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Polymeric Drugs: Some Studies on Collagen-Kanamycin Conjugates

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ABSTRACT

Kanamycin, a broad-spectrum antituberculosis antibiotic, was coupled to three different collagen substrates using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, glutaraldehyde, and periodiate-oxidized kanamycin possessing aldehyde functional groups. Kanamycin-treated collagen fibers have exhibited marked differences in their chemical contraction and relaxation, swelling, and shrinkage-temperature behavior compared to untreated collagen fibers. In an in vitro study, a release of 26% of the bound kanamycin from collagen-kanamycin complex was noticed.

INTRODUCTION

The traditional single-dose method of administering a drug or a pharmaceutical agent is the most convenient of the various methods developed. However, this method has certain drawbacks, such as failure to maintain its action over long periods, difficulty in localizing its actions, and discrepancies in maintaining the plasma level [1]. This has led us to think about a novel approach, namely controlled-release technology. In a controlled-release system, a biologically active agent is released slowly at a predetermined rate toward the

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diseased site. Much work has been carried out on the development of controlled polymeric drug delivery systems [2-5]. The advantages of controlled-release technology over the traditional single-dose method have been reviewed by various researchers [6-9].

Various polymeric drugs based on biodegradable and nonbiodegradable synthetic polymers have been prepared and studied [10]. However, biodegradable polymeric systems exhibit certain advantages over nonbiodegradable systems because they undergo backbone cleavage during biodegradation, thus releasing the biologically active agent. This approach has also been extended to natural polymers. One among them is collagen, a biopolymer, which is biocompatible and biodegradable. However, much attention has not been focussed on the preparation and characterization of collagen-drug complexes. Among the few collagen-drug complexes prepared, the drug has been incorporated into the matrix either by physical entrapment or by ionic binding [11-13].

In this paper we report covalent coupling of kanamycin, an antituberculosis agent, onto collagen by three independent methods.

EXPERIMENTAL

Materials

Collagen prepared from the middle corium of buffalo hide was used as the source of insoluble collagen. Collagen powder was prepared by the procedure followed in the Central Leather Research Institute, Madras, India [14]. The material was checked for purity by determining its hydroxyproline [15] and arginine [16] contents by standard procedures.

Collagen membranes crosslinked with glutaraldehyde and hexamethylene diisocyanate (HMDI) were gift samples from Prof. M. Chvapil, University of Arizona, U.S.A. Collagen fibers were isolated from 4-month-old albino rat tail tendons by the procedure of Trelstad et al. [17].

Kanamycin acid sulfate (Sigma, U.S.A.), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma, U.S.A.), and glutaraldehyde (E. Merck, Germany) were used as supplied. All other reagents used were of reagent grade.

Coupling Methods

With Carbodiimide

To 100 mg of collagen substrate in a 50-mL beaker, the required amount of EDC and 3 mL of distilled water were added. After 30 min, 100 mg of kanamycin was added, and the total volume was made up to 10 mL with 0.2 M sodium phosphate solution. The pH was adjusted to

the required value by adding either dilute alkali or dilute acid. The reaction was allowed to proceed at 25°C for the required time. The reaction products were centrifuged or filtered, washed with cold distilled water, and the washings were made up to a known volume.

With Glutaraldehyde

To 100 mg of collagen substrate in a 50-mL beaker, 3.5 mL of 0.1% glutaraldehyde solution was added. After 60 min of pretanning, 30 mg of kanamycin was added, and the pH was adjusted to 5.5 with 0.01 N HCl. The total volume was maintained at 10 mL and the reaction was allowed to proceed at 20°C for 60 min. The reaction products were washed with ice-cold water and stored.

With Oxidized Kanamycin

Oxidation of Kanamycin. The oxidation of kanamycin was carried out by the procedure of Hurwitz et al. [18] as in the case of daunomycin. To 5 g of kanamycin dissolved in 125 mL of 0.1 M phosphate buffer saline of pH 7.2, 100 mL of 0.1 M sodium periodate was added, and the solution was incubated for 1 h at room temperature in the dark. After the required time, 1 M glycerol was added to a final concentration of 0.05 M to consume any excess periodate. The reaction product was precipitated with 95% ethanol, filtered, and dried in vacuo. The extent of oxidation of kanamycin was determined according to the procedure reported earlier by estimating the aldehyde content [19].

