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Irreversible Cellular Damage Following a Commonly Employed Iodination Procedure

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INTRODUCTION

^{131}Iodine has been widely used as a label that can be attached to soluble proteins. Antibodies, hormones, and blood proteins have been labeled in this manner (3, 6, 9). Because the functional characteristics of the protein under study remains unaltered, investigators are not able to comment without qualification on the integrity of the complete iodinated protein. The studies in the present report indicate that intact cells are a sensitive test system for evaluating the biological effects of the labeling procedure. They are more sensitive indicators than proteins because the intact cell is a complex unit depending on correlated dynamic processes for maintenance of the normal state. Any measurable alteration in these dynamic processes may indicate deleterious effects, whereas in protein only changes in the part of the molecule that is specific for the test system would be detectable. Deleterious effects are described even when concentrations of reactants are lower than those reported to produce no detectable effects on protein specificity.

The present report describes the conditions required for the iodination of intact dog erythrocytes (RBC), lymphocytes and rat thymocytes using the method of Greenwood, *et al*, (3). Criteria of effects of the procedure on the cells are 1) change in erythrocyte mean corpuscular volume (MCV), 2) *in vivo* life span of labeled erythrocytes, 3) growth of labeled lymphocytes *in vitro*, and 4) morphology of labeled cells.

MATERIALS AND PROCEDURES

Composition of Solutions: The composition of all solutions used is tabulated in Table I.

Preparation of Cell Suspensions: Thymocytes were prepared just prior to use in labeling experiments. Rats (University of Rochester strain, a derivative of the Wistar strain) were given a lethal dose of ether; the thymus was removed and

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placed in a vial containing 10 ml. of phosphate-buffer, cut into pieces, and the cells expressed by gentle squeezing of the cut pieces with forceps. The resulting suspension was filtered through glass wool into a centrifuge tube. Thymocytes were concentrated by centrifuging at 450 g (International Model 5, rotor number 241) for five minutes at room temperature (23°C.). The supernatant fluid was discarded and the wet cellular sediment was resuspended in phosphate-buffer to give a concentration of 5×10^7 cells per ml.

Lymphocytes were obtained from the peripheral blood by the method of Cassen, Hitt and Hays (2). The lymphocytes were further concentrated by diluting them 1 to 3 with buffer and layering the cell suspension on polyvinylpyrolidone (PVP) solution for one hour, a procedure which causes aggregation and sedimentation of RBC, leaving lymphocytes in suspension.

TABLE I

COMPOSITION OF SOLUTIONS[2] AND SOURCE OF REAGENTS

Solute	*Solvent*	*Concentration*	*Source*
KI	Buffer[1]	50,400 μg/ml.	Std. reagent USP
Chloramine T	Buffer	500,125 μg/ml.	Eastman Organic Chemicals Rochester, New York
$Na^{131}I$	Buffer	2 and 200 μc/ml.	Oak Ridge, Oak Ridge, Tennessee
$Na^{125}I$	Buffer	2 and 200 μc/ml.	Nuclear Science Engineering Corp., Pittsburgh, Pennsylvania
NADPH	Buffer	1 μmole/ml.	Calbiochem, Los Angeles, California
Sodium Ascorbate	Buffer	1 mg./ml.	Std. reagent USP
Sodium Nitrite	Buffer	100% Saturated (stock)	Std. reagent USP
Phytohemagglutinin (PHA)	Buffer	~ 3 μg./ml.	[4]
Trypsin	Tis-U-Sol[3]	4.5 mg./ml.	Calbiochem, Los Angeles, California
TC-199			Difco, Detroit, Michigan
Polyvinylpyrrolidone (PVP)	Distilled Water	21 gm. %	General Aniline and Film Corp., New York, New York

[1]Buffer = .0014 M $NaH_2PO_4 \cdot H_2O$, .0135 M Na_2HPO_4, 0.134 M NaCl, .005 M KCl and .001 M EDTA; pH = 7.4 and 300 milliosmolar.

[2]All solutions must be freshly prepared.

[3]Tis-U-Sol 289 milliosmolar, Baxter Laboratories, Inc., Morton Grove, Illinois.

[4]Prepared according to the method of Rigas and Johnson, (8).

Intact RBC were obtained from donor dogs by centrifuging whole blood (EDTA used as anticoagulant) at 450 g for 15 minutes at room temperature. The buffy coat and plasma supernatant were discarded and the RBC were washed in 25 ml. of phosphate-buffer and centrifuged as before. The wash supernatant was discarded and the erythrocytes were suspended in enough phosphate-buffer to give a concentration of 1×10^9 cells per ml.

