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ACKNOWLEDGMENTS AND ADDRESSES

Received July 20, 1972, from the *Drug Research Laboratories, Health Protection Branch, Department of National Health and Welfare, Ottawa, Canada.*

Accepted for publication September 27, 1973.

The author acknowledges the valuable discussions with Dr. W. N. French of these laboratories and the assistance in programming of Mr. H. Yule and Mr. R. Mackey of the Division of Statistics and Information Science.

Figures 1–4 were included, by permission of the *Journal of Pharmaceutical Sciences*, in material presented to the 1973 Land O'Lakes Conference on Pharmaceutical Analysis, Land O'Lakes, Wis.

Use of 2-(4′-Hydroxybenzeneazo)benzoic Acid to Study the Binding of L-Thyroxine to Serum Albumins

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Abstract □ A study was made to investigate the use of the dye 2-(4′-hydroxybenzeneazo)benzoic acid as a potential agent that would reflect the binding of L-thyroxine to serum albumins. The dye is a strong visible absorbing material which interacts with serum albumins to give characteristic spectrophotometric peaks and, as such, provides the basis for an extremely convenient assay to measure amounts, free and bound, after their separation in the presence of serum albumin and potential competitive inhibitors. The results obtained showed that the dye and L-thyroxine compete for the same binding site on bovine and rat serum albumins; thus the dye can be used to gauge the displacement of L-thyroxine from serum albumin by potential competitive inhibitors. Additionally, a method was introduced to evaluate statistically differences, between data sets, in y-intercepts obtained by linear regression. The method used should have general application.

Keyphrases □ L-Thyroxine binding to serum albumins—measured using 2-(4′-hydroxybenzeneazo)benzoic acid, spectrophotometry □ Serum albumin binding of L-thyroxine—measured using 2-(4′-hydroxybenzeneazo)benzoic acid, spectrophotometry □ 2-(4′-Hydroxybenzeneazo)benzoic acid—used to measure L-thyroxine binding to serum albumins

2-(4′-Hydroxybenzeneazo)benzoic acid (I) is a dye that interacts with serum albumins to give characteristic spectrophotometric peaks (1). The intensity of these peaks can be related to the extent of binding of the dye to serum albumin and, therefore, could represent an extremely convenient means of studying displacement reactions. The binding of I to serum albumins and its displacement from serum albumin by chlorophenoxyacetic acids were studied previously (2, 3). Moriguchi *et al.* (4) also studied the binding of I to bovine serum albumin and described a procedure that utilized I in determining the binding of acidic drugs. Drugs studied with bovine serum albumin were substituted benzoic acids (5) and sulfonamides (6). This study was made to investigate the use of I as a potential agent that would reflect the binding of L-thyroxine to serum albumins. The objective was to determine if I and L-thyroxine are bound to the same site on serum albumin so that the dye could be used to gauge the displacement of L-thyroxine from serum albumin. The results using bovine and rat serum albumins are presented here.

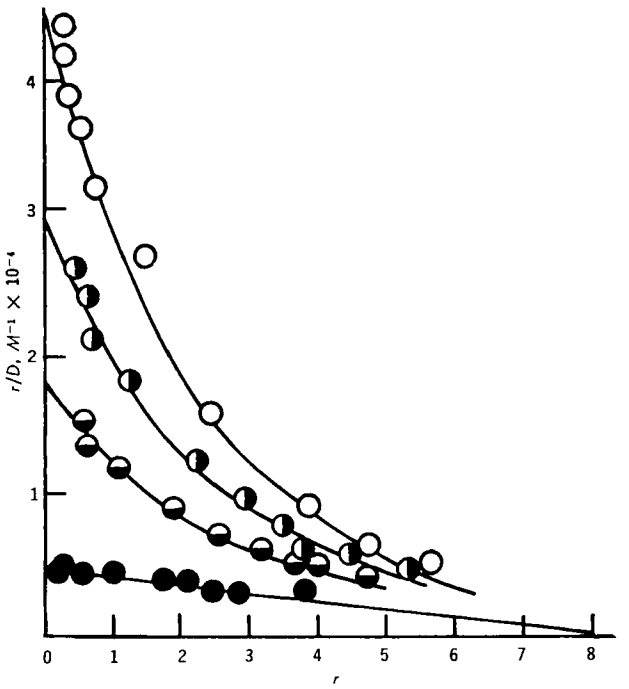

Figure 1—*Scatchard plot for the binding of I to bovine serum albumin (1.0 × 10⁻⁴ M) in the presence of various total amounts of* L-*thyroxine. Key:* ○, *I alone;* ◑, *3.33 × 10⁻⁵ M* L-*thyroxine;* ◒, *6.66 × 10⁻⁵ M* L-*thyroxine; and* ●, *16.66 × 10⁻⁵ M* L-*thyroxine.*

