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Synthesis of a Nitrobenzeneboronic Acid Substituted Polyacrylamide and Its Use in Purifying Isoaccepting Transfer Ribonucleic Acids[†]

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ABSTRACT: Highly purified isoaccepting species of transfer ribonucleic acid (tRNA) were prepared by use of a polyacrylamide substituted with nitrobenzeneboronic acid functional groups. This method exploits the well-known ability of boronic acids to complex with RNA *cis*-diols. tRNA isoacceptors were obtained by enzymatically acylating a mixture of tRNA species with a single amino acid and passing the mixture over a solid-state nitrobenzeneboronic acid at pH 6.5 or 7.0. Pure aminoacyl-tRNA eluted at the column liquid volume, and unacylated tRNA species were bound. The bound species were recovered by lowering the pH of the eluant to 4.5. This procedure is uncomplicated, rapid, and applicable to nearly all tRNA isoacceptors. It does not chemically modify the tRNA(s) of interest or adversely affect their ability to be aminoacylated. Since boronic acids must be ionized to complex with *cis*-1,2-diols, boronic acid derivatives were prepared which ionize at a pH compatible with the stability of the aminoacyl bond. Two isomeric benzeneboronic acids with pK_as of 6.8 and 7.4 were synthesized by introducing electron-withdrawing nitro groups into the aromatic ring. The addition of succinyl side chains permitted the nitrobenzeneboronic acids to be coupled to aminoethylpolyacrylamide. The properties of the nitrobenzeneboronic acid substituted acrylamide were illustrated by enriching phenylalanyl-tRNA at pH 7.0 to greater than 95% purity (1.63 nmol of phenylalanine accepted per A_{260} unit of tRNA) annd seryl-tRNA isoacceptors at pH 6.5 to essentially theoretical purity (1.58 nmol of serine accepted per A_{260} unit of tRNA).

Boronic acids immobilized on solid supports are increasingly utilized for separating molecules with coplanar *cis*-diol groups. Since the synthesis by Weith et al. (1970) of a derivatized cellulose capable of forming complexes with sugars and nucleic acids, the uses of boronic acids attached to insoluble polymers have included separating ribonucleotides from deoxyribonucleotides (Moore et al., 1974), isolating 3′-terminal polynucleotide fragments from large RNAs (Rosenberg, 1974), separating 3′-modified polynucleotides from unmodified ones (Hecht, 1977), assaying adenylate cyclase (Hageman & Kuehn, 1977), and purifying nucleosidyl peptides from tryptic digests (Annamalai et al., 1979).

This report describes the synthesis of immobilized nitrobenzeneboronic acids which are particularly suited for separating aminoacyl-transfer RNA from unacylated species. Nitration of benzeneboronic acids lowers their pK_as so that they ionize at a pH compatible with the stability of the aminoacyl bond. The work of Rosenberg et al. (1972), Schott et al. (1973), and Duncan & Gilham (1975) demonstrated that a boronic acid potentially could be used to separate aminoacyl-transfer RNA from unacylated species. tRNA isoacceptors could be purified by enzymatically acylating a mixture of tRNA species with a single amino acid and passing the tRNA over a boronic acid column. Aminoacyl-tRNA would elute at the column liquid volume, and unacylated material would be bound. The principal liability of these materials was the high pH (8.7) required to effect complex formation between the boronate anion and the RNA *cis*-diol. Substantial hydrolysis of aminoacyl-tRNA bonds occurred on the alkaline columns.

McCutchan et al. (1975) significantly improved the performance of dihydroxyborylaminoethylcellulose (DBAE-cellulose)[1] by treating it with acetic anhydride. Acetylation blocked most residual positive charge on the derivatized cellulose and permitted *cis*-diol-ended tRNA to be selectively bound at pH 7.7. (At pH values lower than 7.5, some aminoacylated tRNA was anomalously retained by the column.)

The synthesis of nitrobenzeneboronic acids was undertaken because the properties of DBAE-cellulose have been found to be variable and to deteriorate with time, presumably by loss of acetyl groups (unpublished experiments; Smoll & Singhal, 1976; S. Hecht, unpublished experiments). Nitrobenzeneboronic acid substituted polyacrylamide was found to be suitable for purifying isoaccepting transfer RNAs at a pH as low as 6.5.

Singhal et al. (1980) have recently described a reversed-phase boronate matrix which is also capable of separating aminoacyl-tRNA from uncharged tRNAs at a slightly acidic pH.

Experimental Procedures

Synthesis of 2-Nitro-3-succinamidobenzeneboronic Acid (X) and 3-Succinamido-4-nitrobenzeneboronic Acid (XI). Figure 1 summarizes the route by which compounds X and XI were prepared. The pK_a of a substituted benzeneboronic acid was lowered by nitration of the aromatic ring. A succinyl side chain was then added to two isomeric nitrobenzeneboronic acids to enable them to be coupled to aminoethylpolyacrylamide.

