# Interaction of Streptonigrin with Metals and with DNA

**KOPPAKA V. RAO**

Received August 28, 1978, from the *Department of Pharmaceutical Chemistry, College of Pharmacy, J. Hillis Miller Health Center, University of Florida, Gainesville, FL 32610.* Accepted for publication January 10, 1979.

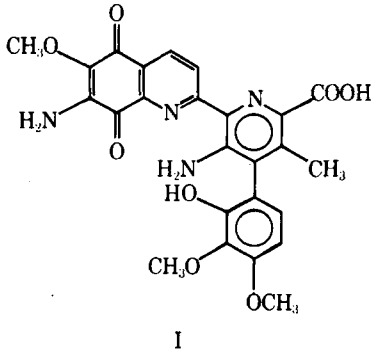
**Abstract** □ The antitumor antibiotic, streptonigrin, interacted with zinc, copper, and manganese but not with calcium or magnesium, as indicated by spectral shifts and difference spectra. The titration data showed the formation of 1:1 complexes, and further titration continued to show spectral shifts until a molar ratio for zinc to streptonigrin of 5–10 to 1 was reached. Streptonigrin interacted with DNA only in the presence of a metal ion such as zinc. Streptonigrin titration with DNA at varying zinc molar equivalents revealed that one antibiotic molecule required 5–7 moles of zinc and 20–25 moles of DNA-phosphorus for complexation. Similar values were obtained from gel permeation chromatography.

**Keyphrases** □ Streptonigrin–interaction with metals and with DNA, complexation, spectrophotometry, gel permeation chromatography □ Antibiotics—streptonigrin, interaction with metals and with DNA □ Antineoplastic agents—streptonigrin, interaction with metals and with DNA □ DNA—interaction with streptonigrin and with metals □ Metals—complexation with streptonigrin and with DNA

Streptonigrin (I), an antitumor antibiotic and a *Streptomyces flocculus* metabolite (1, 2), shows high activity against various experimental neoplasms (3–5). Extensive clinical trials showed that it was active against certain lymphomas (6), and a double-blind comparison with chlorambucil showed comparable activity, with I being slightly more toxic in the GI tract (7).

Strong streptonigrin involvement in DNA metabolism in various cellular systems manifests itself in chromosomal aberration and fragmentation and in drug absorption by nuclear fractions (8–10). Mizuno and Gilboe (11), who studied the *in vitro* interaction of I and DNA, showed that two complexes were formed, one unstable to dialysis and the other stable, with a stoichiometry of 1:2000 for streptonigrin–DNA nucleotides. However, a sedimentation behavior, melting-profile, and gel permeation chromatography study (12) indicated that no complex was formed between streptonigrin and DNA. In that study, I showed no inhibition of DNA-dependent RNA polymerase, unlike other DNA-interacting antibiotics such as actinomycin or daunomycin. Thus, streptonigrin binding to DNA is controversial; this behavior would be somewhat unusual in view of its demonstrated involvement in DNA metabolism.

The complexing ability of streptonigrin toward certain metal ions was explored in this laboratory. Preliminary spectrophotometric studies showed that I readily formed complexes with ions such as $Zn^{2+}$, $Cu^{2+}$, and $Mn^{2+}$ but not with $Ca^{2+}$ or $Mg^{2+}$. Streptonigrin–metal complexes readily interacted with DNA to form clearly demonstrable and relatively stable complexes. This paper describes some characteristics of the interaction of I with metals such as $Zn^{2+}$ and of the complexes such as streptonigrin–zinc with DNA.



I

## EXPERIMENTAL

Tromethamine buffer (0.01 *M*) of appropriate pH was used. Streptonigrin was dissolved in dioxane, approximately three equivalents of sodium bicarbonate were added, and the solution was diluted with water to $2–5 \times 10^{-4}$ *M*. Since I solutions made in tromethamine buffer (pH 7) decomposed gradually, the experiment was completed within 1 hr after the dilution with tromethamine.

