The Binding of Cr\(^{51}\) to Hemoglobin
I. In Vitro Studies

By Howard A. Pearson

In 1950, Gray and Sterling described the use of Na\(_2\)Cr\(^{51}\)O\(_4\) as an agent for labeling human red cells. They found that when incubated with intact red cells, the radioactive substance traversed the cell membrane and became firmly attached to intraerythrocytic hemoglobin. This property was at first employed for blood volume determinations. Since most of the radioactivity remains with the red cell until removed from the circulation, Na\(_2\)Cr\(^{51}\)O\(_4\) was then found to be useful in estimations of red cell life span. Because of a convenient physical half-life and predominant gamma ray emission permitting accurate scintillation counting, survival studies with Cr\(^{51}\) have become the method of choice for this type of investigation.

Despite wide use, there are a number of potential inaccuracies in the Cr\(^{51}\) technic. First, a non-specific elution of Cr\(^{51}\) from Hb A occurs at an estimated 1 per cent per day. Therefore, mathematical corrections must be employed to convert the measured RBC Cr\(^{51}\) T/2 to mean red cell survival. The rather wide range of normal values of 25–35 days for the RBC Cr\(^{51}\) T/2 also suggest that there may be variable rates of elution in different individuals. Whether disease states may influence Cr\(^{51}\) binding or elution is largely unknown.

In their original studies, Gray and Sterling demonstrated that the Cr\(^{51}\) activity was associated with the globin rather than with the heme component of the hemoglobin molecule. The last decade has seen a tremendous increase in knowledge of the chemistry and structure of hemoglobin. In the human hemoglobins, chemical variations occur only in the protein moiety or globin. The heme groups of all of the human hemoglobin variants are apparently identical.

Globin from normal adult hemoglobin (Hb A) consists of two pairs of chemically distinguishable polypeptide chains designated \(\alpha\) and \(\beta\) chains. Hb A, therefore, is represented \(\alpha^2\beta^2\). Most of the abnormal hemoglobins result from point substitutions of single amino acids in one or another polypeptide chain. Hbs S and C result from substitution of a valine or lysine respectively in place of a glutamic acid in the \(\beta\) chain of Hb A. These species, therefore, can be schematically designated \(\alpha^2\beta^2\) and \(\alpha^2\beta^2\). Fetal hemoglobin (Hb F) has two normal \(\alpha\) chains, while the second set are chemically quite different and have been designated \(\gamma\) chains. Thus, Hb F may be represented \(\alpha^2\gamma^2\). Hb H, an unstable variant usually occurring in certain thalassemia syndromes, has a unique structure. It is a tetramer of normal \(\beta\) chains and is represented...
as $\beta^A$ (6). The ready availability of such chemically altered hemoglobin variants has been utilized in these studies of the site of Cr$^{51}$ binding to hemoglobin.

Since many of the clinical conditions associated with hemoglobin variants are manifested as hematologic disease, variability of binding or elution of Cr$^{51}$ from these species might appreciably affect the interpretation of red cell survival studies. This communication reports a series of in vitro experiments in which the site and strength of the Cr$^{51}$ hemoglobin combination have been investigated. Some of these results have been previously reported in preliminary form.(7,8)

METHODS

Starch block electrophoresis was performed using barbital buffer pH 8.6 and phosphate buffer pH 7.0. Agar gel electrophoresis utilized a modification of the method of Robinson et al. Separation of the polypeptide chains of hemoglobin was accomplished by column chromatography on CG-50 cationic resin, and continuous molarity gradient from 2-8 M, with urea pH 1.9, as described by Wilson and Smith. Acid dissociation and recombination of hemoglobin utilized the techniques of Singer and Itano.

Cr$^{51}$ tagging was accomplished in most instances by incubating red cells with Na$_2$CrO$_4$ at pH 7.0. Unless otherwise specified, a ratio of about 0.5-1.5 moles of chromium to 1000 moles of hemoglobin was maintained, since this is the concentration generally used in clinical studies. The tagged cells were washed and hemolyzed with distilled water and toluene to obtain radioactive hemolysates. Pure Hbs A, S, C, F, when required, were separated by starch block electrophoresis before tagging with Cr$^{51}$.

Radioactivity was quantitated by counting in a 3-inch NaI well-type crystal with scaler. Sufficient counts were made to insure a relative standard deviation of less than 5 per cent.

