THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 4, Issue of January 28, pp. 2581-2588, 1994
Printed in U.S.A.
© 1994 by The American Society for Biochemistry and Molecular Biology, Inc.

The Binding Properties of Two Antitumor Ruthenium(III) Complexes to Apotransferrin*

(Received for publication, April 26, 1993, and in revised form, July 19, 1993)

Felix Kratz‡, Markus Hartmann, Bernhard Keppler, and Luigi Messori§

From the §Laboratory of Inorganic and Bioinorganic Chemistry, Department of Chemistry, University of Florence, 50121 Florence, Italy and the Department of Inorganic Chemistry, University of Heidelberg, 6900 Heidelberg 1, Federal Republic of Germany

The interaction of two ruthenium(III) complexes exhibiting high anticancer activity, namely *trans*-indazolium(bisindazole)tetrachlororuthenate(III) (ru-ind) and *trans*-imidazolium(bisimidazole)tetrachlororuthenate(III) (ru-im), with human serum apotransferrin has been investigated through spectroscopic and chromatographic techniques with the ultimate goal of preparing adducts with good selectivity for cancer cells. Whereas the binding of ru-im to human serum apotransferrin takes several hours, ru-ind, the less toxic complex, gives rise to a well defined 2:1 complex within a few minutes. We have ascertained that ru-ind binding occurs around the iron binding sites; binding does not occur in the absence of bicarbonate, and this anion dictates the kinetic and mechanistic characteristics of protein binding of ru-ind. The two ruthenium(III) complexes do not behave as iron(III) complexes, *e.g.* Fe(EDTA) or Fe(nitrilotriacetate), which lose their respective ligands when binding apotransferrin, but the *N*-heterocycles remain attached to the metal in the protein-bound species. Reversion of binding is obtained by acidification in the presence of chelators such as citrate or ATP. In comparison with cisplatin and its deactivation by serum proteins, our results indicate that other metal complexes such as ru-ind could use transferrin as a drug delivery system. Furthermore, the rapid protein binding of ru-ind seems to be related to a lower toxicity while still exhibiting high antitumor activity.

During the last decade a number of ruthenium(III) complexes have been synthesized by Keppler *et al.* (1) and tested as anticancer drugs. The most promising complexes have the general formula *trans*-HL^+ $(Ru(III)L_2Cl_4)^-$ in which L is a *N*-heterocycle. The complexes with indazole[1], $HInd^+(RuInd_2Cl_4)^-$ (Fig. 1*A*), or imidazole, $HIm^+(RuIm_2Cl_4)^-$ (Fig. 1*B*), show a high antitumor activity in the autochthonous colorectal carcinoma model of rats, a model that simulates the colon cancer of humans very well. Recently, it has been shown by Kratz *et al.* (2) that the most promising complex, $HInd^+(RuInd_2Cl_4)^-$, which is far less toxic in long term application than the imidazole analogue, binds within a few minutes to the serum proteins albumin and transferrin. Furthermore, it has been demonstrated that the apotransferrin-bound complex exhibits a significantly higher antitumor activity against human colon cancer cells when compared with the albumin-bound or "free" species.[2] This result suggests that apotransferrin can act as a natural carrier of the drug to the tumor tissues because of the high affinity between this metal transport protein and the large number of transferrin receptors on the surface of tumor cells (4). Indeed, it has been previously reported that transferrin is responsible for the selective delivery of radioactive $^{67}Ga(III)$ complexes to tumor tissues (5); in addition, it has recently been demonstrated that an experimental transferrin conjugate with adriamycin exhibits antitumor activity *in vitro* as well as *in vivo* (4).

In light of these facts, it is tempting to exploit the transferrin cycle as a "natural" route for selective delivery of cytostatic drugs to cancer cells. We therefore decided to investigate the interaction of the two ruthenium(III) complexes with apotransferrin in more detail and to establish the chemical differences of the resulting adducts with the aid of CD, UV-visible, and NMR spectroscopies as well as HPLC. This should make it possible to correlate the protein binding properties of the two ruthenium(III) complexes with their antitumor activity and toxicity.

EXPERIMENTAL PROCEDURES

Materials—The ruthenium(III) complexes were synthesized as described earlier (1) and were used in all the experiments (except in NMR measurements the solid was added) from a freshly prepared 5×10^{-4} M aqueous solution for $HInd(RuInd_2Cl_4)$ (ru-ind) and a 1×10^{-2} M aqueous solution for $HIm(RuIm_2Cl_4)$ (ru-im). Human serum albumin (98% crystalline, research grade, M_r 66,500) and human serum apotransferrin (apoTf) (98% crystalline, essentially iron free, M_r 80,000) were purchased from Sigma, and the latter was purified according to standard procedures (6). In all the experiments a physiological buffer was used so that the final concentrations were 0.004 M NaH_2PO_4, 0.1 M NaCl, and 0.025 M $NaHCO_3$ (pH 7.4). When studying the pH-dependent properties of the drug/protein adducts, the pH was adjusted to the desired value by the addition of hydrochloric acid. The aqueous solutions of citric acid and ATP were 0.1 M.

Methods—The CD spectra were performed on a Jasco 200D spectropolarimeter, and the visible spectra were performed on a Cary 17D spectrophotometer at room temperature.

The ^1H NMR experiments were performed on a Bruker MSL 200 spectrometer operating at 1.4 tesla, typically using 0.5-ml samples in

* This work has been supported by the Dr. Mildred Scheel Shiftung der Deutschen Krebshilfe, Federal Republic of Germany. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Anorganisch-Chemisches Institut, der Universität Heidelberg, Im Neuenheimer Feld 270, 69120 Heidelberg, Federal Republic of Germany. Tel.: 49-6221-56323; Fax: 49-6221-564955.

[1] The abbreviations used are: Ind, indazole; Im, imidazole; ru-ind, $HInd(RuInd_2Cl_4)$; ru-im, $HIm(RuIm_2Cl_4)$; HPLC, high pressure liquid chromatography; apoTf, apotransferrin; LMCT, ligand to metal charge transfer.

[2] M. R. Berger, B. K. Keppler, L. Messori, and F. Kratz, manuscript in preparation.

This is an Open Access article under the CC BY license.

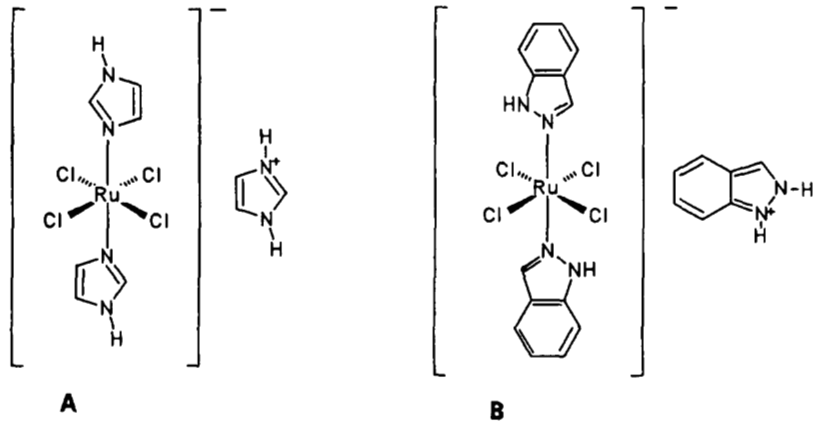


FIG. 1. **Structures of the two ruthenium(III) complexes.** *A*, ru-im; *B*, ru-ind.

