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# THE BINDING OF ATROPINE TO BOVINE SERUM ALBUMIN*

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**Abstract**—The binding of atropine to bovine serum albumin (BSA) as a model system was studied by ultrafiltration, spectrophotometry, and biological assay. Binding of atropine increased with an increase in pH from 5 to 8; the number of binding sites was approximately 20 at pH 6 and 100 at pH 8. Exhaustively acetylated BSA still bound some atropine when studied by ultrafiltration, although spectrophotometry did not show the interaction with atropine. Cysteine, which fully inhibited the spectrophotometric interaction of atropine with BSA, reduced the binding only partially, when determined by ultrafiltration. The requirement of the free amino groups of the protein for the interaction could be demonstrated by spectrophotometry. The participation of other functional groups of the protein was discussed. Atropine-protein binding and its inhibition by cysteine was demonstrated biologically on the isolated rat colon.

EARLY investigations by Storm van Leeuwen and Zeijdner,[1] and Beutner,[2] and more recent studies of Tønnesen[3] demonstrated that atropine binds to plasma proteins. These authors considered the influence of atropine binding on the transport, inactivation, and elimination of this drug.

This investigation concerns atropine binding to native and modified bovine serum albumin. It was undertaken in the hope that such studies in a model system of known constitution might further our understanding of the interaction of this drug with its pharmacological receptors.

## MATERIALS AND METHODS

Crystalline bovine serum albumin was purchased from Pentex, Inc., Kankakee, Ill. Exhaustively acetylated BSA (Ac-BSA), showing essentially complete elimination of ninhydrin-positive groups, was generously supplied by Dr. Clarke Davison, George Washington University Medical School, Washington, D.C. Atropine sulfate neutral, USP, Eimer & Amend, New York, N.Y. was recrystallized from ethanol;[4] mp 189° to 190°; $[\alpha]D^{25} = -2{\cdot}22$. Cysteine hydrochloride was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, and acetylcholine bromide (ACh) from Eastman Organic Chemicals, Rochester, N.Y. All other chemicals used in the following studies were of reagent grade purity either from Merck and Co., Inc., Rahway, N.J., or from

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the Fisher Scientific Co., Silver Spring, Md. Acetate and phosphate buffer solutions were made up in carbon dioxide-free, glass-distilled water.[5]

*Determination of protein-bound drug*

The binding of atropine to BSA was studied by ultrafiltration. The concentration of free atropine was determined in the ultrafiltrate by measuring the extinction at 258 mμ in a Beckman DU spectrophotometer.[6] The amount of bound drug was calculated from the difference between the initial and the experimentally found free drug concentration. The protein concentrations were calculated by correcting the weight for water content, which was determined by drying a separate sample at 105°. A molecular weight of 69,000 was assumed for BSA to calculate its molar concentration.[7]

Ultrafiltration was carried out in Toribara tubes[8, 9] by centrifugation at 2,500 rpm, at various temperatures, and hydrogen ion concentrations. The Visking dialysis tubing (24/32) was washed thoroughly to remove impurities and dried with a cotton-filtered air current. Before use it was rehumidified in a vapor chamber at room temperature. After having ascertained that atropine was not bound to the dialysis tubing at any pH or drug concentration investigated, this control was dispensed with. Four Toribara tubes were run simultaneously in all ultrafiltration experiments. One tube contained 6·0 ml of 1% BSA in the appropriate buffer solution without atropine (control) and each of the other three tubes held 6·0 ml of 1% BSA with increasing amounts of atropine. Preliminary experiments showed that at the centrifugal force employed, both the water and the atropine molecules were filtered at about the same rate. Ultrafiltration at room temperature was started 1 hr after mixing the protein and drug solutions, and 3 hr of centrifugation was necessary to collect about 0·6 ml of ultrafiltrate (0·1 of the initial volume). When working at lower temperatures the solutions were adjusted to the proper temperature before mixing and left standing overnight before centrifugation. This time was chosen for convenience, although preliminary experiments showed earlier equilibration. Four hours of centrifugation was necessary at 15° and 5 hr at 5°. When ultrafiltration was carried out at room temperature, a slight rise in temperature (3° to 5°) invariably took place during centrifugation. For the experiments at lower temperature an International refrigerated centrifuge was used. All experiments were repeated at least three times under identical conditions and the results did not vary more than ±1%.