RESULTS

A. Strength of Binding of Cr$^{51}$ to Various Hemoglobins

It was observed that when Cr$^{51}$-tagged hemoglobin solutions were electrophoresed, radioactivity decreased with time. Several experiments were performed using pure solutions of radioactive Hbs S, C, A, and F (hereinafter a radioactive hemoglobin is signified by an asterisk after the letter symbol for the hemoglobin). In each experiment 0.2 ml. aliquots of the individual types of radioactive hemolysate were inoculated into a broad starch block at pH 8.6. A constant current of 48 ma and 250 volts was applied, and individual hemoglobin bearing segments were cut from the block at varying intervals. The defect was refilled with fresh starch, and the current again started. The hemoglobin was eluted into a constant volume and assayed for Cr$^{51}$ activity and hemoglobin concentration.

The results of a number of such experiments are depicted in figure 1 in which per cent of original radioactivity is plotted against time. For all of the hemoglobins tested, a biphasic pattern of loss of radioactivity was seen. The curves for $A^*$, $C^*$, and $S^*$ were very similar with a rapid loss of approximately 20 per cent of the total activity in the first 6-8 hours and then a more gradual loss. At 24 hours about 65 per cent of the original activity was still present.

The curve seen with Hb F* was quantitatively quite different. The initial rapid loss of radioactivity was 45 per cent at 6 hours. At 24 hours only 45 per cent of the original radioactivity remained. Much of the “eluted” radioactivity appeared in the anodal electrode compartment.
These experiments confirm other reports demonstrating different in vitro rates of elution of Cr\(^{51}\) from Hbs F\(^*\) and A\(^*\). Since the marked chemical difference of Hbs A and F reside in only the \(\beta\) and \(\gamma\) polypeptide chains, respectively, these differences in elution rates might indicate \(\beta\) and \(\gamma\) chains to be important in Cr\(^{51}\) binding. The chemical difference in Hbs A, S, and C is the substitution of only one amino acid, and so it is not surprising that similar elution curves are observed. Finally, the biphasic type of curve observed in all varieties might possibly indicate that two different Cr\(^{51}\) sites or binding mechanisms are present.

**B. Site of Binding of Cr\(^{51}\) to Hemoglobins**

1. **Pattern of Cr\(^{51}\) radioactivity in Hb H disease:** The red cells of a patient with Hb H disease were tagged with Fe\(^{59}\) and Cr\(^{51}\) as part of erythrokinetic studies reported elsewhere\(^1\), and the hemolysate separated by starch block electrophoresis in phosphate buffer, pH 7.0. At this pH, Hb H moves anodally while Hb A moves toward the cathode. This permits a sharp separation in 3 hours, minimizing denaturation of the unstable Hb H. The H and A components were eluted from the block and quantitated spectrophotometrically at 540 m\(\mu\), and for Fe\(^{59}\) and Cr\(^{51}\) activity by gamma ray spectrometry.

As seen in table 1, Hbs H and A constituted 18 per cent and 82 per cent of the total pigment. The Fe\(^{59}\) activity was similarly distributed with 19 per cent and 81 per cent being in the H and A components, respectively. However, when the H and A components were counted for Cr\(^{51}\) at two different chrom-
Binding of Cr\(^{51}\) to Hemoglobin

Table 1

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>Total Hemoglobin (%</th>
<th>Fe(^{55}) (cc./min.)</th>
<th>Fe(^{59}) (% total activity)</th>
<th>Cr(^{51}) (cc./min.)</th>
<th>Cr(^{51}) (% total activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µc. Cr(^{51}) used to tag RBC</td>
<td>H</td>
<td>18</td>
<td>16</td>
<td>20.5</td>
<td>1107</td>
</tr>
<tr>
<td>100 µc. Cr(^{51}) used to tag RBC</td>
<td>A</td>
<td>82</td>
<td>62</td>
<td>79.5</td>
<td>2178</td>
</tr>
</tbody>
</table>

ium-to-hemoglobin ratios, the activity associated with the Hb H fraction was found to be approximately two times greater than would be expected from the hemoglobin concentration.

Since each molecule of Hb H (\(\beta^4\)) contains twice as many \(\beta\) chains as does Hb A (\(\alpha^2\beta^2\)), a possible explanation for the nearly twofold increase in radioactivity would be that Cr\(^{51}\) tags largely to \(\beta\) chains. Increased Cr\(^{51}\) tagging to Hb H has been recently confirmed by other investigators,\(^{15,16}\) using different conditions of electrophoresis.

