this interval, a was 58 ± 9 percent of the control value ($N = 7$)]. It is therefore likely that a decrease in the amplitude of the elementary permeabilty event had also occurred.

In conclusion, it appears that one can obtain a different permeability event not only by modifying the structure of the agonist (*1*), but also by modifying the structure or conformation of the receptor. In our study, the reduction of a specific disulfide bond located near the anionic receptor site (*2, 3*) sufficed to produce a markedly reduced permeability event. This result indicates that a partial inactivation of the basic excitation unit is possible. This would occur if the receptor were comprised of several subunits (*12*) which could be separately affected by DTT treatment.

E. M. LANDAU
D. BEN-HAIM
Department of Physiology and Pharmacology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel

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10. In two preparations, one treated with DTT for 7 and the other for 15 minutes, a decreased to 69 and 64 percent of control values, respectively. In two other preparations, one tested for 20 minutes and the other for 30 minutes, the respective values of a were 50 and 37 percent of controls. In the last experiment, treatment with DTNB for 60 minutes produced a recovery of a to 95 percent of control values (D. Ben-Haim, F. Dreyer, K. Peper, in preparation.
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Hemoglobin Switching in Sheep and Goats: Occurrence of Hemoglobins A and C in the Same Red Cell

Abstract. *Sheep and goats switch from the synthesis of hemoglobin A ($\alpha_2\beta_2^A$) to hemoglobin C ($\alpha_2\beta_2^C$) when made anemic. We have demonstrated the existence of the asymmetrical hybrid hemoglobin, $\alpha_2\beta^A\beta^C$, in the circulating red cells of anemic sheep. These erythroid cells, therefore, synthesized both A and C hemoglobin simultaneously. Thus, the switch appears to be mediated by selective gene expression rather than by a clonal or cellular selective mechanism.*

Hemoglobin synthesis in goats and certain sheep represents a unique model for the study of regulation of gene expression in eukaryotic cells. The erythroid cells of these animals switch from the synthesis of hemoglobin A ($\alpha_2\beta_2^A$) to hemoglobin C ($\alpha_2\beta_2^C$) in vivo in response to anemia, hypoxia, or erythropoietin injection (*1*) and in vitro in tissue culture in the presence of erythropoietin (*2, 3*). Gabuzda *et al.* (*4*) showed that the switch in β globin synthesis in vivo is detectable in the marrow 3 to 5 days after erythropoietin injection. Barker *et al.* (*3*) have found a similar time course in tissue culture. We interpret these data to mean that expression of the β globin gene (or genes) is determined early in erythroid cell maturation. To explore further this model of gene regulation, we found it desirable to ascertain whether control of globin synthesis is exerted by selective stimulation of cells capable of making only one type of β globin (cell selection), or whether both β chains are synthesized simultaneously in each cell (intracellular regulation).

Our study was facilitated by our knowledge of the fact that asymmetrical hybrids of hemoglobin exist in solution (*5*). Hemoglobin is a tetrameric molecule consisting of two α globin subunits and two β globin subunits. The tetramer readily disassociates into dimers as follows: $\alpha_2\beta_2 \leftrightarrow 2\ \alpha\beta$. In a mixture of hemoglobins, reassociation of unlike dimers gives rise to asymmetrical hybrids. For example, one might expect in a mixture of hemoglobins A ($\alpha_2\beta_2^A$)

and C ($\alpha_2\beta_2^C$) to find the asymmetrical hybrid, $\alpha_2\beta^A\beta^C$. By demonstrating this hybrid in intact cells of chronically anemic sheep, we have obtained evidence indicating that regulation of the genes for the β globins occurs by an intracellular mechanism.