Calf thymus DNA (sodium salt, highly polymerized, 8% phosphorus) was purchased[1]. A weighed sample was dissolved in water by gentle stirring with a magnetic stirrer at room temperature for 2–3 hr and was diluted to $1.0–1.5 \times 10^{-2}$ *M* solutions, based on DNA–phosphorus. The solutions were dispensed in 2–4-ml portions in test tubes and kept frozen until use.

The complexation was followed by a recording spectrophotometer[2] with silica cells of 1-cm path length. Metal complexes were studied in alcoholic and in aqueous solutions. For obtaining a Job plot, various molar mixtures of I and the metal ion with constant total molarity were prepared and their spectra were recorded. Corresponding reference solutions with I, but without the metal, also were prepared and their spectra were recorded. The difference in the absorbance between the two at the $\lambda_{max}$ was plotted against the mole fraction.

Titrations were carried out by adding the metal-ion solution from a microburet[3] to the sample taken in the cell containing a magnetic stirring bar. The spectra were recorded against a distilled water (or alcohol) blank. In an alternative procedure, a I solution was taken in both the reference and sample cells, and the sample cell was titrated with the metal-ion solution. The spectra so obtained were the "difference spectra." Correction for the volume change was made by the addition of an identical volume of water to the reference cell.

DNA complexation was studied in aqueous solution with or without a buffer. Both titration methods, using either water or streptonigrin–$Zn^{2+}$ solution as reference, were employed for measuring complexation.

Complex formation also was demonstrated through gel permeation chromatography by direct mixture chromatography and by an adaptation of the Hummel–Dreyer method (13). A cross-linked dextran[4] column (8 × 600 mm) was prepared and equilibrated with the streptonigrin–$Zn^{2+}$ solution ($4 \times 10^{-5}$ *M* with respect to I and $4 \times 10^{-4}$ *M* with respect to $Zn^{2+}$). The DNA sample ($6.4 \times 10^{-3}$ *M*, DNA–phosphorus) was diluted with the appropriate amount of the ligand–metal solution and applied to the column, which was then developed by more of the same solution. Fractions (3 ml) were collected, and their absorbance was read at 385 nm.

## RESULTS AND DISCUSSION

In Fig. 1, curve A is a portion of the spectrum (300–450 nm) of $4 \times 10^{-5}$ *M* I in 0.01 *M* tromethamine buffer, pH 7.5, with $\lambda_{max}$ 363 nm and $\epsilon$ = 15,000. Curve B shows the shift of the $\lambda_{max}$ to 385 nm ($\epsilon$ = 17,000) in the presence of $4 \times 10^{-4}$ *M* $Zn^{2+}$. Curve C is the spectrum of a solution of I ($4 \times 10^{-5}$ *M*), $Zn^{2+}$ ($4 \times 10^{-4}$ *M*), and DNA ($3.2 \times 10^{-3}$ *M*) with $\lambda_{max}$ 410 nm and $\epsilon$ = 16,300. An identical solution, but without $Zn^{2+}$, gave curve

[1] ICN Pharmaceuticals, Cleveland, Ohio.
[2] Beckman model 25.
[3] Micrometric Instrument Co., Cleveland, Ohio.
[4] Sephadex G50, Pharmacia Fine Chemicals.

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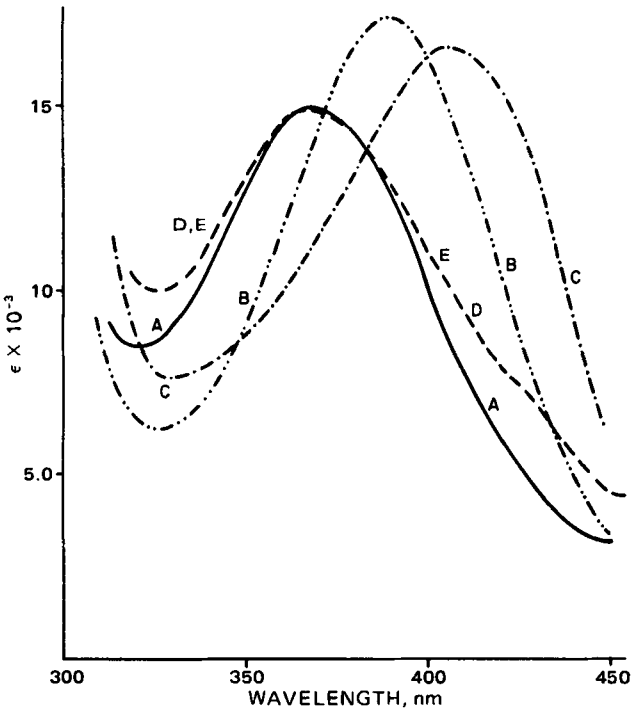