The spectrophotometric interaction of atropine with protein was studied by the method reported for atropine-amino acid complex formation.[10]

The biological assay of atropine in the presence and absence of BSA was carried out on the ACh-induced contractions of the isolated rat colon, suspended in Locke's solution of pH 6·5, according to the method of Luduena and Lands.[11]

## RESULTS

*Ultrafiltration experiments*

It is known that at a fixed drug concentration the ratio of bound to total drug usually increases with increasing protein concentration.[12] In our experiments the concentration of BSA was kept constant at $14{\cdot}3 \times 10^{-5}$ M. The initial concentration of atropine was $4{\cdot}0 \times 10^{-3}$ M, except when the effect of varying drug concentrations was investigated.

*Effect of pH and temperature.* In the initial experiments in the lower pH range (4 to 5·5), 0·1 M acetate buffer was used; in the higher pH range (5 to 8), 0·1 M phosphate buffer was used. At pH 5 and 5·5 atropine binding was studied in both buffers and identical results were obtained. The pH of the drug-protein-buffer solutions was measured before and after ultrafiltration and never varied beyond the limits of accuracy of the instrument (Beckman model G pH meter). The pH-dependence of the binding of atropine to BSA is shown in Fig. 1. Near the isoelectric point of albumin (pH 5) atropine was not bound; binding increased on both sides of the isolectric point. In the range of the hydrogen ion concentrations studied, the fraction bound was highest at pH 8·0. Higher alkalinity was avoided since atropine hydrolyzes easily at higher pH.[13]

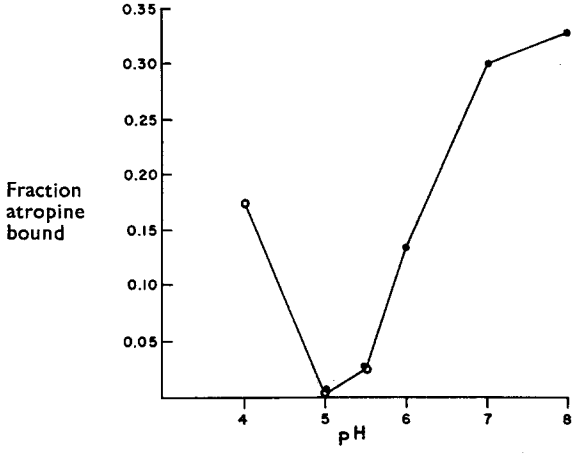


FIG. 1. Dependence on pH of the binding of atropine to BSA. Buffers: 0·1 M acetate from pH 4 to 5·5(○) and 0·1 M phosphate from pH 5 to 8(●). Temperature 28° to 30°. Initial atropine concentration $4{\cdot}0 \times 10^{-3}$ M, BSA $14{\cdot}3 \times 10^{-5}$ M.

The binding of atropine at various temperatures was investigated at pH 8·0 in 0·075 M phosphate buffer, varying the atropine concentration from $1{\cdot}0 \times 10^{-3}$ to $8{\cdot}0 \times 10^{-3}$ M. The protein concentration was kept constant at $14{\cdot}3 \times 10^{-5}$ M. Binding at pH 8·0 was also compared with binding at pH 6·0 at room temperature, the temperature of the solution being 28° to 30°, as measured at the end of the centrifugation. The results obtained are summarized in Table 1.