(*A*) *3-Acetamidobenzeneboronic Acid* (*II*). The hemisulfate salt of 3-aminobenzeneboronic acid (Seaman & Johnson, 1931) was purchased from Aldrich Chemical Co., Milwaukee, WI, and converted to its free base essentially as described by

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[1] Abbreviations used: NBB-polyacrylamide, nitrobenzeneboronic acid substituted polyacrylamide; DBAE-cellulose, dihydroxyborylaminoethylcellulose; BD-DEAE-cellulose, benzoylated diethylaminoethylcellulose; DCC, *N,N′*-dicyclohexylcarbodiimide; 1 A_{260} unit, amount of substance in 1.0 mL of 0.05 M sodium acetate and 0.01 M $MgCl_2$, pH 4.5, which produces an absorbance of 1.0 when measured at 260 nm in a 10-mm light path; Me_2SO, dimethyl sulfoxide; Me_4Si, tetramethylsilane; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; Pipes, piperazine-*N,N′*-bis(2-ethanesulfonic acid).

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Weith et al. (1970). The salt (25 g) was dissolved in 250 mL of deoxygenated water by titrating it to pH 7.0 with 10 M NaOH. The solution was evaporated to dryness in vacuo at 35 °C. The residue then was triturated with dioxane (ca. 200 mL) which had been freshly distilled over lithium aluminum hydride; the extracts were filtered directly into a flask containing 50 mL of well-stirred acetic anhydride and held at 60 °C for 1 h. Water (25 mL) was then added, and the solution was evaporated at 50 °C in vacuo. The sample was dissolved in water and evaporated again at 50 °C to dryness. Brown impurities were removed from the acetamidobenzeneboronic acid by two treatments with 0.5-g portions of Nuchar C-190N in water. The decolorized product was recrystallized from 150 mL of hot water to yield 17.8 g (74%) of white platelike crystals, mp 260 °C. It was not active in a solid-state diazo-coupling test (Johnson, 1978).

(*B*) *Nitration of 3-Acetamidobenzeneboronic Acid* (*II*). Nitration of II was performed in acetic anhydride with fuming nitric acid as described by Torssell (1957). Finely powdered acetamidobenzeneboronic acid (17.8 g) was added in small portions to a solution of 133 mL of acetic anhydride and 6.25 mL of HNO_3 (density 1.5 g/mL, light yellow) stirred at −15 °C in an ethanol-dry ice bath. When the entire sample had been dissolved, the temperature was slowly raised to 0 °C. After nitration for 3 h, the reaction mixture was poured into 500 mL of crushed ice and water. The yellow precipitate which formed was filtered off on Whatman 42 paper, rinsed with ice water, and dried.

(*C*) *Separation of Nitro Isomers*. The dried nitrated products (19.4 g) were extracted 5 times with 200-mL portions of refluxing xylenes (Torssell, 1957) and filtered through Whatman 2 paper on a hot Büchner funnel. The xylene-insoluble fraction, containing 3-acetamido-6-nitrobenzeneboronic acid (V), was harvested and recrystallized from hot water. The resulting very light yellow needles (5.2 g, 23%) charred at >275 °C and gave a negative diazo-coupling test.

The xylene filtrate was chilled at −20 °C to produce an orange precipitate which was collected and dried. The dried precipitate (now yellow), containing the 2- and 4-nitro isomers (III and IV), was recrystallized from hot water to yield 9.3 g (42%).

The 2- and 4-nitro isomers were separated from one another by fractional crystallization from hot ethyl acetate. When the ethyl acetate was slowly cooled to room temperature, grainy yellow crystals formed. The crystals (III) were harvested and recrystallized from water to give yellow leaves which decomposed gradually with blackening above 190 °C. The ethyl acetate filtrate was concentrated to dryness and the residue recrystallized from hot water. The resulting very fine yellow needles (IV) decomposed between 220 and 230 °C with charring. Neither III or IV showed a positive diazo-coupling test.

(*D*) *Thin-Layer Chromatography of Nitrobenzeneboronic Acids*. Compounds II–V each migrated as a single spot when chromatographed on MN-polygram polyamide 6 purchased from Brinkmann Instruments, Inc. Saturated solutions of each compound were prepared with absolute ethanol. Samples (0.1 μL) were applied to polyamide 6, and the plate was developed with water–CH_3CN (9:1 v/v). The compounds and their R_f values were as follows: II, 0.30; III, 0.54; IV, 0.23; V, 0.25. Although IV and V have similar mobilities, III clearly was absent both from the xylene-insoluble fraction V and from its coextracted compound, IV.

(*E*) *Deacetylation of 3-Acetamidonitrobenzeneboronic Acids*. A mixture of 2- and 4-nitro-3-acetamidobenzeneboronic acid (9.3 g) was added to 100 mL of vigorously stirred 2 N HCl preheated in an oil bath to 90 °C. (At temperatures significantly above 90 °C, dihydroxyboryl groups as well as acetyl groups are removed.) After 10 min, the deep red solution was titrated to pH 4 with 10 N NaOH. The precipitate was harvested 5 h later and was dried (yield 6.42 g). A diazo-coupling test of the deacetylated isomers was positive.

