Spectroscopic and Immunochemical Studies with Nitrobenzoxadiazolealanine, a Fluorescent Dinitrophenyl Analogue†

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ABSTRACT: The fluorescent nitro compound 4-(α-*N*-L-alanine)-7-nitrobenz-2-oxa-1,3-diazole (NBDA) is a structural and functional analogue of the 2,4-dinitrophenyl group (DNP). It binds to all induced anti-DNP antibodies examined and to several monoclonal immunoglobulins with nitroaromatic specificity. The fluorescence of NBDA is quenched upon binding to these proteins. Similar quenching of NBDA fluorescence is observed in the presence of aromatic amino acid analogues, and also upon binding to serum albumin and apomyoglobin. NBDA does not bind to immunoglobulins of unrelated specificity or to bovine trypsinogen. The absorption and fluorescence characteristics of NBDA in different solvents reveal large changes which correlate with medium polarity. A few important exceptions, however, exist, suggesting that NBDA is not a simple polarity probe and that its spectral properties are sensitive to specific binding interactions. The observed spectral parameters of NBDA when bound to immunoglobulins clearly indicate that binding does not occur through hydrophobic interactions only and suggest the formation of specific interactions such as a charge-transfer complex and hydrogen bonds. The IgA myeloma protein 460 binds NBDA with an association constant of 3.2×10^5 M^{-1} (at 25 °C). The bound hapten undergoes full quenching of its fluorescence and marked changes in its absorption spectrum. A large induced circular dichroism in the bound hapten's absorption is also observed. NBDA is the first environment-sensitive fluorescent probe reported to bind specifically to a homogeneous immunoglobulin. It may also be used to detect and characterize antinitroaromatic antibodies, even in crude preparations, and possibly on cell surfaces.

Nitroaromatic molecules have been extensively used as haptens both because they form good immunogens (when attached to protein carriers) and can be monitored spectroscopically. The specific interaction between these haptens and elicited antibodies or monoclonal immunoglobulins was the subject of many studies. These included binding measurements (Velick et al., 1960; Eisen et al., 1968), thermodynamic characterization (Barisas et al., 1971; Johnston et al., 1974; Halsey and Biltonen, 1975), affinity labeling (Singer and Thorpe, 1968; Haimovich et al., 1970; Goetzl and Metzger, 1971), kinetic mapping (Pecht et al., 1972; Haselkorn et al., 1974), differential absorption spectroscopy (Little and Eisen, 1967; Eisen et al., 1968), and circular dichroism (Glaser and Singer, 1971; Rockey et al., 1972). Fluorescence measurements, which often provided valuable information about ligand site interactions (Edelman and McClure, 1968; Brand and Gholke, 1972), were not effective in the investigation of these systems, since nitroaromatic compounds are, in general, nonfluorescent. The quenching of protein fluorescence, used extensively in the affinity measurements, does not report on the immediate environment of the hapten in the site, as it results from long-range (dipole–dipole) energy transfer from tryptophan residues in the whole Fab region (Velick et al., 1960).

The 4-amino derivatives of 7-nitro-benz-2-oxa-1,3-diazole (NBD)[1] were previously reported to be a rare example of fluorescent nitroaromatic compounds (Ghosh and Whitehouse, 1968). The fluorescence of these derivatives was found to be environment sensitive, and some of them were used to probe the active sites of proteases (Kenner and Aboderin, 1971; Stuchbury et al., 1975), to study hydrophobic binding sites on ribosomes (Kenner and Aboderin, 1971), and to follow ribosomal reassembly (Huang and Cantor, 1975). In all these cases NBD fluorescence was found to be enhanced upon binding, indicating an apolar environment of the ligand.

We conceived the use of NBD as a fluorescent nitrophenyl analogue in the same way that ε-ATP serves as a fluorescent analogue for ATP (Secrist et al., 1972). For this we used 4-(α-*N*-L-alanine)-NBD (NBDA) which has better water solubility and higher quantum yield in this medium than other amino NBD derivatives previously used and also contains no chromophore other than NBD itself. In two preceding communications we briefly described the binding of NBDA to nitroaromatic-specific immunoglobulins (Lancet and Pecht, 1975) and its use to elucidate the mechanism of hapten binding to immunoglobulin MOPC-460 (Lancet and Pecht, 1976). Here we report a full study of the interaction of NBDA with anti-DNP antibodies and with homogeneous immunoglobulins having nitroaromatic specificity, as reflected in the changes of absorption and fluorescence which NBDA undergoes upon binding. Based on an extensive spectropic characterization of this ligand, the results are interpreted in terms of the elementary interactions formed in nitroaromatic binding sites. These binding interactions were previously proposed to include the formation of a charge-transfer complex (Little and Eisen, 1967), and also hydrophobic interactions of nonspecific nature (Parker and Osterland, 1970). The observation that NBDA fluorescence is *quenched* upon binding to immunoglobulins is inconsistent with the site being merely a hydrophobic pocket and lends strong support to the notion of charge-transfer complexation. Information about other possible interactions is also obtained.

One of the DNP-specific homogeneous immunoglobulins which is reported here to bind NBDA is the IgA MOPC 460. This well-studied protein comprises a unique model system in that: (a) it was proposed to bind different ligands at not fully overlapping sites, thus possibly being an example for antibody

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[1] Abbreviations used: NBD, 7-nitro-benz-2-oxa-1,3-diazole; NBDA, 4-(α-*N*-L-alanine)NBD; MBD, 4-(*p*-methoxybenzylamino)-NBD; NBD-Cl, 4-chloro-NBD; DNP, 2,4-dinitrophenyl; PBS, 0.01 M phosphate buffer with 0.15 M NaCl, pH 7.4; CD, circular dichroism; NATA, *N*-acetyltryptophanamide; Ans, 8-anilino-1-naphthalenesulfonate.

multispecificity (Rosenstein et al., 1972); (b) kinetic studies have shown that upon hapten binding it undergoes a conformational transition which may be related to the triggering of antibody effector functions (Lancet and Pecht, 1976). NBDA is used here to obtain further information on the nature of interactions in the binding site of this protein.

