Chadha, J. S. (1970), *Chem. Phys. Lipids 4,* 104.
Chen, P. S., Jr., Toribara, T. Y., and Warner, H. (1956), *Anal. Chem. 28,* 1756.
Eyring, H., and Jhon, M. S. (1969), Significant Liquid Structures, New York, N.Y., Wiley.
Gaffney, B. J., and Chen, S. (1977), *Methods Membr. Biol. 8,* 291.
Janiak, M. J., Small, D. M., and Shipley, G. G. (1976), *Biochemistry 15,* 4575.
Kawato, S., Kinosita, K., and Ikegami, A. (1977), *Biochemistry 16,* 2319.
Ladbrooke, B. D., and Chapman, D. (1969), *Chem. Phys. Lipids 3,* 304.
Lentz, B. R., Barenholz, Y., and Thompson, T. E. (1976a), *Biochemistry 15,* 4521.
Lentz, B. R., Barenholz, Y., and Thompson, T. E. (1976b), *Biochemistry 15,* 4529.
Luna, E. J., and McConnell, H. M. (1977), *Biochim. Biophys. Acta 466,* 381.
Mabrey, S., and Sturtevant, J. M. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3862.
Mabrey, S., and Sturtevant, J. M. (1977), *Biochim. Biophys. Acta 486,* 444.
Moore, W. J. (1962), Physical Chemistry, Englewood Cliffs, N. J., Prentice Hall, Chapter 5.
Nagle, J. F., and Wilkinson, D. A. (1978), *Biochim. Biophys. Acta* (in press).
Rand, P. R., Chapman, D., and Larsson, K. (1975), *Biophys. J. 15,* 1117.
Robles, E. C., and Van den Berg, D. (1969), *Biochim. Biophys. Acta 187,* 520.
Seltz, H. (1934), *J. Am. Chem. Soc. 56,* 307.
Shinitzky, M., and Barenholz, Y. (1974), *J. Biol. Chem. 249,* 2652.
Shinitzky, M., Dianoux, A.-C., Gitler, C., and Weber, G. (1971), *Biochemistry 10,* 2106.
Sklar, L. A., Hudson, B. S., and Simoni, R. D. (1977), *Biochemistry 16,* 819.
Suurkuusk, J., Lentz, B. R., Barenholz, Y., Biltonen, R. L., and Thompson, T. E. (1976), *Biochemistry 15,* 1393.
Teale, F. W. J. (1969), *Photochem. Photobiol. 10,* 363.
Tsong, T. Y., and Kanehisa, M. I. (1977), *Biochemistry 16,* 2674.
Vaughan, D. J., and Keough, K. M. (1974), *FEBS Lett. 47,* 158.
Weber, G. (1953), *Adv. Protein Chem. 8,* 415.

Binding of Recrystallized and Chromatographically Purified 8-Anilino-1-naphthalenesulfonate to *Escherichia coli lac* Repressor†

Sheldon S. York,* Robert C. Lawson, Jr., and Dilip M. Worah

ABSTRACT: 8-Anilino-1-naphthalenesulfonate (Ans), recrystallized from water as the magnesium salt, contains a fluorescent impurity representing 0.3% of the absorbance at 351 nm. This impurity can be removed by Sephadex LH-20 chromatography. The chromatographic and spectral properties of this impurity suggest that it is bis(Ans), a dimer of Ans. This bis(Ans) impurity makes a significant contribution to the fluorescence increment observed when *lac* repressor is added to recrystallized Ans. This occurs because bis(Ans) binds much more tightly to this protein than does Ans. The dissociation constant divided by the number of binding sites per subunit is 3.1×10^{-6} M for bis(Ans); the corresponding value for Ans is $> 1 \times 10^{-4}$ M. Because of their differing absorption spectra, the impact of this bis(Ans) impurity is especially large with excitation wavelengths above 400 nm. Users of recrystallized Ans should consider the potential consequences of this impurity whenever working with a protein to which Ans binds weakly.

The fluorescent compound 8-anilino-1-naphthalenesulfonate has been commonly used as a probe of protein structure (Brand & Gohlke, 1972). This probe binds noncovalently to a variety of proteins, typically with a large decrease in the wavelength of maximum fluorescence emission and a large increase in the fluorescence quantum yield. These changes are favored by binding sites that are nonpolar (Stryer, 1965) and act rigidly within a nanosecond time scale (Gafni et al., 1977).

We have used Ans[1] to probe structural changes within the *lac* repressor of *Escherichia coli* (Worah et al., 1978). During the course of this study, we came to realize that Ans, purified by recrystallization of the magnesium salt from water, contains an impurity that contributes significantly to the fluorescence enhancement observed in the presence of *lac* repressor. We report a method for removing this impurity from recrystallized Ans that has allowed us to directly evaluate the effects of this impurity by comparing recrystallized and purified Ans. This impurity has been isolated and tentatively identified as bis(Ans), a dimer of Ans first synthesized by Rosen & Weber (1969).

We will discuss the various factors which enable this bis(Ans) impurity to make a prominent contribution to the fluorescence increment observed when a protein is added to recrystallized Ans. This study serves as a caution to the many investigators using Ans to consider the potential consequences of this bis(Ans) impurity.

Klungsøyr (1971) reported removing some darkly colored impurities from Ans by passage through a Sephadex G-25

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[1] Abbreviations used: Ans, 8-anilino-1-naphthalenesulfonate; bis(Ans), bis[4,4′-(8-anilino-1-naphthalenesulfonate)]; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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column. This study, however, did not identify these impurities, nor did it consider the contribution these impurities might make to the observed fluorescence. Very recently, Farris et al. (1978) have reported the use of Sephadex G-75 as a chromatographic material for removing traces of Ans from bis(Ans).

