ANALYTICAL BIOCHEMISTRY **149**, 91–104 (1985)

Analytical Evaluation of the Purity of Commercial Preparations of Cibacron Blue F3GA and Related Dyes

DOUGLAS HANGGI AND PETER CARR

Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455

Received February 6, 1985

The composition and purity of three commercial preparations of the widely used affinity chromatography ligand Cibacron Blue F3GA have been evaluated by TLC and by paired-ion reversed-phase HPLC and were found to contain several chromophoric species. Stepwise synthesis of the reported dye structure showed that only one commercial preparation contained any actual Cibacron Blue F3GA, and that it was present only in minor amounts. In all three preparations the major component appears to be the dichlorotriazinyl precursor of Cibacron Blue F3GA. Commercial samples of the related dyes Procion Blue MX-3G and Procion Blue MX-R are also highly heterogeneous. In addition, our experiments suggest that TLC results must be evaluated carefully to ensure that catalytic surface activity of alumina and silica has not created ghost bands.

Cibacron Blue F3G-A and related triazine dyes have found extensive application in affinity chromatographic purifications of enzymes and other biopolymers. The hundreds of published applications of these dyes in various purification schemes have been extensively reviewed (1–6). In many of these schemes Cibacron Blue F3GA or its analogs have been used as affinity ligands for dehydrogenases or kinases, providing a more stable and far more cost-effective alternative to the use of natural cofactors such as NAD^+, AMP, and ATP. In other cases, the selective purifications of proteins such as interferons and albumins on Cibacron Blue columns appear to result from a unique combination of ionic and hydrophobic sites. A detailed interpretation and the correct assignment of the sources of these interactions is complicated by the highly heterogeneous nature of commercial preparations of these dyes, and inconsistencies in the methods of purification, if any, used in various studies. In addition, variations in the actual chemical composition of the major components between dye preparations are common. Venkataraman (7) has warned that considerable structural variances are tolerated within the textile industry, for which these dyes were manufactured, and that structural homogeneity between dye preparations should not be taken for granted. Since neither Cibacron Blue F3GA nor its dichlorotriazine analog is any longer being manufactured, the considerable stores of the dyes which remain are likely to be contaminated with hydrolysis products even in cases where a homogeneous product was originally present (8). In the context of affinity chromatography, the importance of these factors emerges from the consideration that small changes in the structure can cause large differences in the degree of bioselective interactions. Biellmann *et al.* (9) have reported that a specially purified sample of the *meta*-sulfonate isomer of Cibacron Blue F3GA did not bind to liver alcohol dehydrogenase, while a mixture of the *meta* and *para* isomers did result in binding of the enzyme and inhibition of its activity. As high-performance affinity chromatographic techniques begin to evolve, these factors take on increased importance. Heterogeneity of the affinity ligands due to

0003-2697/85 $3.00

impure dye samples may be partially responsible for the widened and/or unusually shaped peaks, and occasional ghost peaks observed in high-performance affinity chromatography both in our lab and in the literature (5,10). To assess the effects of these impurities we began to investigate the analysis and purity of commercial preparations of Cibacron Blue F3GA and similar dyes as a prelude to assessment of the reported purification schemes for these dyes. In the process we synthesized the dyes ourselves and discovered significant errors in the structural assignment for currently available preparations of some of these dyes. These errors call into question some of the conclusions made concerning the effect of ligand structure in dye ligand affinity chromatography.

EXPERIMENTAL

Materials and equipment. In this work we have investigated several commercial preparations of Cibacron Blue F3GA including those from Sigma (Reactive Blue 2, St. Louis, Mo.), Polyscience (Warrington, Pa.), and Pierce (Rockford, Ill.), in addition to preparations synthesized in this lab. Other anthraquinone containing dyes were also investigated, including Procion Blue MX-R and bromaminic acid (BA)[1] purchased from Polyscience and Pfalz-Bauer (Stamford, CT) respectively, Procion Blue MX-3G donated by ICI America, and the blue chromophore of Cibacron Blue (ASSO) and the dichlorotriazinyl analog of Cibacron Blue (IIA) which we synthesized. The dyes used in this study and their reported structures are shown in Fig. 1. Cyanuric chloride (sTCT) and 2,5-diaminobenzenesulfonic acid (DABS) used in the synthesis of ASSO and structure IIA were obtained from Sigma and Aldrich (Milwaukee, Wisc.) while sulfanilic (SA) and metanilic (MA) acids were Aldrich and Eastman Organics preparations, respectively. The various copper catalysts used in these experiments (cuprous and cupric chloride, powdered copper metal) in the synthesis of ASSO were purchased from Mallinckrodt (St. Louis, Mo.), while the tetrabutylammonium chloride ion-pairing agent for reversed-phase HPLC was obtained from Sigma. All other reagents were of at least reagent-grade quality.

Except for methanol and water used in HPLC experiments all solvents were reagent grade and were obtained through commercial sources. Deuterated water and methanol were obtained from Aldrich, while HPLC solvents were purchased from Mallinckrodt, and were filtered through 0.45-μm nylon filters prior to use.

Thin-layer chromatographic analyses were carried out on silica gel plates from Fisher (Redi-Plates, Pittsburgh, Pa), Analtech (HE-TLC-HLF, Newark, Del.) and Whatman (LK6DF plates, Clifton, N. J.), on reversed-phase silica gel plates from Analtech (RPS plates) as well as on alumina TLC sheets from Macherey-Nagel (Alox N/UV). Semi-preparative-scale purifications were carried out on Baker 60–200-mesh silica gel, Fisher 80–200-mesh neutral alumina A-950, and 30-μm Nucleosil 100 silica (Macherey-Nagel) derivatized with trichlorooctadecylsilane (Petrarch Systems, Bristol, Pa.) by standard methods (11).

HPLC experiments were carried out on either of two systems. The gradient system was comprised of a Perkin–Elmer Series 3B gradient pump module with dynamic mixer (Norwalk, Conn.), a Rheodyne Model 7040 injector (Cotati, Calif.), and a detection system made up of a Hitachi 100-10 variable-wavelength detector (Mountainview, Calif.) and an optional Perkin–Elmer LC-15 fixed-wavelength (254-nm) detector in line. The isocratic system consisted of an Altex 110A pump with pulse dampener (Beckman Instruments, Berkeley, Calif.), a Rheodyne 7120 injector, and a Perkin–Elmer LC-15

[1] Abbreviations used: BA, bromaminic acid; ASSO, 1-amino-4-(4'-aminophenylamino)-2,3'-anthraquinonedisulfonic acid chromophore; sTCT, cyanuric chloride; DABS, 2,5-diaminobenzenesulfonic acid; SA, sulfanilic acid; MA, metanilic acid; PIC, paired-ion chromatography; TBAC, tetrabutylammonium chloride.