5-mm tubes. In order to improve detection of hyperfine shifted signals, short relaxation delays of the order of 50–100 ms were used. Each spectrum typically consisted of 32,000–64,000 scans. The free induction decays, of 8000 data points each, were processed using line-broadening factors of the order 10–30 Hz.

Proton-decoupled ^{13}C NMR spectra were recorded on the same instrument operating at 50.3 MHz. Typically 1000–2000 scans for free complex samples and 10,000 scans for the protein samples were collected by using a 45° flip angle, a pulse delay of 2 s, and a spectral window of 10,000 Hz. Quadrature detection was employed, and the spectra were processed with a line broadening of 5–10 Hz. The chemical shifts are referenced to tetramethylsilane.

HPLC studies were performed with the Perkin-Elmer series 410 LC pump and a LC-95 UV-visible spectrophotometer detector. The columns used were: (*a*) Bio-Sil SEC 250 (300 × 7.8 mm) from Bio-Rad; mobile phase, 0.15 M NaCl, 0.01 M NaH_2PO_4, 5% CH_3CN, pH 7.0; (*b*) Nucleosil (300–7 μm) diole column (250 × 8 mm); mobile phase, 70% CH_3CN and 30% of aqueous 0.005 M KH_2PO_4.

RESULTS

UV-visible Spectra—When the mentioned ruthenium(III) complexes are added to the physiological buffer, characteristic reactions take place that can be followed by changes in the UV-visible spectra. In the case of ru-ind, a light brown complex, we observe formation of a blue-green precipitate within a few minutes, which can be kept in solution by adding a few drops of ethanol. However, no precipitation occurs if apotransferrin is present indicating that binding to the protein takes place. The UV-visible spectra of ru-ind in water, in buffer/5% ethanol, and in the presence of apotransferrin after 20 min of incubation are shown in Fig. 2*A*. The original complex exhibits two bands at 357 and 421 nm. These lie in the typical range of ligand to metal charge transfer (LMCT) transitions of octahedral ruthenium(III) complexes with heterocycles such as $Ru(NH_3)_5(Im)^{3+}$ (7). Both the blue-green product in buffer/ethanol as well as ru-ind bound to apotransferrin exhibit a significant broadening and a shift to lower wavelength of these bands, and a new band at 585 nm appears. Although the molar extinction coefficients are quite high (ϵ values between 1000 and 3000 M^{-1} cm^{-1}) for this low lying band, we nevertheless assign it to a d–d transition[3] assuming a significant lowering of the symmetry of the ruthenium(III) complex after reaction. ϵ values for d–d transitions of low symmetry octahedral ruthenium(III) complexes have been reported ranging from 600 to 6200 M^{-1} cm^{-1} (8).

In contrast, ru-im does not form any precipitate with the physiological buffer; the changes in the visible spectra take much longer to develop, about 12 h at $T = 37$ °C. The charge transfer bands at 347 and 395 nm broaden, and a new band appears at 470 nm, which again is tentatively assigned to a d–d transition (Fig. 2*B*). However, the latter band is not observed in the presence of apotransferrin, the spectrum in this case being very similar to that of the original complex.

CD Spectra—The interaction of ru-ind and ru-im with apotransferrin was also followed through circular dichroism spectroscopy. Circular dichroism spectroscopy is a technique particularly suited to prove specific binding of small chromophoric complexes to chiral macromolecules of high molecular weight (9). Stepwise CD titrations of apotransferrin with increasing amounts of ru-ind are shown in Fig. 3*A*. Characteristic bands are seen around 360, 435, and 505 nm, and the spectra are characteristic of the formation of a specific adduct with the protein; saturation is reached after 2 eq of ru-ind have been added. Further additions of ru-ind do not produce significant changes in the CD spectra.

Because the reaction of ru-im with apotransferrin is much slower, we did not perform a continuous titration as in the case of ru-ind but added the complex at a 2:1 ratio; the resulting spectrum after 8 h is shown in Fig. 3*B*. Two characteristic CD bands, a negative one at 405 and a positive one at 345 nm, are seen. In contrast to ru-ind, further additions of ru-im cause a further increase in spectral intensity, and no saturation is observed up to a 5:1 ratio.

To find out whether the two binding sites for iron(III) in apotransferrin are relevant for the binding of the ruthenium(III) complexes, these sites were blocked with aluminum(III) ions, and the ruthenium(III) complexes were added.

In the case of ru-ind no CD spectra were observed even after 8 h, indicating that the iron(III) binding sites in transferrin are important for the specific binding of the first 2 eq. We also found that the reaction of ru-ind with apotransferrin does not take place in the absence of bicarbonate in the buffer indicating that this anion is primarily involved in the substitution reactions taking place (see below).

In the case of ru-im we found a rather different behavior. Indeed, preincubation with aluminum(III) ions does not prevent development of the characteristic spectrum after 8 h. We also found that the presence of bicarbonate is not critical for the binding process.

We carried out further CD experiments in which we added stoichiometric amounts of the bisnitrilotriacetate iron(III) complex to apotransferrin, loaded with either ru-im or ru-ind at a 2:1 ratio. A few minutes after addition of the iron(III) complex, the characteristic CD spectra of the bound ruthenium(III) complexes disappeared and were replaced by the typical CD spectrum of iron(III) transferrin characterized by two intense bands at 450 and 315 nm.

We also followed the reversibility of the binding of the two

[3] An electron transition between occupied low lying D-orbitals and unoccupied high lying D-orbitals.

