Nuclear Magnetic Relaxation Study of Intermolecular Complexes. The Mechanism of Penicillin Binding to Serum Albumin[1a]

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A study of the protein binding of penicillin was carried out using nuclear magnetic relaxation techniques in order to identify the portions of the penicillin molecule involved in the interaction. Addition of serum albumin to penicillin G solutions causes marked broadening of the penicillin spectral peaks. The relatively large increment in the relaxation rate of the phenyl peak indicates that this portion of the molecule is the binding site.

Introduction

The possibility of using differential changes of relaxation times in the proton magnetic resonance spectra of organic molecules to derive information on the nature of binding sites has thus far received little attention in the chemical literature. The weak, readily reversible interaction of penicillin with serum albumin[2,3] appeared to us as a suitable model for exploring this possibility.

The objective of our investigation was to determine which portion of the penicillin molecule is involved in the binding to serum albumin. *A priori*, several alternatives exist: There is an anionic group which could be important in electrostatic binding; there are several potential hydrogen bonding sites both on the ring structures and on the side chain; and there is a large aromatic hydrocarbon side chain that could be involved in hydrophobic bonding. The usual methods for the study of binding, such as equilibrium dialysis, ultrafiltration, and assay of drug activity, measure only the extent of binding, but fail to give information concerning the mechanism. The findings presented in this paper have shown that such information can be obtained from the differences in the changes of relaxation times which accompany complex formation. A detailed discussion of the theoretical basis is given elsewhere.[4] The findings indicate that it is the aromatic portion of the penicillin molecule which is primarily involved in the binding of the drug to the protein.

Experimental

N.m.r. spectra were obtained using a Varian Associates DP60 high-resolution spectrometer operating at a frequency of 60 Mc.p.s. and were recorded with the Varian G-10 recorder using field sweep rates of approximately 25 mgauss per min. Chemical shifts were measured using the side band technique with a Hewlett Packard Model 200CD wide range oscillator and an HP 522B electronic counter.

All solutions for n.m.r. studies were made in D_2O. Drug concentrations are all expressed in moles/liter (M) and protein concentrations in % (w./v.), *i.e.*, in grams of protein/100 ml. of final solution volume. The following materials were obtained from commercial sources: D_2O, BioRad Corp.; crystallized bovine plasma albumin, Armour Laboratories; γ-globulins, bovine fraction II, California Biochemical; 2-phenoxyacetamide and phenoxyacetic acid, Eastman Organic Chemicals. In addition, the following penicillins were kindly supplied: potassium penicillin G by the Squibb Institute, potassium penicillin V by Eli Lilly and Co., and 6-aminopenicillanic acid by Bristol Laboratories.

Acetylpenicillin was synthesized by the N-acylation of 6-aminopenicillanic acid (6APA) with acetic anhydride following the general method of Perron, *et al.*[5] It was separated as the potassium salt from methyl isobutyl ketone by addition of a 50% solution of potassium 2-ethylhexanoate in 1-butanol and was recrystallized from a water–acetone mixture. The product was a white powder which decomposed at 204° with evolution of gas after having begun to discolor at 195°. *Anal.* Calcd. for $C_{10}H_{13}N_2O_4SK$ (mol. wt. 296): N, 9.46. Found: N, 9.07. Calcd. for 6APA: N, 11.0. The n.m.r. spectrum shows the AB system which is characteristic of the complete penicillins containing the amide linkage. This can be readily distinguished from the spectra of 6APA and the common penicillin breakdown products which show an upfield shift of one of these peaks.

The values of $1/T_2$ given for the various n.m.r. peaks were obtained from the spectral line widths using the formula

$$1/T_2 = \pi(\Delta\nu)$$

where $\Delta\nu$ is the line width at one-half maximum peak height. Because of this they contain contributions from certain instrumental artifacts. Under the conditions used here, line widths of less than 1 c.p.s. were not observed. However, the correspondingly long relaxation times of these peaks made possible the measurement of T_1 using the direct saturation method. Throughout the study it was assumed that $T_1 = T_2$. This assumption is reasonable on theoretical grounds and it was verified in those cases in which a direct comparison was possible.

(1) (a) Presented in part at the annual symposium of the Division of Medicinal Chemistry, June 1964, Minneapolis, Minn., and the symposium on Helical Molecules at the 148th National Meeting of the American Chemical Society, Chicago, Ill., Sept. 1964; (b) from the Ph.D. Thesis of J. J. Fischer, Harvard University, 1964; (c) to whom all correspondence should be addressed.

(2) B. F. Chow and C. M. McKee, *Science*, 101, 67 (1945).

(3) R. Tompsett, S. Shultz, and W. McDermott, *J. Bacteriol.*, 53, 581 (1947).

(4) O. Jardetzky, *Advan. Chem. Phys.*, 7, 499 (1964).

