Interactions of Xanthine Molecules with Bovine Serum Albumin

By M. L. EICHMAN†, D. E. GUTTMAN, Q. VAN WINKLE, and E. P. GUTH

The binding of a number of structurally related xanthine compounds by bovine serum albumin was investigated in an attempt to elucidate some of the structural specificities of the interaction. The study included caffeine, theophylline, 8-nitrotheophylline, 8-chlorotheophylline, theophylline-7-acetic acid, 8-methoxycaffeine, 8-ethoxycaffeine, 8-chlorocaffeine, 8-methylcaffeine, theobromine, 8-nitrotheobromine, uric acid, 1,3,7-trimethyluric acid, uracil, and 1,3-dimethyluracil. Under the conditions employed, interactions between the protein and uric acid, uracil, and dimethyluracil could not be detected. The remaining compounds were bound to varying degrees and evidence was obtained to indicate that a single site on the protein was responsible for the major portion of the binding. Anionic species were generally found to possess much stronger interactive tendencies than those which had no formal charge.

THE ABILITY of serum proteins, particularly albumin, to reversibly bind numerous small molecules has been recognized for many years (1). The majority of earlier investigations were of a qualitative nature. Only in recent years have attempts been made to quantitate the results and to explain the nature of the equilibria and forces involved in the interactions (2, 3). Concomitant with advances in the theoretical aspects of these interactions, there has been an increase in the study of the associations of drug molecules with proteins. Steroid hormones (4), barbiturates (5), sulfonamides (6, 7), penicillins (8), salicylates (9), and other therapeutic agents have been shown to interact with serum albumins.

Goldstein (1), and Brodie and Hogben (10) have discussed the significance of such behavior in the transport, deposition, and elimination of drugs in the body. In addition, it appears that the pharmacologic activity of many medicinal agents is intimately related to their abilities to combine with specialized functional proteins. Elucidation of the factors involved in such interactions may thus contribute to the understanding of drug action.

The interaction of xanthine compounds with components in blood serum had been noted in the early investigations of Pak (11) and Aiello (12). More recently, Schack and Waxler (13) demonstrated that theophylline can be bound by the plasma proteins of rabbits. In the present study, the bindings of caffeine and a number of structurally related compounds by bovine serum albumin (BSA) were quantitatively investigated in an attempt to determine the dependency of the interaction on structural and environmental variables. The compounds employed are listed in Table I. The results will show that the interaction of xanthine derivatives with BSA was dependent on the nature of substituents at the 1, 7, and 8 positions, the acid strength of the compound, and on the hydrogen-ion concentration of the system.

EXPERIMENTAL PROCEDURE

Materials.—Bovine serum albumin, fraction V (BSA), purchased from Armour Laboratories (lots T16812 and U17912), was used throughout this study. Electrophoretic analysis of lot T16812 indicated an albumin content of 97.5%. A molecular weight of 69,000 was assumed. Both lots of albumin were found to exhibit similar binding tendencies for caffeine. Reagent grade mono, di, and trisodium phosphate salts were employed to buffer the solutions and to obtain an equilibrium ionic strength of 0.16. The xanthine derivatives were either purchased or prepared by standard synthetic procedures. All xanthine and albumin solutions were prepared imediately prior to use.

Dialysis Studies.—Equilibrium dialysis experiments, essentially as described by Hughes and Klotz (14), were used to investigate the binding of xanthines by BSA. Ten-milliliter samples of albumin in phosphate buffer were accurately pipetted into bags prepared from Visking cellophane casing. The bags were placed into tubes, each containing a different concentration of the xanthine derivative. The range of concentrations employed was from 5×10^{-5} M to 2×10^{-2} M. A series of control tubes in which buffer solution was used in place of protein solution was similarly prepared. The bags prevented the passage of albumin into the external solutions, but were permeable with respect to the xanthine molecules. Binding of xanthines by the dialysis membrane was found to be insignificant. Equilibrium was established by gentle mechanical

