concentrations of these drugs abolishing the synthetic and the nerve action potential are roughly identical. In both cases, the excitation threshold is raised (see Ref. 11 for this effect in nerves). With nerves, it is well established that the sodium channels, responsible for the rising phase of the action potential, are generally more sensitive than the potassium channels to the presence of local anesthetics (10, 12, 13); with excitable bilayers, the observations presented in Fig. 1 suggest that the analogous channels, the anionic channels, responsible for the rising phase of the action potential also are affected preferentially by the presence of the drugs.

At this stage of the investigation, no attempt was made to define the kind of molecular effect local anesthetics might exert on the anionic channels of bilayers [*i.e.*, these drugs, for example, may act directly on these channels or indirectly through an effect on the lipid matrix (14)]. Rather, the hypothesis that local anesthetics may specifically affect the gating mechanism of excitable membranes in general is emphasized. Procaine does exert some effect on the sodium gating currents of nerves (5), thus affecting their molecular mechanism of gate formation. Another observation consistent with this idea is that tetrodotoxin, which exerts its effects on the "component" of the channels responsible for sodium selectivity of nerves [*i.e.*, this poison selectively blocks the sodium conductance (15) without affecting the sodium gating currents (16)] had no effect on the synthetic action potential of bilayers[8] (where different ion selectivities are involved).

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[8] D. O. Rudin, Eastern Pennsylvania Psychiatric Institute, Philadelphia, PA 19129, personal communication.

Binding of Butylated Hydroxyanisole to Human Albumin Using a Novel Dynamic Method

R. EL-RASHIDY and SARFARAZ NIAZI ˣ

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Abstract □ To study the interaction of butylated hydroxyanisole with various body tissues, a fully automated dynamic method was developed for the determination of plasma protein binding constants at 37°, using membrane filtration equipment designed for dissolution rate studies. Appropriate equations were derived for the calculation of the free drug concentration from comparative diffusion rates across sealed dialysis sacs. A monoexponential equation described the diffusion in the absence of proteins, and a biexponential equation was fitted to diffusion from the drug–protein complex. The Scatchard and double-reciprocal plots were developed for butylated hydroxyanisole. A high degree of reproducibility was obtained for the calculation of protein binding constants ($K = 2.4 - 2.9 \times 10^4$ and $n = 1.4 - 1.32$). The magnitude of these binding-constants suggests that any change in protein binding can have a significant effect on the distribution of butylated hydroxyanisole throughout the body, such as may be brought about by the common variations in the amount ingested.

Keyphrases □ Butylated hydroxyanisole—binding to human albumin studied using membrane filtration equipment □ Binding—butylated hydroxyanisole to human albumin, studied using membrane filtration equipment □ Antioxidants—butylated hydroxyanisole, binding to human albumin studied using membrane filtration equipment

Phenolic antioxidants, butylated hydroxyanisole and butylated hydroxytoluene, are widely used in various products (1) to which the American public is exposed frequently. Although these compounds are generally regarded as safe (GRAS list), they cause enzyme induction (2–9), accumulate in the body tissues (9–13), exhibit dose-dependent elimination (10, 14, 15), affect cellular growth and organization (16–26), and induce various other effects (27). However, no tissue interaction studies have been reported that characterize the toxicity of these compounds.

This paper reports plasma protein binding characteristics of butylated hydroxyanisole using membrane filtration equipment designed for dissolution rate studies. This novel method allows quick estimation of binding parameters at physiological temperature and is totally automated. Because of its advantages over other methods

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Table I—Protein Binding Parameters of Butylated Hydroxyanisole

