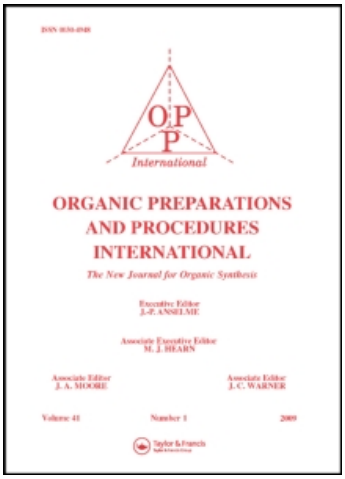

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SYNTHESIS OF 1,1'-BIS(2-SULFOETHYL)PURPURATE (BISEP) TRISODIUM AND TRIPOTASSIUM SALTS. MEMBRANE IMPERMEANT CALCIUM INDICATORS FOR MUSCLE CONTRACTION STUDIES

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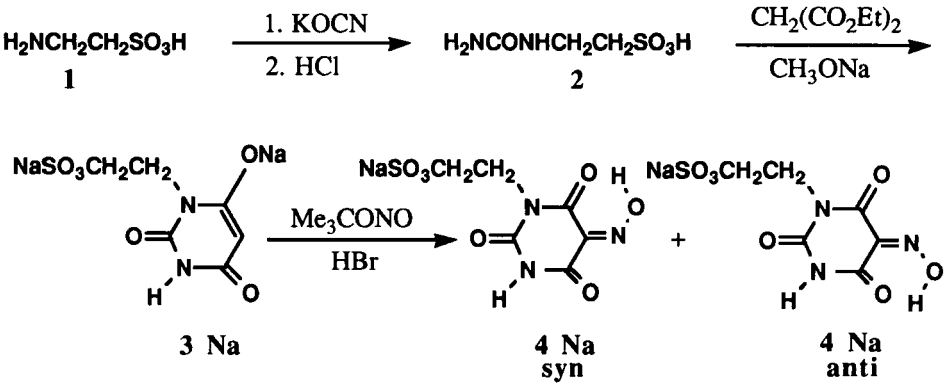
Earlier research in this laboratory has led to the synthesis of new highly water-soluble calcium indicators which are membrane-impermeant and, as indicators of the purpurate (murexide) type, provide both the required rapid indicator response and the relatively low calcium affinity best suited for measuring the calcium transients that are encountered in work on muscle contraction.[1,2] The most satisfactory of these were found to be purpurate-1,1'-diacetic acid (PDAA) tripotassium salt[2,3] and 1,1'-dimethylpurpurate-3,3'-diacetic acid (DMPDAA) triammonium salt,[2] by use of which it was possible to define more closely than with previous methods the average amplitude of the peak contraction-inducing calcium ion concentration in studies on frog cut twitch fibers.[1,2] To be described here is the synthesis of sodium and potassium salts of another water-soluble purpurate-based calcium indicator which was likewise expected to be membrane impermeant and possibly better able to retain the desired impermeance in slightly more acidic media. In the structure of the new purpurate derivatives, the carboxymethyl groups of PDAA are replaced by sulfoethyl groups which, except in very strongly acidic solutions, would be present as alkylsulfonate anions and expected therefore to confer membrane impermeance upon the molecule at any pH likely to be encountered in a biological environment. The new calcium indicators are tripotassium and trisodium salts of 1,1'-*bis*(2-sulfoethyl)purpurate (BISEP) (**6 K** and **6 Na**).

The synthetic sequence began with N-(2-sulfoethyl)urea (**2**),[4,5] which is prepared by treatment of taurine (**1**) with potassium cyanate. Condensation of **2** with diethyl malonate in the presence of methanolic sodium methoxide provided the disodium salt of 1-(2-sulfoethyl)barbituric acid (SEBA, **3 Na**). Nitrosation of this product with *t*-butyl nitrite in the presence of hydrobromic acid yielded 1-(2-sulfoethyl)violuric acid (SEVA) (**4**), which crystallized from the reaction mixture as the monosodium salt (**4 Na**). This sodium salt was very conveniently transformed into the trisodium salt of the symmetrical purpurate (**6**) by a "one-pot" procedure in which **3** was reduced to the sodium salt of 1-(2-sulfoethyl)-uramil (SEU, **5 Na**) by treatment with hydrogen sulfide. Excess hydrogen