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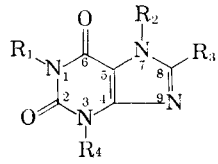
TABLE I.—STRUCTURES AND MOLAR ABSORPTIVITIES OF SOME XANTHINE DERIVATIVES



Compound	R_1	R_2	R_3	R_4	λ max., mμ	Molar Absorptivity × 10^{-4}
Caffeine	CH_3	CH_3	H	CH_3	272	0.956
Theophylline	CH_3	H	H	CH_3	272	1.020
Theobromine	H	CH_3	H	CH_3	273	1.008
8-Methoxycaffeine	CH_3	CH_3	OCH_3	CH_3	281	1.326
8-Ethoxycaffeine	CH_3	CH_3	OCH_2CH_3	CH_3	281	1.365
8-Methylcaffeine	CH_3	CH_3	CH_3	CH_3	275	1.148
8-Chlorocaffeine	CH_3	CH_3	Cl	CH_3	277	1.154
8-Nitrotheophylline	CH_3	H	NO_2	CH_3	387	1.196
8-Chlorotheophylline	CH_3	H	Cl	CH_3	277	1.310
Theophylline-7-acetic acid	CH_3	CH_2COOH	H	CH_3	274	0.975
8-Nitrotheobromine	H	CH_3	NO_2	CH_3	273	1.218
Uric acid	H	H	OH	H	291	1.136
Trimethyluric acid	CH_3	CH_3	OH	CH_3	295	1.636

shaking at 9 ± 1° for 30 hours. All xanthine compounds were studied at pH 6.85. In addition, the interactions of caffeine, theophylline, 8-nitrotheophylline, and 8-chlorotheophylline were also investigated over a wide pH range. At equilibrium, the external solutions were analyzed for the xanthine derivative and the exact pH determined from measurements on the albumin solution in each tube. Solutions of 0.4% albumin were employed in studies of the binding of 8-chlorotheophylline and 8-nitrotheophylline. Albumin solutions, 2%, were used in all other studies.

Analytical Methods.—The concentration of xanthine derivative in each solution was determined spectrophotometrically with a Beckman model DU spectrophotometer. For each compound, the wavelength of maximum absorption was employed. The experimentally determined molar absorptivities are listed in Table I. Appropriate dilutions were made where necessary so that all spectrophotometric readings were recorded below 0.8 absorbance units. Suitable blanks were employed to correct for spectral contributions of dialyzable components in the albumin and from soluble substances in the dialysis membrane. The albumin concentrations were determined spectrophotometrically at 278 mμ. The molar absorptivity of albumin was determined to be 4.603×10^4. This value was not corrected for nonalbumin impurities present in the protein. The pH values of the protein solutions were determined at 25° with a Beckman model G pH meter after each dialysis experiment.

THEORY AND RESULTS

A cursory examination of the data reveals marked differences in the tendencies of the compounds investigated to associate with BSA. This is illustrated in Table II, where the percentage of each xanthine bound by a 2% albumin solution is tabulated. The values are seen to range from 3% for the total amount of theobromine bound, to 98% for 8-nitrotheophylline and 8-chlorotheophylline. These figures were determined from dialyses of 10 ml. of 1×10^{-4} *M* solutions of each xanthine against 10 ml. of BSA solution.

TABLE II.—PERCENTAGE OF EACH XANTHINE DERIVATIVE BOUND BY 2% ALBUMIN SOLUTIONS

Compound	% Bound
Caffeine	17
Theophylline	20
Theobromine	3
8-Chlorocaffeine	58
8-Methoxycaffeine	67
8-Ethoxycaffeine	46
8-Methylcaffeine	40
8-Chlorotheophylline	98
8-Nitrotheophylline	98
Theophylline-7-acetic acid	44
8-Nitrotheobromine	3
Uric acid	6
1,3,7-Trimethyluric acid	83
Uracil	Negligible
1,3-Dimethyluracil	Negligible