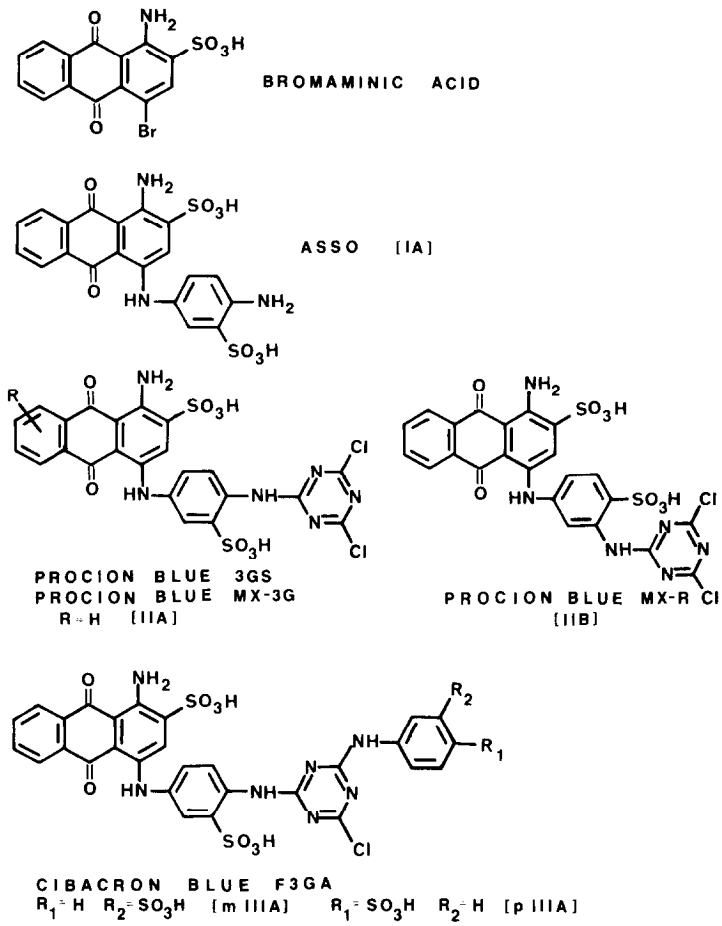

FIG. 1. Anthraquinone dyes investigated in this study and their reported structures.

detector with either a 254 or a 550-nm filter installed. Both systems were connected for data acquisition and storage to an IBM Model 9000 laboratory computer (Danbury, Conn.). Analytical separations were performed on a Hewlett–Packard 10-cm × 4.6-mm-i.d. column packed with Hypersil ODS silica. Semipreparative reversed-phase separations used a 15-cm × 10-mm-i.d. column hand packed with 30-μm Nucleosil ODS silica prepared in this lab.

Visible spectrophotometric characterizations of the dyes were performed on a Hewlett–Packard 8450A diode array spectrophotometer, while infrared spectra of Cibacron Blue fractions were obtained on a Perkin–Elmer Model 298 ir spectrophotometer from potassium bromide pellets. Proton NMR spectra were obtained on a 300-MHz Nicolet NT-300 (Freemont, Calif.) instrument.

Procedures. TLC analyses of the available commercial dyes were attempted on the silica and alumina gel plates listed above using the mobile phases reported in the literature (12–17) with particular emphasis on the solvent systems of Weber *et al.* (13) (48:7, THF:H_2O) and of Seelig (15) (50:40:40:40:20, methyl ethyl ketone:H_2O:triethylamine:pyridine:toluene), which have been reported to resolve several chromophoric bands in Cibacron Blue F3GA. In addition a mobile phase of 2:1 acetonitrile:H_2O was used in alumina TLC experiments. When necessary, the TLC plates were activated by drying

overnight at 120°C. Following development, the position and color of the chromatographic bands were noted and plates were examined under an uv lamp to detect fluorescent bands.

Dye samples were also evaluated by HPLC using the so-called reversed-phase paired-ion chromatography or PIC technique (18), using tetrabutylammonium chloride (TBAC) as the ion-interaction reagent. The weak solvent for this work was a 5 mM solution of TBAC in an aqueous phosphate buffer. The strong solvent was pure methanol. For the first screening of dyes a gradient from 0 to 100% methanol (100 to 0% TBAC) over 1 h at a 1-ml/min flow rate was used to determine the limits of retention for the various dye preparations. On the basis of these results a 45:55 v:v mixture of 5 mM TBAC in an aqueous phosphate buffer (10 mM, pH 7.0) with methanol was used as the mobile phase for the Hypersil ODS column used in this work for the final isocratic evaluation of commercial dyes preparations.

Detection at 254 nm was used for universal detection of aromatic solutes, while 610-nm detection (550 nm on the LC-15 detector) was used to selectively detect blue chromophores. Dye samples for chromatography were prepared by dissolving the dyes in a minimum amount of methanol and diluting with a buffered, aqueous TBAC solution so that the final TBAC/dye ratio was greater than 100. All retention results were verified by varying the concentration of the dyes in order to check for isotherm nonlinearity effects. In general dyes were run at the lowest concentrations that would allow easy detection of the peaks (e.g., 0.01 AUFS).

Synthesis of structures IA, IIA, and the *meta* and *para* isomers of IIIA were performed in order to verify the identity of the materials which elute at the retention times of the actual dyes. The synthetic scheme for these products beginning from bromaminic acid is shown in Fig. 2. In the first step, bromaminic acid is condensed with 2,5-diaminobenzenesulfonic acid (DABS) in the presence of a copper catalyst to yield ASSO (IA), according to the procedure of Wojtkiewicz and Kraska (19). In this procedure a solution of 7.64 g bromaminic acid in 75 ml water is added to 250 ml water containing 5.0 g sodium carbonate, 4.0 g sodium sulfite, and 7.6 g DABS. To this mixture 0.3 g cupric chloride is added and the solution is stirred at room temperature under nitrogen atmo-

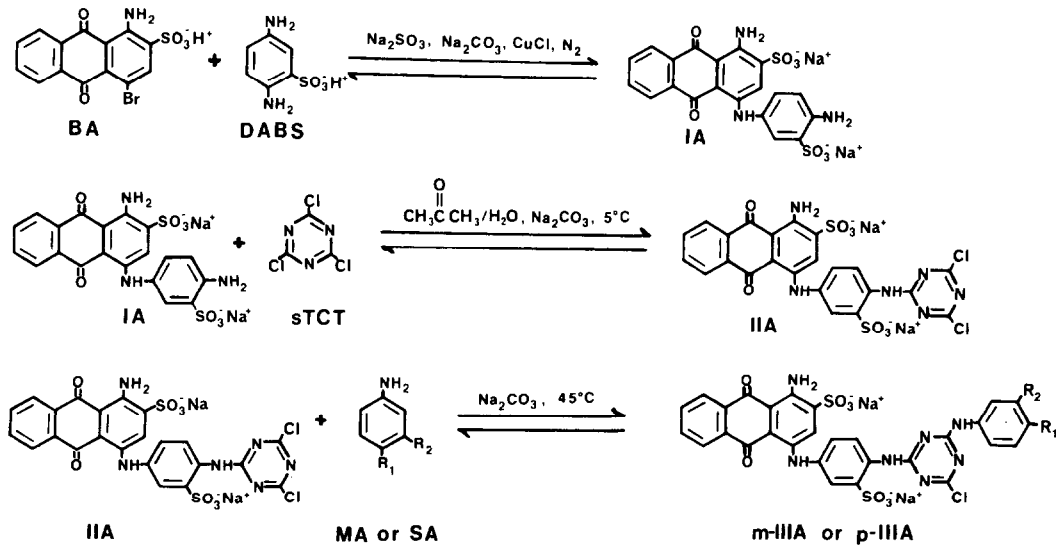

FIG. 2. Synthetic scheme for the preparation of Cibacron Blue-type dyes.