Iodination Procedure: Several parameters of the iodination procedure were evaluated to find the optimal conditions for labeling cells. The various combinations used in the experiments are summarized in Table II and figure 1. In each case, 0.1 μC of ^{131}I (or ^{125}I) and the carrier, KI (Table II, column 1 and figure 1) were added to the cell suspensions. Immediately thereafter chloramine T (column 2) was pipetted into the suspension and the test tube gently shaken to insure mixing. Cells were then centrifuged for 10 minutes, the supernatant discarded and the cells washed three times in 2 ml. of phosphate-buffer. Radioactivity in the cell suspensions was determined after each wash, using a thalium-activated sodium iodide well-type counter and was expressed as the percentage of the initial amount present before addition of chloramine T. (0.1 μC ^{131}I is equivalent to 10^5 counts per minute with a background of 10^2 counts per minute.)

Criteria of Labeling Effects: Two methods were used to test the viability of thymocytes after labeling: a) stained blood films were examined by light

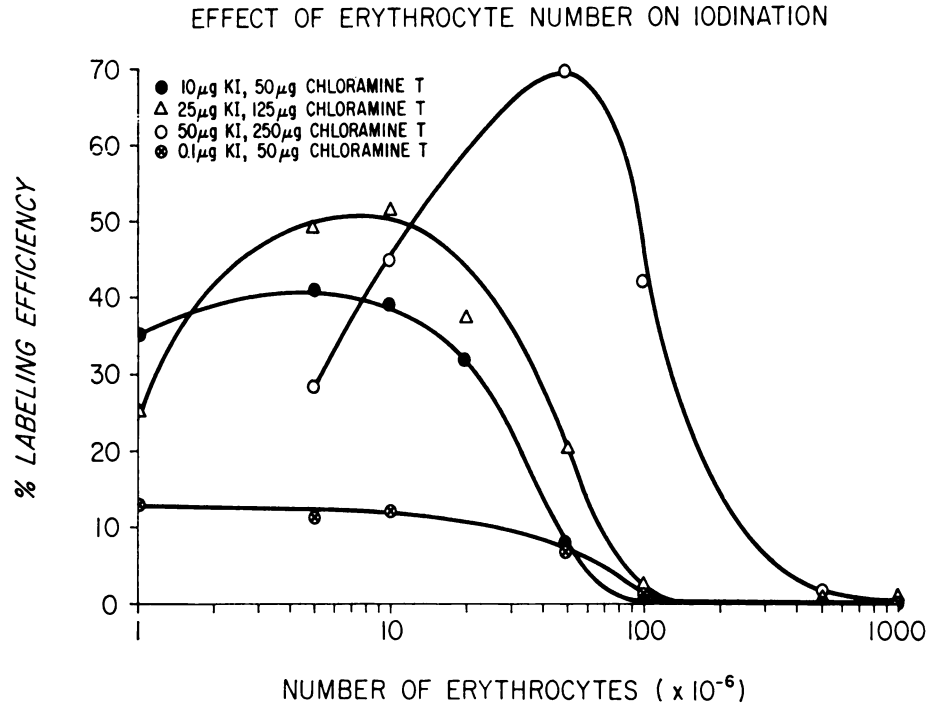

Fig. 1.

microscopy and b) susceptibility of the labeled cells to lysis by trypsin was determined according to the method of Hirata (5).

TABLE II

EFFECTS OF VARYING AMOUNTS OF CHLORAMINE T AND IODIDE ON THYMOCYTE, ERYTHROCYTE AND LYMPHOCYTE LABELING

Carrier Iodide-127 μg	*Chloramine T* μg	*Type of Cell*	*No. Cells*	*Percent of Total Radioactivity on Thymocytes and Lymphocytes*[1]
3–100	0	Thymocyte	5×10^7	Trace[2]
100	100	Thymocyte	5×10^7	7.8 ± 2.0 (6)
	200	Thymocyte	5×10^7	18.3 ± 2.0 (6)
	400	Thymocyte	5×10^7	43.2 ± 1.8 (6)
	800	Thymocyte	5×10^7	44.3 ± 1.5 (6)
	1600	Thymocyte	5×10^7	44.4 ± 2.0 (6)
50	50	Thymocyte	5×10^7	10.1 ± 5.0 (6)
	100	Thymocyte	5×10^7	12.5 ± 3.0 (6)
	200	Thymocyte	5×10^7	23.8 ± 1.4 (6)
	400	Thymocyte	5×10^7	26.5 ± 2.0 (6)
	800	Thymocyte	5×10^7	24.2 ± 3.0 (6)
25	25	Thymocyte	5×10^7	3.1 ± 2.0 (6)
	50	Thymocyte	5×10^7	7.8 ± 2.0 (6)
	100	Thymocyte	5×10^7	14.2 ± 2.7 (6)
	200	Thymocyte	5×10^7	16.1 ± 3.0 (6)
	400	Thymocyte	5×10^7	15.6 ± 3.4 (6)
10	50	Lymphocyte	1×10^7	16.9 (1)
3	12	Thymocyte	5×10^7	5.9 ± 1.0 (6)
1	5	Lymphocyte	1×10^7	3.4 ± 2.9 (3)