(*F*) *Succinamylation of 3-Acetamidobenzeneboronic Acids*. Succinyl dichloride (Aldrich Chemical Co.) was redistilled at 40 °C, ca. 10 mmHg, after traces of water had been removed as a benzene azeotrope. The deacetylated isomers (6.42 g) were dissolved by heating them to 60 °C in 275 mL of dry dioxane. After the solution had cooled to room temperature, succinyl dichloride (3.9 mL, 35.3 mmol) was added. The reaction mixture was stirred for 2.5 h and then was poured into 1 L of ice water and titrated to pH 2.5 with 5 N NaOH. The volume was reduced in vacuo to 250 mL. The precipitate was collected and recrystallized from 500 mL of ethanol–water (3:5 v/v) and dried over P_2O_5. Succinimides VIII and IX were obtained in 25% yield (2.79 g) based on acetamidonitrobenzeneboronic acid. Anal. Calcd for $C_{10}H_9BO_6N_2$: C, 45.48; H, 3.44; N, 10.61. Found: C, 45.47; H, 3.51; N, 10.52 (Huffman Laboratories, Inc.).

Compounds VIII and IX were converted to their corresponding succinamic acids, X and XI, by hydrolysis. NaOH (10 M) was added to a suspension of VIII and IX in water until the sample dissolved and the pH of the solution remained constant at 10. HCl was then added to pH 2.5, and the product was recrystallized from hot water.

Coupling of Nitrobenzeneboronic Acids to Aminoethylpolyacrylamide. Aminoethylpolyacrylamide-150 (AE-P-150, 1.0 mequiv/g) was purchased from Bio-Rad Laboratories. The derivatization of AE-P-150 with nitrobenzeneboronic acid groups was accomplished by water-soluble carbodiimide-promoted amide formation. *N*-Cyclohexyl-*N′*-β-(methylmorpholinio)ethylcarbodiimide *p*-toluenesulfonate was obtained from Aldrich Chemical Co. The coupling reaction was carried out in a medium containing dioxane–water (1:2 v/v) under conditions similar to those described by Weith et al. (1970).

Nitrobenzeneboronic acids X and XI, 0.5 mequiv, were dissolved in a minimum volume of dioxane–water (1:2 v/v). Well-hydrated AE-P-150 (0.5 mequiv of aminoethyl groups, free base form) was equilibrated with dioxane–water (1:2) and compacted by centrifugation in a plastic tube. After the supernatant was decanted, the gel was suspended in the ligand solution. The well-stirred suspension was adjusted to an apparent pH of 6.0 with 1 N HCl and then cooled to 0 °C. Carbodiimide reagent (0.55 mequiv) was added, and the mixture was briskly stirred for 4 h while the apparent pH was maintained at 6.0 by addition of HCl. The suspension was then brought to room temperature and stirred for an additional 4 h. The gel was rinsed extensively with water and then briefly with 0.2 N NH_4OH by centrifugation and decantation. The efficiency of coupling of the ligands to polyacrylamide was determined from the ultraviolet absorbance of the pooled washings. The apparent ϵ_{260} was 3780 for the nitrated isomers in 0.2 N NH_4OH; the ϵ_{260} was 344 for the hydrated carbodiimide reagent. (A new apparent ϵ_{260} value must be determined for each batch of nitrated ligands prepared. The ratio of the 2-nitro of the 4-nitro isomer may vary slightly from one synthesis to the next.)

Sources of tRNAs. Fractions of tRNA enriched for phenylalanine and serine acceptor activity were prepared in the course of other research activities. These samples were convenient for investigating the properties of nitrobenzeneboronic acid columns. Unfractionated tRNA from *Saccharomyces cerevisiae* was the gift of M. Miyazaki. Yeast tRNA enriched for phenylalanine acceptance (0.32 nmol/A_{260} unit) was obtained by chromatography on BD-DEAE-cellulose (Gillam et al., 1967).

Unfractionated tRNA from *Escherichia coli* was isolated from a 2000-L culture of strain K-12 M-72. *E. coli* tRNA enriched for serine acceptance (0.26 nmol/A_{260} unit) was obtained after chromatography on BD-DEAE-cellulose and DEAE-Sephadex A-50 (Johnson, 1978).

E. coli tRNA lacking its 3′-terminal adenosine was produced by periodate oxidation followed by β elimination of 3′-phosphate-ended tRNA (tRNA-Cp). Oxidized tRNA was bound to a solid-state primary amine, aminoethylpolyacrylamide. tRNA-Cp, uncontaminated by oxidized tRNA, was then recovered from the gel by imidazole-catalyzed β elimination as described by Johnson (1974).

Aminoacylation of Transfer RNA. The microscale assay of Kirkegaard (1969) was used to assess the purity of tRNA fractions. $tRNA^{Phe}$ was aminoacylated at 37 °C in a reaction mixture containing 1.0 mM ATP, 0.4 mM CTP, 0.25 M KCl, 0.01 M $MgCl_2$, 0.5 mM EDTA, 0.10 M NH_4Pipes, pH 7.3, 50 μM [^{14}C]phenylalanine (45 μCi/μmol) (Amersham/Searle), and highly purified phenylalanyl-tRNA synthetase. $tRNA^{Ser}$ was assayed similarly except KCl concentration was 0.06 M, [^{3}H]serine (Amersham/Searle) concentration was 500 μCi/μmol, and highly purified seryl-tRNA synthetase was used.