2. Dissociation-recombination studies: When hemoglobin is placed in acid or alkaline solutions, there is asymmetric dissociation into free \(\alpha_2\) and free \(\beta_2\) units. When the pH is returned to neutrality, the polypeptide chains recombine in a random fashion to reform intact hemoglobin.\(^{12}\) Three per cent solutions of tagged hemolysate from a normal adult and from a cord blood specimen from a 7-month premature fetus containing 95 per cent Hb F, as measured by alkali denaturation and agar gel electrophoresis, were mixed with equal amounts of hemolysate from a patient with homozygous Hb C disease. These mixtures were divided into control and reaction portions. The latter was successively dialyzed against sodium acetate buffer, pH 4.7, for varying lengths of time between 12-24 hours, and then returned to neutral pH by dialysis against water. The control was dialyzed against distilled water for a comparable period of time. Control and reaction mixtures were then separated by starch block or agar electrophoresis. The hemoglobin components were eluted and assayed for hemoglobin and radioactivity.

Depending upon the site of chromium binding to hemoglobin, alternative results from such experiments can be postulated. Figure 2A shows what would occur if Cr\(^{51}\) tagged primarily to the \(\beta\) chains, in an experiment in which Hb A\(^*\) was mixed with non-radioactive Hb C. Since radioactive marker (\(\beta^4\)) and chemical marker (\(\beta^2\)) would be on the same chain, no interchange would be possible. Hence, no increase in radioactivity of the Hb C fraction would be seen in the reaction as compared to the control mixture. On the other hand, if Cr\(^{51}\) tagged only to \(\alpha\) chains or to both \(\alpha\) and \(\beta\) chains nonspecifically (fig. 2B), then, after dissociation and recombination, a significant increase in radioactivity of the Hb C fraction should occur. In a similar way the binding of Cr\(^{51}\) to fetal hemoglobin could be studied using Hb F\(^*\) and non-radioactive Hb C.

Results of various recombination studies are listed in table 2. There were no significant changes in the percentage of radioactivity associated with the Hb C fraction after dissociation and recombination procedures, supporting the
Fig. 2.—Alternative schemes for Cr²⁺ activity partition after dissociation and recombination of Hbs A* and C.

hypothesis of β chain binding. The results with Hb F* and Hb C were less clear-cut because of lower total counts and a higher proportion of radioactivity in the Hb C fraction in both control and reaction mixtures. However, since no greater increase in radioactivity of the Hb C fraction was seen after dissociation and recombination procedures, γ chain binding of Cr²⁺ is suggested.

It is noteworthy that some radioactivity was always associated with the Hb C fractions in the control mixtures. Approximately 3 per cent of this in the A*-C mixtures may be accounted for by the Hb A₂ present in the normal hemolysate. Since the δ₂ chains of Hb A₂ are chemically quite similar to β chains,¹⁷ it is not surprising that Hb A₂ apparently binds Cr²⁺ equally as well as does Hb A₁. To study the significance of the activity greater than this, mixtures of Hb A* and Hb C were allowed to passively stand in test tubes for increasing periods of time before being separated by electrophoresis. A steady increase in radioactivity of the C fraction was seen (table 3). When Hb F*
Table 3

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Hemoglobin</th>
<th>cc./min.</th>
<th>Cr⁵¹ Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>A⁺</td>
<td>1080</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>32</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>F⁺</td>
<td>955</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>38</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>A⁺</td>
<td>567</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>24</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>F⁺</td>
<td>734</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>141</td>
<td>16</td>
</tr>
<tr>
<td>24</td>
<td>A⁺</td>
<td>1168</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>153</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>F⁺</td>
<td>813</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>231</td>
<td>22</td>
</tr>
</tbody>
</table>

and Hb C mixtures were similarly studied, the increase in radioactivity of the Hb C. fraction was considerably greater and more rapid. This probably represents diffusion of Cr⁵¹ molecules from the originally tagged hemoglobin molecules with a retagging of other hemoglobin molecules (including Hb C) in the solution. This type of passive transfer is suggested by an increasing radioactivity of the Hb C fractions with increasing time. The increase is much greater with Hb F⁺, again indicating weaker binding or a greater elution rate.

3. Chromatographic studies: Approximately 500 mg. of Hb A⁺, S⁺, and C⁺ were applied to columns and separated into the component polypeptide chains by the acid urea gradient method of Wilson and Smith. Approximately 300 10-12 ml. fractions were collected. The odd number fractions were counted for radioactivity and the even number fractions measured for protein content in a Beckman DU spectrophotometer at 280 μ.

