Preparation of a Chemically Characterized ^{99m}Tc-Penicillamine Complex

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In the reaction labeling penicillamine with ^{99m}Tc, several different chemical states of ^{99m}Tc were observed to occur with slight changes in the labeling conditions, such as the concentrations of penicillamine, $^{99m}TcO_4^-$, stannous chloride, and hydrogen ion. We have studied the effects of these conditions on the formation of the monomer complex that we call Complex I, in which ^{99m}Tc is coordinated with the penicillamine as $^{99m}TcO^{+2}$. Our approach is based on the thesis that Complex I formation competes with $^{99m}TcO^{+2}$ hydrolysis, which leads to other ^{99m}Tc complexes. On this basis, the specific labeling conditions for Complex I formation have been established. Highly reproducible in vivo behavior in mice and rats was obtained with this chemically controlled Complex I.

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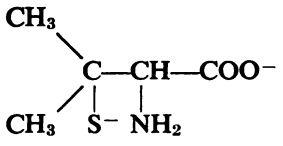
Technetium-99m-labeled penicillamine (^{99m}Tc-Pen) was introduced as a cholescintigraphic agent (*1*). As previously reported, the formation of various labeled compounds with different chemical characteristics was confirmed, and intravenously injected ^{99m}Tc-Pen compounds detected in the bile of rats were limited to two complexes (*2*). In the present paper, these complexes will be referred to as Complex I and Complex II. Our findings emphasize the need to consider the effects of the chemical state of ^{99m}Tc in the labeling compound upon its behavior in vivo.

Although many questions remain, ^{99m}Tc labeling of Pen seems to proceed through the reaction scheme shown in Fig. 1. At the first stage of the reaction, $^{99m}TcO_4^-$ is reduced to a tetravalent ^{99m}Tc species and Complex I is formed through the basic reaction between a tetravalent $^{99m}TcO^{+2}$ ion and Pen as expressed in Reaction A (*2*):

$$^{99m}TcO^{+2} + 2Pen \rightleftharpoons {}^{99m}TcO(PEN)_2^{-2}, \quad (A)$$

where PEN is given in the following formula:



The tetravalent state of technetium has been suggested in the reaction labeling kethoxal-bis(thiosemicarbazone) (KTS) with ^{99m}Tc (*3*). Some recently published papers have also described the formation of tetravalent ^{99m}Tc-labeled compounds (*4,5*).

Independently of Reaction A, other reactions occur at the same time. These reactions take place after the tetravalent ^{99m}Tc species has been hydrolyzed and form other ^{99m}Tc-Pen complexes such as Complex II, III, and so on, in which ^{99m}Tc is assumed to be coordinated with Pen in variously hydrolyzed states or other valence states. Thus, the hydrolysis of $^{99m}TcO^{+2}$ also proceeds at the initial stage of the reaction, as expressed in Reaction B:

$$^{99m}TcO^{+2} + 2H_2O \rightleftharpoons {}^{99m}TcO(OH)_2 + 2H^+. \quad (B)$$

This step is considered one of the most important affecting the labeling reaction. Based on these considerations, the effects of the reaction conditions, such as the pH and the concentrations of Pen, $SnCl_2$, and $^{99m}TcO_4^-$, on the formation of Complex I

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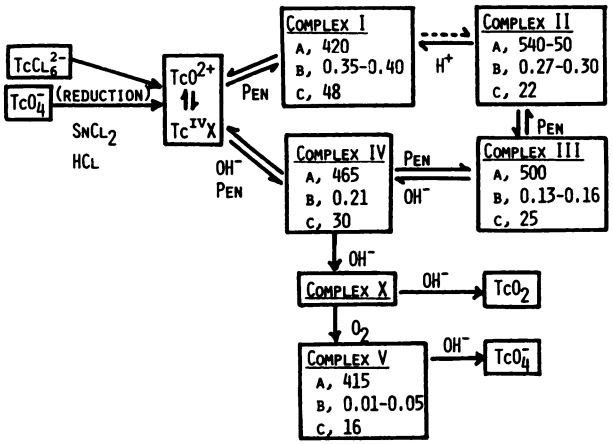

FIG. 1. Labeling products of ^{99m}Tc-Pen reaction. (A) Wavelength of absorption maximum (nm). (B) R_f value on thin-layer chromatography (n-butanol–acetic acid–water, 4:1:1). (C) Elution time (min) in Sephadex G-15 column chromatography (flow rate = 108 ml/hr).

were examined as the first step in the study of ^{99m}Tc-Pen complexes.