Phenylalanyl-tRNA synthetase was prepared from *S. cerevisiae* harvested in the late log phase and lysed with toluene (Millward, 1967). After ammonium sulfate fractionation, preformed gradient ion exchange gel filtration (Johnson & Bock, 1974), and affinity chromatography on the first column described by Remy et al. (1972), 1 A_{280} unit of synthetase incorporated 590 nmol of [^{14}C]phenylalanine into tRNA in 10 min. No contamination of this preparation by ribonuclease was detected when labeled oligonucleotides, [^{3}H](Ap)$_5$A and [^{3}H](Up)$_5$, were incubated with the synthetase and analyzed by thin-layer chromatography on poly(ethylenimine) plates (Johnson, 1978).

Seryl-tRNA synthetase was prepared from *E. coli* harvested in the late log phase and disrupted by sonication. After ammonium sulfate fractionation, preformed gradient ion exchange gel filtration, and DEAE-cellulose chromatography, 1 A_{280} unit of synthetase incorporated 270 nmol of [^{3}H]serine into tRNA in 10 min. No ribonuclease was detected in this preparation by the labeled oligonucleotide assay.

Purification of Aminoacyl-tRNA on NBB-Polyacrylamide. (*A*) *Preparation of Samples.* After incubation at 37 °C for 20 min, the aminoacylation mixture was quenched by adding 1 volume each of iced 0.25 M KOAc, pH 5.0, and buffer-saturated phenol. The phenol-extracted sample was then precipitated at −20 °C with 2.5 volumes of ethanol, dialyzed extensively against 0.4 M KCl at 8 °C, and reprecipitated with ethanol (McCutchan et al., 1975). The precipitate was dissolved in cold 0.05 M *N*-methylmorpholine hydrochloride, pH 7.0 or 6.5, and diluted with 9 volumes of column equilibration buffer.

(*B*) *Operation of Columns.* Aminoacyl-tRNA was applied to a column of NBB-polyacrylamide (0.45 × 12 cm) equilibrated with 0.05 M *N*-methylmorpholine hydrochloride (pH 7.0 or 6.5, as specified), 0.10 M $MgCl_2$, 1.0 M NaCl, and 20% Me_2SO at 8 °C [buffer of McCutchan et al. (1975)]. *N*-Methylmorpholine, practical grade (Eastman Organic Chemicals), was redistilled before use. Me_2SO, "Spectranalyzed", was obtained from Fisher Chemical Co.

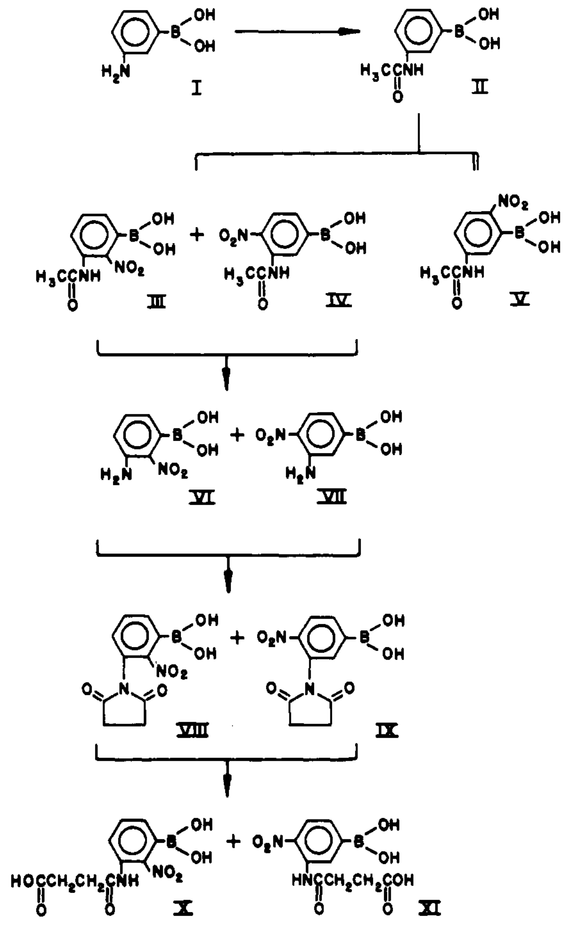

FIGURE 1: Reaction scheme for the synthesis of nitrobenzeneboronic acids of low pK_a.

Bound tRNA was recovered from the column by lowering the pH to 4.5 with 0.05 M NaOAc, 0.01 M $MgCl_2$, 1 M NaCl, and 20% Me_2SO. New NBB-polyacrylamide will bind small amounts of RNA irreversibly. These nonspecific binding sites were saturated by washing the resin with a solution of crude tRNA at pH 7.0 and then purging it with pH 4.5 buffer. tRNA was recovered from NBB-polyacrylamide in high yield (≥95%) after this treatment.