(5) Y. G. Perron, W. F. Minor, C. T. Holdrege, W. J. Gottstein, J. C. Godfrey, L. B. Crast, R. B. Babel, and L. C. Cheney, *J. Am. Chem. Soc.*, 82, 3934 (1960).
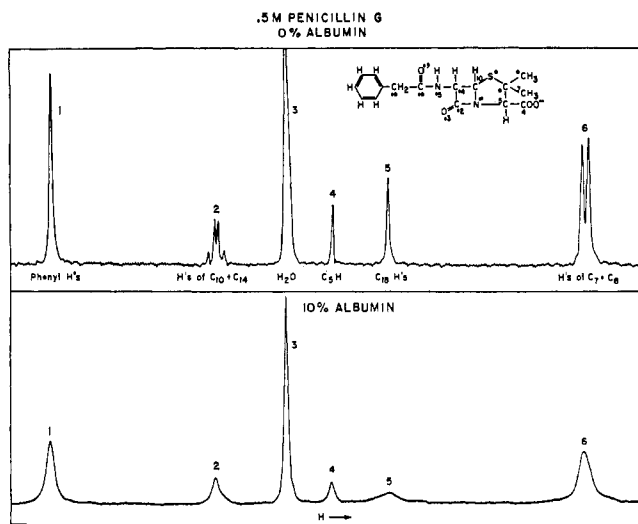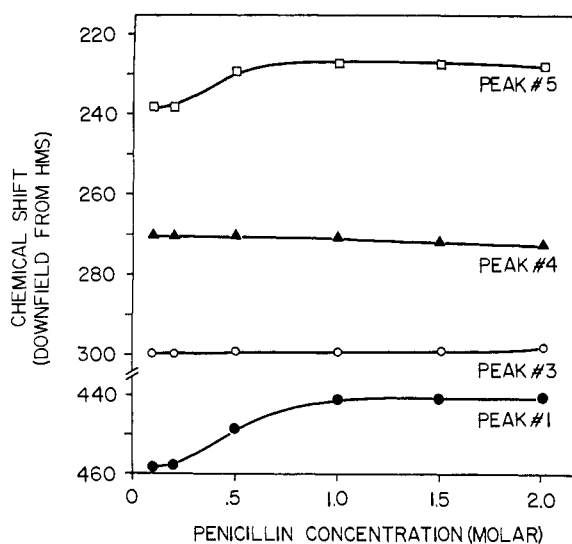
Figure 1.

Figure 2.

Each reported value of $1/T_1$ or $1/T_2$ is the mean of at least four and usually six or eight separate measurements. The standard error of the mean is included in parentheses.

All pH measurements were made using a Beckman Model G pH meter. The results given are the actual meter readings, uncorrected for the presence of D_2O.

Results and Discussion

A. Origin of the Relaxation Effects. The proton magnetic resonance spectrum of 0.5 *M* potassium penicillin G in D_2O is shown in Figure 1a. The key to the interpretation of this spectrum is the quartet, no. 2, which could be due only to the two hydrogens on C-10 and C-14. This is a typical AB system with relative chemical shifts of 3.6 c.p.s. and a coupling constant of 3.9 c.p.s. The phenyl peak is easily identified by its characteristic low-field position. Peak 6 is due to the two nonequivalent methyl groups, peak 5 to the two protons on C-18, and peak 4 to the single proton on C-5. Peak 3 is the water contaminant in the D_2O. For simplicity, measurements of line widths will be confined to the single, nonsplit peaks 1, 4, and 5.
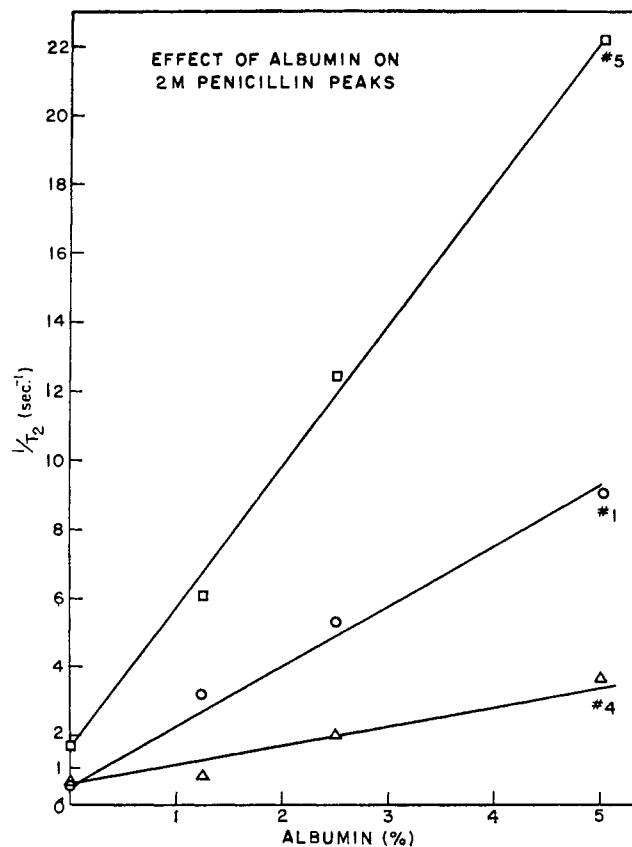

Figure 3.

A typical example of the effect of albumin on line width is shown in Figure 1b. All the lines are broadened, but the extent of broadening varies considerably. *A priori* the possibility exists that the observed broadening is nonspecific rather than an indication of a drug–protein complex. Alternative broadening mechanisms include an increase in solution viscosity, an increase in penicillin–penicillin intermolecular interactions upon addition of protein, and direct intermolecular relaxation by the protein even in the absence of complex formation; each of these has been ruled out by appropriate experiments.

The first of the control experiments was carried out to determine the effect of drug–drug interactions using a series of solutions of penicillin G at varying concentrations. The results of measurements of chemical shifts and relaxation rates are recorded in Figure 2 and Table I, respectively. Marked changes in both of these

Table I. Concentration Dependence of $1/T_2$ Values (sec.$^{-1}$) of Penicillin G Spectral Lines

Concn., *M*	Peak 1	Peak 4	Peak 5
0.1	0.55[a]	0.66[a]	2.0[a]
0.5	5.84 (0.11)	4.52 (0.15)	8.39 (0.24)
1.0	9.9 (0.24)	5.75 (0.5)	12.3 (0.28)
1.5	12.7 (0.32)	6.3 (0.28)	18.5 (0.49)
2.0	19.4 (0.11)	9.0 (0.31)	34.2 (0.8)

[a] Estimated from T_1 measurements.

parameters occur as the limit of solubility, approximately 2 *M*, is approached. However, changes com-
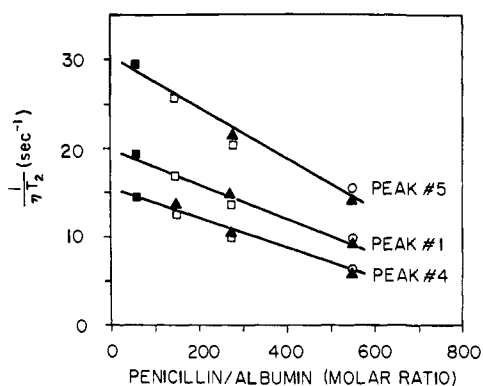
Figure 4.

