Comparison of Several Indium-111 Ligands in Labeling Blood Cells: Effect of Diethylpyrocarbonate and CO_2

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The effect of oxine sulfate, oxine sulfonate, tropolone, and Merc (2 mercaptopyridine-1-oxide) were compared with oxine, with respect to their capability of labeling blood cells when complexed to indium-111 (^{111}In). Indium-111 oxine sulfate performed similarly to [^{111}In]oxine with regard to cell labeling capability. Indium-111 oxine sulfonate had no labeling ability. Indium-111 tropolone and Merc were not superior to [^{111}In]oxine as cell labeling agents. Carbon dioxide (CO_2) and a CO_2 generating compound, diethyl pyrocarbonate, dramatically improved the cell labeling ability in plasma of [^{111}In]tropolone and Merc. In the case of oxine, this improvement was less distinct. Theoretical aspects of the CO_2 cell labeling stimulating effect are discussed in terms of intra- and extracellular transferrin and lactoferrin iron (indium) binding capacity. Indium-111 tropolone behaved favorably with respect to inhibition of leukocyte migration, compared with oxine and Merc. Combined with the property of easy cell labeling and good solubility in water, also in the complexed state, tropolone must be regarded as the most suitable cell labeling ligand.

J Nucl Med 28:1020–1026, 1987

Since the first report of the usefulness of indium-111 [^{111}In] oxine as a leukocyte labeling agent, by McAfee and Thakur in 1976 (*1*), cell labeling has proven to be useful in the diagnosis of abscesses (*2,3*). Indium-111 oxine nonselectively labels cellular blood components. Granulocytes (*4,5*), lymphocytes (*6*), platelets (*7–10*), and erythrocytes (*11*) are labeled after separation. These cells have been used to locate abscesses, myocardial infarctions, thrombus formation, rejection of transplanted organs, etc. Labeled erythrocytes have been used for blood volume measurement, cardiac, liver and spleen blood-pool activity, and for detection of intermittent gastrointestinal bleeding (*12,13*).

Acetylacetone, an alternative for oxine (*14*), was dropped after studying it in paired comparisons (*15*). Tropolone was proposed as an alternative for oxine and, as evidenced in the literature, proved to be more enduring than acetylacetone. Investigators reported easier labeling than oxine, especially when plasma residues were present (*16,17*). In addition, 2-mercaptopyridine-1-oxide (Merc) was proposed (*18*) as a labeling agent for cell suspensions containing plasma.

This study presents some comparisons of oxine, tropolone, and Merc in their cell labeling abilities, and focuses special attention on the buffer systems used to dissolve the labeling complexes. The presence of CO_2 seems to be important in the level of labeling efficiency.

MATERIALS AND METHODS

The following materials were used: [^{111}In]Cl_3 solution, consisting of carrier-free [^{111}In]Cl_3 in 0.1 N hydrochloric acid*. TRIS-acetate buffered [^{111}In]oxine*, a commercial preparation with 25 μg oxine and 1 mCi (37 MBq) ^{111}In/ml.

Besides [^{111}In]oxine, preparations were made containing different oxine concentrations, ranging from 2.5 μg to 50 μg/ml and containing 0.024 N HCl, 5.6 mg sodium chloride, 4.1 mg sodium acetate, and 3.025 mg TRIS. The pH was 7 and the isotonicity 280 mOsmol/kg.

TRIS-acetate buffered [^{111}In]tropolone, TRIS-acetate buffered [^{111}In]Merc, TRIS-acetate buffered [^{111}In]oxine sulfate, and TRIS-acetate buffered [^{111}In]oxine sulfonate† contained the same buffer constituents as the [^{111}In]oxine described above. Preparations were made with different ligand concentrations ranging from 2.5 μg to 50 μg/ml.

Cell suspensions of bovine erythrocytes and human eryth-

Received Feb. 4, 1986; revision accepted Oct. 22, 1986.

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rocytes were used. Human leukocyte and platelet suspensions were prepared according to Hardeman (*19*).

