

# Erythrocyte radiolabelling: in vitro comparison of chromium, technetium, and indium in undamaged and heat damaged cells

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**SUMMARY** The stability of the commonly used red cell radio labels chromium-51, indium-(111 or 113m), and technetium-99m, within intact red cells and stroma and their distribution within the cell were compared in undamaged and heat damaged red cells in relation to the clinical use of heat damaged cells in the assessment of splenic function.

Chromium-51 labelled haemoglobin both in undamaged and heat damaged cells; indium predominantly labelled haemoglobin in undamaged cells but labelled stroma in heat damaged cells, even when the cells were labelled before heating; technetium-99m predominantly labelled haemoglobin in undamaged cells but only labelled stroma in heat damaged cells if these were heated before labelling. Indium was more firmly bound by stroma prepared from heat damaged cells, and technetium-99m showed a high rate of elution both from cells and stroma, although this rate was lower for heat damaged cells.

The rate of clearance from blood of radiolabelled heat damaged red cells is used as an index of splenic function.<sup>1-5</sup> The kinetics of such clearance are complex, not only because of uptake at other sites in the body,<sup>2,6</sup> but also because splenic uptake itself is the result of several kinetic processes.<sup>4,5</sup> The stability of the radiolabel within the cells, the fragility of the cells, the stability of the label within fragments following disintegration of the cells, and the fate of such fragments, if labelled, all superimpose on the complexity of the overall clearance curve. With the ultimate aim of determining which of the commonly used isotopes chromium-51, indium-111 or indium-113m, and technetium-99m, used to label red cells, would be preferable for splenic function studies, we sought to determine the stability of these isotopes within heat damaged red cells, to identify their predominant site of deposition (cytosol or stroma), and to determine the stability within stroma of those isotopes deposited there.

## Material and methods

Blood was obtained from normal subjects using acid citrate dextrose (ACD) (NIH-A) as anticoagulant. The red cells were isolated and washed in physiological saline.

Labelling was performed with chromium-51,<sup>7</sup> technetium-99m,<sup>8</sup> indium-111,<sup>9</sup> or indium-113m,<sup>10</sup> using published methods. For indium labelling acetylacetone<sup>10</sup> or tropolone<sup>11</sup> was used as the ligand. Undamaged and heat damaged cells and stroma were studied. Damage was produced by heating 2 ml packed cells in a glass bottle at 49.5°C for 20 minutes. Stroma was prepared from heat damaged or undamaged cells by hypotonic lysis in excess distilled water, followed by centrifugation at 2000 g for 20 minutes. The percentage of activity bound to cells or stroma in suspension was determined by gamma counting (Packard, model 5360) activity in aliquots removed before and after centrifugation of the suspension at 2000 g for 20 minutes. Bound activity is then equal to the difference in counts divided by the precentrifugation count ( $\times 100$ ). The necessary correction for the haematocrit of the suspension was made only when it was 1% or more.

In some experiments carbon tetrachloride was added to the lysed cells before centrifugation to generate separate aqueous and lipid phases. Activity previously present in the suspension that had not been bound to stroma was then recovered in the aqueous phase.

Several experimental procedures were adopted to determine: firstly, the distribution of each isotope within the red cell; secondly, the effect of heat damaging on this distribution; and thirdly, the stability of the label in cells—fragmented or intact.

#### HEAT INDUCED LYSIS AND ELUTION

Aliquots of 100  $\mu$ l were removed at 10 minute intervals from packed cells undergoing heat damage. They were transferred to volumes of 20 ml 1.2% saline at room temperature, and cell bound activity was measured. For the determination of cell lysis the haemoglobin concentration was measured in two 5 ml aliquots of this suspension, one before and the other after centrifugation (Gallenkamp colourimeter 13). Cell bound haemoglobin was then calculated in the same way as that for the cell bound activity.

#### LABELLING EFFICIENCY OF LABELLED LYSATES

Depending on the isotope, considerable activity was found in stroma. Because of the possibility that stroma becomes labelled after hypotonic lysis as a result of redistribution of isotope from cytosol, the ability of labelled particle free lysate to transfer its label to fresh stroma or cells was tested. Thus after hypotonic lysis of the labelled cells the lysate was separated from the stroma by centrifugation and added to fresh cells or stroma.

#### DISTRIBUTION OF LABEL FOLLOWING LYSIS IN INCREASING WATER VOLUME

As a further examination of the above possibility hypotonic lysis was performed in volumes of water to give ratios of red cell volume to water volume of  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ , and  $10^{-4}$ . Distribution of label between stroma and cytosol was then measured.