±Standard error of the mean.
[1]p.b. Lymphocytes for culturing.
[2]< 3X background.

In addition to the experiments summarized in Table II, thymocytes were iodinated in the same manner using 3, 6, 12, 25 and 50 μg carrier iodide with and without ^{131}iodine. The ratio of chloramine T to iodide was maintained at four throughout, except in two control determinations in which cells were exposed to (a) 400 μg of iodide with no chloramine T added and (b) 400 μg of chloramine T with no iodide added. Another suspension of cells was not iodinated, but

was subjected to the same manipulations and served as an additional control. One ml. of trysin (4.5 mg./ml.) was added to 5×10^7 cells suspended in one ml. of Tis-U-Sol and this suspension was incubated at room temperature for 30 minutes. Decrease in cell count was determined. Cover glass films of cells were prepared before and after exposure to trypsin, stained with Wright's blood stain and examined by light microscopy.

Lymphocytes labeled with 1.0 or 10 μg of carrier iodine (Table II) and 50 μg of chloramine T were exposed to phytohemagglutinin according to the method of Nowell (7). Lymphocytes exposed to the same manipulations but not to the iodinating reagents were cultured as a control. The cultures were sampled at 24 and 72 hours, treated with trypsin and observed under phase microscopy. Cover-slip films were prepared, stained with Wright's blood stain and examined for morphological transformation of lymphocytes.

Cell volume frequency distribution histograms on all erythrocyte samples were made using a Model B Coulter Counter, with an automatic particle size distribution plotter. Treated cells were diluted with phosphate-buffer to give approximately 10^5 cells per ml. Instrument settings were: amplification = 1; aperture current = 2; amplification trim = 70. A 50 μ orifice was used to minimize coincidence counting, and improve resolution of the histograms.

Mean corpuscular volume (MCV) determinations on all samples were made

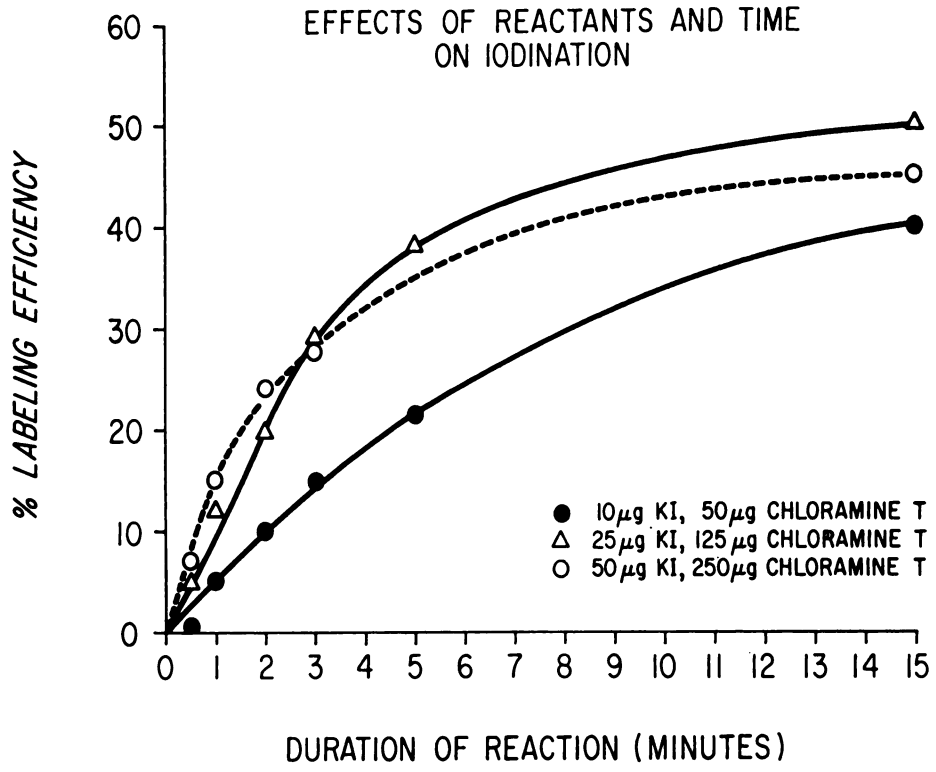

Fig. 2.

using an automatic MCV computer, an accessory to the Coulter Counter. Twenty-five MCV readings were recorded for each sample. The counter and computer were standardized against RBC of known MCV.