(*C*) *Assessment of tRNA Purity.* Pooled tRNA fractions were deacylated by dialyzing them against 0.1 M Tris-HCl, pH 8.0, at 37 °C for 90 min. The pH was then lowered to 5.0 by dialysis against acetate buffer. Samples, if sufficiently concentrated, were collected by precipitation with ethanol; if too dilute, they were concentrated severalfold by lyophilization and then were precipitated. tRNA was reannealed before its amino acid acceptance was determined.

Results and Discussion

Ligand Synthesis. Introduction of electron-withdrawing nitro groups into the aromatic ring of a benzeneboronic acid has been employed to synthesize two isomers with pK_as near 7. These isomers were prepared in a form suitable for covalent attachment to an acrylamide gel, as outlined in Figure 1.

3-Aminobenzeneboronic acid (I) was protected from excessive oxidation by acetylation (II) and then nitrated with fuming HNO_3 in acetic anhydride. Acetic anhydride shifts the distribution of nitration products from predominantly 3-acetamido-6-nitrobenzeneboronic acid (V) to nearly equal yields of compounds III–V (Torssell, 1957).

Table I: Ionization and Spectral Characteristics of Substituted Benzeneboronic Acids

compound[a]	pK_a[b]	ϵ_{max}[c]	ϵ_{min}[c]
II	8.3	$\epsilon_{240} = 8800$	$\epsilon_{223} = 700$
III	7.4	no λ_{max} ($\epsilon_{220} = 7100$)	
IV	6.8	$\epsilon_{229} = 10500$ $\epsilon_{289} = 4700$	$\epsilon_{259} = 2900$
V	8.8	$\epsilon_{229} = 7900$ $\epsilon_{310} = 5250$	$\epsilon_{258} = 2800$

[a] Numerical designations refer to Figure 1. [b] pK_as were determined potentiometrically by titration with 0.10 N HCl. [c] Spectra were obtained in 0.2 M NH_4OH.

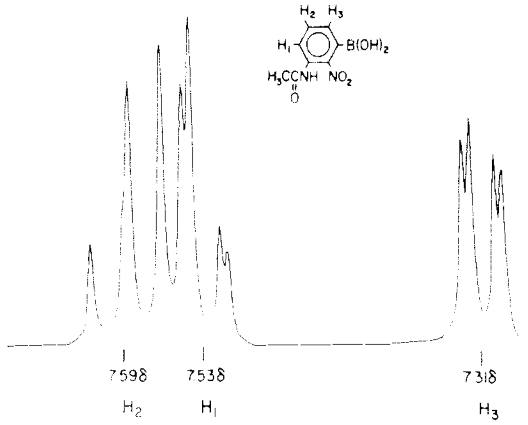

FIGURE 2: Nuclear magnetic resonance spectrum of the aromatic protons of 2-nitro-3-acetamidobenzeneboronic acid. Compound III was dissolved at 100 mg/mL in 100 atom % Me_2SO-d_6 and contained Me_4Si as an internal standard. A total of 100 scans were accumulated on a Bruker WH 270 NMR for Figures 2 and 3. The resolution of each spectrum was 0.367 Hz/point.

Nitration of benzeneboronic acid can be expected to substantially lower its pK_a since the Hammett equation reaction parameter, ρ, for ionization of benzeneboronic acid is +2.1 (Jaffé, 1953; Leppla, 1969). This high value of ρ indicates that ionization of benzeneboronic acids is more greatly affected by electron-withdrawing substituents than are most reactions which follow the Hammett equation.

The products of the nitration of 3-acetamidobenzeneboronic acid were characterized spectrally, and their pK_as were determined by titration with HCl (Table I). Isomers III and IV have pK_as 0.9 pH unit and 1.5 pH units lower, respectively, than the parent compound, II; the pK_a of isomer V is elevated by 0.5 pH unit.

The structure of compound V was deduced by Torssell (1957) by eliminating the dihydroxyboryl group with $AgNO_3$ and showing that the product was identical with *p*-nitroacetanilide. Compounds III and IV, treated in the same manner, each yielded *o*-nitroacetanilide and therefore were indistinguishable. The structures of compounds III and IV are assigned here by NMR spectroscopy at 270 MHz.

Compound III possesses nine protons which have chemical shifts with respect to tetramethylsilane as follows: δ 2.04, 3 singlet acetyl protons; δ 8.35, 2 singlet dihydroxyboryl protons; δ 10.06, 1 singlet amide proton. The three aromatic protons produce an ABX spectrum where the AB subspectra partially overlap. This region is shown in detail in Figure 2. The aromatic protons H_1, H_2, and H_3 have chemical shifts of δ 7.53, 7.59, and 7.31, respectively. Spin coupling constants are $J_{12} = 8.089$, $J_{13} = 1.468$, and $J_{23} = 7.356$ Hz. Isomer III is identified, therefore, as 2-nitro-3-acetamidobenzeneboronic acid.