**Figure 1**—*Effects of $Zn^{2+}$ and DNA on the absorption spectrum of I. Key: A, spectrum of I ($4 \times 10^{-5}$ M) in 0.01 M tromethamine buffer, pH 7.5; B, A + $Zn^{2+}$ ($4 \times 10^{-4}$ M); C, A + B + DNA ($3.2 \times 10^{-3}$ M); D, A + DNA ($3.2 \times 10^{-3}$ M); and E, C + edetate sodium.*

D with $\lambda_{max}$ and $\epsilon$ values essentially the same as those of I. To demonstrate that $Zn^{2+}$ was essential for the I complexation with DNA, the solution that gave curve C was treated with edetate sodium, which produced a spectrum similar to that of D. Without zinc, DNA caused a slight spectral shift in the 410–450-nm region.

The complexation could be observed in two other ways. The reddish-brown I solutions essentially remained unchanged when treated with either $Zn^{2+}$ or DNA separately; but when both were present, the color changed to brownish yellow. Addition of edetate sodium to this solution brought back the original reddish-brown color. In the second method, ethanol addition (5–6 volumes) to a I-DNA solution gave a white stringy DNA precipitate. Similar addition to streptonigrin-$Zn^{2+}$-DNA gave a dark-brown stringy precipitate even with 2–3 volumes of ethanol. The precipitate, after being washed with the solvent, gave the characteristic complex spectrum (curve C).

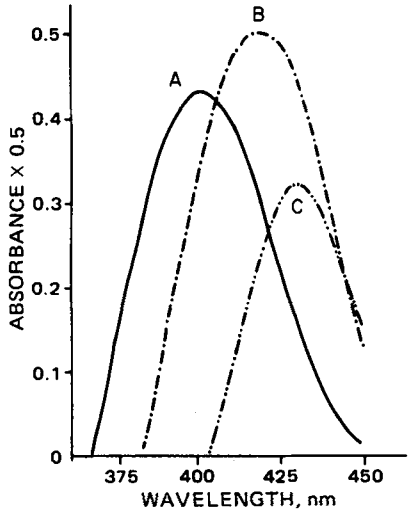


**Figure 2**—*Difference spectra. Key: A, spectrum of streptonigrin-$Zn^{2+}$ complex read against a reference of streptonigrin; B, streptonigrin-$Zn^{2+}$-DNA complex read against a reference of streptonigrin-DNA; and C, streptonigrin-$Zn^{2+}$-DNA complex read against a reference of streptonigrin-$Zn^{2+}$.*

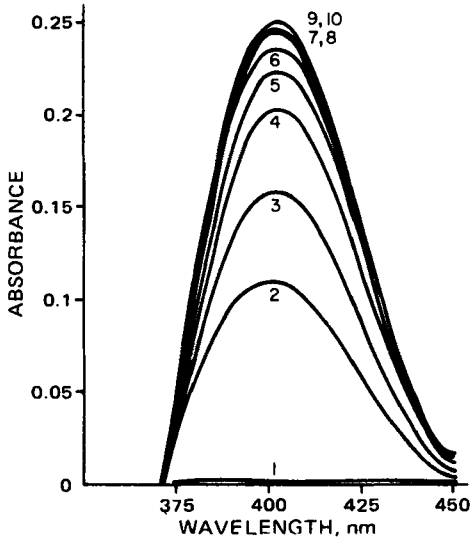


**Figure 3**—*Difference spectra similar to Fig. 2A resulting from titration of I with $Zn^{2+}$. The numbers 1–10 represent the zinc-streptonigrin molar ratios.*

Figure 2 shows the difference spectra.