Experimental Section

Materials. NBD-alanine (NBDA) was synthesized using the following procedure: 1×10^{-2} mol of 4-chloro-NBD (NBD-Cl) dissolved in 120 mL of ethanol was mixed with 5×10^{-2} mol of L-alanine in 80 mL of aqueous $NaHCO_3$ (0.25 M) at 50 °C. The reaction was allowed to proceed for 2 h maintaining a pH of 8.0 by occasional addition of solid $NaHCO_3$. The dark solution was diluted with water to 500 mL and passed through Amicon membrane UM20E under 5 atm, to get rid of polymerization products. The filtrate was evaporated to 50 mL, the pH was adjusted to 8.0, and aliquots of 15 mL were chromatographed on a 2.5 × 20 cm column of DEAE-cellulose (Cellex D, Bio-Rad) equilibrated with Tris buffer, 0.05 M, pH 8.0. The first minor peak of absorption at 450 nm was discarded and a second, major peak was collected. The solution was acidified with HCl to pH 2.5 and extracted with two portions of ethyl acetate, and the organic phase was dried on anhydrous $MgSO_4$ and evaporated. The red powder thus obtained gave a single fluorescent spot in thin-layer chromatography and its elemental analysis corresponded to that of NBDA. NBD-Cl was synthesized according to the procedure of Boulton et al. (1966) from 2,6-dichloronitrosobenzene (Holmes and Bayer, 1960).

Solvents used were of analytical or spectroscopic grade. All other materials were analytical grade. Human serum albumin (Cohn fraction V), bovine trypsinogen (crystallized), and horse skeletal muscle myoglobin were products of Sigma. Apomyoglobin was prepared according to the procedure of Teale (1959). Protein 460 and protein 315 were isolated from ascitic fluid of Balb/c mice bearing the corresponding tumors (MOPC-460 and MOPC-315) using the procedure of Goetzl and Metzger (1970). Plasmacytoma MOPC-315 was generously provided by Dr. M. Potter; plasmacytoma MOPC-460 was kindly provided by Dr. H. Eisen. Goat anti-DNP antibodies were obtained by immunizing animals with DNP-keyhole limpet hemocyanine (3 mg per animal in complete Freund's adjuvant). Separation was made on ε-N-DNP-lysine Sepharose 4B column and the antibodies were eluted with 0.1 M acetic acid. Goat antibody preparations were from single bleedings of the individual animals No. 8 and 44. Pooled rabbit anti-DNP antibodies were kindly donated by Dr. J. Haimovich, protein 25 (obtained from the mouse myeloma XRPC 25) was a gift of Dr. D. Givol, and pentameric IgM_{WAG} and IgM_{MAR} were gifts of Dr. H. Metzger. All binding and spectroscopic measurements in an aqueous medium were performed in 0.01 M phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS). In the case of binding to trypsinogen, an 0.05 M potassium acetate buffer, pH 5.0, containing 0.1 M KCl and 0.025 M $CaCl_2$ was used.

Methods. Absorption spectra were recorded on Cary Model 15 or 118 double-beam spectrophotometers at 25 °C. Difference spectra were measured in rectangular tandem cells (Hellma) with the reference cuvette containing the protein and ligand separately and the sample cuvette containing their mixture. NBDA extinction coefficients are based on $\epsilon_{476} = 24000\ M^{-1}\ cm^{-1}$ in PBS as determined by weight. CD measurements were performed on a Cary 60 spectropolarimeter. Fluorescence spectra and titrations were recorded in the ratio mode on Perkin-Elmer MPF3 or MPF44A spectrofluorometers equipped with a thermostated cell compartment (25 °C). It was found unnecessary to use corrected spectrum in the wavelength range employed. The quantum yield of NBDA in ethanol was calculated to be $\phi = 0.24$ using the ratio of integrated emission bands with fluorescein in 0.01 M aqueous NaOH as a reference ($\phi = 0.85$, Parker and Rees, 1960). Quantum yields of NBDA in other media were calculated using peak height ratio to that in ethanol, the shape and relative width at half height being practically identical in all solvents. All spectral parameters in each solvent were determined in the same (1×10^{-5} M) NBDA solution. The values of dielectric constants were taken from Weast (1970).[2]

Fluorometric titrations were performed by adding aliquots of one solution to the other solution with a micrometric syringe (Hamilton), measuring the fluorescence intensity at a constant wavelength. In the case of protein 460 titrations were performed continuously adding one solution from a motor-driven micrometric syringe–titrator to another stirred solution. All binding measurements were made at 25 °C.

The binding constant, maximal quenching, number of active sites per protein molecule, and the heterogeneity index were calculated from titration data by means of a general nonlinear least-square parameter fit program using the algorithm of Powell (1971). This procedure is very sensitive to small changes of the parameters, does not require the a-priori knowledge of any of the parameters, and contains no approximation. It must be stressed, however, that the determination of the number of active binding sites is possible only since the *hapten* fluorescence is monitored (see Discussion).

Results

Spectroscopic Characterization of NBDA. The absorption spectra of NBDA in H_2O, ethanol, and 1-butanol is shown in Figure 1. These spectra represent the NBD chromophore only, in contradistinction to derivatives used in previous studies (Kenner and Aboderin, 1971), where benzyl or methoxy benzyl groups were part of the ligand. Four transitions are seen, at about 470, 340, 270, and 230 nm with relative intensities of roughly 1.0, 0.3, 0.1, and 0.6, respectively. The intense longer wavelength absorption band is seen to be most influenced by the nature of the solvent and was, therefore, monitored in all the studies reported here. The fluorescence characteristics of NBDA were also found to be different for each of these solvents, both in the wavelength of maximal emission and in the quantum yield.

To examine further the applicability of NBDA as an environment probe, we measured its absorption and emission spectra in 25 different solvents. The results are summarized in Figure 2 (A–D) where the spectral properties are plotted against the dielectric constant of the solvent. These properties are found to vary over a wide range, and to be correlated with the polarity of the solvent: increasing polarity leads to lower quantum yield and higher extinction coefficient, as well as to longer wavelength for both the absorption and emission maxima. These correlations generally agree with those observed by Kenner and Aboderin (1971) and may be used to determine the properties of nitroaromatic binding sites, as discussed below.

Binding Measurements. The structural similarity between 4-amino-NBD and the corresponding DNP derivative is clearly seen in the formulas in Figure 1. The phenyl ring of NBD has

[2] The value for dimethoxybenzene was taken as equal to that of the corresponding mono derivative, in line with the general observation that meta di and mono derivatives have almost identical dielectric constants.