Cell labeling was carried out as follows: A 0.5-ml cell suspension was added to a 10-ml Wheaton vial. Then, 0.05 ml TRIS buffer 0.2*M* pH 8.0 was added. Under gentle mixing, 0.10 ml labeling complex (pH 3.0–3.5) was added. The mixtures were incubated for 20 min at pH 7 and room temperature. A sample was taken with a hematocrit capillary and centrifuged at 300 *g*. The capillaries were cut with a glass-saw just above the packed cells, and the separating ends were placed in counting tubes. The percentage labeling was determined with a counter using an NaI crystal.

In all cell labeling runs, paired comparisons were made between different ligands. Those incubations in all cases were run in duplicate and mean values ± the lower and higher values are given by the bars in Figures 1–4, and 8.

RESULTS

Oxine, oxine sulfate, tropolone, oxine sulfonic acid, and commercial [^{111}In]oxine were compared. Figure 1 shows the ^{111}In labeling yields using the ligands. It is worthwhile to note that the concentrations indicated in Figure 1 were concentrations of the labeling ligands. In the final reaction mixtures those concentrations were a factor of six lower. The lower part of Figure 1 represents exactly the same experiment repeated 1 day later. It can be deduced that the reproducibility of the labeling method is good. Further, there is higher labeling yield at lower ligand concentrations, due to wash-out of indium at higher ligand concentrations. Oxine sulfate behaves as well as oxine. There was not much difference between oxine and tropolone. Oxine sulfonic acid had no labeling ability at all, and oxine corresponds well to the commercial [^{111}In]oxine preparation.

When compared at low concentration (2.5 μg–25 μg/ml), in a saline environment, there is some difference between tropolone and oxine, as shown in Figure 2. At 25 μg/ml, tropolone is slightly better than oxine, whereas, at very low concentrations (2.5 μg and 5 μg/ml) oxine is better than tropolone. The correspondence between laboratory-made and commercial preparations was good.

Figure 3 shows a striking similarity of merc, oxine, and tropolone at concentrations ranging from 2.5 μg to 25 μg/ml. Merc, at 25 μg/ml, is slightly inferior to the other two ligands, oxine and tropolone. No dramatic differences were noticed if blood cell suspensions without plasma residue were labeled with ^{111}In complexed to either tropolone, Merc, or oxine.

Leucocyte and platelet labeling was then performed in 85% plasma environment. Contrary to the reports in the literature (*16,17*) no significant differences between the labeling ligands were observed on labeling in plasma (Fig. 4). Labeling yields in all cases were poor, with the commercial [^{111}In]oxine preparation showing a maximum labeling yield of 30%–40%.

Acidification of the Plasma

According to Wolf (*20*), metal binding of transferrin in plasma requires the presence of bicarbonate anions under physologic conditions. This indicated that acidification of the plasma should shift the CO_2/hydrogen carbonate equilibrium to the CO_2 side, thereby, pre-