MATERIALS AND METHODS

Pertechnetate in saline solution was regularly eluted from a Mallinckrodt generator every morning, and a 0.5–1 mCi/ml solution was used for the labeling reaction. Long-lived $^{99}TcO_4^-$ was obtained from New England Nuclear Corp. All chemicals and solvents were of reagent grade.

The analytic methods employed were thin-layer chromatography, electrophoresis, and Sephadex column chromatography. The thin-layer chromatography strips used were Toyo Kasei silica gel spot film and the solvent was n-butanol–acetic acid–water (4:1:1). For the electrophoresis, Toyo filter paper No. 50 was used and the solvent was phosphate buffer (0.2 *M*, pH 7.0). The Sephadex column chromatography was performed on a 2.0 × 17-cm column of Sephadex G 15, and 0.15 *M* NaCl solution was used for the elution at a flow rate of 108 ml/hr. The radioactivity of ^{99m}Tc on the thin-layer chromatography and Sephadex column chromatography was measured with the apparatus described previously (*3,6*).

Preparation of Complex I. The $^{99m}TcO_4^-$ eluate, 10^{-2} *M* DL-Pen, and 0.2 *M* acetate buffer solution (pH 5.5–6.0) were mixed in a 3:1.5:3 volume ratio, after which 0.5 volume of 4×10^{-5} *M* $SnCl_2$ [freshly prepared in a nitrogen atmosphere, in 0.1 *N* HCl] was added. After stirring for 10 min, the mixture was filtered through a 0.22-μm Millipore filter.

In vivo distribution study. Using 25–30-gm mice (ddY), the in vivo distribution of the ^{99m}Tc was studied after intravenous injection of Complex I.

Biliary excretion study. These Wistar experiments (*3*) used male rats weighing 250–300 gm.

RESULTS

Effect of Pen concentration. Preliminary experiments established that when 2–6 ml of $^{99m}TcO_4^-$ eluate was used, 1 ml of the Pen at greater than 10^{-3} *M* concentration would be necessary for the formation of Complex I. When the concentration of Pen was lower than 10^{-4} *M*, a considerable amount of $^{99m}TcO_4^-$ was detected.

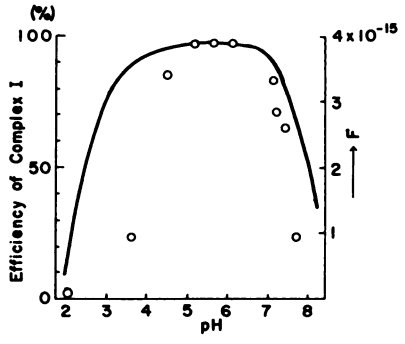

FIG. 2. Effect of pH on Complex I formation. Circles represent labeling efficiencies determined by Sephadex column chromatography. [Labeling conditions: 4×10^{-5} *M* $SnCl_2$ (0.5 ml) was added to mixture of 10^{-2} *M* Pen (1.5 ml) and $^{99m}TcO_4^-$ (3 ml); pH was adjusted with acetate buffer or HCl (3 ml).] Curve corresponds to calculated F-value ($K_{OH}[H^+]^2[PEN]^2$).