In view of these results, it was considered desirable to characterize quantitatively each of the systems studied by one or more equilibrium constants and to determine the number of sites on the protein molecule participating in the interactions. In this way, a clearer understanding of the interactions may be achieved so that logical comparisons between compounds can be made. The mathematical analysis of binding data of the type presented here has been described and discussed by Klotz (2, 15) and Scatchard (16). If only one class of sites on the protein is responsible for the binding, the number of moles of small molecule bound per mole of protein can be related to the concentration of the free small molecule by

$$r = \frac{nk(\mathrm{A})}{1 + k(\mathrm{A})} \qquad \text{(Eq. 1)}$$

where r = moles of small molecule bound per mole of protein, n = number of binding sites available on each protein molecule, k = association constant for

the reaction, and (A) = concentration of unbound small molecule. The values of n and k can be graphically determined by plotting the data in accordance with a rearranged form of Eq. 1

$$r/(A) = nk - rk \qquad \text{(Eq. 2)}$$

A plot of $r/(A)$ *vs.* r will result in a straight line of slope $-k$ and ordinate and abscissa intercepts of nk and n, respectively.

When both the protein and the small molecule possess a formal charge, an electrostatic correction factor must be applied. In such cases, a modified formula, as suggested by Scatchard (16), can describe the system

$$[r/(A)]\ f = nk - rk \qquad \text{(Eq. 3)}$$

where $f = e^{2wZ_PZ_A}$, Z_P = the charge on the protein, Z_A = the charge on the small molecule, and w = an electrostatic parameter. Tanford, *et al.* (17), evaluated w to be equal to 0.026 at $\Gamma/2 = 0.16$. At pH 6.85, Z_P was taken as -7 and Z_A was -1 for the anionic xanthines and 0 for the neutral molecules. If the xanthine molecule is uncharged, f is unity, and Eq. 3 becomes identical to Eq. 2.

A general description of the system where two or more classes of binding sites on the macromolecule participate in the interaction is given by Eq. 4. In this case, each class of sites will possess a different affinity for the small molecule

$$r/(A) = \frac{n_1k_1}{1 + k_1(A)} + \frac{n_2k_2}{1 + k_2(A)} + \ldots + \frac{n_ik_i}{1 + k_i(A)} \qquad \text{(Eq. 4)}$$

Such behavior is indicated by curvature of the $r/(A)$ *vs.* r plot. It can be shown that the abscissa and ordinate intercepts in this case will be Σn and Σnk, respectively. In order to evaluate constants from nonlinear data of this type, a method described by Scatchard, *et al.* (18), and Karush (19) was employed, in which only two classes of sites on the protein were assumed to account for the binding. Our results indicated Σn to be equal to three. The data were therefore evaluated for several compounds with the assumption that the two classes of binding sites contain one and two groupings, respectively. The curves calculated on this basis agree well with those drawn through the experimental points. The values for k_1 and k_2 are listed in Table III. Where no values are given for k_2, secondary interactions have been found to be negligible and the bindings are therefore adequately represented by k_1. This was the case for caffeine and its 8-substituted derivatives. Their k values were obtained by linear extrapolation to the ordinate axis in the $r/(A)$ *vs.* r plots, as illustrated in Fig. 1. In contrast, secondary interactions were quite prominent in the case of 8-chlorotheophylline, theophylline-7-acetic acid, and trimethyluric acid. The values for both k_1 and k_2 were estimated for these compounds.