Materials and Methods

Materials. The purification of *lac* repressor is described in Worah et al. (1978). This protein was dialyzed for 3 to 4 h at 4 °C, with buffer changes every hour, against a standard buffer used for all fluorescence experiments. This buffer consisted of 0.1 M Tris-HCl, pH 8.0, at 25 °C, and 0.3 mM dithiothreitol.

The magnesium salt of Ans (Eastman) was recrystallized six times from water. Throughout this and subsequent procedures, Ans was kept dark. For many experiments, recrystallized Ans was further purified by passage of 8 mg (2 mL × 4 mg/mL in standard buffer) through a 1 × 14 cm Sephadex LH-20 column equilibrated with standard buffer. Ans emerged from this column after approximately 14 column volumes of standard buffer had passed through. The peak fraction contained 6×10^{-4} M Ans.

The bis(Ans) impurity was isolated from 40 mg of recrystallized Ans (4 mL × 10 mg/mL in standard buffer) on a 0.9 × 8 cm Sephadex LH-20 column equilibrated with standard buffer. After loading Ans, the column was washed with 375 mL of standard buffer and 125 mL of 1 mM Tris-HCl, pH 8.0, at 25 °C, 0.3 mM dithiothreitol to remove all traces of Ans. The bis(Ans) impurity was then completely eluted with 50 mL of water.

Bis(Ans) was obtained from Regis Chemical Co. EM Reagents silica gel plates (No. 5763, 0.25 mm thick) were used for TLC. All other chemicals were reagent grade.

Fluorescence Measurements. All fluorescence measurements were performed on a Perkin-Elmer MPF-3 fluorescence spectrophotometer, using a 4-mm pathlength cuvette thermostated to 25 °C. Typical excitation and emission spectral band widths for experiments employing Ans were 7 and 6 nm, respectively. The stability of the fluorimeter during the course of an experiment was monitored using 1×10^{-6} M quinine sulfate (Sigma) in 0.1 N H_2SO_4. Fluorescence spectra were not corrected for variations with wavelength in the lamp intensity, photomultiplier response, or optical efficiency.

The fluorescence intensity arising from Ans complexed with repressor was calculated as follows. Fluorescence intensity = $(F_{RAns} - F_{Ans})$ (inner filter correction factor) $- (F_R - F_B)$, where F_{RAns} is the fluorescence intensity of a mixture of repressor and Ans in buffer, F_{Ans} is the fluorescence intensity of the same concentration of Ans in buffer, F_R is the fluorescence intensity of the same concentration of repressor in buffer, and F_B is the fluorescence intensity of buffer alone. Corrections were also made for dilution effects where appropriate. Subtracting the fluorescence intensity of an equal concentration of Ans from that of the mixture of repressor and Ans is justified because under the experimental conditions employed, very little Ans is actually bound to the repressor (see Results). The term $(F_R - F_B)$ corrects for contributions made by repressor alone to the total fluorescence intensity. Both solutions containing Ans experienced an inner filter effect that was absent with repressor or buffer alone. The inner filter correction factor was calculated from the relationship (Brand & Witholt, 1967): $2.3 A_\lambda \Delta l/(10^{-A_\lambda l})(1 - 10^{-A_\lambda \Delta l})$, where A_λ is the absorbance of the solution in a 1-cm pathlength at the exciting wavelength, Δl (0.3 cm) is the width of the cuvette observed by the detection system, and l (0.05 cm) is the distance through which the exciting light travels before it reaches this region of the solution.

Fluorescence spectra were recorded with 275 μL of sample. Titrations where the concentration of Ans was progressively increased were performed by adding aliquots of Ans, totaling from 20 to 35 μL, to 250 μL of initial solution. Titrations where the concentration of repressor subunits was progressively decreased were performed as follows. The initial solution (250 μL) contained the highest repressor concentration. Aliquots were then progressively removed and replaced with 1.3×10^{-5} M Ans.

The concentrations of *lac* repressor, Ans, and bis(Ans) were determined spectrophotometrically, using the following values. *lac* repressor: $A_{280nm}^{1mg/mL} = 0.59$ (Huston et al., 1974), subunit mol wt = 37 500 (Beyreuther et al., 1973). Ans: $\epsilon_{351nm} = 6000$ M^{-1} cm^{-1} (Ferguson & Cahnmann, 1975). Bis(Ans): $\epsilon_{385nm} = 16\,790$ M^{-1} cm^{-1} (Farris et al., 1978).

Results

Recrystallized Ans Can be Further Purified by Sephadex LH-20 Chromatography. The magnesium salt of Ans (Eastman), after six recrystallizations from water, contains small amounts of a fluorescent contaminant that can be seen by thin-layer chromatography. When 50 μg of recrystallized Ans is spotted on a silica gel plate and developed with a chloroform–methanol–acetic acid solvent (14:5:1), two fluorescent spots are clearly visible: one very bright with a R_f of 0.55 attributed to Ans, and one faint with a R_f of 0.22. All other commercial sources of Ans that we have tested, some of them reportedly highly purified, have also shown this contaminant.

This impurity in recrystallized Ans can be removed by passing the Ans through a Sephadex LH-20 column equilibrated with standard buffer. Using a 1 × 14 cm column, Ans is eluted in about 14 column volumes of buffer, at which time at least two different fluorescent impurities remain on the column. One of these impurities migrates 10% of the way down the column in this time, while the other remains at the top of the column. The Ans recovered from this column is pure as judged by TLC.