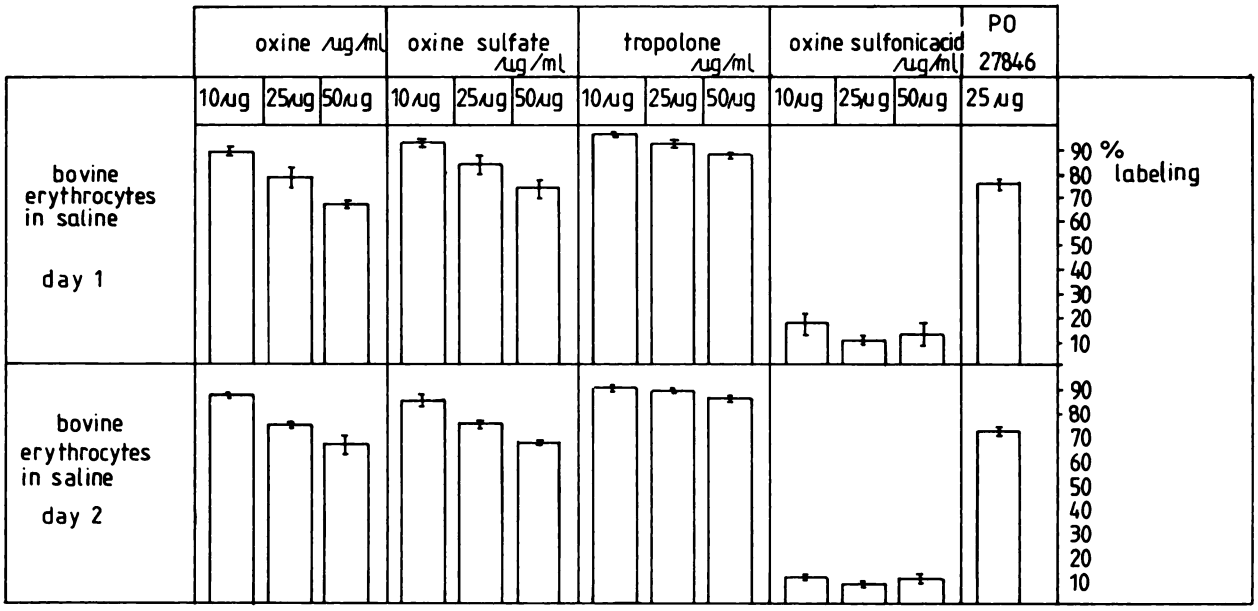

FIGURE 1
Comparison of oxine, oxine sulfate, tropolone, oxine sulfonic acid, and commercial [^{111}In]oxine, with respect to cell labeling efficiency. Average values of two independent incubations. PO 27846 is commercial [^{111}In]oxine. Cell count was 3×10^8 cells/ml. (Bars represent range for lower to higher values.)

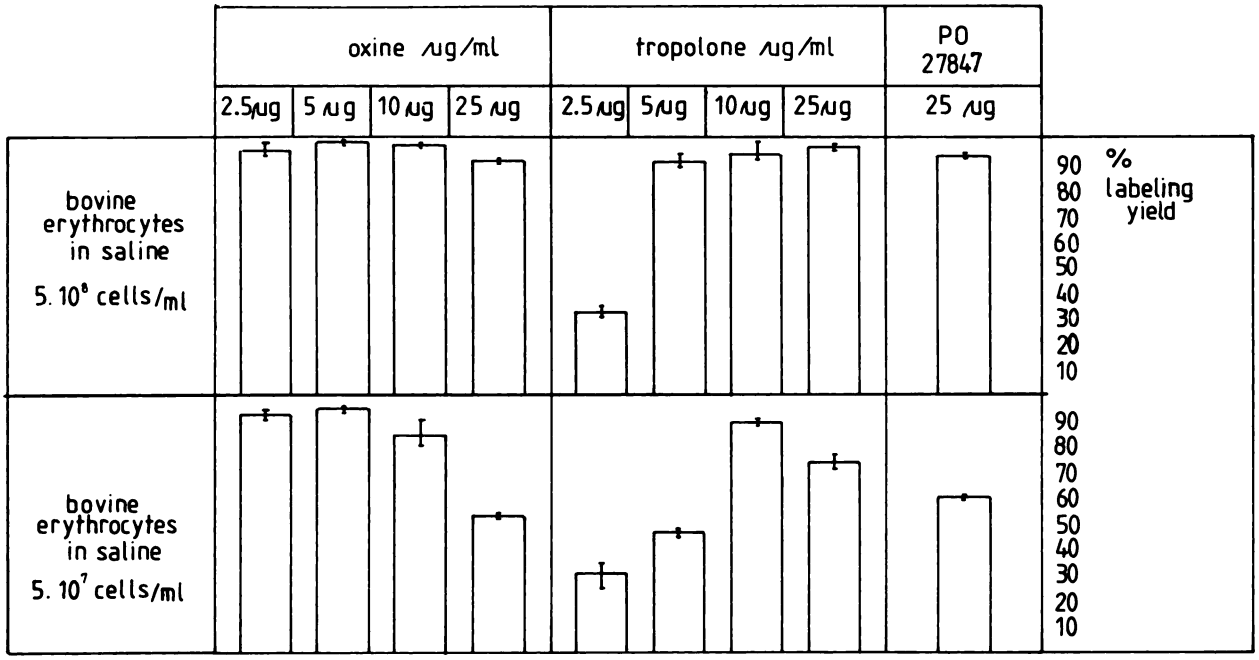

FIGURE 2
Comparison of oxine, tropolone, and commercial [^{111}In]oxine, with respect to cell labeling efficiency. Average values of two independent incubations. PO 27847 is a commercial [^{111}In]oxine. (Bars represent range for lower to higher values.)

venting indium binding to transferrin and improving cell labeling yields in plasma.