For all hemoglobins tested there was a large loss of radioactivity in the initial fractions of eluate long before the first protein-containing fractions appeared. Most of the radioactivity that was associated with protein-bearing fractions was seen in the second, or α chain, protein peak. Figure 3 depicts a typical result obtained with Hb A⁺. The patterns of radioactivity observed with Hbs. S⁺ and C⁺ were similar. This confirms the findings of Heisterkamp and Ebaugh et al.¹⁸ but not those of Chernoff.¹⁹

When Hb F⁺ was separated chromatographically (fig. 4), a very large initial loss of radioactivity occurred, and no significant radioactivity was found in either α- or γ chain-bearing fractions. Relatively weak binding of Cr⁵¹ to Hb F and rapid elution in acid solutions again hampered studies of Hb F⁺.

4. Tagging of isolated α, β and γ polypeptide chains. Fractions of eluates from Wilson-Smith chromatographic separation of Hbs A and F were chosen to include only the main protein peaks in order to exclude cross-contamination of other chains (for example, on figure 3, fractions #125–#175 would be chosen as relatively pure α chains and fractions #230–#275 as β chains).

These fractions were dialyzed free of urea and concentrated by dialysis.
Fig. 3.—Chromatographic separation of Hb A₁⁺. The protein partition (O.D. at 280 mλ) is compared to the distribution of radioactivity.

against Carbowax. Aliquots of α, β, and γ fractions were adjusted to the same optical density and then placed in dialysis bags and immersed for 12 hours in several beakers containing aqueous solutions of Na₂Cr⁵¹O₄ at pH 7.0. The fractions were then dialyzed against distilled water to eliminate unbound Cr⁵¹. The radioactivity of the various chains after dialysis is listed in table 4.

There was a marked preferential binding of Cr⁵¹ to β chains when compared to the α chains. With increasing concentrations of Cr⁵¹, this discrepancy became more pronounced. When α and γ chains were studied, results were much less striking although the γ chain always showed increased binding of Cr⁵¹.

C. Distribution of Cr⁵¹ among Normal Minor Components of Hemoglobin

When Cr⁵¹-tagged hemolysate from normal persons is separated by electrophoresis, three more or less distinct components are observed. The major component is called Hb A₁ and constitutes about 90 per cent of the total pigment. Hb A₂, the slow minor fraction, constitutes about 3 per cent in normal

*Union Carbide Chemicals Co.
Fig. 4—Chromatographic partition of protein and radioactivity of Hb F*.
Table 4

<table>
<thead>
<tr>
<th>Cr(^{51}) (μc., c. 10 mL)</th>
<th>Polypeptide Chain</th>
<th>cc./min.</th>
<th>Ratio β/α</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>α</td>
<td>1163</td>
<td>3.5/1</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>4148</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>α</td>
<td>2410</td>
<td>21/1</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>50,734</td>
<td>γ/α</td>
</tr>
<tr>
<td>10</td>
<td>α</td>
<td>1071</td>
<td>1.13/1</td>
</tr>
<tr>
<td></td>
<td>γ</td>
<td>1215</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>α</td>
<td>2459</td>
<td>1.16/1</td>
</tr>
<tr>
<td></td>
<td>γ</td>
<td>2861</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>α</td>
<td>9651</td>
<td>1.48/1</td>
</tr>
<tr>
<td></td>
<td>γ</td>
<td>14,755</td>
<td></td>
</tr>
</tbody>
</table>

persons. Hb A\(_3\), the fast minor component, constitutes 7–10 per cent in fresh hemolysate, although in old hemolysate it may increase considerably. The synthesis of Hbs A\(_1\) and A\(_2\) is under genetic control. The nature of Hb A\(_3\) is less certain and it has been postulated that it is a senescent form or breakdown product of Hb A\(_1,2\) or perhaps by conjugation with glutathione.\(^{21}\)

When these various components obtained from Cr\(^{51}\)-tagged hemolysate are assayed for radioactivity, a marked difference of partition between the radioactivity and hemoglobin is observed. The A\(_3\) component, constituting only 7–10 per cent of the total hemoglobin spectrophotometrically, contains about 40 per cent of the radioactivity. This unusual partition of radioactivity has been noted by a number of observers, and has been interpreted by some as indicating a unique property of Hb A\(_3\).\(^{22}\) No difference of this distribution is observed if the red cells or hemolysate are tagged or if Na\(_2\)Cr\(^{51}\)O\(_4\) or Cr\(^{51}\)Cl\(_3\) is used, as has been reported by Malcolm and Ranney\(^{23}\) (fig. 5). The gross molecular structure of Hb A\(_3\) was, therefore, studied to see if it possessed unique polypeptide chain configuration.

