Studies on Abnormal Hemoglobins

II. Their Identification by Means of the Method of Fractional Denaturation

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In the preceding communication we have reported that alkali resistant hemoglobin causes in the hereditary hemolytic syndromes (sickle cell anemia, Mediterranean anemia and in some instances of hereditary spheroctysis) and also in some acquired hematologic disorders (leukemia, aregenerative anemia and malignant disease of the marrow). The hypothesis was advanced that these abnormally denaturing fractions may represent a continued production of fetal pigment beyond the physiologic age limit in the former conditions, and a reactivation of such a mechanism in the latter. In order to furnish further support for this concept, the various resistant pigments were tested by means of “fractional denaturation.” This term is used here for a procedure which follows the progress of the denaturation process by permitting the alkaline reagent to react with identical portions of the same hemoglobin solution for intervals of increasing length of time and determining the remaining amounts of unaffected pigment. Similarities in the degradation rates of fetal hemoglobin and the other various resistant compounds might, then, indicate their identity, whereas differences would be suggestive of dissimilarities in their physico-chemical structures.

Material and Methods

The blood specimens used in this study were obtained from the same patients as mentioned in the companion paper.

In performing the fractional denaturation procedure, the method employed is identical with that described in detail for the determination of the one minute denaturation values, except that the reaction is allowed to proceed in aliquot portions of the hemoglobin solution over longer periods of time. Since the selection of the appropriate time intervals depends upon the concentration of the resistant fraction in the solution, the one minute denaturation value was always established prior to the performance of the test. This value represents the total amount of the abnormally denaturing hemoglobin in a given sample (the normal pigment being completely destroyed within one minute) minus the small

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quantity which has been removed simultaneously with the normal compound. Thus, when
the one minute value was, for example, 60 per cent, the longest time interval was set for
one hour; when it was 20 per cent, for forty minutes; and when it was only 5 per cent, the
last determination was carried out at twenty minutes. In each instance, at least 8 to 10
aliquot portions of the hemoglobin solution was then denatured at convenient periods
between these time limits. When the one minute value was less than 5 per cent, 0.2 ml.
instead of 0.1 ml. of the hemoglobin solution was used in performing the denaturation tests
in order to increase the accuracy of the spectrophotometer readings. Comparative studies
have demonstrated that adding double the amount of the hemoglobin solution to the alka-
line reagent does not affect the results of the procedure. With such small concentrations of
resistant pigment, the upper time limit was set at ten to fifteen minutes, with intermediate
determinations carried out at two minute intervals.

Brinkman and Jonxis\(^3\) have pointed out that when the logarithms of the percentages of
the unaltered hemoglobin concentrations found at varying intervals are plotted
against time, a straight “line of disappearance” is observed, provided that a single type of resistant

![Fractional denaturation of 3 specimens containing various concentrations of fetal hemoglobin.](image)

\[
\text{slope } \Delta = \frac{\log \% \ hgb \ at \ t_1 - \log \% \ hgb \ at \ t_2}{t_2 - t_1}
\]

where \(t_1\) and \(t_2\) represent the times at which denaturation was stopped to obtain the
concentrations of unaffected hemoglobin. Calculation of the \(\Delta\) values for the individual graphs
has the advantage of permitting comparative evaluation of the results. It should be em-
phasized, however, that the method of fractional denaturation is relatively inexact and
that only those graphs where the majority of the determined “points” fall on a straight
line are useful in estimating the slope values. In our experience, the limits of the technic
are such that variations in \(\Delta\) of \(\pm 0.15 \times 10^{-2}\) must be considered within the range of ex-
perimental error. Figure 1 depicts examples of fractional denaturation of 3 specimens con-
taining fetal hemoglobin in various concentrations.
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Results

1. Fractional Denaturation of Fetal Hemoglobin

Fourteen specimens containing fetal hemoglobin were investigated. Ten of these consisted of samples of cord blood, while the remaining 4 were obtained from infants during the first six months of life. The one minute denaturation values showed a spread from 84.5 to 3.8 per cent; the Δ values were found to range from 1.6 to 1.9 × 10⁻². In figure 2 all the individual “lines of disappearance” have been arranged in such a manner that their starting points are identical in order to achieve better visualization of the spread of their respective slopes. No characteristic differences in the Δ values of the bloods obtained from the umbilical cords and from infants were noted.