Figure 5.

parable to those observed on addition of albumin to dilute penicillin solutions are not observed until a penicillin concentration of 1–1.5 M is reached. Furthermore, the changes in the relaxation rates are paralleled by changes in chemical shifts. This is not the case upon addition of albumin, as may be seen from Table II. It is therefore unreasonable to assume that the

Table II. Chemical Shifts[a] of 0.5 M Penicillin G–Albumin Solutions

Albumin concn., %	Peak					
	1	2[b]	3	4	5	6[b]
0	448.	343.5	298.5	270.5	228.5	107.5
5	447.7	343.8	298.8	269.9	227.6	108.9
10	447.9	343.8	299.	270.4	228.1	108.2

[a] Downfield shifts relative to hexamethyldisiloxane as an external standard. Estimated accuracy ±0.5 c.p.s. [b] Center of the multiplet.

broadening of penicillin peaks observed in penicillin–albumin solution could be accounted for by interactions between drug molecules.

To study the effects upon the penicillin spectrum of addition of albumin to the solution, an array of concentrations was chosen. For each penicillin concentration (0.01, 0.05, 0.1, 0.2, and 0.5 M) the following albumin concentrations were used: 0, 1.25, 2.5, 5, and occasionally 10 and 15%.

The results of a typical series of measurements for a fixed penicillin and varying albumin concentrations are shown in Figure 3. The values for 0% albumin are derived from T_1 measurements, the remaining values from measurements of line width. Although peak 5 is the broadest, it is readily seen that the relaxation rate of peak 1 is changed by a larger factor than the relaxation rate of either peak 4 or 5. It is this relative increment, rather than the absolute extent of broadening, which allows the interpretation in terms of preferential stabilization of the aromatic ring.[4]

Figure 4 shows that the relaxation rate is dependent on the penicillin–albumin ratio rather than on the albumin concentration itself. This finding is exactly opposite to what one would expect if the broadening were due to one of the nonspecific mechanisms mentioned previously. Here addition of more penicillin to a given penicillin–albumin solution decreases the width of the lines. However, more penicillin increases
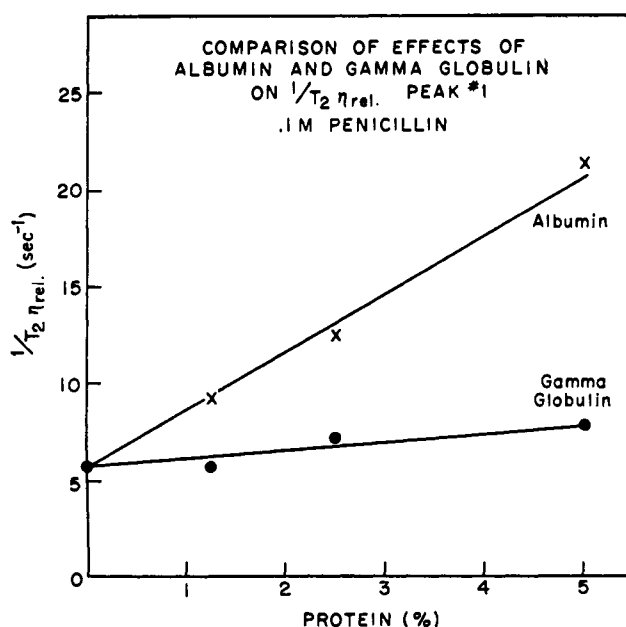
the viscosity of the solution, increases the possibility of drug–drug interactions, and certainly should not decrease any intermolecular relaxation effects of the protein on the drug. The only feasible alternative is to attribute the broadening to a specific interaction between drug and protein. The results indicate that this interaction must be saturable; that is, a given albumin molecule can interact with only a limited number of penicillin molecules at one time.

The possibility that the observed changes are attributable to a change in viscosity is ruled out by the fact that the viscosity of a 5% solution of albumin used in this experiment in D_2O is only 1.25 times as large as the viscosity of pure D_2O.[6] This is much too small a change to be the sole cause of the observed effects. Further evidence for the specificity of this effect arises from experiments with γ-globulin, which is known not to bind penicillin.[2,3] Figure 5 shows that when correction is made for viscosity changes γ-globulin has little effect on the line widths.

In order to interpret adequately the effects of albumin on chemical shifts and line widths, it is necessary to have an estimate of the fraction of penicillin molecules bound to albumin. Because of the very large ratio of penicillin to albumin, approximately 200:1, one would expect a priori that the bound fraction would be small, since the number of binding sites is believed to be less than 5.[7] However, such estimates are based on experiments at low (10^{-4} M) penicillin concentrations.

To obtain data directly applicable to the n.m.r. solutions dialysis experiments were carried out using 0.01 M penicillin concentrations. Results with a 5% albumin solution show 1.2 μmoles of penicillin bound by 1.4 μmoles of albumin. This finding can be considered at best a rough estimate, but with the great penicillin excess it is a reasonable indication that there

(6) Viscosity measurements were performed in this laboratory by V. Seery. The figure used for calculating the corrections in Figures 4 and 5 was the viscosity of the solution relative to the viscosity of D_2O, η_{rel}.
(7) W. Scholtan and J. Schmid, Arzneimittel-Forsch., 12, 741 (1962).

are never more than 2 or 3 molecules of penicillin bound per molecule of albumin.