sphere for 8 h. Cuprous chloride, cupric sulfate, or finely divided copper metal can be substituted in place of cupric chloride as the catalyst. A crude ASSO product is obtained after clarification with charcoal and salting out of the dye. The blue ASSO may be separated from the orange-yellow impurities either by column chromatography on alumina using a 4:1 THF:H_2O eluant or by a three-step extraction procedure. In the former case the ASSO can be concentrated into the aqueous phase by mixing the THF:H_2O solution with either toluene or chloroform. In the extraction, the blue ASSO chromophore and one yellow component of the crude mixture are extracted from neutral or mildly basic aqueous solution into chloroform upon addition of TBAC. ASSO is then isolated by discarding the yellow aqueous phase, and reextracting the ASSO from the chloroform layer into a fresh aqueous hydrochloric acid solution. The aqueous ASSO solution from either method can then be neutralized and salted out to recover the reagent, or may be used directly to proceed with the synthesis of structure IIA.

In the remaining steps, cyanuric chloride is reacted with ASSO under conditions that only allow the substitution of one chlorine on the triazine ring so as to yield the dichlorotriazinyl product IIA. This product may then be salted out from solution or the synthesis may be continued directly to the IIIA form. In the latter case, sulfanilic or metanilic acid is then added and the temperature increased so as to react the triazine ring at a second chloro position to yield *p*-IIIA and *m*-IIIA respectively. The actual reaction conditions were taken as being those of the isomeric dyes Procion Blue MX-R and Procion Brilliant Blue H-GR (20). The critical conditions for these steps are the solution basicity and a temperature of approximately 0° and 40–60°C, respectively, for the two triazinyl substitution reactions. In the first step, a stoichiometric amount of a solution of cyanuric chloride (1.9 g in 8 ml acetone and 10 g ice) is added to a concentrated aqueous solution (0.05 M) of ASSO at 0–5°C. The resulting solution is stirred at 0–5°C for 1 h while 2 M sodium carbonate solution is added to keep the pH in the range of 5–7. In the final reaction step, an excess of a solution of metanilic or sulfanilic acid (0.33 M) in water is added to the solution of IIA prepared previously. The temperature is increased to 40–60°C, and sodium carbonate is again added to maintain a pH of 5–7. Reaction progress can be monitored chromatographically using reversed-phase PIC. An alternate set of reaction conditions for the same synthetic scheme in Fig. 2 is given by Senn and Zollinger (21). Synthetic products IIA and IIIA can be precipitated from solution by salting out the dyes using sodium chloride or nitrate. The dyes can be separated from unreacted cyanuric chloride by washing with diethyl ether (21), and from unreacted metanilic and sulfanilic acids by reversed-phase paired-ion chromatography using a mobile phase of approximately 25:75 TBAC:H_2O to concentrate the dye on the column, followed by elution with methanol.

The triazinyl hydrolysis products of IIA and IIIA were generated from the original dyes by incubation at room temperature in 0.5 M sodium hydroxide for 2 h. Under these conditions the hydrolysis reactions in Fig. 3 were expected. All the synthetic dyes and their hydrolysis products were separated by TLC and HPLC under the conditions discussed earlier to determine their R_f values and retention volumes.

The two major fractions from the Sigma preparation of Cibacron Blue F3GA were isolated by column chromatography on silica gel using a 20:48:7 ethyl acetate:THF:H_2O (13), or by elution from alumina gels using a solvent gradient from 99:1 acetonitrile:water to 40:60 acetonitrile:water. The efficiency of the purifications was checked using the analytical PIC method.

RESULTS

Results of the HPLC chromatography of the samples of the synthesized dyes support

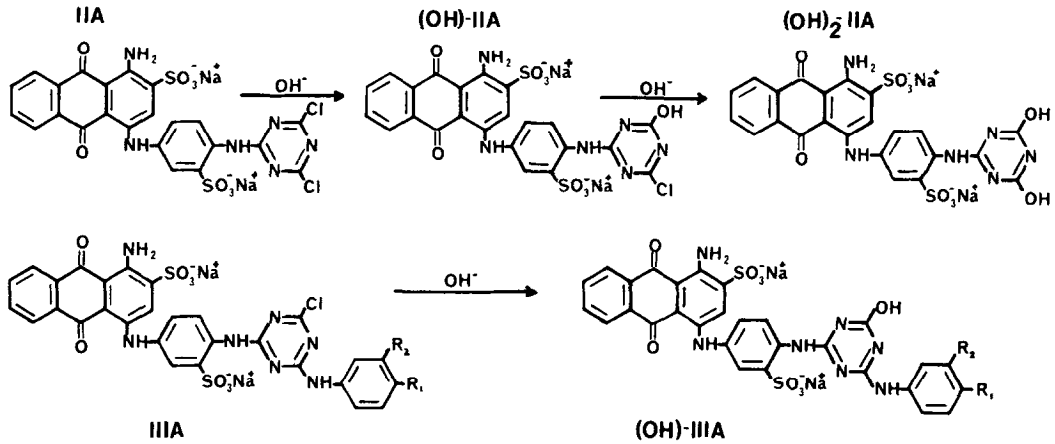

FIG. 3. Hydrolysis reactions for the Cibacron Blue-type chlorotriazinyl dyes.

the validity of the synthetic approach used in this work, and are shown in Fig. 4. While identification of unknown compounds solely on the basis of retention volumes generally does not lead to positive identification of components, the unique agreement of con-

