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THE INFLUENCE OF ACETYLCHOLINE ON THE BINDING OF ATROPINE TO BOVINE SERUM ALBUMIN*

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Abstract—The effect of acetylcholine on atropine binding to native bovine serum albumin and to *p*-hydroxymercuribenzoate-treated bovine serum albumin was studied by various methods. Depending on the pH, the nature of the buffer, and the molar proportion of the two drugs, atropine binding was differently influenced by acetylcholine, which either decreased or increased the binding.

The facilitating action of acetylcholine on atropine binding could be attributed to an alteration of the reactivity of the —SH group of the protein.

CLARK[1] classified atropine-acetylcholine antagonism as a competitive effect. This concept was challenged by Schild[2] and more recently by Marshall,[3] who interpreted this antagonism as noncompetitive. The data of Chen and Russel,[4] who applied the Lineweaver-Burk modification of the Michaelis-Menten theory to drug antagonism, when carefully analyzed, did not permit a clear-cut classification of this agonist-antagonist pair as either competitive or noncompetitive. We therefore investigated the effect of acetylcholine on the binding of atropine to bovine serum albumin as a model system, in an attempt to obtain further information on the nature of the acetylcholine-atropine interaction.

MATERIALS AND METHODS

Crystalline bovine serum albumin (BSA) was purchased from Pentex, Inc., Kankakee, Ill. The protein was free of cholinesterase activity, as determined manometrically.[5] *p*-Hydroxymercuribenzoate-treated BSA (PMB-BSA) was generously supplied by Dr. Clarke Davison, George Washington University Medical School, Washington, D.C. Atropine sulfate, USP, Eimer & Amend, was recrystallized from ethanol,[6] mp 189° to 190°. Choline chloride and acetylcholine bromide (ACh) were obtained from Eastman Organic Chemicals, Rochester, N.Y. The latter compound was recrystallized at least every 2 weeks from absolute ethanol-ether.[7] All other chemicals were of reagent grade purity either from Merck and Co., Inc., Rathway, N.J., or from the Fisher Scientific Co., Silver Spring, Md.

Acetate, phosphate, and citrate-phosphate buffer solutions were made up in carbon dioxide-free, glass-distilled water.[8]

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Determination of protein-bound drug

The binding of atropine to BSA was studied both by ultrafiltration and by equilibrium dialysis in the absence and presence of ACh. The concentration of free atropine was determined in the ultrafiltrate or the diffusate by measuring the extinction at 258 mμ in a Beckman DU spectrophotometer.[9] Hestrin's colorimetric method[10] was used to analyze for ACh. The hydroxamation was performed with hydroxylamine buffered with Na_2CO_3 to pH 9·2 because, at this pH, atropine did not interfere with the ACh determination. The amount of bound drug was calculated from the difference between the initial and the experimentally found free drug concentration. The protein concentration was calculated by correcting the weight for water content, a molecular weight of 69,000 for BSA assumed.[11]

Ultrafiltration in Toribara tubes[12] was carried out as described in the previous paper.[13] Four Toribara tubes, each containing 6·0 ml of solution, were centrifuged simultaneously. The details of a typical run were as follows: tube 1, 1% BSA; tube 2, 1% BSA with atropine; tube 3, 1% BSA with ACh; tube 4, 1% BSA with atropine and ACh. All experiments were repeated at least three times, and the results did not vary more than ±1%.

Equilibrium dialysis was performed according to McMenamy and Oncley.[14] The cellophane tubing was prepared as described previously for ultrafiltration.[13] The solution inside the bag was 4·0 ml of 3% buffered BSA, outside was 8·0 ml of buffered atropine alone, buffered ACh alone, or atropine mixed with ACh. Appropriate controls of three types were set up: one control contained each of the specified drug solutions on the outside and buffer on the inside; the second contained buffer on the outside and protein on the inside; and the third, buffer on both sides of the bag. At room temperature, equilibrium was reached within 6 hr. The solutions outside the membrane were analyzed for the free drug concentration, and the bound drug concentration was calculated as described for ultrafiltration. Each tube was set up in triplicate and each experiment repeated at least twice.

Spectrophotometric interaction of atropine with protein was studied by the method reported previously for atropine-amino acid complex formation.[15]

RESULTS

Ultrafiltration experiments

Effect of pH. The effect of pH on atropine binding in the presence of ACh was compared with that previously found in the absence of ACh,[13] keeping the atropine concentration constant at 4×10^{-3} M (Fig. 1). Two buffers were used, from pH 4 to 5·5, 0·1 M acetate; and from pH 5 to 8, 0·1 M phosphate. In the pH region in which both buffers were used the results were identical. The pH's of the drug-protein-buffer solutions were the same before and after ultrafiltration.