Recrystallized and Column Purified Ans Fluoresce Differently in the Presence of lac Repressor. The addition of *lac* repressor to either recrystallized or column purified Ans enhances the fluorescence intensity and blue shifts the emission maximum (Figure 1). Greater enhancements are observed with recrystallized Ans, especially when exciting at 410 nm. The emission spectrum due solely to the complex formed between column purified Ans and repressor was derived from the data given in Figure 1B, as described in Materials and Methods, and is shown in Figure 2. The emission maximum of this complex is 468 nm, as compared with 515 nm for Ans alone.

The difference between the spectrum recorded with repressor and recrystallized Ans and that recorded with repressor and column purified Ans represents the emission spectrum of the impurity within recrystallized Ans complexed with repressor. This spectrum, calculated from the data given in Figure 1B, is also shown in Figure 2. Its emission maximum, 482 nm, is higher than that of the column purified Ans–repressor complex. Because of this, the relative contributions made by column purified Ans and impurity to the emission spectrum of the recrystallized Ans–repressor complex, obtained by adding the two spectra shown in Figure 2, depend on the emission wavelength. This dependence is shown in Figure 2, where the ratio of fluorescence due to the impurity–repressor complex vs. the fluorescence due to the column purified Ans–repressor complex is plotted. This ratio increases twofold

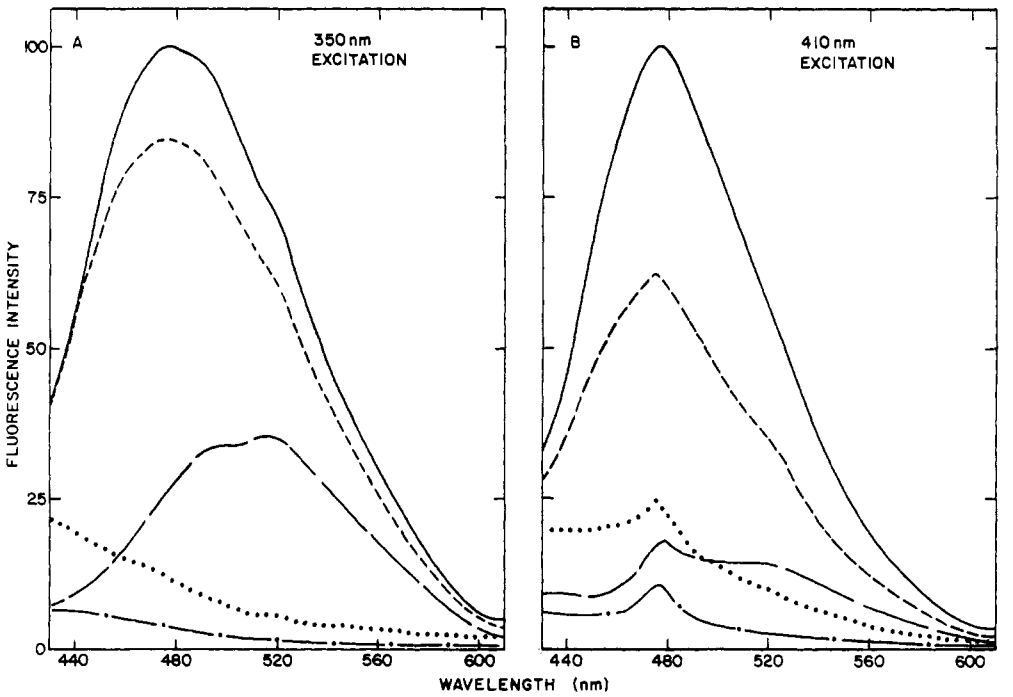

FIGURE 1: Emission spectra of recrystallized and column purified Ans mixed with *lac* repressor. (A) Excitation wavelength = 350 nm; (B) excitation wavelength = 410 nm. [Ans] = 3.0×10^{-5} M; [repressor subunits] = 1.8×10^{-5} M in A and 2.2×10^{-5} M in B. Repressor + recrystallized Ans (—); repressor + column purified Ans (- - -); column purified Ans (— —); repressor (• • •); standard buffer (— • —). The emission spectra of recrystallized Ans are virtually the same as column purified Ans and are not shown. For each excitation wavelength, fluorescence intensities have been normalized to give a maximum of 100. Had B been plotted on the same relative fluorescence intensity scale as A, the maximum fluorescence intensity would have been 54. A Raman scattering peak is observed at 475 nm with 410-nm excitation.

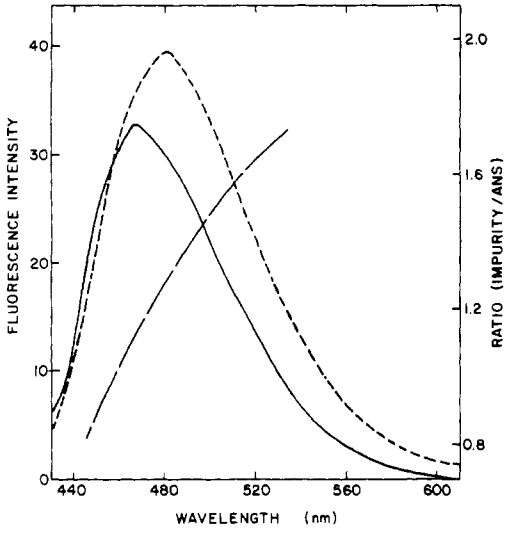

FIGURE 2: Emission spectra of column purified Ans (—) and impurity within recrystallized Ans (- - -) complexed with *lac* repressor, obtained from data presented in Figure 1B (410-nm excitation). Both spectra have been corrected for inner filter effects (correction factor = 1.013). The ratio of fluorescence intensities (impurity/Ans) is plotted (— —).

