[Chem. Pharm. Bull. 22(2) 385—389 (1974)]

UDC 547.475.2.04 : 577.322.23

Drug Interactions. I. Binding of Ascorbic Acid and Fatty Acid Ascorbyl Esters to Bovine Serum Albumin[1]

Tyotaro Tukamoto, Shoji Ozeki, Fujio Hattori, and Takashi Ishida

Faculty of Pharmaceutical Sciences, Nagoya City University[2]

(Received June 11, 1973)

The interaction of ascorbic acid and some monofatty acid ascorbyl esters to bovine serum albumin was investigated by a dynamic dialysis method in an attempt to elucidate some of the structural specificities of the interaction. The chemicals used were ascorbic acid, ascorbyl octanoate, ascorbyl decanoate, and ascorbyl benzoate. Analysis of the binding data showed that the albumin possesses a single binding site for these chemicals, and that there are marked differences in their binding strength, which increases in order of ascorbic acid, benzoate, octanoate, and decanoate. The affinity of binding was also found to depend on the hydrophobic character of their acid residues, as expressed by a partition coefficient. The thermodynamic parameters indicate that these interactions are exothermic and occur spontaneously under the experimental conditions used. In addition electrostatic attraction, the interaction of hydrophobic groups between the protein and ascorbates was shown to be involved in the formation of complexes. Finally, the role of the hydrophobic interaction was indicated from the consideration of values for the positive change of entropy.

Many therapeutically useful drugs are bound to plasma protein and this binding may profoundly affect their properties.[3] The presence of bound ascorbic acid in blood and tissues has previously been demonstrated by several investigators.[4] Electrophoreotic studies have shown that a combination occurs between bovine serum albumin (BSA) and ascorbic acid.[5] Other indirect evidences consist of observations that the stability of ascorbic acid in aqueous solution was much increased by the presence of proteins.[6] However, the report on the direct evidence for the binding of ascorbic acid to BSA has not yet appeared. In the present series of work, we made comparative and quantitative studies on the binding of ascorbic acid and fatty acid ascorbyl esters with serum albumin by the use of dynamic dialysis, and were able to clarify the relationship between the binding force and chemical structure.

Experimental

Materials——Bovine serum albumin, Fraction V (Armour Co.), was used in this study, and its molecular weight was assumed as 69000. The ascorbyl octanoate (6-O-octanoyl-L-ascorbic acid), ascorbyl decanoate (6-O-decanoyl-L-ascorbic acid), and ascorbyl benzoate (6-O-benzoyl-L-ascorbic acid), were synthesized accord-

1) Presented at the 93rd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1973.
2) Location: *Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan.*
3) A. Goldstein, *Pharmacol. Rev.*, **1**, 102 (1949).
4) M.M. Eidelman and F.Y. Godon, *Biokhimiya*, **14**, 58 (1949) [*Chem. Abstr.*, **43**, 5072 (1949)]; W.N. Sumerwell and R.R. Sealock, *J. Biol. Chem.*, **196**, 753 (1952); W.H. Hastings and C.F. Spencer, *J. Marine Res.*, **11**, 241 (1952) [*Chem. Abstr.*, **47**, 2898 (1953)]; W.R. Lewis, *Arch. Biochem. Biophys.*, **89**, 21 (1960); M.C. Malakar and B.C. Guha, *Naturwissenschaften*, **48**, 645 (1961); M.C. Malakar, *Nature*, **198**, 185 (1963); J.H. Rose and S.B. Itscorte, *Proc. Soc. Exp. Biol. Med.*, **113**, 648 (1963) [*Chem. Abstr.*, **59**, 14373 (1963)]; M.C. Malakar, *Naturwissenschaften*, **51**, 88 (1964); A. Tohtz and A. Kemper, *Acta Biol. Med. Ger.*, **12**, 365 (1964) [*Chem. Abstr.*, **61**, 3465 (1964)]; M.C. Malakar, *Sci. Cult.* (Calcutta), **29**, 109 (1965) [*Chem. Abstr.*, **64**, 10148 (1966)]; R. Fiddick and H. Heath, *Biochim. Biophys. Acta*, **136**, 206 (1967).
5) F.W. Putnam, in "The Proteins," 2nd Ed., ed. by H. Neurath, Vol. III, Chapt. 14, Academic Press, Inc., New York, 1965, p. 198.
6) T. Tukamoto and S. Ozeki, unpublished data.

