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The Effect of Red Cell Aging on Chromium-51 Binding and in Vitro Elution[1]

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The possibility of non-uniform uptake of ^{51}Cr by red cells of different ages has been considered by several authors (1, 2). It has been shown, for example, that chromates are preferentially incorporated into rabbit and human reticulocytes (3). Variation of ^{51}Cr elution also has been demonstrated both *in vivo* and *in vitro*. It has been suggested that the *in vivo* elution may be accelerated in hemolytic diseases (4). The *in vitro* elution of ^{51}Cr from Hb F is also more rapid than from Hbs. A, S, or ^{5}C.

In the present work the variation of ^{51}Cr incorporation as well as the *in vitro* elution, with red cell age was studied. The difference in relative osmotic fragility of old and young cells (7) as well as their variation in density (7, 8) were used to obtain hemoglobin solutions derived from cells varying in their mean age. The effect of altering the osmotic fragility of the same cell population on the *in vitro* elution of label was also studied.

MATERIAL AND METHODS

The Labeling Procedure:

Fresh blood was obtained from four normal healthy adults and in one experiment blood from a patient with polycythemia rubra vera was used. Labelling was performed with $Na_2{}^{51}CrO_4$ of a specific activity of approximately 118 mC/mg. (Radiochemical Center, Amersham), in a ratio of 1.5 to 2 moles of Cr to 1000 moles of hemoglobin.

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The labeling procedure was essentially that described by Mollison (10) using a phosphate buffered ACD, pH 6.8. The labeled red cells were washed five times in five volumes of normal saline. The radioactivity in the last saline was of the order of 100 cpm/ml. An 85% suspension of the labeled red cells in saline was prepared.

Fractionation of labeled cells by graded saline hemolysis:

Graded hemolysis of labeled cells was performed by adding one ml of red cell suspension to 5 ml of saline solutions of concentrations that were previously shown to produce 0 to 100% hemolysis. The specific activity of the supernatant hemoglobin solutions was measured. The radioactivity in the 1.0% saline supernate was subtracted as blank from the activity of the hemolysates of all other tubes.

Centrifugational red cell separation:

Labeled red cells were fractionated into top and bottom cells by repeated centrifugation using the method described by Prankard (7) except that heparin was used as an anticoagulant instead of ethylenediamine tetra-acetic acid.

The separated top and bottom cells were then washed and hemolyzed. The hemoglobin solutions were used either for the determination of hemoglobin specific activity or in the elution experiments.

Fe^{59} Labeling of Red Cells:

Three subjects who were hematologically normal but had epitheliomata of tongue, foot and lip respectively were given 500 μC of ^{59}Fe intravenously as labeled plasma. Heparinized blood samples were obtained for fifteen days after the injection. Each blood sample was divided into three parts: The first was labeled with ^{51}Cr as described above; the second was treated similarly except that 10 μg of ^{52}Cr as $Na_2{}^{52}CrO_4$ were added instead of the radiochromate. Two-tenth ml of chromium-free saline was added to the third part which, otherwise, was treated similarly. Each of the three red cells suspensions were then fractionated either by graded saline hemolysis or by centrifugation and the specific activity of hemoglobin in the various solutions determined.

Determination of the ^{51}Cr or ^{59}Fe Specific Activity of Hemoglobin:

Hemoglobin solutions were first partially purified as described by Marks and Johnson (6).

Radioactivity measurements were done in a 1⅞″ well-type scintillation counter. Hemoglobin concentrations were determined colorimetrically as cyanomethemoglobin 12, 13 at 540 mμ.

The specific activity was expressed for either ^{51}Cr or ^{69}Fe in arbitrary units of cpm/3ml of solution divided by the optical density.

The Strength of Binding of ^{51}Cr to Hemoglobin:

The strength of ^{51}Cr hemoglobin binding was tested by measuring the temporal decrease of the specific activity of labeled hemoglobin solution during paper electrophoresis.