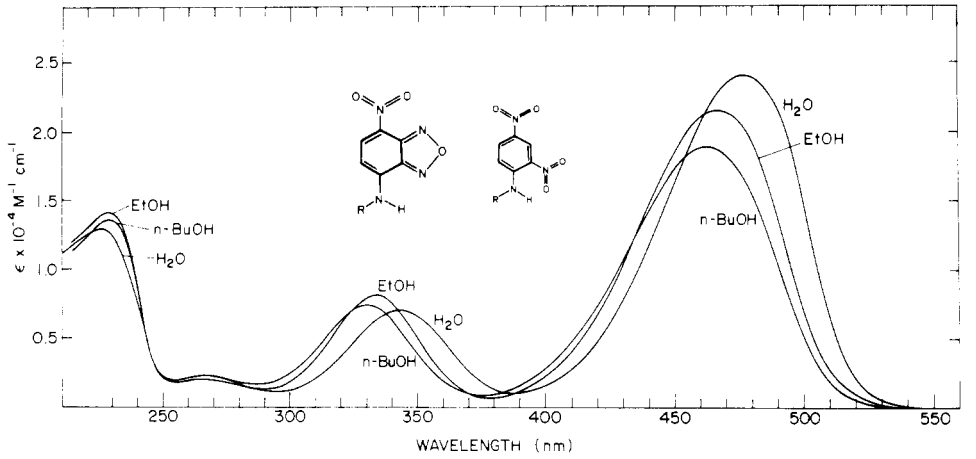

FIGURE 1: Absorption spectra of NBDA in water (pH 7), ethanol, and butanol. Spectra were taken at an NBDA concentration of 2.8×10^{-5} M, and values of ϵ are based on $\epsilon_{476} = 24\,000\ M^{-1}\ cm^{-1}$ in H_2O. Insert: Structural formula of 4-amino-NBD (left). For NBDA, R = $-CH(CH_3)COO^-$. For comparison the formula of the analogous 1-amino-DNP is also shown (right).

TABLE I: The Binding and Fluorescence Changes of NBDA with Different Proteins.[a]

No.	Protein	Q_{max} (%)	λ_{em}^{max} (bound)	K (M^{-1})	n[c]
1	Protein 460	96.9	540	3.0×10^5	2.0
2	Protein 315	36.0	543	5.0×10^4	1.8
3	Goat 8 anti-DNP	97.5	525	1.3×10^7	1.05
4	Goat 44 anti-DNP	67.6	527	2.6×10^5	2.0
5	Rabbit anti-DNP	96.0	540	1.9×10^7	1.4
6	Human serum albumin	60.9	531	1.5×10^4	1.0
7	Horse apomyoglobin	~30[b]	535	$\sim 1.0 \times 10^4$[b]	

[a] All values in this table were obtained in titrations in which the protein was added to NBDA and the fluorescence of the latter was followed (Figure 3). λ_{em}^{max} is accurate to ±2 nm and is obtained by extrapolation as described in the legend to Figure 4. In all other parameters the estimated error is ±5%. [b] Estimated from two titration points. [c] n = average number of NBDA binding sites per protein molecule.

a nitro group at a position corresponding to the 4-nitro group of 1-amino-2,4-dinitrophenyl and an oxidiazole ring closing at a position which corresponds to the 2-nitro group. In terms of molecular structure, these two compounds differ from each other only in that NBD has one nitrogen atom more and one oxygen atom less than DNP. The general dimensions and shape of the two molecules are almost identical as found by molecular model building, and their chemical nature is similar, both having phenyl rings substituted with electron attracting oxygenated nitrogens. We therefore considered the possibility that NBDA could serve as an analogue for DNP and bind to sites specific for it or for related nitroaromatic compounds (Lancet and Pecht, 1975).

In preliminary experiments, NBDA was added to concentrated solutions (>5×10^{-5} M sites) of different DNP binding immunoglobulins (in PBS), and its fluorescence (excitation at 475 nm, emission 480 to 600 nm) was compared with that of the free ligand. Three different anti-DNP antibody preparations (pooled from rabbit and from goats No. 8 and 44), three DNP binding homogeneous immunoglobulins (mouse myeloma IgA proteins 315, 460, and 25), and one *p*-nitrophenyl-specific human Waldenstrom protein (IgM_{WAG}) were checked for their binding of NBDA. In the presence of the first five proteins NBDA underwent a fluorescence change, while the last two did not cause any effect. In contrast to the fluorescence enhancement expected for binding to a site that is less polar than H_2O (cf. Figure 2A), NBDA underwent *quenching* of its fluorescence in all five cases where a change was observed. Preliminary experiments were performed also with a preparation of rabbit antibodies induced against NBDA (conjugated at its free carboxyl to keyhole limpet hemocyanine) and binding was observed followed by >80% quenching of NBDA fluorescence. Antibodies and immunoglobulins of other specificities were checked as well, and none gave any fluorescence change of NBDA. These included the phosphorylcholine binding IgA proteins HOPC-8, TEPC-15, and McPC-603, rabbit antibodies induced against 1-dimethylaminonaphthalene-5-sulfonate (DNS), human IgM_{MAR}, and bovine normal immunoglobulin, all at around 10^{-4} M sites concentration. As the fluorescence of NBDA is found to be very sensitive to environment changes, the lack of spectral effect in the presence of protein 25, IgM_{WAG}, and the heterologous immunoglobulins most probably means that they do not bind this ligand.

The interaction of NBDA with immunoglobulins was further investigated by performing fluorometric titrations in which proteins were added to a solution of NBDA, following the fluorescence of the latter. Figure 3 depicts representative titrations of this kind. The values of maximal quenching, association constant, and number of reactive binding sites per protein molecule were obtained using a parameter fit procedure (see Methods), and are listed in Table I, No. 1-5. In all cases the data fitted a simple binding isotherm, namely, homogeneous binding (heterogeneity index of 1.0).

Representative fluorescence spectra of NBDA when fully bound to immunoglobulins are given in Figure 4. It is seen that the extent of quenching and the shift in the wavelength of maximal emission vary from one protein to another. This indicates a difference of the interactions in the binding sites of these proteins. The emission parameters of NBDA when fully

bound to proteins are listed in Table I.

Other proteins were also checked for their effects on NBDA fluorescence. Human serum albumin and horse apomyoglobin, both known for their ability to bind aromatic molecules such as anilinonaphthalenesulfonate (Ans) at hydrophobic sites (Weber and Laurence, 1954; Stryer, 1965), were found to react with NBDA. Again, NBDA fluorescence was found to be quenched, in contrast to the enhancement expected upon binding due to mere hydrophobicity of the sites. A fluorometric titration with human serum albumin is shown in Figure 3A and the data for both proteins are given in Table I, No. 6 and 7. Bovine trypsinogen was checked as well and did not give rise to any spectral change of NBDA. This experiment was done under the conditions at which Kenner and Aboderin (1971) observed binding of methoxybenzyl-NBD (MBD) to this protein, giving rise to a threefold enhancement of the ligand's fluorescence. The possible reasons for this discrepancy are discussed below.