Hybrid hemoglobins cannot be isolated by conventional electrophoresis or chromatography because, during separation, the hybrid disassociates into unlike dimers, each of which sorts with its parent hemoglobin. Recently, such hybrids have been demonstrated by isoelectric focusing of deoxygenated hemoglobins on polyacrylamide gels (*6*, *7*). The equilibrium constant for the disassociation of deoxyhemoglobin into dimers is several orders of magnitude lower than that for oxyhemoglobin (*8*). Therefore, in our experiments, the hemoglobin in the intact cells was deoxygenated prior to isoelectric focusing in order to enhance the integrity of tetramers and prevent the dissipation of asymmetrical hybrids during separation.

Sheep homozygous for hemoglobin A were made anemic by phlebotomy. Bleeding was continued for several weeks to ensure that the cells examined had been formed during a period of simultaneous synthesis of hemoglobins A and C. The total amount of blood removed was equivalent to five to ten times the blood volume of the animal. After this prolonged bleeding, substantial amounts of both hemoglobins A and C remained in the circulating cells. To obtain additional evidence that both hemoglobins were synthesized simultaneously, we obtained bone marrow aspirates for animal 960 (Fig. 1c) and animal Y-34 (Fig. 1d) on day 195 and day 56 of bleeding, respectively. The cells were incubated with [^{3}H]leucine, and the globin chains were resolved by column chromatography (*9*). For animal 960 the ratio of β^A to β^C synthesis was 1 : 1, and for animal Y-34 the ratio was 3 : 1.

Red cells were washed twice with normal saline and stored in Alsever's solution (*10*). The cells were prepared for isoelectric focusing by washing them once more with saline and then suspending them in a solution of normal saline and isotonic phosphate (4 : 1) buffer, *p*H 7.0, to give a hemoglobin concentration of 10 mg/ml. One milliliter of the cell mixture was placed in a sealed flask under a stream of hydrated nitrogen for 15 minutes. Isotonic sodium dithionite was added (3.5 percent by volume) just before the sample was applied to the gel. The general technique of isoelectric focusing of hemoglobins was that described by Drysdale *et al.* (*11*) as modified by Bunn and McDonough (*7*). A portion (35 μl) of cell suspension was applied anaerobically to each gel. Cell lysis occurred immediately after application. The hemoglobins were focused to their isoelectric points with ampholines of *p*H 6.0 to 8.0.

Each focusing experiment included duplicate or triplicate controls consisting of (i) an equal mixture of cells containing pure hemoglobin A or pure hemoglobin C and (ii) an equal mixture of hemoglobins A and C in the form of stroma-free lysates. These were de-