Although a similar dependence on pH obtained in the presence of ACh (Fig. 1, solid line) as in its absence (dashed line) it can be clearly seen that ACh affected the amount of atropine bound at various hydrogen ion concentrations. From about pH 5 to 6·5, atropine binding was increased; above pH 7 and below pH 5 it was decreased in the presence of ACh, although ACh itself was not bound to the protein.

The influence of ACh and its hydrolysis products. In order to ascertain the specificity of the facilitating effect of ACh on atropine binding, the influence of choline was

studied. The results obtained in 0·1 M phosphate buffer at pH 6·0 are summarized in Table 1.

The effect of choline differed markedly from that of ACh. At a proportion of atropine to ACh of 1 to 0·5, ACh increased the binding of atropine by 30%, whereas choline in the same proportion did not alter it. In a proportion of 1 to 1, the facilitating effect of ACh disappeared, and choline decreased the binding by 39%. Similar results were obtained with 0·1 M acetate instead of phosphate buffer.

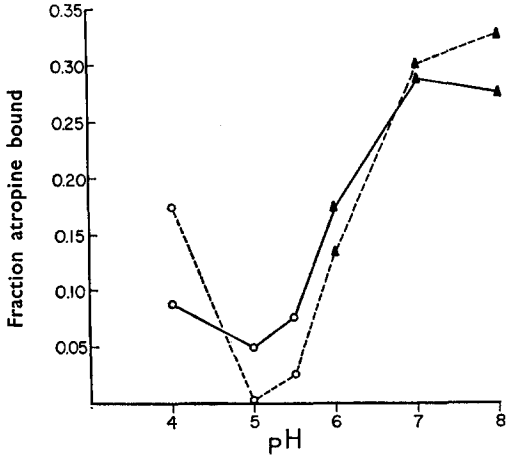

FIG. 1. Influence of pH on the binding of atropine to BSA in the presence of ACh (solid line) and in its absence (dashed line). Buffers: 0·1 M acetate, pH 4 to 5·5 (○); 0·1 M phosphate, pH 5 to 8 (▲). Initial atropine concentration 4×10^{-3} M, acetylcholine 2×10^{-3} M, and BSA $14{\cdot}3 \times 10^{-5}$ M.

TABLE 1. THE INFLUENCE OF ACH AND CHOLINE (CH) ON THE BINDING OF ATROPINE (A) TO BOVINE SERUM ALBUMIN

A: 4×10^{-3} M; BSA: $14{\cdot}3 \times 10^{-5}$ M; 0·1 M phosphate buffer, pH 6·0, 28° to 30°.

Molar ratio		Increase (+) or decrease (−) of bound atropine* (%)
$\frac{A}{ACh} =$	1/0·5	+30
	1/1	0
$\frac{A}{Ch} =$	1/0·5	0
	1/1	−39

* The fraction of atropine bound in the absence of ACh or Ch was set equal to 100%.

Effect of the nature of the buffer. Specific buffer effects on the binding of organic anions to proteins have been reported.[16] We therefore compared atropine binding in the presence and absence of ACh with acetate, phosphate and citrate-phosphate buffers (Table 2).

A striking difference appeared when the pH dependence of atropine binding in phosphate buffer was compared with that in citrate-phosphate buffer. At pH 5, near the isoelectric point of albumin, hardly any atropine was bound in the absence of ACh in phosphate buffer. In citrate-phosphate buffer more atropine was bound at pH 5·0 than at pH 6·0, although the facilitating effect of ACh was absent at the lower

TABLE 2. THE EFFECT OF VARIOUS BUFFERS AND ACh ($2{\cdot}0 \times 10^{-3}$ M) ON THE BINDING OF ATROPINE ($4{\cdot}0 \times 10^{-3}$ M) TO BSA ($14{\cdot}3 \times 10^{-5}$ M) AS A FUNCTION OF pH

Buffer concentration: 0·1 M, 28° to 30°.

	Fraction of atropine bound			
	Phosphate		Citrate-Phosphate	
	ACh		ACh	
pH	Absent	Present	Absent	Present
5·0	0·01*	0·06*	0·16	0·15
6·0	0·14	0·18	0·10	0·20
7·0	0·30	0·30	0·30	0·30

* The same results were obtained in 0·1 M acetate buffer.

