Fluorocarbon Aerosol Propellants V: Binding Interaction with Human Albumin

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Abstract □ Binding of trichloromonofluoromethane, dichlorodifluoromethane, and dichlorotetrafluoroethane was studied in aqueous 5% human albumin solution, using the partition coefficient method in sealed serum bottles. The partition coefficient and the fraction of fluorocarbons bound were highly dependent on fluorocarbon concentrations. The average binding sites per molecule of albumin were 2.17, 0.30, and 0.42 and the binding association constants were 1.11×10^3, 1.73×10^3, and $5.06 \times 10^3\ M^{-1}$, respectively. At the lowest concentration studied, 62.3, 25.5, and 65.6% were found bound to albumin, respectively. This appears to represent the first extensive study on any gas–albumin interaction.

Keyphrases □ Fluorocarbon propellants—binding to human serum albumin determined using partition coefficient method in sealed serum bottles □ Trichloromonofluoromethane—binding to human serum albumin □ Dichlorodifluoromethane—binding to human serum albumin □ Dichlorotetrafluoroethane—binding to human serum albumin □ Binding of fluorocarbon propellants to human serum albumin—determined using partition coefficient method in sealed serum bottles □ Interactions, gas–albumin—binding of fluorocarbon propellants to human serum albumin

Fluorocarbons such as trichloromonofluoromethane (bp 23.7°), dichlorodifluoromethane (bp −29.8°), and dichlorotetrafluoroethane (bp 4.1°) have been widely used as aerosol propellants in various pressurized aerosol packages. In 1973, an estimated 3 billion units were manufactured in this country (1). The possible acute and chronic toxicities of these previously regarded "inert" propellants have been the subjects of intensive studies in recent years (1–6).

Based on the partition coefficient study, it was shown previously that these volatile or gaseous fluorocarbons were much more soluble in human plasma than in water or normal saline (7). It was postulated that such a solubility enhancement might be partly due to the binding of the fluorocarbons to plasma proteins. This postulation was subsequently confirmed by the fluorocarbon–human albumin binding study with one single concentration, using the partition coefficient method (8). In that study, a surprisingly high degree of binding of the three fluorocarbons to the purified human and bovine albumins was found. Since the possible effect of concentration variation on the extent of protein binding for numerous nonvolatile compounds has been well established, it was decided to extend the investigation by using a wide range of fluorocarbon concentrations. This approach also allows the number of the binding sites per molecule of protein and the protein-binding association constant to be calculated.

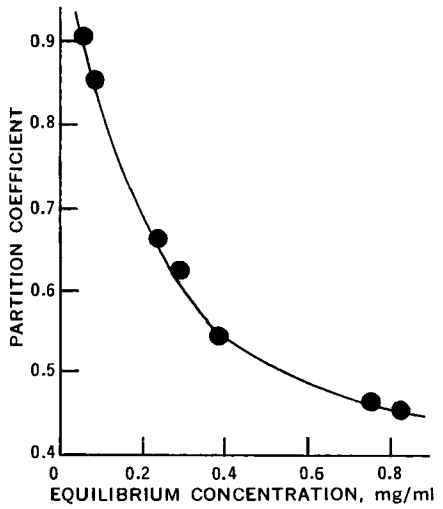

Figure 1—*Effect of concentration of trichloromonofluoromethane (both bound and unbound form) on its partition coefficient between 5% human albumin solution and air.*

EXPERIMENTAL

Materials—Only the three most widely used fluorocarbon propellants, trichloromonofluoromethane[1] (mol. wt. 137.4), dichlorodifluoromethane[1] (mol. wt. 120.9), and dichlorotetrafluoroethane[1] (mol. wt. 170.9), were studied. The crystallized and lyophilized human albumin (mol. wt. 69,000) was purchased commercially[2].