Results with $Cu^{2+}$ and $Mn^{2+}$ were similar to those with $Zn^{2+}$. No spectral shifts were observed when I solutions were treated with $Mg^{2+}$ or $Ca^{2+}$ ions or when DNA was added in the presence of these ions in the pH 6.0–8.0 range.

**Metal Complexation**—I titration with $Zn^{2+}$ solution, in the presence or absence of a buffer, gave difference spectra of increasing intensity (Fig. 3). A plot of the difference spectrum intensity against the I mole fraction is shown in Fig. 4. The slope change occurring at mole fraction 0.5 shows the existence of a 1:1 complex. The same result was obtained with the continuous variation (Job) method (Fig. 5). The experiments with $Zn^{2+}$, $Cu^{2+}$, and $Mn^{2+}$ were carried out in aqueous as well as in ethanolic solutions with the same results shown in Fig. 3. Values from these experiments were used to calculate the formation constants for the complexation reaction shown in Table I according to Scheme I:

$$\text{streptonigrin} + Zn^{2+} \rightleftharpoons \text{streptonigrin-}Zn^{2+}$$

*Scheme I*

where the formation constant $K$ = [streptonigrin-$Zn^{2+}$]/([streptonigrin]/[$Zn^{2+}$]). The concentration of each component was obtained from the spectrophotometric data according to the relationship:

$$K = \frac{(\epsilon_l - \epsilon_c)}{C_M(\epsilon_c - \epsilon_l) - (A - \epsilon_c C_l)} \qquad \text{(Eq. 1)}$$

where $\epsilon_c$ and $\epsilon_l$ were the absorptivities of the complex (which was the absorptivity at very high DNA/streptonigrin-$Zn^{2+}$ ratios) and of the ligand, respectively; $C_M$ and $C_l$ were the total initial $Zn^{2+}$ and streptonigrin

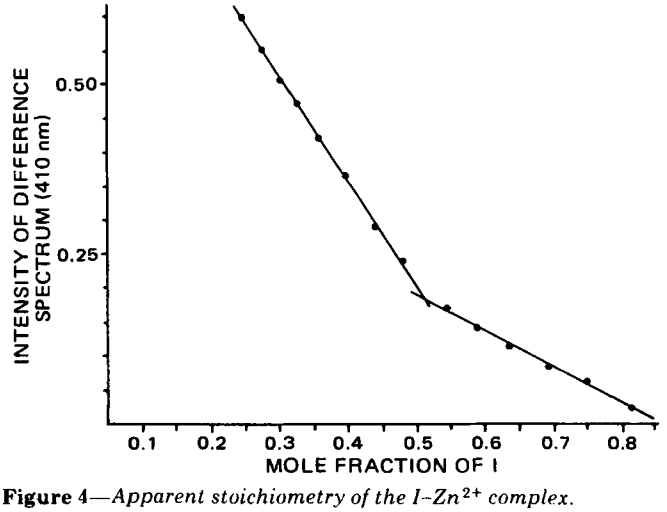


**Figure 4**—*Apparent stoichiometry of the I-$Zn^{2+}$ complex.*

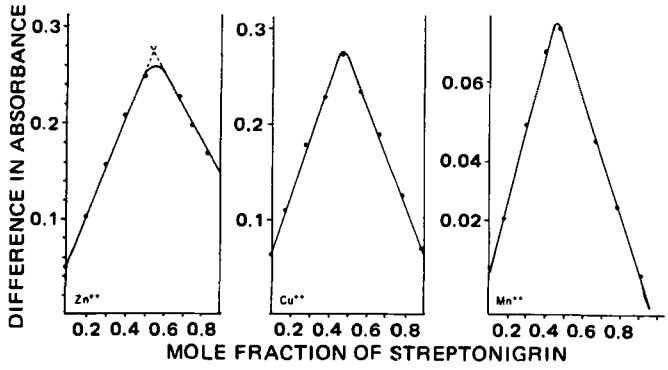


**Figure 5**—*Job plots of streptonigrin–metal complexes. The ordinates represent the difference between the absorbance of streptonigrin and that of the complex at the mole fraction value represented in the abscissa. The total molarity (ligand plus metal) was constant.*

concentrations, respectively; and $A$ was the observed absorbance value.