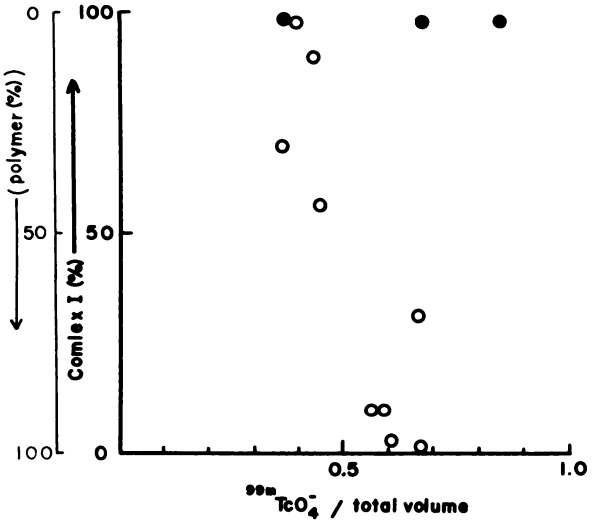

FIG. 3. Effect of $^{99m}TcO_4^-$ concentration on Complex I formation. Concentration of $^{99m}TcO_4^-$ is expressed as ratio of added $^{99m}TcO_4^-$ volume to total volume of labeling solution. Efficiency was determined by Sephadex column chromatography.

Labeling conditions. (○) 10^{-4} to 10^{-5} *M* $SnCl_2$ (1 ml) was added to a mixture of 10^{-2} *M* Pen (1–2 ml) and various volumes of $^{99m}TcO_4^-$ at pH 1–2; pH was then adjusted to 5–6. (●) 10^{-5} *M* $SnCl_2$ (0.5–1 ml) was added to a mixture of 10^{-2} *M* Pen (1–2 ml), with various volumes of $^{99m}TcO_4^-$, and acetate buffer (3 ml at pH 5–6).

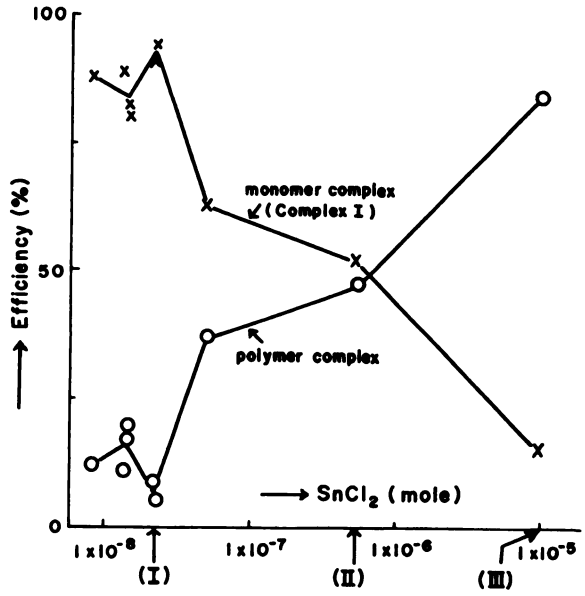

FIG. 4. Effect of $SnCl_2$ concentration on formation of Complex I and polymerized complex. Efficiency was determined by Sephadex column chromatography. [Labeling conditions: $SnCl_2$ (0.5 ml) at various concentrations (1.6×10^{-5} to 2×10^{-2} M) was added to mixture of 10^{-2} M Pen (1.5 ml), $^{99m}TcO_4^-$ (3 ml) and acetate buffer solution (3 ml, pH 6.0).] Labeling solutions prepared, using 2.1×10^{-9} (I), 5.5×10^{-7} (II), and 10^{-5} mole (III) of $SnCl_2$, were used for biliary excretion studies in rats (Fig. 7).

Effect of pH. Labeling solutions were made at various pH: $^{99m}TcO_4^-$ (3 ml), 10^{-2} *M* Pen (1.5 ml), and acetate buffer or HCl solution (3 ml) were mixed; then 0.5 ml of 4×10^{-5} *M* freshly prepared $SnCl_2$ in 0.1 *N* HCl was added and stirred for 10 min. The labeling solution was analyzed by Sephadex column chromatography after filtration through a 0.22-μm Millipore filter. The highest efficiency for Complex I formation is obtained at pH 5–6, and the efficiency falls off rapidly when labeling is performed at a higher or lower pH (Fig. 2, circles).