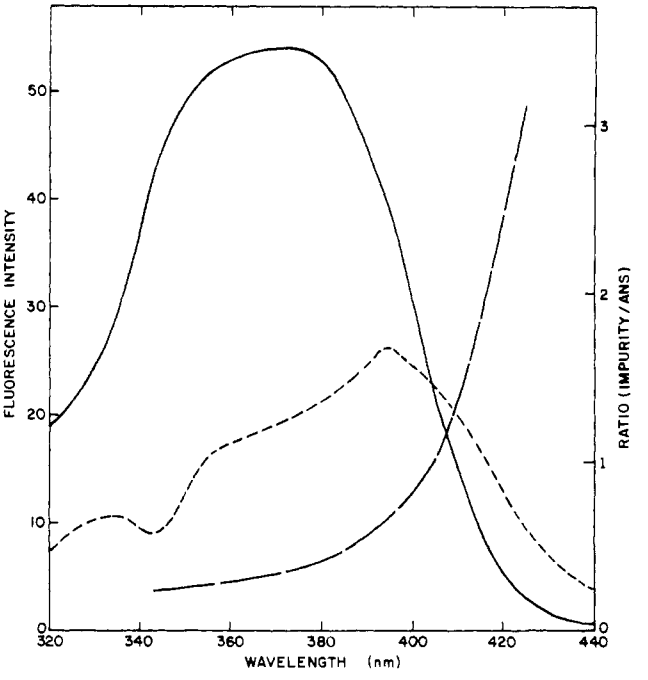

FIGURE 3: Excitation spectra of column purified Ans and impurity within recrystallized Ans complexed with *lac* repressor. The spectrum with column purified Ans (—) was obtained as described in Materials and Methods. The spectrum with impurity (- - -) represents the difference between spectra of recrystallized and column purified Ans mixed with repressor. Emission wavelength = 470 nm; [Ans] = 3.0×10^{-5} M; [repressor subunits] = 2.2×10^{-5} M. Both spectra have been corrected for inner filter effects, which vary with the excitation wavelength (correction factor = 1.086 at 350 nm and 1.013 at 410 nm). The ratio of fluorescence intensities (impurity/Ans) is plotted (— —).

when the emission wavelength changes from 445 to 535 nm; with an emission wavelength of 470 nm, this ratio is 1.2.

The emission maxima obtained by replotting the data shown in Figure 1A are essentially the same, 467 nm for the column purified Ans-repressor complex and 484 nm for the impurity-repressor complex (spectra not shown). The major difference when exciting at 350 nm is that the impurity contributes relatively little fluorescence, the ratio of the contributions from impurity to column purified Ans being only 0.28 at an emission wavelength of 470 nm.

Excitation spectra of the column purified Ans-repressor and impurity-repressor complexes are shown in Figure 3. The excitation maximum of the column purified Ans-repressor complex is about 370 nm, as compared with 357 nm for free

Ans. In contrast, the excitation maximum for the impurity–repressor complex is 395 nm. Because of this rather large difference in excitation maxima, there is a great variation in the relative contributions at different exciting wavelengths made by column purified Ans and impurity to the excitation spectrum of the recrystallized Ans–repressor complex. This latter spectrum can be obtained by adding the two spectra shown in Figure 3. The ratio of fluorescence due to the impurity–repressor complex vs. the fluorescence due to the column purified Ans–repressor complex varies from 0.26 when exciting at 350 nm to 3.1 when exciting at 425 nm (Figure 3).

Recrystallized Ans Contains bis(Ans). The impurities within recrystallized Ans that are removed by Sephadex LH-20 chromatography can themselves be recovered from this column. The impurity which migrates slowly in standard buffer can be rapidly eluted with water. The amount eluted is sufficient to contribute 0.3% of the absorbance at 351 nm of recrystallized Ans. It appears to be the contaminant in recrystallized Ans observed on TLC, for its R_f is also 0.22. The other impurity resolved on the Sephadex LH-20 column can be rapidly eluted with ethanol. It contributes less than 0.1% to the absorbance at 351 nm of recrystallized Ans.

The impurity eluted with water closely resembles bis(Ans). The two comigrate on Sephadex LH-20 columns and TLC plates. The absorption spectrum of this impurity from 230 to 450 nm is quite similar to that of bis(Ans). The large fluorescence enhancement of bis(Ans) in the presence of *lac* repressor provides a more sensitive means of comparing these two substances. Both the excitation and emission spectra of bis(Ans) and impurity complexed with *lac* repressor are shown in Figure 4. Under these conditions, the fluorescence intensities of bis(Ans) and impurity alone are negligible. While the fluorescence intensity obtained from impurity complexed with repressor is only three-fourths that obtained with bis(Ans), the shapes of the spectra are identical. It appears that this impurity isolated from recrystallized Ans consists principally of bis(Ans), but does contain another substance which contributes to the absorbance at 385 nm but not to the fluorescence in the presence of *lac* repressor.