The Interaction of NBDA with Protein 460. NBDA is found to bind to protein 460 with a relatively high association constant of 3.0×10^5 M^{-1}, and 96.9% maximal quenching of its fluorescence (Table I). This interaction was further characterized. A titration was performed in which NBDA was added to the protein, following the fluorescence of both reactants. In parallel, NBDA was also added to normal bovine immunoglobulin (nonspecific titration). NBDA quenched the protein (tryptophan) fluorescence, (via nonradiative energy transfer) to a maximum of 45%. Correction for quenching due to light absorption was made using the nonspecific titration data. At each point in the titration the fluorescence of NBDA was also found to be lower than that in the corresponding point of the nonspecific titrations. The extent of quenching of either reactant's fluorescence enabled an independent calculation of their respective fractional saturations at each point in the titration. An association constant of 3.5×10^5 M^{-1} with 1.9 active binding sites per protein molecule and a heterogeneity index of 1.0 were obtained in either mode of detection.

Inhibition experiments were also performed, in which increasing amounts of 2,4-dinitronapth-1-ol or ϵ-*N*-DNP-lysine were added to a mixture of protein 460 and NBDA. NBDA fluorescence was found to increase gradually, indicating its displacement from the protein-460-binding site.

Absorption difference spectra of bound vs. free NBDA measured in the presence of increasing concentrations of protein 460 (in PBS) are shown in Figure 5. A fitting procedure was carried out where absorbance changes at 33 wavelengths (in the range 410 to 525 nm) were plotted against the calculated concentration of bound hapten and a value of the association constant was sought so as to maximize the linearity of these plots. The minimal deviation from linearity was obtained with $K = 3.6 \times 10^5$ M^{-1} (insert of Figure 5). The slope of these plots gives the value of $\Delta\epsilon$ at each wavelength, and the difference spectrum thus constructed is shown in Figure 6. The spectrum of protein-460-bound NBDA (ϵ_b, calculated by adding $\Delta\epsilon$ to ϵ of the free ligand) is also shown. At wavelengths lower than 410, $\Delta\epsilon$ and ϵ_b represent extrapolations of those measured at the highest ligand saturation using the value of K obtained above.

The circular dichroism (CD) spectrum of free NBDA was measured and found to be very close to zero at all wavelengths (Figure 7). However, upon addition of protein 460, a large induced CD spectrum was observed. Figure 7 shows the observed effect in terms of both $\Delta\epsilon_{CD}$ and g_{abs} as a function of wavelength.

Interaction of NBDA with Aromatic Molecules. The fluorescence of NBDA was measured in the presence of aromatic

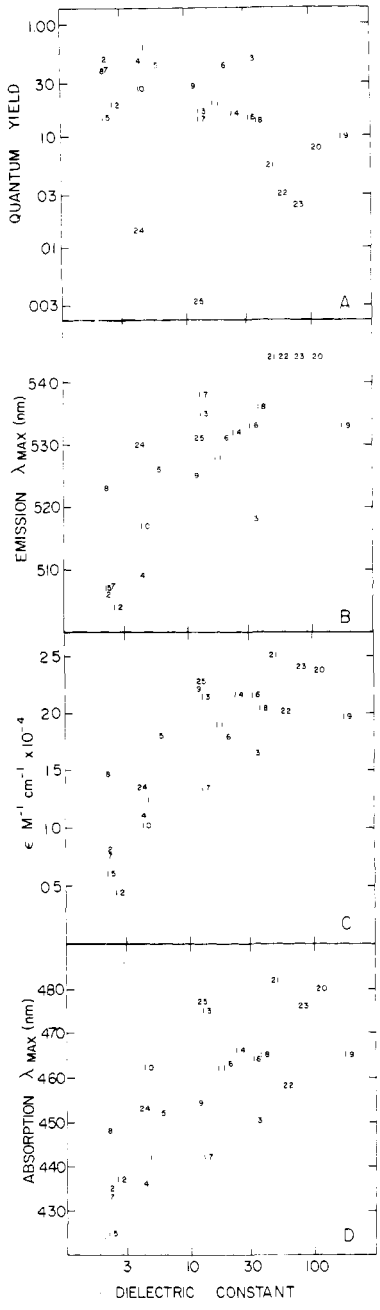

FIGURE 2: Correlation between the spectral properties of NBDA and the dielectric constant of its solvent. (A) Quantum yield; (B) wavelength of maximal emission; (C) extinction coefficient at maximal absorption; (D) wavelength of maximal absorption. C and D are for the longest wavelength absorption band. The center of the leftmost digit of a number (corresponding to a solvent in the list) denotes the x,y coordinates. Enumeration is in the order of decreasing NBDA quantum yield. Correlation coefficients are calculated as $\rho = \Sigma(y_i - \bar{y})(x_i - \bar{x})/\Sigma(y_i - \bar{y})^2\Sigma(x_i - \bar{x})^2]^{1/2}$, where x and y are values on the abscissa and ordinate, respectively, subscript i denotes an individual value, and a bar denotes the mean value. The values obtained are: quantum yield, $\rho = -0.38$ with points no. 24, 25; $\rho = -0.67$ when these points are omitted. Emission maximal wavelength, $\rho = 0.81$; extinction coefficient, $\rho = 0.80$; absorption maximal wavelength, $\rho = 0.75$. Logarithmic scales were used for the dielectric constant and quantum yield due to the wide range of values involved. A table listing the values of the dielectric constants and all spectral parameters is given as supplementary material for this paper (see paragraph concerning supplementary material at the end of this paper). Solvents are as follows: (1) chloroform; (2) benzene; (3) nitromethane; (4) diethyl ether; (5) ethyl acetate; (6) acetone; (7) toluene; (8) dioxane; (9) pyridine; (10) methoxybenzene (anisole); (11) butanol; (12) carbon disulfide; (13) benzyl alcohol; (14) ethanol; (15) carbon tetrachloride; (16) methanol; (17) acetic acid; (18) ethylene glycol; (19) *N*-methylformamide; (20) formamide; (21) dimethyl sulfoxide; (22) formic acid; (23) water; (24) *m*-dimethoxybenzene; (25) *m*-hydroxytoluene (*m*-cresol).