Table III.—Association Constants for the Binding of Xanthine Derivatives by Albumin

Compound	$n_1 = 1$, $k_1 \times 10^5$	$n_2 = 2$, $k_2 \times 10^{-4}$	pH
Caffeine	0.0154	0.003	4.80
Caffeine	0.0168	...	6.85
8-Methylcaffeine	0.0520	...	6.85
8-Ethoxycaffeine	0.0750	...	6.85
8-Chlorocaffeine	0.125	...	6.85
8-Methoxycaffeine	0.158	...	6.85
Theophylline	0.0160	0.004	5.80
Theophylline	0.0161	0.009	6.85
Theophylline	0.0198	0.021	7.23
Theophylline	0.0253	0.037	7.85
8-Nitrotheophylline	2.64	1.27	6.85
8-Chlorotheophylline	3.07	1.10	6.85
Theophylline-7-acetic acid	0.0592	0.061	6.85
Theobromine	0.0024	...	6.85
8-Nitrotheobromine	0.0020	...	6.85
Uric acid	0.0075	...	6.85
1,3,7-Trimethyluric acid	0.344	1.21	6.85
Uracil	Negligible	...	6.85
1,3-Dimethyluracil	Negligible	...	6.85

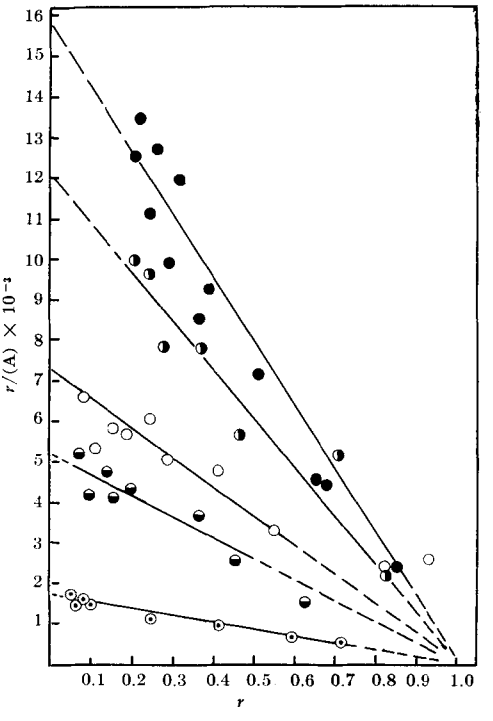


Fig. 1.—The binding of caffeine and 8-substituted caffeines by BSA. ●, 8-methoxycaffeine; ◑, 8-chlorocaffeine; ○, 8-ethoxycaffeine; ◒, 8-methylcaffeine; ⊙, caffeine.

DISCUSSION

The ability of caffeine to form complexes with a large number of structurally different small molecules has been well documented (20, 21). On this basis, it was expected that several different groupings on the albumin macromolecule might serve as binding sites for caffeine. From the study illustrated in Fig. 2, it was apparent, however, that the principal interaction with BSA involved only one site on the protein. The low magnitude of k_2 indicated that secondary associations were of minor significance. Information pertaining to the nature of the primary site was adduced from the influence of pH on the interaction. This is illustrated in Fig. 3. The

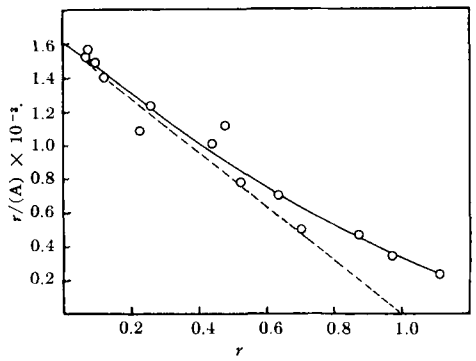


Fig. 2.—The interaction of caffeine with BSA at pH 4.80. – – –, primary interaction; ——, primary plus secondary interactions.