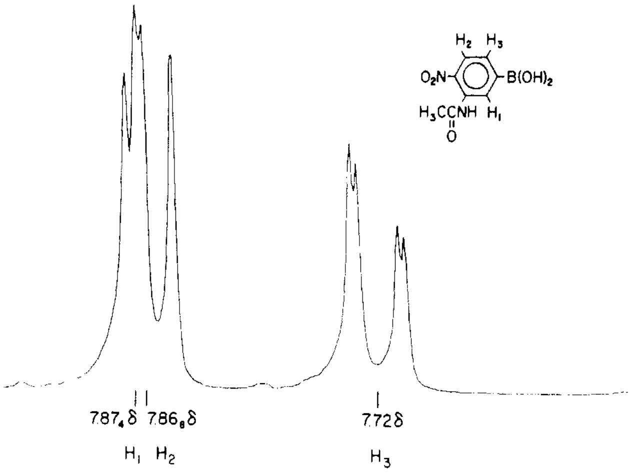

FIGURE 3: Nuclear magnetic resonance spectrum of the aromatic protons of 3-acetamido-4-nitrobenzeneboronic acid. Compound IV was dissolved at 50 mg/mL in 100 atom % Me_2SO-d_6 and contained Me_4Si as an internal standard.

The spectrum of compound IV also reveals nine protons: δ 2.04, 3 singlet acetyl protons; δ 8.47, 2 singlet dihydroxyboryl protons; δ 10.25, 1 singlet amide proton. The portion of the spectrum due to the three aromatic protons is shown in Figure 3. The AB subspectra of this ABX system are degenerate. Proton assignments are δ 7.78_4 (H_1), 7.86_8 (H_2), and 7.72 (H_3). The spin–spin coupling of H_1 and H_2 is negligible. Compound IV is identified as 3-acetamido-4-nitrobenzeneboronic acid.

Compounds III and IV were converted to succinamic acids (X and XI) to enable them to be coupled to aminoethylpolyacrylamide. After the protecting acetyl groups of III and IV were removed with acid, the resulting arylamines (VI and VII) were rederivatized with succinyl dichloride. (Succinic anhydride in pyridine failed to react with these amines.) Succinyl dichloride should not be added in excess of isomers VI and VII. An excess augments the elimination of dihydroxyboryl groups, resulting in *o*-nitrosuccinamylbenzene.

Once the acid chloride reacts with the arylamine, it cyclizes to give a succinimide, VIII or IX. This cyclization was demonstrated by the NMR spectrum of the resulting mixed isomers in Me_2SO-d_6 (data not shown). Unsplit methylene protons were seen at δ 2.91, but no amide proton was detected downfield. The methylene protons were in essentially equivalent environments, as would be expected for succinimides but not for succinamic acids. The elemental analysis of VIII + IX is consistent with this interpretation (see Experimental Procedures).

When compounds VIII and IX are treated with dilute base, the desired succinamic acids are obtained. The NMR spectrum of X + XI includes four protons as a doublet of triplets in the methylene region and one amide proton at δ 10.285. Titration of X + XI demonstrated the presence of ionizable groups of pK_a ca. 7.0 and 4.4, as expected for boronic acids and carboxylic acids, respectively.

Coupling of Ligands to an Insoluble Polymer. Nitrobenzeneboronic acids were coupled to aminoethylpolyacrylamide by water-soluble carbodiimide-promoted amide formation. Weith et al. (1970) demonstrated the utility of this approach by linking a high pK_a boronic acid to aminoethylcellulose with 62% efficiency. Since the low pK_a nitrated ligands are only sparingly soluble in water at pH 6, the coupling was carried out in a medium containing 33% dioxane by volume. (When dioxane or other organic solvents exceed 50% by volume, the gel beads dehydrate, shrink drastically, and become far less reactive.) At 4 °C and an apparent pH

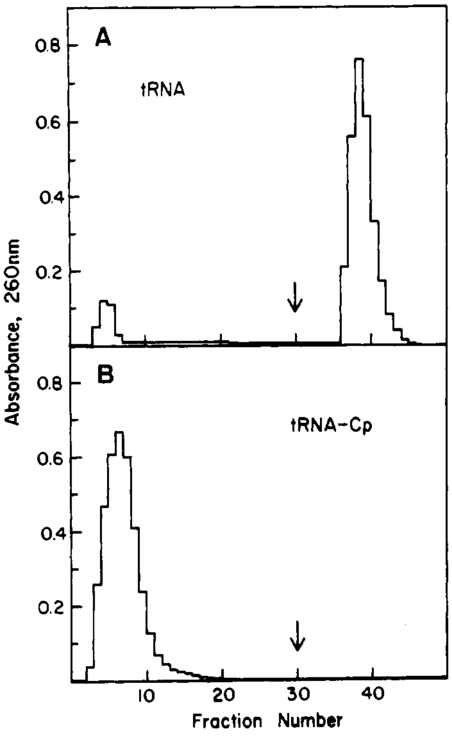

FIGURE 4: Nitrobenzeneboronic acid substituted polyacrylamide selectively binds the *cis*-3′-diol of tRNA. (A) Crude, unfractionated *E. coli* tRNA was applied to NBB-polyacrylamide at pH 7.0 as described under Experimental Procedures. Fractions of 1.4 mL were collected every 20 min. The arrow marks the beginning of elution with pH 4.5 buffer. The absorbance values plotted have been corrected for the absorbance of each buffer at 260 nm. (B) Unfractionated tRNA from which the 3′-terminal adenosines had been removed (tRNA-Cp) was chromatographed in the same manner.

of 6, 30% of the gel amino groups were derivatized with the nitrobenzeneboronic acids.