![Figure 2](image)

**Fig. 2.—** Fractional denaturation of fetal hemoglobin (14 specimens studied).

2. Fractional Denaturation of the Resistant Hemoglobin Fractions in Sickle Cell Anemia

Hemoglobin solutions prepared from the blood of 11 patients with sickle cell anemia were studied. The one minute denaturation values varied from 4.6 to 20.4 per cent, the range of the Δ values from 1.3 to 3.0 × 10⁻². The spread of the various slopes of the resistant fractions may be seen from figure 3 where, again, the individual graphs have been adjusted to a common starting point. Four of the “lines of disappearance” fall within the limits established for fetal hemoglobin, whereas the others are strikingly outside of this range. Since in all the examined hemoglobin solutions the abnormal fractions reveal a straight line denaturation pattern, it must be concluded that the alkali resistant pigment in a given patient behaves as a homogenous compound. On the other hand, from the considerable differences in the slope values of different patients, it must
be inferred that the structural alterations in the hemoglobin molecules responsible for their resistance to alkali denaturation are not of a sharply defined, invariable nature. Thus no specific fractional denaturation pattern can be established for the abnormally resistant portion of sickle cell anemia hemoglobin.

Whether the \( \Delta \) value for a certain individual with sickle cell anemia remains constant over a prolonged period of time has not yet been determined. Although short term follow-up studies seem to indicate such a behavior, extended observations are needed.

3. Fractional Denaturation of the Resistant Hemoglobin Fractions in Mediterranean Anemia and in Hereditary Spherocytosis

Calculation of \( \Delta \) could be performed in only 2 cases of Mediterranean anemia and was found to be 1.7 and \( 1.9 \times 10^{-2} \) respectively, with corresponding one minute denaturation values of 11.7 and 22.6 per cent. These \( \Delta \) values, therefore, lie within the range of fetal hemoglobin (figure 4).

It has been mentioned in the preceding communication\(^1\) that only 1 out of 4 families with hereditary spherocytosis showed an alkali resistant hemoglobin fraction. In the 2 members of this family—with one minute denaturation values of 4.8 and 5.2 per cent, respectively—\( \Delta \) was noted to be \( 1.1 \times 10^{-2} \) in both. Four months later, a \( \Delta \) of \( 1.2 \times 10^{-2} \) was again obtained in one of these subjects. These findings demonstrate that the composition of the alkali resistant hemoglobin in these 2 individuals must be distinctly different from the fetal pigment.

4. Fractional Denaturation of the Resistant Hemoglobin Fractions in Acute Leukemia and in Chronic Aregenerative Anemia

Determination of \( \Delta \) in 2 cases of acute leukemia showed slopes of \( 1.7 \times 10^{-2} \) in both (figure 5). Their one minute denaturation values were 4.3 and 3.2 per
cent. In the 13 year old boy with chronic aregenerative anemia, discussed in the companion paper, \( \Delta \) was calculated to be \( 1.9 \times 10^{-2} \) with a one minute denaturation value of 5.0 per cent (figure 5). Thus, in these 3 instances of acquired hematologic disorders, the resistant pigment again shows the same denaturation pattern as fetal hemoglobin.

![Figure 4](image4.png)

**Fig. 4.—Fractional denaturation of the resistant hemoglobin in Mediterranean anemia and in hereditary spherocytosis.**

**Fig. 5.—Fractional denaturation of the resistant hemoglobin in acute leukemia and in chronic aregenerative anemia.**

**Discussion**

By means of the method of fractional denaturation it has been established that fetal hemoglobin shows a characteristic rate of alkaline degradation under the given experimental conditions. In the few instances of Mediterranean anemia and of the acquired disorders with a resistant hemoglobin fraction (chronic aregenerative anemia and leukemia) in which the procedure was carried out, an identical pattern was demonstrable. In sickle cell anemia, however, the resistant
fractions of only 4 out of 11 specimens behaved like typical fetal pigment, whereas the others revealed either faster or slower rates of denaturation. The alkali resistant hemoglobin in 2 instances of hereditary spherocytosis also showed considerable deviations in their “lines of disappearance” from those obtained with the fetal compound.

