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# COMMUNICATIONS

## In-vitro interaction of mitomycin C and streptomycin A with collagen

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**Abstract**—Mitomycin C and streptomycin A were studied for their interaction with soluble collagens from sepia, fish and rat skins using UV absorption spectroscopy at pH 7·4 and 3·0 and equilibrium dialysis. Both the drugs bound to collagen as shown from association constants and could be quantitatively recovered by prolonged dialysis against water showing formation of dissociable bonds between drug and collagen. At pH 7·4, mitomycin C showed greater binding affinity than streptomycin A with collagen from all three species. The reverse trend was seen at pH 3·0. Binding capacity of sepia and fish skin collagens for streptomycin A at pH 3·0 was found to be significantly greater than for mitomycin C.

Collagen has been used as a biodegradable carrier for the sustained release of mitomycin C, it being possible to regulate the release of drug for 10 days (Bloomfield et al 1978; Yamahira et al 1985). More recently (Ramesh et al 1986, 1989) we found an in-vitro interaction of bleomycin with collagens from three different species, i.e. skin of sepia, fish and rat. The binding capacity of bleomycin was found to be greatest at pH 3·0 with sepia skin collagen. The study has now been extended to mitomycin C and streptomycin A to find a particular collagen which would be more suitable as a biodegradable carrier. The aim of the present study was to investigate the binding behaviour of these drugs with collagens representing vertebrates (rat), lower vertebrates (fish) and invertebrates (sepia) at two different pH values.

### Materials and methods

*Materials.* Mitomycin C was purchased from Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan and was used without further purification. Streptomycin A was purchased locally and was also used without further purification.

*Preparation of soluble collagen.* Soluble collagen from sepia skin was prepared according to Kimura et al (1981). Sepia skin was carefully removed and cut into small pieces. The tissue (200 g wet weight) was washed with cold distilled water and then with 0·5 M sodium acetate. After the final washing, the tissue was suspended in 1·5 L of 0·5 M acetic acid mixed with 100 mg pepsin (2900 units $mg^{-1}$) and incubated at 1°C for 48 h with stirring. The digest was then centrifuged at 10000 *g* for 1 h. The supernatant was precipitated with 5% NaCl. The precipitate was collected by centrifugation and dissolved in 0·5 M acetic acid and then dialysed several times against 0·2 M disodium hydrogen phosphate. The dialysate was centrifuged and the residue was dissolved successively in 0·5, 0·2 and 0·01 M acetic acid. Dialysate thus obtained was lyophilized and used in the present study.

Fish skin collagen and rat skin collagen were prepared by the method of Piez et al (1963). The purity of collagens was determined by amino acid analysis and was found to be 96–98%.

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*Dissolution of collagens in buffers.* Phosphate buffer (0·05 M) pH 7·4 was prepared. Sufficient sodium chloride was added to give a final NaCl concentration of 0·15 M and the required pH (pH 7·4) was obtained by adding NaOH pellets. Acetate buffer (pH 3·0) was prepared by standard methods. Each collagen was dissolved in phosphate buffer (pH 7·4) and acetate buffer (pH 3·0, 0·1–2 mg $mL^{-1}$) to give a final concentration of collagen between 0·033 and $0{\cdot}66 \times 10^{-5}$ M. Mitomycin C and streptomycin A were also dissolved in phosphate and acetate buffers to give a concentration of $5 \times 10^{-5}$ M for stock solutions. The mol. wt of collagen was assumed to be 300000 daltons.

*UV absorption.* The absorbance of mitomycin C at 217·8 nm and of streptomycin A at 306·2 nm was determined in the presence of collagen. Binding studies were carried out at therapeutic concentrations ($0{\cdot}15$–$2{\cdot}5 \times 10^{-5}$ M) of mitomycin C and streptomycin A. For each value of the drug, concentration of collagen was varied between 0·05 mg $mL^{-1}$ ($0{\cdot}016 \times 10^{-5}$ M) and 1·5 mg $mL^{-1}$ ($0{\cdot}5 \times 10^{-5}$ M). Acetate buffer (pH 3·0) and phosphate buffer (pH 7·4) with the same concentration of collagen were used as blanks. All determinations were in triplicate. Net decrease in absorbance of the drug at each addition of collagen was taken as the value for binding between the drug and collagen. The values $S_u$ (unbound drug concentration) and $S_b$ (bound drug concentration) were calculated from the extinction coefficients for the unbound drug, $E_1$ (in the absence of collagen), for completely bound drug $E_2$ and fraction of unbound drug, $F_u$, as described by Klotz (1946).