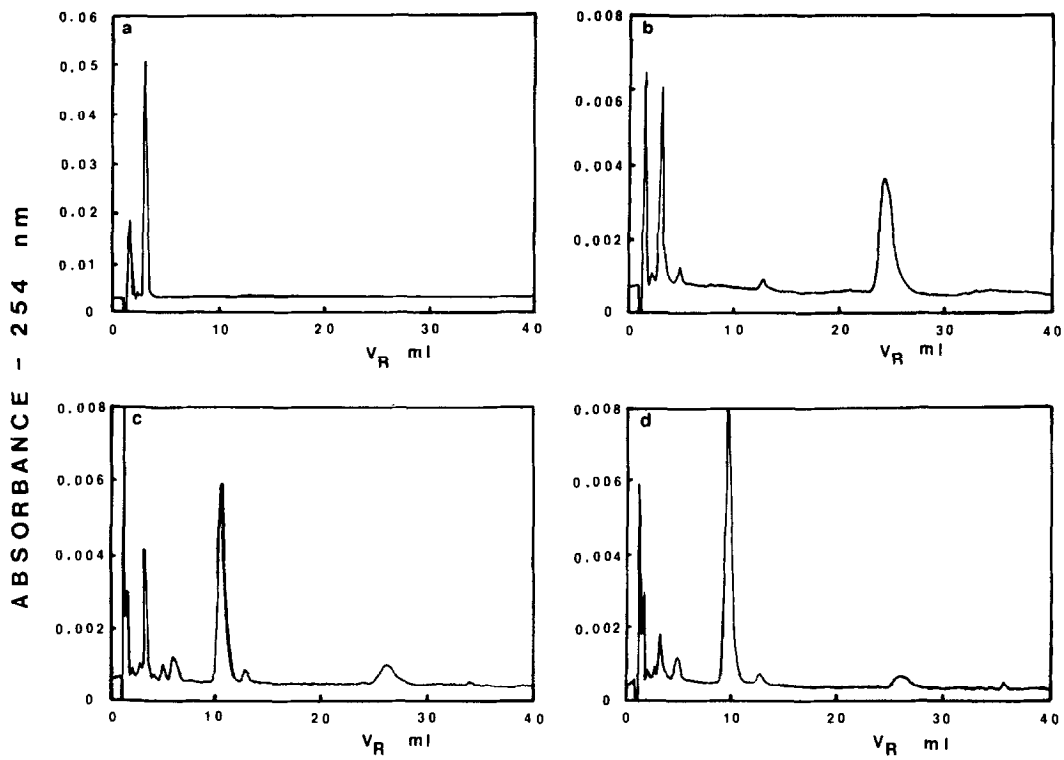

FIG. 4. Reversed-phase paired-ion HPLC chromatograms of the reaction products from the synthetic scheme in Fig. 2 illustrating the successive reactions in the synthesis of Cibacron Blue-type dyes. Chromatographic conditions: 10 cm Hypersil 5-μm ODS column, 45% (5 mM TBAC, 10 mM phosphate, pH 7.0), 55% methanol, 1 ml/min, 254-nm detection. (a) ASSO (IA) synthesis, (b) IIA synthesis, (c) *m*-IIIA synthesis, and (d) *p*-IIIA synthesis.

trolled synthetic, derivatization, and degradation studies with chromatographic results for the limited number of compounds involved in this case permits the assessment of structural identity of these dyes from chromatographic results with a high degree of confidence. A chromatogram of the synthesized ASSO sample is shown in Fig. 4a. In this chromatogram the peak at a retention volume of 3.0 ml represents ASSO and is blue in color. The virtually unretained band is believed to be composed of a mixture of bromaminic acid and a blue-fluorescing oxidation product of the DABS. Upon reaction with cyanuric chloride, the ASSO peak almost quantitatively disappears concurrent with the emergence and growth of the peak (IIA) at a retention volume of approximately 25 ml. The chromatogram in Fig. 4b shows the decrease in the ASSO peak intensity and the emergence of the IIA peak after 1 h of reaction (approximately 80% completion). The minor peak at 12.7 ml is not blue, and possibly corresponds to a DABS–triazine product. Chromatographic results for the reaction of structure IIA with metanilic and sulfanilic acids are shown in Figs. 4c and d, respectively. The IIA peak diminishes as the peaks at 10.4 and 9.6 ml grow for structures *m*-IIIA and *p*-IIIA, respectively. The chromatographic results for the hydrolyzed samples further suggest the remaining identifications in Table 1.

Results of the HPLC chromatography of the three commercial preparations of Cibacron Blue F3GA are shown in Fig. 5. In all three preparations the major peak (V_R = 24–25 ml) matches the retention of the synthetic structure IIA. On the basis of both TLC and HPLC results, the preparations from Sigma and Polyscience are virtually indistinguishable. The Cibacron Blue preparation from Pierce differs from those of Sigma and Polyscience by the presence of the two peaks at 10.4 and 9.6 ml, which were identified above as structures *m*-IIIA and *p*-IIIA, respectively.

The major peak at 25 ml for all the Cibacron samples was identified as structure IIA on the basis of its retention, and the identity was confirmed by several experiments. When purified fractions of this peak isolated from the Sigma preparation were reacted with metanilic acid under mildly basic conditions, a peak at 10.4 ml was found to have developed when the reaction product was chromatographed, corresponding to the synthesis of *m*-IIIA from IIA. During the course of this reaction, the peak at 25 ml decreased in size while the peaks at 5.2 and to a lesser extent at 10.4 ml increased. Under more basic conditions and/or at elevated temperatures only the 5.2-ml peak formed with the decrease in the 25-ml peak. Under mild hydrolysis conditions the 25-ml peak is converted to the 5.2-ml peak, while under stronger conditions both the 25-ml and the 5.2-ml peaks are converted into a single peak with a retention volume of 4.2 ml. The identification of the major peak in Cibacron Blue samples as the dichlorotriazinyl dye IIA is further supported by the failure to detect aniline, sulfanilic acid, or metanilic acid in purified samples of the 5.2- and 25-ml peaks of commercial Cibacron preparations following reductive hydrolysis of the purified frac-

TABLE 1

CIBACRON BLUE INTERMEDIATES RETENTION VOLUMES[a]

V_R	k'	Peak identification	Comment
1.2	0.14	Sulfanilic acid	
1.4	0.33	Bromaminic acid	Yellow/red color
1.4–1.5	0.33–0.43	?	Blue color
2.1	1.00	?	Not blue
3.0	1.86	ASSO (IA)	Blue color
4.2	3.00	$(OH)_2$-IIA	Blue color
4.8	3.56	?	Not blue
5.2	3.95	(OH)-IIA	Blue color
8.0	6.62	(OH)-p-IIIA	Blue color
8.4	7.00	(OH)-m-IIIA	Blue color
9.6	8.14	*p*-IIIA	Blue color
10.4	8.90	*m*-IIIA	Blue color
12.7	11.1	?	Not blue
25	22	IIA	Blue color

[a] Based on chromatography on the 10-cm × 4.6-mm Hypersil 5-μm ODS column with a 45:55 (5 mM TBAC, 10 mM phosphate, pH 7.0):methanol mobile phase at 1.0-ml/min flow rate.