TABLE 3. THE BINDING OF ATROPINE TO BSA ($14{\cdot}3 \times 10^{-5}$ M) AS A FUNCTION OF THE ATROPINE CONCENTRATION IN THE PRESENCE AND ABSENCE OF ACh

Molar proportion of atropine: ACh = 1:0·5; pH 6·0, 0·1 M phosphate buffer, 28° to 30°

Initial atropine concentration (mole/L $\times 10^3$)	Final free atropine concentration (mole/L $\times 10^3$) (A)	Bound atropine concentration (mole/L $\times 10^3$)	Mole atropine bound/mole albumin r	$1/r$	$1/(A) \times 10^{-3}$
		ACh absent			
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
		ACh present			
8·00	6·45	1·55	10·80	0·093	0·155
5·32	4·10	1·22	8·40	0·118	0·245
4·00	3·00	1·09	7·00	0·143	0·333
2·66	1·97	0·69	4·85	0·205	0·505
2·00	1·44	0·56	3·90	0·256	0·692

pH. At pH 6 the increase by ACh in the binding of atropine to BSA was more pronounced in citrate-phosphate than in phosphate buffer. At pH 7 both the difference of the buffer effects and the facilitating action of ACh were absent.

Binding as a function of the atropine concentration. Atropine concentrations were varied from $2{\cdot}0 \times 10^{-3}$ to $8{\cdot}0 \times 10^{-3}$ M, with the BSA concentration constant at $14{\cdot}3 \times 10^{-5}$ M. In order to determine the influence of ACh on the binding, the molar proportion of atropine to ACh was kept constant at 1 to 0·5. Ultrafiltration at room

temperature was performed in 0·1 M phosphate buffer of pH 6·0. The temperature of the solutions at the end of the centrifugation was 28° to 30°. The results obtained are summarized in Table 3 and graphically presented according to Klotz[16] in Fig. 2. Since collision effects predominated in the binding of atropine to BSA, both in the presence and absence of ACh, a straight line fitted the data.

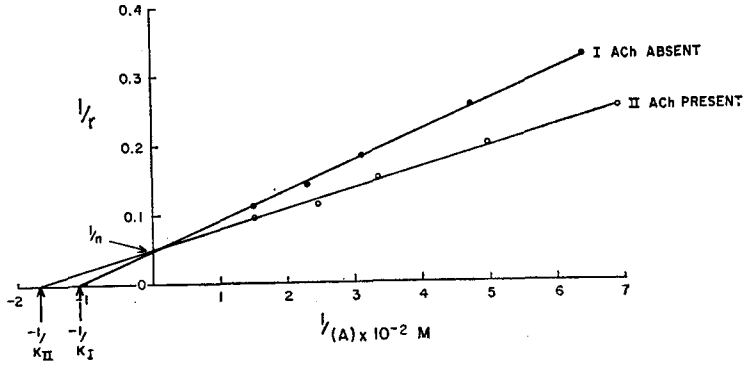

FIG. 2. The effect of ACh on the binding of atropine to BSA ($14{\cdot}3 \times 10^{-5}$ M); 0·1 M phosphate buffer, pH 6·0, temperature 28° to 30°. The molar proportion of atropine to ACh was kept constant at 1 to 0·5.
Curve I: ACh absent, $1/n = 0{\cdot}05$, $-1/K_I = -1{\cdot}16 \times 10^{-2}$.
Curve II: ACh present, $1/n = 0{\cdot}05$, $-1/K_{II} = -1{\cdot}64 \times 10^{-2}$.

The number of available binding sites for atropine at pH 6·0 was found to be 20 also in the presence of ACh (Fig. 2); the dissociation constant in the absence of ACh equalled $0{\cdot}86 \times 10^{-2}$ and in its presence $0{\cdot}62 \times 10^{-2}$ (Fig. 2). The first association constants and the free energy changes, derived by the usual equations[16] were, in the absence of ACh: $k_1 = 2{\cdot}3 \times 10^3$, $-\Delta F = 4{\cdot}65$ kcal/mole; in the presence of ACh: $k_1 = 3{\cdot}3 \times 10^3$, $-F = 4{\cdot}9$ kcal/mole. Thus the association constant in the presence of ACh was somewhat higher than in its absence; the difference found for ΔF was negligible.

Equilibrium dialysis

In order to corroborate the finding of the facilitating effect of ACh on the binding of atropine to BSA, equilibrium dialysis was used. In these experiments the BSA concentration was kept constant at $42{\cdot}9 \times 10^{-5}$ M, the atropine concentration at $6{\cdot}0 \times 10^{-3}$ M, and the ACh concentration at $3{\cdot}0 \times 10^{-3}$ M. The studies were performed at room temperature on 0·1 M phosphate buffer of pH 6·0. Equilibrium was reached within 6 hr. The results, obtained by ultrafiltration, were confirmed. A 25% increase of atropine binding was caused by ACh. ACh, however, was not bound to BSA either in the presence or absence of atropine.