Effect of volume ratio of $^{99m}TcO_4^-$ saline to the total labeling solution. In these experiments, $SnCl_2$ solution (1 ml, 10^{-4} to 10^{-5} *M*) was added, with continuous stirring under nitrogen atmosphere, to the mixture containing 10^{-2} *M* Pen (1–2 ml) and various volumes of $^{99m}TcO_4^-$ eluate at pH 1–2. Finally, the solution was rapidly adjusted to pH 5–7 by using alkaline solution.

Analysis by Sephadex column chromatography shows that Complex I formation is heavily dependent on the volume of $^{99m}TcO_4^-$ employed (Fig. 3). The abscissa expresses the ratio of $^{99m}TcO_4^-$ eluate volume to the total volume of the labeling solution. Complex I formation is no longer observed at ratios higher than 0.6 (open circles), whereas the formation of polymer complexes such as Complex II, III, etc. (Fig. 1), tends to increase at the higher ratios.

When the labeling is performed in acetate buffer solution (pH 5–6), the effect of $^{99m}TcO_4^-$ volume is not observed (solid circles). In these procedures, the formation of $^{99m}TcO_4^-$ and $^{99m}TcO_2$ was negligible. A similar finding resulted when concentrated HCl was used instead of $SnCl_2$ to reduce the $^{99m}TcO_4^-$, as reported by Tubis et al. (*1*).

Effect of the concentration of $SnCl_2$. Various concentrations of $SnCl_2$ solution were made under a nitrogen atmosphere. Each of these solutions (0.5 ml) was added to a mixture of 10^{-2} *M* Pen (1.5 ml), $^{99m}TcO_4^-$ eluate (3 ml), and acetate buffer (3 ml, pH 6.0). The other labeling conditions were kept optimal for the formation of Complex I.

Complex I is formed with high efficiency when 1.6×10^{-5} to 6×10^{-5} *M* (8×10^{-9} to 3×10^{-8} mole) of $SnCl_2$ solution is used (Fig. 4). The figure shows further that increased $SnCl_2$ results in decreased formation of Complex I and, simultane-

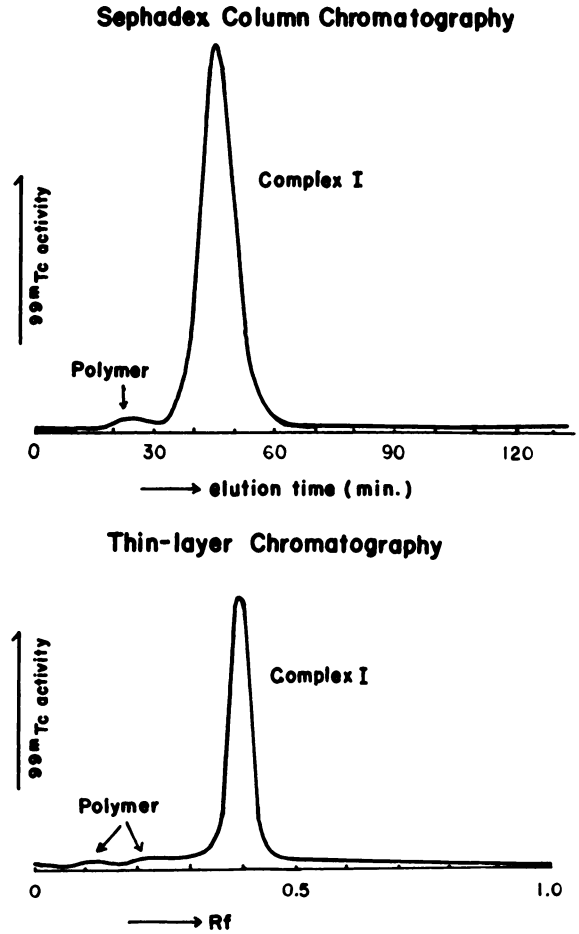

FIG. 5. Typical results of Complex I on Sephadex column chromatography and thin-layer chromatography. Sephadex column chromatography: Sephadex G-15, 2.0 × 17-cm column, 0.15 M NaCl elution solvent, 108 ml/hr flow rate. Thin-layer chromatography: Toyo Kasei silica gel spot film, n-butanol–acetic acid–water, 4:1:1.

ously, increased formation of polymer Complexes II, III, etc. (Fig. 1).