Table I. Melting Points and Elementary Analyses of Fatty Acid Ascorbyl Ester

Chemical	mp (°C)	Analysis (%) Calcd. C	Calcd. H	Found C	Found H
Ascorbyl octanoate	73— 75	55.62	7.34	55.21	7.80
Ascorbyl decanoate	99—100	58.17	7.93	57.78	8.24
Ascorbyl benzoate	183—184	55.72	4.32	55.39	4.78

ing to the procedures reported by Swern, *et al.*[7] Melting points[8] and results of elementary analyses of these compounds are shown in Table I. Ascorbic acid and all other chemicals of analytical grade were obtained from commercial sources, and used without further purification. The bags for dynamic dialysis were prepared from 20/32 Cellophane tubing obtained from the Visking Co. All tubings were heated (90°, 1 hr) in distilled water, rinsed with distilled water, and soaked in an appropriate buffer until use. The same phosphate buffer (pH 7.45 and ionic strength 0.16) was used throughout this study.

Dialysis Procedure——Binding of ascorbic acid and ascorbyl esters with serum albumin was studied by dynamic dialysis at 5° and 20°. The apparatus used by Meyer and Guttman[9] for studying the protein binding of small molecules, which is based on the determination of the rate of dialysis of a small molecule from a protein-containing compartment, was modified slightly in this experiment as follows: The dynamic dialysis was carried out in a nitrogen atmosphere to prevent oxidative degradation of ascorbic acid and ascorbyl esters. The apparatus consists of two parts; one is a wide-mouthed bottle (about 250 ml capacity) in combination with a thermostatic water bath and a water circulation, which provides excellent temperature stability, and the other is a four-holed Teflon stopper which was made to fit the bottle. The four-holed stopper is provide with four glass tubings (T_1, T_2, T_3, and T_4); T_1 is an inlet tubing (i.d. 0.2 cm) which extends as far as possible into the bottle, and oxygen-free nitrogen is introduced through it at the rate of 60 ml/min. T_2 is an outlet tubing (i. d. 0.2 cm) and T_3 is a samplingvent (i. d. 1.0 cm). The upper end of T_4 (i. d. 1.0 cm) is provided with a two-holed Teflon stopper bearing an inlet tubing (i. d. 0.2 cm), which is supported at a sufficient height to give a clearance of 1 cm above the surface of the water, and an outlet tubing (i. d. 0.2 cm). Nitrogen gas is also introduced at the rate of 20 ml/min through the inlet tubing fitted to T_4. Hydrated Cellophane dialysis tubing was knotted at one end to form a bag (length, 7 cm) and was attached with a rubber band to the lower end of T_4. Into this bag, 7 ml of the chemical or the chemical-protein solution were placed. The bag was immersed in 200 ml of the buffer solution. All the experiments were conducted at constant temperature, pH, liquid volume, bag size, stirring rate, and nitrogen flow rate. The concentration of the chemicals used was 1×10^{-2}M, and that of BSA, 2×10^{-4}M. The dynamic dialysis procedure was carried out by withdrawing a 100 ml sample from the external solution every 30 min and replenishing by the same volume of fresh buffer saturated with oxygen-free nitrogen gas. Each sample withdrawn was analysed by spectrophotometry, and the total amount of the chemical dialysed at any sampling time was determined. From the difference between this value and the total chemical concentration before sampling, the total concentration (Dt) remaining in the dialysis bag was calculated.

Partition Coefficient——Partition coefficient (P) was determined using isoamyl alcohol and appropriate phosphate buffer as the solvent pair. For the partitioning, isoamyl alcohol saturated with buffer solution and buffer solution saturated with isoamyl alcohol were used. A small amount (0.1%) of $Na_2S_2O_3$ was added to the buffer solution to avoid the oxidation of ascorbic acid and ascorbyl esters.