Spectrophotometric study of atropine binding to BSA and PMB-BSA

The interaction of atropine with BSA shown by spectrophotometry was reported in the previous paper.[13] Since it had been observed that ACh partly counteracted the inhibition by cysteine of the atropine-histidine reaction,[17] it was thought of interest

to compare the effect of ACh on the interaction of atropine with native BSA with the interaction of the alkaloid with BSA in which the -SH function was blocked by *p*-hydroxymercuribenzoate (PMB-BSA).

In Fig. 3 the reaction rates of the increase in extinction at 295 mμ were plotted. As can be seen, the initial reaction rate was greater with PMB-BSA (curve 3) than with BSA (curve 1); and ACh, which increased the reaction rate with BSA (curve 2), had no effect on the rate with PMB-BSA (curve 4). At equilibrium the differences in ΔE_{295} became less pronounced. When the binding of atropine to PMB-BSA was studied by ultrafiltration, ACh also had no facilitating effect, and PMB-BSA in the absence of ACh bound as much atropine as BSA in its presence.

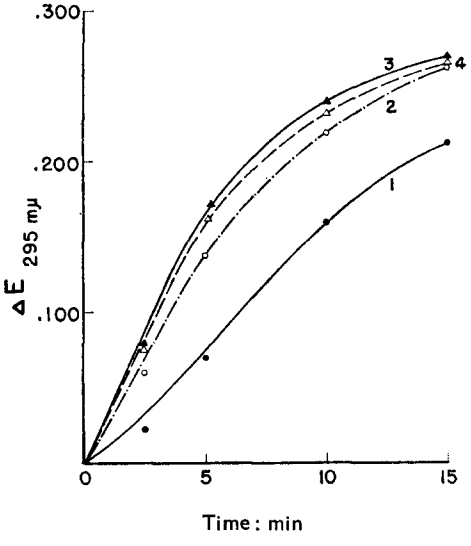

FIG. 3. Reaction rate of the interaction of atropine with BSA and PMB-BSA in the presence and absence of ACh. Temperature 25°, 0·1 M phosphate buffer, pH 6·5.
Curve 1: 5×10^{-3} M atropine + 1% BSA.
Curve 2: 5×10^{-3} M atropine + 1% BSA + $2{\cdot}5 \times 10^{-3}$ M ACh.
Curve 3: 5×10^{-3} M atropine + 1% PMB-BSA.
Curve 4: 5×10^{-3} M atropine + 1% PMB-BSA + $2{\cdot}5 \times 10^{-3}$ M ACh.

DISCUSSION

The binding of atropine to BSA was reported and discussed in the previous paper.[13] The binding was influenced by ACh. The effect of ACh depended on the pH, on the nature of the buffer employed, and on the molar proportion of the two drugs. At both pH 4 and pH 8 ACh markedly decreased the binding of atropine, whereas at pH 5 and pH 6, in phosphate or acetate buffer, ACh increased it. In the presence of citrate the facilitating effect of ACh was confined to pH 6. Facilitation of atropine binding could not be demonstrated with the hydrolysis products of ACh e.g. choline and acetate. ACh apparently had this action without being itself bound to the protein in detectable amounts.

It is realized that a direct transfer of the results of protein binding studies *in vitro* to a biological system is not permissible. Nevertheless, the observed decrease in atropine binding by ACh might be considered analogous to the well known biological antagonism of these two drugs and could be looked upon as the result of their competition for binding sites on the protein.

This, however, is not a specific effect of ACh, since choline also decreased atropine-protein binding. The facilitating effect of ACh on atropine-protein binding is apparently more specific and constitutes a synergistic action. In some biological systems synergism between ACh and atropine has been reported,[18–21] for which the proportion of atropine to ACh was crucial, as we found also in our model system.

The rate of the spectrophotometric interaction of atropine with PMB-BSA was found to be higher than the rate of the interaction of atropine with native BSA. This might suggest an inhibitory effect of the free –SH group of the native protein in analogy to the inhibition of the atropine-amino acid interaction by –SH amino acids which could be reversed by *p*-hydroxymercuribenzoate.[15] ACh increased the rate of atropine-BSA interaction but had no effect on atropine-PMB-BSA. Thus it appears from the spectrophotometric data that the facilitating effect of ACh might be connected with the –SH function of BSA. A decrease by ACh in the reactivity of –SH groups has been demonstrated by amperometric titration.[22]

Although the facilitating effect of ACh on the binding of atropine to native BSA was observed by ultrafiltration as well as by spectrophotometry, the results obtained by these two methods should not be equated, since the spectrophotometric interaction of atropine with protein and its binding, as determined by ultrafiltration, showed a quantitative difference as reported in the previous paper.[13]

Since ACh showed a dual effect, facilitation and inhibition, on atropine-protein interaction even *in vitro*, it is not surprising that the classification of the interaction of these two drugs, based on studies in complex biological systems, *in vivo* and in isolated organs, is still controversial.

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