Although a 1:1 complex could be demonstrated readily in the preceding experiments, further I titration with $Zn^{2+}$ showed progressive increases in the difference spectrum intensity. The increase rate showed a slope change at a 5–10-mole ratio for $Zn^{2+}$ to streptonigrin. This continued until a 20–25-mole ratio was reached, beyond which point it remained constant. This unusual metal to ligand ratio is difficult to rationalize merely on the basis of the streptonigrin structure. To assess the ionic strength influence on the metal complexation, a titration was carried out in the presence of sodium chloride (0.1 $M$). This lowered the end-point, with the constant value being obtained at a mole ratio of 10–15:1 for $Zn^{2+}$ to streptonigrin.

The streptonigrin structure has many potential sites for complexation with zinc: the picolinic acid system, the 2,2-pyridylquinoline system, the aminoquinone system, the 2-(3-amino-2-pyridyl)quinoline system, and the amino and phenolic functions of rings C and D. The strongest are probably the first two. However, they cannot all be involved in complexation at the same time because some atoms or functions taking part in complexation are common to several systems. Also, after one proton has been displaced to form a complex, additional proton removal is a more difficult operation. A more likely explanation for the observed large metal–ligand ratios is in terms of mass action; *i.e.*, a sufficiently large metal ion excess must be present to form a relatively stable complex involving more than one metal atom. The term "apparent stoichiometry" is used in expressing this relationship until a more detailed picture of the interactions emerges.

**DNA Complexation**—Although the necessity for zinc was clearly demonstrated for streptonigrin binding with DNA, stoichiometry determination must consider the variable metal to ligand ratios indicated. For this reason, I titration with DNA was carried out at various $Zn^{2+}$ to streptonigrin ratios (Fig. 6A). In the absence of $Zn^{2+}$, the difference spectrum was nonexistent. In the presence of varying $Zn^{2+}$, spectrophotometric titrations with DNA could be carried out to stable end-

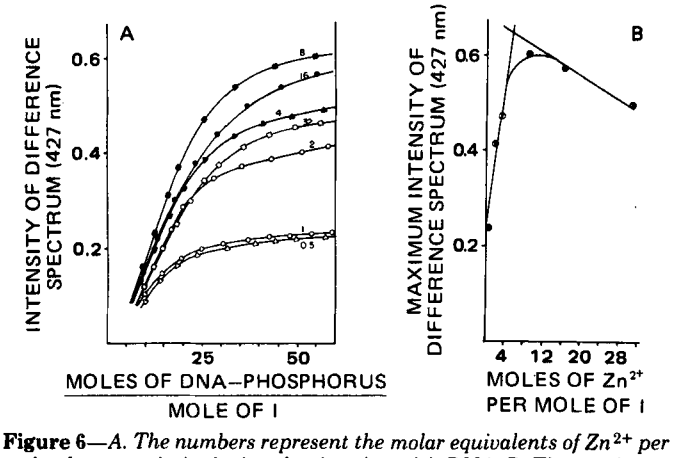


**Figure 6**—*A. The numbers represent the molar equivalents of $Zn^{2+}$ per mole of streptonigrin during the titration with DNA. B. The maximum intensity values from Fig. 6A are plotted against the molar ratio of $Zn^{2+}$-streptonigrin.*