#### STABILITY OF LABEL

Labelled cells were resuspended in excess saline or autologous plasma. After 30 minutes and one hour aliquots were removed and the percentage cell bound activity (c) determined. Elution (k) was determined by least squares regression analysis based on the equation:

$$C = e^{-Kt}$$

or

$$\ln C = -Kt$$

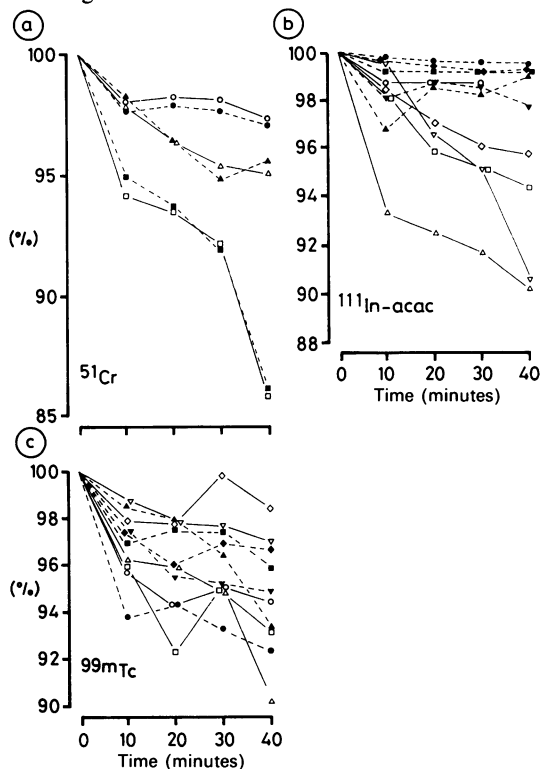
The percentage of activity that is cell bound at  $t = 0$ ,  $C(0)$ , inversely reflects the quantity of isotope released from the cell on resuspension.

#### ELUTION FROM STROMA

Because technetium and indium label stroma, the release of these two isotopes on resuspension of stroma and the subsequent elution rate were determined, as described for intact cells. The percentage of remaining activity lost from the stroma with further centrifugation and resuspensions (washes) and the final activity left on the stroma after a total of two washes were also determined.

#### ASSOCIATION OF HAEMOGLOBIN WITH CYTOSKELETON

To check the possibility that the change in distribution of isotope induced by heating is simply the result of increasing binding of haemoglobin to heat damaged stroma the association of haemoglobin with cytoskeleton in heat damaged v undamaged cells was determined. Stromal associated haemoglobin was determined by comparing the haemoglobin concentrations in two identical hypotonically lysed red cell suspensions, one before and the other after centrifugation.



Time course of percentage of radioactivity (closed symbols) and haemoglobin (open symbols) remaining particle bound during heating of radiolabelled red cells at 49.5°C; cells labelled (a) with Cr-51; (b) In-111-acetylacetonate; and (c) Tc-99m. Corresponding paired symbols represent one subject's blood.

Table 1 Mean (SE) distribution of radiolabel between stroma and cytosol (a) after hypotonic lysis (percentage total activity bound to stroma); (b) after hypotonic lysis and carbon tetrachloride extraction (total activity minus activity in aqueous phase (per cent))

	Undamaged		Heat damaged			
	Hypotonic lysis	Hypotonic lysis and carbon tetrachloride extraction	Damaged, then labelled		Labelled, then damaged	
	(a)	(b)	(a)	(b)	(a)	(b)
Chromium	12 (4)	7 (2)	13 (6)	10 (3.5)	8 (5)	8 (2.5)
Technetium	16 (10)	8 (8)	65 (11)	73 (6)	29 (13)	22 (9)
Indium (acetylacetone)	26 (8)	20 (4)	78 (5)	77 (2)	75 (5)	73 (4.5)
Indium (tropolone)	24 (11)	19 (3)	73 (8)	75 (2)	77 (6)	75 (6)

Mean ± SE (n = 6-8).

**Results**

**HEAT INDUCED ELUTION AND LYSIS**

The Figure shows the percentages of haemoglobin and radiolabel remaining cell bound at different times after heating for indium and chromium. With chromium it is evident that the free isotope liberated was equal to the free haemoglobin liberated. The same was essentially true for technetium, although the results were more variable. With indium, however, more free haemoglobin was liberated than isotope, indicating that most of the isotope remained in association with the stroma after heat induced lysis.