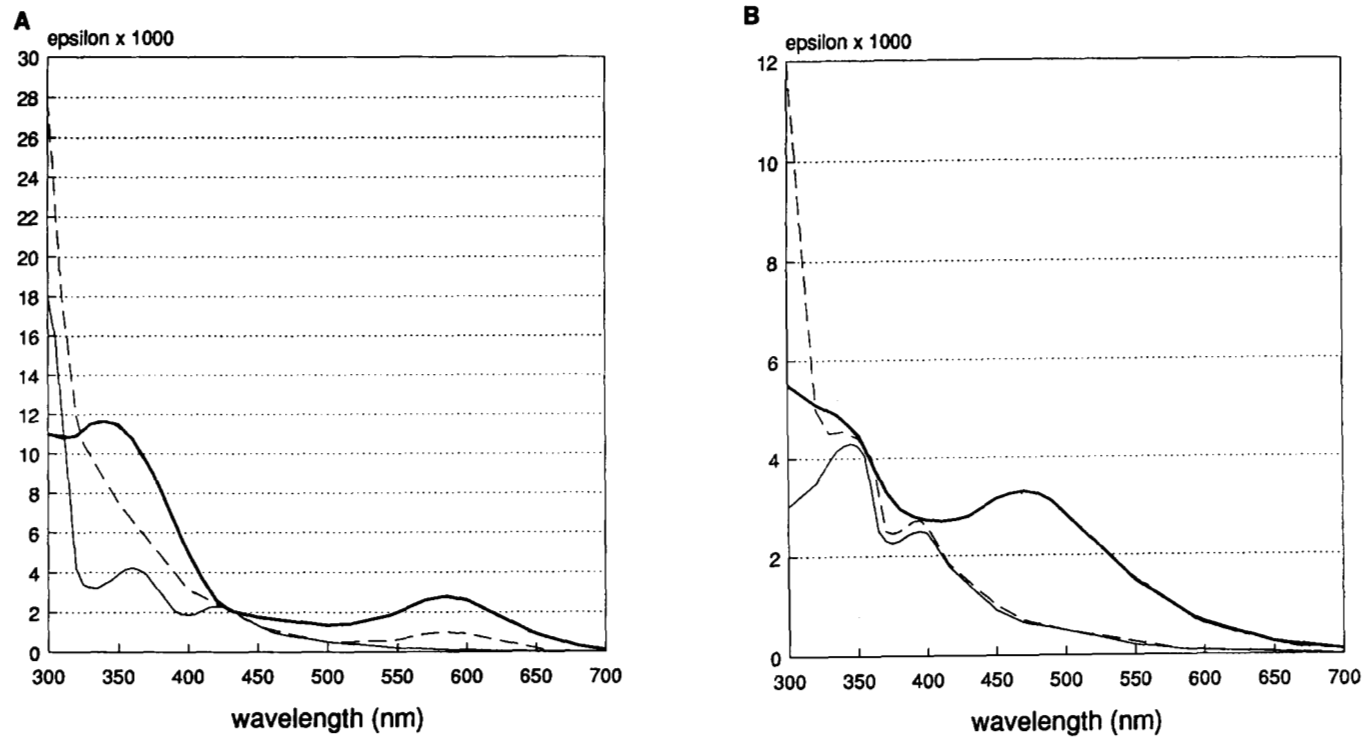


FIG. 2. *A*, UV-visible spectra of ru-ind in water (——), ru-ind in buffer/5% ethanol (▬), and ru-ind bound to apoTf (- - -) after 20 min. The concentration of ru-ind and apoTf was 5×10^{-5} M. *B*, UV-visible spectra of ru-im in water (——), ru-im in buffer (▬), and ru-im bound to apoTf (- - -) after 12 h. The concentration of ru-im and apoTf was 5×10^{-5} M.

apotransferrin-bound ruthenium(III) complexes (ratio 2:1) in which we gradually lowered the pH from 7.4 to 4.0 using 0.1 M HCl. An immediate decrease in the intensity of the CD spectra was observed; at pH 4.0 the intensity of the CD spectra had decreased to about a fifth of the original intensity. When the pH was lowered with either citric acid or ATP, the decrease of the CD spectra was far more pronounced, both CD spectra disappearing at pH 5 after a few minutes (data not shown). These results indicate that the ruthenium(III) complexes bind reversibly to apotransferrin under the above mentioned conditions.

HPLC—An appropriate method of investigating the *kind* and *rate* of reaction between the ruthenium(III) complexes and apotransferrin is by separation of the two on a HPLC system. Fig. 4, *a–c*, shows chromatograms of ru-ind with apotransferrin using a size-exclusion column. The separation was followed at 280 nm (aromatic region) and 360 nm (LMCT band) after the complex was added to apotransferrin at T = 37 °C in the physiological buffer.

Fig. 4*a* shows a chromatogram of ru-ind by itself; it can be clearly seen that the complex anion $(RuInd_2Cl_4)^-$ (12.5 min) is separated from the cation $(HInd)^+$ (15.8 min). Fig. 4*b* shows the chromatogram of ru-ind and apotransferrin after only 3 min of incubation. The original complex (12.5 min) has disappeared, but two intermediate complexes appear (peaks at 10.3 and 11 min), and there is already binding to apotransferrin (*A*) that is seen with the emergence of a peak at 360 nm at the position of apotransferrin (5.5 min). After 5 min (Fig. 4*c*) the reaction is complete, all of the complex now being bound to apotransferrin. The important thing to note is that this reaction takes place only in the presence of bicarbonate. Without bicarbonate in the buffer there is no formation of intermediates and no binding to apotransferrin. It should be mentioned that the reaction sequence of ru-ind with human serum albumin under the same conditions is the same as for apotransferrin with respect to kinetics and mechanism when followed through HPLC (data not shown).

When the same HPLC investigation was performed on the ru-im complex, a different behavior was observed. Fig. 5, *lower panel*, shows ru-im reacted with apotransferrin after 3 min followed at 254 and 340 nm (LMCT); in contrast to ru-ind, only one peak is seen for ru-im at 10 min. This suggests that the complex exists in solution as a stable ionic pair, the size-exclusion column not being able to separate the imidazolium ion from the complex anion. Another thing that can be observed is that after this short time interval there is neither binding to apotransferrin nor detection of intermediates. Fig. 5, *upper panel*, shows the chromatogram of ru-im and apotransferrin after 2 h of incubation. The original complex is still present although the signal intensity is reduced to about one-half of the original value, and a clear signal at 5.5 min, the position of apotransferrin, is now observed at 340 nm. A further thing to note is that no signal for a free imidazolium ion appears in the chromatogram indicating that the original imidazolium (bisimidazole)tetrachlororuthenate complex rearranges into a Tris-imidazole complex before or during binding to the protein. This assumption is supported by the hydrolysis studies of ru-im in water and in the buffer, using a diole column (see below). Protein binding is complete after approximately 5 h when the chromatogram shows only the apotransferrin peak at both wavelengths. These observations indicate that protein binding of ru-im takes place without the formation of intermediates.