Coupling Procedure. To 100 mg of the collagen substrate was added 10 mL of the periodate-oxidized drug solution (10 mg/mL) in 0.15 M potassium carbonate buffer of pH 9.5. The solution was mixed and incubated at 37°C for 1 h. Sodium borohydride was added to a final concentration of 0.3 mg/mL, and the total volume was made up to 20 mL. The reaction was allowed to proceed for 2 h. The reaction products were filtered and washed thoroughly with cold distilled water.

Estimation of Bound Kanamycin

The conditions adopted by Dubois et al. [20] for the estimation of sugars was followed to determine the kanamycin bound to collagen. After coupling of kanamycin to collagen, the unbound kanamycin in the wash liquor was estimated, and the amount of kanamycin bound was computed from the difference. These values were obtained from the standard values of kanamycin ranging from 20 to 100 μg. Alternatively, the bound kanamycin was estimated by an indirect method. In this, a known weight of collagen-kanamycin complex was hydrolyzed with 6 N HCl at 105-108°C in vacuo, and the collagen content was computed from arginine values using standard procedures [16].

Structural Stability of Collagen Fibers

Chemical Contraction and Relaxation

This was carried out according to the procedure of Chvapil and Zahradnik [21] using 2.5 M KI solution.

Swelling Rate

The swelling rate of collagen fibers was studied by using a calibrated eyepiece micrometer grid. Fibers of uniform thickness were placed under the micrometer eyepiece, and the initial thickness of the fibers was noted. Some 0.001 N hydrochloric acid was placed on the fiber, and the swelling of the fiber was noted for the required time. The rate of swelling was determined by the difference in the thickness of the fiber before and after adding hydrochloric acid.

Shrinkage Temperature (T_S°)

The shrinkage temperature of collagen fibers was determined by a method similar to that of Nutting and Borasky [22]. For each fiber, six determinations were made and the mean value was taken.

In Vitro Release of Kanamycin from Collagen-Kanamycin Complex

To 2 mg of collagen kanamycin complex weighed into a 100-mL conical flask was added 50 mL of 0.2 M citrate of tris buffer of varying pH (5.0-9.0). The flasks were incubated at 37°C. After the required time, 5 mL of the above buffer was pipetted out into a Pyrex tube and the released kanamycin was estimated by the procedure discussed earlier. The values were computed from a standard absorbance curve of kanamycin.

RESULTS AND DISCUSSION

Deaths due to contagious diseases are very common in India. Tuberculosis is a prime disease among them. Efforts are being made to eradicate this communicable disease. Hence, collagen-based slow-release polymeric systems using kanamycin, a broad-spectrum antituberculosis antibiotic, have been investigated in the present study. As a part of this study, in this paper we report preliminary results obtained on the preparation and characterization of collagen-kanamycin complexes.

The kanamycin molecule produced by streptomyces kanamyceticus contains 6-deoxy-6-amino-D-glucopyranose and 3-deoxy-3-amino-D-glucopyranose linked by α-glycosidic bonds to the 4th and 6th positions