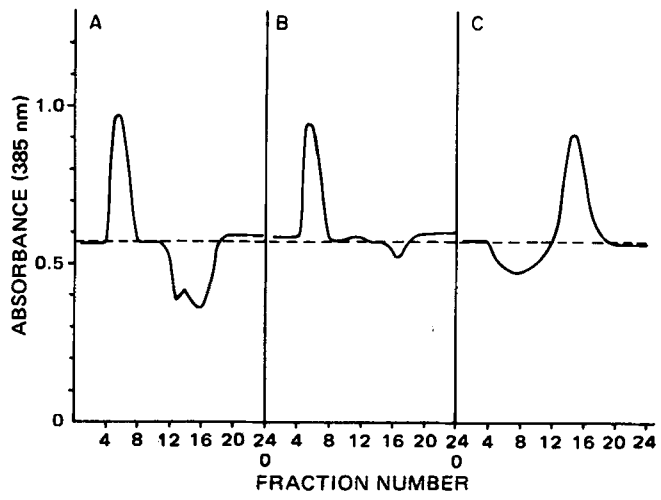


**Figure 7**—*Gel-chromatography of I-DNA in the presence (A and B) and absence (C) of $Zn^{2+}$. A. The column was equilibrated with streptonigrin–zinc ($4 \times 10^{-5}$ M and $4 \times 10^{-4}$ M, respectively) and the DNA sample ($6.4 \times 10^{-3}$ M of DNA-phosphorus) was added. Elution was with the same streptonigrin–$Zn^{2+}$ solution. B. Same as A but the DNA sample contained $3.2 \times 10^{-4}$ M of streptonigrin. C. Same as A but without $Zn^{2+}$.*

points. At $Zn^{2+}$ to streptonigrin ratios of 0.5:1 and 1:1, the curves were of the same order of magnitude. They showed that complexation took place between DNA and 1:1 streptonigrin–$Zn^{2+}$. At higher metal to ligand ratios, the difference spectrum intensity increased progressively until a maximum was reached at a zinc to streptonigrin ratio of 8:1. At still higher ratios, the difference spectrum intensity began to decrease.

In Fig. 6B, the maximum difference spectrum intensity was plotted against the $Zn^{2+}$ to streptonigrin ratio. Extrapolation of the linear portions gave an intersection point that corresponded to a ratio of 6–7:1 for $Zn^{2+}$ and streptonigrin. This value gave the maximum difference spectrum intensity change, suggesting that at this ratio of $Zn^{2+}$ to streptonigrin, complexation with DNA was at the maximum. Titration of I with DNA at what appears to be an optimum $Zn^{2+}$-streptonigrin ratio of 7:1 showed the pattern commonly seen during complex formation, and titration could be performed to a relatively stable end-point. From several titrations, a value of 20–25 was obtained for the apparent stoichiometry, *i.e.*, 1 mole of streptonigrin/20–25 moles of DNA-phosphorus in the presence of 7 moles of $Zn^{2+}$. Thus, the interaction of I and DNA in the presence of $Zn^{2+}$ parallels that of I and $Zn^{2+}$, where several moles of metal ion per mole of I were required for maximum interaction, as judged by the spectral shifts and intensity changes.

The streptonigrin–$Zn^{2+}$–DNA complex prepared in a 1:7:25 ratio could be diluted up to 10-fold without appreciable dissociation (as shown by the Beer's law plot). The complex was stable to dialysis for 4 days at 5°. The presence of $Zn^{2+}$ in the complex was readily demonstrable by the $\lambda_{max}$ (405–410 nm), as well as through the shift induced by edetate sodium to 365 nm.

Gel permeation chromatography was used for further characterization. When a I-DNA mixture was chromatographed on a cross-linked dextran column, the two appeared as sharp bands at their appropriate elution volumes. In the presence of $Zn^{2+}$ (10 molar excess), two bands representing bound and free I could be seen during initial column development. However, as the elution progressed, they emerged as a very broad band. This behavior was not unexpected because of the continuous equilibrium alteration during elution.

The existence of the complex between streptonigrin and $Zn^{2+}$ and DNA could be seen more clearly by the chromatographic procedure described previously (13) (Fig. 7A). As the DNA emerged from the column, the peak represented not only DNA but also the apparent stoichiometric amount of streptonigrin–$Zn^{2+}$. Since the introduced sample did not have the

**Table I—I-Metal Complex Formation Constants**

| Complex | $K$ at 22° |
|---|---|
| I-$Zn^{2+}$ | $1.45 \pm 0.5 \times 10^5\ M^{-1}$ |
| I-$Cu^{2+}$ | $1.27 \pm 0.5 \times 10^5\ M^{-1}$ |
| I-$Mn^{2+}$ | $2.03 \pm 0.5 \times 10^4\ M^{-1}$ |

required ligand–metal complex concentration, it must have been taken up by the DNA from the surrounding solution. The negative peak that followed the DNA peak represented the ligand–metal complex removed from the solution. The two peak areas were equal.