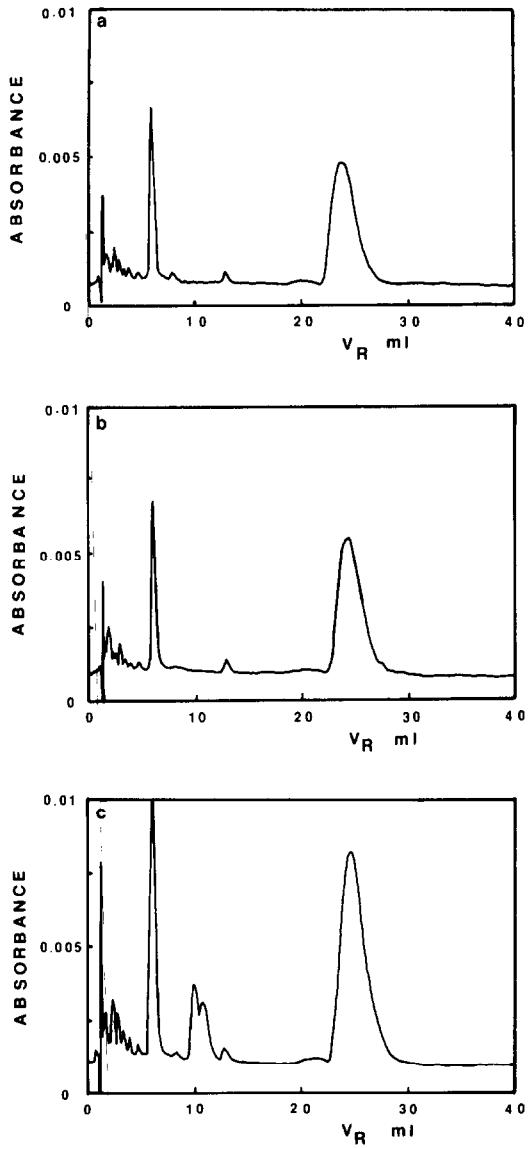

FIG. 5. Chromatograms of samples of the commercial preparations of Cibacron Blue F3GA by reversed-phase paired-ion HPLC. The conditions were the same as denoted in Fig. 4. (a) Reactive Blue 2 Sigma; (b) Cibacron Blue F3GA Polyscience; (c) Cibacron Blue F3GA Pierce.

tions (10). Finally, the ^1H NMR spectra of purified fractions from the commercial Cibacron Blue preparations were consistent with the proposed assignments as structure IIA and the monohydroxylated triazine derivative (OH)-IIA. In the preparations from Sigma and Polyscience, no Cibacron Blue F3GA as designated by Panchartek *et al.* (10) was identified in the commercial preparations, while in the Pierce preparation the Cibacron Blue F3GA peaks (*m*- and *p*-IIIA) represented much less than 10% of the blue dye present in the sample.

The gradient elution HPLC results for the commercial products Cibacron Blue F3GA, Procion Blue MX-3G, and Procion Blue MX-R are shown in Fig. 6. In these chromatograms the absorbance at 610 nm was used to selectively detect only blue components in the preparations. The gradient slope in these chromatograms was 1.67% increase of strong solvent per minute. In the top chromatogram the Pierce preparation of Cibacron Blue F3GA is shown for comparison with the isocratic results in Fig. 5. The chromatograms are comparable with the exception of the small, highly retained peak in the gradient run which elutes as background in the isocratic mode. The strong retention of this peak indicates significantly greater hydrophobicity for this compound, possibly being a partially desulfonated derivative. In addition the isomers of IIIA are not resolved in the gradient run. The second chromatogram in Fig. 6 is a sample of Procion Blue MX-3G. The heterogeneity of the preparation is apparent. Some ASSO, but no IIA, was identified in this product by isocratic chromatography. The final chromatogram in Fig. 6 shows that the sample of Procion Blue MX-R is also extremely heterogeneous. In addition to the blue compounds observed here, a strong yellow-fluorescing colorless band is present.

The TLC results for the analyses of these dyes were in general far less definitive. On the silica gels used in this study, only the solvent system of Seelig (15) yielded significant retention and resolution of the components in Cibacron Blue F3GA samples. The TLC procedure of Weber *et al.* (13) using a THF:water eluant yielded some retention and resolution of chromophores, but was found to be highly irreproducible. In both cases some evidence for catalytic reaction

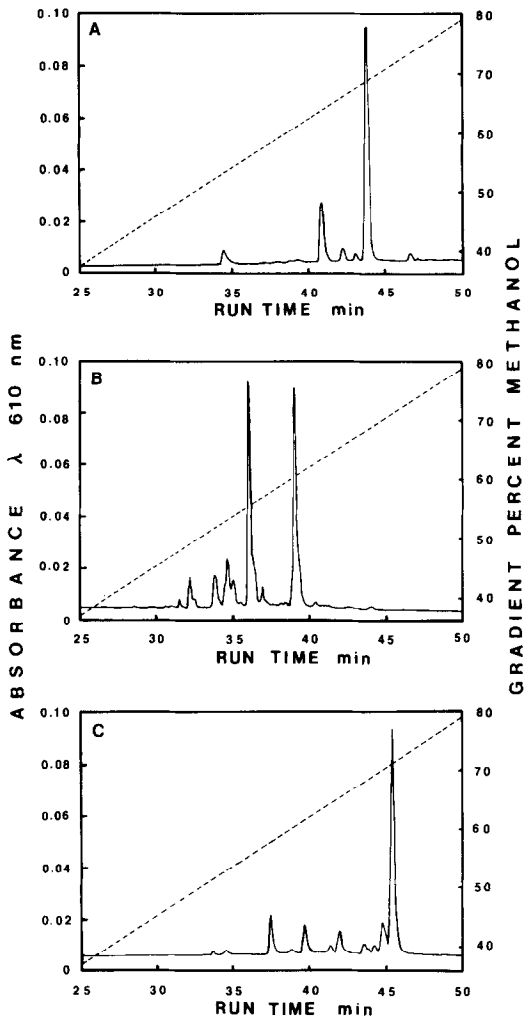

FIG. 6. Gradient elution chromatograms of sample of commercial preparations of Cibacron Blue-type dyes. (A) Cibacron Blue F3GA from Pierce Chemical, (B) Procion Blue MX-3G from ICI America, and (C) Procion Blue MX-R from Polyscience. The absorbance at 610 nm was monitored during a linear methanol gradient at a flow of 1 ml/min.

during chromatography was noted for compounds containing chlorotriazines. Purified samples of the chlorotriazinyl compounds chromatographed as multiple blue bands by TLC, while ASSO samples resulted in a single blue band. Samples of structure IIA produced all the bands noted in the original Sigma Cibacron Blue sample when chromatographed by TLC, while the TLC of the purified minor fraction from the Cibacron preparations resulted in all original bands but the IIA band.

The basicity of the eluting solvent (50:40:40:40:20 methyl ethyl ketone:H_2O:triethylamine:pyridine:toluene) used by Seelig is not directly responsible for the induced reactions, in that the chromatography of a sample of Cibacron Blue incubated for 2 h in the solvent was indistinguishable from that of the original dye preparation. Our experiments also indicated that solutions of Cibacron Blue in THF:water and THF:methanol solvents were unstable with time, but the source of this instability was not pursued. Alumina gel TLC using ACN:water solutions showed similar catalytic effects. Band discoloration, presumably due to air oxidation, also readily occurs after both alumina and silica TLC in a few days.

The visible spectra of the synthesized and purified dyes are shown in Fig. 7. Spectra were taken of dye solutions at dye concentrations of 0.1 to 50 μM in 10 mM sodium phosphate buffer, pH 7.00.