The results of chromatographic separation of the polypeptide chains of 200 mg. Hb A\(_3\) are depicted in figure 6; “α” and “β” chains were present in about equal proportions. The partition of radioactivity was similar to the Hb A\(_1\) separation with a majority of the protein-associated activity being in the “β” chain fraction. Because of the small quantity of Hb A\(_3\) applied to the column, both protein and Cr\(^{51}\) curves were of less magnitude than obtained with Hb A\(_1\). However, the ratio of optical density to corrected counts per minute was similar.

Pure Hb A\(_1\), A\(_2\), and A\(_3\) were prepared from starch block eluates and adjusted to the same concentration. Aliquots of each were placed in separate dialysis bags and dialyzed for 24 hours against solutions of Na\(_2\)Cr\(^{51}\)O\(_4\) in the same container at pH 7.0. They were then dialyzed against distilled water to eliminate unbound Cr\(^{51}\). Results are listed in table 5. Under these conditions Hbs A\(_1\), A\(_2\), and A\(_3\) had approximately equal affinity for Cr\(^{51}\).
It has been noted elsewhere that Cr\textsuperscript{51} changes the electrophoretic mobility of Hb A, making it more electrically negative.\textsuperscript{24-26} Since the Cr\textsuperscript{51}-Hb A\textsubscript{1} complex is more electronegatively charged than Hb A\textsubscript{1} alone, it is more strongly attracted to the anode during electrophoresis and becomes concentrated in the vanguard of A\textsubscript{1}; namely, in the Hb A\textsubscript{3} fraction. This probably explains the consistently high specific activity of Hb A\textsubscript{3} which has been repeatedly observed.

**SUMMARY AND CONCLUSIONS**

By a number of different technics, confirmatory evidence has been obtained that Cr\textsuperscript{51} activity of Hb A\textsubscript{1} tagged with Na\textsubscript{2}Cr\textsuperscript{51}O\textsubscript{4} in a ratio of approximately 1 mole chromium to 1000 mole of hemoglobin is largely associated with the $\beta$ polypeptide chains. No significant difference in the site or strength of chromium binding could be demonstrated in abnormal hemoglobins resulting from point amino acid substitutions (Hbs S and C).

The results obtained with Hb F were very suggestive of $\gamma$ chain binding, but an apparently weak Hb F-Cr\textsuperscript{51} bond or increased elution rate markedly hampered such studies.
The propensity of Cr\textsuperscript{51} for preferential tagging of β chains was seen not only when hemoglobin was tagged intact, but also when isolated β chains were tagged. This may indicate that there is a specific locus or chemical configuration within the β chains where Cr\textsuperscript{51} tagging occurs. Knowledge of the exact total amino acid sequence of α, β, and γ chains should soon be available and may permit a precise identification of such a Cr\textsuperscript{51} binding site.

Whether the results of this type of highly artificial manipulation of hemoglobin has significance in Cr\textsuperscript{51} red cell survival studies in clinical situations where large amounts of Hb F occur is uncertain. Conflicting results of red cell survival studies of the fetal red cells suggests that it may\textsuperscript{27,28} Further investigation of this problem is currently in progress.

**SUMMARIO IN INTERLINGUA**

Per medio de un numero de differente technicas evidentia confirmatori esseva obtenite que le activitate de Cr\textsuperscript{51} de hemoglobina A\textsubscript{1}, quando marcate
con Na$_2$Cr$_{5+}$O$_4$ in un proportion de approximativemente 1 mol de chromo a 1000 mol de hemoglobina, es predominantemente associate con le catenas polypeptidic, $\alpha$. Non esseva possibile demonstrar nile significative differentia in le sito o le fortiad del ligaminage de chromo in hemoglobinas anormal resultante ab substitutiones in punctos de amino-acido (hemoglobina S e hemoglobina C).

Le resultatos obtenite in caso de hemoglobina F suggereva fortemente ligaminage de catena $\gamma$, sed un apparentemente debile ligamine de Cr$^{5+}$ con hemoglobina F o un elevate elution disturbava iste genere de studio fortemente.

Le propensitate de Cr$^{5+}$ pro le marcage preferential de catenas $\alpha$, $\beta$, e $\gamma$ pote esser expectate sin grande retardo e va forsan permitter un precise identification del sito de ligaminage de Cr$^{5+}$.

Il es incerte si le resultatos de iste typo de altemente artificial manipulation de hemoglobina es de signification pro le uso d Cr$^{5+}$ in studios del supervivencia de erythrocytos in situationes clinic in que grande quantitates de hemoglobina F occurre. Le possibilitate es suggerite per confligente resultatos in studios de supervivencia de erythrocytos fetal. Investigationes additional del problema es currentemente in progresso.

REFERENCES

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The Binding of Cr$^{51}$ to Hemoglobin I. In Vitro Studies

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