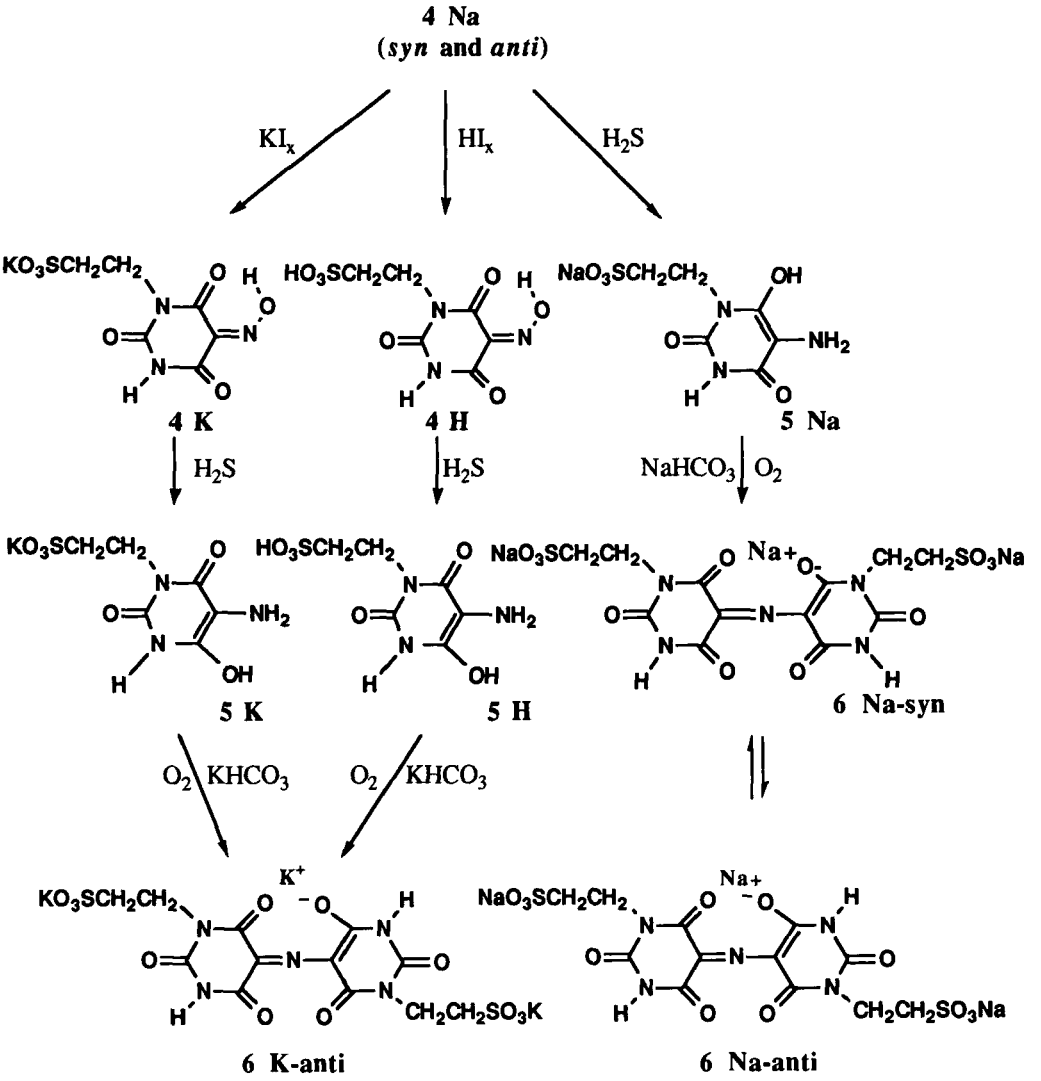


sulfide was then removed and the uramil **5** was oxidized to **6 Na**, 1,1'-Bis(2-sulfoethyl)purpurate (BISEP) trisodium salt, by drawing air through the solution after the required amount (one-half equivalent) of sodium bicarbonate had been added.