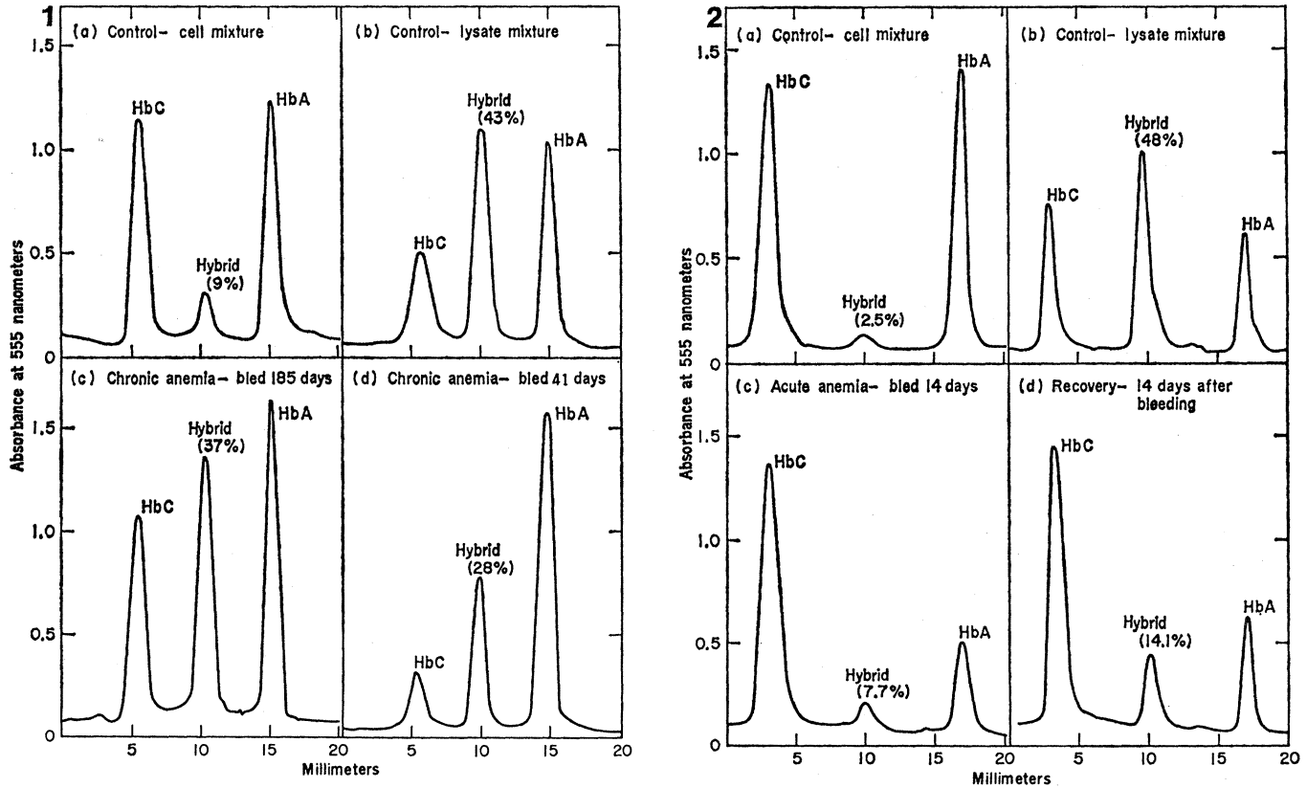

Fig. 1 (left). Isoelectric focusing of intracellular hemoglobins from sheep cells. The gels were scanned at 555 nm with a Gilford recording spectrophotometer. The area under each peak was measured to quantify the amount of hemoglobin. (a) An artificial mixture containing equal amounts of hemoglobin A (HbA) red cells and HbC red cells. (b) A portion of the above cell mixture was lysed, and the hemoglobin was allowed to equilibrate in air prior to deoxygenation and focusing. (c) Blood from sheep 960 after being bled intermittently over a period of 185 days to maintain anemia (hematocrit, 16 to 20 percent). (d) Blood from animal 34 after being bled over a period of 41 days. Fig. 2 (right). (a and b) Same as in Fig. 1, a and b. (c) Blood from animal 962 after being bled over a 14-day period. The hematocrit was maintained at 10 to 12 percent. (d) Blood from animal 961 obtained 14 days after bleeding stopped. At the end of the bleeding period, the HbC of this animal was greater than 95 percent.

oxygenated and focused exactly by the procedure followed for the experimental samples. The first control was necessary in order to demonstrate that the precautions taken were sufficient to prevent the formation of hybrid after lysis of the cells. The amount of the hybrid found in the control mixture varied from 2.5 to 12 percent, but was consistent within 3 percent in replicate gels in the same experiment. A similar amount of hybrid was present in cell-free mixtures of deoxygenated hemoglobin and decreased with the addition of 2,3-diphosphoglycerate or reduction of *p*H (*6, 7*).