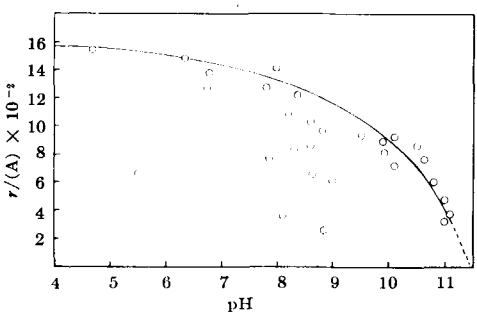


Fig. 3.—The effect of pH on the binding of caffeine.

gradual decrease in binding which occurred between the pH values of 4.8 and 8.5 may be the result of slight changes in the structure of the albumin. The marked decrease in association above pH 8.5 may be rationalized either by assuming that a specific interaction occurred between caffeine and a protonated site on the protein or by considering a binding site to be involved which was either blocked or abolished by a configurational change which resulted from the neutralization of a protonated group on the protein. Ionizable substituents on albumin include carboxyl, imidazole, amino, phenolic, guanidino, and sulfhydryl groups. Since the protonated • amino groups of lysine dissociate in the pH range where the major decrease in binding occurred, it is quite possible that neutralization of such a group is related to the marked decrease in the interaction.

The xanthine precursors, uracil and 1,3-dimethyluracil, as well as the dimethylxanthines, theophylline and theobromine, were investigated for binding activity in order to elucidate further the factors involved in the interaction. It was initially considered that the pyrimidinedione nucleus might be responsible for the binding. However, investigations with uracil and dimethyluracil revealed that neither compound was capable of interaction. The interactions of caffeine and theophylline as compared with that of theobromine emphasize the importance of N_1 substitution. The strengths of binding exhibited by the former compounds were considerably greater than that of the latter. It appears therefore, that not only are both rings required in order for significant binding to occur, but, in addition, substitution at the N_1 position is also a necessary prerequisite.

In contrast to caffeine, theophylline exhibited an increase in interaction as the pH was raised above 4.8, as shown in Fig. 4. From the association constants, k_1 and k_2, listed in Table III for several pH values, it is seen that this increase is the result of both greater primary and secondary interactions. The differences in the binding behaviors of caffeine and theophylline can be attributed to the ionization of theophylline (pKa 8.7). The fraction of theophylline in its anionic form becomes significant within the pH region where the rise in association occurred. It appears, therefore, that the negatively charged form of theophylline is more strongly bound than the uncharged molecule. At higher pH values the sharp decrease in the binding of theophylline parallels that observed with caffeine and is most probably related to the release of a proton from a specific acidic group on the protein.

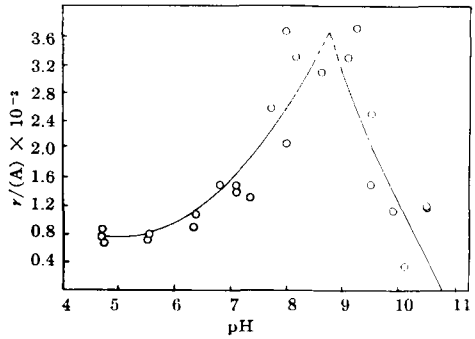


Fig. 4.—The effect of pH on the binding of theophylline.

The interactions of 8-substituted caffeines are graphically represented in Fig. 1. It is seen that in all cases association appears to involve one site on the protein. Marked differences in binding strength are, however, apparent. For example, the association constant for the 8-methoxycaffeine–BSA interaction is approximately ten times that found for the analogous caffeine system. Although it is difficult to interpret these results, the involvement of both steric and electronic factors are considered likely. Greater van der Waals attraction forces, resulting from the larger molecular size of the small molecule may account for the increased binding of the substituted derivatives. Another possible explanation lies in the ability of the small molecule to fit into a receptor site on the protein. Lumry and Eyring (22) have indicated that the molecule which would provide the best fit for the binding site would be expected to produce the strongest interaction. The 8-substituted caffeines, because of their larger sizes, may be more capable of filling the available space.