The purpurate dye **6** in the form of its tripotassium salt **6 K** was obtained from the monosodium salt of 1-(2-sulfoethyl)violuric acid (SEVA) (**4 Na**) by two different procedures. In the first of these, the SEVA potassium salt (**4 K**) was obtained from the monosodium salt with the aid of a potassium-loaded ion-exchange resin (shown as K-Ix in the reaction chart). Following hydrogen sulfide reduction of the potassium salt **4 K**, the SEU potassium salt (**5 K**) precipitated from the reaction mixture and was collected by filtration. It was then dissolved in water containing one-half equivalent of potassium bicarbonate and oxidized to the tripotassium purpurate **6 K** by drawing air through the solution. In the second procedure, the SEVA free acid **4 H** was prepared from the sodium salt by acidification with an ion-exchange resin (shown as H-Ix in the reaction chart) and conversion to the potassium salt was performed at the 1-(2-sulfoethyl)uramil (**5**) stage of the synthetic sequence by adding the appropriate quantity of potassium bicarbonate to the reaction mixture following the hydrogen sulfide reduction of the SEVA free acid (**4 H**) to the SEU free acid (**5 H**). Removal of excess hydrogen sulfide and oxidation of the uramil to the tripotassium salt of the purpurate (**6 K**) was accomplished by drawing air through the solution as described above for the trisodium salt.

In order to characterize adequately the hygroscopic SEBA and SEVA intermediates they were converted into their pyridine salts, which were were stable and easily recrystallized. The BISEP sodium and potassium salts were isolated from the aqueous reaction mixtures in which they were formed by addition of absolute ethanol to cause precipitation. The salts could then be purified by reprecipitations from aqueous solutions by addition of methanol or ethanol, which led to the separation of hydrated salts showing UV-visible spectra corresponding in every respect to the purpurate chromophore and giving values of molar absorptivity of 15,000 M^{-1} cm^{-1} or more. Results of elemental analysis of the potassium salt corresponded to a sesquihydrate, that of the sodium salt to a dihydrate. The ^{1}H NMR spectra of the BISEP salts in deuterium oxide showed, as expected, only the two 2-proton triplets from the 2-sulfoethyl groups. A ^{13}C NMR spectrum of the BISEP

trisodium salt (**6 Na**) showed the separate resonance for each of the six differently-situated carbon atoms which would be expected for the BISEP structure if it is assumed to be a symmetrical resonance hybrid. Interestingly, one of these signals, that near 1.23 ppm attributable to the carbons at the 3 and 3'-ring position which are joined to the ring-linking nitrogen, showed a very slight



splitting into signals at 122.69 and 122.91 ppm. These were of unequal amplitude and may be an indication of the presence of unequal amounts of the geometrical isomers represented by formulas **6 Na-syn** and **6 Na-anti** (corresponding isomers are probably present also in the BISEP potassium salt). Since in a crystalline alkali metal purpurate an oxygen from each ring of the purpurate anion has been found to be bonded to the central alkali metal ion,[6] interconversion of *syn*- and *anti*-isomers of the indicated type would presumably not be so rapid as to prevent observation of a discrete signal from each isomer in the NMR spectrum.

The sodium and potassium BISEP salts are very similar to the PDAA and DMPDAA salts in their indicator response to calcium ion.[1,2] Complete conversion to the calcium purpurate in aqueous solution shifts the absorption maximum from 526 nm to 485 nm with an isosbestic point at 512 nm. The dissociation constant, K_D, for the calcium complex of the BISEP indicator **6 K** was 1.17 mM in aqueous solution at pH 7. Preliminary experiments with cut frog twitch fibers indicate that the BISEP salt **6 K** has membrane impermeance similar to that displayed by PDAA salts, and would be suited to similar application in studies of muscle contraction,[7] in which murexide and tetramethylmurexide are unsatisfactory because they are not adequately confined by membrane barriers.[1,2] BISEP might be expected to retain its membrane impermeance better than PDAA when the pH falls below neutrality.

EXPERIMENTAL SECTION

Microanalyses are by Atlantic Microlab, Inc., Norcross, Georgia. Spectra were determined with the following instruments: UV-VIS spectra on a Hewlet Packard 8452 diode array spectrophtometer; IR spectra on a Nicholet Model 5DXB FT-IR spectrophotometer; NMR spectra on an IBM NR/ 300 FTNMR spectrometer. With NMR spectra abbreviations used are the following: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad signal.

Preparation of N-(2-Sulfoethyl)urea (Taurocarbamic Acid) (2)[4].- The procedure was a modification of the method used by Salkowski[4] and by Gabriel.[5] Taurine (**1**) (10 g; 80 mmol) and potassium cyanate (6.5 g; 80 mmol) were dissolved in 20 ml of water. The solution was placed in an open crystallizing dish and heated on a steam bath until the water had evaporated to leave a colorless crystalline residue, which was recrystallized by dissolving it in 15 ml of water and adding 80 ml of absolute ethanol slowly with stirring. After 1 hr the resulting white crystals were collected by filtration, washed on the filter with absolute ethanol, and dried in a vacuum desiccator. The yield of the potassium salt was 16 g (96%). The free acid was obtained by dissolving this quantity of potassium salt in water (24 ml) and acidifying with concentrated hydrochloric acid (5 ml), then inducing crystallization by slow addition of absolute ethanol (95 ml) with stirring. The white crystals were filtered from the mixture and washed on the filter with absolute ethanol. The yield was 12 g (88%), mp. 225-230°with rapid heating. Gabriel[5] records softening at ~180°, mp 190-198° with slow heating.

