A NUCLEAR MAGNETIC RESONANCE STUDY OF THE BINDING OF ACETYLSALICYLIC ACID TO HUMAN SERUM ALBUMIN

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The binding of acetylsalicylic acid to human serum albumin has been studied by nuclear magnetic resonance methods. The results indicate that acetylsalicylic acid exchanges rapidly with at least one site on human serum albumin, and that the nuclear spin relaxation of the acetyl protons of bound acetylsalicylic acid is characterized by a correlation time of the magnitude expected for the rotational correlation time of a human serum albumin molecule. The calculated rotational correlation time of the ASP·HSA complex is 4×10^{-8} seconds. Limits are also set on the rate constants involved.

The binding of small molecules such as salicylates, sulphonamides, and penicillin to serum albumin has been studied by equilibrium dialysis (1-3), ultrafiltration (4,5), spectrophotometric (6), gel filtration (7), and nuclear magnetic resonance (8-10) methods. The biological implications of this binding have been the subject of considerable discussion (11, 12). In this regard, recent studies have indicated that acetylsalicylic acid (ASA) acetylates human serum albumin (HSA) and a variety of other body constituents *in vitro* and *in vivo* (13-15). The present study was undertaken to elucidate the details of the binding of ASA to HSA, which precedes acetylation, by NMR methods. The pioneering NMR studies by Jardetsky and co-workers (8-10) exemplify the advantages of NMR methods in studying the interactions involved in intermolecular complexes.

This communication presents the results of the measurements of the nuclear spin relaxation times, T_1 and T_2, and the chemical shift of the acetyl protons of ASA as a function of HSA concentration at two resonance frequencies. The results are interpreted in terms of the Swift and Connick equations for the exchange of nuclear spins between two environments (16); in this case ASA free in solution and ASA bound to HSA. The results indicate that ASA exchanges rapidly with at least one site on HSA, and that the relaxation of the acetyl protons of bound ASA is characterized by a correlation time of the magnitude expected for the rotational

correlation time of a HSA molecule. The results are used to calculate the rotational correlation time of the ASA·HSA complex and to set limits on the rate constants for the formation and lifetime of the ASA·HSA complex.

Experimental. Acetylsalicylic acid was obtained from Aldrich, kept dry in a dessicator, and used without further purification. All ASA solutions ([ASA] $\simeq 1.7 \times 10^{-2}$M) were freshly prepared before use. For linewidth measurements and measurements of T_1 by the direct method, these solutions were made up in 0.1M phosphate buffer, pH = 7.36. For the measurements of T_1 and T_2 by the $T_{1\rho}$ method, the ASA was dissolved in D_2O (Bio Rad 99.88% lot #5811), which was then brought to pH (meter reading) = 7.4 ± 0.1 by the addition of small amounts of K_3PO_4.

Human serum albumin was obtained from Pierce Biochemicals, lot #4334, and used without further preparation for most measurements. No change in the measured relaxation times was observed if the HSA was first dialyzed against 0.1M EDTA - 0.1M phosphate buffer, pH = 6.6, to remove possible trace metal contaminants[1] and then against 0.1M phosphate buffer, pH = 7.4; or if the solutions were made 2.6×10^{-3} in EDTA. Solutions were made up by adding freshly prepared ASA solution to weighed amounts of HSA immediately before the spectra were recorded or relaxation times measured. The final concentration of HSA was in the range $0 - 5 \times 10^{-4}$M based upon a molecular weight of 70,000. All measurements were made within 15 mins. This time is much less than the time required for significant acetylation [24 hours at 37°C. (13)]. No change in the spectra was observed for periods as long as 1 hr. The ambient temperatures of the spectrometers were: HA-100, 33°C; A-60, 28°C; T-60, 35°C. Temperature studies were done using a Varian model V4341/V6057 temperature controller. Temperatures were determined from the chemical shift of a methanol sample placed in the probe before and after each measurement.