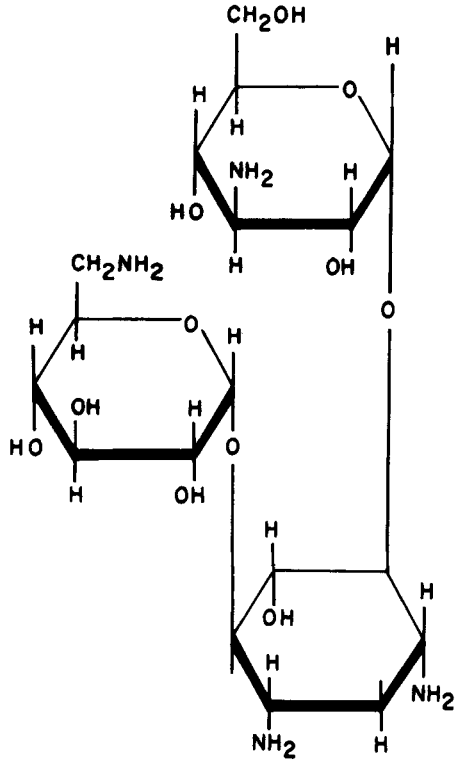
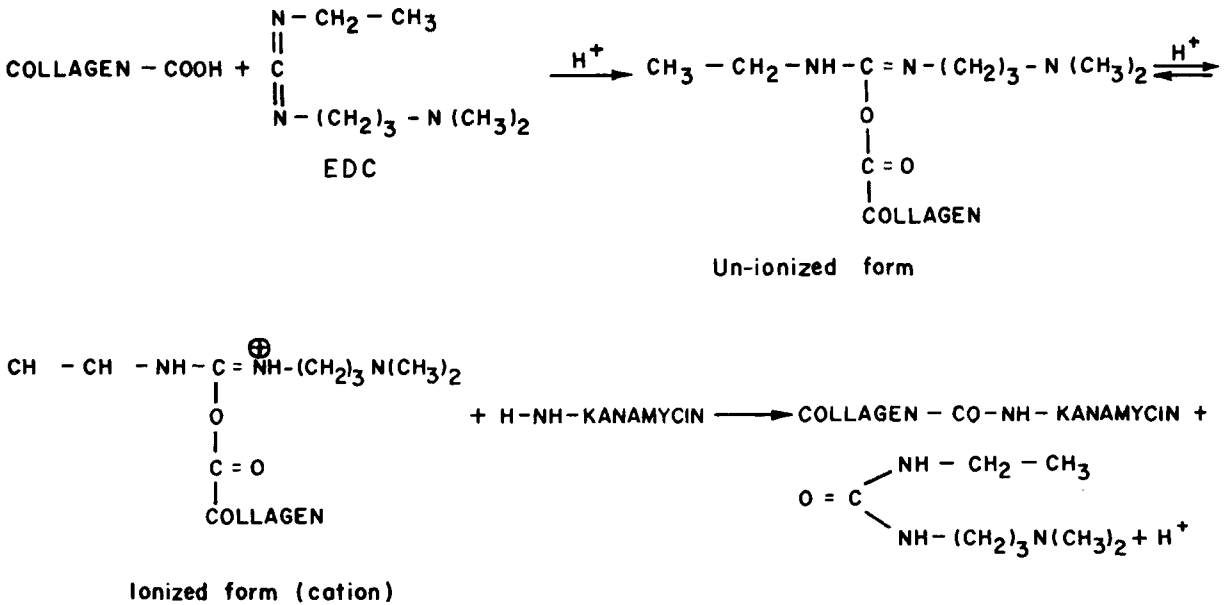


FIG. 1. Structure of kanamycin.

of 2-deoxystreptamine (Fig. 1). Three independent methods of coupling were used in the preparation of collagen-kanamycin complex to check the viability of coupling. In the first method, carbodiimide (EDC) was used as a coupling agent because of its advantages and reactivity with various functional groups [23-25]. In the second method, glutaraldehyde was used because of its high reactivity with amino groups of either of the substrates. In the last method, sodium periodate-oxidized kanamycin possessing aldehyde functional groups was used for coupling reactions with collagen. The extent of oxidation of kanamycin, determined by the hydroxylamine method, was found to be 58% [19]. Further, the presence of the carbonyl function was evidenced by the IR spectrum. A strong absorption band at 1700 cm^{-1} established the presence of aldehyde carbonyl groups in oxidized kanamycin.

The biodegradability of a matrix is dependent on the extent of crosslinking. An increase in crosslinking of collagen by any means slows down the degradation of the collagen substrates, thereby causing the slow release of drug from the matrix [26]. Hence, in the present



SCHEME 1. Coupling of kanamycin to collagen by using carbodiimide.

study, collagen membranes crosslinked with hexamethylene diisocyanate and glutaraldehyde were used for the coupling reaction. For comparison and also as a representative among various collagen products, uncrosslinked hide powder was used throughout the experiments.

During reactions, accessibility between collagen substrates, EDC, and kanamycin as provided by swelling the collagen substrate in sodium phosphate solution in a heterogeneous medium. The reactions involved during coupling using three methods are shown in Schemes 1 and 2.

