Binding Constant of Neutral Red with Riboflavin Binding Protein

CHEM 445 - 025L

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ABSTRACT

Through spectrophotometric techniques, the dissociation constant, K_D, of the Riboflavin Binding Protein - Neutral Red complex was determined to be $(2.77 \pm 0.12) \times 10^{-5}$ M and $(2.77 \pm 0.14) \times 10^{-5}$ M, for two different trials. These values exhibit a high degree of agreement with one another but are one magnitude greater than the literature value [4] of $(2.2 \pm 0.3) \times 10^{-6}$ M. The reasons for this discrepancy are largely unknown.

INTRODUCTION

Ultraviolet/Visible (UV/VIS) spectroscopy is an analytical technique used to determine the optical properties of a solution of interest [1]. In this experiment, the absorbance of the Riboflavin Binding Protein (RFBP) complexed with a surrogate compound, Neutral Red (NR), was studied. This surrogate compound played the role of Riboflavin (or Vitamin B_{12}) and thus, allowed for the quantification of the likelihood of RFBP to bind to Vitamin B_{12} [2].

By measuring the absorbance at λ_{max} ~550 nm for subsequent additions of RFBP to a buffered solution of NR, the relationship between the concentration of the complex and the absorbance was established. From the absorbance, the degree of dissociation was calculated using the following equation:

$$\alpha = \frac{\Delta A}{\Delta A_{max}} = \frac{[RFBP \bullet NR]}{[RFBP]_0}$$

which, along with the data collected from the experiment, allowed for the determination of the dissociation constant of the complex:

$$K_D = \frac{[NR][RFBP]}{[RFBP \bullet NR]} = \frac{[NR][RFBP]}{\alpha[RFBP]_0}$$

Furthermore, as a check on the technique employed in this experiment, Beer-Lambert plots were generated for stock solutions of NR and RFBP, which were expected to yield linear results due to the linear relationship between the absorbance and the concentration of the absorbing species [3].

RESULTS AND DISCUSSION

Table 1: Neutral Red Data Used for Beer's Law Plot

Trial	$V_{NR,\ total}$ (µL)	[NR] M	$A\{\lambda_{max}\}$
1	0	0.000E+00	0.0000
	200	7.709E-06	0.0787
	200	7.709E-06	0.0767
	400	1.413E-05	0.1664
	400	1.413E-05	0.1643
	600	1.957E-05	0.2415
	600	1.957E-05	0.2413
	800	2.423E-05	0.3060
	800	2.423E-05	0.3067
2	0	0.000E+00	0.0000
	200	7.709E-06	0.0903
	200	7.709E-06	0.0912
	400	1.413E-05	0.1801
	400	1.413E-05	0.1814
	600	1.957E-05	0.2474
	600	1.957E-05	0.2462
	800	2.423E-05	0.3068
	800	2.423E-05	0.3062

$V_{buffer} = 2000\ \mu L$; $[NR]_{stock} = 8.480 \times 10^{-5}$ M

Dilution Factor {NR} $= \frac{V_{NR,\ total}}{V_{NR,\ total} + V_{buffer}}$

Table 1 presents the data associated with NR that was consequently used to generate a Beer-Lambert plot. Looking at the absorbance, for a given trial, it is apparent that there is some level of agreement between the duplicate measurements (to within ± 0.001 AU). However, cross-comparison of the values obtained from the two trials, shows that there is an even lower level of agreement between the absorbance values that correspond to a given concentration of NR. These apparent discrepancies may be due to the lack of experience that the students might have had with precise delivery of aliquots using micropipettes and operation of a spectrophotometer.