Labeling procedure for Complex I. Based on the foregoing results, the recommended labeling procedure for Complex I was established (see Materials and Methods). This procedure consistently yielded greater than 95% efficiency. The results of Sephadex column chromatography and thin-layer chromatography are shown in Fig. 5. These results and the in vivo behavior of the product show that Complex I is in the same chemical state as the ^{99m}Tc-labeled Pen described by Tubis et al. (*1*).

In vivo distribution of Complex I. The body distribution was studied in mice using Complex I by intravenous injection. The highly reproducible result is shown in Fig. 6. The ^{99m}Tc appears very rapidly in the gallbladder and is present throughout the course of the study.

Biliary excretion of ^{99m}Tc with ^{99m}Tc-Pen complexes. As previously shown (Fig. 4), increased $SnCl_2$ causes a decrease in Complex I formation, along with an increase in other ^{99m}Tc-Pen complexes. In order to study the biliary excretion of ^{99m}Tc-Pen complexes in greater detail, the bile of male rats was collected by the bile-duct cannulation method, using labeling compounds prepared with various amounts of $SnCl_2$ as plotted in Fig. 4. Curves I, II, and III in Fig. 7 show the results obtained from labeling compounds prepared with 2.1×10^{-8} mole, 5.5×10^{-7} mole, and 10^{-5} mole of $SnCl_2$, respectively.

In the presence of 2.1×10^{-8} mole of $SnCl_2$, almost all the ^{99m}Tc is in the form of Complex I. Thus, Curve I can be taken as the ^{99m}Tc excretion pattern of Complex I. Further, as is indicated by Curves II and III in Fig. 7, the total ^{99m}Tc activity recovered in bile decreases with increasing amounts of $SnCl_2$.

DISCUSSION

Since the Complex I formation reaction competes with $^{99m}TcO^{+2}$ hydrolysis, a high concentration of Pen is an essential factor in the formation of Complex I. In fact, analysis of the labeling solution containing a low concentration of Pen showed considerable residual $^{99m}TcO_4^-$. This result was consistent with the similar reaction mechanism described in Ref. *2*: in the presence of an amount of $SnCl_2$ sufficient for the reduction of $^{99m}TcO_4^-$, a reduced ^{99m}Tc species was initially formed and the reaction then proceeded to form the unstable hydrolyzed species. Residual $^{99m}TcO_4^-$ was formed through a reoxidation pathway (Fig. 1).

As shown in Fig. 2, the effect of pH on the formation of Complex I is prominent, and the highest labeling efficiency is obtained at pH 5–6. In an at-

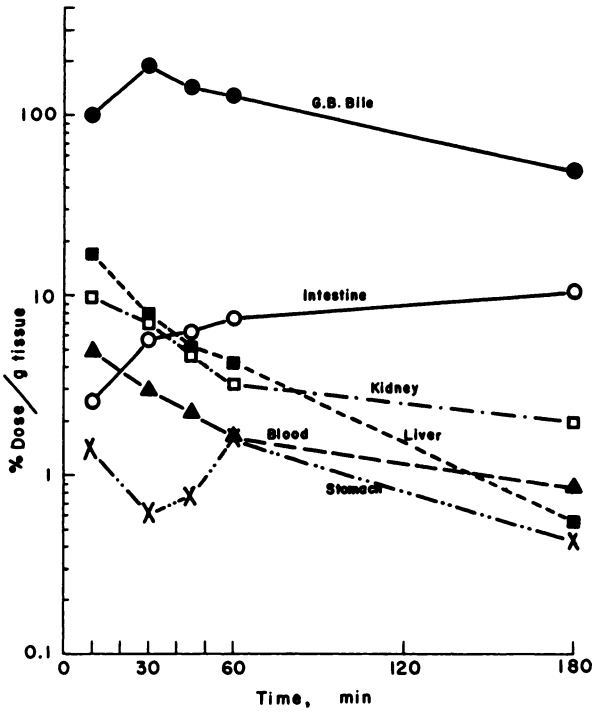

FIG. 6. Organ distribution of ^{99m}Tc in mice after intravenous injection of Complex I. Each point is mean value for 4–6 animals.