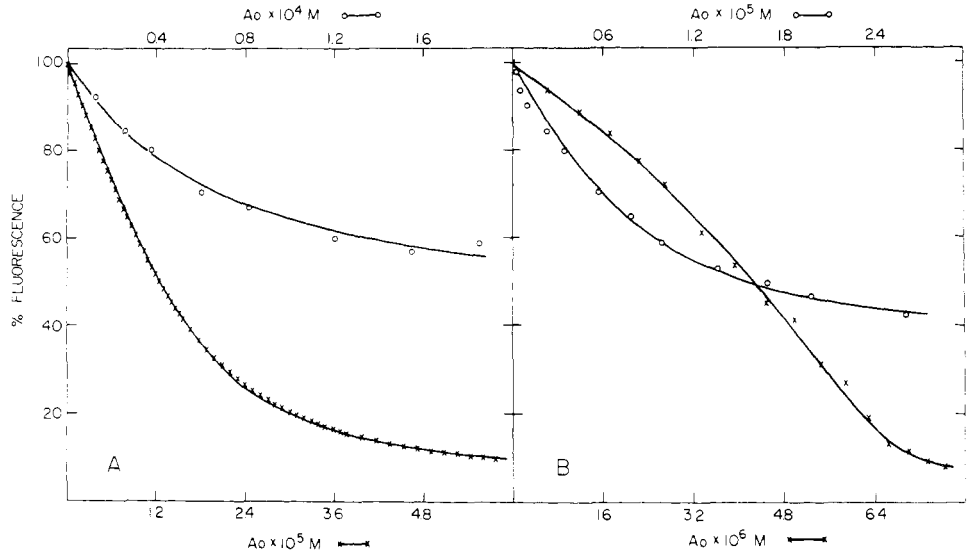

FIGURE 3: Quenching curves obtained in fluorometric titrations in which proteins were added to NBDA and the ligand fluorescence was followed (excitation 475 nm, emission 540 nm). A_0 is total added protein molar concentration. Points are experimental and curves are calculated with the best fit parameters listed in Table I (see Experimental Section). A: (X) MOPC-460, initial NBDA concentration $H_i = 1.6 \times 10^{-5}$ M; (O) HSA, $H_i = 7.3 \times 10^{-6}$ M. B: Anti-DNP; (X) rabbit, pooled, $H_i = 5.7 \times 10^{-6}$ M; (O) goat 44, $H_i = 7.3 \times 10^{-6}$ M. The hapten is diluted with added protein down to a value of 0.60–0.80 of the initial. The apparent sigmoidal shape of curve (X) in B is due to this dilution effect. Dilution is taken into account in the fit procedure, and all calculated curves represent binding with a single, homogeneous association constant.

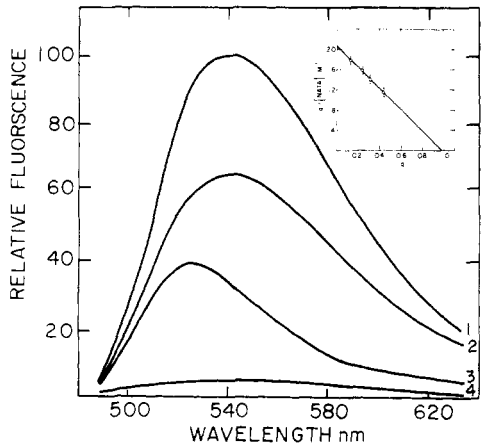

FIGURE 4: Fluorescence spectra of NBDA (in PBS), free (1) and fully bound to protein 315 (2), anti-DNP, goat 44 (3), and protein 460 (4). Spectra 2, 3, and 4 are originally obtained at an incomplete saturation of the hapten and corrected for free hapten contribution using the data of Table I and spectrum 1. Insert: A modified scatchard plot of a fluorometric titration where *N*-acetyltryptophanamide (NATA, 0.7–4.0 $\times 10^{-2}$ M) is added to NBDA (1×10^{-4} M). q is the fraction of NBDA fluorescence quenched and is proportional to the concentration of bound NBDA. [NATA] is the total concentration of NATA, but, as it is in 100-fold excess over NBDA, it also represents the free NATA concentration to a very good approximation. The slope of this plot yields $K = 21$ M^{-1} and the intercept gives $q_{max} = 0.98$.

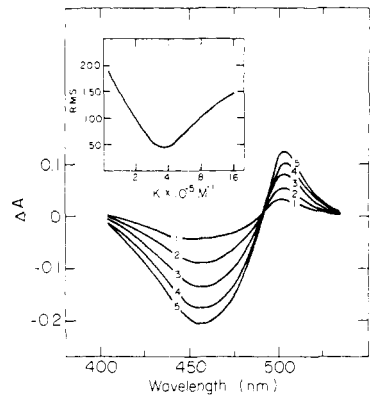

FIGURE 5: Difference absorption spectra of protein-460-bound vs. free NBDA (3.09×10^{-5} M) at different protein concentrations. Total protein 460 site concentrations in molar sites and hapten saturation fraction (calculated with $K = 3.6 \times 10^{-5}$ M^{-1}) are respectively: (1) 1.33×10^{-5} M, 0.198; (2) 2.72×10^{-5} M, 0.394; (3) 4.57×10^{-5} M, 0.615; (4) 7.76×10^{-5} M, 0.834; (5) 3.15×10^{-4} M, 0.979. In the inset RMS is the root-mean-square deviation of the experimental points from the line calculated with the particular value of K (see results for details). A minimal error is found with $K = 3.6 \times 10^5$ M^{-1}.

amino acid analogues in PBS. NBDA was titrated with *N*-acetyltryptophanamide (NATA) and the results are shown in the form of a modified scatchard plot (insert of Figure 4). From this graph a value of 21 M^{-1} is obtained for the association constant, and NBDA fluorescence is found to be quenched to a maximal value of 98%. An interaction of the same type occurs also when NBDA is added to a saturated phenol solution in H_2O (~1.0 M, pH 7) or to a 0.35 M aqueous solution of imidazol: NBDA fluorescence is quenched by 86% and 36%, respectively.

Discussion

Throughout this study it was found that the fluorescence of protein-bound NBDA is quenched relative to that in water. As the quantum yield of NBDA was shown here to decrease with increasing polarity of its environment, the observed quenching could be attributed to very polar binding sites. This, however, is inconsistent with the finding that, even in extremely polar solvents such as formamide and *N*-methylformamide (No. 20 and 19 in Figure 2A), NBDA has higher fluorescence than in water. Very high site polarity also does not agree with the behavior of the other spectral parameters of protein-bound NBDA, as discussed below. The quenching of NBDA fluorescence should therefore be attributed to properties of the site other than its polarity.

From Figure 2A it is apparent that only in two solvents, *m*-dimethoxybenzene and *m*-cresol (No. 24 and 25 in this figure), does NBDA have fluorescence lower than in water. The quantum yield of NBDA in these two solvents clearly lies outside the observed correlation, being 50–100 times lower than that in other solvents with comparable dielectric constants. This behavior seems to be related to the electron-rich

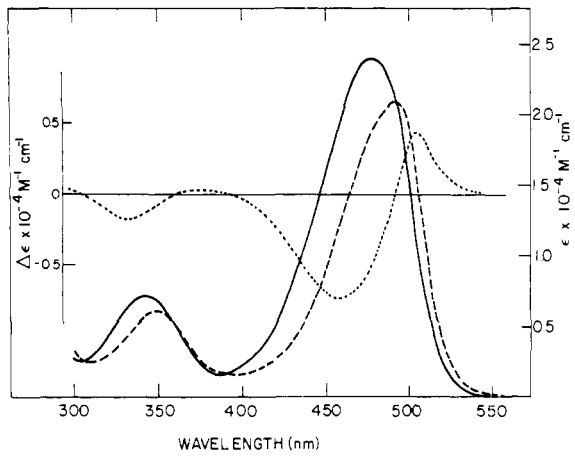

FIGURE 6: Absorption spectra in PBS of free (—) and protein-460-bound (- - -) NBDA and their difference (• • •). The difference spectrum and protein bound spectrum above 410 nm are calculated from the data of Figure 5 (see Results).