This bis(Ans) impurity is responsible for the additional fluorescence recrystallized Ans exhibits when mixed with *lac* repressor, compared with column purified Ans. The emission and excitation spectra of this additional fluorescence, shown in Figures 2 and 3, closely resemble the spectra for bis(Ans) bound to repressor. This conclusion was verified by adding back the bis(Ans) impurity to column purified Ans so that it once again contributed 0.3% of the absorbance at 351 nm. This mixture behaved the same as recrystallized Ans when added to repressor.

lac Repressor Has a Much Lower Affinity for Ans Than for Bis(Ans). Why should the bis(Ans) impurity, which contributes only 0.3% of the absorbance at 351 nm of recrystallized Ans, contribute so much to the fluorescence increment upon addition of *lac* repressor? The relatively small fluorescence increase obtained by adding *lac* repressor to column purified Ans offers an explanation. Perhaps very little Ans is bound to the protein under our experimental conditions. By binding more tightly, similar amounts of the bis(Ans) impurity may be bound to the protein. To answer this question, we have investigated the binding of both recrystallized and column purified Ans to the repressor, by carrying out two titrations, one where the concentration of Ans is varied while the concentration of repressor subunits is fixed at 1.3×10^{-5} M, and one where [repressor subunits] is varied while [Ans] is fixed at 1.3×10^{-5} M (Ingham & Suelter, 1975).

Using column purified Ans, the [repressor subunits] varied

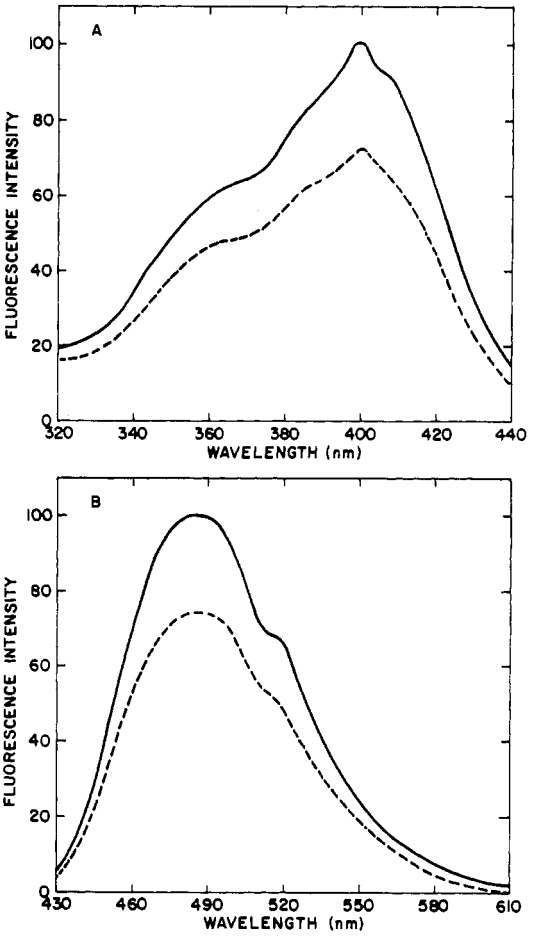

FIGURE 4: Comparison of bis(Ans) (—) and impurity isolated from recrystallized Ans (- - -) when complexed with *lac* repressor. (A) Excitation spectra, emission wavelength = 485 nm; (B) emission spectra, excitation wavelength = 385 nm. [Repressor subunits] = 1.1×10^{-5} M. Bis(Ans): $A_{385nm} = 0.0286$, [bis(Ans)] = 1.70×10^{-6} M. Impurity: $A_{385nm} = 0.0295$. Spectra were recorded with 4-nm slit widths and corrected for minor contributions from buffer, repressor, and inner filter effects.

and [Ans] varied plots coincide, whether exciting at 350 or 410 nm (Figure 5). These coincident straight lines indicate that the values of both K_{DA} and K_{DA}/n_A, where K_{DA} is the dissociation constant for Ans and n_A is the number of Ans binding sites per subunit, are large in comparison with the maximum concentrations of the varied species (approximately 45 μM). In order to reach this conclusion it is essential to carry out both titrations. A very large value of n_A would ensure a straight [Ans] varied plot over the concentration ranges employed, regardless of the value of K_{DA}. Similarly, a straight [repressor subunits] varied plot would result if n_A were 0.25 (one Ans binding site per tetramer) and K_{DA} was small enough to ensure stoichiometric binding. It is the coincidence together with the linearity of these plots which proves that the *lac* repressor has a low affinity for Ans.

The data presented in Figure 5 can be used to place a lower limit on the values of both K_{DA} and K_{DA}/n_A. If either of these values were less than 1×10^{-4} M, one of the plots would fall outside 10% error bars to the data. Since this represents the estimated maximum error in this data, the values of K_{DA} and K_{DA}/n_A must both be greater than 1×10^{-4} M. Therefore, under the conditions of our experiments, only a small fraction of the Ans is bound to the protein. This explains the relatively small fluorescence increment observed when repressor is added to Ans (Figure 1).