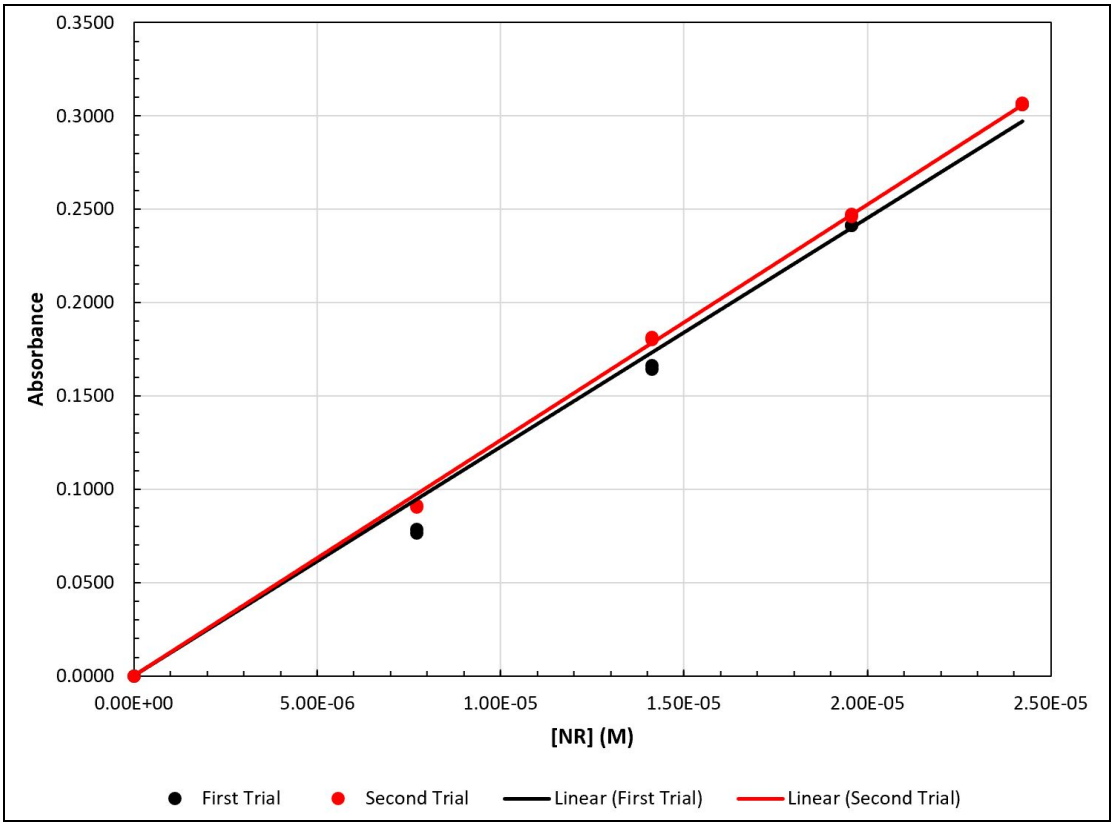

Figure 1: Plot of Absorbance vs. Concentration of NR for Two Different Trials

Figure 1 shows a comparison of the two data sets presented in Table 1. Each data set was fitted with a linear trendline, delineating the expected linear relationship between absorbance and concentration. As can be seen from the figure, the two curves appear to coincide in the range of concentrations [0.00, 7.00 μM]. However, for higher concentrations, the two curves appear to deviate further from each other. This is expected as the absorbance values were not reproducible. Furthermore, it is apparent that there is an appreciable level of spread, where three data points associated with the first trial fell off their trendline.

Table 2: Slope and Standard Error From Regression

Trial	Slope (M^{-1})	Standard Error (M^{-1})
First	12270	211
Second	12618	71

Table 2 displays the data obtained from running a regression. The discrepancy between the slopes and the high values of the standard error serve as indication to the imprecision with which this experiment was conducted. Ideally, the two curves should coincide, and therefore have identical slopes. Nonetheless, there is still some level of agreement. From here, the analysis could be extended to solutions of the RFBP•NR complex. Since if Beer's Law applies to solutions of NR, it ought to apply to solutions of a complex containing NR.