Result and Discussion

Dynamic Dialysis

The results of the dynamic dialysis experiment are shown in Fig. 1. The dialysis run in the absence of BSA showed that the diffusions were first-order with respect to all the chemicals, and that adsorption by the dialysis bag was negligible.[10] Thus it may be concluded that the dialytic behavior obeyed the expected rate law[9]:

7) D. Swern, A.J. Stirton, J. Turer, and P.A. Wells, *Oil & Soap* (Chicago), **20**, 224 (1943) [*Chem. Abstr.*, **38**, 501 (1944)].
8) Melting points are uncorrected.
9) M.C. Meyer and D. Guttman, *J. Pharm. Sci.*, **57**, 1627 (1968).
10) M.C. Meyer and D. Guttman, *J. Pharm. Sci.*, **59**, 33 (1970).

$$\frac{-\mathrm{d}(Dt)}{\mathrm{d}T} = K(Df) \qquad (1)$$

where $-\mathrm{d}(Dt)/\mathrm{d}T$ is the rate of loss of the chemical molecule from the dialysis bag, K is the first-order rate constant which characterizes the diffusion process and which incorporates the area and thickness of the membrane, and Df is the concentration of unbound chemical molecule in the dialysis bag. It is apparent from this graph that the rate of dialysis of the chemical was markedly decreased in the presence of BSA. The rate decreasing effect of BSA shows that complex formation occurs reversibly between the chemical and BSA in the BSA compartment. The rate of the chemical outflowing from the compartment is directly proportional to Df. From the instantaneous rates and from the first-order rate constant obtained from the dialysis of the chemical in the absence of BSA, the concentration of Df, and hence of Db, was calculated from Eq. 1 for various periods. The number of moles of the chemical bound per mole of BSA ($\bar{\nu}$) was then calculated for these periods. Data of typical dynamic dialysis experiments for ascorbyl decanoate and BSA at 5° in phosphate buffer of pH 7.45 are shown in Table II.

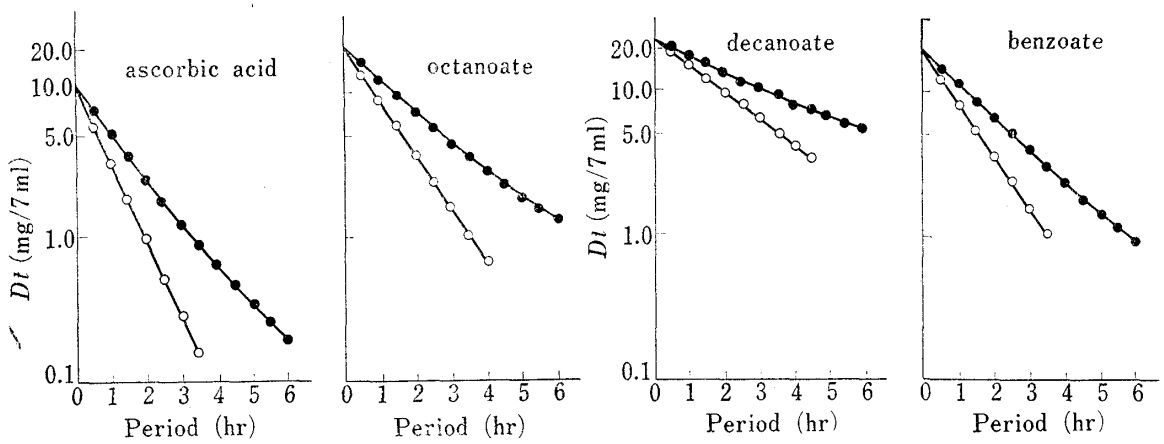

Fig. 1. Dynamic Dialysis Experiments of Ascorbic acid and Fatty Acid ascorbyl Esters to BSA at pH 7.45 and 5°

—○—: absence of BSA, —●—: presence of BSA

TABLE II. A Typical Dynamic Dialysis Experiment of Ascorbyl Decanoate and BSA at 5° in Phosphate Buffer (pH 7.45)

Total chemical concn. (mg)	Free chemical concn. (mg)	Bound chemical concn. (mg)	$\bar{\nu}$	$\bar{\nu}/Df\times10^{-3}$
18.2	10.90	7.30	15.75	3.34
14.2	8.08	6.12	13.22	3.78
10.9	5.90	5.00	10.80	4.23
8.52	4.46	4.05	8.75	4.53
6.62	3.36	3.25	7.03	4.83
5.16	2.54	2.62	5.65	5.14

