Binding of betamethasone and dexamethasone by collagen

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While steroid-protein interactions have been reported (Daughaday, 1959; Villee & Engel, 1961; Sandberg, Rosenthal & others, 1966; Westphal, 1971), few reports have appeared on the binding of steroids by collagen (Eik-Nes, Schellman & others, 1954; Menczel & Maibach, 1972). Such a study provides the opportunity to investigate a protein very different from the much-studied serum albumin. This I have done using the dynamic dialysis technique of Meyer & Guttman (1968) to determine the binding parameters (number of sites, n, and their corresponding association constants, K) of the disodium phosphate ester salts of betamethasone and dexamethasone in solutions of collagen at pH 3 and 7 under isothermal conditions.

Acid soluble calf-skin collagen (ASC) was obtained from the skin of a two-week old Jersey bull using Gross's method (1958) as modified by Piez, Eigner & Lewis (1963). The ASC was purified by phosphate precipitation (Gross, 1958), lyophilized and stored over silica gel at 2°. Purity was checked by amino-acid analysis, chromatography and ultracentrifugation (Cooper & Davidson, 1965; Davidson, & Cooper 1967) whilst ash, moisture and nitrogen determinations were carried out according to Eastoe & Courts (1963). The molecular weight of the ASC was taken as 300 000 (Boedtker & Doty, 1955; 1956; Hannig & Engel, 1961; Lewis & Piez, 1964). Phosphate solutions (0·05 M) were prepared from phosphoric acid. Sufficient sodium chloride was included to give a final sodium chloride concentration of 0·15 M and the required pH obtained by the addition of sodium hydroxide pellets. Stock solutions of protein were prepared by dissolving approximately 2 mg ml^{-1} of lyophilized ASC in 0·05 M phosphate solution, pH 3·0, containing 0·15 M sodium chloride at 5° with intermittent stirring over 48 h and the solutions were clarified by centrifugation at 32 000 *g* for 1 h at 5°. The concentration of collagen solutions were obtained from the $[\alpha]_{365}^{15}$ value which was determined as − 1330·56 (Kanfer, 1975). This value compared very favourably with that found by Drake, Davison & others (1966).

The apparatus used for dynamic dialyses was based on that described by Meyer & Guttman (1968). All binding studies were conducted to give a final collagen concentration < 1 mg ml^{-1} in order to prevent the possibility of aggregation with subsequent precipitation of collagen fibrils (Wood & Keech, 1960). The use of 0·05 M phosphate buffer together with the relatively high neutral salt and low protein concentration reduces the possibility of interference from the Donnan effect (Donnan, 1924) during dialysis (Rosenberg & Klotz, 1960). The concentrations of corticosteroids were monitored spectrophotometrically at 241 nm.

The data from plots of Dt (total drug concentration) versus time from the semi-log plot in the presence of protein were fitted to an empirical tri-exponential equation (Meyer & Guttman, 1968) which has recently been shown to be the function of choice regarding treatment of dynamic dialysis data (Kanfer & Cooper, 1976). The binding data, computed from the dialytic rates as described by Meyer & Guttman (1968; 1970), were used to generate Scatchard plots (Scatchard, 1949). The binding parameters were evaluated from these Scatchard plots with the aid of a non-linear regression digital computer program (Marquardt, 1963; Meyer, personal communication) adapted for use with an ICL 1901A digital computer.

Fig. 1 depicts the Scatchard plots for the binding of betamethasone and dexamethasone to 0·084 % collagen at pH 3. The binding parameters (Table 1) were derived

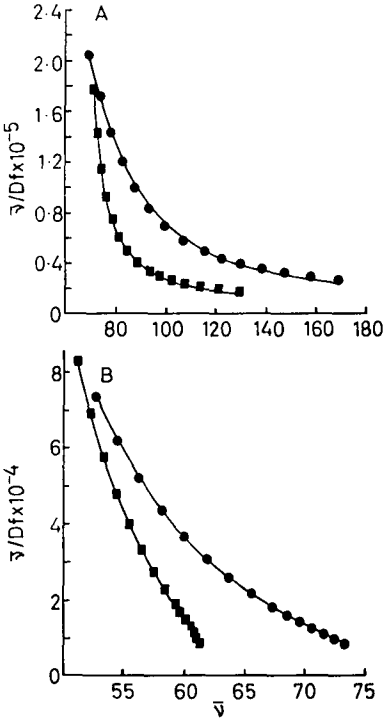

FIG. 1. Scatchard plots for the binding of corticosteroids to collagen at A–pH 3·0, B–pH 7·0 and 20°. 0·084% collagen: ■ dexamethasone; ● betamethasone. The solid curves were generated from the appropriate binding parameters. $\bar{\nu}$ = average number of moles of corticosteroid bound by one mole of collagen; Df: concentration of unbound corticosteroid.