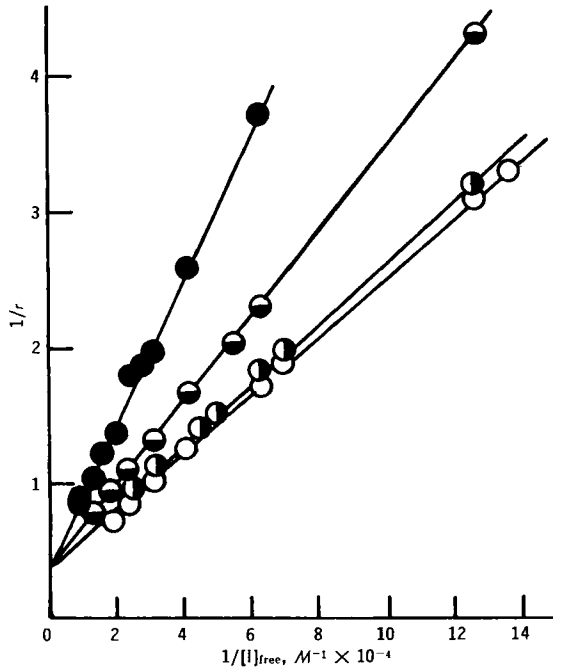

Figure 2—*Relationship between 1/*r (*moles bovine serum albumin per mole I*) *and the reciprocal of free I concentration at low* r *values in the presence of* L-*thyroxine. Key:* ○, *no thyroxine;* ◑, *1.66 × 10⁻⁵ M* L-*thyroxine;* ◒, *3.33 × 10⁻⁵ M* L-*thyroxine; and* ●, *6.66 × 10⁻⁵ M* L-*thyroxine.*

EXPERIMENTAL

Materials—Bovine serum albumin[1] (Fraction V, fatty acid poor), 2-(4′-hydroxybenzeneazo)benzoic acid[1], rat serum albumin[2] (Fraction V), L-thyroxine[3], and dimethyl sulfoxide[3] were used as purchased without further purification. All final solutions were made with 0.1 *M* sodium phosphate buffer, pH 7.4, using analytical grade chemicals. The dimethyl sulfoxide stock solution of L-thyroxine (1.0×10^{-3} *M*) was diluted with appropriate amounts of phosphate buffer to give the desired final concentrations.

Competitive Binding between I and L-Thyroxine to Bovine Serum Albumin—The binding of I to this albumin in the presence and absence of L-thyroxine was determined by varying the concentration of I from 7.0×10^{-5} to 1.6×10^{-3} *M* at constant bovine serum albumin and L-thyroxine concentrations. The albumin concentration was held at 1.0×10^{-4} *M* (using molecular weight 67,000) in all studies, and four fixed concentrations of L-thyroxine were used (1.66×10^{-5}, 3.33×10^{-5}, 6.66×10^{-5}, and 16.66×10^{-5} *M*). Ultrafiltration at ambient temperatures was used to separate the free amount of I. Twelve milliliters of solution was placed in the ultrafiltration cell[4]. The ultrafiltrate (1.2 ml) was collected and analyzed spectrophotometrically after suitable dilution. In the absence of L-thyroxine, five separate determinations were made where the concentration of free I was determined from the absorbance of the ultrafiltrate at 350 nm. The ϵ for I is 17,682 at this wavelength in phosphate buffer. In the presence of L-thyroxine, the ultrafiltrate was analyzed spectrophotometrically at 400 nm after adjusting the pH of the solution to 10.2 with 2 *N* sodium hydroxide. At pH 10.2, L-thyroxine does not contribute to the 400-nm absorbance of I. The ϵ for I at 400 nm is 21,630.

Competitive Binding between I and L-Thyroxine to Rat Serum Albumin—The binding of I to this albumin in the presence and absence of L-thyroxine was determined by varying the concentration of I from 5.0×10^{-5} to 1.6×10^{-3} *M* at constant rat serum albumin and L-thyroxine concentrations. The albumin concentration was held at 1.0×10^{-4} *M* in all studies, and three fixed concentrations of L-thyroxine were used (3.33×10^{-5}, 6.66×10^{-5}, and 16.66×10^{-5} *M*). Ultrafiltration was used to determine the amount of free I by spectrophotometric analysis at 350 nm in the absence of L-thyroxine and at 400 nm in the presence of L-thyroxine, as already described for the competitive binding to bovine serum albumin.