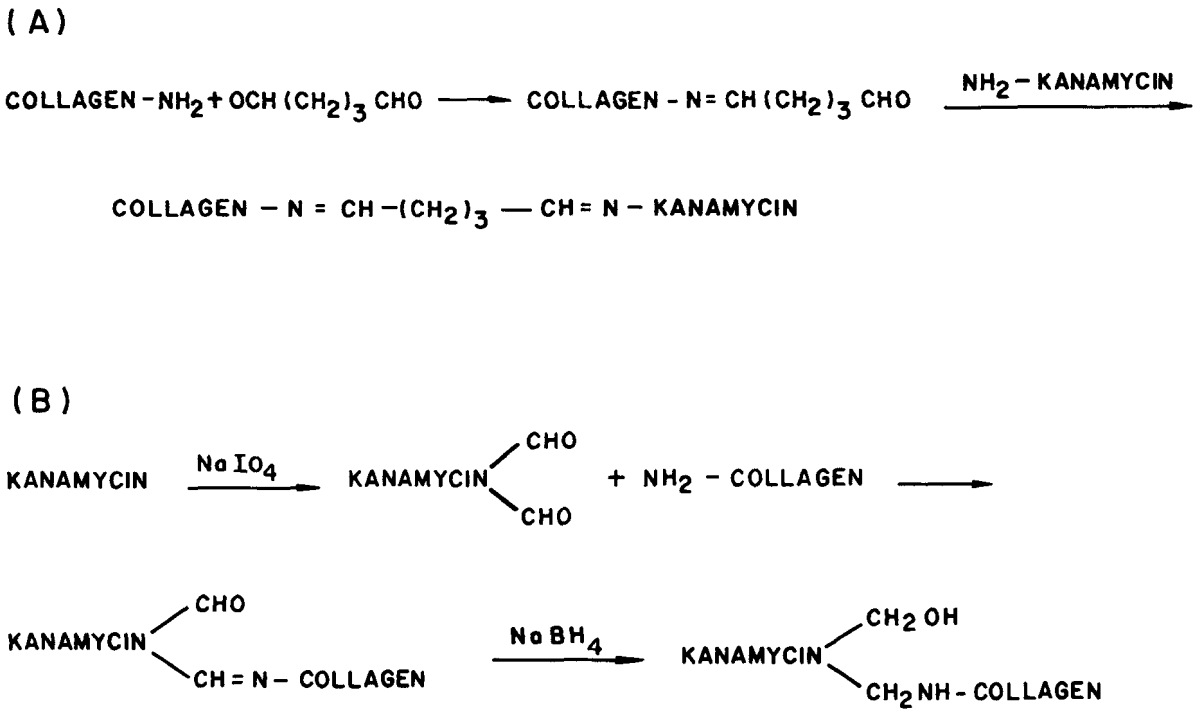
The amount of kanamycin bound to various collagen substrates was estimated both directly and indirectly. After trying several methods, it was noticed that kanamycin produces a color with phenol and sulfuric acid as to the amino sugars. Hence, this method was followed to estimate the kanamycin bound to collagen when EDC and oxidized kanamycin, respectively, were used for coupling. However, as an indirect method, the kanamycin bound was determined by estimating the collagen content from the arginine content of the collagen-kanamycin complex after hydrolysis with 6 N HCl [16]. This method was particularly followed when glutaraldehyde had been used as a coupling agent because, unlike other coupling agents, glutaraldehyde interferes during estimation by the phenol-sulfuric acid method [20].

INFLUENCE OF COUPLING VARIABLES

The kanamycin content in collagen-drug complex is dependent on such factors as EDC, concentration, reaction time, and pH. Hence, some of these coupling variables were investigated to study their effect on the binding of kanamycin.

EFFECT OF EDC CONCENTRATION

From Table 1 it can be seen that the binding of kanamycin increases with increasing EDC concentration. A fourfold increase in binding was observed at 100 mg/10 mL EDC. Presumably, this may be due to the activation of more carboxyl groups on collagen under our experimental conditions. A further increase in EDC concentration had no effect on binding. Since kanamycin contains only a limited number of amino groups, an appreciable number of intermediates produced by the activation of carboxyl groups of collagen might have reacted with kanamycin, thereby attaining saturation. Obviously, an excess of intermediates produced has no effect on further binding.



SCHEME 2. Coupling of kanamycin to collagen by using (A) glutaraldehyde and (B) oxidized kanamycin.