The number of binding sites and the dissociation constants were derived by the graphical solution of Klotz's equation:[12]

$$\frac{1}{r} = \frac{1}{(A)} \times \frac{K}{n} + \frac{1}{n}$$

where $r$ = mole bound drug/mole total protein, $K$ = dissociation constant at equilibrium, $n$ = total number of available, independent binding sites on the protein molecule, and $(A)$ = free drug concentration. If collision-effects predominated and electrostatic repulsion between successively attached molecules had a negligible role,

the plot of $1/r$ on the ordinate versus $1/(A)$ on the abscissa would be a straight line. The intercept on the ordinate, obtained by extrapolation, is numerically $1/n$ and the intercept on the abscissa $-1/K$. The data for the binding of atropine at different pH's and temperatures fitted such a straight line (Fig. 2). More atropine was bound at the lower temperature. The data obtained at 5° differed only slightly from those at 15° (Table 1). The number of binding sites, found to be 100 at pH 8, was not affected

TABLE 1. THE EFFECT OF TEMPERATURE AND PH ON THE BINDING OF VARIOUS CONCENTRATIONS OF ATROPINE TO $14{\cdot}3 \times 10^{-5}$ M BSA

| Initial atropine concentration (mole/L $\times 10^3$) | Final free atropine concentration (mole/L $\times 10^3$) | Bound atropine concentration (mole/L $\times 10^3$) | Mole atropine bound/mole albumin $r$ | $1/r$ | $1/(A) \times 10^{-3}$ |
|---|---|---|---|---|---|
| 0·075 M phosphate buffer, pH 8·0, 5° | | | | | |
| 8·0 | 5·45 | 2·55 | 17·80 | 0·06 | 0·18 |
| 4·0 | 2·66 | 1·34 | 9·35 | 0·11 | 0·38 |
| 2·0 | 1·39 | 0·61 | 4·25 | 0·24 | 0·72 |
| 1·0 | 0·58 | 0·42 | 2·94 | 0·34 | 1·72 |
| 0·075 M phosphate buffer, pH 8·0, 15° | | | | | |
| 8·0 | 5·24 | 2·76 | 19·00 | 0·05 | 0·19 |
| 5·2 | 3·35 | 1·85 | 12·95 | 0·077 | 0·298 |
| 4·0 | 2·58 | 1·42 | 9·98 | 0·10 | 0·39 |
| 3·0 | 1·92 | 1·08 | 7·50 | 0·13 | 0·52 |
| 2·0 | 1·30 | 0·70 | 4·90 | 0·20 | 0·77 |
| 1·0 | 0·58 | 0·42 | 2·90 | 0·35 | 1·72 |
| 0·075 M phosphate buffer, pH 8·0, 28° to 30° | | | | | |
| 8·0 | 6·02 | 1·98 | 13·70 | 0·07 | 0·166 |
| 5·2 | 4·00 | 1·20 | 8·32 | 0·120 | 0·230 |
| 4·0 | 2·96 | 1·14 | 7·25 | 0·143 | 0·345 |
| 3·0 | 2·20 | 0·80 | 5·60 | 0·18 | 0·46 |
| 2·0 | 1·46 | 0·54 | 3·72 | 0·27 | 0·68 |
| 1·0 | 0·75 | 0·25 | 1·74 | 0·58 | 1·33 |
| 0·1 M phosphate buffer, pH 6·0, 28° to 30° | | | | | |
| 8·00 | 6·66 | 1·34 | 9·30 | 0·103 | 0·150 |
| 5·32 | 4·35 | 0·97 | 6·60 | 0·146 | 0·230 |
| 4·00 | 3·26 | 0·74 | 5·25 | 0·190 | 0·310 |
| 2·66 | 2·11 | 0·55 | 3·84 | 0·260 | 0·474 |
| 2·00 | 1·56 | 0·44 | 3·06 | 0·326 | 0·640 |

by temperature. The association constants, calculated according to statistical theory,[12] were larger at 15° than at 30°; $\Delta F$ was identical. The number of available binding sites at pH 6·0 was reduced to 20, the first association constant was slightly smaller than at pH 8 at the same temperature, and the difference in $\Delta F$ was negligible.

The association constants and the free energy changes were derived from the experimental data of Table 1 and Fig. 2 by the usual equations[12] and are summarized in Table 2.