An alternative method of amide formation, the production of the *N*-hydroxysuccinimide esters of the nitrated ligands with DCC in dry dioxane, followed by coupling in an aqueous medium at pH 9, was not successful. This approach has been used to couple an unnitrated ligand, 3-succinamidobenzeneboronic acid, to aminoethylpolyacrylamide with 90% efficiency (Gilham, 1981; unpublished experiments). *N*-Hydroxysuccinimide esters may promote the recyclizing of the succinamyl side chains of the nitrobenzeneboronic acids to give compounds VIII and IX of Figure 1.

Use of NBB-Polyacrylamide To Purify Isoaccepting tRNAs. At a pH near its pK_a, NBB-polyacrylamide selectively binds tRNAs by their *cis*-3′-diols. When crude, unfractionated tRNA was applied to a column at pH 7.0, most of the sample was retained until the pH of the eluant was lowered to 4.5 (Figure 4). A small peak of ultraviolet-absorbing material commonly elutes at the column liquid volume when a crude sample is used. This material presumably consists of tRNA degradation products containing 3′-phosphates and perhaps some aminoacyl-tRNA. When the same unfractionated tRNA was applied at pH 4.5, the entire sample emerged at the solvent front.

Crude tRNA lacking its terminal adenosine (tRNA-Cp) is incapable of complexing with polymer-linked nitrobenzeneboronic acid groups. tRNA-Cp, applied to a column in the same pH 7.0 buffer used for tRNA binding, was not retarded. No ultraviolet-absorbing material eluted when the eluant pH was lowered to 4.5 (Figure 4).

The capacity of NBB-polyacrylamide for tRNA approaches 2 mg/g of dry gel at pH 7.0. For tRNA isoacceptor purification, a sample of approximately 1 mg of tRNA/g at pH 7.0 and 0.5 mg of tRNA/g at pH 6.5 usually was applied to the column.

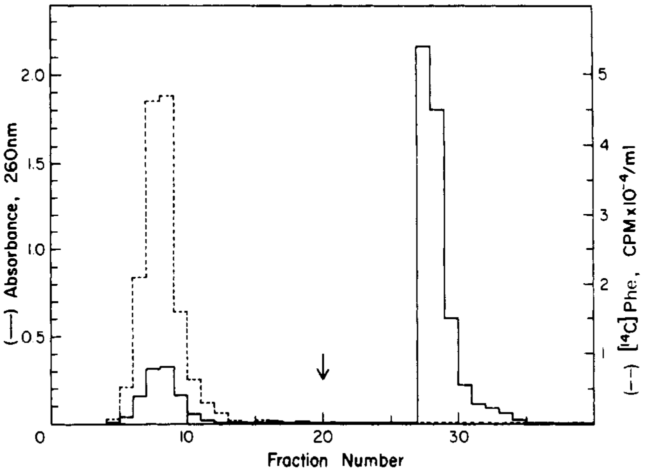

FIGURE 5: Purification of yeast phenylalanyl-tRNA on NBB-polyacrylamide. Yeast tRNA (10 A_{260} units) (enriched for tRNA^{Phe} on BD-DEAE-cellulose) was acylated with [^{14}C]phenylalanine by a highly purified, nuclease-free phenylalanyl-tRNA synthetase as described under Experimental Procedures. The column was equilibrated at pH 7.0; elution with pH 4.5 buffer was begun at the arrow. Fractions of 1.4 mL were collected every 20 min. Radioactivity was monitored by counting 10 μL of each fraction in a water-miscible scintillation fluid at 77% efficiency. Each liter of scintillation fluid contained 5 g of PPO, 0.3 g of dimethyl-POPOP, 700 mL of toluene, and 300 mL of 2-ethoxyethanol.