TABLE 1. Effect of EDC Concentration on Binding of Kanamycin to Collagen[a]

EDC, mg	Drug bound,[b] mg/100 mg
20	3.26
40	6.86
60	9.88
80	12.46
100	14.49
120	14.38

[a]Collagen = 100 mg, kanamycin = 100 mg, pH = 4.75, Time = 2 h, 25°C, total volume = 10 mL.
[b]Values calculated from the concentration difference of kanamycin before and after the reaction.

EFFECT OF REACTION TIME

Binding of kanamycin to collagen increased with increasing time. A twofold increase in binding was observed for every 20 min up to 60 min. The binding at 60 min and 120 min is more or less the same, longer reaction time having no effect on the degree of binding (Table 2).

EFFECT OF pH

The binding of kanamycin increased with increasing pH up to 5.5 and then decreased. Theoretically, a certain concentration of hydrogen ion is required to cause the protonation of carbodiimide and at the same time to make available the maximum number of unionized amino groups for the kanamycin. Maximum binding occurs at pH 5.5 (Table 3).

Binding Capacity of Kanamycin to Various Collagen Substrates

With Carbodiimide

It can be seen from Table 4 that the binding of kanamycin to various collagen substrates is more or less the same. However, collagen mem-

TABLE 2. Effect of Time on Binding of Kanamycin to Collagen[a]

Time, min	Drug bound,[b] mg/100 mg
20	3.36
40	7.04
60	14.49
120	14.62

[a]Collagen = 100 mg, kanamycin = 100 mg, EDC = 100 mg, pH = 4.75, 25°C, total volume = 10 mL.
[b]Values calculated from the concentration difference of kanamycin before and after the reaction.

TABLE 3. Effect of pH on Binding of Kanamycin to Collagen[a]

pH	Drug bound,[b] mg/100 mg
3.10	6.86
4.00	10.48
4.75	14.49
5.50	15.41
6.50	12.49

[a]Collagen = 100 mg, kanamycin = 100 mg, EDC = 100 mg, time = 2 h, 25°C, total volume = 10 mL.
[b]Values calculated from the concentration difference of kanamycin before and after the reaction.

TABLE 4. Binding Capacity of Collagen to Various Collagen Substrates

Type of collagen substrate	Drug bound, mg/100 mg		
	a[a,b]	b[c,d]	c[b,e]
Collagen powder	14.49	16.50	8.44
Collagen membrane (HMDI crosslinked)	14.78	17.04	8.14
Collagen membrane (glutaraldehyde crosslinked)	8.48	11.66	2.14
Rat tail tendon	15.01	15.49	7.86

[a]Collagen = 100 mg, kanamycin = 100 mg, EDC = 100 mg, pH = 4.75 ± 0.01, time = 2 h, 25°C, total volume = 10 mL.
[b]Values calculated from the concentration difference of kanamycin before and after the reaction.
[c]Collagen = 100 mg, glutaraldehyde = 3.5 mL, kanamycin = 30 mg, pH = 5.5, time = 1 h, 25°C, total volume = 10 mL.
[d]Values calculated from the arginine content of the collagen-kanamycin complex.
[e]Collagen = 100 mg, oxykanamycin = 200 mg, pH = 9.5, time = 2 h, 37°C, total volume = 20 mL.

branes crosslinked with glutaraldehyde exhibit less binding. Since only the carboxyl groups of collagen are expected to participate in the binding of kanamycin, it is not clear why glutaraldehyde-tanned collagen should show a decrease in binding. Probably the highly crosslinked nature of the substrate prevents swelling and thus impairs the accessibility of the reagent to the carboxyl groups of collagen. Further, glutaraldehyde is prone to polymerization, and the polymerized molecules attached to the collagen may also mask carboxyl groups of collagen.

With Glutaraldehyde

As in the case of EDC coupling the glutaraldehyde binding of kanamycin to collagen substrates of different types is more or less the same except for the collagen membrane crosslinked with glutaraldehyde. The glutaraldehyde-tanned collagen membrane was not freshly tanned but had been tanned and kept for a long time. In glutaraldehyde tanning, the ϵ-amino groups of collagen are irreversibly modified and, at the same time, some reactive aldehyde groups are also introduced into collagen. It is possible that these free aldehyde groups in the modified collagen are modified on prolonged keeping due to oxidation,

etc., so that the modified substrate contains very few free amino and aldehyde groups, resulting in less binding after the coupling reaction (Table 4).