*Spectrophotometric study of atropine-protein interaction*

*Native bovine serum albumin.* A reaction of atropine similar to that reported with amino acids[10] was observed with protein, which resulted in an increase in extinction from 290 to 330 mμ and provided the means to study atropine-protein interaction

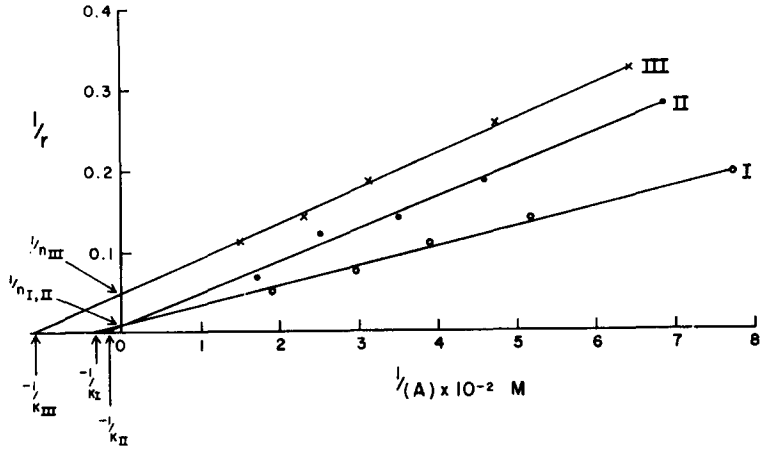


FIG. 2. The effect of temperature and pH on the binding of atropine to $14{\cdot}3 \times 10^{-5}$ M BSA. (Buffer: phosphate, pH 8, 0·075 M; pH 6, 0·1 M.)
Curve I: pH 8, 15°; $1/n_{I} = 0{\cdot}01$; $-1/K_{I} = -0{\cdot}393 \times 10^{2}$.
Curve II: pH 8, 28° to 30°, $1/n_{II} = 0{\cdot}01$; $-1/K_{II} = -0{\cdot}262 \times 10^{2}$.
Curve III: pH 6, 28° to 30°; $1/n_{III} = 0{\cdot}05$; $-1/K_{III} = -1{\cdot}16 \times 10^{2}$

TABLE 2. THE EFFECT OF TEMPERATURE AND PH ON THE BINDING CONSTANTS* FOR ATROPINE-BSA

| | pH 6 (0·1 M phosphate) 28° to 30° | pH 8 (0·075 M phosphate) 28° to 30° | 15° |
|---|---|---|---|
| $n$ | 20 | 100 | 100 |
| $K \times 10^{-2}$ | 0·86 | 3·82 | 2·54 |
| $k_1 \times 10^{3}$ | 2·30 | 2·60 | 3·90 |
| $-\Delta$F kcal/mole | 4·65 | 4·75 | 4·75 |

* $n$ = number of binding sites, $K$ = dissociation constant, $k_1$ = first association constant.

spectrophotometrically. Curve I in Fig. 3 shows the difference extinction spectrum of atropine in the presence of BSA. The maximum increase in extinction appeared at 300 mμ. Curve II represents the difference extinction spectrum when cysteine was present. It can be seen that cysteine inhibited the atropine-protein interaction in a manner similar to the atropine-histidine reaction.[10]

In order to corroborate the spectrophotometric findings, inhibition of the atropine-protein interaction by cysteine was also studied by ultrafiltration at pH 6·5 in 0·1 M phosphate buffer at room temperature. Mixtures of atropine ($4 \times 10^{-3}$ M) with BSA ($14{\cdot}3 \times 10^{-5}$ M) were ultrafiltered simultaneously in the presence and absence

of cysteine ($4 \times 10^{-3}$ M). Cysteine was preincubated with atropine for 60 min before the addition of BSA. The bound fraction of atropine in the presence of cysteine was 0·13 and in its absence 0·23. Thus cysteine, in equimolar concentration to atropine, caused only a 41·3% inhibition of atropine binding to BSA when determined by ultrafiltration, although it almost completely inhibited the spectrophotometric interaction.