This theory was tested by incubating erythrocytes suspended in plasma with oxine, tropolone, and Merc at different pH. Figure 5 shows the relationship between pH and labeling yield. In the case of oxine and tropolone, it appeared that an inverse correlation between pH and labeling yield exists; for Merc this correlation was less distinct. The pH effect on labeling efficiency has been previously cited by other investigators (*21*).

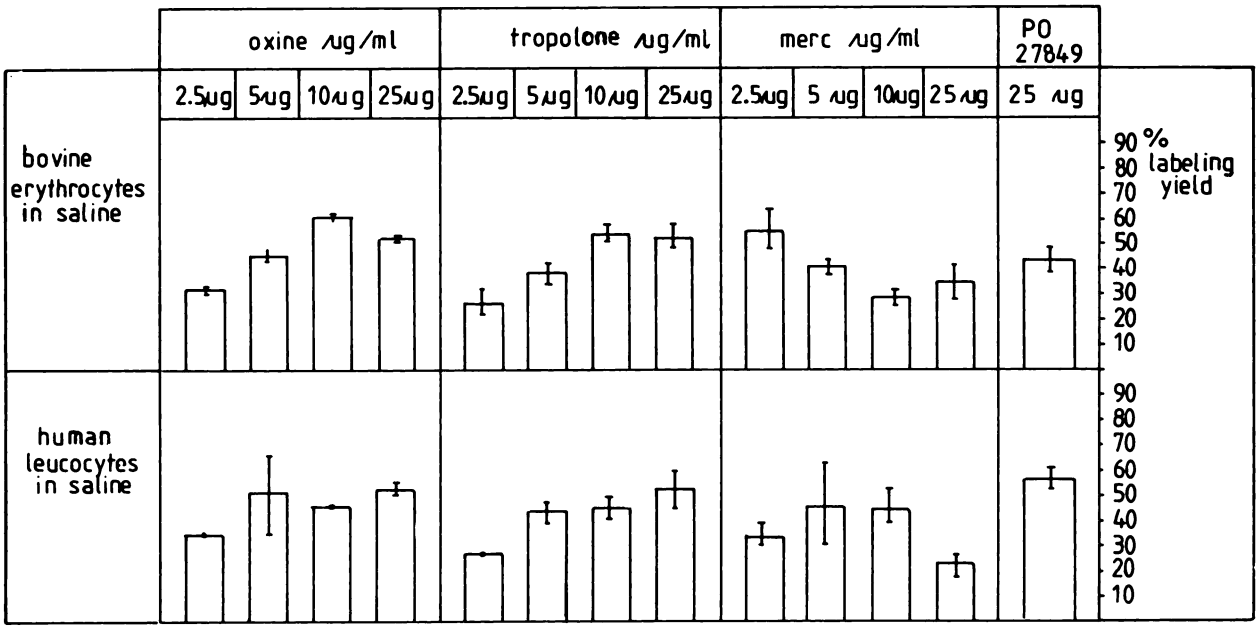

FIGURE 3
Comparison of oxine, tropolone, Merc, and commercial [^{111}In]oxine, with respect to cell labeling efficiency. Average values of two independent incubations. PO 27849 is a commercial [^{111}In]oxine. Cell count was ~10^7 cells/ml. (Bars represent range for lower to higher values.)