BSA concentration $=2\times10^{-4}$M

The binding data obtained were used to construct the Scatchard plot of Fig. 2. Here the abscissa is $\bar{\nu}$ and the ordinate $\bar{\nu}/Df$, the concentrations of Df and Db being transformed into molar units. With a single species of binding site, the Scatchard plot gives a linear relationship between $\bar{\nu}$ and $\bar{\nu}/Df$ with a slope of $-k$ (k=association constant) and intercept on the $\bar{\nu}$ axis equals n, the number of binding sites per molecule of BSA. The present plots for ascorbyl decanoate at 5° and 20° indicate that there is only one type of binding site.

All other chemicals investigated also followed this straight line relationship. The binding parameters are summarized in Table III.

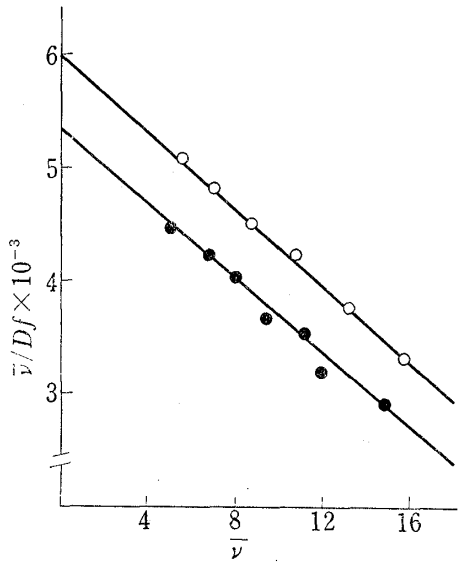

Fig. 2. Binding of Ascorbyl Decanoate to BSA

—○—: at 5°, —●—: at 20°

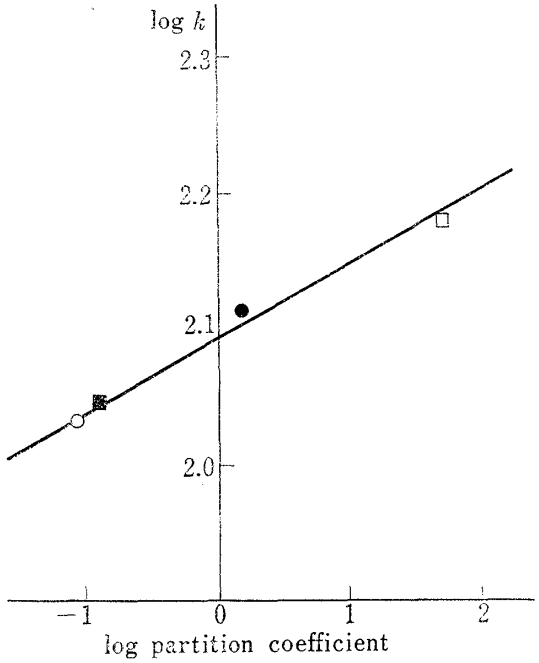

Fig. 3. Correlation of Binding Constant with Partition Coefficient

—○—: ascorbic acid, —□—: ascorbyl decanoate,
—●—: ascorbyl octanoate, —■—: ascorbyl benzoate

Table III. Summary of Binding Constants

Chemical	Temp. (°C)	n	$kn\times10^{-3}$	$k\times10^{-2}$
Ascorbic acid	5	34	3.87	1.14
	20	34	3.67	1.08
Ascorbyl octanoate	5	35	5.07	1.45
	20	35	4.65	1.33
Ascorbyl decanoate	5	34	5.95	1.75
	20	35	5.32	1.52
Ascorbyl benzoate	5	35	4.16	1.19
	20	35	3.88	1.11