Hours	C_{fo}, mg/ml	D_{fo}, mg	ΣD_s	D_{ti}[a], mg	$\frac{dD_{ti}}{dt}$ [b]	D_b, mg	$1/C_{fi}$[c], M	$\bar{v}$	$1/\bar{v}$	$\bar{v}/C_{fi}$, M
0.0	0.0	0.0	0.0	2.5000	−1.1288	1.68957	1110.52	5.0061	0.1998	5559.41
0.5	0.00331	0.497	0.0	2.0030	−0.9054	1.37175	1425.74	4.0644	0.2460	5794.84
1.0	0.00612	0.918	0.00828	1.5737	−0.7263	1.11770	1973.68	3.3117	0.3020	6536.24
1.5	0.00828	1.242	0.02358	1.2344	−0.5826	0.91341	2803.83	2.7064	0.3695	7588.28
2.0	0.00990	1.485	0.04428	0.9707	−0.4673	0.74892	4058.08	2.2190	0.4506	9004.97
2.5	0.01030	1.545	0.06903	0.8860	−0.3748	0.61247	3290.32	1.8147	0.5510	5971.03
3.0	0.01098	1.647	0.09478	0.7582	−0.3006	0.50481	3551.84	1.4957	0.6686	5312.60
3.5	0.01170	1.755	0.12223	0.6228	−0.2412	0.41950	4426.96	1.243	0.8045	5502.55
4.0	0.01240	1.860	0.15148	0.4885	−0.1934	0.35145	6566.95	1.0413	0.9603	6838.38
4.5	0.01296	1.944	0.18248	0.3735	−0.1552	0.29707	11775.48	0.8802	1.1361	10364.86
5.0	0.01332	1.998	0.21488	0.2871	−0.1245	0.25292	26331.19	0.7494	1.3344	19732.40
5.5	0.01335	2.003	0.24818	0.2488	−0.09983	0.21616	27573.53	0.6405	1.5613	17660.13

[a] $D_{ti} = 2.559e^{-0.44099t}$. [b] $dD_{ti}/dt = -1.12876e^{-0.44099t}$. [c] $k_{12} = 0.6681\ hr^{-1}$; $k_{21} = 0.02226\ hr^{-1}$.

(28–30), this technique is highly recommended for protein binding studies.

EXPERIMENTAL

Materials—Butylated hydroxyanisole[1], human plasma albumin[2], dialysis tubing[3], and monobasic and dibasic potassium phosphates[4] were obtained commercially.

Equipment—Membrane filtration equipment[5] customarily used for dissolution rate studies was modified for protein binding studies (Fig. 1). The modifications included removal of the filter membrane and introduction of a dialysis sac (10.16 × 3.18 cm) sealed on both ends with sterile disposable clamps[3] in the dissolution chamber. In each sac, 10 plastic beads (diameter of 8 mm) were placed to facilitate mixing during agitation.

Each solution chamber was filled with 150 ml of pH 7.4 phosphate buffer, maintained at 37 ± 1°, and 20 plastic beads were added. A sample of 2.5 ml was automatically drawn from the solution chamber every 30 min, and this volume was instantaneously and automatically replaced by phosphate buffer maintained at the same temperature in the external solvent reservoir.

Protein Binding Study—A solution of butylated hydroxyanisole was prepared at 0.5 mg/ml (2.777 mM) in 4% (0.375 mM) albumin in phosphate buffer. A 5-ml aliquot of this solution was placed in the dialysis sac to which 10 plastic beads were added, and the sac was sealed with clamps. The sac was then placed in the solution chamber containing 150 ml of phosphate buffer, and the chamber was rotated at 1.2 rpm (normal peristaltic speed).

A 2.5-ml sample, drawn periodically, was analyzed at 288 nm using a UV spectrophotometer[6]. The standard curve was prepared in phosphate buffer and gave a molar absorptivity of 5×10^3 at $r^2 > 0.99$.

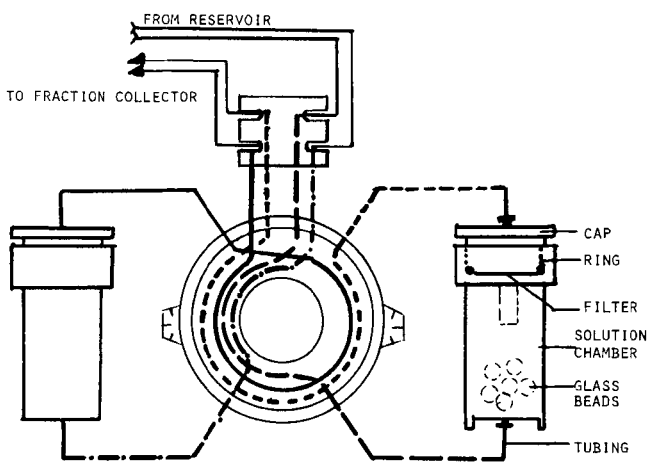

Figure 1—*Flow diagram of solubility simulator.*

[1] BHA, Nutritional Biochemical Corp., Cleveland, OH 44128.
[2] Sigma Chemical Co., St. Louis, MO 63178.
[3] Arthur H. Thomas Co., Philadelphia, Pa.
[4] Fisher Scientific Co., Fair Lawn, N.J.
[5] Sartorius membrane filter, GMbH 34 Göttingen/BRD Postfach, Germany.
[6] Beckman DBGT, Beckman Instruments, Fullerton, Calif.