Some T_2's were determined from measured linewidths at 60 MHz and 100 MHz on Varian A-60 and HA-100 spectrometers. The contribution to the observed linewidths from magnetic field inhomogeneities was accounted for by subtracting the linewidth of internal tert-butanol ($\lesssim$0.4% v/v). External hexamethyldisilazane was used as a lock signal on the HA-100. T_1's and T_2's were also measured by the $T_{1\rho}$ method at 100 MHz on a Varian HA-100 spectrometer while the spectrometer was locked on external benzene. Details of these

1. Sarkar and Wigfield were unable to completely free commercial HSA of Cu(II) contaminants by dialysis (17). However, the addition of 3×10^{-4}M Cu(II) to an ASA-HSA solution produced only a small broadening ($\sim$20%) of both the ASA and tert.-butanol protons. 3×10^{-4}M Cu (II) represents a 100-fold excess over the maximum suspected contamination.

measurements are presented elsewhere (18,19). T_1's were also measured by the direct method (20) at 60 MHz on a Varian T-60 spectrometer.

Results and Discussion. The linewidth of the acetyl resonance of ASA broadens dramatically as HSA is added to the solution. Typical spectra are presented in Fig. 1. The values of T_1 and T_2 at 60 MHz and 100 MHz for the acetyl protons of ASA as a function of $[HSA]_o/[ASA]_o$ are presented in Fig.2. The broadening at 60 MHz appears to be slightly larger than that at 100 MHz, but this only reflects the difference in ambient temperatures between the two spectrometers as can be seen from Fig.3. In Fig.3 the relaxation times T_1 and T_2 at 100 MHz for the acetyl protons of ASA in the presence of HSA are presented as a function of temperature. The value of $1/\pi\ T_2$(ASA·HSA) at 100 MHz and 33°C is 60 Hz.

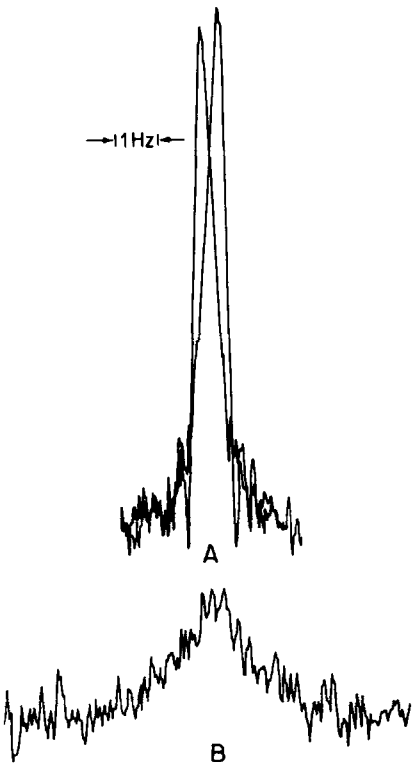

Fig. 1: Linewidth of the acetyl protons of acetylsalicylic acid at 100 MHz. A) 1.64×10^{-2} M ASA, B) 1.64×10^{-2} M ASA, 3.28×10^{-4} HSA. A forward and a reverse sweep are superimposed in A).

The observed acetyl proton resonance of ASA as a function of HSA concentration is small. The value of δ(ASA·HSA) - δ(ASA) = 0.17 ppm (upfield).

The first conclusion is that ASA binds reversibly to HSA as evidenced by the fact that the acetyl proton resonance of ASA is dramatically broadened

as a function of HSA concentration. Since the broadening for all rates of exchange is proportional to P = [ASA·HSA]/[ASA]<<1(16), a limit on the dissociation constant for the reaction

$$ASA + HSA \underset{}{\overset{K_D}{\rightleftharpoons}} ASA \cdot HSA \qquad (1)$$

can be set (assuming a one-to-one complex) using a computer program designed to fit the observed linewidths as a function of initial concentrations of reactants with a K_D and a bound linewidth (18,21). The fit is equally good for all values of $K_D \lesssim 1 \times 10^{-3}$M; hence $K_D(max) = 1 \times 10^{-3}$M. This $K_D(max)$ is approximately one order of magnitude less than the value of K_D for penicillin binding to bovine serum albumin (9).