The elution of label from the hemoglobin of the 15 to 20 per cent of the least osmotically resistent red cells was compared with that of the 10 to 15 per cent most resistent cells. The hemoglobin solutions were prepared as follows:

Four milliliters of 0.2 per cent saline were added to 5 ml of the ^{51}Cr labeled red cell suspension, mixed by inversion five times and after standing for 10 minutes the tube was centrifuged for 15 minutes. The supernate was then removed and purified as mentioned above. This procedure was found to hemolyze 15 to 20 per cent of red cells and this solution will be subsequently referred to as Hb_f (*i.e.* hemoglobin solution derived from relatively fragile cells). Three milliliters of 0.2 per cent saline were then added to the sediment mixed by inversion and allowed to stand for 10 minutes. After spinning for 15 minutes the supernate was discarded and the sediment washed three times in 20 volumes of saline. It represented 10 to 20 per cent of the original red cell mass and was then homolyzed in four volumes of distilled water and the hemolygate purified as described above. It will be referred to as Hb_r (*i.e.* hemoglobin solution derived from the most osmotically resistent cells).

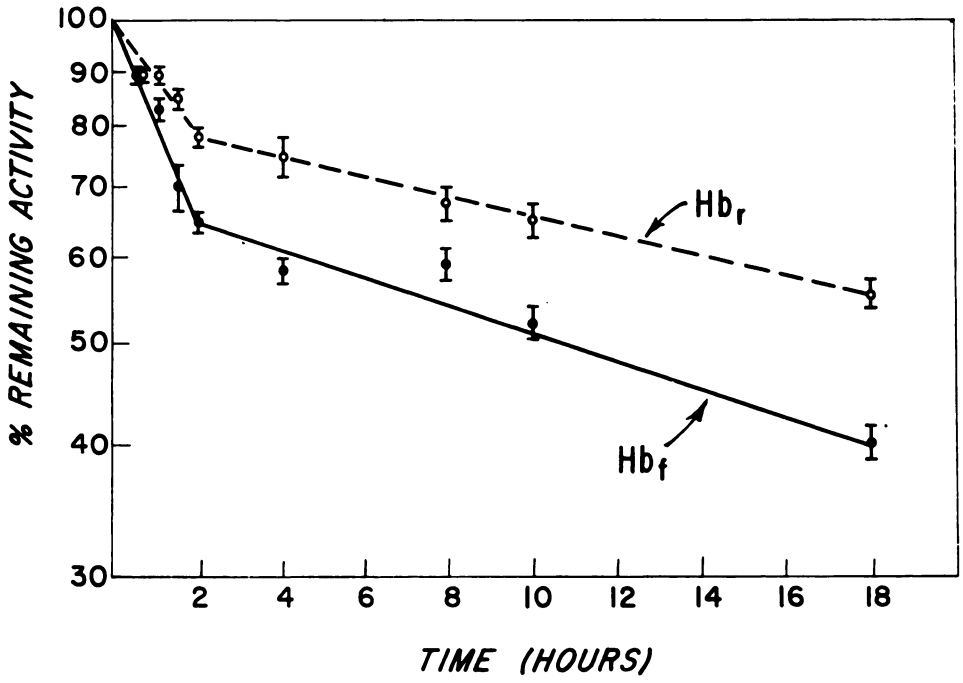

Fig. 1. The specific activities of hemoglobin solutions obtained by graded saline hemolysis. The specific activity of the fully hemolyzed blood is taken as 100 per cent.

Aliquots of Hb_f and Hb_r solutions (0.030 ml.) of the same order of optical density and radioactivity were applied on separate strips of Whatman #1 filter paper. Sixteen strips were run each time: eight of each hemoglobin solution. Electrophoresis was carried out using veronal buffer pH 8.6 (ionic strength 0.05) and a current of 8mA and 140 V. Electrophoresis running was carried out for 0.5, 1, 1.5, 2, 4, 8, 10 and 18 hours; each period being represented by at least eight strips. At the end of electrophoresis each hemoglobin band was eluted with 4 ml Drabkin's reagent. Aliquots of the eluates were then counted, their optical densities determined and their specific activities were expressed as cpm/3ml divided by the optical density. The "initial specific activity" of each hemoglobin solution was determined by applying aliquots of each on four strips of filter paper previously moistened with the buffer and eluting them immediately before electrophoresis. The specific activity of the eluates after electrophoresis was expressed as per cent of the "initial specific activity" of the corresponding hemoglobin solutions. This assumed that the radioactivity was initially bound to hemoglobin. This assumption could be confirmed by plotting the specific activities of the eluates after 10, 20, 30, 40, 50, 90 and 100 minutes running on a semilogarithmic paper. The values could be fitted to a straight line intercepting with the zero time at a value corresponding to the "initial specific activity" as determined by the above method.