**DISTRIBUTION OF RADIOLABEL BETWEEN STROMA AND CYTOSOL**

Distribution determined by hypotonic lysis and centrifugation was essentially the same as that recorded when the stroma was extracted into carbon tetrachloride before centrifugation (Table 1) and was in line with the distribution suggested by the heat induced elution results described above. With chromium about 90% of the label was in the cytosol, whether the lysed cells were previously undamaged or not. With technetium about 85% of the activity was found in the cytosol liberated from undamaged cells. If the cells were heat damaged, however, the distribution was different. Thus about 75% of the activity was in the cytosol when the cells were labelled before they were heated, or 30%, if the cells were heated before being labelled. With indium about 80% of the activity

was in the cytosol of undamaged cells, whereas only 25% was in the cytosol when the cells were damaged (irrespective of the order of heating and labelling and irrespective of the ligand used).

**EFFECT OF RED CELL:WATER VOLUME ON DISTRIBUTION**

Increasing the volume of water for hypotonic lysis had no appreciable effect on the subsequent distribution of radiolabel between stroma and cytosol (Table 2), suggesting that the stroma was not labelled as a result of uptake of isotope from solution immediately after lysis.

**LABELLING EFFICIENCY OF LABELLED LYSATE**

In line with the preceding observation, stroma free lysates, prepared from previously labelled cells, were unable to label fresh cells (after adjustment of osmolarity to physiological with hypertonic saline) or stroma (without adjustment of osmolarity), unless further ligand was added. Restoring the acetylacetone concentration in the lysate to 0.19% gave a labelling efficiency of 48 ((SE) 5)%, to 0.38%, an efficiency of 54 (2)%, and to 0.95%, an efficiency of 64 (0.6)%. No differences were detected between "undamaged" and "heat damaged" lysates, or between undamaged and heat damaged cells or stroma.

**ASSOCIATION OF HAEMOGLOBIN WITH STROMA**

In undamaged cells 8 (0.6)% of the haemoglobin was associated with stroma in so far as it was sedimented

Table 2 Effect of ratio of labelled red cell:water (used for hypotonic lysis) volume on distribution of radiolabel between stroma and cytosol (values expressed as percentage of total activity bound to stroma after lysis)

Volume of cells (ml)	Volume of water (ml)	Indium		Technetium
		Undamaged	Heat-damaged (before labelling)	Heat-damaged (before labelling) (mean SE)
0.1	1	22 (n = 2)	80 (n = 2)	52 (4) (n = 3)
0.1	10	21 (n = 2)	79 (n = 2)	57 (n = 2)
0.1	100	25 (n = 2)	80 (n = 2)	52 (n = 3)
0.01	100	37 (n = 2)	81 (n = 2)	48 (0.6) (n = 3)

Table 3 Loss of isotope from stroma, prepared from undamaged and heat damaged cells after resuspension of stroma in saline or plasma

	Indium (mean SE)		Technetium (mean SE)	
	Saline	Plasma	Saline	Plasma
<b>Undamaged:</b>				
First resuspension	18 (3) (n = 11)	42 (5) (n = 6)	44 (8) (n = 6)	26 (1.5) (n = 4)
Second resuspension	3.3 (1.2) (n = 8)	10.3 (0.5) (n = 4)	35 (8) (n = 4)	
Third resuspension	2.6 (1.2) (n = 7)	12.0 (3) (n = 4)	20 (4) (n = 3)	
Stroma	84 (4) (n = 8)	42 (10) (n = 4)	18.5 (6) (n = 4)	
<b>Heat damaged (before labelling):</b>				
First resuspension	11 (2.4) (n = 8)	10 (2) (n = 4)	8 (2.5) (n = 4)	7 (2) (n = 4)
Second resuspension	2.2 (0.7) (n = 4)		7.6 (n = 2)	
Third resuspension	2.9 (0.6) (n = 4)		5.6 (n = 2)	
Stroma	92 (2) (n = 4)		74 (n = 2)	

Results expressed as mean  $\pm$  SE (n in brackets).

Table 4 Elution rate of isotope from labelled red cells and stroma (%/hour) resuspended in saline or plasma

	Stroma		Cells	
	Saline	Plasma	Saline	Plasma
<b>Indium:</b>				
Undamaged	3.1 (1.5) (n = 11)	8.4 (3) (n = 6)		
Heat damaged (before labelling)	0.8 (1.2) (n = 8)	3.6 (1.8) (n = 4)		
<b>Technetium:</b>				
Undamaged	10.6 (3.2) (n = 4)	12.8 (1) (n = 3)	4.9 (3) (n = 5)	9.4 (n = 2)
Heat damaged (before labelling)	8.3 (2.5) (n = 5)	6.2 (1.5) (n = 4)	4.7 (1) (n = 3)	6.3 (n = 2)

The loss of activity from indium and chromium labelled cells (undamaged and heat damaged (before labelling)) was negligible at less than 1%/hour.

by centrifugation after hypotonic lysis. The corresponding value for heat damaged cells was similar at 8.3 (0.3)%.