Effects of Reducing Agents on Iodination Procedure: Iodination is mediated by oxidation of iodide. It was thought that some of the deleterious effects observed following iodination might be due to oxidation of cellular constituents. For this reason, several physiological and chemical reducing agents were evaluated with respect to their ability to prevent or reverse erythrocyte volume changes. Furthermore, by their addition, the effect of exposure time on the efficiency of iodination could also be assessed.

Reducing agents, nicotinamide adenine dinucleotide phosphate reduced (NADPH), sodium ascorbate, and glutathione reduced (GSH) were added to separate suspensions containing 1×10^7 RBC and 0.1μc ^{131}iodide at the times shown in figure two. The reducing agents were in 3 molar excess to chloramine T. Cells were then washed; radioactivity was determined, and cell volume frequency distribution histograms and MCV were obtained. Photomicrographs were taken of iodinated cells using phase microscopy.

Comparison of an Iodination Effect with Oxidation by Sodium Nitrite: In order to see if an increased MCV could be produced by an oxidizing agent alone, as well as iodination, cells were exposed to $NaNO_2$. An erythrocyte-nitrite mixture containing 1×10^7 cells and 3 ml. of a 25%, 50% and 100% saturated $NaNO_2$ solution was allowed to stand at room temperature for one minute. The suspension was centrifuged for 10 minutes at 450 g, the supernatant discarded and cells were resuspended in 2 ml. of buffer. A 1:50 dilution of this suspension was made in buffer for cell volume distribution plotting and MCV determination.

Erythrocytes were labeled using 200μC ^{131}iodide or ^{125}iodide, 0.1μg ^{127}iodide as a carrier and 50μg chloramine T. To obtain a large quantity of cells labeled under optimal conditions, ten separate 1 ml. suspensions of erythrocytes were labeled, washed twice, pooled and resuspended in a final volume of 5 ml. buffer from which a 0.5 ml. sample was removed for MCV and radioactivity determinations. The remaining suspension was infused into the brachial vein of the same dog from which the cells had been previously removed. A second batch of cells was labeled according to an identical protocol, except that 10μg of carrier ^{127}iodide was used.

Peripheral blood samples were taken from the external jugular vein at 4 minutes, 10 minutes, 30 minutes, 1, 2, 3, 4, 21 and 36 hours to determine the rate of disappearance of labeled cells from the circulation.

RESULTS

Labeling Efficiency with ^{131}I: The results of thymocyte and lymphocyte labeling experiments are tabulated in column 5 of Table II. The erythrocyte labeling experiments are summarized in figure one, and figure two.

Four major variables that influence labeling efficiency have been identified. The first of these is the absolute amount of carrier iodide. Under the conditions described for thymocyte labeling, (Table II), the efficiency increases with increasing amounts of carrier iodide when it is in constant ratio to chloramine T.

The second variable is the ratio of chloramine T to carrier iodide. As this ratio increases up to a value of 4, the labeling efficiency increases; beyond this there is no further significant increase. The third variable is the time during which cells are exposed to reactants, the efficiency increasing with longer exposures (Fig. 2). The reducing agents, NADPH, ascorbate or GSH were equally effective in terminating the iodination reaction. The fourth variable is the number of cells (Fig. 1). There is an optimal number of cells for maximal labeling efficiency using a given amount of reactant. Labeling efficiency decreases if either more or fewer cells are used.

When concentrations of either thymocytes or erythrocytes and reactants are optimal for labeling, there is no loss of label when cells are washed repeatedly or dialysed for 24 hours against phosphate buffer. When the conditions are not optimal, radioactivity is lost with each wash up to the third. Thereafter, no more radioactivity can be washed off, nor is any lost during dialysis against buffer for 24 hours. The greater the deviation from optimal conditions, the more radioactivity is lost by washing.