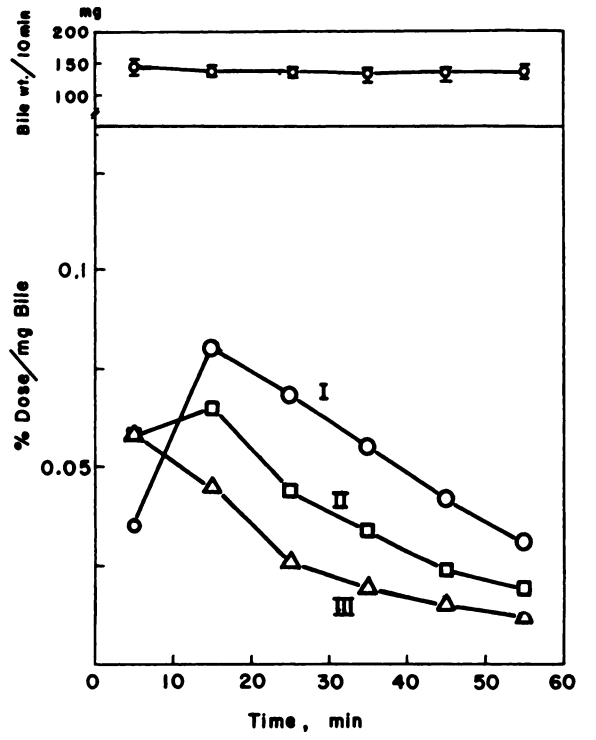

FIG. 7. Biliary excretion of ^{99m}Tc and bile volume in rats, at different time intervals after intravenous injection of solutions prepared using amounts of $SnCl_2$ specified for Curves I, II, and III in Fig. 4. [Curve I: $SnCl_2$ (I), Complex I 95%, polymer 5%; Curve II: $SnCl_2$ (II), Complex I 52%, polymer 48%; Curve III: $SnCl_2$ (III), Complex I 15%, polymer 85%.] Each point is mean value for 4–6 animals.

tempt to understand this result, the following considerations are offered, based on the competition between Reactoins A and B. Under labeling conditions where total Pen concentration (C_{Pen}) is greater than the total concentration of ^{99m}Tc (C_{Tc}), the ratio of the concentration of Complex I, $[TcO(PEN)_2^{-2}]$, to C_{Tc} is given by the expression

$$\frac{K_f K_{OH}[H^+]^2[PEN]^2}{1 + K_{OH}[H^+]^2 + K_f K_{OH}[H^+]^2[PEN]^2}. \quad (1)$$

Here, K_f and K_{OH} are the stability constant and hydrolysis constant for Reactions A and B, respectively.

Equation 1 shows that Complex I, in which K_f is expected to be rather high, may be formed with highest efficiency in the pH range that achieves the maximum value of $K_{OH}[H^+]^2[PEN]^2$ (the F-value). The hydrolysis constant was estimated to be $K_{OH} = 10^{3.7}$ (*7*) and the concentration of the dissociated form of Pen, [PEN], could be obtained using the acid dissociation constants of Pen: $pK_{a1} = 2.5$, $pK_{a2} = 8.32$, and $pK_{a3} = 10.31$ (*8*). The F-value could then be calculated for various pH levels.

The result calculated for the maximum F-value in Fig. 2 (pH 5–6) agrees with experiment. Complex I is formed almost quantitatively in this pH range. Thus, the results in Fig. 2 indicate that the competition between Reactions A and B is the most important feature of the labeling reaction, as previously proposed.

Moreover, based on the experimental result ($[TcO(PEN)_2^{-2}]/C_{Tc} \approx 1$ at $[H^+] = 10^{-5} - 10^{-6}$), the stability constant, log K_f, can be estimated from Eq. 1 to be greater than 16. For other Pen complexes with such bivalent metal ions as Co^{+2}, Ni^{+2}, and Cu^{+2}, the stability constants are reported to be 17.1, 23.2, and 21.7, respectively (*8,9*). Thus, our estimate for the bivalent $^{99m}TcO^{+2}$ ion, similarly classified as a transition-metal ion, can be taken as a reasonable value. Although it is necessary to specify the reaction conditions for Complex I formation, this complex, once formed, remains very stable throughout a wide range of environments. This characteristic is understandable in view of the extremely high stability constant for this complex.