This result is of considerable importance. It shows that essentially the same number of binding sites are used at the high concentrations necessary for the n.m.r. studies as are used in the pharmacologically interesting range. The possibility can thus be rejected that binding at high concentrations brings into prominence many additional sites.

The lack of an effect of albumin on chemical shifts is now readily understood, since any observable changes would necessarily be small. The maximum likely effect due to interactions other than the breaking of chemical bonds can be estimated at 200 c.p.s., caused by placing a proton into the diamagnetic region of an unsaturated ring structure.[8] In the limit of rapid exchange, one peak would be observed with a shift that was the weighted average of the two. Since only approximately 1% of the molecules are in the bound form, the change would be at most 2 c.p.s. For broad lines this would be very difficult to detect. For very slow exchange rates, two separate peaks would result. However, since one would arise from so few molecules it would not be detected.

On the same basis one can rule out exchange broadening as the mechanism responsible for the observed changes in widths. Even if the exchange rate were in the correct range, approximately 10–200 c.p.s., to produce this phenomenon, the small fraction, $^1/_{1000}$, in the one state would make the effect negligible.[9] It will be shown below that the results of the albumin line width studies are incompatible with such a slow exchange rate, and indeed one is dealing here with an example of exchange which is very fast in terms of averaging chemical shifts.

B. The Model of the Penicillin–Albumin Interaction. To explain the results of the penicillin–albumin experiments quantitatively, we propose the following kinetic model. Despite the great simplicity of this treatment, the predicted results are in remarkable agreement with the observations.

Assume that the penicillin molecules in albumin solutions can exist in only one of two forms, bound or free, and that the relaxation rate for the bound state is much faster. This is reasonable considering the nature of relaxation processes. Three possible cases, depending on the rate of exchange between the states, can then be distinguished.

Case I: $1/T_{exchange} < (1/T_2)_{free} < (1/T_2)_{bound}$

In this case two superimposed lines, one broad and one narrow, will be seen. Such a result clearly does not occur here.

Case II: $(1/T_2)_{free} < 1/T_{exchange} < (1/T_2)_{bound}$

The nuclei of the bound molecule will relax as usual, but those of the free molecules will be influenced by the exchange rate. When the molecules are predominantly free (approximately 99% in our case) the relaxation rate of the observed peaks will be determined by the exchange rate. All peaks will have approximately

equal rates. This is certainly not the case for the penicillin–albumin solutions, since the methylene peak is much broader than the others.

Case III: $(1/T_2)_{free} < (1/T_2)_{bound} < 1/T_{exchange}$

This is the fast exchange limit. The relaxation rates for the bound and free forms will be averaged with the result:

$$1/T_2 = (1/T_2)_{free} + B[(1/T_2)_{bound} - (1/T_2)_{free}] \quad (1)$$

where B is the fraction of the total penicillin that is bound. Each peak will have a different relaxation rate depending on its $(1/T_2)_{free}$ and $(1/T_2)_{bound}$ values. The same value of B will, of course, apply to each. It is this case that applies to the experiments in this section.

Use of a simple mass action model to determine B will make it possible to calculate $(1/T_2)_{bound}$ from eq. 1. Let P and A be the total penicillin and albumin concentrations, respectively, and assume that there are n noninteracting binding sites on each protein molecule. Then

$$K = \frac{P(1 - B)(nA - PB)}{PB} \quad (2)$$

which can be arranged to give

$$P = \frac{K}{B - 1} + \frac{nA}{B} \quad (3)$$

If the line width *vs.* albumin concentration is plotted for each of the five values of penicillin concentration, it is possible to use this series of graphs to establish for any penicillin peak all those combinations of penicillin and albumin concentration that will produce some arbitrarily chosen width. The locus of these penicillin–albumin combinations that produce a given line width can then be plotted on a graph of penicillin *vs.* albumin concentration. Such a graph is shown in Figure 6. The results for two different widths are presented for each of three different penicillin peaks.

From eq. 1 it can be seen that lines of constant width obtained as described above will also be lines of constant B. Equation 3 then predicts that these lines will be straight with slope n/B, and will intersect the $A = 0$ axis at $-K$ (for $B \ll 1$).[10] The straightness of the lines in Figure 6 and the fact that they do all extrapolate to roughly the same point are support for the model chosen. K can be estimated at 0.01–0.02 M.

In order to obtain values for $(1/T_2)_{bound}$ from eq. 1, it is necessary to know B. Since only n/B values[11] can be obtained from Figure 6, these calculations will be carried out using $n = 1$. The results can then be altered to fit any n value to a good approximation by

$$(1/T_2)_{bound,n} = (1/T_2)_{bound,n=1}/n \quad (4)$$

(8) C. E. Johnson and F. A. Bovey, *J. Chem. Phys.*, **29**, 1012 (1958).

(9) J. A. Pople, W. G. Schneider, and J. H. Bernstein, "High-resolution Nuclear Magnetic Resonance," McGraw-Hill Book Co., Inc., New York, N. Y., 1959, p. 222.

(10) As we mentioned above, a certain error due to instrumental limitations is introduced when using line width to obtain $1/T_2$ values. On a line of constant width as plotted in Figure 6 this error would be the same for all points. No correction is needed for the value of K obtained in this way, but the value of n/B will be slightly in error. This error becomes less significant for broad lines, and the choice of the widths used in Figure 6 was influenced by this consideration.