Preparation of 1-(2-Sulfoethyl)barbituric Acid (SEBA) (3).- N-(2-sulfoethyl)urea (**2**) (7.5 g; 44.4 mmol) was added to a stirred solution in methanol (100 ml) containing diethyl malonate (16.88 g, (16 ml); 105 mmol) and sodium methoxide (8.27 g, 153 mmol (35 ml of a 25% w/w solution in methanol (Aldrich)). The mixture, which contained suspended near-white solids in increasing amounts as the reaction proceeded, was stirred and heated under reflux for 24 hrs. The mixture was cooled and the solids were collected by filtration, then washed on the filter with methanol. The yield was 11.8 g (95%) of the disodium salt **3 Na**, which could be assayed for purity by taking the UV spectrum in water. Typically, the crude product showed a molar absorptivity value (ε) of 14,000 at 260 nm. It was purified for use in the next step by crystallization from water-methanol. A solution of

the crude product (6 g) in water (13 ml) when diluted by addition of methanol (40 ml) yielded white crystals of the disodium salt (4 g; 66% recovery) showing a value for ε of 16,000 at 260 nm (water) and undergoing decomposition at *ca.* 360°. For further characterization of this intermediate, a crystalline salt was prepared from the free acid (**3 H**) derived from **3 Na** by acidification with an ion exchange column. A 3 x 10 cm column of Amberlite IRA-118H was washed with 6 N hydrochloric acid then with distilled water until the eluate reached pH 5-6. The SEBA salt **3 Na** (0.5 g, 1.86 mmol) was dissolved in 5 ml of water and loaded on the column. Following elution with 80 ml of water the eluate was taken to dryness in a rotary evaporator with the aid of additions of absolute ethanol. The SEBA free acid (**3 H**) was obtained as a clear light-yellow non-crystalline residue in the form of a syrup or gum. When the free acid (0.42 g, 1.86 mmol) was mixed with pyridine (0.3 g (0.3 ml), 3.7 mmol), the pyridinium salt was produced as an orange-yellow powder, which was suspended in hot absolute ethanol (20 ml) and brought into solution by addition of the required minimum amount of water with continued heating. The salt separated as yellow-orange crystals, mp 223-225°, as the solution cooled. The ultraviolet spectrum showed peaks at 218 and 258 nm, and a value for ε of 12,000 at 258 nm (water). ^{1}H NMR (D_2O): δ 3.18 (t, 2H, J = 7.5 Hz, $NCH_2\underline{CH_2}SO_3$-), 4.23 (t, 2H, J = 7.5 Hz, $NCH_2\underline{CH_2}SO_3$-), 8.07 (t, 2H, pyridine), 8.62 (t, 1H, pyridine), 8.78 ppm (d, 2H, pyridine).
Anal. Calcd. for $C_{11}H_{13}N_3O_6S$: C, 41.90; H, 4.16; N, 13.33. Found: C, 41.87; H, 4.17; N, 13.33