To obtain a better understanding of the reaction of the complexes in the absence of apotransferrin, we followed the reaction of both complexes in physiological buffer using a diole column. Fig. 6 shows the chromatograms of ru-ind in the physiological buffer at 37 °C after different times followed at 280 nm. At 0 min the chromatogram shows the peaks of the indazolium ion $(HInd)^+$ and of the complex anion (Ru-

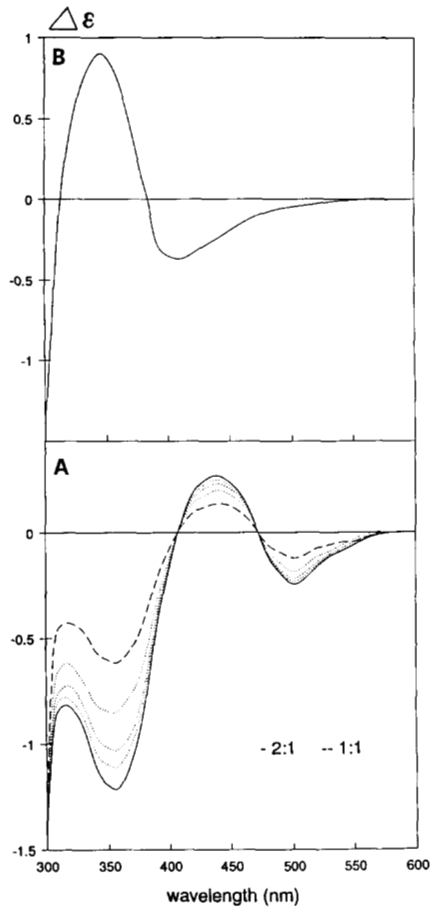


FIG. 3. *A*, CD spectra in the visible region showing the stepwise titration of apoTf (1×10^{-4} M) with ru-ind; *B*, CD spectrum in the visible region of ru-im plus apoTf (1×10^{-4} M) at a 2:1 ratio, obtained after 12 h of incubation.

$Ind_2Cl_4)^-$. After only 1-min incubation two new peaks (*A* and *B*) are observed that disappear with time and are not revealed in the chromatogram after 4 min in which a new peak (*C*) is seen. After 10 min peak *C* is no longer observed but instead four new peaks (*D*, *E*, *F*, and *G*) appear, and the peak of the complex anion $(RuInd_2Cl_4)^-$ has totally disappeared.

In contrast, the reaction of ru-im in the buffer is much slower. After 6 h two new peaks appear while the peak of the anion has diminished (Fig. 7, *A* and *B*); after 12 h the main peak is seen at position *B*, and only small peaks are observed at positions *A*, $(HIm)^+$ and $(RuIm_2Cl_4)^-$. The results of previous hydrolysis studies of ru-im using the same column are almost identical to those performed in the buffer (10); in these studies the peak at position *B* has been identified as the Tris complex $RuIm_3Cl_3$.

Finally, the reversibility of protein binding of ru-ind and ru-im was studied under acidic conditions (pH 4) in the presence of the chelating agent citric acid using the size-exclusion column. Fig. 8 shows the results after a 100-fold excess of citric acid was added to the ru-ind/apotransferrin adduct so that the resulting pH value was 4. After 1-h incubation nearly all of the bound complex is released from the protein, and a new signal appears at 10.2 min (labeled as *Ru?*) that is probably due to a ru-ind/citrate adduct; a similar chromatogram with the new signal appearing at 7 min is obtained when a 100-fold excess of ATP is used (pH 5, data not shown). If the pH is lowered solely with the aid of hydrochloric acid, no new peak is observed in the chromatogram, and the original peak of ru-ind/apotransferrin has not

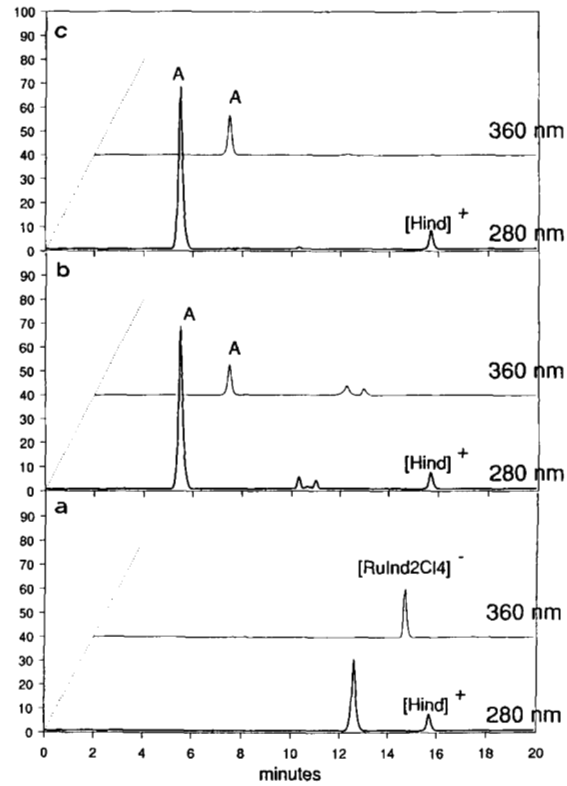


FIG. 4. **Chromatograms of ru-ind (*a*), ru-ind plus apoTf after 3 min (*b*), and ru-ind plus apoTf after 5 min (*c*).** All chromatograms were performed on a size-exclusion column and recorded at 280 and 360 nm, respectively. Ru-ind was incubated with apoTf in the physiological buffer at $T = 37$ °C at a ratio of 1:1, the concentration of apoTf being 1×10^{-4} M. The peak of apoTf is labeled *A*.

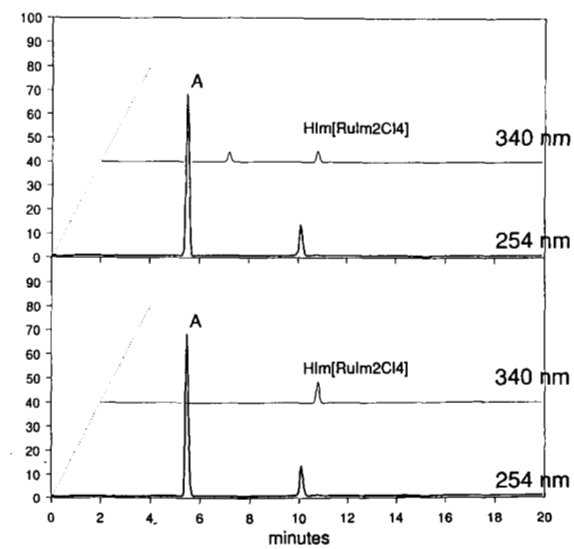


FIG. 5. **Chromatograms of ru-im plus apoTf after 3 min (*lower panel*) and ru-im plus apoTf after 2 h (*upper panel*).** The chromatograms were performed on a size-exclusion column and recorded at 254 and 340 nm, respectively. Ru-im was incubated with apoTf in the physiological buffer at $T = 37$ °C at a ratio of 1:1, the concentration of apoTf being 5×10^{-4} M. The peak of apoTf is labeled *A*.

decreased in intensity. This demonstrates that a chelator-like citrate or ATP is needed to release the bound ruthenium(III) complex from the protein. Reversibility of binding was also

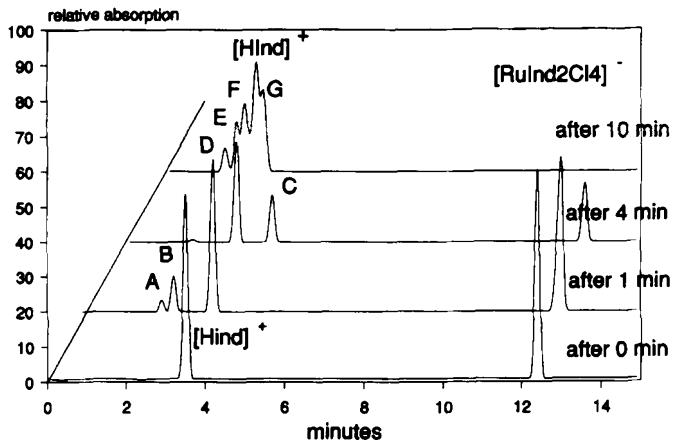


FIG. 6. **Chromatograms of ru-ind in the physiological buffer at 37 °C on a diole column after different incubation times.** The concentration of ru-ind was 5×10^{-4} M.