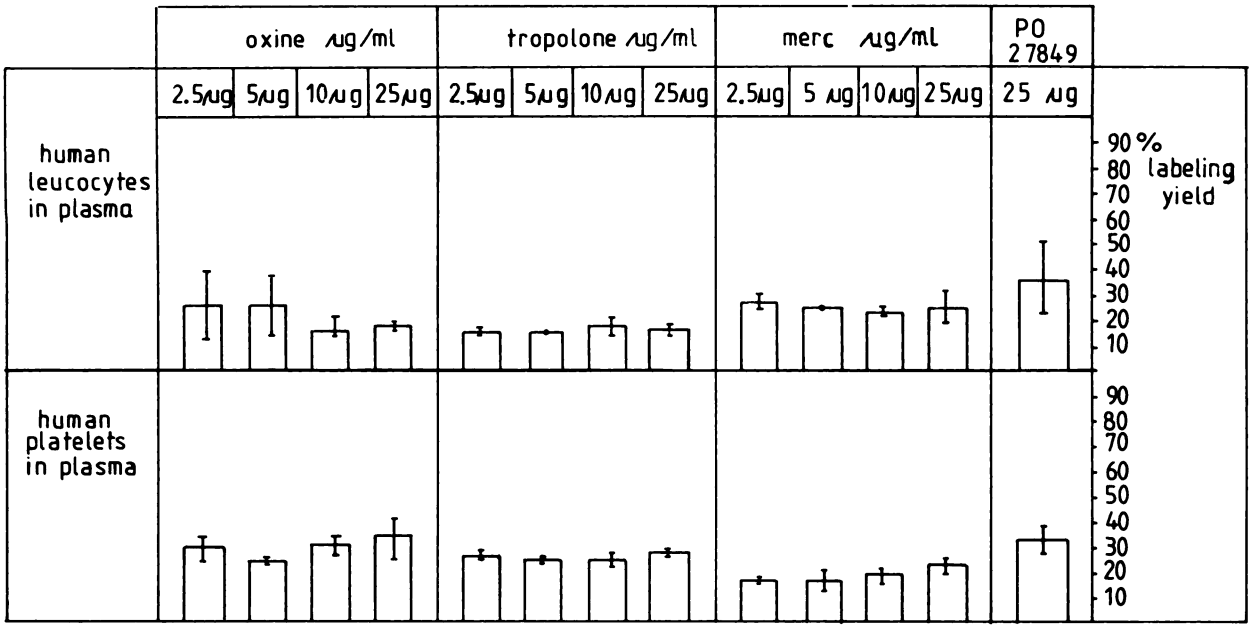

FIGURE 4
Comparison of oxine, tropolone, Merc, and commercial [^{111}In]oxine, with respect to cell labeling in plasma. Average values of two independent incubations. PO 27849 is a commercial [^{111}In]oxine. Cell count was ~5 × 10^6 cells/ml. (Bars represent range for lower to higher values.)

Effect of Diethyl Pyrocarbonate and CO_2

Diethyl pyrocarbonate (DEP) is a compound capable of inhibiting iron binding to transferrin (*22*). We tried DEP as an additive in indium cell labeling experiments using oxine, tropolone, and Merc. DEP was added in varying amounts to human erythrocytes suspended in 30% plasma and labeling ligand 1 µg/ml incubation mixture.

Figure 6 indicates an incubation time of 20 min and 1 hr (left and right sides, respectively). With [^{111}In] tropolone, a dramatic increase was observed from 15% labeling yield without DEP to ~60% at a concentration