*Equilibrium dialysis.* Binding constants of soluble collagens (sepia, fish and rat skin) were also determined by equilibrium dialysis (Milch & Murry 1961). In this method ground-glass stoppered tubes (2·5 × 6 cm) were used. Binding was studied at different therapeutic concentrations of mitomycin C or streptomycin A ($0{\cdot}15$–$2{\cdot}5 \times 10^{-5}$ M) in each dialysis tube. Keeping the drug concentration constant, the collagen concentration was varied from $0{\cdot}016$ to $0{\cdot}50 \times 10^{-5}$ M. Each tube was dialysed against the same concentration of mitomycin C or streptomycin A. The bound ligand $S_b$ inside the dialysis tube is calculated from the difference between the total quantity of drug applied and the amount determined in the fluid outside the dialysis tube.

$$S_b = S_t - S_u$$

*Presentation of binding data.* Binding constants $K_B$, $V_{max}$ and number of binding sites, n, were calculated from $S_b$, $S_t$ and $S_u$ as described earlier (Schellman et al 1954; Milch & Murry 1961).

### Results

*UV and equilibrium dialysis studies.* Binding affinity of mitomycin C is demonstrated as reciprocal plots ($1/\bar{v}$ and $1/s$) using the spectrophotometric method (Fig. 1a) and by equilibrium dialysis (Fig. 1b). Fig. 1c, d demonstrate the binding affinity as