The standard error of 20 duplicate determinations of the specific activity of eluted hemoglobin bands was 6.0 per cent. Eighty-five per cent of the activity of any hemoglobin band could be eluted by 4.0 ml of Drabkin's reagent and only 50 per cent of the remaining activity could be eluted with 0.1 N HCl. On counting the filter paper other than the hemoglobin band after electrophoresis, it was found to give a count rate slightly above background.

The elution of label from hemoglobin solutions prepared from the top and bottom layers of centrifuged cells was similarly studied.

The influence of red cell fragility on in vitro elution:

A sample of fresh heparinized blood was centrifuged and the red cells were resuspended in their own plasma after it has been made slightly hypotonic by adding one part distilled water to 12 parts plasma. The mixture was then incubated for 24 hours at 37° C. The red cells were then labeled as usual, an aliquot was hemolyzed, and the rest was fractionated by centrifugation into top and bottom layers. The elution of label after two and four hours of electrophoresis of hemoglobin solutions of the hemolyzates of mixed cells, top and bottom layers cell were then measured. Fresh blood from the same subject was used, as control.

The effect of ACD solution on the in vitro elution of label:

The effect of washing the red cells in two different ACD solutions on the *in vitro* elution of label from Hb_f, Hb_r and total hemolyzate was studied. The first treatment was that described above. In the second treatment the buffered ACD was replaced by an ACD solution having the following composition: disodium citrate (anhydrous) 2 g., dextrose (anhydrous) 3 g., water to 120 ml.

RESULTS

The Specific Activity of Hemoglobin Solutions Obtained by Graded Saline Hemolysis:

Taking the specific activity of the fully hemolyzed samples as 100% the ^{51}Cr label was found to be uniformly distributed amongst all supernatant solutions except those obtained after less than 1.0% hemolysis of normal blood and 5.0% hemolysis in the case of polycythemia vera, which showed significantly higher specific activities (Fig. 1).

The ^{59}Fe labeled red cells showed a fragility pattern, with respect to age, similar to that previously reported (6). In the blood of the three subjects studied, no measurable ^{59}Fe radioactivity was detected up to the eleventh day after injection, in solutions obtained after less than 1.0% hemolysis; ^{59}Fe activity started to appear in such solutions on the fifteenth day. On the tenth day after the injection the mean ratios of the specific activities of the fully hemolyzed blood: Hb_f:Hb_r solutions were 1.0 : 0.59 : 1.5. This means that Hb_f solution contained 6.0% of the cells whose age varied from 0 to 10 days, and the corresponding figure for Hb_r solution was 15 per cent. The ^{59}Fe experiments showed that the relation of red cell age to saline osmotic fragility did not seem to alter as result of labeling with either stable or radiochromate.

The specific activity of red cell layers of centrifuged cells:

The top red cell layer showed a slightly higher ^{51}Cr specific activity than the bottom layer in a ratio of 100 : 94. Radioiron studies confirmed the relative