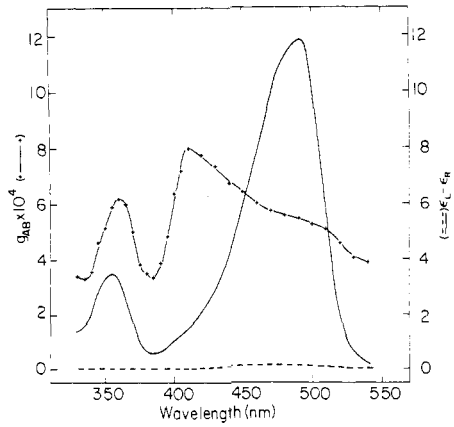

FIGURE 7: CD spectra of free (- - -) and protein-460-bound (—) NBDA, in terms of $\Delta\epsilon_{CD} = \epsilon_L - \epsilon_R$. $\Delta\epsilon_{CD}$ is the actual molar extinction coefficient difference for left-handed (ϵ_L) and right-handed (ϵ_R) circularly polarized light and is related to the molar ellipticity $[\theta]$ by $[\theta] = 3300\ \Delta\epsilon_{CD}$. Also shown is the CD spectrum in terms of $g_{abs} = \Delta\epsilon_{CD}/\epsilon_b$ (+ — +), where ϵ_b is the molar extinction coefficient of protein-460-bound NBDA (Figure 6). The spectrum was taken at the concentrations of point no. 5 in Figure 5 and $\Delta\epsilon_{CD}$ calculated assuming $K = 3.5 \times 10^5\ M^{-1}$. The spectrum is corrected for the contribution of the protein below 330 nm.

aromatic ring of *m*-cresol and *m*-dimethoxybenzene that makes them good charge-transfer donors. Charge-transfer complexation with NBDA as an acceptor may thus be responsible for its decreased fluorescence (cf. Foster, 1969, p 85). The observation of similar fluorescence quenching in the presence of the aromatic amino acid analogues lends support to the notion that aromatic residues play the role of charge-transfer donors in protein binding sites. The use of NBDA as a fluorescent probe thus provides a strong and independent confirmation for the original suggestion of Little and Eisen (1967), based on differential absorption spectroscopy, that a charge-transfer complex is formed with tryptophan residues in the site of nitrophenyl-binding antibodies. NBDA fluorescence quenching does not, however, allow distinction between different aromatic donors. In the case of apomyoglobin, no tryptophans are located in the heme crevice, (Kendrew, 1962), where NBDA presumably binds, and the charge-transfer donor must be either one of tyrosine, phenylalanine, or histidine. In all other cases it may indeed be tryptophan.

The quantum yield of NBDA does not correlate very well with the dielectric constant of the solvent even when the two charge-transfer donor solvents (No. 24 and 25) are ignored (ρ = 0.67 as compared with around 0.8 for the other spectral parameters, see Figure 2). This implies that additional properties of the solvent may affect the fluorescence. Indeed, the quantum yield of NBDA in the hydrogen bonding solvents No. 14, 17, 18, and 22 is significantly lower than that in the non-hydrogen-bonding solvents No. 6, 9, 3, and 21 which, respectively, have similar dielectric constants (Figure 2A). Part of the decrease in NBDA fluorescence upon binding to immunoglobulins may therefore be attributed to hydrogen bonds formed between a proton donor group in the site and the nitro or oxadiazole oxygens of NBDA. Thus partial quenching might imply charge-transfer complexation only, while 90% or higher quenching could suggest additional hydrogen-bond formation. The pair of charge-transfer donor solvents (No. 24 and 25 in Figure 2A) seems to represent just this situation, where *m*-cresol, being also a proton donor, causes a much higher quenching of NBDA fluorescence than *m*-dimethoxybenzene. In this context it is noteworthy that a higher NBDA fluorescence quenching occurs in the sites of proteins which also have higher affinity toward this ligand (Table I).

The emission wavelength of NBDA (Figure 2B) is found to be well correlated with solvent polarity. The blue-shifted fluorescence of NBDA in the sites of goat 44 anti-DNP, serum albumin, and apomyoglobin (Table I) therefore clearly indicates an apolar site, whereas the absence of such shift suggests higher site polarity (e.g., protein 315). In cases where almost total quenching occurs, the uncertainty in the position of the emission maximum is large and no definite conclusions may be drawn.

The absorption parameters (Figures 2C and 2D) are also found to correlate well with solvent polarity; yet their interpretation for protein-460-bound NBDA (Figure 6) is less straightforward. The decreased extinction coefficient indicates an apolar site, while the pronounced red shift implies high site polarity. It is, however, very probable that the site is indeed apolar and that a charge-transfer absorption band at long wavelengths (Foster, 1969, p 33) gives rise to the red shift. This is consistent with the observation (Figure 2D) that, in the charge-transfer donor solvents *m*-dimethoxybenzene (No. 24), *m*-cresol (No. 25), and also anisol (No. 10), the wavelength of NBDA maximal absorption is longer than in other solvents with the same dielectric constant.

It has been proposed (Parker and Osterland, 1970) that nitroaromatic haptens bind to immunoglobulins through what may be described as non-specific interactions with hydrophobic-pocket-type sites. The results of our study suggest that the binding of nitroaromatics to proteins never occurs through hydrophobic interactions only, as this would certainly bring upon an enhancement of the bound NBDA fluorescence. Rather, it is suggested that the nitroaromatic binding site has at least one aromatic residue which forms a specific charge-transfer complex with the hapten, tryptophan being the best candidate (cf. Little and Eisen, 1967) but other aromatic residues not necessarily being excluded. The site most probably also contains proton-donor group(s). Aliphatic hydrophobic residues may be in contact with the nitroaromatic hapten, giving rise to the general apolar nature of the site; yet they do not play a major role in the binding interactions. A typical nitroaromatic binding site is that of protein 315 (Eisen et al., 1968). For this protein it has indeed been suggested (Padlan et al., 1977) that tryptophan 93L is the charge transfer donor, asparagines 36L and 36H are the hydrogen bond donors, and that the hydrophobic residues phenylalanine 34H and leucine 103H are also in contact with the dinitrophenyl hapten.