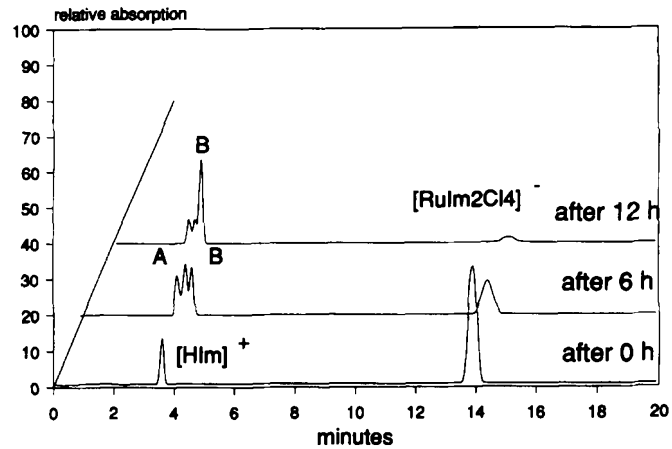


FIG. 7. **Chromatograms of ru-im in the physiological buffer at 37 °C on a diole column after different incubation times.** The concentration of ru-im was 5×10^{-4} M.

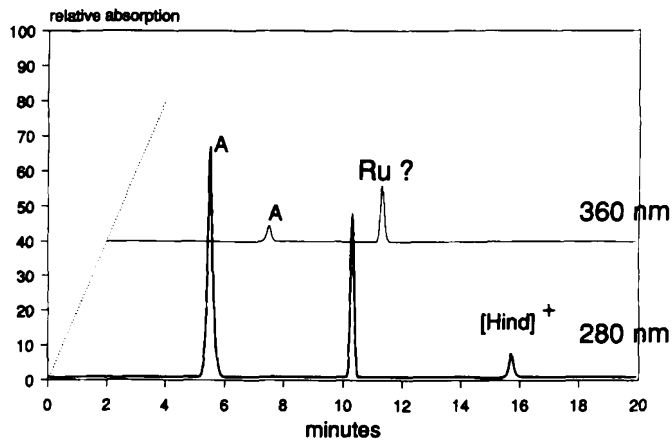


FIG. 8. **Chromatogram of ru-ind bound to apoTf after 1 h of incubation with a 100-fold excess of citric acid (pH 4).** The peak *Ru?* is probably a Ru(III)-indazole-citrate complex. The concentration of apoTf was 1×10^{-4} M.

shown in the case of ru-im under the same conditions (data not shown).

It should be noted that the same chromatogram is observed when citrate is added to ru-ind bound to albumin, demonstrating that there is reversibility of binding with both proteins under these conditions.

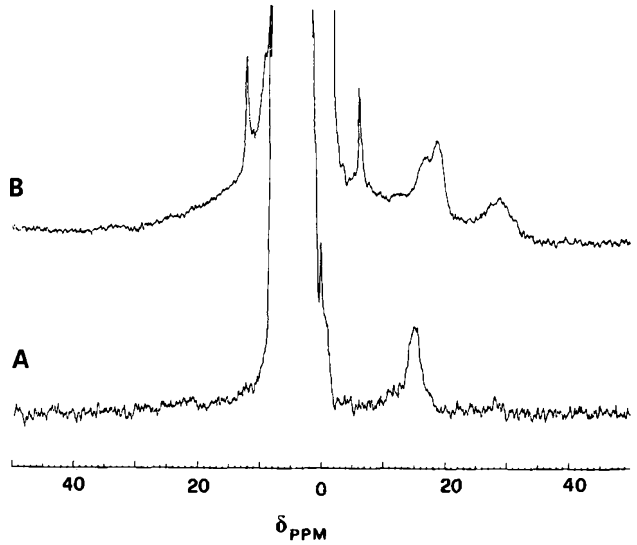


FIG. 9. **200-MHz ^{1}H NMR spectra of 5 mM ru-ind dissolved in methanol-d_4 (*A*) and the blue-green precipitate (which forms upon reaction of ru-ind in the physiological buffer) redissolved in methanol-d_4 (10 mg/ml) (*B*).**

^{1}H NMR Studies—ru-ind is fairly soluble in water but exhibits a higher solubility in methanol and dimethyl sulfoxide. The ^{1}H NMR spectrum of ru-ind dissolved in methanol-d_4 at 298 K is shown in Fig. 9*A*. The spectrum is characterized by a broad hyperfine signal in the upfield region at about −15 ppm. As stated above, upon reaction with the physiological buffer ru-ind forms a blue-green precipitate that can be redissolved in methanol-d_4. The ^{1}H NMR spectrum of this product is profoundly different from that of ru-ind being characterized by a number of hyperfine signals with both upfield and downfield shifts in the range of 15 to −40 ppm (Fig. 9*B*). If solid ru-ind is added to the physiological buffer in the presence of apotransferrin at a 2:1 complex-protein ratio, the blue-green color forms, but there is no precipitation. No hyperfine shifted ^{1}H NMR signals are detected in this case. Only by following the reaction between ru-ind and apotransferrin during the first minutes after mixing is it possible to detect the broad upfield feature at −15 ppm characteristic of nonhydrolyzed ru-ind (data not shown). The signal quickly disappears as the substitution reaction proceeds. A reasonable explanation of this behavior that is consistent with the previous spectroscopic results is that upon reaction in the buffer the newly formed ruthenium(III) complexes bind to the protein giving rise to a tight slow rotating adduct with broad and nondetectable NMR signals.