In figure two, each point represents the time each reducing agent (NADPH, ascorbate, and GSH) was added and the percent labeling efficiency for the reducing agents as a group. It is evident these reducing agents can effectively stop

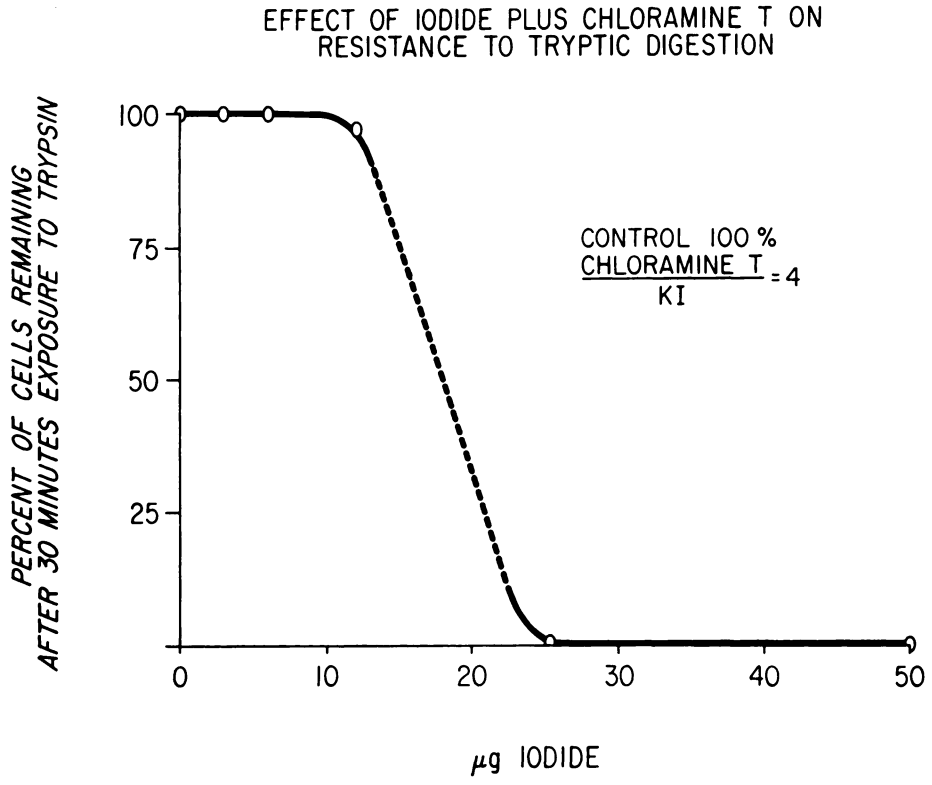

Fig. 3.

the iodination reaction at zero time. When any of the reducing agents are added at progressively later stages after initiation of the iodination reaction, the amount of incorporation up to that point can be determined. By 15 minutes exposure of the cells to reactants, the iodination is essentially complete.

Effect of Labeling on Cell Viability: The effect of trypsin on the cell count immediately after thymocyte labeling is shown in figure three where the change in cell count is expressed as per cent of the trypsin-treated control suspension. Iodide or 1μC of ^{131}I alone did not render the cells susceptible to trypsin. Four hundred micrograms of chloramine T alone had about the same effect on the susceptibility as 12μg of iodide and 50μg of chloramine T together. Suspensions of labeled thymocytes shows no change in cell count or morphology at any concentration of iodide together with chloramine T until after exposure to trypsin.

Labeled lymphocytes in tissue culture were susceptible to tryptic digestion 24 hours after the culture was planted and the cells remaining at 72 hours showed no signs of morphological change or mitosis, typical of normal lymphocytes in PHA cultures (transformation). The per cent iodination of these lymphocytes is shown in Table II. Control lymphocyte cultures showed typical transformation and were resistant to the proteolytic agent at both 24 and 72 hours.