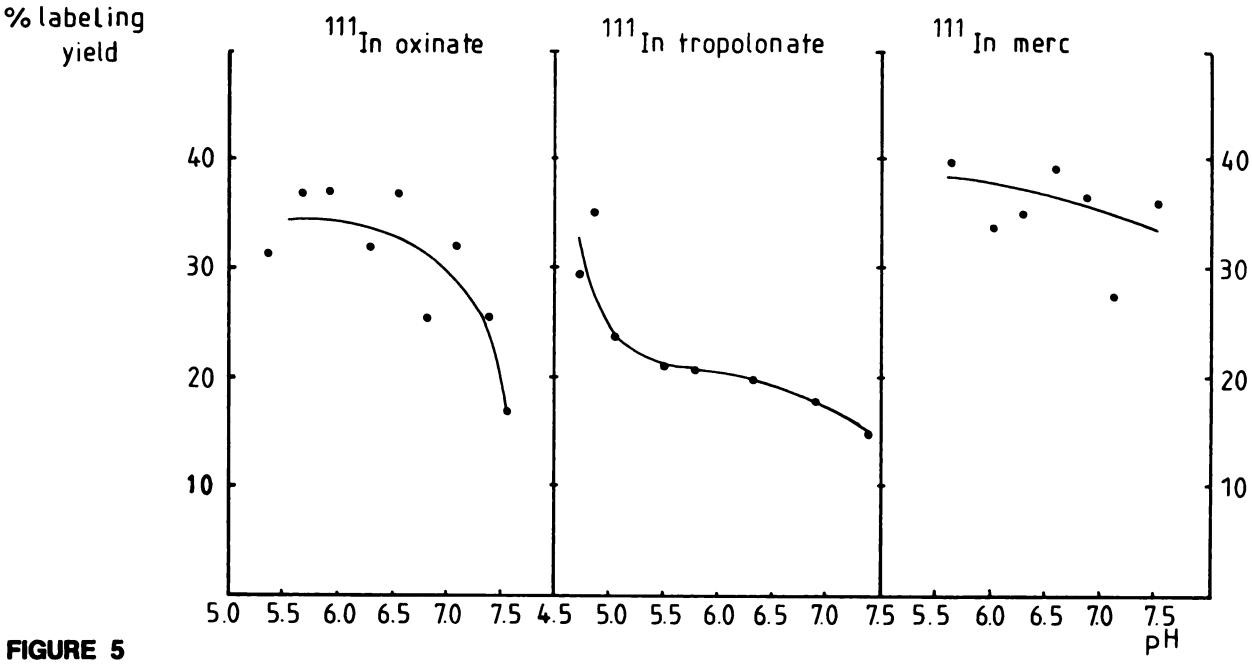

FIGURE 5
Relationship between pH and cell labeling yield of ^{111}In complexed to oxine, tropolone, and Merc.

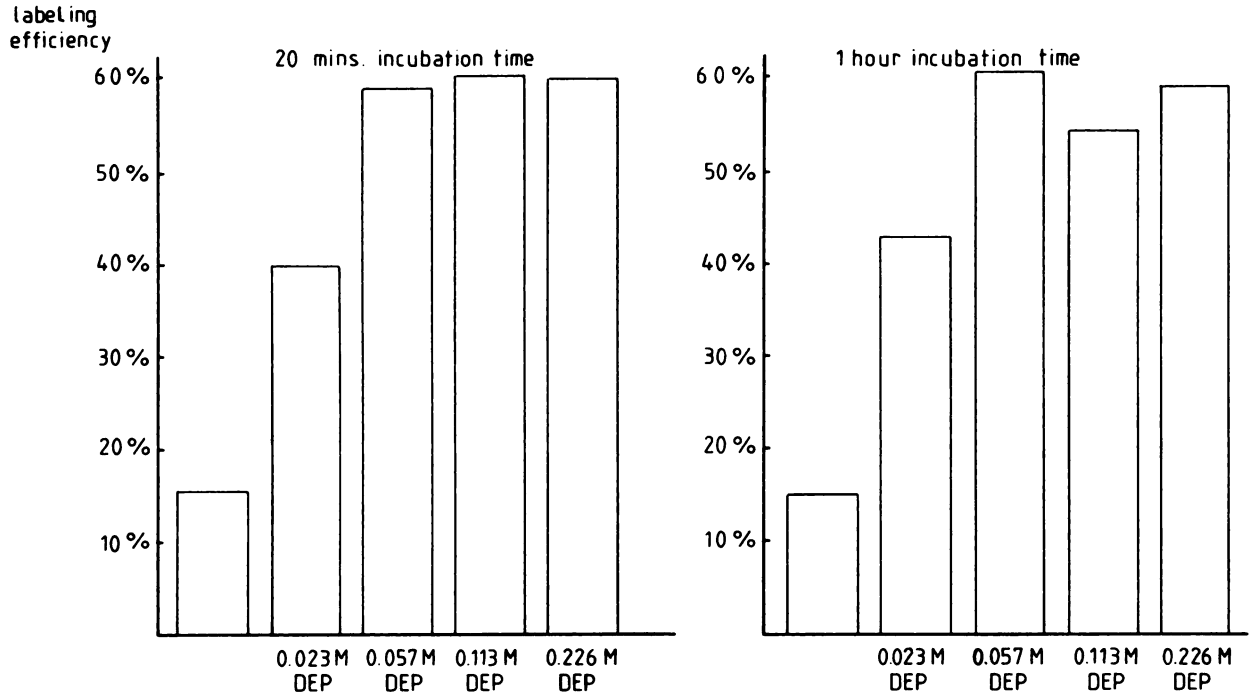

FIGURE 6
Effect of DEP on [^{111}In]tropolone labeling efficiency. Human erythrocytes were suspended in 30% plasma. Final suspension concentration of tropolone was 1 μg/ml.

of 0.057 molar DEP. When [^{111}In]Merc was used, there were similar increases in labeling efficiencies.