The binding of anilinenaphthalenesulfonate (Ans) to many

nitrophenyl-specific immunoglobulins (Parker and Osterland, 1970) is not, in our opinion, an indication that nitroaromatics bind nonspecifically to proteins. This finding seems to be informative mainly concerning the nature of Ans itself, providing further evidence for the tendency of this ligand to bind nonspecifically at hydrophobic sites which also bind other ligands specifically (cf. Stryer, 1965). Nitroaromatics apparently have much lower tendency of this kind, as seen from the lack of NBDA binding to many heterologous immunoglobulins. Even in the case of the typical hydrophobic sites of serum albumin and apomyoglobin, where weak binding of NBDA is observed, the affinity does not arise from mere hydrophobicity of the site but from more specific interactions as suggested by the observed quenching of NBDA fluorescence. The notion that hydrophobic interactions play a minor role in the binding of nitroaromatic haptens to immunoglobulins is further supported by the large and negative values of both the enthalpy and entropy changes associated with these reactions (Barisas et al., 1971; Johnston et al., 1974). A similar conclusion, namely, that "the dinitrobenzene group cannot be considered as a simple nonpolar, hydrophobic moiety", was reached in the solvent-transfer study of Halsey and Biltonen (1975).

NBDA is found to bind to all three studied induced anti-DNP antibody preparations. The measured binding constants for the different antibodies span a range of two orders of magnitude, and the NBDA emission properties also differ from one preparation to the other. It is thus possible to characterize anti-DNP preparations on the basis of their reaction with NBDA. It is, however, important to note that the binding specificity for DNP and for its analogue NBDA is not identical. This is expressed in the general observation that only part of any group of DNP-specific immunoglobulins binds the heterologous hapten (compare Richards et al., 1975). Thus, out of three homogeneous immunoglobulins with anti-DNP activity only two are found to bind NBDA. Also, in two of the three induced anti-DNP antibody preparations only a fraction of the available antibody sites is reactive with NBDA. As all sites are reactive towards DNP (isolation was made on a DNP column with mild elution), this partial activity should represent specificity differences. The homogeneity of NBDA binding affinity observed for these antibodies apparently reflects the limited number of individual immunoglobulins in their NBDA-specific fraction. Interestingly, in the only anti-DNP preparation (from goat 44) where all available sites bind NBDA, some binding heterogeneity is indicated by the deviation from the fitted line of the first points in the titration (Figure 3B). The specificity differences between DNP and NBDA are also apparent in their respective binding constants to the three mouse myeloma proteins: for DNP-lysine the affinity decreases in the order protein 315 > protein 25 > protein 460 (Johnston et al., 1974; Sharon and Givol, 1976), while for NBDA the order is protein 460 > protein 315 $\gg$ protein 25.

The relatively strong binding of NBDA to protein 460 makes it the first reported case of a monoclonal immunoglobulin binding a fluorescent environment-sensitive probe. Unfortunately, the fluorescence is being almost totally quenched, thus preventing measurements of the detailed fluorescence properties of the bound ligand. However, the large quenching observed upon binding enables accurate affinity measurements. The quenching of NBDA fluorescence and the red shift in its absorption suggest the formation of a charge-transfer complex, presumably with a tryptophan in protein-460-binding site. The total NBDA fluorescence quenching may also indicate hydrogen-bond formation and the absorption hypochromic effect implies an apolar environment in this site. Using the value of $\epsilon_{470} = 1.6 \times 10^4\ M^{-1}\ cm^{-1}$ for the bound NBDA (Figure 6)[3] and the correlation line (Figure 2C), a value of 15–40 is obtained for the effective dielectric constant in the site, as compared with 78 in water and less than 3 for nonpolar solvents. The site therefore has an intermediate polarity, in line with the notion that both apolar and polar residues contribute to the binding. The large negative difference absorption spectrum ($-\Delta\epsilon/\epsilon_{free} = 0.49$ at 490 nm) comprises a useful way to follow the binding in both static and kinetic measurements (Lancet and Pecht, 1976). The observed large induced circular dichroism of the protein-460-bound NBDA implies that it senses an asymmetric environment in the binding site. It is notworthy that $\Delta\epsilon_{CD}$ is positive throughout the spectrum, which is similar to that observed for other nitroaromatic haptens with protein 460 (Rockey et al., 1972) but markedly different from that measured for polynitrophenyls in the presence of protein 315 (Glaser and Singer, 1971; Rockey et al., 1972).

The binding of NBDA to protein 460 as monitored by each of three different spectral parameters (i.e., the fluorescence of either reactant and the hapten absorption) consistently gave $K = 3.3 \pm 0.3 \times 10^5\ M^{-1}$ at 25 °C. This consistency means that the spectroscopic results directly represent the actual binding properties of the ligand. The value of association constant is identical with that for 2,4-dinitronaphthol, the best known ligand for protein 460 ($K = 3.2 \pm 0.8 \times 10^5\ M^{-1}$ at 25 °C; Johnston et al., 1974). The observed inhibition of NBDA binding by dinitronaphthol and DNP-lysine means that all bind at identical or overlapping sites. This observation remains to be evaluated in the context of the reported multispecificity of protein 460 (Rosenstein et al., 1972).

In a previous work Kenner and Aboderin (1971) reported the enhancement of fluorescence intensity of the 4-methoxybenzylamino derivative of NBD (MBD) upon binding to bovine trypsinogen. Our results show that NBDA undergoes no fluorescence change in the presence of 1.4×10^{-4} M bovine trypsinogen. The discrepency arises, in our opinion, from the presence of the methoxybenzyl moiety in the ligand employed by Kenner and Aboderin. Methoxybenzyl is a good charge-transfer donor, and an internal complex could therefore be formed in MBD. This is supported by the observed low quantum yield of MBD in H_2O (0.005) as compared with that of NBDA (0.024), and also by the longer wavelength of maximal absorption, 480 nm as compared with 476 nm for NBDA. Upon binding to trypsinogen, the spectral parameters of MBD approach those of NBDA in H_2O: the emission quantum yield increases to 0.016, and the unusually red- shifted emission peak (553 nm for free MBD) moves to 541. It is thus possible that mainly the methoxybenzyl group interacts with trypsinogen, and that upon binding the NBD moiety senses an aqueous environment. This possible interpretation does not, however, diminish the applicability of MBD as a spectral probe or contradict in any way the conclusions drawn in the study of Kenner and Aboderin.