In Fig. 2, marked deviation occurs between the experimental and calculated values in the range pH 2–4, where technetium hydrolysis prevails over Complex I formation. This suggests that in this pH range, the reaction proceeds rapidly toward the formation of a ^{99m}Tc complex with a polymerized ^{99m}Tc state, considering the tendency of almost all hydroxo complexes to undergo olation (*10*). Thus, in this pH range, the reaction time is an additional important factor to be considered. In fact, spectrometric studies using the ^{99}Tc isotope clearly showed that in this pH range changes in standing time led to remarkable differences in the labeled products.

Moreover, the effect of the polymerization of the hydroxo complex on the labeling product is suggested in Fig. 3. The effect of $^{99m}TcO_4^-$ solution volume on the labeled product is observed in a strongly acid medium, whereas this effect is not observed in the buffered solution at pH 5–6 optimal for Complex I formation. It is reasonable that an increased concentration of the hydrolyzed ^{99m}Tc species results in an increased formation of the polymerized ^{99m}Tc species, and consequently in the decreased formation of Complex I. When $^{99}TcO_4^-$ is reduced by concentrated HCl, the reduced technetium species are in various hydrolyzed forms, depending on the concentration of technetium (*11*). Even in a highly acidic medium, the monomeric technetium species is formed only in a carrier-free ^{99m}Tc solution (*11*). In view of these data, under the reaction conditions present at pH 1–2, where the hydrolysis may easily occur, the effect of the concentration of the reduced ^{99m}Tc on its polymerization reactions may not be negligible even at carrier-free ^{99m}Tc levels. The effect of technetium concentration on the labeling product has been also reported by Smith et al. (*12*).

Thus, the results in Fig. 3 should show the effect of ^{99m}Tc concentration on the labeling products. In practice, however, it is difficult to achieve a constant concentration of ^{99m}Tc in the labeling reaction, since its concentration in the generator's eluate depends on standing time since prior elution (*13*). These facts lead us to conclude that any use of the reduced technetium species in an acidic medium must be avoided in the reproducible preparation of Complex I.

The reducing agent may be generally considered as an unavoidable factor complicating ^{99m}Tc labeling procedures. As recently reported (*4*), when $SnCl_2$ is used as the reducing agent, the technetium oxidation state (+3 or +4) depends on the reaction pH and the way the complexing agent coordinates with technetium. Under the conditions recommended above, the tetravalent ^{99m}Tc complex is found to be formed when $SnCl_2$ is used as the reducing agent. Increased concentrations of $SnCl_2$ clearly result in the increased formation of the polymerized ^{99m}Tc species, indicating an additional important effect of $SnCl_2$ on the ^{99m}Tc labeling reaction (Fig. 4). The highly reproducible data on organ distribution in mice (Fig. 6) and on biliary excretion of ^{99m}Tc (curve I in Fig. 7) are seen when Complex I is prepared using a low concentration of $SnCl_2$ (See Materials and Methods). On the other hand, completely different ^{99m}Tc excretion patterns (curves II and III in

Fig. 7) are obtained when labeling is performed at higher concentrations of $SnCl_2$. This different ^{99m}Tc excretion is attributable to the other ^{99m}Tc-Pen complexes formed at higher $SnCl_2$ concentrations.

In conclusion, Complex I can be effectively prepared when the chemical conditions are carefully controlled, particularly the concentrations of Pen, $^{99m}TcO_4^-$, and $SnCl_2$ and the reaction pH. Under proper control, a highly reproducible behavior in vivo was obtained with this chemically defined ^{99m}Tc-labeled compound. Our findings on the behavior of ^{99m}Tc may provide a foundation generally applicable to the development of effective labeling procedures for other ^{99m}Tc-labeled radiopharmaceuticals.

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