Figure 7 shows an increase in labeling efficiency from 25% to ~50% for [^{111}In]oxine, which was lower than when tropolone and Merc were used as ligands.

In the next experiment, the incubation mixture consisting of human erythrocytes in plasma was labeled with [^{111}In]tropolone and flushed with CO_2. Figure 8 shows a significant rise in labeling efficiency from 15% to over 60% after flushing with CO_2. From these results, we inferred that CO_2 is the active principle in the stimulating effect of DEP. The pH in the incubation

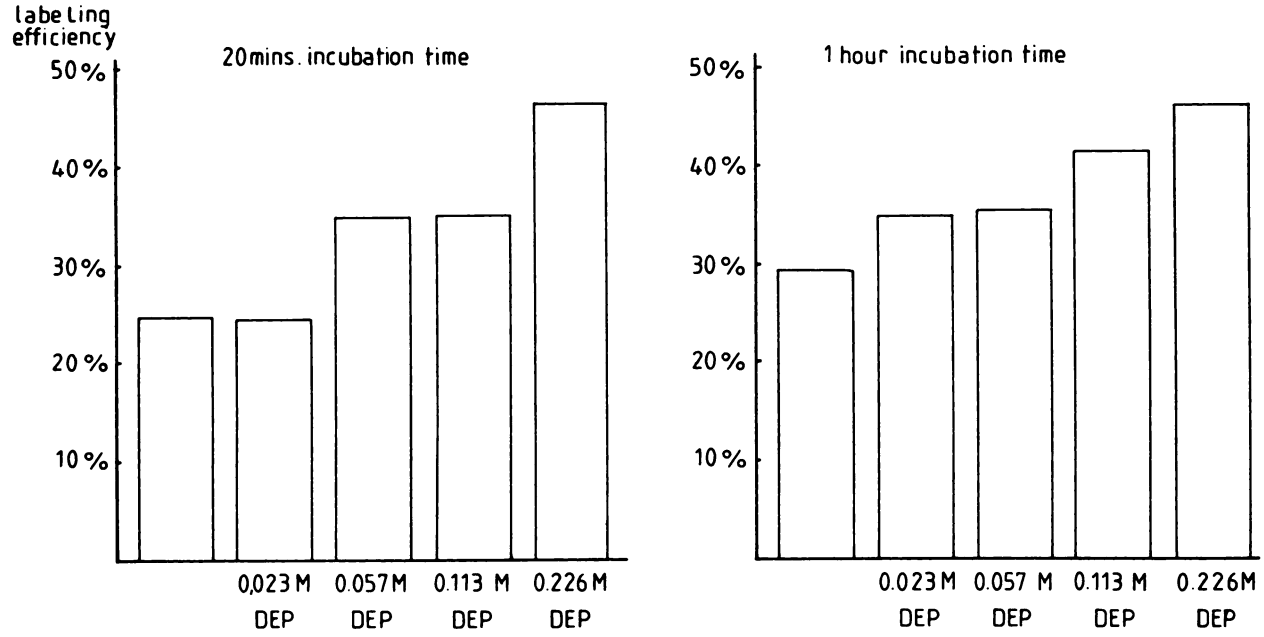

FIGURE 7
Effect of DEP on [^{111}In]oxine labeling efficiency. Human erythrocytes were suspended in 30% plasma. Final suspension concentration of oxine was 1 μg/ml.