DISCUSSION

Various paper or thin-layer chromatographic systems have been used in attempts to analyze the chromophoric components of commercial preparations of the Cibacron Blue-type dyes (12–17,22). While many schemes showed no polydispersity in the dye preparations, three studies in particular indicated that Cibacron Blue F3GA samples from a variety of sources contained as many as 15 colored components, and that different preparations did not necessarily contain the same individual components (13–15). In those cases where no polydispersity was noted, it is not at all clear that the solvent systems, e.g., pure methanol (24), would be capable of resolving similar chromophores, and care should be taken in assuming that these dyes are pure. On the other hand, our experience indicates that TLC results for the chlorotriazine dyes can be grossly misleading due to

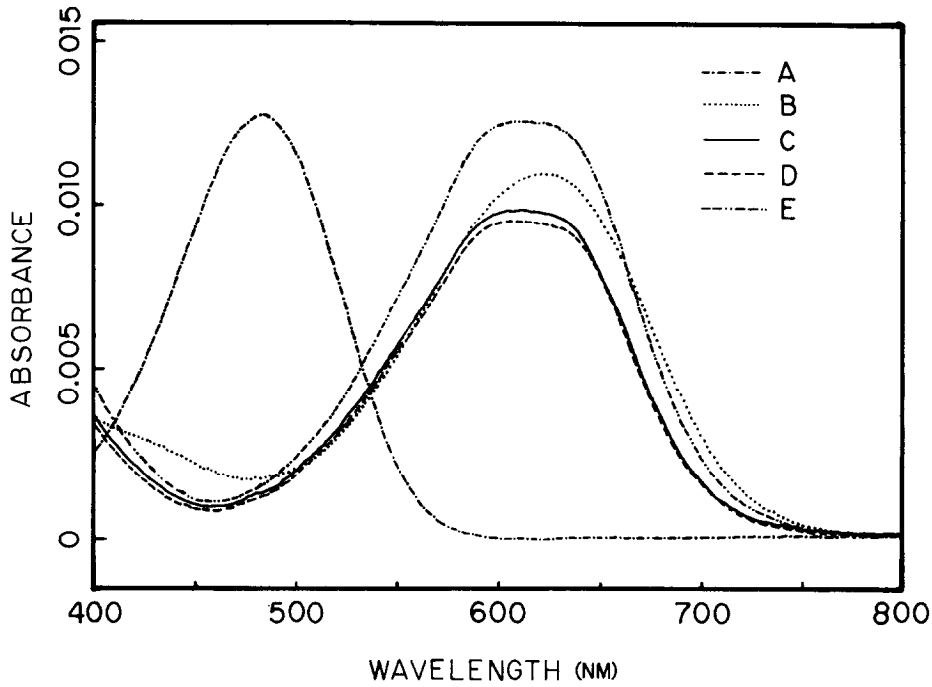

FIG. 7. Visible spectra of Cibacron Blue and its precursors. (A) Bromaminic acid, (B) ASSO, (C) structure IIA, (D) structure *m*-IIIA, and (E) structure *p*-IIIA. Solutions were approximately 1 micromolar in 10 mM phosphate buffer, pH 7.0, pathlength was 1 cm. (Bromaminic acid 2 μM)

the strong catalytic reactions which increase the number and concentration of hydrolysis product bands observed in these experiments. The source of these catalytic effects, observed on both alumina and silica TLC gels, may involve residual metals, ionic sites, and the nature of the binder used in plate manufacturing. The latter aspect probably also plays a significant role in the success of the actual separation of the components in the dye preparations as evidenced by the poor performance of the widely cited Weber TLC procedure (13) on our plates. The TLC binders are not the sole problem, as similar difficulties were noted in attempts to separate the dye preparations by HPLC on silica and alumina gels. Far more reproducible separations were obtained on reversed-phase supports, using ion-pairing reagents to achieve retention, suggesting that the source of these catalytic effects are either masked or are removed through the preparation of a hydrocarbonaceous bonded phase.

We recommend the use of reversed-phase paired-ion chromatography (PIC) for the analysis of purity and identity of these dyes in place of the traditional TLC methods. The PIC method has proven extremely reproducible and has shown virtually no catalytic effects, in contrast to the TLC methods. The PIC method provides excellent separations of the dye components in both isocratic and gradient elution modes on the basis of variations in the hydrophobicity and ionic charge density and distribution of the solute dyes. In addition, quantitation is far simpler in the HPLC method than in TLC.

The chromatographic retention volumes of the dye intermediates in the PIC method are consistent with expectations based on the structures of the components and the anticipated results of the synthetic scheme in Figs. 2 and 3. ASSO is more retained than either bromaminic acid or DABS by virtue of the increased hydrophobicity of the added ring structure and the increased interaction of

both sulfonates with the ion-pairing agent adsorbed on the support (23). Addition of the dichlorotriazine ring to yield IIA results in a large increase in retention due to the hydrophobicity of the dichloro ring and presumably due to silanophilic interactions between the ring nitrogens and residual silanols (24). The decrease in retention for the *meta* and *para* isomers of IIIA is consistent with steric constraints restricting the degree to which the sulfonates can all simultaneously interact with the surface-adsorbed ion-pairing reagents assuming a dynamic ion-exchange retention scheme (23). Hydrolysis of a chloro group to a hydroxyl group decreases retention not only through the decrease in the overall hydrophobicity of the substituent, but also from the possibility of ionization of the hydroxyl group whose pK_a may be near neutrality (25).

The reproducibility of retention volumes in paired-ion chromatography is heavily influenced by the pH and the ionic strength of both the injected sample and the mobile phase. The effect of pH changes on the fully chlorinated triazines is expected to be slight over the pH range above approximately pH 5 since the pK_a values of the sulfonic acid and amine groups in Cibacron Blue should be less than approximately 1 and 4, respectively. Under these conditions both the amine and the sulfonate groups should be fully deprotonated above pH 5, and no net change in the degree of ionization should affect retention in this region. This is consistent with the results obtained in 10 mM acetate (pH 5.0)- and phosphate (pH 7.0)-buffered TBAC mobile phases. Hydroxytriazines, however, may be partially or fully ionized in this region and thereby show a decrease in retention. An increase in ionic strength similarly decreases the retention of ionic species due to the partial blocking of ionic interactions between solute anions and adsorbed TBAC molecules. Thus for samples with increased pH and ionic strength, it is expected that the sample solutes will experience less net interaction with the stationary phase at the head of the column until the unretained sample solvent bolus has been completely resolved from the solute band. The net effect is that there can be some differences in the actual retention of solutes injected as samples with different pH or ionic strength. The effect is expected to be more apparent on highly retained solutes and upon the hydroxytriazinyl derivatives. This is observed in the case of structure IIA in which the retention varies between approximately 24 and 25 ml. This problem is particularly manifested in the interpretation of hydrolysis experiments in which the hydrolysis is carried out in 0.5 M base, followed by neutralization, so that even after dilution the neutral salt/solute ratio in the chromatographic samples was still higher than for the unhydrolyzed samples. In addition, commercial preparations of the dyes are packaged with up to approximately 50% by weight phosphate buffer, which may vary both the hydrolysis pH as well as that of the final sample. As a result, it must be understood that the retention volumes and capacity factors reported in Table 1 for structure IIA and the hydroxytriazines are only approximate values and that some variation between runs was expected and observed. In most cases the variations in the solute retention was small relative to the selectivity between nearest peaks, so that identification of peaks by retention volume was not compromised, although the exact identification of peaks whose isocratic retentions were less than 5 ml is less well defined.