Table 3: Protein - Neutral Red Complex Data Used for Beer's Law Plot

Trial	$V_{RFBP,\ total}$ (μL)	[RFBP] (M)	[NR] (M)	A{550 nm}	ΔA{550 nm}	ΔA_{corr} {550 nm}
1	0	0.00E+00	2.83E-05	0.0813	0.0000	0.0000
	0	0.00E+00	2.83E-05	0.0828	0.0000	0.0000
	50	5.56E-06	2.78E-05	0.1027	0.0207	0.0220
	50	5.56E-06	2.78E-05	0.1030	0.0210	0.0223
	100	1.09E-05	2.74E-05	0.1227	0.0407	0.0433
	100	1.09E-05	2.74E-05	0.1227	0.0407	0.0433
	150	1.61E-05	2.69E-05	0.1394	0.0574	0.0613
	150	1.61E-05	2.69E-05	0.1404	0.0584	0.0623
	200	2.12E-05	2.65E-05	0.1461	0.0641	0.0692
	200	2.12E-05	2.65E-05	0.1455	0.0635	0.0686
	250	2.61E-05	2.61E-05	0.1493	0.0673	0.0736
	250	2.61E-05	2.61E-05	0.1487	0.0667	0.0730
	300	3.08E-05	2.57E-05	0.1460	0.0640	0.0714
	300	3.08E-05	2.57E-05	0.1458	0.0638	0.0712
2	0	0.00E+00	2.83E-05	0.0598	0.0000	0.0000
	0	0.00E+00	2.83E-05	0.0604	0.0000	0.0000
	50	5.56E-06	2.78E-05	0.0787	0.0186	0.0196
	50	5.56E-06	2.78E-05	0.0785	0.0184	0.0194
	100	1.09E-05	2.74E-05	0.0953	0.0352	0.0371
	100	1.09E-05	2.74E-05	0.0951	0.0350	0.0369
	150	1.61E-05	2.69E-05	0.1111	0.0510	0.0539
	150	1.61E-05	2.69E-05	0.1115	0.0514	0.0543
	200	2.12E-05	2.65E-05	0.1234	0.0633	0.0671
	200	2.12E-05	2.65E-05	0.1243	0.0642	0.0680
	250	2.61E-05	2.61E-05	0.1258	0.0657	0.0703
	250	2.61E-05	2.61E-05	0.1262	0.0661	0.0707
	300	3.08E-05	2.57E-05	0.1267	0.0666	0.0721
	300	3.08E-05	2.57E-05	0.1263	0.0662	0.0717

Dilution Factor {NR} = $\frac{V_{NR,\ stock}}{V_{NR,\ stock} + V_{buffer} + V_{RFBP,\ total}}$

Dilution Factor {RFBP} = $\frac{V_{RFBP,\ total}}{V_{NR,\ stock} + V_{buffer} + V_{RFBP,\ total}}$

V_{buffer} = 2000 μL; $V_{NR,\ stock}$ = 1000 μL;

$[NR]_{stock} = 8.480\times10^{-5}$ M; $[RFBP]_{stock} = 3.390\times10^{-4}$ M

Table 3 presents the data associated with the protein-NR complex, that was consequently used to construct a Beer-Lambert plot. As can be seen from the table, the duplicate values of absorbance, for a given concentration, display a reasonable level of agreement, as they should. The entries in the [RFBP] column were calculated using the following formula:

$$[\text{RFBP}] = \frac{V_{RFBP,\,total} \times [RFBP]_{stock}}{V_{buffer} + V_{NR,\,stock} + V_{RFBP,\,total}} \quad \text{(Eq. 1)}$$

$$\text{where } [\text{RFBP}]_{\text{stock}} = \frac{m_{RFBP}}{0.001 \times M_{RFBP}} \; ; \; M_{RFBP} = 29{,}200 \; \frac{g}{mol}$$