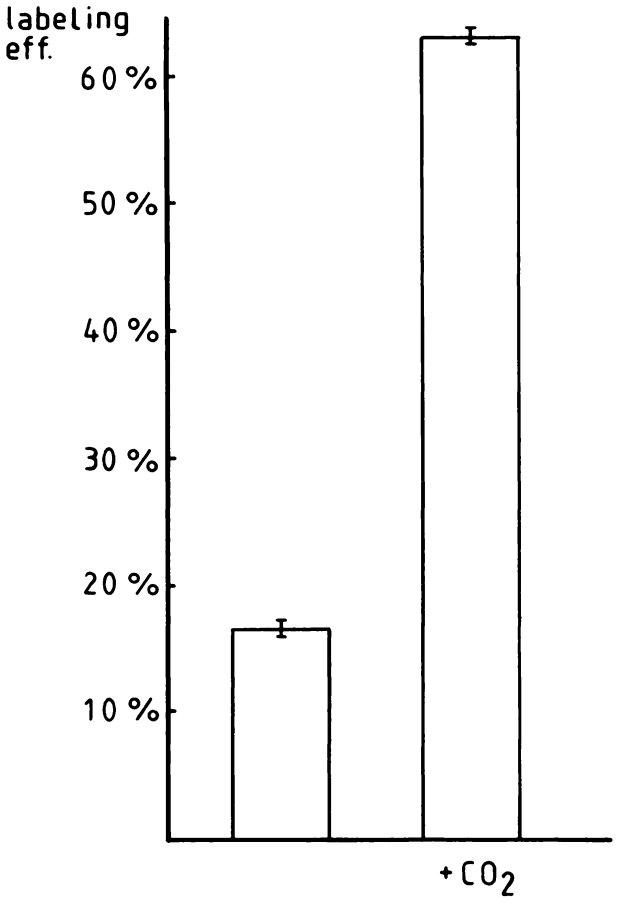

FIGURE 8
Effect of CO_2 flushing on [^{111}In]tropolone cell labeling efficiency. Human erythrocytes were suspended in 30% plasma. Final concentration of tropolone was 1 μg/ml. Average value of two independent incubations with bars representing range from lower to higher values.

mixture, when influenced by addition of CO_2 and DEP, dropped from 7.5 to 6.0.

DISCUSSION

It is evident that oxine sulfate and oxine (chloride) behaved similarly in cell labeling studies, an expected result because of their chemical similarity. Comparing tropolone and merc with oxine, using an acetate TRIS buffer to dissolve the ligands complexed to ^{111}In, revealed only minor differences in the absence of plasma. If plasma residue >30% was present, neither [^{111}In] tropolone nor [^{111}In]Merc could exceed the performance of [^{111}In]oxine with respect to cell labeling efficiencies.

Surprisingly, differences in favor of tropolone and Merc are noticed when CO_2 or CO_2-generating compounds, such as DEP, are added to the incubation mixtures. Increases in labeling yields are dramatic. A doubling or tripling effect is observed in the presence of plasma, which is significantly more than can be obtained by only slightly acidifying the incubation mixtures.

Based on the theory that hydrogen carbonate ions are necessary for binding metal to transferrins in plasma, the stimulating effect of CO_2 was unexpected. By addition of CO_2, we would favor binding of indium to transferrin by supplying hydrogen carbonate ions at the expense of In binding to cells.

This phenomenon is explained by assuming that after passing the double lipoid membrane of the cell, In is captured by protein-like structures that biochemically resemble transferrin or lactoferrin, which also needs hydrogen carbonate ions for binding iron or indium. Iron-free apotransferrins, or lactoferrins, do occur intracellularly as part of the endocytosis process of iron transferrins (*23*), and, leukocytes especially, contain a significant amount of lactoferrin (*24–26*).

We believe that addition of CO_2 to the cell incubation mixture favors hydrogen carbonate anion availability intracellularly only. Extracellularly, there is already a saturated situation as plasma is buffered by the presence of excess hydrogen carbonate and CO_2. In fact, addition of CO_2 should shift the partition equilibrium of indium ions more to the cell moiety than to the plasma moiety. In the absence of CO_2 this equilibrium shifts to the plasma. This theory was sustained by cell labeling experiments in complete absence of plasma, where we observed stimulation of cell labeling efficiency by flushing the incubation mixture with CO_2.

All the ^{111}In ligands evaluated in this study were stimulated by this CO_2 effect with markedly quantitative differences. This must be attributed to subtle differences in complexing constants and (or) lipophilic-hydrophilic properties.

When making a choice, we tend to select tropolone as the suitable ligand, based on simplified and improved cell labeling abilities, favorable behavior in the leukocyte migration test (*27*), and relatively good solubility in water after being complexed to indium.

ACKNOWLEDGMENTS

The authors thank A.A. van Dulmen, W.J. Mercer, and D.W. Wester for critically reading the manuscript.

NOTES

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† Oxine sulfonic acid was dissolved in a solution of 0.1 *M* sodium hydroxide and acidified to the appropriate pH.

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