#### LOSS OF ACTIVITY ON WASHING OF STROMA OR CELLS AND ELUTION RATE

Tables 3 and 4 summarise the percentages of activity liberated into the medium after washing of stroma (labelled with indium and technetium) and the elution rates of activity from such stroma and from labelled intact cells.

Chromium and indium remained firmly bound to intact cells, both undamaged and heat damaged, whereas technetium eluted at 5%–10% per hour. The activity (technetium and indium) on undamaged stroma was less firmly bound than that on heat damaged stroma, detaching more readily on washing and eluting more rapidly. Activity was also more readily removed from stroma when the medium was plasma rather than physiological saline.

The labelled undamaged or heat damaged cells were lysed in water, the stroma pelleted, and the lysate removed. The labelled stroma was resuspended in saline or plasma. The values shown in Table 3 under first resuspension were the percentages of the total activity in the suspension not bound to the stroma—that is, the percentage activity released on the first resuspension. The stroma was pelleted again and resuspended in more saline or plasma and this time the values shown (Table 3) were the percentages of

remaining activity not bound to stroma after this second and third suspension—that is, the percentage activity released with each resuspension. "Stroma" indicates the percentage of the initial activity still bound to stroma after the successive washes.

#### Discussion

In this study we found that chromium binds to components in the cytosol of the red cells. Others<sup>12</sup> have described the binding site as the globin moiety of haemoglobin, and our results are consistent with this. Technetium is also thought to bind predominantly to globin,<sup>13,14</sup> and our results were also consistent with this, with regard to undamaged cells. With respect to heat damaged cells, however, the distribution seemed to be different, with much more activity present in the stroma. Indium has previously been reported to bind to both haemoglobin and stroma.<sup>15</sup> Again, our results were consistent with this finding.

Although Rao and Dewanjee<sup>15</sup> found only about 40% of the activity in the haemolysate of undamaged cells labelled with indium tropolonate, they, like us, found about 75% in the haemolysate from cells labelled with indium acetylacetonate, at a concentration similar to that used in our study. When cells were heat damaged, however, we found a quite different distribution, with only about 25% in the cytosol. Interestingly, this distribution seemed to be unaffected not only by the ligand used (acetylacetone or tro-

polone), but also by the order in which the cells were labelled and heated. This curious finding suggested a possible artefact in that such a distribution may not have been present before lysis but may have been the result of an avid uptake of free isotope (still complexed with lipid soluble ligand) by the stroma from the lysate after lysis. This possibility was, however, excluded by showing that labelled lysates were unable to label fresh cells or stroma and by finding that the relative volumes of cells and water (used for lysis) had no effect on apparent distribution. The transfer of label from cytosol to stroma induced by heating could not be explained by the fixation of labelled haemoglobin to stroma as the amount of haemoglobin associated with stroma was not increased after heat damaging.

As an appreciable fraction of indium and, in the case of heat damaged cells, technetium, was associated with stroma, it seemed worthwhile to evaluate the stability with which these isotopes labelled stroma. Indium was very firmly bound, particularly to stroma from heat damaged cells, when evaluated as an elution rate or as activity released on washing. Activity detached from stroma more readily in plasma than in saline, reflecting the avidity of indium for transferrin. Technetium was also more firmly bound by heat damaged than by undamaged stroma, as reflected by stability during washing, although as most of the activity in the undamaged cell is in the cytosol, the activity apparently released on the first resuspension of stroma may be overestimated.

Given that the heat damaged red cell clearance curve is corrected for plasma bound activity, the fate of stroma determines the relevance of stromal binding. About 90% of stroma is cleared by the liver,<sup>16</sup> almost certainly much more rapidly than splenic clearance of intact heat damaged cells. When there is lysis in the preparation before injection, the clearance of indium labelled heat damaged cells would, in addition to splenic uptake, contain a component of liver stromal uptake, a negligible component, however, due to elution. Technetium labelled cell clearance should also contain a component resulting from elution. The clearance of chromium labelled cells, on the other hand, would be predicted to be free of components, both of stromal uptake and elution. Any lysis occurring in vivo would be registered as intact cell clearance, although this would be common to all three labels. This implies that chromium is the best label, notwithstanding its poorer counting statistics. On the other hand, the washing of technetium or indium labelled heat damaged cells in isotonic rather than hypertonic saline, and the use of minimal centrifugation speeds before injection decreases the component due to liver uptake after injection,<sup>5</sup> pre-

sumably as a result of the inclusion of less labelled stroma. Using this approach, indium should then be as good as chromium.

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