(11) It should be noted that the values of B obtained from these slopes (assuming $n = 1$ or 2) are in general agreement with the results of the dialysis experiments. They range from approximately one-one-hundredth to one-six-hundredth.
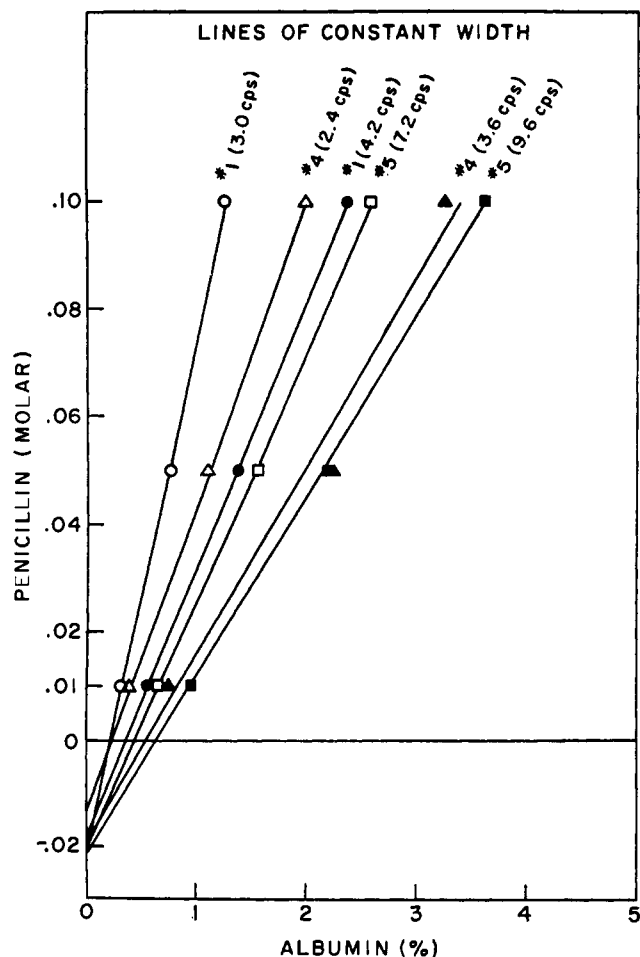
Figure 6.

The calculation of the $(1/T_2)_{bound}$ values in Table III was carried out using the $1/T_1$ values for the free drug, the widths for the observed $1/T_2$ values, and the slopes of

Table III. Calculation of Relaxation Rates for Bound Penicillin

Peak	Width, c.p.s.	$1/T_2$, sec.$^{-1}$	$1/T_{2free}$	n/B	$1/T_{2B}$	Av. $1/T_{2B}$	T_{2free}/T_{2B}
1	3.0	9.4	0.555	621	5550	4990	9000
1	4.2	13.2	0.555	350	4430		
4	2.4	7.55	0.667	372	2560	2380	3560
4	3.6	11.3	0.667	207	2200		
5	7.2	22.6	2.0	332	6850	6725	3360
5	9.6	30.2	2.0	234	6600		

the lines of Figure 6 for the n/B values. Two calculated values of $(1/T_2)_{bound}$ are obtained for each of the three peaks, and the agreement is excellent.[12]

It should be emphasized, however, that the primary interest here is not in the absolute values of $1/T_2$

(12) It should be noted that the largest $(1/T_2)_{bound}$ value calculated is approximately 7000 c.p.s. (This would be only 3500 c.p.s. if $n = 2$ were used, etc.) To be dealing with the fast exchange limit, case III above, and thus to justify the use of eq. 1, requires an exchange rate fast compared to this value. One would expect this condition to be met for weak interactions of the type discussed here. An activation energy for breaking the complex of less than 10 kcal. per mole would ensure averaging of these relaxation times, if the reaction rate is assumed to equal $kT/h \exp(-\Delta F^*/kT)$.[13]

(13) F. H. Johnson, H. Eyring, and M. J. Polissar, "The Kinetic Basis of Molecular Biology," John Wiley and Sons, Inc., New York, N. Y., 1954, p. 98.
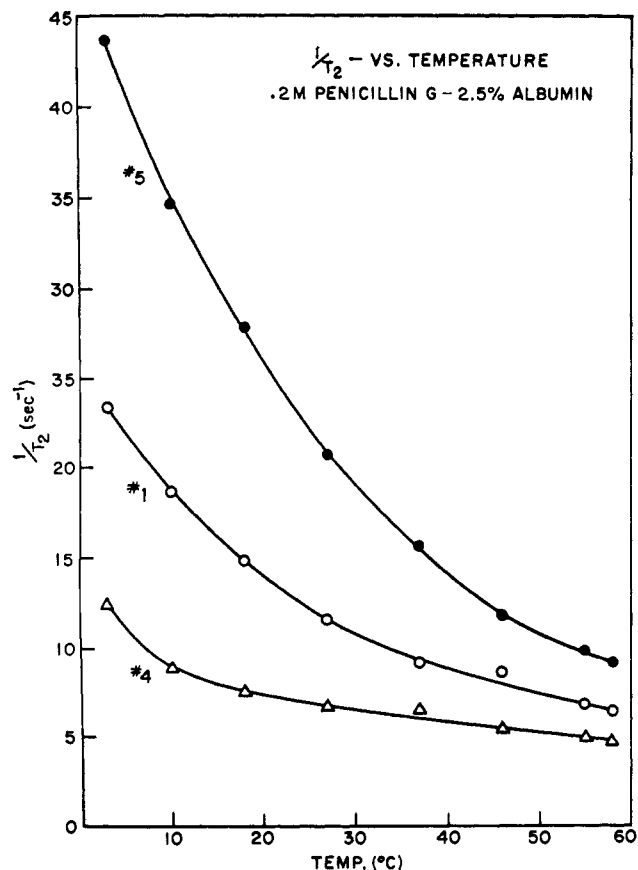

Figure 7.

for the bound molecules, because this reflects to a large extent the intrinsic differences in relaxation of the different groups, methylene, phenyl, etc. To compare the changes in correlation times that accompany binding, it is necessary to compare the ratios of $(1/T_2)_{bound}$ to $(1/T_2)_{free}$ for each of the peaks.[4,14] The results of this comparison are also shown in Table III. It now becomes obvious that the phenyl group is much more affected by binding than are the other groups. Its relaxation rate changes by a factor of approximately 9000, while that of the methylene changes by approximately 3600 and the CH group by 3400.