These results indicate that there are marked differences in binding strength, which increased in the order ascorbic acid, benzoate, octanoate, and decanoate. The difference in the affinity can be attributed to the hydrophobic region in the molecule. The k values[11] (2.4×10^3 for heptanoic acid, 1.2×10^3 for octanoic acid, 6×10^4 for decanoic acid, 2.3×10^5 for dodecanoic acid, and 1.5×10^4 for benzoic acid) for the strong binding site in free fatty acids or benzoic acid are much greater than those of the ascorbyl esters investigated. The differences in k values of free organic acids do not depend on acid strength, which differs but slightly among organic acids. The longer the hydrophobic carbon chain of organic acids are, the more favorably they interact with BSA, and the range of this increase is very large. From these considerations, it is likely that the hydrophobic region in the ascorbate molecule

11) J.D. Teresi and J.M. Luck, *J. Biol. Chem.*, **194**, 823 (1952); C. Davison and P.K. Smith, *J. Pharm. Exp. Thrap.*, **133**, 161 (1961); J. Reynolds, S. Herbert, and J. Steinhardt, *Biochemistry*, **7**, 1357 (1968).

lies away from the flat portion of the receptor surface in contrast with organic acids, contributing to the van der Waals attractions of the overall binding. At the pH used (7.45), the hydroxyl group at 3-position of the ascorbic acid moiety is 99.9% ionized and it is therefore assumed that one important element of the ascorbic acid-BSA interaction may be an ionic bond to the cationic site on the receptor surface as has previously been postulated for large organic anions.[1,12] The carbonyl oxygen atom of the ascorbic acid moiety might well participate in hydrogen bond formation with an appropriate receptor group (*e.g.*, -NH of a peptide bond), thus further stabilizing the interaction. A good rectilinear correlation between log k and log P (isoamyl alcohol/buffer solution) is shown in Fig. 3 which was illustrated by the method of least squares. Increase in the hydrophobic chain of the acid residues is seen to increase both the oil/water partitioning and the binding affinity. This leads to the conclusion that the hydrophobic region helps to stabilize the chemical-BSA complex through the hydrophobic bonding.

Thermodynamic Result

It is possible to estimate the standard enthalpy change, $\Delta H°$, for the binding from Eq. 2:

$$\log \frac{k \text{ at } 5°}{k \text{ at } 20°} = \frac{-\Delta H°}{2.303\boldsymbol{R}}\left(\frac{1}{278}-\frac{1}{293}\right) \tag{2}$$

The standard free energy change, $\Delta G°$, for complex formation is estimated from

$$\Delta G° = -\boldsymbol{R}T \ln k \tag{3}$$

and the entropy change, $\Delta S°$, is obtained by substituting $\Delta H°$ and $\Delta G°$ into the Gibbs-Helmholtz equation:

$$\left(\frac{\partial \Delta G°}{\partial T}\right)_p = -\Delta S° \frac{\Delta G° - \Delta H°}{T} \tag{4}$$

Table IV. Thermodynamic Parameters of Binding

Chemical	ΔH (kcal/M)	ΔG at 20° (kcal/M)	ΔS (e.u./M)
Ascorbic acid	−0.584	−2.724	+7.28
Ascorbyl octanoate	−0.931	−2.846	+6.53
Ascorbyl decanoate	−1.521	−2.914	+4.77
Ascorbyl benzoate	−0.750	−2.740	+6.79

Thermodynamic parameters for the binding reaction are given in Table IV. The decrease in binding strength of BSA for ascorbates with increasing temperatures is characteristic of an exothermic reaction and has been reported for many protein interactions.[13] The negative sign for $\Delta G°$ means that the binding process is spontaneous. All the $\Delta S°$ on binding are positive. This would appear anomalous, since the association of the chemical and protein should result in an increased orderliness of the system, and a number of other factors must be considered. The exothermic nature of the binding, due to increase in the number of ionic bonds and hydrogen bonds accompanying them is believed to exceed the endothermic melting of the so-called icebergs,[14] resulting in net negative $\Delta H°$. The melting of the iceberg around ascorbate and BSA molecules will result in an increase in randomness and the disordering effect probably exceeds the ordering effects, resulting in the net possitive $\Delta S°$.

12) M.C. Meyer and D. Guttman, *J. Pharm. Sci.*, **57**, 895 (1968).
13) R.A. O'Reilly and P.E. Kowitz, *Clin. Invest.*, **46**, 829 (1967).
14) H.S. Frank and M.W. Evans, *J. Chem. Phys.*, **13**, 507 (1945).