The resolution of the dye components in this work was also found to be fairly independent of changes in the size or geometry of the quaternary ammonium salt used as the ion-pairing agent. Similar results were obtained using decyltrimethyl and hexadecyltrimethyl ammonium chlorides, although the actual retentions varied in these cases. Tetramethyl and tetraethyl salts did not provide sufficient retention or resolution of all the components. Bromide and iodide quaternary salts were also found to be unsatisfactory, consistent with expectation (26). Chromato-

graphic samples prepared without an excess of ion-pairing agent or in solute concentrations in excess of the column capacity often yielded low-retention ghost peaks, which were easily identified by increasing the ion-pairing agent concentration in the sample and by decreasing the solute concentration injected onto the column.

The PIC results obtained in this work emphasize the degree of impurity of the commercial dye preparations and the general confusion regarding the actual identities of the dyes themselves. The widely reported structure of Cibacron Blue F3GA was determined by Panchartek *et al.* (22) to consist of a mixture of the two sulfonic acid isomers of structure IIIA shown in Fig. 1. This structure has been assigned the dye Constitution Index number 61211, and has also been reported as that of the dyes sold commercially as Reactive Blue 2 and Procion Blue HBS (27). Our identification of the major component in the current commercial preparations of Cibacron Blue F3GA as the dichlorotriazinyl dye IIA contradicts the structure determined by Panchartek *et al.* (22) based on the dye's patent description and on hydrolysis experiments. This discrepancy probably results from changes in the commercial manufacturing processes instituted subsequent to the Panchartek study which have resulted in incomplete reaction conditions for the second substitution step, or from a decision to alter the production scheme by eliminating the second triazinyl substitution. In the latter case such a decision may have been prompted by the increased reactivity of the dichloro dye relative to the monochloro dye, while the structural changes would have resulted in little difference in the textile dying applications which made up the primary market for the dye. Since studies investigating the dependence of the biological interactions upon the dye structures have used the structure given by Panchartek *et al.* (22) for Cibacron Blue, e.g., (9,12,28–32), errors in correlating the sources of these interactions with structure may have been made if the dye samples corresponded to the currently available preparations. It is entirely possible that the preparations of Cibacron Blue F3GA used in the above-noted studies did correspond to the structure of Panchartek, but our results indicate that this should not be taken for granted. In addition, even after immobilization for affinity studies, the potential for undesired further reactions still exists for the dichlorotriazinyl dye. These could result in undesired nonspecific adsorption of amine and hydroxyl compounds on the dyes. These problems would not be anticipated on the basis the monochloro dye structure of Panchartek, but should be considered.

In addition, the dichlorotriazine dye shown as structure IIA in Fig. 1 has been reported variously in the affinity chromatography literature as Procion Blue M-3GS (33) and by Dean and co-workers as Procion Blue MX-3G (1,28,29), although in another source Procion Blue MX-3G is reported to contain an extra sulfonate group on the anthraquinone ring (30), while in (5) structure IIA is misidentified as Procion Blue MX-R. The overall decreased retention of the peaks in the Procion Blue MX-3G dye sample relative to that of the IIA peak in the Cibacron Blue sample, as shown in Figs. 6a and b for gradient elution experiments, suggests that the Procion Blue MX-3G sample is not composed of structure IIA, and raises questions concerning the validity of conclusions made upon the assumption of structure IIA. The stronger retention of the blue 1-amino-4-(4′-aminophenylamino)-2,3′-anthraquinone-disulfonic acid chromophore (structure IA, Fig. 1), denoted variously in the literature as ASSO (12) and sulfoparablue (31), further suggests that the polysulfonated anthraquinone dye structure in (30) is more likely for this Procion Blue MX-3G sample. From Fig. 6c it is also clear that the assignment of structure IIA to Procion Blue MX-R in (5) is also mistaken.

The heavy reliance on chromatographic methods in the determination of the identities of individual components is a result of the

difficulties involved in the spectroscopic identification and characterization of these dyes due to a variety of chemical and physical properties. Infrared spectra of the two major fractions isolated from commercial preparations of Cibacron Blue F3GA (IIA and (OH)-IIA) obtained from KBr pellets show the characteristic pattern for a 1,4-diaminoanthraquinone-2-sulfonic acid (34), but provide little other structurally diagnostic information. Proton NMR spectra of the dye components in deuterated solvents were found to vary from run to run. The general patterns and integration studies of the spectra obtained from the two major fractions of Cibacron Blue F3GA in the region of 7–8.5 ppm were consistent with the signals expected from the primary amino and the eight ring protons in IIA and (OH)-IIA (35). Secondary amino protons yield signals considerable farther downfield (35). The difficulty in exactly interpreting the details of the NMR spectra and the variation between runs is believed to result from a strong concentration and pH-dependent self-association phenomenon of the dye. This self-association has been previously reported in the strong nonlinearity in Beer–Lambert law plots for Cibacron Blue F3GA (31). The nonideality is greatly reduced in solvents of high ionic strength, e.g., 0.1 M sodium phosphate buffer, pH 7.0. The sulfonated dyes did not melt or decompose below 300°C so that electron impact mass spectrometry did not yield structural information.

The purification of commercial preparations of the triazine dyes from added buffers, stabilizers, and fillers has been reported by precipitation techniques (30,36,37). These techniques were found to be necessary presteps when commercial preparations were reacted, as in the hydrolysis studies. Semipreparative-scale purification of individual dye components TLC or by silica or hydroxypropylated sepharose column chromatography have been reported (13,14,38) to isolate fractions from Cibacron Blue F3GA preparations. In these cases the actual components were not positively identified or characterized. The purification scheme for Cibacron Blue F3GA given by Weber *et al.* (13) was found useful for separating dichloro IIA from its monohydroxy form. Alumina-based separations were even more selective, although on the alumina supports strong adsorption or covalent bonding of the blue dyes to the support occurred in some instances and the partially modified surface may have been responsible for the high separation efficiency we observed on the alumina phase. In our studies fractions obtained from semipreparative chromatography on silica and alumina columns were generally pure when analyzed by HPLC, although some surface catalytic activity did form secondary bands which needed to be resolved. In addition, semipreparative PIC separations can be applied to the separations of solutes with varying charge density. In general the ambiguities in the purity and structure in the commercial preparations discourage us from continued purifications of commercial preparations. In-lab syntheses of these dyes are relatively straight forward and the products and purification needs are simpler and better defined. These same purification procedures can then be readily adapted to the specific needs of a given reaction step.