Figure 7B shows the behavior when an additional 20 equivalents of streptonigrin–$Zn^{2+}$ were introduced with the DNA sample. The negative peak was nearly absent, although the positive peak height due to the complex was the same as before. The apparent stoichiometry determined from several such elution curves, in which the negative peak was nearly absent, was 1 mole of streptonigrin/20–25 moles of DNA–phosphorus in the presence of 7 *M* equivalents of $Zn^{2+}$.

The column behavior in the absence of $Zn^{2+}$ is shown in Fig. 7C. The initial negative peak suggested that the DNA displaced I from its position on the column and emerged first. The displaced streptonigrin appeared as the subsequent positive peak. This finding further indicates that complexation did not occur in the absence of $Zn^{2+}$.

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# 3-Quinuclidinyl Benzilate Hydrolysis in Dilute Aqueous Solution


**LARRY A. HULL, DAVID H. ROSENBLATT, and JOSEPH EPSTEIN** ˣ



Received March 9, 1977, from the *Environmental Research Division, Chemical Systems Laboratory, Department of the Army, Aberdeen Proving Ground, MD 21010.* Accepted for publication January 9, 1979.

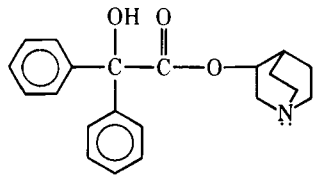


**Abstract** □ The hydrolysis kinetics of 3-quinuclidinyl benzilate were determined over the pH 0–14 range at different temperatures in solutions buffered to a constant pH. Analysis of data extrapolated to zero buffer concentrations permitted construction of an overall rate expression for pH 0–14 and 0–100°. Reaction mechanisms are discussed.



**Keyphrases** □ Quinuclidinyl benzilate—hydrolysis in dilute aqueous solution, kinetics, pH, temperature □ Aminoalcohol esters—quinuclidinyl benzilate, hydrolysis in dilute aqueous solution, kinetics □ Hydrolysis kinetics—quinuclidinyl benzilate in dilute aqueous solution, pH, temperature


3-Quinuclidinyl benzilate (I), like atropine, is a muscarinic cholinergic antagonist of considerable pharmacological interest (1). Among the properties of greatest pharmaceutical significance is its hydrolytic behavior in dilute aqueous solution. In the present research, sufficient information was gained to permit hydrolysis rate prediction in the absence of high general base or salt concentrations at any desired temperature (0–100°) or pH (0–14).



I

## EXPERIMENTAL

**Reagents and Materials**—Technical grade I was dissolved in chloroform, decolorized with charcoal, and extracted into aqueous hydrochloric acid. The acidic solution was made slightly basic and back-extracted with chloroform. Solvent evaporation left a solid residue, which was recrystallized twice from ethyl acetate to give a product of at least 98% purity.

*Anal.*—Calc. for: C, 74.75; H, 6.87; N, 4.15; O, 14.23. Found: C, 74.6; H, 7.1; N, 4.3; O, 14.4.

Compound I-HCl was prepared by addition of hydrogen chloride-saturated ether to a chloroform solution of I; the crystalline precipitate was dried carefully. All solutions were made with carbon dioxide-free distilled water. Commercial grade buffers were used without additional purification. Ether was purified of peroxide by filtration through an alumina column.

**Instrumentation**—A spectrophotometer[1] with 1-cm quartz cells or 10-cm silica cells was used for absorptiometric determinations. The pH was determined with a pH meter[2]. Solvent contamination with stopcock grease was avoided through the use of separators with polytef stopcocks for liquid–liquid extractions. The reaction solutions were kept at a constant temperature with water baths[3], or, in some cases, the reacting mixtures were sealed in glass vials immersed in appropriate refluxing solvents that kept the temperature constant.

**Procedure**—Products were isolated from a pH 11.5 reaction mixture that was kept 4 hr at 60°. Only benzilic acid and 3-quinuclidinol were found; these compounds were identical to authentic samples according to IR and melting-point analyses.

[1] Beckman DK-2.
[2] Beckman model G.
[3] Forma 2095S and Aminco 4-8600.

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