Analogous ^{1}H NMR experiments were performed on ru-im, for which previous ^{1}H NMRs have been reported (1). If dissolved in the physiological buffer, the compound is far more soluble and stable than ru-ind. The ^{1}H NMR spectrum at 37 °C of ru-im dissolved in the physiological buffer at 1 mM concentration is shown in Fig. 10*A*. The spectrum exhibits two narrow hyperfine shifted signals at −15 and −20 ppm well outside the diamagnetic envelope, which were previously assigned to the ortho-like protons of the coordinated imidazole (11). If we dissolve the same amount of ru-im in a 1 mM solution of human serum apotransferrin in the same buffer, we obtain the spectrum shown in Fig. 10*B* within the first 30 min, which is virtually identical to the previous spectrum. With time the intensity of the hyperfine shifted signals markedly decreases (Fig. 10*C*) until they eventually disappear after approximately 5 h. Again, the disappearance of the characteristic ^{1}H NMR features of the free complex is probably due to

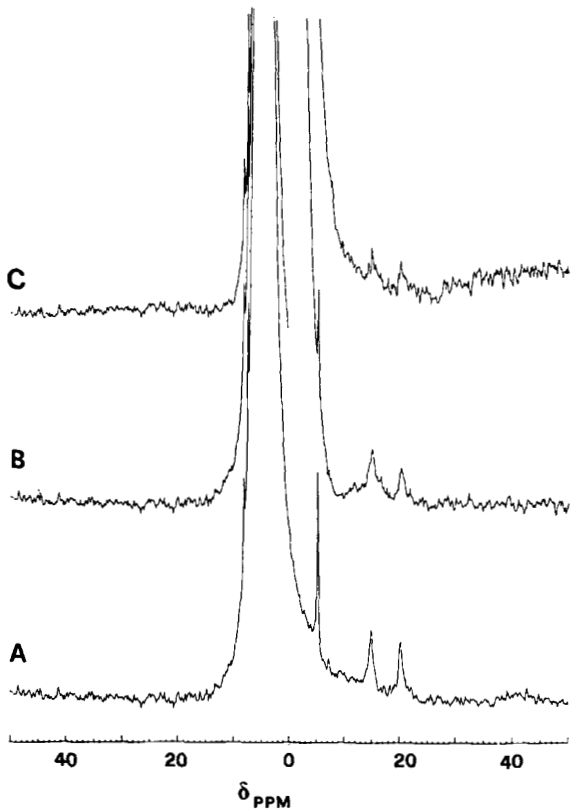


FIG. 10. **200-MHz ^{1}H NMR spectra at 37 °C.** *A*, 1 mM ru-im in the buffer; *B*, 1 mM ru-im plus 0.5 mM apotransferrin in the same buffer recorded during the first hour after mixing; and *C*, sample B after 2 h.

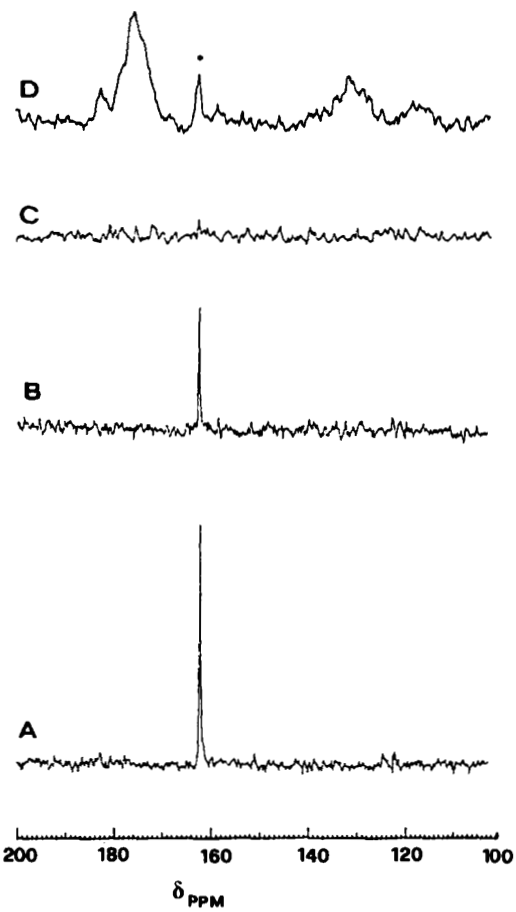


FIG. 11. **50.2-MHz ^{13}C NMR spectra.** *A*, 1 mM ^{13}C-enriched bicarbonate dissolved in a deuterated buffer containing sodium phosphate (0.004 M) and sodium chloride (0.1 M, pH 7.4); *B*, sample A after the addition of an equimolar amount of ru-im; *C*, sample A after the addition of 1 eq of ru-ind and removal of the blue-green precipitate; and *D*, 1 mM apotransferrin and 2 mM sodium bicarbonate in the deuterated buffer (pH 7.4). The line of free bicarbonate is labeled with an *asterisk*. The spectrum is not modified after the addition of 2 eq of ru-ind and development of the characteristic green color.

progressive binding to apotransferrin and subsequent broadening of the hyperfine resonances.

^{13}C NMR Spectra—To understand the role of bicarbonate in the reactions of ru-im and ru-ind within the physiological buffer and in the presence of apotransferrin, we followed the above reactions through ^{13}C NMR spectroscopy using 90% ^{13}C-enriched sodium bicarbonate.

The ^{13}C NMR spectra of ^{13}C-enriched sodium bicarbonate in the buffer both in the absence and in the presence of an equimolar amount of ru-im are shown in Fig. 11, *A* and *B*. It must be noted that addition of an equimolar amount of ru-im causes a decrease in the intensity of the $H^{13}CO_3$ peak but no significant change in line width nor in position. This might suggest that part of the bicarbonate is directly bound to ruthenium(III) under slow exchange conditions on the NMR time scale.

In the case of ru-ind we observe a markedly different behavior. Indeed, after precipitation the ^{13}C NMR line of free bicarbonate disappears indicating that bicarbonate is part of the blue-green products (Fig. 11*C*).

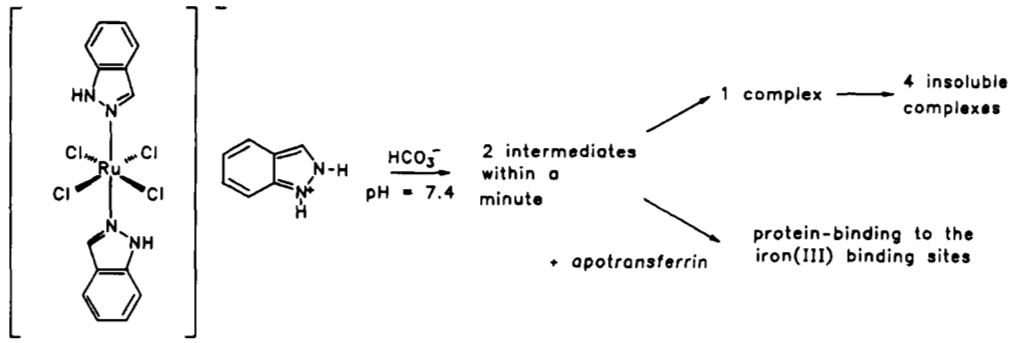
We also investigated the behavior of ^{13}C-enriched bicarbonate in the presence of apotransferrin before and after the addition of ru-ind. The ^{13}C NMR spectrum of a 1 mM solution of human serum apotransferrin in the physiological buffer prepared without bicarbonate (pH 7.4) in the presence of 2 mM ^{13}C-enriched bicarbonate is shown in Fig. 11*D*. The ^{13}C NMR line of free bicarbonate is still detectable at 161.4 ppm. Interestingly, after the addition of 2 eq of ru-ind and formation of the characteristic ru-ind/apotransferrin adduct, there is no change in the ^{13}C NMR peak of free bicarbonate (peak labeled with *asterisk*) ruling out that it is directly bound to the ruthenium(III) center or that it is playing the role of a synergistic anion.