The mass of a sample of the protein was weighed on a microbalance and determined to be 9.9×10^{-3} g. Following that, a 1000μL aliquot of the buffer was delivered to the Eppendorf tube, containing the sample, to create a solution of concentration 3.390×10^{-4} M, which is $[\text{RFBP}]_{\text{stock}}$. Additionally, the concentration of the NR stock solution was provided and given to be 8.480×10^{-5}. Since with each addition of volume (of RFBP) the mixture became more dilute, the total concentrations of both RFBP and NR were recalculated for each sequential addition. Additional information provided in Table 3 is both $\Delta\text{A}\{550\text{ nm}\}$ and $\Delta\text{A}_{\text{corr}}\{550\text{ nm}\}$, where both of these quantities were calculated using the following equations:

$$\Delta\text{A}\{550\text{ nm}\} = A\{550\,nm\} - A\{550\,nm, w/o\,RFBP\} \quad \text{(Eq. 2)}$$

$$\Delta\text{A}_{\text{corr}}\{550\text{ nm}\} = A\{550\,nm\} - A\{550\,nm, w/o\,RFBP\} \times \left(\frac{3000\;\mu L}{(3000 + V_{RFBP,\,total})\mu L}\right) \quad \text{(Eq. 3)}$$

In the above two equations, the absorbance without RFBP corresponds to the entries in the table where the concentration of RFBP is zero. Since two values of absorbance were collected for each concentration, $A\{550\,nm, w/o\,RFBP\}$ refers to the average of those two values. Furthermore, it is important to note that the absorbance was recorded at 550 nm, as this the λ_{max} of the complex.