This close agreement between the experimental results and the predictions of a quite simple model which is both *a priori* reasonable and internally consistent is strong support for the contention that the observed broadening is due to the penicillin–albumin binding. The quantitative pattern of broadening seen with the various penicillin–albumin concentrations is completely incompatible with any of the nonspecific relaxation mechanisms. The very striking selective effect of the binding on the phenyl group indicates that it is intimately involved in the binding process, while other portions of the molecule retain freedom of motion to a much greater extent.

C. *Corollary Experiments.* (a) *Temperature Studies.* The effect of variations in temperature on the n.m.r. spectra of penicillin and penicillin–albumin solutions has been studied over a range of temperature 0–60°, limited by the freezing of the solutions on

(14) O. Jardetzky and C. D. Jardetzky, *Methods Biochem. Analy.*, 9, 235 (1962).

Table IV. Effects of pH on $1/T_2$ Values of Penicillin G Spectral Lines for 0.1 M Penicillin-2.5% Albumin Solutions

pH	Peak 1	Peak 4	Peak 5
6.1	10.7 (0.16)	6.7 (0.15)	20.7 (0.06)
6.35	10.37 (0.12)	6.5 (0.15)	18.8 (0.4)
6.6	10.2 (0.24)	5.98 (0.34)	18.8 (0.2)
6.7	9.2 (0.08)	5.78 (0.18)	17.2 (0.3)
7.1	9.17 (0.12)	5.34 (0.06)	17.2 (0.5)
7.75	9.17 (0.23)	5.7 (0.18)	16.3 (0.42)
7.9	8.92 (0.19)	5.53 (0.21)	16.4 (0.21)
8.3	8.9 (0.15)	5.1 (0.126)	16.2 (0.4)

Table V. Effects of Ionic Strength on the $1/T_2$ Values (sec.$^{-1}$) of Penicillin G Spectral Lines

NaCl concn., M	Peak 1	4	5	1	4	5
	0.1 M penicillin			0.1 M penicillin-2.5% albumin		
0	5.21 (0.3)	4.21 (0.23)	5.21 (0.15)	11.7 (0.14)	6.33 (0.14)	20.5 (0.23)
0.187	5.09 (0.4)	4.05 (0.18)	4.05 (0.18)	11.1 (0.4)	6.0 (0.46)	21.2 (0.09)
0.375	4.96 (0.23)	4.27 (0.08)	5.08 (0.19)	11.8 (0.90)	6.97 (0.24)	21.7 (0.23)
0.75	5.88 (0.44)	4.59 (0.3)	5.35 (0.18)	13.3 (0.23)	6.9 (0.24)	24.0 (0.47)
1.5	5.18 (0.28)	4.15 (0.26)	5.2 (0)	14.8 (0.09)	8.0 (0.42)	31.5 (0.88)
3.0	5.37 (0.18)	4.33 (0.10)	8.29 (0.22)	25.4 (0.62)	9.99 (0.36)	50.9

one hand and by the precipitation of the protein on the other. No significant changes in the chemical shifts of the penicillin peaks occur in this range for either 0.2 M penicillin or 0.2 M penicillin-2.5% albumin solution, as expected. Similarly, for the solution of 0.2 M penicillin with no albumin there is very little change in the widths throughout the temperature range of 2 to 60°.

In contrast, the effects of temperature variation on the line widths for the penicillin–albumin solution are quite large (Figure 7). Even if the instrumental contribution to the widths of the narrowest lines is estimated as 4 sec.$^{-1}$, the $1/T_2$ values increase by almost tenfold as the temperature is decreased from 58 to 3°. These changes could arise from increases in the $1/T_2$ values for the free and/or the bound forms of the drug and from an increase in the fraction of the drug that is bound. It is evident from the studies of the penicillin alone that the changes in the line widths for the free form are not sufficient to account for these results. It is not possible to determine exactly the relative contribution of the other two factors. If the rigid sphere models were applicable to the motion of the protein, one would again predict that changes in $1/T_2$ values for the bound form would be small, 20%. It is therefore more reasonable to assume that the major effect is due to an increase in the fraction of drug bound as the temperature is decreased. However, in view of the uncertainty of the relative importance of the two mechanisms, calculations of thermodynamic parameters are not warranted at this time.

Within the range of temperature studied, it was not possible to slow the rate of exchange between the free and bound forms sufficiently to produce qualitative changes in the spectra, to correspond to case II or case I.

(*b*) *pH Effects on Penicillin–Albumin Solutions.* To study the effect of changes in pH upon the penicillin–albumin interaction, a solution of 0.1 M penicillin G and 2.5% albumin was chosen. The range over which such a study could be carried out was quite restricted due to the instability of the penicillin.