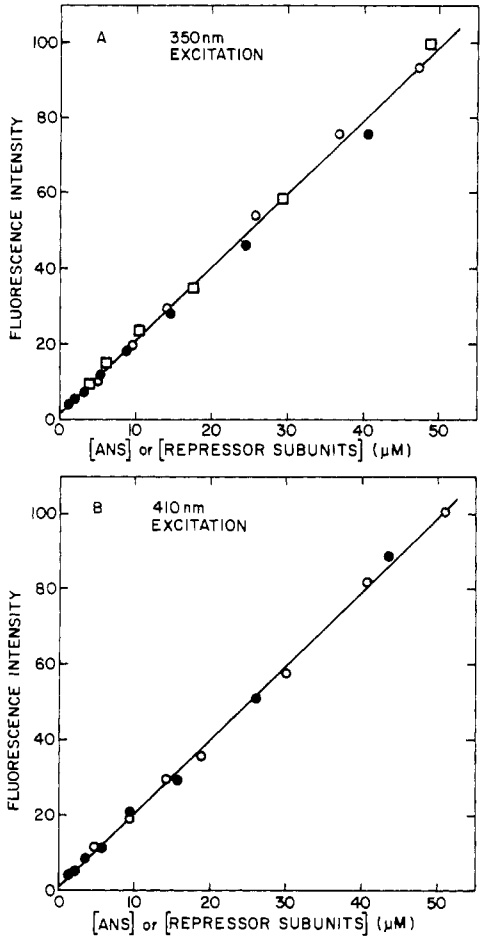

FIGURE 5: Fluorescence emission from column purified Ans complexed with *lac* repressor as [Ans] or [repressor subunits] is varied while the other remains constant at 1.3×10^{-5} M. (●) [Repressor subunits] progressively decreased; (○) [Ans] progressively increased; (□) [Ans] progressively decreased. The fluorescence intensity of the column purified Ans-repressor complex is plotted on a relative scale. (A) Excitation and emission wavelengths, 350 and 470 nm, respectively. (B) Excitation and emission wavelengths, 410 and 460 nm, respectively. The emission wavelength (460 nm) was chosen to avoid contributions from Raman scattering. Both straight lines were fit by a least-squares analysis with correlation coefficients of 0.998 for A and 0.999 for B.

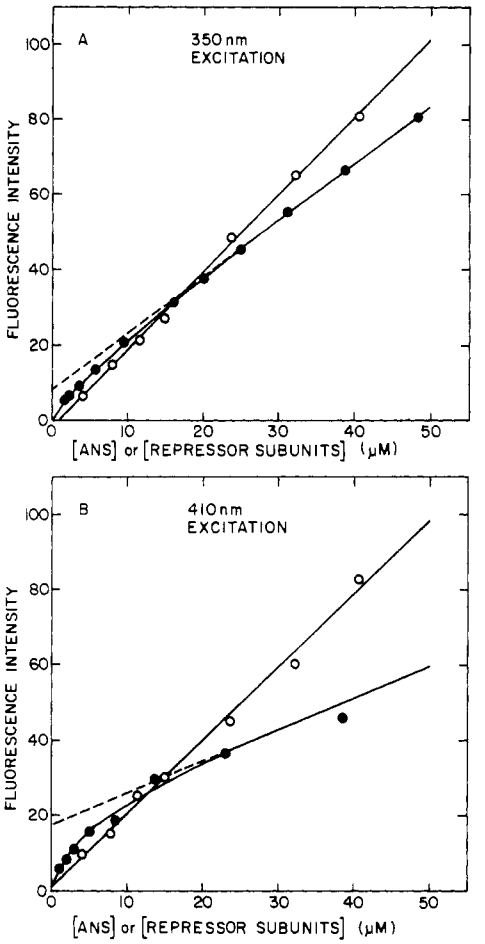

FIGURE 6: Fluorescence emission from recrystallized Ans complexed with *lac* repressor as [Ans] or [repressor subunits] is varied while the other remains constant at 1.3×10^{-5} M. (●) [Repressor subunits] progressively decreased; (○) [Ans] progressively increased. The fluorescence intensity of the recrystallized Ans-repressor complex is plotted on a relative scale. (A) Excitation and emission wavelengths, 350 and 470 nm, respectively. (B) Excitation and emission wavelengths, 410 and 460 nm, respectively. The emission wavelength (460 nm) was chosen to avoid contributions from Raman scattering. The results obtained with an emission wavelength of 470 nm were quite similar. The [Ans] varied plots were fit to straight lines by a least-squares analysis with correlation coefficients of 0.999 for A and 0.996 for B.

In order to determine the values of both K_{DA} and n_A, it would be necessary to continue these titrations to much higher concentrations of repressor and Ans. This has not been possible for the following reasons. The *lac* repressor precipitates at concentrations above 3 mg/mL in standard buffer. Thus, the highest concentration of repressor subunits obtainable in these experiments is 8×10^{-5} M. At high concentrations of Ans, inner filter effects become prohibitively large. With the 4-mm pathlength cuvette and an excitation wavelength of 350 nm, the inner filter correction factor is 1.146 at 5×10^{-5} M Ans. This problem can be alleviated only partially by exciting at 410 nm, where the absorbance is 16% of what it is at 350 nm.

Using recrystallized Ans, the [repressor subunits] varied and [Ans] varied plots differ, the differences being greater when exciting at 410 nm than when exciting at 350 nm (Figure 6). These differences are caused by the bis(Ans) impurity within recrystallized Ans. The initial rapid increase in fluorescence intensity when the concentration of repressor subunits is increased results primarily from the binding of the bis(Ans) impurity to repressor. At concentrations of repressor subunits higher than 2.5×10^{-5} M, essentially all of this impurity is bound, and the slow linear increase in fluorescence intensity from thereon is due solely to the binding of Ans. The y intercept obtained by extrapolating this linear increase (Figure 6, dashed lines) gives the fluorescence enhancement from complete binding of the bis(Ans) impurity within 1.3×10^{-5} M Ans to the repressor.

The curvature in these [repressor subunits] varied plots can be analyzed quantitatively to yield the value of K_{DI}/n_I, where K_{DI} is the dissociation constant for the bis(Ans) impurity and n_I is the number of sites per subunit where this impurity binds. The fluorescence increment due solely to the bis(Ans) impurity can be calculated by subtracting the contribution of Ans, which is obtained from the slope of the linear portion of the curve, from the total fluorescence increase. A double-reciprocal plot of this data then yields the value of K_{DI}/n_I, which is 3.1×10^{-6} M for the curved plot shown in Figure 6B. The corresponding value for Ans is at least 30 times larger. A least-squares analysis of this double-reciprocal plot indicates that the data fits a straight line very well (correlation coefficient = 0.987), reinforcing our earlier conclusion that a single impurity, i.e., bis(Ans), is responsible for this fluorescence increase.