In conclusion, we would like to summarize some advantages of NBDA as a nitrophenyl fluorescent analogue, which may be important for further studies. (1) NBDA provides a useful analytical tool for the detection of nitroaromatic specific proteins. It enables a fast and reliable assay for the presence or absence of binding activity by merely adding it to the protein solution in question and monitoring its fluorescence changes.

[3] At the wavelength of 470 nm, the charge transfer band appears to contribute very little assuming that it has a Gaussian shape and extrapolating the positive part of the difference spectrum in Figure 6. This is also roughly where the maximal absorption is found when the extrapolated charge transfer band is subtracted from the bound NBDA spectrum, and is within the range expected for the maximal absorption of NBDA in a medium with a dielectric constant of 15–40 (Figure 2D).

It is not necessary to perform a complete titration and no corrections for nonspecific effects are required. (2) The use of NBDA extends the range of measurable binding constants between proteins and nitroaromatics toward the lower limit. Titrations in which the protein is added to NBDA following the latter's fluorescence are possible even when the association constant is very low ($<10^4$ M^{-1}). This is not practical when employing the classical method of following protein fluorescence quenching, since for hapten concentrations of around 10^{-3} M the nonspecific quenching is too large to be corrected for. (3) NBDA permits the detection of nitroaromatic specific antibodies in the presence of a large excess of other proteins. Again, this is not possible when protein fluorescence is followed, as stated already by Velick et al. (1960). (4) The observed spectral changes of NBDA are proportional to the fractional saturation of the ligand rather than to that of the protein. As the total ligand concentration is usually known while that of the protein reactive sites is often not, the advantage of following the spectral properties of the ligand is obvious. Thus, following NBDA fluorescence it is possible to calculate the number of active binding sites per molecule in an antibody preparation (cf. Table I), which is not possible when following tryptophan fluorescence. (5) It is possible to follow the interaction of antibodies with proteins or cells bearing many NBD groups on their surface, as no spectral interference due to the carrier will occur. This may help to resolve questions relating to the effects of multivalency on antibody–antigen interactions.

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Supplementary Material Available

A table that lists the actual values of the spectral parameters of NBDA in different solvents and the dielectric constants of these solvents as shown in Figure 2 (1 page). Ordering information is given on any current masthead page.

References

Barisas, B. G., Sturtevant, J. M., and Singer, S. J. (1971), *Biochemistry 10,* 2816–2821.

Boulton, A. J., Ghosh, P. B., and Katritzky, A. R. (1966), *J. Chem. Soc. B,* 1004–1011.

Brand, L., and Gohlke, J. R. (1972), *Annu. Rev. Biochem. 41,* 843–868.

Edelman, G. M., and McClure, W. O. (1968), *Acc. Chem. Res. 1,* 65–70.

Eisen, H. N., Simms, E. S., and Potter, M. (1968), *Biochemistry 7,* 4126–4134.

Foster, R. (1969), Organic Charge-Transfer Complexes, London, Academic Press.

Ghosh, P. B., and Whitehouse, M. W. (1968), *Biochem. J. 108,* 155–156.

Glaser, M., and Singer, S. J. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2477–2479.

Goetzl, E. J., and Metzger, H. (1970), *Biochemistry 9,* 1267–1278.

Haimovich, J., Givol, D., and Eisen, H. N. (1970), *Proc. Natl. Acad. Sci. U.S.A. 67,* 1656–1661.

Halsey, J. F., and Biltonen, R. L. (1975), *J. Solution Chem. 4,* 275–283.

Haselkorn, D., Friedman, S., Givol, D., and Pecht, I. (1974), *Biochemistry 13,* 2210–2222.

Holmes, R. R., and Bayer, R. P. (1960), *J. Am. Chem. Soc. 82,* 3454–3456.

Huang, K.-H., and Cantor, C. R. (1975), *J. Mol. Biol. 97,* 423–441.

Johnston, M. F. M., Barisas, B. G., and Sturtevant, J. M. (1974), *Biochemistry 13,* 390–396.

Kendrew, J. C. (1962), *Brookhaven Symp. Biol. 15,* 216–228.

Kenner, R. A., and Aboderin, A. A. (1971), *Biochemistry 10,* 4433–4440.

Lancet, D., and Pecht, I. (1975), *Isr. J. Med. Sci. 11,* 1393.

Lancet, D., and Pecht, I. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3549–3553.

Little, J. R., and Eisen, H. N. (1967), *Biochemistry 6,* 3119–3125.

Padlan, E. A., Davies, D. R., Pecht, I., Givol, D., and Wright, C. (1977), *Cold Spring Harbor Symp. Quant. Biol. 41,* 627–637.

Parker, C. A., and Rees, W. T. (1960), *Analyst 85,* 587.

Parker, C. W., and Osterland, C. K. (1970), *Biochemistry 9,* 1074–1082.

Pecht, I., Haselkorn, D., and Friedman, S. (1972), *FEBS Lett. 24,* 331–334.

Powell, M. J. D. (1971), in Harwell Subroutine Library, Atomic Energy Research Establishment, Harwell, U.K. (Subroutine VA04A).

Richards, F. F., Konigsberg, W. H., Rosenstein, R. W., and Varga, J. M. (1975), *Science 187,* 130–136.

Rockey, J. H., Montgomery, P. C., Underdown, B. J., and Dorrington, K. J. (1972), *Biochemistry 11,* 3172–3181.

Rosenstein, R. W. R., Musson, R. A., Armstrong, M. Y. K., Konigsberg, W. H., and Richards, F. F. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 877–881.

Secrist, J. A., III, Barrio, J. R., Leonard, N. J., and Weber, G. (1972), *Biochemistry 11,* 3499–3506.

Sharon, J., and Givol, D. (1976), *Biochemistry 15,* 1591–1594.

Singer, S. J., and Thorpe, N. O. (1968), *Proc. Natl. Acad. Sci. U.S.A. 60,* 1371–1378.

Stryer, L. (1965), *J. Mol. Biol. 13,* 482–495.

Stuchbury, T., Shipton, M., Norris, R., Malthouse, J. P. G., Brocklehurst, K., Herbert, J. A. L., and Suschitzky, H. (1975), *Biochem. J. 151,* 417–432.

Teale, F. W. J. (1959), *Biochim. Biophys. Acta 35,* 543.

Velick, S. F., Parker, C. W., and Eisen, H. N. (1960), *Proc. Natl. Acad. Sci. U.S.A. 46,* 1470–1482.

Weast, R. C., Ed. (1970), Handbook of Chemistry and Physics, 50th ed, Cleveland, Ohio, The Chemical Rubber Co.

Weber, G., and Laurence, D. J. R. (1954), *Biochem. J. 56,* xxxi.