A change in line width is seen for each of the peaks between pH values 6.1 and 6.7, but this is quite small compared to the total effect of the albumin (see Table IV). Certainly complex formation is not prevented to any great extent, but perhaps a slight change in the equilibrium constant takes place. The pK of penicillin G is 2.8, so there should be no significant effect on the form of this molecule over the pH range studied. On the other hand, bovine serum albumin contains 16 histidine residues per molecule, the imidazole groups of which are titrated generally in the range pH 6–7.[15] Since the pH effect on broadening is so small, it seems possible to conclude that the penicillin–albumin binding does not depend strongly on an ionic interaction between the positively charged histidine and the carboxyl group of the penicillin. The change in total charge of approximately 16, which occurs through the pH range tested, would on the other hand be expected to alter the conformation of the protein to some extent. It is therefore not too surprising that a small change in the binding occurs.

(*c*) *Effects of Ionic Strength on Penicillin and Penicillin–Albumin Solutions.* To study the effect of ionic strength on the penicillin–albumin interaction, the 0.1 M penicillin G–2.5% albumin solution was again chosen. This and a control solution of 0.1 M penicillin alone were examined in the presence of varying amounts of NaCl, 0–3 M. The results of line-width measurements are presented in Table V. There is a marked increase in the line width with increasing ionic strength for the penicillin–albumin solution, while the effect for the penicillin alone is much less. The chemical shifts of both solutions change in an identical manner.

The enhancement of broadening upon addition of salt is useful in deciding upon a mechanism to explain the penicillin–albumin binding. Since the energy of electrostatic interactions varies inversely with dielectric constant, one would expect an ionic bond between the drug and protein to be weakened by addition of salt. The above findings therefore rule against this as a possible mechanism.

On the other hand, it is well known that salt tends to increase the interactions between hydrophobic groups, for example, the salting out effect as seen for solutions of slightly soluble organic molecules in water.[16] This suggests that the penicillin–albumin

(15) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Academic Press Inc., New York, N. Y., 1958, p. 534.
(16) J. T. Edsall and J. Wyman, in ref. 15, pp. 263–282.

interaction is hydrophobic in nature and agrees completely with the finding in section B that the phenyl group, a very hydrophobic structure, is most strongly affected by the binding.

(d) Inhibition Studies. The specificity of the binding sites on the albumin molecules was further examined in a series of inhibition studies. In these experiments an attempt was made to displace penicillin G from the albumin by adding a large excess, usually 4–5-fold, of another penicillin or some similar molecule. The structure of the compounds used is shown below:
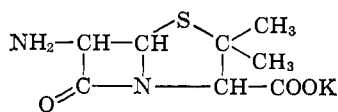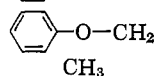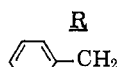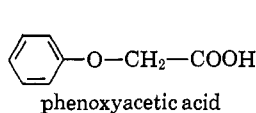

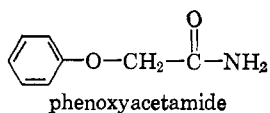penicillin   R

G   phenyl-CH₂

V   phenyl-O-CH₂

acetyl   CH₃
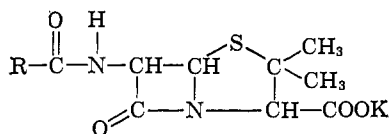
6APA

phenoxyacetic acid   phenoxyacetamide

In principle a study of this kind could be done using dialysis experiments, but in practice it would be quite difficult. Using the n.m.r. spectrum as the assay for binding, it is only necessary to choose the inhibiting substance carefully so that not all of the peaks of the two substances overlap.

The general experimental design was the same for all the inhibiting substances tested. Spectra of the following solutions were obtained: (1) penicillin G alone and the inhibitor alone, serving as controls for the combined solutions; (2) penicillin G and the inhibitor together, as a control for interaction between the two substances; (3) penicillin G and albumin, as a control for the effect of the inhibitor; (4) the inhibitor and the albumin (if changes in the line widths of the inhibitor peaks occurred which were comparable in magnitude to those seen for the analogous peaks of penicillin G (in the same concentration range), it was assumed that the inhibitor also was bound to the protein. If these were not seen, then such interactions were assumed not to have taken place; (5) penicillin G, inhibitor and albumin, to assay for inhibition of binding.

The first inhibiting substance investigated was another penicillin, V, which is also known to bind to albumin.[17] In the spectrum of this compound the methylene peak is displaced to a lower-field position relative to that of penicillin G. It is then possible to distinguish these peaks in the spectrum of a mixture of the compounds, although unfortunately the other peaks are super-

(17) R. C. Anderson, C. C. Lee, H. M. Worth, and K. K. Chen, *Antibiotics Annual*, 540 (1956).

Table VI. Inhibition Studies-$1/T_2$ Values (sec.$^{-1}$)

a. Penicillin V

	CH_{2_G}	CH_{2_V}
0.1 M Peni G	5.2 (0.11)	
0.5 M Peni V		8.98 (0.18)
0.1 M Peni G 0.5 M Peni V	6.75 (0.16)	9.12 (0.07)
0.1 M Peni G 2.5% Alb	22.0 (0.57)	
0.5 M Peni V 2.5% Alb		17.9 (0.59)
0.1 M Peni G 0.5 M Peni V 2.5 % Alb	11.73 (0.31)	16.8 (0.54)

b. 6APA

	CH_{2_G}	CH_{6APA}
0.5 M 6APA 2.5% Alb		4.5 (0.15)
0.5 M 6APA 0.1 M Peni G	5.18 (0.09)	Overlap
0.5 M 6APA 0.1 M Peni G 2.5% Alb	26.1 (0.3)	Overlap

c. Ac-Peni

	CH_{2_G}	$CH_{Ac-Peni}$	$CH_{3_{Ac-Peni}}$
0.1 M Ac-Peni		4.05 (0.09)	3.95 (0.1)
0.1 M Ac-Peni 2.5% Alb		4.8 (0.09)	5.09 (0.11)
0.5 M Ac-Peni 2.5% Alb		4.24 (0.18)	4.61 (0.18)
0.5 M Ac-Peni 0.1 M Peni G	4.99 (0.18)	3.95 (0.24)^a	4.24 (0.18)
0.5 M Ac-Peni 0.1 M Peni G 2.5% Alb	22.3 (0.16)	4.15 (0.15)^a	4.52 (0.15)

d. Phenoxyacetamide (POAAm)