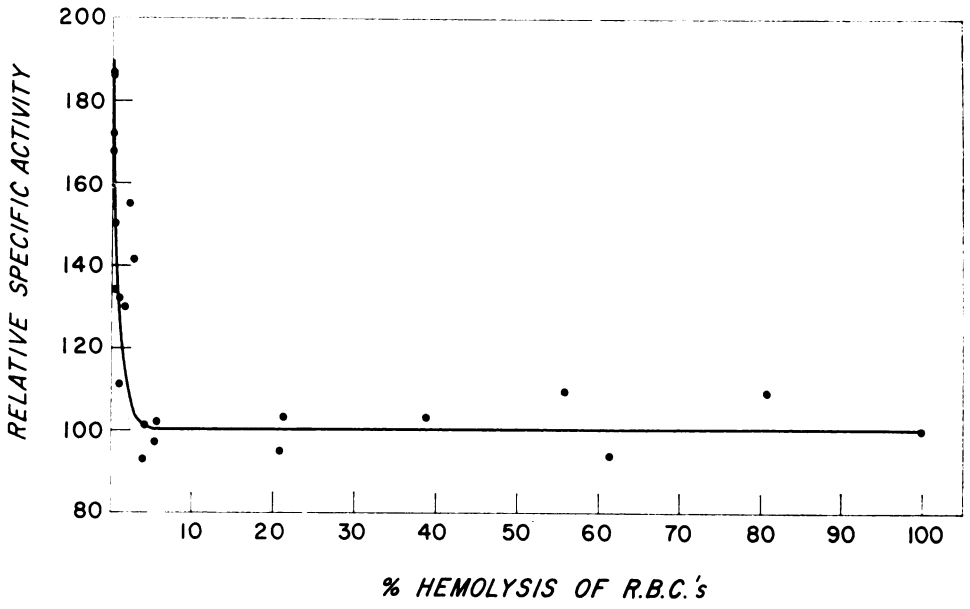

Fig. 2. The temporal change of the specific activity of Hb_f and Hb_r during paper electrophoresis (see text).

TABLE I

^{51}Cr ELUTION FROM THE TOP AND BOTTOM LAYERS OF FRESH AND INCUBATED BLOOD

Time of Electrophoresis	*Fresh Blood*			*Incubated Blood*		
	Mixed	*Top*	*Bottom*	*Mixed*	*Top*	*Bottom*
	Elution: per cent of the initial specific activity					
2 hours	14 ± 3.4	13 ± 2.0	18.4 ± 1.8	30 ± 1.2	28 ± 1.6	32.8 ± 2.0
4 hours	23 ± 3.0	20 ± 3.7	37 ± 2.7	33 ± 2.8	28 ± 4.0	37.5 ± 3.4

enrichment of the top layer with young cells. Double labelling of cells with ^{51}Cr did not alter these density relationships. On the tenth day after the injection the mean ratios of the ^{59}Fe specific activity of hemoglobin of the mixed cells: top cells: bottom cells was 1 : 2.3 : 0.30.

The In Vitro Elution of ^{51}Cr from Labeled Hemoglobin:

The specific activity of hemoglobin dropped steadily during electrophoresis. An initial rapid rate of elution was always observed followed by an appreciably slower rate (Fig. 2). A biphasic elution curve was also reported during starch gel electrophoresis of ^{51}Cr-labeled hemoglobin (5).

It was assumed that all ^{51}Cr was bound to hemoglobin prior to electrophoresis. This was justified by two findings: first the estimated "initial specific activity" coincided with the zero-time intercept of the first two hours specific activity curve when the specific activities were expressed in the arbitrary units of cpm/3ml/optical density. Second: it was demonstrated that any free radiochromate applied on the filter paper would migrate toward the anodal compartment, at a much faster rate than the hemoglobin band.

The rate of spontaneous elution of label during simple contact of labeled hemoglobin with the buffer-moistened paper was estimated directly. The two hours elution rate of freshly applied bands was determined by running them electrophoretically immediately after their application on paper. Other bands were left in contact with the paper in the electrophoresis chamber for periods varying from 2-18 hours and then the current was switched on for two hours; the two hours elution rates being then estimated, and compared with that of the fresh bands. The difference between the two rates could be taken as a measure of spontaneous elution independent of the current applied. It could be shown using this method that a slow spontaneous elution would occur over the 18 hours of the

electrophoretic running experiment and this could account for 15 to 20 per cent of the total elution observed. In the subsequent analysis and comparisons, the total elution rate after electrophoresis was taken as a measure of the strength of binding of radiochromate to hemoglobin.

The following results were obtained from the elution experiments:

1) The elution of label was significantly greater from Hb_f than from Hb_r solutions (Fig. 2). In either case the specific activity curve showed a biphasic pattern on semilogarithmic plotting. The initial specific activities of both solutions did not differ significantly.

2) The elution of ^{51}Cr from the hemoglobin solution derived from the bottom cells after centrifugations was significantly greater than from the hemoglobin of the top cells. As indicated before the initial activity of the top cells was slightly higher (Table I).

