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A Model for Ultracentrifugal Quantification of [^{35}S]Bromosulphthalein-Binding to Plasma Proteins in the Presence of Radioimpurities[1] (38617)

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Bilirubin uptake from blood by the liver is proportional to the concentration of the non protein-bound moiety (1). Bromosulphthalein (BSP) appears to behave similarly because its hepatic uptake is influenced by both levels and affinities of BSP-binding proteins in plasma (2). Quantifying BSP's protein-binding may clarify certain poorly-understood aspects of BSP dynamics *in vivo* (3, 4). To be clinically applicable, such studies must be done at molar ratios of BSP-to-plasma proteins found when BSP is injected intravenously to test liver function. That is difficult because the levels of unbound BSP are too low to be measured by spectrophotometry or neutron activation.[2] To attain the required analytical sensitivity, we used [^{35}S]BSP. However, we were unsuccessful in purifying the labeled BSP adequately. Therefore, we developed a mathematical model to determine picomole-quantities of unbound [^{35}S]BSP in the presence of radioimpurity.

Materials and Methods. A common pool of aseptically-collected heparinized dog plasma, stored at 2°, was sampled for all trials. Total protein concentration in the plasma was 5.82 g/100 ml, as determined by the biuret reaction. Protein fractions were separated by electrophoresis on Sephraphore III cellulose acetate strips (Gelman Instrument Co., Ann Arbor, MI) in Tris–barbital buffer (pH 8.8). The strips were stained in ponceau S, cleared by dehydration, and scanned with a densitometer. Peak-integration showed alpha-1 globulins and albumin, the principal BSP-binding plasma proteins (5), composed 4.5% and 37.9%, respectively, of the total protein.

We attempted to purify [^{35}S]BSP (Amersham-Searle Corp., Arlington Heights, IL) by gel filtration on Sephadex G-10, anion exchange chromatography, and by thin-layer chromatography on silica (4:1:2 (v/v) *n*-butanol:acetic acid:water). To ascertain the extent of purification, we ultracentrifuged a solution of the [^{35}S]BSP in plasma and measured radioactivity of the protein-free supernatant. We then replaced the supernatant by Krebs–Ringer buffer (pH 7.40) and repeated the process. As BSP has a high affinity for certain plasma proteins, supernatant removal should not significantly alter the total [^{35}S]BSP remaining in the centrifuge tube, and thus, in a pure sample of [^{35}S]BSP, the measured radioactivity in the supernatant after each spin should not fall. However, we obeserved a fall in supernatant radioactivity with sequential spins, which indicated that an impurity of relatively low binding affinity remained in our preparation.

Using a Packard Tri-Carb liquid scintillation spectrometer and correcting both for quench by internal standardization and the 87-day half-life of ^{35}S, we measured radioactivity within a standard deviation of 0.32%. Samples of 0.1 or 0.4 ml were counted in a cocktail of 2.5 ml of NCS solubilizer (Amersham-Searle Corp.) and 10 ml toluene containing 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-*bis*[2-(5-phenyloxazolyl)]benzene per liter. The molar concentration of the same [^{35}S]BSP sample was determined spectrophotometrically in phosphate buffer at pH 11.0. The specific activity was 52.5 mCi/mmole.

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[2] A thermal neutron flux, of 1.8 × 10^{12} neutrons/cm^2/sec for 0.5 hr in a Triga Mark II reactor generated radioactive [^{89}Br]BSP. However, in plasma samples, concomitant ^{24}Na-formation interfered prohibitively with radioactivity measurements.

TABLE I. DECREASING SUPERNATANT RADIOACTIVITY OVER CONSECUTIVE ULTRACENTRIFUGATIONS[a] OF MIXTURES OF CANINE PLASMA AND [^{35}S]BSP.[b]

Trial no.	Radioactivity (dpm/ml in thousands) of: Uncentrifuged mixture[c]	Supernatant after n ultracentrifugations[d] $n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$
1	6504	10.819	7.302	5.460	4.422	4.018	3.724	3.612
2	6861	11.120	7.403	5.627	4.824	4.149	3.881	—
3	7188	11.576	8.167	5.931	8.815[e]	4.384	4.154	—

[a] After each centrifugation, supernatant was replaced by buffer (pH 7.40) and sedimented protein was resuspended. Centrifugations were at 5° and at 226,000 *g* for 24 hr.