With Oxidized Kanamycin

From Table 4 it is evident that the binding of kanamycin to various collagen substrates is almost the same after stabilization with sodium borohydride. A decrease in binding in the membrane crosslinked with glutaraldehyde may be attributed to the nonavailability of sufficient amino groups of collagen, as discussed previously.

The influence of bound kanamycin on the structural stability of collagen has been studied by chemical contraction and relaxation, rate of swelling, and shrinkage temperature of kanamycin-treated collagen fiber and untreated collagen fibers. Since oxidized kanamycin directly participates in the reaction (i.e., in the absence of any coupling agent), fibers treated with oxidized kanamycin were chosen for these studies.

Chemical Contraction and Relaxation. It has been reported that contraction and relaxation of collagen fibers in certain high ionic strength solutions is modified characteristically. Hence, contraction and relaxation of collagen fibers in the presence of a lyotropic agent have been used to study the interaction between amino-sugars and collagen (contraction phase) and between individual collagen fiber structures (relaxation phase) [21]. As seen from Fig. 2, oxykanamycin-treated fibers showed only a minimal contraction during 120 min of incubation in 2.5 M potassium iodide solution and no relaxation. This may be due to the crosslinking of oxykanamycin with collagen. Like other aldehydes, oxykanamycin may also bring about the crosslinking of collagen, resulting in a pronounced change in its contraction and relaxation behavior. In contrast to treated fiber, the untreated collagen fiber showed both contraction and relaxation phases.

Rate of Swelling. The swelling of oxykanamycin-treated fiber gradually increases with time. The swelling rate has attained almost a constant value after 5 min. This may be attributed to the participation of kanamycin in crosslinking, thereby inhibiting further swelling. Unlike treated fiber, untreated collagen fiber showed a continuous increase in swelling with time (Fig. 3).

Shrinkage Temperature. The oxykanamycin-treated collagen fiber showed a shrinkage temperature of 68°C while untreated fibers showed 53°C. This increase in shrinkage temperature may once again be attributed to intermolecular crosslinking of collagen with kanamycin.

In Vitro Release of Kanamycin from Collagen-Kanamycin Complex

A continuous increase in the release of kanamycin from collagen-kanamycin complex has been observed with increasing time as well