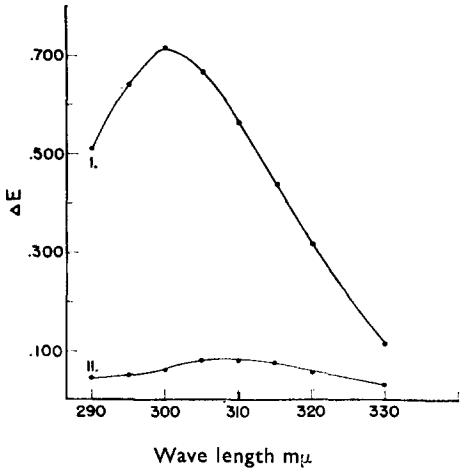


FIG. 3. The reaction of atropine (A = $5 \times 10^{-3}$ M) with native BSA ($14{\cdot}3 \times 10^{-5}$ M) and its inhibition by cysteine (C = $5 \times 10^{-3}$ M). Extinction spectra were taken after equilibrium was established (3 hr reaction time); 0·1 M phosphate buffer, pH 6·5, 25°.
Curve I: Difference spectrum of A in the presence of BSA:
$\Delta E = (O.D._{BSA+A}) - (O.D._{BSA}+O.D._{A})$.
Curve II: Difference spectrum of A in the presence of C and BSA
$\Delta E = (O.D._{BSA+C+A}) - (O.D._{BSA+C} + O.D._{A})$

*Acetylated bovine serum albumin.* It had been found that N-acetylated amino acids did not interact with atropine.[10] It was therefore thought of interest to study the binding of atropine to Ac-BSA spectrophotometrically and to compare the findings with ultrafiltration. Ac-BSA failed to give an increase in the extinction of atropine. Instead, a slight decrease was obtained at 295 mμ ($\Delta E = -0{\cdot}100$; 1% Ac-BSA, $5 \times 10^{-3}$ M atropine, pH 6·5 0·1 M phosphate buffer, 25°). When studied by ultrafiltration Ac-BSA still bound about one-fourth as much atropine as the native protein; the concentration of bound atropine was $0{\cdot}18 \times 10^{-3}$ mole per liter with Ac-BSA and $0{\cdot}74 \times 10^{-3}$ mole per liter with native BSA (1% protein, $4 \times 10^{-3}$ M atropine, pH 6·0, 0·1 M phosphate buffer, 28° to 30°).

*Biological demonstration of atropine-albumin binding*

The binding of atropine to BSA and its inhibition by cysteine was also investigated biologically on the rat colon with acetylcholine as the contractile agent (Fig. 4). Equivalent doses were taken from two solutions and appropriately diluted just before use: (1) atropine sulfate in Locke's solution of pH 6·5, diluted with Locke's solution (A); and (2) atropine sulfate (0·7 mg/ml) pre-exposed for 3 hr to BSA (15 mg/ml) in

Locke's solution, appropriately diluted with Locke's solution containing 15 mg/ml of BSA (A-BSA). Since BSA (5 mg/ml) was incorporated into the bath fluid to prevent dissociation when A-BSA was tested, this concentration of BSA was also added to the acetylcholine controls. As can be clearly seen, the contractions of the rat colon by a constant, repeated dose of acetylcholine were inhibited to a lesser extent (about 50%) by atropine in the presence of BSA than in the absence of the protein (Fig. 4, part I).