Treatment of Binding Data—The concentration of bound I in the ultrafiltration studies was determined by taking the difference between the total amount added and the amount found free after ultrafiltration. Corrections were made for the small amount of ligand bound to the ultrafiltration membrane. Scatchard plots were constructed and computer analyzed for binding parameters assuming that two independent classes of binding sites exist on the serum albumin. The computer-generated constants gave *n* (maximum number of sites) and *K* (association constant) values for each class.

Statistical Analysis of Linear Regression Intercepts (7)—To determine whether or not the intercept values for two sets of regression data are the same, use was made of the Student *t* test. In this test, the difference between the average intercepts for two linear regressions is divided by the pooled standard deviation for the data sets:

$$t = \frac{I_1 - I_2}{s_{\text{pooled}}} \qquad \text{(Eq. 1)}$$

In Eq. 1, I_1 is the mean intercept for data set 1, I_2 is the mean intercept for data set 2, and s is the pooled standard deviation for the two data sets. In a regression analysis of y on x, the variance in the intercept, s_I^2, for a data set is given by:

$$s_I^2 = MSE \cdot G/N \qquad \text{(Eq. 2)}$$

where N is the number of observations, G (that takes into account the effect on error variance of the distance of the intercept from the mean $\bar{X}$) is given by:

$$G = 1 + \frac{Nx^{-2}}{\sum_{i=1}^{N}(x_i - \bar{x})^2} \qquad \text{(Eq. 3)}$$

[1] Nutritional Biochemical Corp., Cleveland, Ohio.
[2] Pentex Inc., Kankakee, Ill.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Amicon model 12 cell with a PM-10 membrane.

Table I—Statistical Analysis of Linear Regression Intercepts at $p = 0.01$

System	Number of Observations, N	Mean Square Error[a]	Calculated F Ratio for Mean Standard Error[b]	Theoretical F Ratio for Mean Standard Error[c]	Variance in Intercept[d], s_I^2	Calculated t[e]	Theoretical t[c]
In bovine serum albumin							
I alone	11	3.06×10^{-3}	—	—	8.73×10^{-4}	—	—
I + thyroxine (1.66×10^{-5} *M*)	9	2.09×10^{-4}	14.62	5.61	8.87×10^{-5}	0.69	2.92
I + thyroxine (3.33×10^{-5} *M*)	11	2.70×10^{-3}	1.13	5.35	7.17×10^{-4}	1.18	2.88
I + thyroxine (6.66×10^{-5} *M*)	14	8.41×10^{-3}	2.75	5.11	1.34×10^{-3}	0.78	2.83
In rat serum albumin							
I alone	6	1.04×10^{-4}	—	—	1.55×10^{-4}	—	—
I + thyroxine (3.33×10^{-5} *M*)	5	6.81×10^{-5}	1.53	28.70	9.02×10^{-5}	2.27	3.50
I + thyroxine (6.66×10^{-5} *M*)	10	2.77×10^{-3}	26.62	7.01	1.34×10^{-3}	1.97	3.05
I + thyroxine (16.66×10^{-5} *M*)	8	1.35×10^{-4}	1.29	9.15	1.71×10^{-4}	1.80	3.17

[a] Calculated by Eq. 4. [b] Mean standard error for I/mean standard error for system. [c] From Ref. 9, degrees of freedom $= N - 2$. [d] Calculated by Eq. 2. [e] From Eq. 1.

and MSE is the mean square error given as:

$$MSE = \sum_{i=1}^{N}(y_i - y_{calc})^2/(N - 2) \qquad \text{(Eq. 4)}$$

The numerator of Eq. 4 is the sum of squares of the errors *(SSE)*, where y_{calc} is the regression calculated value for y. The pooled standard deviation used in Eq. 1 is defined as:

$$s_{pooled} = \sqrt{s_{I,1}^2 + s_{I,2}^2} \qquad \text{(Eq. 5)}$$

where subscripts refer to individual data sets. Substituting Eq. 2 into Eq. 5 gives:

$$s_{pooled} = \sqrt{MSE_1 \cdot G_1/N_1 + MSE_2 \cdot G_2/N_2} \qquad \text{(Eq. 6)}$$