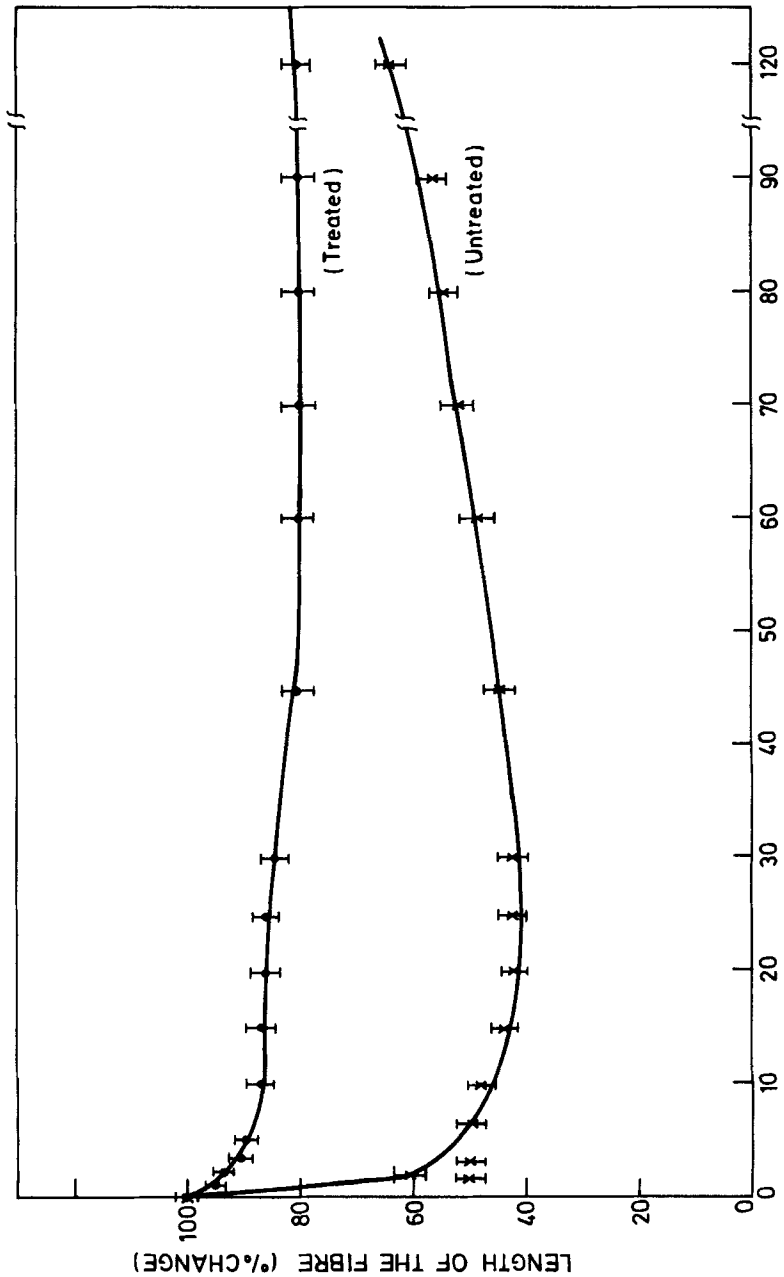


FIG. 2. Chemical contraction and relaxation of untreated and kanamycin-treated collagen fibers.

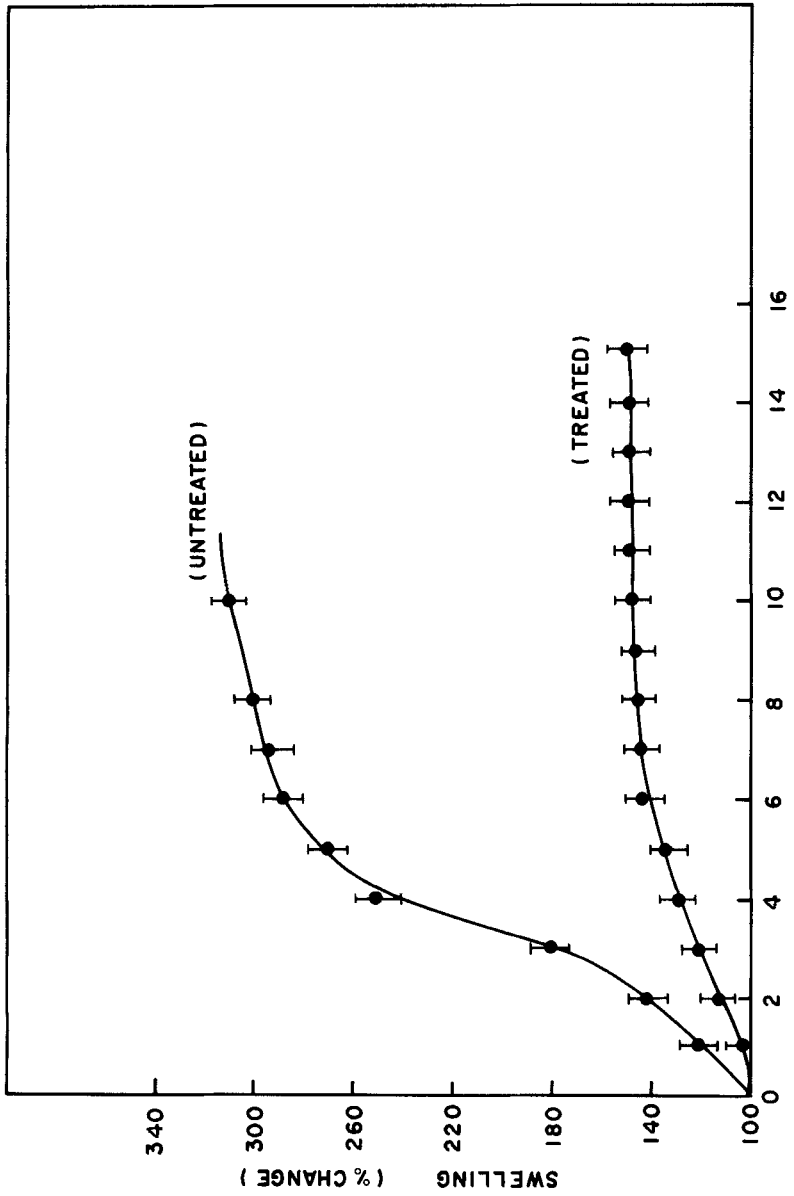


FIG. 3. Swelling rate of kanamycin-treated and untreated collagen fibers.