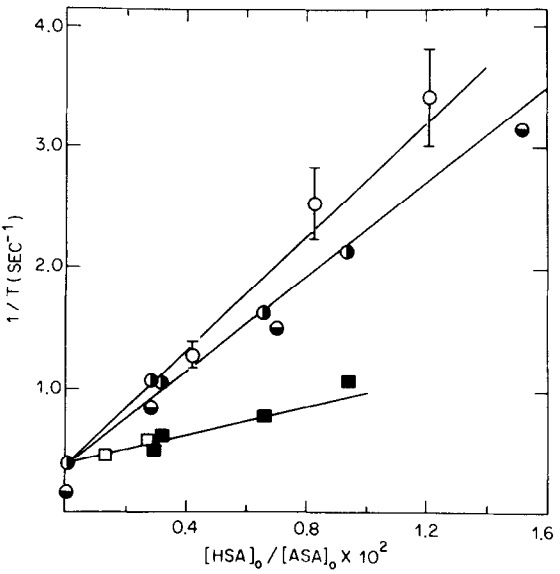

Fig. 2: Relaxation times for the acetyl protons of acetylsalicylic acid as a function of $[HSA]_o/[ASA]_o$:

$1/T_2$ at 60 MHz from linewidth 0;
$1/T_2$ at 100 MHz by $T_{1\rho}$ method ◐;
$1/T_2$ at 100 MHz from linewidth ●;
$1/T_1$ at 60 MHz by direct method □;
and $1/T_1$ at 100 MHz by $T_{1\rho}$ method ■.

The second interesting feature is that the observed relaxation times $1/T_1$ and $1/T_2$ of the acetyl protons of ASA in the presence of HSA are not a function of resonance frequency. As discussed by Sykes, Schmidt and Stark (22), there are two limits of the general equations for the observed relaxation times $1/T_1$ and $1/T_2$ in the presence of chemical exchange between two sites (16, 23, 24) which satisfy the conditions that $1/T_1$ and $1/T_2$ are not a function of resonance frequency and that $1/T_2 - 1/T_1 > 0$. The two

limits are

1) the slow exchange limit

$$1/T_2 - 1/T_2(ASP) = P/\tau \quad (2)$$

$$1/T_1 - 1/T_1(ASP) = P/T_1(ASP\cdot HSA) \quad (3)$$

with

$$\tau << T_1(ASP\cdot HSA) \quad (4)$$

and 2) the fast exchange limit

$$1/T_2 - 1/T_2(ASP) = P/T_2(ASP\cdot HSA) \quad (5)$$

$$1/T_1 - 1/T_1(ASP) = P/T_1(ASP\cdot HSA) \quad (6)$$

with

$$1/T_2(ASP\cdot HSA) > 1/T_1(ASP\cdot HSA) \quad (7)$$

where τ is the lifetime of the ASP·HSA complex ($1/\tau = k_{-1}$), $T_1(ASA)$ and $T_2(ASA)$ are the relaxation times of the acetyl protons of free ASA, $T_1(ASA\cdot HSA)$ and $T_2(ASA\cdot HSA)$ are the relaxation times of the acetyl protons of ASA bound to HSA, and Δ is the chemical shift of the acetyl protons of ASA bound to HSA.

The situation $1/T_2(ASA\cdot HSA) > 1/T_1(ASA\cdot HSA)$ could result if the relaxation of the bound ASA is dominated by an interaction with a long correlation time (25). This means that $(\omega_o\tau_c)^2 >> 1$, where τ_c is the correlation time of the relaxation mechanism for the bound ASA and ω_o is the resonance frequency. Under these conditions (25, 26, 27)

$$1/T_2(ASA\cdot HSA) = 9/10(h^2\gamma^4/r_o^6)\tau_c \quad (8)$$

$$1/T_1(ASA\cdot HSA) = 6/5(h^2\gamma^4/r_o^6)\tau_c/(\omega_o\tau_c)^2 \quad (9)$$

where γ is the gyromagnetic ratio for protons and r_o is the internuclear distance between the protons. While $1/T_1(ASA\cdot HSA)$ is still a function of resonance frequency, the magnitude of $P/T_1(ASA\cdot HSA)$ is generally so small that the frequency dependence of the observed relaxation time $1/T_1$ (equations 3 and 6) is unobservable within experimental error. $1/T_1(ASA)$ and $1/T_2(ASA)$ are equal and frequency independent.

The slow and fast exchange limits are distinguished by their temperature dependences; in case (1)

$$1/T_2 - 1/T_1 = p(1/\tau - 1/T_1(ASA\cdot HSA)) \simeq Pk_{-1} \quad (10)$$

which should increase with increasing temperature; in case (2)

$$1/T_2 - 1/T_1 = P(1/T_2(ASA.HSA) - 1/T_1(ASA\cdot HSA)) \simeq P/T_2(ASA\cdot HSA)\alpha\tau_c \quad (11)$$

which should decrease with increasing temperature. If the relaxation is dominated by intramolecular dipole-dipole interactions (28), τ_c will be the rotational correlation time of the ASA·HSA complex. Under the $[HSA]_o/[ASA]_o$ experimental conditions used $P = [HSA]_o/[ASA]_o$ and is temperature independent.