The total amount of drug bound to proteins can be calculated from:

$$D_t = D_b + D_{fi} + D_{fo} \qquad \text{(Eq. 1)}$$

where D_t is the total drug in the system, D_b is the amount of drug bound, D_{fi} is the amount of free drug in the protein compartment, and D_{fo} is the amount of free drug in the solution chamber.

The loss of drug from the protein compartment takes place due to the equilibration of free drug across the dialysis sac and is expressed as:

$$\frac{dD_{ti}}{dt} = -k_{12}D_{fi} + k_{21}D_{fo} \qquad \text{(Eq. 2)}$$

where k_{12} and k_{21} are first-order mass transport constants as shown in Scheme I, and D_{ti} is the total drug inside the dialysis bag.

$$D_b \rightleftharpoons D_{fi} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} D_{fo}$$

Scheme I

The values of these rate constants can be determined easily by excluding the protein from the dialysis sac (28); thus:

$$\frac{dD_{ti}}{dt} = \frac{dD_{fi}}{dt} = -k_{12}D_{fi} + k_{21}D_{fo} \qquad \text{(Eq. 3)}$$

which yields the following solution (31):

$$D_{fi} = \frac{D_{fi}{}^0 k_{21}}{k_{12} + k_{21}} + \frac{D_{fi}{}^0 k_{12}}{k_{12} + k_{21}} e^{-(k_{12}+k_{21})t} \qquad \text{(Eq. 4)}$$

where $D_{fi}{}^0 = D_{ti}$ at $t = 0$ and:

$$D_{fo} = \frac{D_{fi}{}^0 k_{12}}{k_{12} + k_{21}} (1 - e^{-(k_{12}+k_{21})t}) \qquad \text{(Eq. 5)}$$

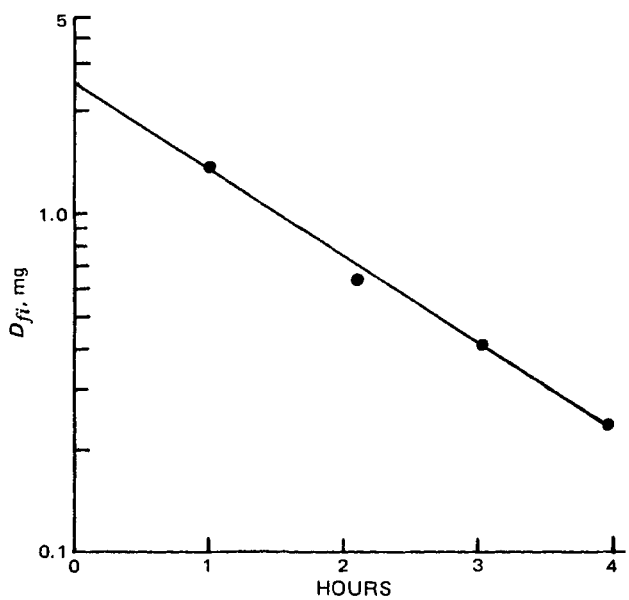

Figure 2—*Loss of free drug from the dialysis sac in the absence of protein.*

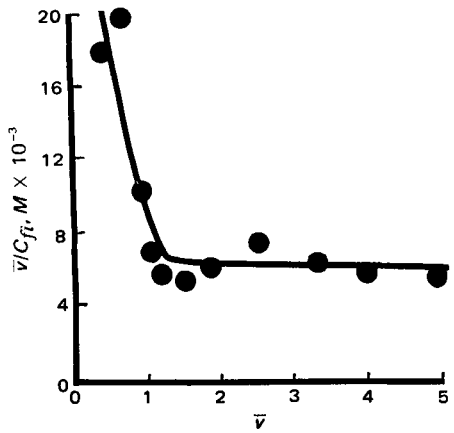

Figure 3—*Scatchard plot. (See text for details.)*

Since D_{fo} can be directly measured as a function of time and:

$$D_{fi} = D_{fi}^0 - D_{fo} \qquad \text{(Eq. 6)}$$

both D_{fi} and D_{fo} are accessible and can be used to calculate the rate constant as shown in Fig. 2, where 2.5 mg of butylated hydroxyanisole was placed inside the dialysis bag and allowed to diffuse.