CONCLUSIONS

In this work we have presented a method of analyzing the purity of commercial preparations of anionic triazine dyes used as affinity chromatographic ligands. The use of reversed-phase paired-ion chromatography can resolve the various dyes and intermediates, and can be used in some cases to purify the dyes. Silica gel and alumina TLC separations of the dyes were found to be less reliable and in many cases overemphasized the heterogeneity of the dyes. Commercial preparations of Cibacron Blue F3GA contained almost exclusively the dichlorotriazinyl analog of Cibacron Blue or its monochloromonohydroxytriazinyl hydrolysis product,

and very little of the purported monochloro dye. The actual Cibacron structures can be readily synthesized and purified to yield the *meta* and *para* isomers of the monochloro dyes. If commercial preparations of these dyes are to be used in affinity work, careful analysis of the purity and structural composition of the preparations is mandatory.

ACKNOWLEDGMENTS

This work was carried out under funding from the National Science Foundation (CHE 8217363), and under fellowship assistance for D.H. by E. I. DuPont de Nemours & Company. We also thank ICI America for the sample of Procion Blue MX-3G used in this work.

REFERENCES

1. Dean, P. D. G., and Qadri, F. (1983) *in* Solid Phase Biochemistry, (Scouten, W. ed.), pp. 79–148, Wiley-Interscience, New York.
2. Kopperschläger, G., Böhme, H.-J., and Hoffmann, E. (1982) *Adv. Biochem. Eng.* **25,** 101–138.
3. Dean, P. D. G., and Watson, D. H. (1979) *J. Chromatogr.* **165,** 301–319.
4. Lowe, C. R., Small, D. A. P., and Atkinson, A. (1981) *Int. J. Biochem.* **13,** 33–40.
5. Small, D. A. P., Atkinson, T., and Lowe, C. R. (1981) *J. Chromatogr.* **216,** 175–190.
6. Fulton, S., and Marios, M. (1980) Dye-Ligand Chromatography, Amicon Corp., Lexington, Mass.
7. Venkataraman, K. (1977) *in* Analytical Chemistry of Synthetic Dyes (Venkataraman, K., ed.), pp. 1–13, Wiley-Interscience, New York.
8. Personal Communications, Technical Services, Ciba-Geigy, Dyestuffs and Chemical Division (Greensboro, N. C.); and ICI America (Charlotte, N. C.), Summer 1984.
9. Biellmann, J. F., Samama, J. P., Brandon, C. I., and Eklund, H. (1979) *Eur. J. Biochem.* **102,** 107–110.
10. Lowe, C. R., Glad, M., Larsson, P.-O., Ohlson, S., Small, D. A. P., Atkinson, T., and Mosbach, K. (1981) *J. Chromatogr.* **215,** 303–316.
11. Evans, M. B., Dale, A. D., and Little, C. J. (1980) *Chromatographia* **13**(1), 5–10.
12. Beissner, R. S., and Rudolph, F. B. (1978) *Arch. Biochem. Biophys.* **189,** 76–80.
13. Weber, B. H., Willeford, K., Moe, J. G., and Piszkiewicz, D. (1979) *Biochem. Biophys. Res. Commun.* **86,** 252–258.
14. Issaly, I., Poiret, M., Tauc, P., Thiry, L., and Herve, G. (1982) *Biochemistry* **21,** 1612–1623.
15. Seelig, Gail (1982), personal communications.
16. Witt, J. J., and Roskoski, R. R. (1980) *Biochemistry* **19,** 143–148.
17. Edwards, R. A., and Woody, R. W. (1977) *Biochem. Biophys. Res. Commun.* **79,** 470–476.
18. Snyder, L. R., and Kirkland, J. J. (1979) Introduction to Modern Liquid Chromatography, 2nd ed., pp. 453–482, Wiley-Interscience, New York.
19. Wojtkiewicz, W., and Kraska, J. (1964) **Pol.** 48,011; *Chem. Abstr.* **61,** 16203f.
20. Schündehütte, K. H. (1972) *in* The Chemistry of Synthetic Dyes, (K. Venkataraman, ed.), Vol. 6, p. 310, Academic Press, New York.
21. Senn, R. Ch., and Zollinger, Hch. (1963) *Helv. Chim. Acta* **86** (III), 781–794.
22. Panchartek, J., Allen, Z. J., and Muzik, F. (1960) *Col. Czech. Chem. Commun.* **25,** 2783–2799.
23. Stranahan, J. J., and Demings, S. (1982) *Anal. Chem.* **54** (13), 2251–2256.
24. Nahum, A., and Horvath, C. (1981) *J. Chromatogr.* **203,** 33–64.
25. Finlay, T. H., Troll, V., Levy, M., Johnson, A. J., and Hodgins, L. T. (1978) *Anal. Biochem.* **87,** 77–90.
26. Schill, G. (1974) *in* Ion Exchange and Solvent Extraction (Marinsky, J. A., and Marcus, Y., eds.), Vol. 6, pp. 1–58, Dekker, New York.
27. Society of Dyers and Colourists (1975) *Colour Index,* 3rd ed., Vols. 3 and 4, Lund, Humphfries, Bradford, & London, London.
28. Qadri, F., and Dean, P. D. G. (1980) *Biochem. J.* **191,** 53–62.
29. Leatherbarrow, R. J., and Dean, P. D. G. (1980) *Biochem. J.* **189,** 27–34.
30. Atkinson, A., McArdell, J. E., Scawen, M. D., Sherwood, R. F., Small, D. A. P., Lowe, C. R., and Bruton, C. J. (1982) *in* Affinity Chromatography and Related Techniques (Gribnau, T. C. J., Visser, J., and Nivard, R. J. F., eds.), pp. 399–409, Elsevier, Amsterdam/New York.
31. Bornmann, L., and Hess, B. (1977) *Z. Naturforsch.* **32,** 756–759.
32. Beissner, R. S., and Rudolf, F. B. (1978) *J. Chromatogr.* **161,** 127–135.
33. Siegel, E. (1972) *in* The Chemistry of Synthetic Dyes (K. Venkataraman, ed.), Vol. 6, pp. 4–208, Academic Press, New York.
34. Miller, R. K. (1977) *in* Analytical Chemistry of Synthetic Dyes (Venkataraman, K., ed.), pp. 197–215, Wiley-Interscience, New York.
35. Foris, A. (1977) *in* Analytical Chemistry of Synthetic Dyes (Venkataraman, K., ed.), pp. 217–254, Wiley-Interscience, New York.
36. Mehta, H. U., Ravikrishnan, M. R., and Chitale, A. J. (1962) *J. Soc Dyers Colour* **78,** 552–558.
37. Apps, D. K., and Gleed, C. D. (1976) *Biochem. J.* **159,** 441–443.
38. Haff, L. A., and Easterday, R. L. (1978) *in* Theory and Practice in Affinity Techniques (Sundaram, P. V., and Eckstein, F., eds.), pp. 23–44, Academic Press, New York/London.