[b] Specific activity of [^{35}S]BSP was 117 dpm/picomole.

[c] Standard deviation of the counts was 0.105%.

[d] Standard deviation of the counts was 0.316%.

[e] Excluded from data analyzed.

We siliconized all glassware and centrifuge tubes with Siliclad (Clay Adams, Parsipanny, NJ), and found no binding of radioligand to equipment. [^{35}S]BSP in Krebs–Ringer buffer centrifuged for 24 hr at 226,000 *g* remained homogeneously distributed when present at concentrations near those of the unbound form encountered in our experiments.

Adding aliquots of 180.0–220.0 mg of 0.83 m*M* solution of [^{35}S]BSP in Krebs–Ringer buffer to 5.0 ml of plasma established molar ratios of ligand-to-protein near those in plasma when standard doses of BSP are injected intravenously to test hepatic function. We adjusted the final volume to 60. ml, and spun 5 ml of the mixture at 226,000 *g* (at the bottom of the tube) in a Titanium-50 fixed-angle rotor for 24 hr at 5°. Of the supernatant, 2.5 ml (protein-negative to biuret) was replaced by Krebs–Ringer buffer (pH 7.40), sedimented protein was resuspended, and the process repeated six or seven spins. Radioactivities of the uncentrifuged mixture and of the supernatant after each centrifugation were determined.

Results and Discussion. The decline in supernatant radioactivity over consecutive ultracentrifugations with supernatant replacements is shown in Table I. Less than 0.2% of the original radioactivity was nonprotein-bound. Most of the unbound radioactivity was rapidly lost during initial centrifugations, the remainder more gradually. Apparently, supernatant removal eliminated a source of unbound radioactivity during the initial centrifugations, leaving a relatively constant concentration of [^{35}S]BSP in the supernatants of later spins. To quantify those observations, we derived a mathematical model that described the progressive decline in supernatant radioactivity so we could determine unbound [^{35}S]BSP.

Mathematical model. V = volume of sample in centrifuge tube. S = volume of [supernatant removed after each spin; $[L_t]_n$ = concentration of total ligand after n spins; $[L_f]_n$ = concentration of unbound ligand after n spins; n = number of ultracentrifugal spins.

We assume: A. The protein content of the centrifuge tube remains constant from spin to spin, i.e., no protein is lost when the supernatant is removed. That assumption is supported by all supernatants being biuret-negative. B. At the molar ratios of ligand-to-protein used, binding sites of only one affinity are significantly involved. For the interaction

$$P + L_f \rightleftarrows PL$$

where P is an active binding site on the protein molecule and L_f is the unbound ligand, we define the affinity constant

$$K = [PL]/([P]\cdot[L_f]).$$

C. In a bovine serum albumin–bilirubin system of constant albumin concentration, the number of active binding sites on albumin was independent of ligand concentration (1). We assume our system to behave

similarly, and thus $[PL]/[L_f]$, and therefore $[L_f]/[L_t]$ is constant.

After the first centrifugation of protein-ligand mixture, S ml of supernatant is removed, representing one-half of the total volume of V ml. This corresponds to $S \cdot [L_f]_1$ amount of ligand removed, leaving an amount equal to $[L_t]_1 \cdot V - [L_f]_1 \cdot S$ in the centrifuge tube. When the sedimented protein is resuspended in buffer to the same volume, the new concentration of total ligand is:

$$[L_t]_2 = [L_t]_1 \cdot \left(1 - \frac{S \cdot [L_f]_1}{V \cdot [L_t]_1}\right). \quad \text{(Eq. 1)}$$

Since $[L_f]_1/[L_t]_1$ is constant:

$$[L_f]_2 = [L_f]_1 \cdot \left(1 - \frac{S \cdot [L_f]_1}{V \cdot [L_t]_1}\right) \quad \text{(Eq. 2)}$$