The second control was designed to measure the maximum amount of hybrid demonstrable in each focusing experiment. In a mixture of hemoglobin tetramers of equal stability, the percentage of hybrid was calculated from the binomial expansion, $a^2 + 2ab + b^2 = 1$. In this equation, a and b are the amounts of the two hemoglobins in the mixture ($a + b = 1$). At equilibrium, $2ab$ is equal to the fraction of the asymmetrical hybrid in the mixture. By this computation the expected amount of hybrid in Fig. 1b is 49 percent and that in Fig. 2b is also 49 percent. Deviation from the expected amount ranged from 1 to 8 percent, although again there was consistency between replicate samples in the same experiment. Bunn and McDonough (*7*) have ascertained that the amount of the hybrid decreases with the duration of focusing. Our experiments were stopped when the bands were sharp enough to allow adequate scanning for quantification (1¼ to 2 hours). The errors introduced by the small amount of hybrid in the mixture of cells and the less-than-predicted amount of hybrid in the lysate mixture tended to cancel each other in measuring the percentage of hybrid in the experimental cells.

Figure 1 illustrates the occurrence of the asymmetrical hybrid hemoglobin in the cells of animals bled over a period of several months. Animal 960 was studied on five occasions from day 164 to 185 of bleeding, and in each instance there was a substantial amount of hybrid present (30 to 40 percent). Similarly, cells from animal Y-34 were studied three times between day 29 and day 54 of bleeding, and 23 to 35 percent of hybrid was found. Two other animals bled over a several-month period were studied once, and the asymmetrical hybrid was identified. Thus we found substantial amounts of hybrid in cells formed during approximately equal synthesis of both β globins.

During acute severe anemia there is prompt cessation of synthesis of β^A and synthesis of β^C appears (*9*). Thus, cells should contain either hemoglobin A (residual cells) or C (newly formed cells) and little hybrid. This result was obtained (Fig. 2c). Similarly, after cessation of bleeding, there is prompt reversion to hemoglobin A synthesis, although in an animal we studied, the presence of 14 percent hybrid (Fig. 2d) suggested that for a period both A and C were synthesized simultaneously and produced a cohort of cells with the hybrid. Garrick *et al.* (*12*) studied goat cells during the recovery phase of anemia by applying immunofluorescent techniques with antibodies specific for hemoglobins A and C. They found that nearly all cells fluoresced with each antiserum and concluded, as we do, that hemoglobin A and C are synthesized in the same cell.

Kabat (*13*) has suggested a model of gene control involving selective gene deletion of closely linked related genes by a process of intrachromosomal crossing over. Allelic structural loci on the two chromosomes would be controlled relatively independently. Application of this model to hemoglobin switching leads to the prediction that a given erythroid cell would synthesize either hemoglobin C or hemoglobin A or an equal mixture of each. This expected prediction is difficult to test by our method of analysis.

However, the hybrid hemoglobin is present in nearly the amount predicted, assuming free equilibration, in the cells of animals making both hemoglobins A and C for long periods. The finding that most cells fluoresced with both antibody to A and antibody to C during the recovery phase of anemia also implies that most cells make both types of hemoglobin, even when hemoglobin C presumably represented a small fraction of that being synthesized (*12*). However, definitive testing of Kabat's interesting hypothesis may require study of sheep heterozygous at the β globin structural locus.

We have presented evidence that regulation of globin genes during hemoglobin switching occurs by an intracellular mechanism. Prior studies have demonstrated that the switch is accompanied by change in functional globin messenger RNA (mRNA) (*9*). Although the amino terminal sequences of the β^A and β^C chains differ substantially, detailed examination has failed to reveal the requirement for a unique protein initiation factor or transfer RNA, and thus translation of these mRNA's seems to occur by a common mechanism (*14*). Recent studies have established that globin mRNA sequences can be transcribed in vitro from erythroid chromatin (*15*), and therefore this level of gene control is accessible to direct examination. Application of techniques to hemoglobin switching may permit definition of the alteration in chromatin which results in the transition from β^A to β^C synthesis.

ARTHUR W. NIENHUIS
Section on Clinical Hematology, National Heart and Lung Institute, Bethesda, Maryland 20014
H. FRANKLIN BUNN
Division of Hematology, Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115

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