Yeast phenylalanyl-tRNA, an aminoacyl-tRNA of average stability, was enriched to near the maximum obtainable purity on NBB-polyacrylamide at pH 7.0. A sample of tRNA obtained after chromatography on BD-DEAE-cellulose and possessing 0.32 nmol of phenylalanyl-acceptor activity per A_{260} unit was used for the purification illustrated in Figure 5. The tRNA first was applied to NBB-polyacrylamide at pH 7.0 in case the sample contained traces of aminoacyl-tRNA and RNA fragments bearing 3′-phosphates. This preliminary procedure proved to be unnecessary since 100% of the ultraviolet-absorbing material bound to the column. The *cis*-diol-ended tRNA was released with pH 4.5 buffer, harvested by precipitation with 1 volume of ethanol, and aminoacylated with [^{14}C]phenylalanine. About 9 A_{260} units were reapplied to the NBB-polyacrylamide. All of the [^{14}C]phenylalanine emerged at the front bound to about 1.5 A_{260} units of tRNA; unlabeled tRNA (ca. 7.1 A_{260} units) was recovered in pH 4.5 elution buffer. Fractions 7-10 and 28-30 were pooled separately for deacylation and assay. The tRNA not bound by the resin accepted 1.63 nmol of phenylalanine/A_{260} unit. The tRNA eluted by pH 4.5 buffer accepted only ≤0.04 nmol of phenylalanine/A_{260} unit. Since yeast tRNA^{Phe} of the highest purity accepts 1.70 nmol of phenylalanine/A_{260} unit, the tRNA^{Phe} obtained by fractionation on nitrobenzeneboronic acid resin is over 95% pure.

A tRNA sample need not be partially purified before using NBB-polyacrylamide. A preliminary chromatograpic step sometimes is advantageous, however, since less resin is required to obtain a given amount of pure isoaccepting tRNAs. The scale of synthesis described under Experimental Procedures is convenient and yields nitrated ligands sufficient to produce 10 g (150 mL) of resin. Approximately 200 A_{260} units of tRNA can be applied at pH 7 to 10 g of resin.

E. coli seryl-tRNA isoacceptors were purified in an analogous manner to illustrate the versatility of this resin. Seryl-tRNAs were chosen because they cannot be purified by derivatization with 2-naphthyloxyacetic acid and chromatog-

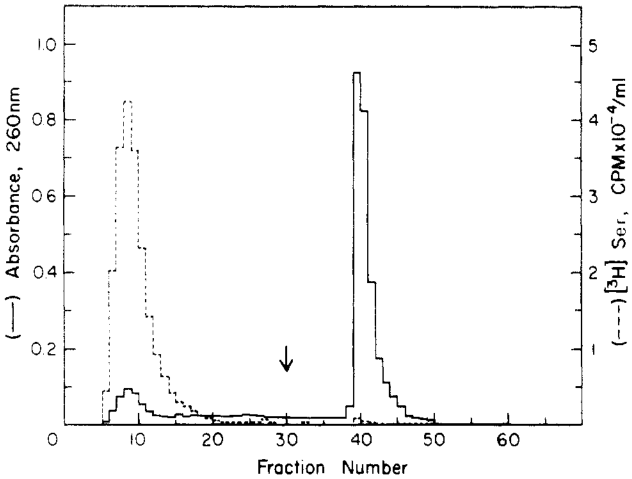

FIGURE 6: Purification of *E. coli* seryl-tRNA on NBB–polyacrylamide. *E. coli* tRNA (5 A_{260} units) (obtained after DEAE-Sephadex A-50 chromatography) was acylated with [^{3}H]serine by a highly purified, nuclease-free seryl-tRNA synthetase as described under Experimental Procedures. The column was equilibrated at pH 6.5; elution with pH 4.5 buffer was begun at the arrow. Fractions of 1.2 mL were collected every 6 min. Radioactivity was monitored by counting 50 μL of each fraction at 26% efficiency in toluene–ethoxyethanol scintillation fluid.

raphy on BD-DEAE-cellulose (Roy et al., 1971). The resin was equilibrated at pH 6.5 rather than 7.0 to demonstrate the feasibility of NBB–polyacrylamide fractionation for purification of the most labile aminoacyl-tRNAs. At pH 6.5, the column capacity was reduced as expected.

A tRNA sample possessing 0.26 nmol of seryl-acceptor activity per A_{260} unit was aminoacylated with [^{3}H]serine. Its elution profile on NBB–polyacrylamide is shown in Figure 6. Nearly all of the [^{3}H]serine emerged in pH 6.5 buffer. [The small amount of tritium which eluted in pH 4.5 buffer probably reflects a limited ability of free serine to complex with the resin. When serine alone (10 nmol, 500 nCi of ^{3}H/nmol) was chromatographed under these conditions, 5.8% of the applied radioactivity eluted in pH 4.5 buffer.] Fractions 7–11 and 40–41 were pooled separately for assay.

The tRNA fraction not bound at pH 6.5 accepted 1.58 nmol of serine/A_{260} unit, whereas the bound fraction assayed at 0.06 nmol/A_{260} unit. *E. coli* seryl-tRNAs have very long chain lengths; tRNA$_1^{Ser}$, and tRNA$_3^{Ser}$ consist of 88 and 93 nucleotides, respectively (Ishikura et al., 1971; Yamada & Ishikura, 1973). Serine-isoaccepting tRNAs have been judged to accept less than 1.60 nmol of serine/A_{260} unit at 100% purity (McCutchan et al., 1975). Seryl-tRNAs obtained after fractionation on nitrobenzeneboronic acid resin, therefore, are near the maximum purity obtainable.

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