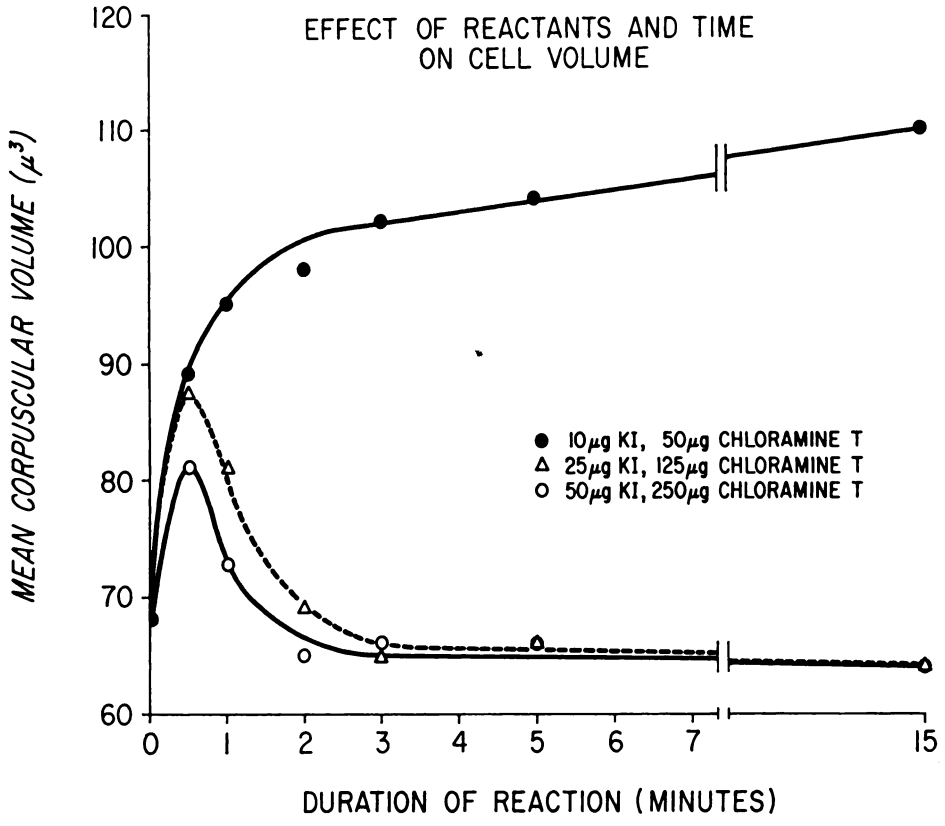

Fig 4.

The effect of labeling on mean corpuscular volume of erythrocytes varied with the concentration of reactants. At the lowest concentration, (10.0μg iodide, 50μg chloramine T), the MCV as observed on the histogram and the MCV computer increased approximately 50% within the first minute after exposure and showed little change thereafter. In contrast, the MCV of cells exposed to higher concentrations of reactants reached a peak value after 30 seconds exposure and then abruptly decreased, reaching a level within the range of the pre-exposure values two minutes later. These changes are depicted graphically in figure four which shows that the highest concentration of reactants produced the smallest increase in MCV one minute after exposure. Because cells were washed before MCV was measured, the data in figure four were actually obtained approximately 15 minutes after the reaction was interrupted. To ascertain that the curves truly represented the MCV at the time shown, one series of MCV measurements was obtained for unwashed cells at intervals ranging from 20 seconds to 15 minutes after interrupting the reaction. These measurements, actually made immediately after the reaction was stopped, gave a curve of the same shape as that shown in figure four and absolute values in the same range. Figure 5 a, b, c shows phase micrographs of erythrocytes treated with 0, 10 and 25 μg KI, respectively, $\left(\frac{\text{chloramine T}}{\text{iodide}} = 5\right)$.

The effect of the oxidizing agent, $NaNO_2$, on MCV of RBC is shown in figure six. The increase in MCV shown as a function of increasing concentrations of $NaNO_2$ demonstrates that this oxidizing agent can also cause an increase in MCV.

Fate of Labeled Cells In Vivo: The severe injury of erythrocytes labeled with either 0.10 or 10μg of iodide and 50 μg of chloramine T was confirmed by a dramatic *in vivo* life span shortening (Fig. 7), as compared to the survival time of normal dog erythrocytes of 100 days. The change in MCV of cells labeled with 10 μg KI was marked, whereas the change in MCV of cells labeled with a .10 μg KI was negligible. In both cases, however, the clearance half-time of the injected labeled cells was 12 hours. No radioactivity appeared in the plasma before two hours.

DISCUSSION AND CONCLUSIONS

Iodine-131 and ^{125}I can be attached with good efficiency to intact thymocytes, lymphocytes and erythrocytes, by a slight modification of an iodination procedure

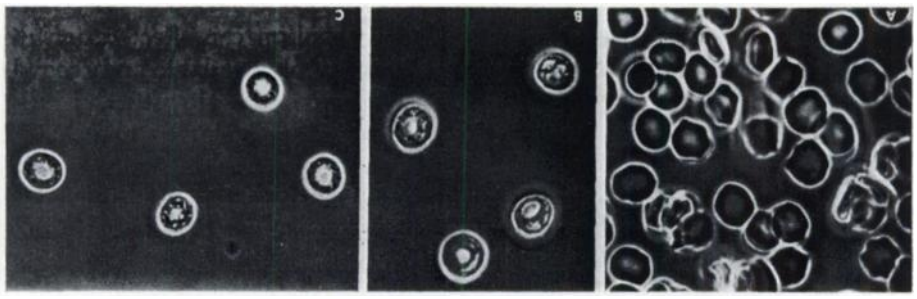

Fig. 5.

used to label soluble protein. These experiments also demonstrate that intact cells can be labeled, using lesser amounts of reactants than those employed when labeling protein.