Preparation of Fluorocarbon–Albumin Solutions—The albumin solution (5% w/v) was freshly made by dissolving albumin into isotonic pH 7.4 phosphate buffer solution, prepared according to the method of Sorensen (9). One milliliter of this albumin solution was then pipetted into a 5-ml serum bottle[3], which was then sealed with a flange-type lacquer-coated stopper and aluminum cap (7). In the previous partition coefficient study (7, 8), the fluorocarbons were first dissolved in normal saline prior to introduction into plasma or protein samples. Due to their limited solubilities in normal saline or phosphate buffer and the high concentrations of the fluorocarbons used in the present study, their gaseous stock preparations were prepared by diluting the pure fluorocarbons from their liquid state (dichlorodifluoromethane and dichlorotetrafluoroethane were stored in pressurized steel cylinders at room temperature and trichloromonofluoromethane was stored in an aluminum container at 5°) into the sealed 50-ml empty serum bottles.

The albumin solutions, containing various amounts of the fluorocarbon, were prepared by transferring approximately 0.05–1 ml from the fluorocarbon stock preparations, using a gastight syringe[4], to the sealed 5-ml serum bottle containing 1 ml of the albumin solution. A 27-gauge disposable hypodermic needle was inserted briefly into the headspace of the serum bottle to reduce the pressure in the headspace to the atmospheric pressure. The three fluorocarbons were studied individually.

Determination of Partition Coefficients between Protein Solution and Air Phase—The fluorocarbon–albumin solution in the sealed serum bottle was shaken in a vortex mixer[5] for 30 min and allowed to stand at room temperature (25 ± 1°) for another 30 min. The preliminary study showed that the equilibration of the fluorocarbons between the protein solution and headspace was

[1] Supplied by E. I. du Pont de Nemours and Co., Wilmington, Del.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Wheaton Scientific, Millville, N.J.
[4] Hamilton.
[5] Vortex mixer, Fisher Scientific Co., Springfield, Mass.

reached under these conditions since the partition coefficient remained the same even after 1 hr of shaking. A much shorter period (less than 5 min) was required to obtain an equilibrium by the same method of shaking when the fluorocarbon dissolved in normal saline was first introduced into the protein solution as used previously (7, 8).

The concentration of the fluorocarbon in the headspace was analyzed directly by injecting an appropriate volume of the sample into the GC column using a 50- or 100-μl gastight syringe[4]. The GC operation conditions were identical to those reported previously (8, 10, 11). A proper dilution of the sample, using the sealed empty serum bottle, was made when the concentration of the fluorocarbon in the headspace was too high and the amount injected would have exceeded the linear response range of the electron-capture detector (10).

After determination of the fluorocarbon in the headspace, its concentration in the albumin solution was analyzed by transferring about 0.2 ml of the solution to a 3- or 5-ml vacuum tube[6] containing a known amount of cyclohexane. The tube was shaken in a shaker[7] for 5 min followed by centrifugation for 5 min in a clinical centrifuge[8]. The fluorocarbon concentration in the cyclohexane phase was determined by drawing 5–10 μl of the solution, using a 10-μl syringe[4], and injecting onto the GC column.

During the transferring of the fluorocarbon–albumin solution to the vacuum tube, the following special procedure was used to avoid loss of the fluorocarbon when the conventional pipet or syringe was employed. A 6.3-cm (2.5-in.) 22-gauge disposable hypodermic needle was cut to remove the hub and sharpened with a file. One end of the needle was inserted into the albumin solution of the inverted serum bottle, and the other end was then immediately inserted into the vacuum tube. When the liquid stopped flowing into the tube, another 23-gauge disposable needle was inserted into the headspace of the serum bottle to equilibrate the whole system to atmospheric pressure. The volume of the liquid transferred was measured by weighing after correction for its density. The exact volume (about 2.8 ml for a 3-ml tube and 4.8 ml for a 5-ml tube) of cyclohexane used in the extraction was also measured by weighing after correction for its density.

The extraction recovery of the fluorocarbons into the cyclohexane was essentially 100%. This recovery can be expected because of their high solubility in this solvent (7) and the relatively smaller volumes of the protein solution and headspace used. Throughout the present study, duplicate and excellent reproducible readings (less than 3% of variation) were obtained for each GC assay.