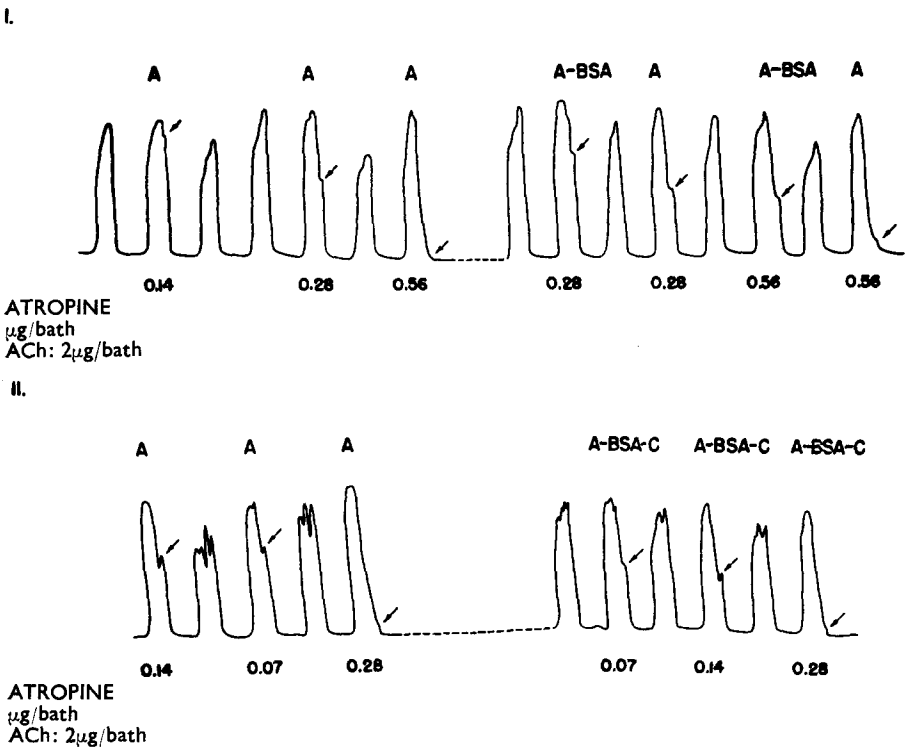


FIG 4. The binding of atropine to BSA and its inhibition by cysteine, as demonstrated by the antagonism of acetylcholine, induced contractions of the rat colon. Bath fluid: Locke's solution, gassed with 5% $CO_2$ in $O_2$, pH 6·5; 37° ± 0·5°; bath volume 30 ml. Acetylcholine (ACH, 0·067 μg/ml of bath fluid), given every 3 min, was left to contract the tissue for 30 sec before the addition of graded amounts of atropine, indicated on the figure. Control contractions to acetylcholine were alternated with contractions inhibited by atropine (A), atropine bound to BSA (A-BSA) or atropine pre-exposed to cysteine before the addition of BSA (A-BSA-C). The effects of A, A-BSA, or A-BSA-C, left in contact with the tissue for an additional 30 sec before washing, are marked by the arrows. Further description of the experimental design in the text.

For the second series of experiments atropine sulfate was preincubated for 1 hr with an excess of cysteine before the addition of BSA. Two solutions were employed: (1) atropine sulfate in Locke's solution of pH 6·5 (A), diluted as described above; and (2) atropine sulfate (0·7 mg/ml) pre-exposed to cysteine (7 mg/ml) for 1 hr before the addition of BSA (15 mg/ml). The three-component mixture was left standing for 3 hr before appropriately diluting with Locke's solution containing cysteine (7 mg/ml) and BSA (15 mg/ml) (A-BSA-C). BSA (5 mg/ml) and cysteine (0·5 mg/ml) were incorporated into the bath fluid when A-BSA-C was tested and were therefore also

added to the acetylcholine controls. As can be clearly seen, cysteine, by inhibiting atropine-albumin binding, restored the original biological activity of atropine (Fig. 4, part II).

## DISCUSSION

It has been postulated that organic cations in general are bound to BSA to a lesser extent than are anions of comparable size.[12, 14] From the data presented in this paper it is evident that atropine, an organic cation, was bound to BSA to a considerable extent, below and above the isoelectric point of this protein. Furthermore, the pH- and temperature-dependence of atropine binding bore some similarities to those of organic anions and neutral molecules.[12, 15–18] Atropine binding increased markedly by raising the pH from 5 to 8, and by lowering the temperature.