When the protein is added to the dialysis bag, the total amount of drug inside the bag can be determined by the following mass balance equation:

$$D_{ti} = D_{ti}^0 - D_{fo} - \sum_{i=1}^{n-1} D_{si} \qquad \text{(Eq. 7)}$$

where $\Sigma_{i=1}^{n-1} D_{si}$ is the cumulative amount of drug lost due to preceding samplings of the system.

Table I reports the total drug inside as a function of time. The decrease in the D_{ti} as a function of time, except for D_{si}, can be represented by Eq. 2; thus:

$$D_{fi} = \frac{k_{21} D_{fo} - \dfrac{dD_{ti}}{dt}}{k_{12}} \qquad \text{(Eq. 8)}$$

The term dD_{ti}/dt can be determined either by drawing tangents at specific time function plots or, more appropriately, by fitting to an exponential equation:

$$D_{ti} = \sum_{i=1}^{n} (A_i e^{-a_i t}) \qquad \text{(Eq. 9a)}$$

$$\frac{dD_{ti}}{dt} = \sum_{i=1}^{n} (-A_i a_i e^{-a_i t}) \qquad \text{(Eq. 9b)}$$

where A_i values are constants, a_i values are hybrid rate constants, and t is time. This determination can be accomplished easily by using the desk-top calculater program described by Niazi (32). The data in Table I were fitted by a monoexponential equation:

$$D_{ti} = 2.559e^{-0.4409t} \qquad (r^2 > 0.98) \qquad \text{(Eq. 10)}$$

and:

$$-\frac{dD_{ti}}{dt} = 1.12876e^{-0.4409t} \qquad (r^2 > 0.99) \qquad \text{(Eq. 11)}$$

With this information, all data required for protein binding analysis can be derived as shown in Table I.

RESULTS AND DISCUSSION

Many studies (28–30) reported protein binding parameters using dynamic methods. In some instances, a sink condition is maintained outside the dialysis sac (28) to avoid the necessity of calculating the back-diffusion rate constant. Although the maintenance of sink conditions simplifies the calculations, it leads to cumbersome experimental procedures and definite theoretical errors since some degree of back-diffusion must be assumed no matter how small the outside concentration.

In the approach presented here, the main emphases are on the simplification of experimentation through automation and quick calculations of protein binding parameters using membrane filtration equipment. This equipment maintains the desired temperature (37 ± 1°) throughout the experiment and provides gentle and uniform mixing of solutions, automated sampling at preprogrammed intervals, and simultaneous replacement of the sample volume with the medium; all of these conditions are in a closed system. Further automation can be achieved by hooking the sampling port to a spectrophotometer, which can transmit impulses to a recorder or an appropriate data analyzer system.

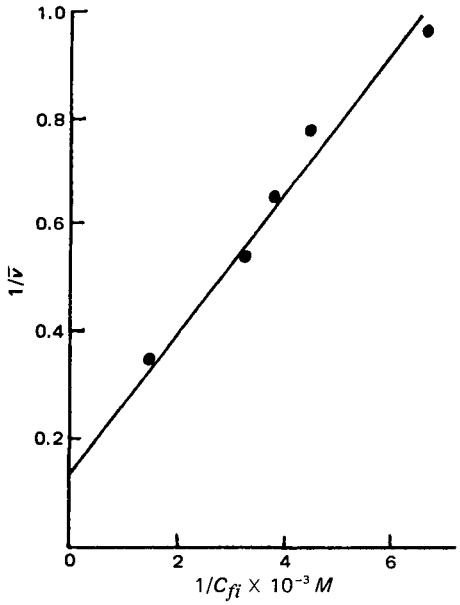

Figure 4—*Double-reciprocal plot using the data points representing the linear part of the Scatchard plot.*

Since the mathematical treatment presented does not require the maintenance of sink conditions, a greater concentration range can be studied. As shown in Table I, the C_{fo}, the outside concentration, varied from 2.62 to about 204% of the inside free concentration, C_{fi}:

$$\% \text{ of } C_{fi} = \frac{C_{fo} \times 5 \times 100}{(D_{ti} - D_{bi})} \qquad \text{(Eq. 12)}$$

Another modification in this method requires accounting for the drug lost from the system because of sampling. Although the total volume sampled each time is small, large numbers of samples can lead to significant errors. For example, after 5.5 hr of sampling, about 10% of the initial amount of drug was removed. If this removal is not considered, the term D_b would be grossly overestimated since it is arrived at by indirect mass balance calculations.