The partition coefficient, P_p, between the protein solution and the air phase was calculated according to the following equation:

$$P_p = \frac{C_p}{C_g} = \frac{C_c V_c}{C_g V_p} \qquad \text{(Eq. 1)}$$

where:

C_p = concentration of fluorocarbon in protein solution at the equilibrium state (weight per unit of volume)
C_g = concentration of fluorocarbon in air phase (*i.e.*, headspace) at the equilibrium state (weight per unit of volume)
C_c = concentration of fluorocarbon in cyclohexane extract
V_c = volume of cyclohexane used for extraction
V_p = volume of protein solution transferred for extraction

RESULTS AND DISCUSSION

Partition Coefficient Study—The partition coefficients of the three fluorocarbons between the 5% human albumin solution and the air phase were found to change with the total fluorocarbon concentration in the albumin solution at the equilibrium state. This finding is clearly demonstrated by a typical plot of the partition coefficient of trichloromonofluoromethane as a function of its total concentration in protein solution (Fig. 1). The partition coefficient decreased markedly and approached the value between the plain pH 7.4 phosphate buffer without protein and the air phase, P_f (8, 12). The P_f values for the three fluorocarbons (0.347 for trichloromonofluoromethane, 0.105 for dichlorodifluoromethane, and 0.032 for dichlorotetrafluoroethane) were independent of their total concentrations in the protein solution. These values are slightly higher than those previously reported for both water and normal saline systems (7). The discrepancies were caused by the depressing effect on the sensitivity of the electron-capture detector in the presence of water when the aqueous sample was injected directly onto the GC column (12) and by the inherent lack of a high degree of accuracy in the low partition media (such as partition coefficients less than 0.2) when the simple headspace method (7) was employed. This latter aspect will be dealt with in a future report.

Table I—Number of Fluorocarbon Binding Sites per Molecule of Human Albumin, n, and of the Binding Association Constant, K

Fluorocarbons	n	K, M^{-1}
Trichloromonofluoromethane	2.18	1.11×10^3
Dichlorodifluoromethane	0.30	1.73×10^3
Dichlorotetrafluoroethane	0.42	5.06×10^3

The concentration-dependent partition coefficient phenomenon could be rationalized by the classical drug–protein interaction. As the fluorocarbon concentrations increase, the protein binding sites become more saturated; therefore, the solubilities or partition coefficients tend to decrease. A detailed analysis of the binding properties will be discussed later. There appears to be some discrepancies between this study and the results published previously (7) that showed a concentration-independent partition coefficient phenomenon in the human blood and plasma systems. This previous finding could be attributed to the much lower concentrations of fluorocarbons used in that study. As will be shown later, the partition coefficients in the human albumin solution actually change only slightly in these lower concentration ranges.

Principles of Partition Coefficient Method to Study Protein Binding—Most reported protein binding studies were primarily limited to the nonvolatile compounds. The most common methods employed in the binding studies were equilibrium dialysis and ultrafiltration (13). Due to the existence of these fluorocarbons at the gaseous state at 25 or 37°, these conventional methods could not be easily adopted for their binding study. Since one can reasonably assume that only the free or unbound form is in equilibrium with the fluorocarbon in the headspace and that the partition coefficient is the same as that between the plain buffer solution without protein and the air phase, P_f, the data from the parti-

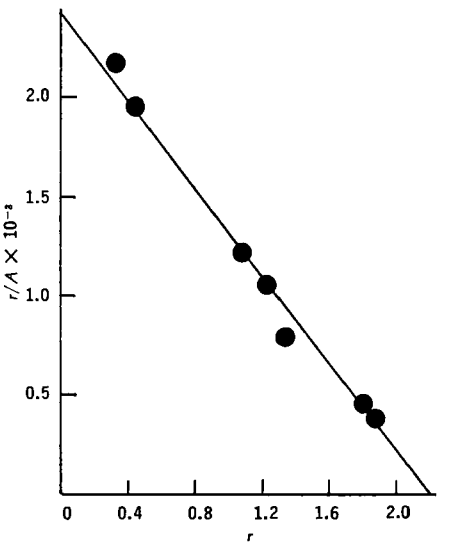

Figure 2—*Scatchard plot for the binding of trichloromonofluoromethane to human albumin.*

[6] Vacutainer, Becton-Dickinson Co., Rutherford, N.J.
[7] Catalog Number 65885, Precision Scientific Co., Chicago, Ill.
[8] Catalog Number 67477, Precision Scientific Co., Chicago, Ill.

tion coefficient study can be used to quantify the percentage of the fluorocarbon bound, the number of binding sites per molecule of protein, and the binding association constant. Mathematically, these values could be derived or determined as follows.