Atropine (p$K$ 10) bears a positive charge throughout the pH range studied. It therefore appears that the increase in binding at higher pH values should be due to the net increase in the negative charge of BSA (increasing the pH, the number of positively charged groups decreases). The number of binding sites ($n$) was determined to be 20 at pH 6 and 100 at pH 8. These values, however, are by no means exact; they were obtained by extrapolation. It had been emphasized that a large uncertainty is involved always in the numerical values of $n$.[19, 20] Nevertheless the difference, found at pH 6 and 8, in the number of available binding sites appears to be much larger than the number of the groups on BSA, titratable in this pH range. Between pH 6 and pH 8 only about 17 to 18 imidazole groups are titrated in BSA.[17] Thus the increased negativity of BSA alone cannot account quantitatively for the large increase in the number of binding sites.

In order to explain the binding of atropine to BSA, other properties of this protein should also be considered. It has been postulated that the remarkable capacity of BSA to combine with many substances is due partly to its configurational adaptability.[19] Configurational changes of BSA may occur as the result of changes in pH, and involve alterations in both secondary and tertiary structures.[21, 22] It might be suggested that at higher pH there exist a large number of configurations capable of binding a large number of atropine molecules, at sites that might have a specific structural complementarity to this small molecule. Thus the observed pH dependence of atropine binding would be the final result of the electrostatic and configurational changes mentioned above which take place simultaneously on the macromolecule.

A single set of equivalent binding sites with the same intrinsic affinity for atropine might be postulated because of the straight lines obtained at both pH 6 and pH 8, when we plot $1/r$ versus $1/A$.[12] However, it cannot be decided whether this is really the case since even in studies in which the heterogeneity of the binding has been detected, the sites have been divided into but two classes,[16, 18] and the results were also subject to considerable error.[17] Even if we assume on the basis of our data that the binding sites for atropine are equivalent, it should be remembered that a single binding site need not mean a single functional group. It can involve several groups participating in the binding of an atropine molecule. Theoretically, atropine can provide four functional groups that might be bound to protein: (1) the positively charged nitrogen, (2) the esteratic group, (3) the alcoholic hydroxyl group, and (4) the phenyl group. Each of these groups, when attached, could contribute a certain amount of energy to the over-all free energy of formation of the atropine-albumin

complex, the sole $\Delta F$ value that could be calculated from our studies. The involvement in the binding of these functional groups of atropine is substantiated as follows.

The free carboxyl groups of the protein can provide a negative center for the positively charged nitrogen of the atropine throughout the pH range studied. The increase in atropine binding with pH increasing from 6 to 8, might indicate the involvement of the unprotonated imidazole nucleus. This, as a strong hydrogen acceptor, could bind the alcoholic hydroxyl of the drug molecule in a manner similar to that already suggested for the amino acid-atropine interaction.[10] In these spectrophotometric studies we postulated an essential role for the free amino group of the amino acids and suggested that it is attached to the esteratic site of the alkaloid. The results of the present spectrophotometric experiments on atropine-protein interaction provided evidence for a similar decisive role of the free amino groups of the protein molecule: BSA showed the characteristic increase in extinction, whereas the acetylated protein (Ac-BSA) did not show this reaction with atropine. It appears, however, that the binding through the amino groups, which are exclusively responsible for the interaction shown spectrophotometrically, is not the only possible way of attaching atropine to the protein, since Ac-BSA still bound some atropine, as determined by ultrafiltration. Furthermore cysteine, in equimolar amounts with atropine, inhibited completely the spectrophotometric reaction of atropine with BSA but decreased atropine binding, as determined by ultrafiltration, only to an extent of about 40%. It has been reported that cysteine inhibited the atropine-histidine interaction by combining through its sulfhydryl group with atropine.[10] A similar mechanism might be involved in the abolition by cysteine of the spectrophotometric reaction between atropine and BSA. However it cannot be decided, from the few experiments performed, by which mechanism cysteine influenced the over-all binding of atropine to BSA, as determined by ultrafiltration or by biological assay.

In contrast to the amino acid-atropine complex,[10] the cholinolytic activity of atropine, when bound to BSA, was decreased substantially. Additional forces might strengthen the reversible attachment of the alkaloid to the protein. The phenyl group, assumed to be essential for the pharmacological action of atropine,[23] might bind to BSA by van der Waal's forces.

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