As recrystallized Ans is progressively added to repressor, the fluorescence intensity due to complex formation increases linearly (Figure 6). Both Ans and the impurity within recrys-

tallized Ans contribute to this increase. The contribution from Ans binding is linear with respect to the concentration of recrystallized Ans because the *lac* repressor's affinity for Ans is so low. This contribution from pure Ans is equal to the slope of the linear portion of the [repressor subunits] varied plot. While the impurity binds much more tightly, its concentration is so low that only a small fraction of its binding sites are occupied. Therefore, the contribution from impurity binding is also linear with respect to the concentration of recrystallized Ans. This contribution from impurity binding can be calculated in the following manner. The extrapolated y intercept of the [repressor subunits] varied plot represents the complete binding of impurity in 1.3×10^{-5} M recrystallized Ans to the repressor. In the [Ans] varied plot, the concentration of repressor subunits is 1.3×10^{-5} M, or only enough to bind 81% of the impurity. Thus, the presence of impurity should contribute $(0.81y$ intercept$)/1.3 \times 10^{-5}$ M to the slope of the [Ans] varied plot. By adding these separate contributions from Ans and impurity, one should arrive at the slope of the [Ans] varied plot. The agreement between the calculated and observed [Ans] varied slopes is excellent. For both Figures 6A and 6B, the calculated slopes are 98% of the observed slopes.

The slopes of the [Ans] varied plot and the linear portion of the [repressor subunits] varied plot shown in Figure 6A can be compared with each other in yet another way. The ratio of these two slopes should be the same as the ratio of the fluorescence increments obtained by adding recrystallized and column purified Ans to the repressor. The agreement between these two ratios is quite good. The ratio determined from Figure 6A is 1.4, which compares favorably with the ratio of 1.3 determined by adding recrystallized and column purified Ans to 1.3×10^{-5} M repressor subunits under the same experimental conditions. For Figure 6B the linear portion of the [repressor subunits] varied plot was drawn to give a ratio of 2.3, the same as observed when recrystallized and column purified Ans were added to 1.3×10^{-5} M repressor subunits under these experimental conditions. The line drawn in this manner fits the data quite well.

Effect of Bis(Ans) Impurity Depends on Repressor Concentration. In our experiments with recrystallized Ans, the concentrations of Ans and bis(Ans) impurity have been much lower than their respective dissociation constants. Under these conditions

$$\frac{F_I}{F_A} = \frac{A_I P_I}{A_A P_A}\left[\frac{K_{DA}/n_A + [\text{repressor subunits}]}{K_{DI}/n_I + [\text{repressor subunits}]}\right] \quad (1)$$

where F_I is the fluorescence from bis(Ans) impurity bound to repressor, A_I is the absorbance of this impurity at the exciting wavelength, P_I is the probability of bound impurity fluorescing at the emission wavelength following excitation, and F_A, A_A, and P_A are the corresponding values for Ans. Knowing F_I/F_A to be 1.3 with 1.3×10^{-5} M repressor subunits and excitation and emission wavelengths of 410 and 460 nm, respectively, and assuming P_I to be equal to P_A, the value of K_{DA}/n_A is calculated to be 1.2×10^{-3} M. The value of F_I/F_A at other repressor concentrations can be obtained from the [repressor subunits] varied plot shown in Figure 6B. These values for F_I/F_A closely follow the curve predicted from eq 1 using the value for K_{DA}/n_A calculated above (Figure 7).

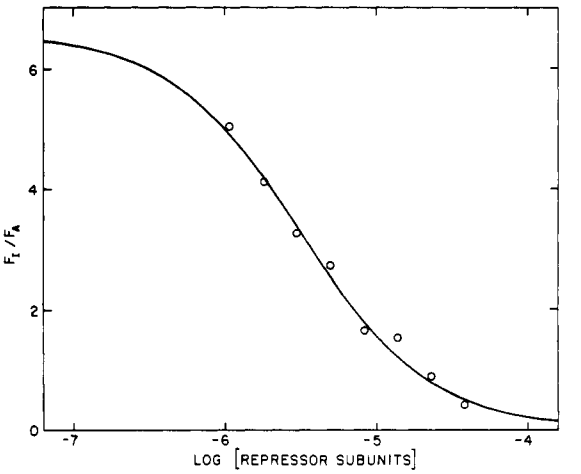

FIGURE 7: Variation of F_I/F_A with repressor concentration. F_I and F_A were obtained from the [repressor subunits] varied plot in Figure 6B, with excitation and emission wavelengths of 410 and 460 nm, respectively. F_A values were obtained from a line drawn through the origin having the same slope as the linear portion of the plot. F_I values were obtained by subtracting these F_A values from the observed fluorescence increase. The solid line is predicted by eq 1 using the following values: $A_I/A_A = 0.017$, $P_I/P_A = 1.0$, $K_{DA}/n_A = 1.2 \times 10^{-3}$ M, $K_{DI}/n_I = 3.1 \times 10^{-6}$ M.

Discussion

Ans is commonly purified by recrystallization of the magnesium salt from water. While better than 99% pure (based on absorbance at 351 nm), this recrystallized Ans contains a trace amount of bis(Ans). Our studies have shown that this bis(Ans) impurity makes a significant contribution to the fluorescence increment obtained by adding *lac* repressor to recrystallized Ans. Even so, its presence went unrecognized for some time. Neither the emission nor excitation spectra of recrystallized Ans bound to repressor revealed the presence of bis(Ans).