After n spins:

$$[L_t]_n = [L_t]_1 \cdot \left(1 - \frac{S \cdot [L_f]_1}{V \cdot [L_t]_1}\right)^{n-1} \quad \text{(Eq. 3)}$$

and

$$[L_f]_n = [L_f]_1 \cdot \left(1 - \frac{S \cdot [L_f]_1}{V \cdot [L_t]_1}\right)^{n-1} \quad \text{(Eq. 4)}$$

In a system of different ligands, the level of each in the supernatant is determined not only by the ligand's total concentration but also by its affinity for binding proteins. In our system, both BSP and impurity are ^{35}S-labeled; thus, supernatant radioactivity tells little about the concentration of either unbound [^{35}S]BSP after n spins ($[B_f]_n$) or radioimpurities ($[X_f]_n$, $[Y_f]_n$, $[Z_f]_n$, etc. Supernatant radioactivity after n spins ($[R_f]_n$) is the sum of the contributions from [^{35}S]BSP and radioimpurities:

$$[R_f]_n = [B_f]_n + [X_f]_n + [Y_f]_n + [Z_f]_n + \cdots\cdots. \quad \text{(Eq. 5)}$$

The concentration of each ligand in the supernatant after n spins is given by that ligand's expression of equation 4.

Data analysis. As [^{35}S]BSP has a high affinity for plasma proteins, in contrast to radioimpurity in our preparation, $[B_f]_n$ does not change significantly over the limited number of centrifugations in our experiment. Furthermore, the assumption of only one kinetically-distinguishable species of radioimpurity in the supernatant served to explain our data. Hence:

$$[R_f]_n = [B_f] + [X_f]_1 \cdot \left(1 - \frac{S \cdot [X_f]_1}{V \cdot [X_t]_1}\right)^{n-1}. \quad \text{(Eq. 6)}$$

We measured $[R_f]_n$ experimentally after each spin, and determined the constants $[B_f]$, $[X_f]_1$, and $[X_t]_1$ by the NONLIN computer program for least-squares parameter estimation of nonlinear equations (written by Carl M. Metzler of the Upjohn Company). A plot of $[R_f]_n$ *versus* n for one ultracentrifugal series, along with the computer-derived components $[B_f]$ and $[X_f]_n$, is shown in Fig. 1, and a summary of the three trials is presented in Table II.

The assumption that $[B_f]$ is constant from spin-to-spin can now be justified. The average $[B_f]/[B_t]$ ratio of 528 × 10^{-6} (Table II) indicates (Eq. 4) that, after seven centrifugations, the net decline in $[B_f]$ is only 0.15%. In contrast, for the average $[X_f]/[X_t]$ ratio of 0.94, the $[X_f]_7$ is 98% less than $[X_f]_1$, which explains the rapid drop in

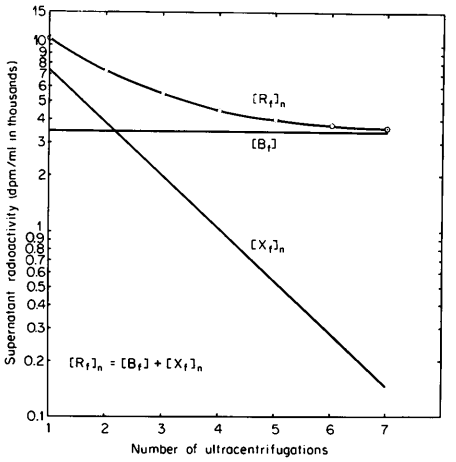

FIGURE 1. Decreasing supernatant radioactivity in mixtures of canine plasma and [^{35}S]SBSP over consecutive ultracentrifugations with supernatant replacement. Observed levels of total supernatant radioactivity (⊙) are plotted along with computer-derived (Eq. 6) estimates of concentrations of unbound [^{35}S]BSP ($[B_f]$), radioimpurity after each spin ($[X_f]_n$), and the sum of those components ($[R_f]_n$).