The interactive tendencies of 8-chloro and 8-nitrotheophylline at pH 6.85 were considerably greater than those of other compounds investigated. The binding curve for 8-chlorotheophylline is shown in Fig. 5 where a theophylline curve is also plotted for comparative purposes. A similar curve was obtained for the 8-nitrotheophylline–BSA system. The marked curvature is indicative of rather

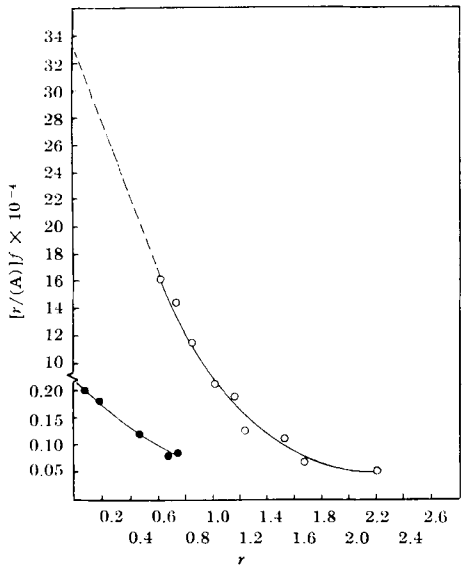


Fig. 5.—The bindings of 8-chlorotheophylline and theophylline by BSA at pH 6.85. O, 8-chlorotheophylline; ●, theophylline.

strong secondary interactions. It may be noted from Table III that the association constants for 8-chlorotheophylline and 8-nitrotheophylline at pH 6.85 are larger than those of other compounds investigated by magnitudes ranging from approximately ten to several hundred.

It is significant in this respect to compare the degrees of ionization at pH 6.85 of the various compounds investigated. It is found that 8-chlorotheophylline (pKa = 5.5) and 8-nitrotheophylline (pKa = 3.6) were completely ionized at this pH value. Furthermore, studies which are illustrated in Fig. 6 demonstrate a marked relationship between the degree of association with BSA and the ionization characteristics of the two compounds. 8-Nitrotheophylline, for example, was completely ionized throughout the pH range investigated and its interactive tendencies remained relatively constant between pH values of 4.8 and 8.5. In contrast, the fraction of 8-chlorotheophylline which existed in the

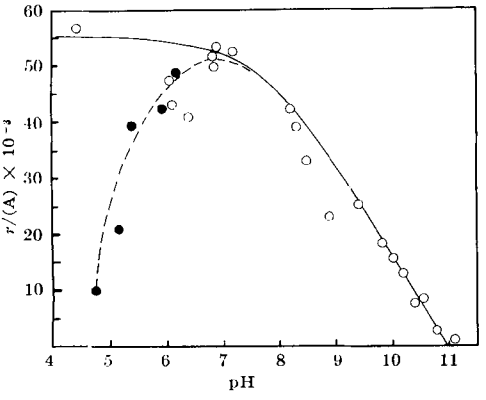


Fig. 6.—The effect of pH on the binding of 8-nitrotheophylline and 8-chlorotheophylline. O, 8-nitrotheophylline; ●, 8-chlorotheophylline.

ionized form increased in the pH range of 4.8 to 6.5 and was the predominant specie at pH values above this. Its association with BSA exhibited a similar pH dependency. Both compounds exhibited the diminution in binding at higher pH values which seems to be a characteristic of xanthine derivative-BSA interactions.

It can be concluded from the studies with theophylline and its 8-substituted derivatives that the anionic forms possess much greater binding tendencies than their nonionic counterparts. The basic nature of the association does, however, appear to be the same. The primary interactions of both types appear to involve one grouping on the protein and in both cases marked inhibition of binding occurred at pH values above approximately 8.5.

In view of these results, it was of interest to investigate the interactions of other anionic xanthine derivatives. Studies were therefore conducted with theophylline-7-acetic acid, trimethyluric acid, and uric acid. The binding of theophylline-7-acetic acid was observed to be greater than that of theophylline but significantly less than that of the 8-substituted theophyllines. This indicated that factors other than the mere presence of a negative charge determine the affinity with which the xanthine molecule is bound by the protein. This is substantiated by the results of studies with uric acid and trimethyluric acid. These compounds, too, are ionized at pH 6.85; their negative charge occurring at N_9. The interaction of uric acid was slight. On the other hand, trimethyluric acid was bound to a greater degree than the 8-substituted caffeine derivatives. Here too, however, its association constant was considerably smaller than those for the 8-substituted theophyllines.