The experiments shown in Figure 6 provided our first indication that recrystallized Ans contained an impurity that was influencing our results. This inference was based upon the following observations. (1) The extent of curvature in the [repressor subunits] varied plots is highly dependent on the excitation wavelength. (2) Both [repressor subunits] varied plots show a linear increase in fluorescence after an initial rapid rise; they are not hyperbolic curves. (3) The magnitude of this initial rise is directly related to the difference between the slopes of the [Ans] varied plot and the linear portion of the [repressor subunits] varied plot. These results can be readily explained by the presence of an impurity, preferentially excited at higher wavelengths than Ans, which binds much more tightly to the protein than does Ans.

This inference was proven correct when column purified Ans was used in place of recrystallized Ans (Figure 5). The curvature in the [repressor subunits] varied plots is gone; these lines are now identical to the [Ans] varied plots. The coincidence of these straight lines proves that the *lac* repressor has a low affinity for Ans. Both K_{DA} and K_{DA}/n_A must be greater than 1×10^{-4} M.

It is important to realize that given only fragments of the information presented in Figure 6, one would not infer the presence of an impurity and might draw very different conclusions about the binding of Ans. For example, given only the [repressor subunits] varied plot shown in Figure 6B, one might conclude that Ans binds rather tightly to the repressor, obtaining a value of K_{DA}/n_A of approximately 1×10^{-5} M from a double-reciprocal plot.

The impact made by the bis(Ans) impurity within recrystallized Ans on the fluorescence increment observed with *lac* repressor depends on many factors. The relative contribution of the impurity is increased by selecting higher emission (Figure 2) and excitation wavelengths (Figure 3). The use of excitation wavelengths above 400 nm, in particular, greatly increases the relative contribution made by the impurity. This

is significant, for many investigators have chosen to excite Ans at these higher wavelengths in order to avoid inner filter effects.

The relative contribution of the bis(Ans) impurity within recrystallized Ans to the total fluorescence increase also depends quite strongly on repressor concentration, as described by eq 1. Of course, the bis(Ans) impurity is capable of making a significant contribution because K_{DI}/n_I is much smaller than K_{DA}/n_A. This contribution will be maximal and independent of the repressor concentration when [repressor subunits] is smaller than either K_{DA}/n_A or K_{DI}/n_I. If [repressor subunits] is much greater than K_{DI}/n_I but still much smaller than K_{DA}/n_A, the ratio of fluorescence from bound impurity to fluorescence from bound Ans will be inversely proportional to [repressor subunits]. When [repressor subunits] is much greater than K_{DA}/n_A, the contribution of impurity to the fluorescence increase will be minimal and independent of the repressor concentration.

In our experiments, the repressor concentration has ranged from about 1×10^{-6} to 5×10^{-5} M subunits, bracketing the value of K_{DI}/n_I, 3.1×10^{-6} M. Consequently, we have observed that the relative contribution of impurity depends quite strongly on repressor concentration (Figure 7).

It is interesting to note that both bovine serum albumin (Rosen & Weber, 1969) and ribosomes (Pochon & Ekert, 1973) bind bis(Ans) considerably more tightly than Ans. We find that the *lac* repressor protein behaves similarly. Should this be a common occurrence, the bis(Ans) impurity within recrystallized Ans that influenced our results might be expected to influence the results of others, whenever the concentration of Ans binding sites falls well below the dissociation constant for Ans. This situation is most easily recognized by a relatively small increase in Ans fluorescence upon addition of protein.

Prolonged exposure of an aqueous solution of Ans to ordinary fluorescent lighting can significantly increase the fluorescence intensity observed with repressor (unpublished experiments). This increase appears to be correlated with an increase in the concentration of the bis(Ans) impurity. These results emphasize the importance of minimizing the exposure of Ans to light!

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References

Beyreuther, K., Adler, K., Geisler, N., & Klemm, A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 3576.

Brand, L., & Gohlke, J. R. (1972) *Annu. Rev. Biochem. 41,* 843.

Brand, L., & Witholt, B. (1967) *Methods Enzymol. 11,* 776.

Farris, F. J., Weber, G., Chiang, C. C., & Paul, I. C. (1978) *J. Am. Chem. Soc. 100,* 4469.

Ferguson, R. N., & Cahnmann, H. J. (1975) *Biochemistry 14,* 287.

Gafni, A., DeToma, R. P., Manrow, R. E., & Brand, L. (1977) *Biophys. J. 17,* 155.

Huston, J. S., Moo-Penn, W. F., Bechtel, K. C., & Jardetzky, O. (1974) *Biochem. Biophys. Res. Commun. 61,* 441.

Ingham, K. C., & Suelter, C. H. (1975) *Arch. Biochem. Biophys. 168,* 531.

Klungsøyr, L. (1971) *Biochemistry 10,* 4875.

Pochon, F., & Ekert, B. (1973) *Eur. J. Biochem. 36,* 311.

Rosen, C.-G., & Weber, G. (1969) *Biochemistry 8,* 3915.

Rosenberg, J. M., Khallai, O. B., Kopka, M. L., Dickerson, R. E., & Riggs, A. D. (1977) *Nucleic Acids Res. 4,* 567.

Stryer, L. (1965) *J. Mol. Biol. 13,* 482.

Worah, D. M., Gibboney, K. M., Yang, L.-M., & York, S. S. (1978) *Biochemistry 17* (following paper in this issue).