From these data the question immediately arises as to the nature and possible significance of the observed dissimilarities. No solution to this problem is readily available since the alterations in the hemoglobin molecule responsible for the resistance to alkali are not understood at present. In addition to the method of alkali denaturation, the embryonic pigment can also be differentiated from the normal adult compound by means of a number of other procedures, e.g., immunologic, crystallographic, spectrophotometric, spreading velocity in a monomolecular film, and affinity for oxygen as expressed in oxygen dissociation curves. However, each of these technics establish only that the two hemoglobins differ from each other but do not elucidate the basic nature of their heterogeneity. Porter and Sanger have demonstrated that variations in the composition of the respective hemoglobin molecules exist in so far as the fetal pigment showed 2.6 terminal valine residues in contrast to the adult compound which has 5 such residues. There were also 47 free lysine amino groups in the fetal, but only 43 in the adult compound. These findings may point to alterations in the number of open peptide chains and, therefore, to differences in the physico-chemical structure of the two protein moieties. If such changes are related to the resistance to alkaline reagents, it seems justified to postulate compounds having structural compositions similar to, but not identical with, that of fetal globin. Such pigments, although resistant to alkali, may then manifest themselves by deviations in their rates of denaturation from that established for fetal hemoglobin and may be called fetal-like.

Such a concept could explain the spectrum of slope values obtained with the hemoglobin solutions of sickle cell anemia patients and would be in keeping with the general experience of biochemists dealing with proteins under pathologic conditions. It has been suggested that “proteins may exist in nature not as pure chemical entities but as ‘families’ of very similar substances.” From the standpoint of this interpretation, the finding of fetal-like hemoglobins in some cases of sickle cell anemia, and of fetal in others, is thus not in disagreement with the hypothesis that the resistant fraction in this disorder may represent a continued production of the embryonic pigment beyond the physiologic age limit. The difficulties with which this hypothesis meets in relation to the electrophoretic abnormality of the hemoglobin molecules in sickle cell anemia (type S hemoglobin) has been broached in the preceding paper.

The occurrence of fetal-like hemoglobins with identical Δ values in 2 members of one family with hereditary spherocytosis may give some insight into the inheritance of pathologic proteins. It is unlikely that this special kind of pigment is specific for the subtype of familial hemolytic jaundice showing a resistant hemoglobin; it will probably be found to represent a peculiar constitutional anomaly in this particular family. Further studies of other similar cases are required to clarify this problem.
The finding of \( \Delta \) values identical with those established for fetal hemoglobin in the patients with acute leukemia and with chronic aregenerative anemia seems to support the interpretation that in these acquired disorders the production of fetal pigment may be reactivated. It should, however, be emphasized that this statement is merely a descriptive one without being based on any knowledge of the biochemical mechanisms involved. It is the elucidation of these mechanisms which may bring about a better understanding of the fundamental processes of hemoglobin synthesis in health and disease.

**Summary**

By means of fractional denaturation, it is possible to follow the progress of alkaline degradation of resistant hemoglobins. A characteristic pattern was established for the fetal compound. The alkali resistant hemoglobin fractions in Mediterranean anemia and in some acquired hematologic conditions (acute leukemia and chronic aregenerative anemia) behaved like the fetal pigment. In sickle cell anemia only 4 out of 11 specimens were found to have fetal hemoglobin, whereas the others seem to have a fetal-like compound. Similarly, in 2 members of one family with hereditary spherocytosis, the resistant pigment was also fetal-like. The significance of these findings for the hypothesis that the resistant hemoglobin fractions in these disorders represent either a continuation or a reactivation of the production of the embryonic pigment is discussed.

**REFERENCES**

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