If MSE_1 is essentially equal to MSE_2, Eq. 6 simplifies to:

$$s_{pooled} = \sqrt{\frac{SSE_1 + SSE_2}{N_1 + N_2 - 4}\left(\frac{G_1}{N_1} + \frac{G_2}{N_2}\right)} \qquad \text{(Eq. 7)}$$

Therefore, to use Eq. 7 (and Eq. 1), it had to be established that the errors (mean square error values) in the two data sets being compared were essentially the same. To determine whether or not the errors were statistically different, the F ratio for the mean square error in the two data sets was calculated and compared to the theoretical F values at $p = 0.01$. If the calculated F ratios were less than the theoretical values, it was concluded that the errors were not statistically different and that the use of Eq. 7 (and Eq. 1) was valid.

RESULTS AND DISCUSSION

Competitive Binding between I and L-Thyroxine on Bovine Serum Albumin—The binding of the azo dye, I, to bovine serum albumin in the presence and absence of fixed amounts of L-thyroxine gave the results plotted in Fig. 1. In the presence of L-thyroxine, the amount of bound dye decreases and more dye is displaced as the concentration of the hormone increases. At a concentration of 16.66×10^{-5} *M* L-thyroxine, it appears that I is dis-

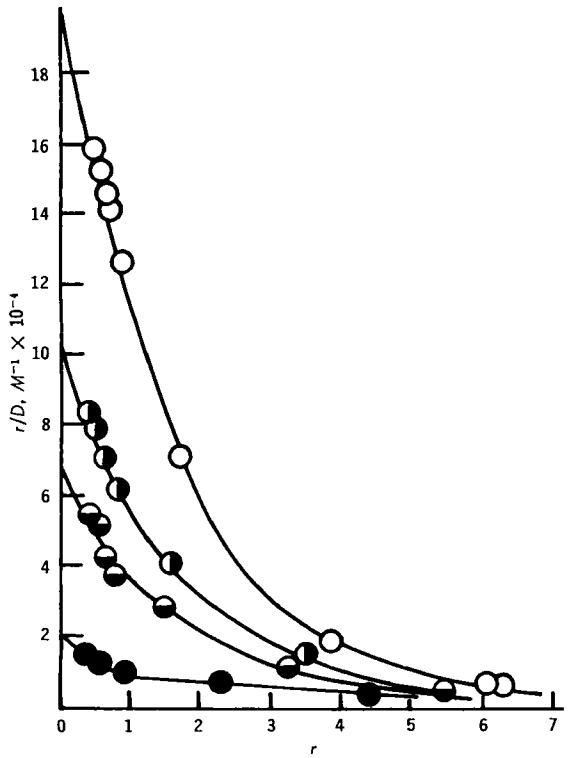

Figure 3—*Scatchard plot for the binding of I to rat serum albumin (1.0×10^{-4} M) in the presence of various total amounts of L-thyroxine. Key:* ○, *I alone;* ◑, *3.33×10^{-5} M L-thyroxine;* ◒, *6.66×10^{-5} M L-thyroxine; and* ●, *16.66×10^{-5} M L-thyroxine.*

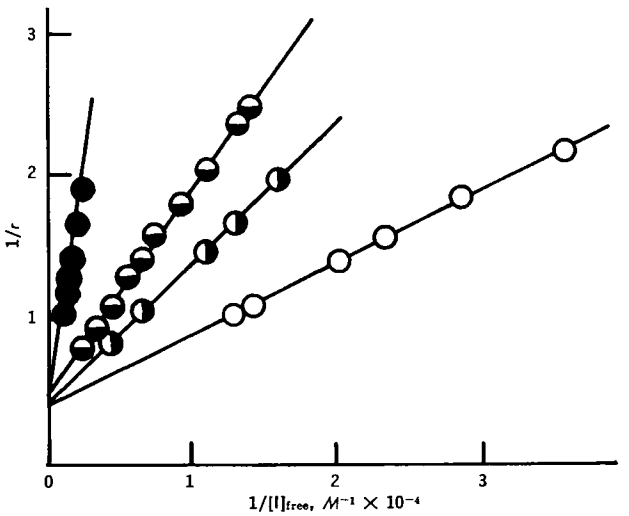

Figure 4—*Relationship between 1/r (moles rat serum albumin per mole I) and the reciprocal of free I concentration at low r values in the presence of L-thyroxine. Key:* ○, *no thyroxine;* ◑, *3.33×10^{-5} M L-thyroxine;* ◒, *6.66×10^{-5} M L-thyroxine; and* ●, *16.66×10^{-5} M L-thyroxine.*