Experimentally, the broadening $1/T_2 - 1/T_1$ decreases with increasing temperature (see Fig. 3) indicating that the ASA exchanges rapidly with a site on HSA but when bound, the relaxation of the acetyl protons of ASA is dominated by an interaction with a long correlation time. Assuming that the relaxation is dominated by intramolecular dipole-dipole interactions between the three protons of the acetyl group (28), that the rotational motion of the macromecule is isotropic (29), that no internal rotations are present (30), a value for τ_c can be calculated from equations 8 and 11.

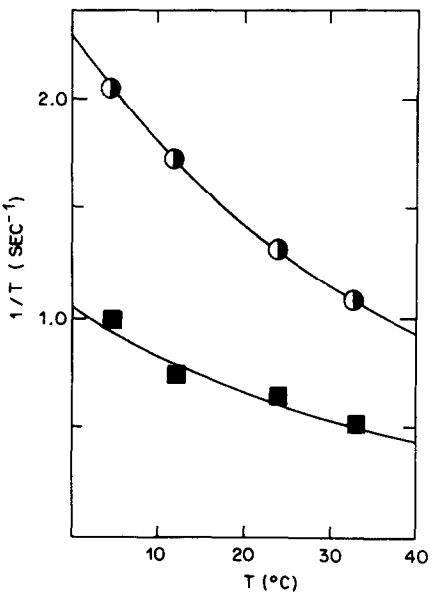

Fig. 3: Relaxation times for acetyl protons of acetylsalicylic acid in the presence of human serum albumin as a function of temperature at 100 MHz;

$1/T_2$ ● ; $1/T_1$ ■; $[HSA]_o/[ASA]_o = (2.86 \pm 0.11) \times 10^{-3}$.

The calculated value of τ_c is 1.0×10^{-8} secs. The assumption of no internal rotation is most probably in error, since the rotation of the acetyl protons of ASA around their three-fold axis would be expected to be much faster than the rotation of the ASA·HSA complex. The calculated τ_c is therefore too short by a factor of $(3 \cos^2\beta - 1/2)$, where β is the angle between the three-fold axis and the internuclear vector (30). Hence

$\tau_c = 4 \times 10^{-8}$ secs (β=90°). This value of τ_c is comparable with the value of τ_c calculated for the aspartate transcarbamylase-succinate complex (M.W. = 100,000) by NMR methods ($\tau_c = 1.1 \times 10^{-8}$ secs) (22), τ_c for aspartate aminotransferase (M.W. = 90,000) determined by flourescence depolarization methods ($\tau_c = 4.3 \times 10^{-8}$ secs) (22, 31)[2], and τ_c for bovine serum albumin ($2 - 20 \times 10^{-8}$ secs) (30). The ASA is therefore bound tightly to HSA so that it experiences a rotational correlation time of the magnitude of that expected for the HSA molecule.

The value of $1/T_1$(ASA·HSA) may be calculated from $(\omega_o \tau_c)^2 = 39$ $\omega_o = (2\pi) \times 10^8$ radians sec^{-1} and equation 9, yielding $1/T_1$(ASA·HSA) = 6 sec^{-1}. Hence, for $P = 1.6 \times 10^{-2}$, P/T_1(ASA·HSA) = 0.1 and no frequency dependence of $1/T_1$ will be observed. The slope of $1/T_1$ with [HSA] may be determined in part by the increasing viscosity of the solution.

The other possible choice for the dominant relaxation mechanism is scalar and dipolar interactions with bound, non-dialyzable paramagnetic metal ions with long electron spin relaxation times (32, 33). While no bound metal ions are thought to be present[1] and the smallness of the bound chemical shift argues against contact with paramagnetic metals as well as the fact that most electron spin relaxation times are shorter than $2 - 20 \times 10^{-8}$ secs (32), this possibility is being investigated further.