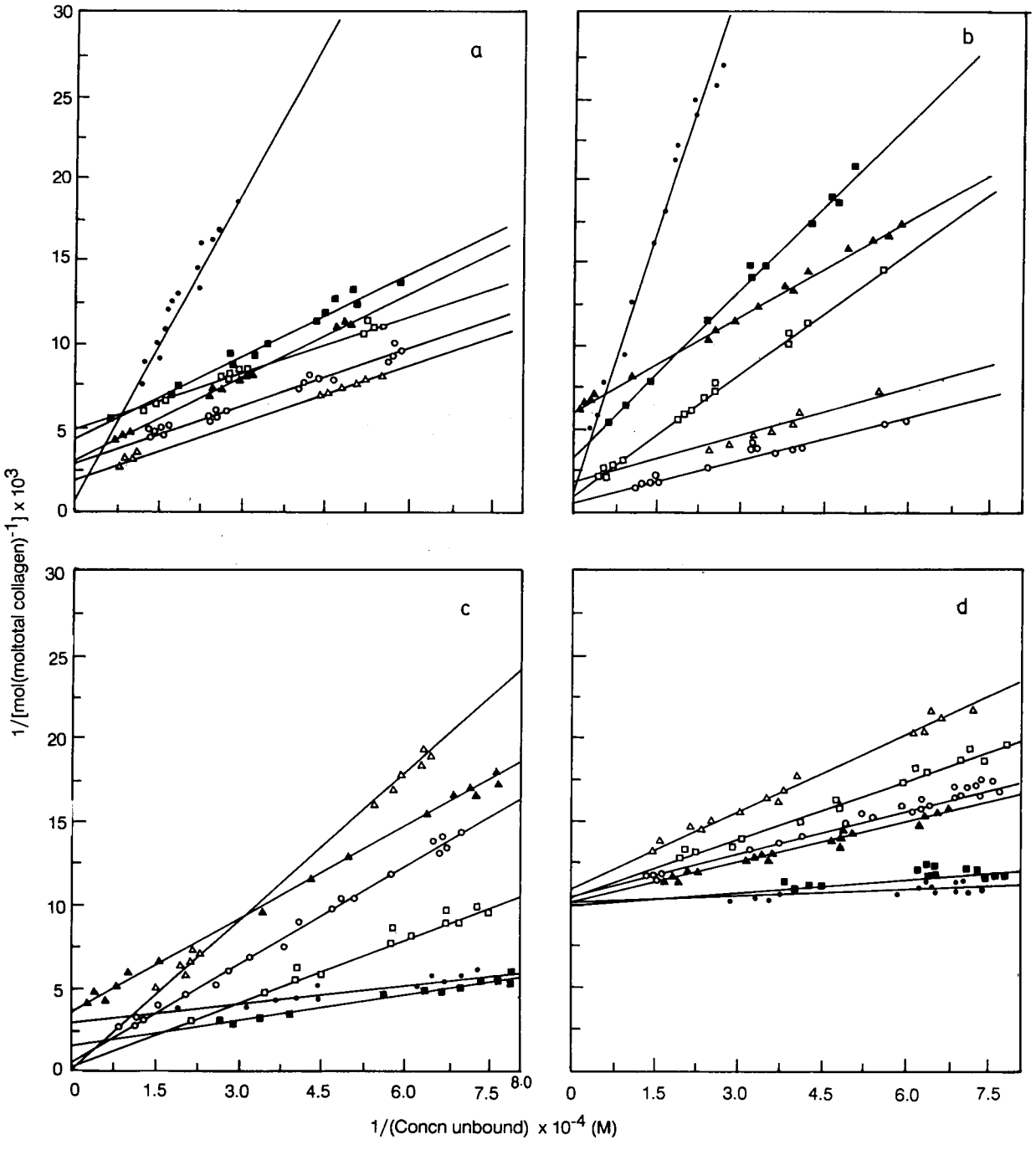


FIG. 1. Binding of mitomycin C (a,b) and streptomycin A (c,d) to acid soluble skin collagen of sepia, fish and rat at pH 3·0 and 7·4 by reciprocal plots; a and c UV spectrometric method, b and d equilibrium dialysis method. ●, Sepia (acetate buffer pH 3·0); ○, sepia (phosphate buffer pH 7·4); ■, fish (acetate buffer pH 3·0); □, fish (phosphate buffer pH 7·4); ▲, rat (acetate buffer pH 3·0); △, rat (phosphate buffer pH 7·4). For a and b, $\bar{v}$ = moles of mitomycin C bound per mole of total collagen: s = concentrations of mitomycin C. For c and d, $\bar{v}$ = moles of streptomycin A bound per mole of total collagen, s = concentration of unbound streptomycin A.

reciprocal plots for streptomycin A by spectrophotometry and equilibrium dialysis, respectively. Reciprocal plots of the binding of drugs to collagens are linear indicating a single type of independent binding site on the collagen molecule. Values of $K_B$ for each drug can be calculated from the slope of each plot. The number of binding sites present on the collagen molecule for each drug are obtained from the reciprocal of the extrapolated ordinate intercept of each plot.

The $K_B$ values and number of binding sites n for the mitomycin C-collagen interaction of each plot are listed in Table 1. Similarly $K_B$ values and number of binding sites of the streptomycin A-collagen interaction of each plot are listed in Table 2.

## Discussion

Our present investigation indicates that mitomycin C and streptomycin A bind collagens of sepia, fish and rat. The results

Table 1. Parameters of binding of mitomycin C to collagen.

| Method of estimation | Source of collagen | pH | $K_B$ $\times 10^{-7}$M | Binding sites (n) |
|---|---|---|---|---|
| UV absorption | Sepia | 3·0 | 0·22 | 1100 |
| | | 7·4 | 0·98 | 370 |
| | Fish | 3·0 | 0·64 | 233 |
| | | 7·4 | 0·95 | 208 |
| | Rat | 3·0 | 0·64 | 360 |
| | | 7·4 | 0·93 | 770 |
| Equilibrium dialysis | Sepia | 3·0 | 0·14 | 625 |
| | | 7·4 | 1·25 | 1250 |
| | Fish | 3·0 | 0·31 | 312 |
| | | 7·4 | 0·49 | 1000 |
| | Rat | 3·0 | 0·55 | 180 |
| | | 7·4 | 1·14 | 490 |

Table 2. Parameters of binding of streptomycin A to collagen.

| Method of estimation | Source of collagen | pH | $K_B$ $\times 10^{-7}$ M | Binding sites (n) |
|---|---|---|---|---|
| UV absorption | Sepia | 3·0 | 3·33 | 286 |
| | | 7·4 | 0·66 | 833 |
| | Fish | 3·0 | 2·51 | 435 |
| | | 7·4 | 0·87 | 833 |
| | Rat | 3·0 | 0·62 | 250 |
| | | 7·4 | 0·38 | 833 |
| Equilibrium dialysis | Sepia | 3·0 | 2·9 | 910 |
| | | 7·4 | 0·69 | 625 |
| | Fish | 3·0 | 2·50 | 833 |
| | | 7·4 | 0·48 | 625 |
| | Rat | 3·0 | 0·71 | 833 |
| | | 7·4 | 0·33 | 475 |

also show that both mitomycin C and streptomycin A can be quantitatively recovered by prolonged dialysis against water, thereby suggesting that the bond formed between the drug and collagen is dissociable. The reciprocal plots (Fig. 1) are linear indicating a uniform type of independent binding site on the collagen molecule.

The UV absorption maximum of the nitroguanidino group (Bonner & Lockhart 1958) is reported to be 264 nm. The shift in the absorption maximum towards a higher wave-length for streptomycin might be due to substitution in the guanidine group as shown in the structure of streptomycin. It is, therefore, likely that the interaction of mitomycin C involves the 4, 7-dione group present in the pyrrole ring.

From the results obtained in the present investigation it appears that collagen obtained from invertebrates and lower vertebrates could be used as a better source for the sustained release of mitomycin C and streptomycin A. In particular, sepia collagen would be ideal for mitomycin C drug delivery systems at physiological pH and both sepia and fish collagens would be better sources for streptomycin C drug delivery systems. Thus collagen may replace polymeric materials such as polyvinyl alcohol for controlled release of mitomycin C and streptomycin A. The biological environment it provides required for controlled release of pilocarpine has already been reported (Vasantha et al 1988).

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