DISCUSSION

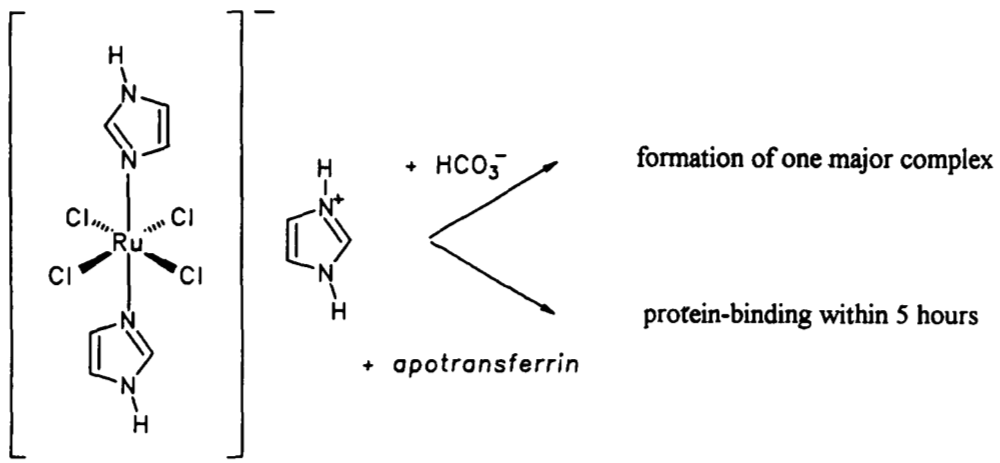
Protein Binding Properties of the Ruthenium(III) Complexes—The knowledge of the transferrin cycle has rapidly increased in recent years (12–14), and this cycle holds promise as an attractive system for strategies of drug targeting to tumor tissues. Indeed, tumor cells exhibit a large demand of iron for their growth and therefore express the transferrin receptor at a high rate (4, 15). As a consequence, transferrin conjugates that retain a good affinity for the transferrin receptor can preferentially interact with cancer cells. Faulk *et al.* (4) have exploited such a strategy to achieve targeting of adriamycin to neoplastic tissues.

These considerations prompted us to prepare conjugates of the newly developed antitumor ruthenium(III) complexes with apotransferrin, to test their antitumor activity on *in vitro* and *in vivo* models, and to understand the differences in the toxicity profile.

In the present paper we have conducted an extensive characterization of the adducts between the ruthenium(III) drugs and apotransferrin, and we have gained insight into the intriguing solution chemistry of the two complexes. The reac-



SCHEME 1



SCHEME 2

tivity pattern of both ru-ind and ru-im with apotransferrin is complicated by the occurrence of concomitant hydrolysis processes and substitution reactions with solute species. Surprisingly, in spite of their structural similarity, ru-im and ru-ind exhibit very different reactivity patterns that are reflected in their respective protein binding abilities.

From the HPLC results as well as the CD and UV-visible spectra, the reaction schemes that can be drawn for the reaction of ru-im and ru-ind in the physiological buffer in the absence or presence of apotransferrin are shown in Schemes 1 and 2.

The major difference between the behavior of the two complexes is a kinetic and mechanistic one. Binding of ru-ind to apotransferrin proceeds through the formation of two intermediates that then bind rapidly to the protein, whereas it takes 5 h at 37 °C for ru-im to bind the protein, and no intermediates are observed during the reaction. In the case of ru-ind, protein binding and formation of the intermediates take place only when bicarbonate is present in the buffer. This is a novel feature of this ruthenium(III) complex and implies that it is not simple hydrolysis but that a number of substitution reactions involving bicarbonate are taking place before binding to apotransferrin. In the absence of protein the intermediates take part in further reactions leading to the four insoluble products that we have not been able to isolate and characterize as yet.

In the case of ru-im, no intermediates and no free imidazole are observed during binding when followed by HPLC. Because ru-im behaves as quite a stable ionic pair, there is probably a slow rearrangement to a Tris-imidazole complex when binding to the protein.

As stressed before, the reaction sequence of ru-ind and ru-im with human serum albumin is the same as for apotransferrin. This indicates that it is the reactions of the complexes within the buffer that are of primary importance for the overall reaction pattern, the protein playing a secondary role.

However, when the resulting intermediates of ru-ind bind to apotransferrin they do so in a specific manner. CD spectroscopy demonstrates that 2 eq of the complex can be bound by apotransferrin indicating that binding takes place around the two iron(III) binding sites of the protein. When these sites were blocked with Al(III), no CD spectra were observed demonstrating that the iron binding sites are indeed involved in the binding of the complex. ^{13}C NMR of [^{13}C]bicarbonate gives indirect evidence that bicarbonate is not part of the bound complex in contrast to the typical metallotransferrin complexes where it acts as a synergistic anion.

Conversely, the CD data do not demonstrate unequivocally that ru-im binds specifically to the iron binding sites of apotransferrin. CD studies of the apotransferrin bound complex using iron nitrilotriacetate could indicate that iron(III) displaces the bound ruthenium(III) complex from the iron binding sites.

However, we would like to point out that we have recently obtained evidence of the nature of the binding sites of ru-im through soaking experiments of this complex with crystals of the strictly related protein, apolactoferrin. Subsequent analysis of the x-ray data clearly shows that the iron(III) binding sites are indeed occupied (see below).

The CD spectra for both ru-im and ru-ind bound to apotransferrin differ from that of ruthenium(III) transferrin, which was prepared by addition of Ru(III)nitrilotriacetate to apoTf and characterized previously (16). In the latter adduct ruthenium(III) simply substitutes for iron(III), whereas in the case of ru-im and ru-ind the respective *N*-heterocycles remain bound to the metal. That this is the case is also verified by

the protein binding studies using HPLC because no new imidazolium or indazolium appeared in the respective chromatograms after binding.

The binding of ru-ind, and probably of ru-im, at the active site region is a novel feature of transferrin chemistry. Computer graphic inspection of the metal binding region of the parent protein apolactoferrin (coordinates kindly provided by E. N. Baker (17)) using the program FRODO has revealed that both complexes can easily enter the open cavity of the protein (18).