There appear to be two variables influencing the amount of elution following labeling, namely deviation from optimal number of cells, and too brief an exposure of cells to reactants. Although these factors affecting elution have been isolated, the chemical nature of the eluted substance itself remains undetermined. The authors feel that the elutable product is the end result of reactions competitive with and inseparable from the iodination reaction. When the optimal number of cells is used, the competitive reactions are minimal and labeling efficiency is maximal. One such competitive reaction is the formation of iodate, either by non-specific oxidation of iodide by the chloramine T or by disproportionation of HOI.

The following schematic summary indicates possible reactions that are consistent with the data presented:

1) Chloramine T + $H_2O \leftrightarrows$ HOCl + Toluene p-sulfonamide

2) $HOCl + I^- \longrightarrow HOI + Cl^-$

3) $HOCl + HOI \xrightarrow{\text{non-specific oxidation}} HIO_3 + Cl^-$

4) $\left.\begin{matrix} HOI \\ HOCl \end{matrix}\right\} \xrightarrow{\text{substate}} \text{Non-Specific oxidation} + \left\{\begin{matrix} I^- \\ Cl^- \end{matrix}\right.$

$\xrightarrow{\text{substrate}}$ Halogenation

$\xrightarrow{\text{disproportionation}} IO_3 + 2I^- + ClO_3 + 2Cl^-$

The chloramine T procedure for labeling proteins with iodine is an oxidation which can be viewed as a change of the iodide to HOI by the chloramine T. This change may be effected through hydrolysis of chloramine T to HOCl, which then oxidizes the iodide to HOI. The positive I can either iodinate the cellular proteins or bring about non-specific oxidation.

The mean corpuscular volume of the erythrocyte depends upon the distribution of anions, cations and water between the suspending medium and the intracellular contents. Maintenance of these distributions depends upon a number of physiological processes which have been extensively studied and reviewed (1). It has also been shown that changes in cell metabolism can effect cation distribution in two ways. First, by varying the energy supply available and second, by altering the membrane resistence to passive diffusion. The alteration in cell volume observed after iodination may be attributed to the presence of HOI or HOCl during the reaction. By oxidation of enzymes or through the incorporation of iodine into protein, these moieties may derange the biochemical processes responsible for the maintenance of normal cell volume.

The possibility of enzyme oxidation is consistent with the observed change in cell volume following exposure to the oxidizing agent, sodium nitrite, and probably accounts for the severe damage to erythrocytes.

All iodinated cells exhibited as initial increase in MCV. In those cells iodinated with 25 or 50 μg of iodide, this response was transient and was followed by a return of cell volume to the normal range. The initial swelling observed when cells are iodinated with 25 and 50 μg iodide may be attributed to the colloidal effects of intracellular protein, in the presence of a non-operative sodium pump. Subsequent reduction of cell volume can be attributed to fixation. During the first 30 seconds when the cell is swelling, the HOI concentration is increasing. Continued exposure of cells to the ever-increasing HOI concentration may perhaps result in fixation of the membrane and intracellular proteins and reduction of erythrocyte volume.

An additional indication of irreversible erythrocyte injury is their rapid removal from circulation. Since no radioactivity appeared in the plasma until two hours after injection, the loss of radioactivity from the blood represents loss of erythrocytes from the circulation and not elution of the label from the erythrocytes.

These experiments show that intact living cells are sensitive indicators of the deleterious effects of iodination. Many investigators have assumed that no alteration of soluble proteins occurs subsequent to the iodination procedure, if few iodine atoms are incorporated. On the basis of the data presented, caution is urged in assuming that retention of protein activity following iodination can be equated with integrity of the entire protein.

Although labeled viable cells could not be prepared using this technique, the procedure may prove useful in preparing labeled particulates of uniform size. For example, labeled non-viable erythrocytes may be used to study clearance