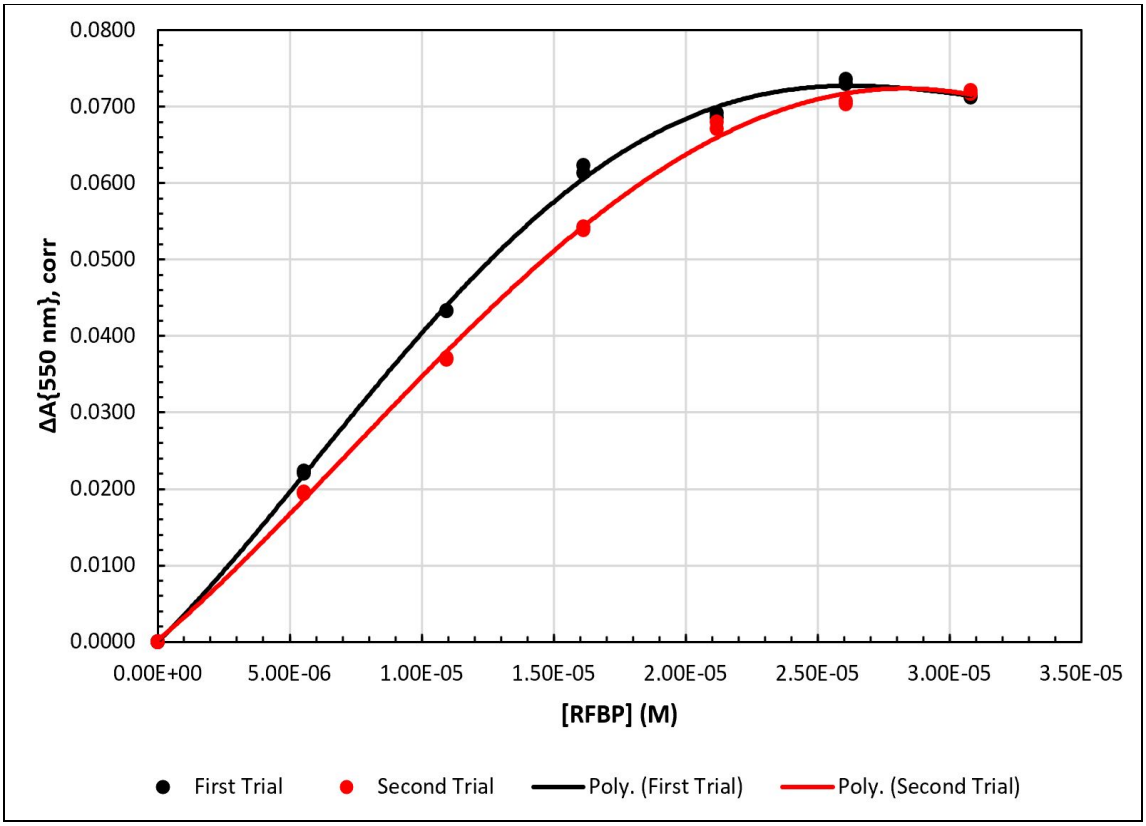

Figure 2: Plot of Corrected Absorbance vs. Concentration of RFBP for Two Different Trials

As is apparent from Figure 2, there is a considerable amount of deviation that arises between the two curves. Ideally, the two curves ought to be superimposable. This is because, for a given concentration, the solution should exhibit similar absorbance. However, from looking at the values tabulated in Table 3, it is obvious that this is not the case. Cross-comparison shows that there is an appreciable level of dissimilarity between the absorbance values for a given concentration in trial 1 versus trial 2. This may be due to the inexperience of the student operating the spectrophotometer. For instance, not waiting till the reading on the device is stable. Furthemore, it is obvious that both data sets do not follow a linear fit. That is, there is significant curvature, especially at the high end of absorbance. This could be due to a variety of reasons. For instance, there could have been an unknown in the sample, that interfered with the measurements. Alternatively, the device might have not operated as expected (i.e. instrumental malfunctioning). Yet, another reason could be imprecision on the part of the student. In other words, the data sets collected do not seem to obey the Beer-Lambert Law.

Table 4: Protein - Neutral Red Complex Data Used for Determination of Dissociation Constant

Trial	[NR] (M)	[RFBP] (M)	[NR][RFBP] (M^2)	ΔA_{corr} {550 nm}	α	[RFBP • NR] (M)
1	2.78E-05	5.56E-06	1.55E-10	0.0220	0.2990	1.66E-06
	2.78E-05	5.56E-06	1.55E-10	0.0223	0.3031	1.68E-06
	2.74E-05	1.09E-05	2.99E-10	0.0433	0.5886	6.44E-06
	2.74E-05	1.09E-05	2.99E-10	0.0433	0.5886	6.44E-06
	2.69E-05	1.61E-05	4.35E-10	0.0613	0.8327	1.34E-05
	2.69E-05	1.61E-05	4.35E-10	0.0623	0.8463	1.37E-05
	2.65E-05	2.12E-05	5.62E-10	0.0692	0.9404	1.99E-05
	2.65E-05	2.12E-05	5.62E-10	0.0686	0.9323	1.98E-05
	2.61E-05	2.61E-05	6.80E-10	0.0736	1.0000	2.61E-05
	2.61E-05	2.61E-05	6.80E-10	0.0730	0.9918	2.59E-05
	2.57E-05	3.08E-05	7.92E-10	0.0714	0.9707	2.99E-05
	2.57E-05	3.08E-05	7.92E-10	0.0712	0.9680	2.98E-05
2	2.78E-05	5.56E-06	1.55E-10	0.0196	0.2718	1.51E-06
	2.78E-05	5.56E-06	1.55E-10	0.0194	0.2690	1.50E-06
	2.74E-05	1.09E-05	2.99E-10	0.0371	0.5154	5.64E-06
	2.74E-05	1.09E-05	2.99E-10	0.0369	0.5126	5.61E-06
	2.69E-05	1.61E-05	4.35E-10	0.0539	0.7474	1.21E-05
	2.69E-05	1.61E-05	4.35E-10	0.0543	0.7530	1.22E-05
	2.65E-05	2.12E-05	5.62E-10	0.0671	0.9305	1.97E-05
	2.65E-05	2.12E-05	5.62E-10	0.0680	0.9430	2.00E-05
	2.61E-05	2.61E-05	6.80E-10	0.0703	0.9758	2.55E-05
	2.61E-05	2.61E-05	6.80E-10	0.0707	0.9814	2.56E-05
	2.57E-05	3.08E-05	7.92E-10	0.0721	1.0000	3.08E-05
	2.57E-05	3.08E-05	7.92E-10	0.0717	0.9944	3.07E-05