TABLE II. BINDING OF [^{35}S]BSP AND RADIOIMPURITY TO PLASMA PROTEINS AND FRACTION OF RADIOIMPURITY IN ^{35}S-LABELED BSP.[a]

Trial no.	$\frac{[B_f]}{[B_t]} \times 10^6$	$\frac{[X_f]}{[X_t]} \times 10^2$	$\frac{[X_t]}{[B_t]+[X_t]} \times 10^5$
1	531 ± 5.6[b]	96 ± 3.2	118 ± 3.4
2	536 ± 17.5	97 ± 10.2	111 ± 9.7
3	517 ± 26.8	90 ± 12.8	121 ± 14.0
Mean	528 ± 9.6[c]	94 ± 5.0	117 ± 5.2

[a] Calculated by the NONLIN program for least-squares analysis of nonlinear equations. Correlation coefficients between observed and calculated values of $[R_f]_n$ were 0.9999, 0.9996, and 0.9994 for trials 1, 2, and 3, respectively ($df = n$-3).

[b] Standard deviation of fitting the model to the data.

[c] Standard errors of the means.

supernatant radioactivity of initial centrifugations.

The highly significant correlation coefficients (Table II; $P \ll 0.01$) justify the model and the assumption of only one kinetically-distinguishable radioimpurity, i.e., impurity with only one binding affinity for plasma proteins. The impurity being 94% unbound (Table II) suggests [^{35}S]sulfate. That [^{35}S]-sulfate may have been generated by detaching [^{35}S]sulfonic acid groups through photolysis, autoradiolysis, or interaction with support materials of our purification methods, as suggested by our inability to completely purify the [^{35}S]BSP. For sulfate, we do not have to assume the nonsaturating behavior characteristic of bilirubin-to-albumin binding (assumption C, above). If we assume, instead, that sulfate is bound very loosely to a large number of sites on serum proteins, then, because of the low [^{35}S]-levels, the concentration of vacant sites is nearly as high as it can get to begin with and it does not change much during the experiment. Hence for the 'sulfate' sites, $[P]$—and thus $[L_f]/[L]$—is constant, and the derivation of equation 6 follows as before.

The [^{35}S]BSP preparation we used was 99.88% pure (Table II). Considering that only 0.12% of radioimpurity overshadowed the level of supernatant [^{35}S]BSP in equilibrium with protein-bound radioactivity, protein-binding studies using nonpurified ^{35}S-labeled BSP (6) may need to be reevaluated.

In our experiment, simplification of the model (Eq. 6) was allowed because (i) BSP is tightly bound to protein, and (ii) only one radioimpurity was kinetically apparent. But use of the model is by no means limited to that situation. The general case of a mixture of several radioactive compounds (Eq. 5) can be solved using simultaneous equations, provided the compounds have different binding affinities ($[L_f]_1/[L_t]_1$ in equation 4). Each additional ligand in a mixture requires determining two new parameters: the initial concentrations of unbound and total ligand, $[L_f]_1$ and $[L_t]_1$, respectively (Eq. 4). Hence, for each additional ligand, one must obtain two more data points ($[R]_n$-values), which requires two more centrifugations. Use of the model may be indicated in any binding study in which ligand purity cannot be achieved or ascertained.

Summary. Solutions of [^{35}S]bromosulphthalein ([^{35}S]BSP) in heparinized canine plasma, in the proportions established *in vivo* after injecting BSP intravenously to test liver function, were ultracentrifuged at 226,000 *g* for 24 hr at 5°. Protein-free supernatant was replaced by Krebs–Ringer buffer (pH 7.40), the protein sediment resuspended, and the mixture recentrifuged. That process was repeated several times, and the radioactivity of each resulting supernatant was measured. Since [^{35}S]BSP could not be adequately purified, supernatant radioactivities reflected both [^{35}S]BSP and radioimpurity. Therefore, a model was derived that (i) interpreted the rapid decrease in supernatant radioactivities of initial centrifugations and the gradual fall thereafter; and

(ii) allowed us to determine picomoles of non-protein-bound [^{35}S]BSP. Results indicated that only 0.053% (SD 0.0013%) of BSP in our system was not protein-bound.

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