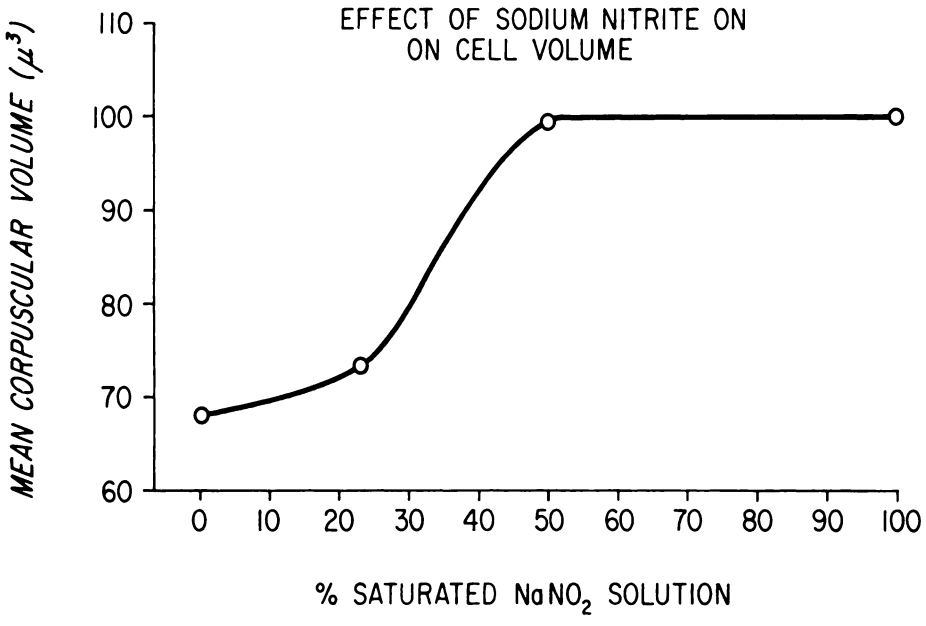

Fig. 6.

by reticuloendothelial system. If ^{125}I is used as the radioactive label, the presence of the ingested cells could be demonstrated by radioautography (4).

SUMMARY

A procedure for the rapid labeling of intact erythrocytes, thymocytes and lymphocytes with ^{125}I or ^{131}I is described. Labeling efficiencies are as high as 70%.

Four factors influencing labeling efficiency are identified, namely number of cells, absolute amount of iodide in constant ratio to chloramine T, chloramine T to iodide ratio, and duration of exposure of cells to reactants.

Profound and irreversible cellular damage is observed even when the amounts of reactants employed are less than those used to label soluble proteins without detectable alteration. This cell damage is thought to be caused by oxidation of cellular constituents.

Although labeled cells are not viable, the technique may prove useful in preparing labeled particles.

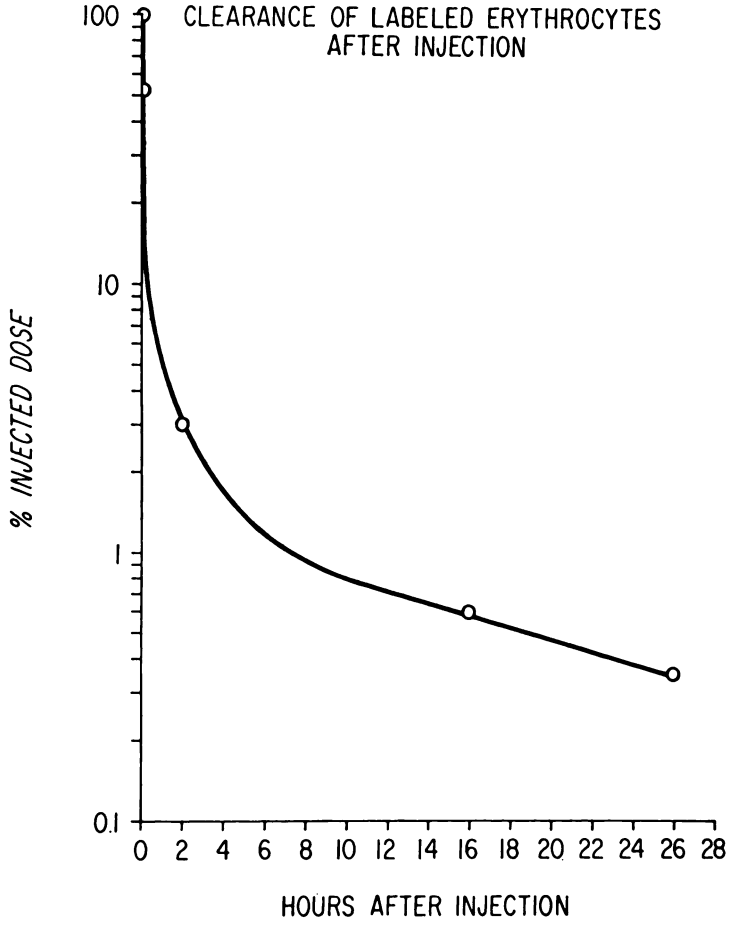

Fig. 7.

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