$$\alpha = \frac{[RFBP \bullet NR]}{[RFBP]_0} \quad \text{(Eq. 4)}$$

$$\alpha = \frac{\Delta A}{\Delta A_{max}} \quad \text{(Eq. 5)}$$

Table 4 shows the concentration of the protein-neutral red complex, which was calculated using the relationship for the parameter, α, as shown in Eq. 4, defined as the ratio of the complex concentration at any concentration of NR to the total concentration of RFBP, $[RFBP]_0$. $[RFBP]_0$ encompasses both the concentrations of free and bound RFBP in solution. The previously calculated [RFBP] is the concentration of the free protein in solution; however, it can be assumed that $[RFBP]_0 \cong [RFBP]$, since only a small amount of the protein is bounded in the complex. Furthermore, since the change in physical properties of the solution is directly dependent on the concentration of the complex, α can alternatively be defined as the ratio of absorbance change to maximum absorbance change, when RFBP is saturated with NR. For this analysis, the corrected absorbance was used as it took into account the dilution of NR, which otherwise would have had a significant impact on the determination of the complex's concentration. From the listed values of ΔA_{corr} in the table, $\Delta A_{corr,\,max}$ was found to be 0.0736 and 0.0721 for trials 1 and 2, respectively. Therefore, using Eq. 5, the α values were calculated and tabulated in Table 4. After α was obtained, the concentration of the protein-neutral red complex, for each absorbance value, was easily determined from Eq. 4. From the calculated values for the concentration of the complex, the dissociation constant was determined.

$$RFBP \bullet NR \rightarrow NR + RFBP \text{ (Reaction 1)}$$

$$K_D = \frac{[NR][RFBP]}{[RFBP \bullet NR]} \text{ (Eq. 6)}$$

$$[NR][RFBP] = K_D[RFBP \bullet NR] \text{ (Eq. 7)}$$

Reaction 1 demonstrates the dissociation reaction of the protein-neutral complex into its free, separate forms. The equilibrium dissociation constant, K_D defined as Eq. 6, can further be rearranged into a linear form, as shown in Eq. 7.