The forward and reverse mass transfer rate constants were obtained by excluding the protein from the sac and fitting the data to Eqs. 4 and 5, using the minicomputer program reported by Niazi (32).

The data fitted to Eq. 4 were plotted in Fig. 2 and showed excellent log linearity ($r^2 > 0.98$). These rate constants were utilized in Eq. 8 to calculate the free drug concentration inside the sac, leading to the complete set of parameters needed for protein binding calculations (Table I):

$$\bar{v}/C_{fi} = nK - K\bar{v} \qquad \text{(Eq. 13)}$$

$$\frac{1}{\bar{v}} = \frac{1}{nKC_{fi}} + \frac{1}{n} \qquad \text{(Eq. 14)}$$

where $\bar{v}$ is the ratio of the moles of drug bound per mole of protein, n is the number of binding sites, and K is the protein binding constant.

The data presented in Table I are plotted according to Eqs. 13 and 14 in Figs. 3 and 4, respectively. Both Scatchard (Eq. 13) and double-reciprocal (Eq. 14) plots gave excellent fits ($r^2 > 0.95$ and $r^2 > 0.98$, respectively). The number of binding sites obtained were 1.40 and 1.32, respectively: K values of 2.4×10^4 and 2.9×10^4 were obtained from Scatchard and double-reciprocal calculations, respectively. The agreement between these two methods of data handling can be considered excellent since the double-reciprocal method normally leads to great variation.

The protein binding constant obtained for butylated hydroxyanisole can be compared with the binding parameters of nafcillin (33), tetracyclines (34), digitoxin (35), digitoxigenin (35), prostaglandins (36), and dimethoxychlorpromazine (37). Most of these drugs show extensive distribution in the body and a wide range of biological half-lives. The magnitude of the binding constant of butylated hydroxyanisole suggests

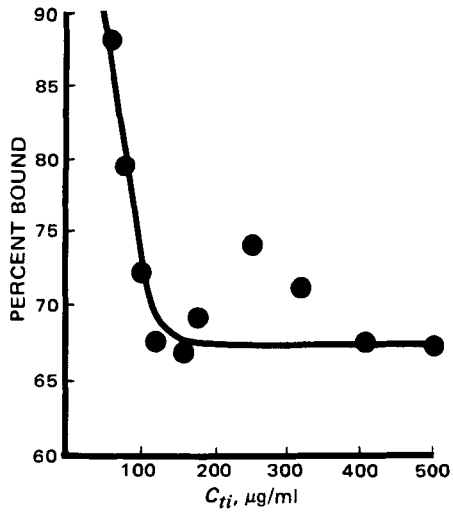

Figure 5—*Percent binding of butylated hydroxyanisole as a function of the total concentration in the protein compartment.*

that any change in protein binding will have a significant effect on the distribution throughout the body (38), such as may be brought about by the variations in the amount of butylated hydroxyanisole ingested.

Figure 5 shows the percent binding of butylated hydroxyanisole as a function of the total concentration in the protein compartment. The minimum binding was about 68% and the maximum binding exceeded 90%. This finding is important since, in normal dosing conditions (2 mg/kg), the total concentration of butylated hydroxyanisole will be well below the range providing minimum binding. For example, a 2-mg/kg dose in a 65-kg individual will give the maximum concentration of about 26 μg/ml, assuming all drug remains in the blood compartment initially. This concentration is well below the concentration at which the percent binding shows a plateau (Fig. 5). Thus, during normal ingestion of butylated hydroxyanisole, a high degree of protein binding can be assumed.

Higher doses of butylated hydroxyanisole have shown nonlinear elimination of the free compound in the urine (15). The increased metabolism can, at least in part, be attributed to possible effects of nonlinearity in protein binding. These studies are currently being conducted and will be reported.

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