In collaboration with E. N. Baker, we have performed soaking experiments of apolactoferrin crystals with both complexes, and subsequent x-ray data were collected. Preliminary x-ray structure analysis already shows that the two ruthenium(III) complexes bind to a histidine at both of the iron binding sites. Both structures are being refined at the moment with an expected resolution of the order of 2.2–3.0 Å and will be published at a later stage.

Implications of Protein Binding for Biological Activity—It is instructive to compare the protein binding properties of ru-ind with those of the clinically established cisplatin. Over 90% of the platinum found in the blood 3–4 h after administration of cisplatin is bound to plasma proteins (19, 20), and the cisplatin protein bound species have no significant antitumor activity (21). The loss of activity of cisplatin when bound to plasma proteins is probably due to the irreversible binding to cysteines of plasma proteins such as albumin (22). In contrast, we have recently shown that ru-ind bound to apotransferrin retains and even exceeds the antitumor activity of the free complex when tested in colon cancer cells.[2]

The potential of using transferrin conjugates for selective drug delivery to tumor tissue should be seen in light of the natural transferrin cycle and iron metabolism. The transferrin cycle and thus the delivery of iron to the cells are believed to be as follows (15): binding of diferric transferrin by transferrin receptors ($K = 10^8 - 10^9$ M^{-1}), internalization of the receptor-ligand complex by endocytosis, and release of iron in the acidic environment (pH 5.0–5.5) of endosomes with the aid of intracellular chelating agents. Most of the apotransferrin is then cycled back to the plasma membrane by the transferrin receptor and finally dissociated into the external medium due to a fall in receptor affinity on encountering the elevated pH of the extracellular medium. Thus, should transferrin conjugates such as those of ru-ind follow the natural transferrin cycle, they should be able to release the bound drug at lower pH. Our HPLC and CD studies have demonstrated that this is the case when ru-ind/apoTf is incubated with a 100-fold excess of citrate or ATP (pH 4–5), nearly all of the ruthenium species being released from the protein after 1 h. Therefore, the prerequisite for drug release after cellular uptake of the conjugate inside the endosome is apparently met. That toxins bound to transferrin can be released to the cells at low pH has been recently shown by Wellhoner *et al.* (3) using acid labile conjugates.

Finally, the animal experiments in the autochthonous colon cancer model in rats have shown that ru-ind is far less toxic than ru-im but also exhibits a slightly higher antitumor activity (1). Our present results indicate that the difference in toxicity might be related to the different protein binding ability of the two compounds assuming that the free complex is formally responsible for the systemic toxicity.

In light of the above results ru-ind/apoTf conjugates are being evaluated for antitumor activity in *in vivo* tumor models with the ultimate goal of developing selective drug delivery systems for clinical trials.

Acknowledgments—We thank Brunella Innocenti and Nadia Mulinacci for assisting with the HPLC work. The support of Dr. Giulio Borghi from Laboratorio di Analisi Fanfani is also acknowledged.

REFERENCES

1. Keppler, B. K., Henn, M., Juhl, U. M., Berger, M. R., Niebl, R., and Wagner, F. F. (1989) *Prog. Clin. Biochem. Med.* **10,** 41–69
2. Kratz, F., Mulinacci, N., Messori, L., Bertini, I., and Keppler, B. K. (1992) *Metal Ions in Biology and Medicine*, Vol. 2, pp. 69–74, John Libbey Limited Eurotext, Paris
3. Wellhoner, H. H., Meville, D. M., Jr., Srinvasacher, K., and Erdmann, G. (1991) *J. Biol. Chem.* **266,** 4309–4314
4. Faulk, W. P., Harats, H., and Berczi, A. (1990) in *Oxidoreduction at the Plasma Membrane* (Crane, F. L., Morre, J. D., and Low, H., eds) Vol. 1, pp. 205–224, CRC Press, Inc., Boca Raton, FL
5. Ward, S. G., and Taylor, R. C. (1988) in *Metal-based Antitumor Drugs* (Gielen, M. F., ed) pp. 1–54, Freund Publishing House Ltd., London
6. Bates, G. W., and Schlabach, M. R. (1975) *J. Biol. Chem.* **250,** 2177–2182
7. Krogh-Jespersen, K., and Schugar, H. J. (1984) *Inorg. Chem.* **23,** 4390–4393
8. Manoharan, P. T., Mehrotra, P. K., Taquikhan, M. M., and Andall, R. K. (1973) *Inorg. Chem.* **12,** 2753–2757
9. Mason, S. F. (1982) *Molecular Optical Activity and the Chiral Discriminations*, Cambridge University Press, Cambridge, United Kingdom
10. Lipponer, K.-G. (1992) *Synthesis, Characterization, Hydrolysis, Kinetics, Stability, and Biological Activity of Antitumor Ruthenium and Cobalt Complexes.* Ph.D. thesis, University of Heidelberg
11. Hagen, W. R., Keppler, B. K., Lipponer, K.-G., Ni Dhubhghaill, O. M., and Sadler, P. J. (1993) *J. Chem. Soc. Dalton Trans.*, in press
12. Crichton, R. R., and Ward, R. J. (1992) *Biochemistry,* **31,** 11255–11264
13. Baker, E. N., and Lindley, P. F. (1992) *J. Inorg. Biochem.* **47,** 147–160
14. Trowbridge, I. S., Collawn, J., Jing, S., White, S., Esekogwu, V., and Stangel, M. (1991) in *Biotechnology of Plasma Proteins: Haemostasis, Thrombosis and Iron Proteins* (Hemker, H. C., ed) Vol. 58, pp. 139–147, S. Karger, AG Basel, Switzerland
15. Cazzola, M., Bergamaschi, G., Dezza, L., and Arosio, P. (1990) *Blood* **75,** 1903–1919
16. Kratz, F., and Messori, L. (1993) *J. Inorg. Biochem.* **49,** 79–82
17. Anderson, B. F., Baker, H. M., Norris, G. E., Rumball, S. V., and Baker, E. N. (1990) *Nature* **344,** 784–787
18. Kratz, F. (1993) in *Metal Complexes in Cancer Chemotherapy* (Keppler, B. K., ed) pp. 391–429, Verlag Chemie, Weinheim, Germany
19. Cole, W. C., and Wolf, W. (1980) *Chem. Biol. Interact.* **30,** 223–235
20. Daley-Yates, P. T. (1985) in *Biochemical Mechanisms of Platinum Antitumor Drugs* (McBrien, D. C. H., and Slater, T. F., eds) pp. 121–146, IRL Press, Oxford
21. Takahashi, K., Seki, T., Nishikawa, K., Minamide, S., Iwabuchi, M., Ono, M., Nagamine, S., and Horinishi, H. (1985) *Jpn. J. Cancer Res.* **76,** 68–74
22. Pizzo, S. V., Swaim, M. W., Roche, P. A., and Gonias, S. L. (1988) *J. Inorg. Biochem.* **33,** 67–76