A limit on the value of k_{-1} may be determined from the condition for the fast exchange limit, τ/T_2(ASA·HSA)$\ll 1$. T_2(ASA·HSA) = 5×10^{-3} sec^{-1} implies $k_{-1} \geq 10^{-3}$ sec^{-1}. Combining this with $K_D(max) \simeq 10^{-3}$M yields k_1 (min) $\simeq 10^6 M^{-1}$ sec^{-1}. Taking k_1(max) as the diffusion controlled limit yields 10^6 $M^{-1}sec^{-1} \leq k_1 \leq 10^9 M^{-1}$ sec^{-1}.

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REFERENCES

1) I. M. Klotz and F. M. Walker, J. Am. Chem. Soc. 70, 943 (1948).
2) A. Lindenbaum and J. Schubert, J. Phys. Chem. 60, 1663 (1956).
3) C. Davison and P. K. Smith, J. Pharmacol. Exp. Ther. 133, 161 (1961).
4) C. J. Moran and W. H. C. Walker, Biochem. Pharmacol. 17, 153 (1968).
5) J. Rieder, Arzneimettel-Forsch 13, 81 (1963).
6) I. Moriguchi, S. Wada, and T. Nishizawa, Chem. Phar. Bull. (Tokyo) 16, 601 (1968).
7) J. N. McArthur and M. J. H. Smith, FEBS Lett. 1, 332 (1968).
8) O. Jardetzky, Adv. Chem. Phys. 7, 499 (1964).
9) J. J. Fischer and O. Jardetzky, J. Am. Chem. Soc. 87, 3237 (1965).

2. Note difference in factor of three between correlation times for NMR and fluorescence depolarization experiments (22, 31).

10) O. Jardetzky and N. G. Wade-Jardetzky, Mol. Pharmacol. 1, 214 (1965).
11) D. M. Woodbury, Pharmacological Basis of Therapeutics, ed. L.S. Goodman and A. Gilman, p. 312-330, 3rd Edition, MacMillan Co., N.Y. 1965.
12) Salicylates; and international symposium. Ed. A. Dixon, B. K. Martin, M. J. H. Smith, and P. H. N. Wood; J. A. Churchill, Ltd., London, 1963.
13) D. Hawkins, R. N. Pinckard, I. P. Crawford, and R. S. Farr, J. Clin. Invest. 48, 536 (1969).
14) D. Hawkins, R. N. Pinckard, and R. S. Farr, Science 160, 780 (1968).
15) R. N. Pinckard, D. Hawkins, and R. S. Farr, Nature 219, 68 (1968).
16) T. J. Swift and R. E. Connick, J. Chem. Phys. 37, 307 (1962).
17) B. Sarkar and Y. Wigfield, Can. J. Biochem. 46, 601 (1968).
18) B. D. Sykes, J. Am. Chem. Soc. 91, 949 (1969).
19) B. D. Sykes and J. M. Wright, Rev. Sci. Instr. 00, 000 (1970).
20) A. L. Van Geet and D. N. Hume, Anal. Chem. 37, 983 (1965).
21) P. G. Schmidt, G. R. Stark, and J. D. Baldeschwieler, J. Biol. Chem. 244, 1869 (1969).
22) B. D. Sykes, P. G. Schmidt, and G. R. Stark, J. Biol. Chem. 000, 0000 (1970).
23) D. E. O'Reilly and C. P. Poole, Jr., J. Phys. Chem. 67, 1762 (1963).
24) Z. Luz and S. Meiboom, J. Chem. Phys. 40, 2686 (1964).
25) A. Abragam, Principles of Nuclear Magnetism. Oxford University Press, London, 1961.
26) D. E. Woessner, J. Chem. Phys. 36, 1 (1962).
27) M. Bloom, M. Lipsicas and B. H. Muller, Can. J. Phys. 39, 1093 (1961).
28) A. S. V. Burgen, O. Jardetzky, J. C. Metcalf, and N. G. Wade-Jardetzky, Proc. Natl. Acad. Sci. 58, 447 (1967).
29) J. L. Oncley, J. Phys. Chem. 44, 1103 (1940).
30) D. Wallach, J. Chem. Phys. 47, 5258 (1967).
31) G. E. Churchich, Biochem. Biophys. Acta. 147, 511 (1967).
32) M. Cohn, Biochem. 2, 623 (1963).
33) N. Bloembergen and L. O. Morgan, J. Chem. Phys. 34, 842 (1961).