3) Incubation of cells in a slightly hypotonic solution resulted in an increase in the osmotic fragility of cells as well as in the rate of label elution. The effect on fragility was more marked on the bottom cells as was shown by Prankard (7) (Table I). Incubation did not, however, alter the rate of uptake of radiochromate by red cells.

4) The elution of label from hemoglobin of cells treated with unbuffered ACD solution was nearly twice that from cells washed in the buffered ACD (Table II). The efficiency of labeling was also greater after the latter treatment.

DISCUSSION

The present data show a significant increase in the rate of *in vitro* elution of ^{51}Cr associated with red cell aging. Since this study of *in vitro* elution involved drastic and artificial manipulations of hemoglobin, it is not possible to infer directly the significance of this finding in *in vivo* work. Some of the presented data, however, suggest some possible relationships to *in vivo* elution. Washing of red cells with buffered ACD, for example, was shown to prolong the half-time of the ^{51}Cr-labelled red cell survival curve *in vivo* (9,10,11). The same treatment resulted also in reduction of the elution rate *in vitro*. Other *in vivo* studies also indicate that ^{51}Cr elution is faster from the more fragile cells of certain hemolytic diseases (4). This might have some relevance to the observed increase in the *in vitro* elution rate associated with the artificial induction of increased red cell fragility reported in this paper.

The factors involved which result in alteration of the chromium elution on varying the labelling procedure are poorly understood largely because the mechanism of chromium uptake is not fully known. It is not clear, for instance why a small change of the pH of the medium in which the red cells are washed, should result in slowing the rate of elution *in vivo* (10). In view of the fact that ^{51}Cr-labelling of erythrocytes can be subject to the influences of a large number of methodological variants, it is essential to give explicitly all the technical details when reporting results obtained in a given red cell survival study. It is also important to draw conclusions on the basis of comparisons with studies performed on a control group employing identical methods.

TABLE II

THE EFFECT OF ACD SOLUTION ON *in vitro* ELUTION OF ^{51}Cr

	Unbuffered ACD	*Buffered ACD*
Time of Electrophoresis	*Elution: Per cent of the initial specific activity*	
2 hours	60 ± 2.0	23 ± 30.0
4 hours	79 ± 2.0	30 ± 2.0

The uniformity of label distribution amongst cells of different osmotic fragility (with the exception of the 1.0% most fragile cells) and the noted higher specific activity of the top cells separated by centrifugation would indicate that the two methods do not yield quite similar cell populations. Both methods, however, can yield populations enriched in either old or young cells; the ^{59}Fe studies suggest that the centrifugational method is more efficient in this respect. Labelling of red cells with radiochromates in ratios of the order of those used in clinical work did not seem to alter the pattern of osmotic fragility or the density variation, with respect to age.

The preferential uptake of ^{51}Cr by young cells has to be considered in the interpretation of the *in vivo* ^{51}Cr-labeled red cell survival curve. It assumes particular significance in conditions associated with an abundance of reticulocytes (3, 8,14). It has to be also seriously considered in cases of H-thalassemia, since hemoglobin H binds about twice as much radiochromate as hemoglobin A (15,16).

The differences of the ^{51}Cr-hemoglobin specific activity between young and old cells might be related to the alterations of the chemical or enzymatic environment or in the metabolic activity associated with cell aging (7,14,18).

SUMMARY

Differential osmotic lysis and centrifugional separation of red cells were used to study the relationship between red cell age and ^{51}Cr-hemoglobin binding and in *in vitro* elution of the label during electrophoresis.

The hemoglobin specific activity was uniform in hemolysates obtained by graded saline hemolysis except for the 1.0% most fragile cells, which showed a higher specific activity. The previously reported higher specific activity of the top cells separated by centrifugation was also shown. Labeling of red cells with radiochromate did not alter the pattern of osmotic fragility and density relationship between old and young cells.

The elution of label during electrophoresis was significantly higher from solutions obtained from old cells than from that of relatively young cells. Wash-

ing of cells with phosphate buffered ACD markedly reduced the elution of label. Artificial induction of increased fragility resulted in a marked increase in the rate of elution.

The significance of these findings in the interpretation of *in vivo* ^{51}Cr-labeled red cell survival curves is discussed.

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