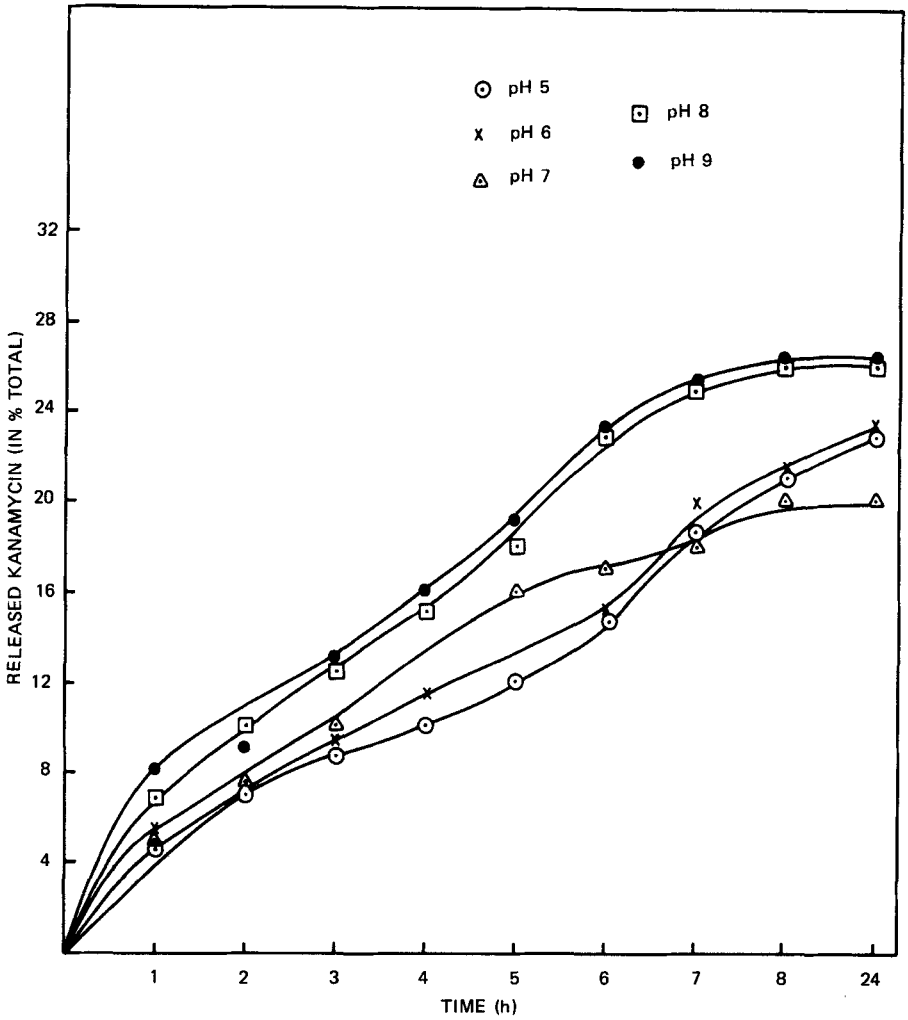


FIG. 4. Release of kanamycin from collagen-kanamycin complex.

as pH. Higher amounts of kanamycin were released at alkaline pH values. A maximum of 26% of bound kanamycin was released after 24 h. This may be due to the partial hydrolysis of the newly formed bonds between collagen and kanamycin and also due to traces of loosely bound kanamycin (Fig. 4).

In conclusion, of the three methods studied, the glutaraldehyde coupling method showed a higher degree of binding to collagen. Hurwitz et al. [18] found a similar trend when daunomycin was coupled

to antibodies. Further, structural studies indicate that kanamycin contributes to intermolecular crosslinking. Chvapil et al. [27] reported a similar observation when various drugs were bound to collagen.

Details of the activity of these collagen-drug complexes will be reported elsewhere.

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