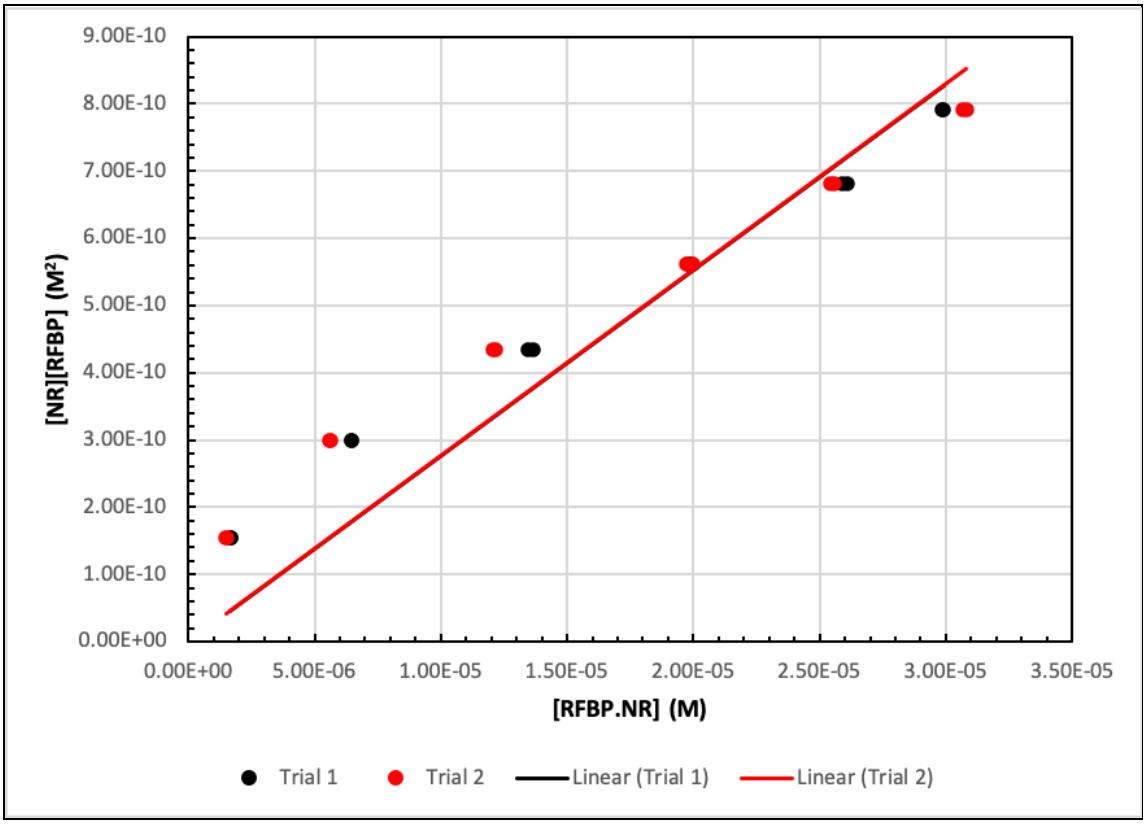

Figure 3: Plot of [NR][RFBP] vs. Concentration of Protein - Neutral Red Complex

By plotting the product of the concentrations of NR and RFBP against the concentration of the complex, with intercept zero, the values of K_D were determined as the slopes of the lines. This plot is shown in Figure 3, where the equations of the two trendlines corresponding to trials 1 and 2 are listed below:

$$\text{Trial 1: } [NR][RFBP] = (2.77 \pm 0.12) \times 10^{-5}\,[RFBP \bullet NR] \quad \text{(Eq. 8)}$$

$$\text{Trial 2: } [NR][RFBP] = (2.77 \pm 0.14) \times 10^{-5}\,[RFBP \bullet NR] \quad \text{(Eq. 9)}$$

K_D was determined to be $(2.77 \pm 0.12) \times 10^{-5}$ and $(2.77 \pm 0.14) \times 10^{-5}$ M for trials 1 and 2, respectively, which are one magnitude greater than the literature value of $(2.2 \pm 0.3) \times 10^{-6}$ M [4]. From visual inspection, the trendlines seem to poorly fit the data points, and by performing a regression analysis, the R^2 values were found to be 0.981 and 0.972, for trials 1 and 2 respectively. Instead, the data points could be fitted with higher order polynomials, which would yield higher R^2 values. However, the value of K_D is constant and therefore, the slope of the curve should not vary. This unexpected behavior might be due to the existence of foreign particles in the solution or malfunctioning of instruments that might have interfered with the procedure.

CONCLUSION

Through this rigorous analysis, many observations were made. From the Beer-Lambert plot for Neutral Red, it was apparent that there was an overall good agreement between the curves obtained for the two independent trials. However, for the Riboflavin Binding Protein, there was a considerable deviation between the curves corresponding to the two trials. Additionally, neither trial exhibited the expected Beer-Lambert linear behavior. All of these discrepancies culminated in the inaccurate values obtained for the dissociation constants. Although the values $(2.77 \pm 0.12) \times 10^{-5}$ M and $(2.77 \pm 0.14) \times 10^{-5}$ M (which correspond to trials 1 and 2, respectively) are in close agreement with one another, both of these values greatly deviate from the literature value. These inconsistencies could be attributed to unknown interference, instrumental malfunctioning, or lack of experience with the use of micropipettes and/or spectrophotometers.

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