placed from its primary site since an apparent linear Scatchard plot exists at this L-thyroxine level, giving $n = 8$ and $K = 640$ by regression analysis which approaches the values of n_2 (7) and K_2 (1310 M^{-1}) for I as determined by a computer fit of the binding of the dye to this albumin. The n_1 and K_1 values for I are 1.7 and 23,000 M^{-1}, respectively. By using values for r (moles bound I per mole albumin) of approximately 1 and less, where the first class of binding sites is primarily affected, the data of Fig. 1 were replotted as $1/r$ *versus* $1/[I]_{free}$ to determine if L-thyroxine and I are competing for the same binding site on bovine serum albumin. If the same extrapolated $1/r$ intercept is obtained for each concentration of L-thyroxine as found for I alone, the conclusion can be made that the same site is involved (8).

Figure 2 shows the effect of three different total concentrations of L-thyroxine on the binding of I to bovine serum albumin. Linear regression analysis of the data of Fig. 2 produces intercepts of 0.38 for I alone and of 0.36, 0.35, and 0.34 for L-thyroxine at 1.66×10^{-5}, 3.33×10^{-5}, and 6.66×10^{-5} M, respectively. The values for the intercepts are not statistically different at $p = 0.01$ as shown by the Student t test (Table I). The validity of the use of the test is described in the *Experimental* section. In the case of 1.66×10^{-5} M L-thyroxine, the t value suggests no difference in intercepts, but statistical methods cannot be used here because the F distribution limit for the variances of the intercepts falls outside the theoretical range. It is assumed that this intercept is not really different since the other concentrations of L-thyroxine give statistically valid interpretations. The conclusion is, therefore, made that L-thyroxine competes with I at the same binding site on bovine serum albumin.

Competitive Binding between I and L-Thyroxine on Rat Serum Albumin—The binding of I to rat albumin in the presence and absence of fixed amounts of L-thyroxine gave the results plotted in Fig. 3. In the absence of inhibitor, computer-generated values were: $n_1 = 2.2$, $n_2 = 8.1$, $K_1 = 83{,}610$ M^{-1}, and $K_2 = 1295$ M^{-1}. In the presence of L-thyroxine, the amount of bound dye decreases and more dye is displaced as the concentration of the hormone increases. At a concentration of 16.66×10^{-5} M L-thyroxine, it appears that I is almost completely displaced from its primary site since an almost linear Scatchard plot exists at this L-thyroxine level. By using values for r (moles bound I per mole rat albumin) of approximately 1 and less, where the first class of sites for I would most likely be involved, the data of Fig. 3 were replotted as $1/r$ *versus* $1/[I]_{free}$ to determine if L-thyroxine and I are competing for the same binding site in rat serum albumin. Figure 4 shows the effect of three different total concentrations of L-thyroxine on the binding of I to the albumin. Linear regression analysis of the data of Fig. 4 produces intercepts of 0.37 for I alone and of 0.41, 0.49, and 0.40 for L-thyroxine at 3.33×10^{-5}, 6.66×10^{-5}, and 16.66×10^{-5} M, respectively. The values for the intercepts are not statistically different at $p = 0.01$ as shown by the Student t test (Table I). In the case of 6.66×10^{-5} M L-thyroxine, the t value suggests no difference in intercepts, but statistical methods cannot be used here because the F distribution limit for the variances of the intercepts falls outside the theoretical range. It is assumed that this intercept is not really different since the other concentrations of L-thyroxine give statistically valid interpretations. The conclusion is, therefore, made that L-thyroxine competes with I at the same binding site on rat serum albumin.

The results of the competitive binding studies between I and L-thyroxine to bovine and rat serum albumins show that I and L-thyroxine compete at the same binding site on both albumins. Therefore, it should be possible to use I as a model to mirror the binding of L-thyroxine to serum albumin. Use of this property of I on the binding of hypolipemic agents to serum albumins is described in a subsequent report (10).

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ACKNOWLEDGMENTS AND ADDRESSES

Received June 11, 1973, from the *College of Pharmacy, Ohio State University, Columbus, OH 43210*

Accepted for publication September 26, 1973.

Supported in part by the Public Health Service, National Heart and Lung Institute, Grant HL-12740.

The authors thank Mr. William Kramer for help in the statistical treatment of data.

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