On the basis of these results it can be stated that, although negatively charged species appeared to be more strongly bound to albumin than neutral molecules, the position of charge is of great significance. Theophylline and its 8-substituted derivatives ionize through removal of the proton from N_7. In view of the greater electronegativity of oxygen as compared to nitrogen, Cavalieri, *et al.* (23), have suggested that the negative charge probably resides on the oxygen on C_6. This shift in position of charge is facilitated by resonance in the molecule. It is consistent with the results to hypothesize that this oxygen participates directly in the binding reaction; perhaps through hydrogen bond formation with a protonated grouping on the protein. The strength of such a bond would be substantially increased if the oxygen were to possess a negative charge. In addition, it may be noted that a methyl substituent on the N_1 position will increase the electron density around the oxygen through an inductive effect.

Although the information which has been provided by this preliminary study is insufficient to characterize completely the interaction between BSA and xanthine derivatives, some interesting observations have been made. Of particular interest is the suggested involvement of only one grouping on the protein molecule. There is evidence to suggest that this grouping is an ϵ amino group of lysine. Since it has been reported that albumin contains 22 lysine residues, it can be speculated that the configuration of the macromolecule is such that only one of the lysine side chains is in an environment which will permit significant interaction with this family of

small molecules. Further studies are being undertaken to substantiate this hypothesis and to determine the nature of the forces which are responsible for the association.

The influence which slight structural modification of the small molecule has on the degree and strength of binding is also rather intriguing. The large differences which were found among the 8-substituted caffeines are particularly interesting. Variations in biological half-lives and in pharmacological activities might be partially explainable on this basis and studies are planned to test this possibility.

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Effects of Tranquilizers on Bacterial Toxemias III

Chlorpromazine

By LEO GREENBERG and JAMES W. INGALLS

Previous papers from this laboratory have indicated the role of reserpine and meprobamate in overwhelming bacterial toxemias experimentally induced in laboratory animals. The present study was primarily concerned with chlorpromazine. In rats and mice, pretreatment with chlorpromazine was shown to prolong significantly the survival of animals inoculated with lethal doses of tetanus or botulinus toxins. Similar prolongations following chlorpromazine pretreatment were demonstrated with *E. coli, S. typhosa,* and *S. marcescens* endotoxins. At identical doses, neither hydroxyzine nor methaminodiazepoxide pretreatment consistently provoked similar responses to toxin challenges.

SINCE 1957, our laboratories have been primarily concerned with the role of tranquilizing drugs in experimental stress induced in laboratory animals through the use of cultures of virulent microorganisms and by various potent microbial products, both of exotoxic and endotoxic type. We have found that reserpine exerted a highly significant life-prolonging effect in tetanus and botulinus exotoxemias and pneumococcal septicemia of mice (1). Similar prolongation of survival time in exotoxemias was found with meprobamate treatment in rats of both sexes as well as mice, although no influence on pneumococcal septicemia was observed (2). Further, the life-prolonging properties of meprobamate were shown to extend to animals inoculated with lethal doses of purified lipopolysaccharide endotoxins of *Escherichia coli* and *Salmonella typhosa* (2). In all cases, the prolongation of survival time was associated with a specific set of experimental conditions, namely the administration of a relatively large dose of tranquilizer approximately 1 hour prior to challenge with an overwhelmingly lethal dose of the toxic agent.

The present study summarizes the results of our investigation of chlorpromazine within this framework of bacterial stress. In the case of chlorpromazine, unlike that previously found with reserpine and meprobamate, the literature is replete with references, both clinical and experimental, indicating an influence of the drug in disease processes. Unfortunately, the direction and magnitude of the influence reported has sometimes been contradictory. Thus, it has been noted that chlorpromazine shortened survival time from pneumococcal (3, 4) or *Salmonella*

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