	CH_G	CH_{2_G}	$CH_{2_{POAAm}}$
0.01 M Peni G	3.77 (0.15)	4.42 (0.09)	
0.01 M Peni G 0.5% Alb	6.66 (0.35)	16.1 (0.46)	
0.04 M POAAm			4.03 (0.18)
0.04 M POAAm 0.5% Alb			5.57 (0.10)
0.04 M POAAm 0.01 M Peni G	3.83 (0.06)	5.28 (0.14)	3.83 (0.23)
0.04 M POAAm 0.01 M Peni G 0.5% Alb	5.21 (0.22)	12.8 (0.49)	5.38 (0.11)

e. Phenoxyacetic acid (POAAc)

	CH_G	CH_{2_G}	$CH_{2_{POAAc}}$
0.04 M POAAc			3.91 (0.1)
0.04 M POAAc 0.01 M Peni G	3.66 (0.16)	4.73 (0.26)	3.83 (0.18)
0.04 M POAAc 0.5% Alb			6.31 (0.2)
0.04 M POAAc 0.01 M Peni G 0.5% Alb	5.11 (0.18)	8.45 (0.45)	6.25 (0.29)

^{a} Slight overlap; but this can be taken as a maximum value.

imposed. Table VIa gives the line widths of the two methylene peaks for the solutions of the various combinations of interest. The spectrum of the penicillin

G and V and albumin mixture shows slightly less broadening of the V peak and much less broadening of the G peak compared with their controls. Thus there is a competition for the binding sites on the albumin molecule.

Table VIb gives the results of an experiment carried out with 6-aminopenicillanic acid (6APA), the penicillin nucleus. 6APA does not interact with albumin itself, and does not inhibit the penicillin G interaction.

A similar experiment was carried out with the acetyl derivative of 6APA which was synthesized specifically for this purpose, since it has the shortest possible side chain while also containing the characteristic amide linkage. The results in Table VIc show this compound is not bound to albumin and does not inhibit the binding of penicillin G.

Tables VId and e show the results of inhibition with two analogs of the penicillin V side chain, phenoxyacetic acid (POAAc) and phenoxyacetamide (POAAm). These were chosen rather than the corresponding penicillin G analogs to prevent overlap in the spectra. The limited solubility of these compounds made it necessary to use a lower concentration range than was used in the preceding three experiments, i.e., 0.01 M penicillin G and 0.04 M inhibitor. Both of the

compounds tested were bound to the albumin (as judged by the increase in width of their methylene peaks) and also inhibited the binding of penicillin G.

The specificity of the inhibition agrees with the results of the experiments reported in the preceding section.

6APA, the nucleus of the penicillin molecule, does not bind or inhibit binding, indicating that its contribution to the observed binding is negligible. Furthermore, acetylpenicillin does not bind or inhibit. It is a complete penicillin containing the characteristic amide linkage but only a very short side chain. If the amide linkage were the binding site, this molecule should be the strongest inhibitor for there would be no possibility of steric hindrance interfering with bond formation.

Penicillin V binds and inhibits, as do the two small analogs of its side chain, POAAm and POAAc. These inhibition experiments clearly demonstrate, by a second, independent method, that the ring structure is the binding site.

Communications to the Editor

An Understanding of the Stereoselectivity of Base-Catalyzed Olefin Isomerization Based on a Thermodynamically More Stable cis-Allylic Anion

Sir:

Base-catalyzed prototropic shifts are often accompanied by unexpected kinetic control to the less stable *cis* form.[1-4]

Existing explanations to account for these stereoselectivities in the homogeneous base-catalyzed isomerizations of ethers, amines, and olefins are not wholly adequate. For example, the observation that 4,4-dimethyl-1-pentene shows considerable kinetic control to a *cis* product[5] makes it highly unlikely that an extension of the proposed metal cation bonding to a hydrogen[1] can account for the data. Similarly, Price's[6] explanation based on the energy difference of conformers is incompatible with the subsequent conclusion that all conformers of 1-butene are equally populated at room temperature.[7]

The rate of base-catalyzed olefin isomerization is a marked function of variations in the base and solvent.[8] In contrast, as reported here, the stereoselectivity shows a pronounced insensitivity to these factors.

The stereoselectivity was examined in a variety of bases and solvents. The pertinent data are summarized in Table I. Cation variations produce rate factors of 450 in going from sodium *t*-butoxide to cesium *t*-butoxide, an effect discussed in a forthcoming publication.[8] Of particular interest to the present study is the fact that the stereoselectivities are essentially the same for all the cations.

In addition, similar ratios of stereoselectivity are obtained with potassium methoxide and *t*-butoxide, although the rates of isomerization are markedly dependent on the anion (KO-*t*-Bu/KOMe = 125).

A striking resemblance to the results of the effect of base is observed in the study of the effect of solvent variations upon the stereoselectivity. Addition of the hydroxylic species *t*-butyl alcohol has essentially no effect upon the stereoselectivity. Indeed the factors which account for the rate decreases[9] cannot have direct bearing upon the stereoselectivity. Finally, base-catalyzed isomerization in several dipolar solvents proceeding at various relative rates once more produces similar stereoselectivities. The effect of solvent

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(9) A. Schriesheim and C. A. Rowe, Jr., *J. Am. Chem. Soc.*, 84, 3160 (1962).