For the determination of the percent bound:

$$\% \text{ bound} = \frac{C_p - C_f}{C_p}(100) \qquad \text{(Eq. 2)}$$

where C_p and C_f are the total concentration (bound and unbound form) and the concentration of the unbound or free form in the protein solution, respectively.

Dividing Eq. 2 by C_g, the concentration in the headspace, one obtains (8):

$$\% \text{ bound} = \frac{C_p/C_g - C_f/C_g}{C_p/C_g}(100) \qquad \text{(Eq. 3)}$$

$$\% \text{ bound} = \frac{P_p - P_f}{P_p}(100) \qquad \text{(Eq. 4)}$$

To determine the number of fluorocarbon molecules bound per molecule of albumin *(r)*:

$$r = \frac{(C_p)(\text{fraction bound})(\text{mol. wt.}_{al})}{C_{al}(\text{mol. wt.}_f)} \qquad \text{(Eq. 5)}$$

where C_p and C_{al} are concentrations of the fluorocarbon and albumin expressed in the same unit, and mol. $wt._f$ and mol. $wt._{al}$ are their molecular weights, respectively.

The number of binding sites, n, and binding association constant, K, can be obtained from the Scatchard plot based on the following relationship (14):

$$\frac{r}{A} = nK - rK \qquad \text{(Eq. 6)}$$

where A is the molar concentration of the free form of the fluorocarbon. The plot of r/A *versus* r yields a straight line. The n value can be determined from the intercept on the abscissa and the nK value from the intercept on the ordinate.

Binding of Fluorocarbons with Human Albumin—Based on the partition coefficient data and Eq. 4, the percentages in the bound form for the three fluorocarbons at various concentrations can be calculated. The percentage bound decreased from 62 to 23, 23 to 8, and 60 to 24 as the total concentration increased from 0.06 to 0.83 mg/ml for trichloromonofluoromethane, from 0.03 to 0.27 mg/ml for dichlorodifluoromethane, and from 0.006 to 0.170 mg/ml for dichlorotetrafluoroethane. These concentration ranges are close to some reported fluorocarbon concentrations encountered in *in vitro* and *in vivo* toxicity studies (1, 15, 16).

A typical Scatchard plot for trichloromonofluoromethane is shown in Fig. 2. The two parameters, n and K, obtained from this and similar plots are summarized in Table I. All plots showed a straight line, which indicates that there are only primary binding sites for all three fluorocarbons. The highest binding constant found for dichlorotetrafluoroethane was not unexpected in view of its higher molecular weight and higher solubility in the lipoid medium (8), which may result in stronger van der Waals interaction and hydrophobic binding. Although the binding constant for dichlorodifluoromethane was slightly higher than for trichloromonofluoromethane, its binding capacity, n, was only about one-seventh of the latter. This finding explains why the percentages bound for dichlorodifluoromethane were much lower compared to trichloromonofluoromethane at similar concentrations.

When lower concentrations (about 130–8000 times lower) were used, the percentages bound were in good agreement with the predicted values, using the parameters listed in Table I. The binding data at these low concentrations are relevant because of the low blood levels that may be encountered clinically (17, 18) or in pharmacokinetic studies (10, 19, 20). The moderate to extensive protein binding found at these lower concentrations could explain in part the higher partition coefficients or solubilities of these fluorocarbons in blood and plasma of humans and other species (7, 20, 23). In light of these results, one can assume that these fluorocarbons may also be bound extensively to other macromolecules in various tissues of the body. This probably contributes partly to the high apparent volume of distribution of dichlorotetrafluoroethane in a dog estimated to be about eight times body weight based on the blood concentration (19). Similar findings for other fluorocarbons were also obtained with other dogs and will be reported.