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Table 1. *Binding of corticosteroids by collagen.*

n_1	K_1 (litre mol^{-1})	n_2	K_2 (litre mol^{-1})	pH	SSQ. of DEV*
Betamethasone disodium phosphate					
78·6	$1·19 \times 10^4$	390	48·4	3·0	$9·3 \times 10^{-1}$
48·5	$1·96 \times 10^4$	31·9	$4·19 \times 10^3$	7·0	$8·6 \times 10^{-1}$
Dexamethasone disodium phosphate					
70·6	$6·62 \times 10^4$	993	8·7	3·0	$4·6 \times 10^{-2}$
43·4	$1·19 \times 10^5$	20·6	$1·09 \times 10^3$	7·0	$8·9 \times 10^{-2}$

* Sums of squares of the deviations. Represent the differences between the experimental values of $\bar{v}$ (average number of moles of corticosteroid bound by one molecule of collagen) and those calculated from the binding parameters.

from the non-linear regression of the Scatchard plots. The solid curves were generated from the appropriate binding parameters. The values obtained indicate that approximately 10 % more primary binding sites are involved in the betamethasone-collagen interaction. The larger number of primary binding sites is, however, associated with a lower binding affinity. The values for n_2 and K_2 implicate a second class of sites of relatively high capacity and low affinity. When the corresponding n and K values obtained from the studies at pH 7·0 are compared (Table 1), a significant reduction of primary binding sites is seen to have occurred. Similarly, the number of secondary binding sites were reduced. The influence of pH on the number of available binding sites reflects the involvement of ionic interactions. At low pH, the corticosteroid is present in solution mainly as the monoanion (Flynn & Lamb, 1970) whereas collagen has a high proportion of cationic sites available at this pH. The reduction in the number of available sites observed at neutral pH may thus be rationalized in terms of the reduction in the number of cationic groups on the collagen molecule at pH 7·0 as well as a possible increase in the repulsive forces due to residual charge effects on the bound corticosteroid molecule which exists predominantly as the dianion at pH 7·0. The Scatchard plots depicting the binding of betamethasone and dexamethasone to collagen at pH 7·0 are illustrated in Fig. 1B. These results follow the same trend observed at the lower pH in that approximately 10 % more binding sites appear to be involved in the betamethasone interaction. The significant difference between the pair of epimeric corticosteroids used, lies in the configuration of the 16-methyl group. The variations in the binding constants between the steroids may thus be attributed to this stereochemical difference. Although the present investigation was concerned with the molecular interactions between collagen and charged corticosteroid ester salts, and in spite of the available evidence which indicates that the interactions are mediated by electrostatic bonding, it cannot be assumed that this type of mechanism is exclusive. Foster (1960) indicated that correlations between the number of anions bound and the number of cationic sites on a protein may be fortuitous, suggesting that the hydrophobic nature of anions plays an important role in the binding to proteins. Davis (1946) also reported that some aliphatic amino-acids have a high affinity for the non-polar portion of anionic molecules, further implicating the role of hydrophobic bonds in interactions of this nature.

The values for the number of binding sites obtained from these studies are however, difficult to reconcile with the number of basic groups present in the collagen molecule under the various pH conditions (Hulmes, Miller & others, 1973). Hence the additional involvement of non-ionic interactions are implicated.

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Indomethacin in low concentration potentiates the actions of some spasmogens on the isolated oestrous rat uterus

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In a number of smooth muscle tissues, the spasmogenic activity of bradykinin has been reported to involve the release of prostaglandins (Piper & Vane, 1969; Palmer, Piper & Vane, 1973; Crocker & Willavoys, 1976). The tissues of the isolated rat uterus synthesize prostaglandins (Williams, 1973) and spontaneous contractions of the rat uterus have been shown to be mediated by their release (Vane & Williams), 1973.

While testing the effectiveness of indomethacin, a prostaglandin synthetase inhibitor (Vane, 1971), in suppressing spontaneous contractions, we noted that indomethacin has another unexpected action on the isolated oestrous rat uterus.

Virgin Wistar rats 180–220 g, were brought to artificial oestrus, 20–22 h after a single subcutaneous injection of stilboestrol (20 μg per 100 g body weight) in 40% (v/v) aqueous ethanol. The animals were killed and exsanguinated. The uterine horns were excised and a length of 1–2 cm was suspended in 1·5 ml de Jalon solution (composition, g litre^{-1}: NaCl, 9; KCl, 0·42; $CaCl_2$, 0·06; $NaHCO_3$, 0·5; glucose, 0·5) at 35°, gassed with O_2/CO_2 (95:5%) and containing atropine sulphate (1 μg ml^{-1}).

Contractions were detected using an isotonic transducer with a load on the tissue of 0·5 g. Tissues were allowed to wash in de Jalon solution for 1 h before testing, to permit full relaxation. A 5 min dose cycle was used. Spasmogens remained in contact with the tissues for 70 s. The concentrations of indomethacin remained in contact with the tissue for the whole of the cycle. Every concentration of bradykinin and indomethacin was tested at least six times. Possible pH changes due to the various concentrations of indomethacin were monitored using a pH meter. Statistical significance of differences was determined using Student's *t*-test for paired differences.

Indomethacin in low concentration was found to potentiate contractions of the rat uterus produced by bradykinin (Fig. 1). Maximum potentiation was reached between 50 s and 10 min after contact of the tissue with indomethacin. The contraction size returned to the control value within 1 h of washing out the indomethacin. Parallel dose-response curves to bradykinin obtained in the presence of increasing concentrations of indomethacin, 0–0·56 μM (0–200 μg litre^{-1}) are shown in Fig. 2. A concentration of indomethacin as low as 0·14 μM produced detectable potentiation of the uterine contractions. The potentiation appears to be non-specific, since initial studies show that both acetylcholine and 5-HT are similarly potentiated by 0·56 μM indomethacin ($P<0·01$, n = 6). Indomethacin itself caused no change in the resting length of the tissue nor did it at any of its concentrations produce detectable alterations of pH.

Indomethacin (2·8 μM) has recently been reported to inhibit contractions of rat intestine produced by brady-

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