Trichloromonofluoromethane has been shown *in vitro* to decrease significantly and irreversibly the invertase activity (21), and dichlorodifluoromethane has also been shown to inhibit markedly the *in vitro* o-diphenol oxidase activity (22). Hydrophobic binding and conformational modification of enzyme molecules have been proposed to explain the results, but no binding constants and binding sites were investigated in the two studies.

From a search of literature, it appears that no detailed binding studies on gas–protein interactions have been reported. The partition coefficient method proposed, using a sealed serum bottle and analyzing by the headspace and extraction method, appears to offer a simple and promising tool for studying the binding interactions between macromolecules and volatile or gaseous compounds.

There is some discrepancy in the percentage of dichlorodifluoromethane bound to the human albumin reported from this study and previous preliminary studies (8); the reason is unknown. Since the results from the previously reported concentration could not be reproduced and the present study employed a very wide range of concentrations, it is concluded that the value obtained from the present study is more reliable.

REFERENCES

(1) *Consumer Report*, **May 1974**, 374.

(2) A. Silverglade, *J. Amer. Med. Ass.*, **222**, 827(1972).

(3) W. S. Harris, *ibid.*, **219**, 1508(1973).

(4) W. L. Chiou, *ibid.*, **227**, 658(1974).

(5) D. M. Aviado and M. S. Belej, *Toxicology*, **2**, 31(1974).

(6) T. Balazs, F. L. Earl, G. W. Bierbower, and M. A. Weinberger, *Toxicol. Appl. Pharmacol.*, **26**, 407(1973).

(7) S. Niazi and W. L. Chiou, *J. Pharm. Sci.*, **63**, 532(1974).

(8) W. L. Chiou and J. H. Hsiao, *Res. Commun. Chem. Pathol. Pharmacol.*, **8**, 273(1974).

(9) K. Diem and C. Lentner, "Scientific Tables," 7th ed., Ciba-Geigy Ltd., Basle, Switzerland, 1971, p. 281.

(10) W. L. Chiou and S. Niazi, *Res. Commun. Chem. Pathol. Pharmacol.*, **6**, 481(1973).

(11) W. L. Chiou and J. H. Hsiao, *J. Pharm. Sci.*, **63**, 1614(1974).

(12) J. H. Hsiao and W. L. Chiou, *ibid.*, **63**, 1776(1974).

(13) A. Goldstein, L. Aronow, and S. M. Kalman, "Principles of Drug Action," 2nd ed., Harper & Row, New York, N.Y., 1974, p. 158.

(14) J. Krasner, *Pediat. Clin. N. Amer.*, **19**, 51(1972).

(15) C. F. Reinhardt, A. Azar, M. E. Maxfield, P. F. Smith, and L. S. Mullin, *Arch. Environ. Health*, **22**, 265(1971).

(16) S. M. Kilen and W. S. Harris, *J. Pharmacol. Exp. Ther.*, **183**, 245(1972).

(17) C. T. Dollery, G. H. Draffan, D. S. Davies, F. M. Williams, and M. E. Conolly, *Lancet*, **2**, 1164(1970).

(18) J. W. Paterson, M. F. Sudlow, and S. R. Walker, *ibid.*, **2**, 565(1971).

(19) W. L. Chiou, *Res. Commun. Chem. Pathol. Pharmacol.*, **7**, 679(1974).

(20) S. Niazi and W. L. Chiou, *J. Pharm. Sci.*, **64**, 763(1975).

(21) D. B. Lund, O. Fennema, and W. D. Powril, *Arch. Biochem. Biophys.*, **129**, 181(1969).

(22) H. C. Warmbier, O. Fennema, and E. H. Martin, *J. Food Sci.*, **37**, 702(1972).

(23) S. Niazi and W. L. Chiou, presented to Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences Meeting, Aug. 1974, Chicago, Ill., Abstract No. 14.

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