Preparation of 1-(2-Sulfoethyl)violuric Acid (SEVA) (4).- 1-(2-Sulfoethyl)barbituric acid (SEBA) sodium salt (**3 Na**) (0.84 g, 3.0 mmol) was dissolved in 3 ml of 3N hydrobromic acid and the solution was cooled to 0°. A solution of *t*-butyl nitrite (0.87 g, 1 ml, 8.4 mmol) in acetic acid (2.5 ml), also at 0°, was then added with stirring at a rate slow enough to keep the temperature from rising above 5°. During the addition, the reaction mixture turned a dark green-brown color. Stirring was then continued overnight without cooling while the dark color faded from the mixture and the product separated as white or light pink crystals. These were collected, washed on the filter with acetone, and dried in a vacuum desiccator. The yield was 0.65g (75%) of 1-(2-sulfoethyl)violuric acid (SEVA) sodium salt (**4 Na**), mp 215-230° dec. with shrinking above 195°, and a molar absorptivity (ε) of 17,000 at 312 nm (water, pH > 10). The compound free acid form (**4 H**) was obtained by use of the acidic form of an Amberlite ion exchange resin. A 3 x 10 cm column of Amberlite IRA-118H was washed with 6N hydrochloric acid, then with distilled water until the eluate reached pH 5-6 and loaded with 0.15 g of the sodium salt of the sulfoethylvioluric acid (**4 Na**) in 2 ml of water. Following elution with 80 ml of water, the eluate was taken to dryness on a rotary evaporator with the aid of additions of absolute ethanol. The residue was obtained as a clear, light-yellow glass, which was dissolved in acetone. When the acetone solution was allowed to evaporate in the atmosphere, yellow-tinted white crystals were obtained (0.13 g, 87%), mp 125-128°, ε = 15,600 at 314 nm (water, pH >10). In order to obtain a more complete characterization of the compound a crystalline pyridine salt was prepared. A sample of the free acid (0.6 g, 2.27 mmol) was suspended in 1 ml of absolute ethanol. When pyridine (0.41 g, 0.42 ml, 5.2 mmol) was added, the suspended pale yellow solid

changed to a white crystalline powder, which was recrystallized from a mixture prepared from absolute ethanol (30 ml) and water (1.5 ml). Upon cooling a hot solution white crystals were obtained, mp. 263-265° dec, ε = 16,500 at 310 nm (water). The ^{1}H-NMR (D_2O) spectrum, which was more complex than originally expected, could be explained on the basis of the presence of both of the possible configurations of the oximino group, giving rise to geometrical (*syn-anti*) isomers having slightly different chemical shifts for protons of corresponding methylene groups of the 2-sulfoethyl group, and consequently giving patterns of NMR signals reflecting overlapping of the pairs of noncoincident triplets which result. ^{1}H NMR (D_2O): δ 3.19 (2 H, symmetrical 4-signal pattern (overlapping triplets), $NCH_2C\underline{H}_2SO_3$-), 4.25 ppm (2H, symmetrical 5-signal pattern (overlapping triplets), $NC\underline{H}_2CH_2SO_3$-), 8.06 (t, 2H, pyridine), 8.62 (t, 1H, pyridine), 8.77 ppm (d, 2H, pyridine).

Anal. Calcd.for $C_{11}H_{12}N_4O_7S$: C, 38.37; H, 3.51; N, 16.27. Found: C, 38.28; H, 3.52; N, 16.20

One Step Preparation of 1,1'-Bis(2-sulfoethyl)purpurate (BISEP) Trisodium Salt (6 Na) from SEVA Mono Sodium Salt (4 Na).- The SEVA sodium salt **4 Na** (0.574 g 2.00 mmol) was suspended in 5 ml of water in a side-arm test tube and the suspension was stirred while hydrogen sulfide was passed in as in a previously described procedure.[3] The initial pink color of the mixture faded to yellowish white after *ca.* 10 minutes, but addition of hydrogen sulfide was continued for another 20 min. Following addition of sodium bicarbonate (0.084 g, 1.0 mmol), air was drawn through the mixture in the test tube by replacing the hydrogen sulfide inlet tube with a similar narrow tube open to the air and then partially evacuating the system with an aspirator. The purple color of the dye began to develop after *ca.*10 min; the UV-visible spectrum of the mixture continued to change under treatment with air for nearly 3 hrs until it closely resemble that of a purpurate ion. At that time the solution was filtered to remove sulfur, absolute ethanol (30 ml) was added with stirring, and the precipitated product was separated by centrifugation, washed in the centrifuge tube with 15 ml of methanol, then with 20 ml of ethyl acetate, and dried in a vacuum desiccator to yield 0.49 g of a product having a molar absorptivity at 526 nm of *ca.*10,000. Purification was accomplished by dissolving the dye in water (8 ml) and precipitating it by addition of absolute ethanol (32 ml). After washing with methanol (20 ml) and ethyl acetate (25 ml) and drying in a vacuum desiccator, a dark red powder (0.322 g) was obtained. Another precipitation performed in the same manner afforded 0.30 g (25% yield) of a product which gave the correct UV-visible and ^{13}C NMR spectra for the expected purpurate. This purified product showed an absorbance in water at 526 nm which would correspond to a value for ε of 14,800 for $C_{12}H_{10}N_5O_{12}S_2Na_3$, the anhydrous molecular formula, or 15,800 for $C_{12}H_{10}N_5O_{12}S_2Na_3 \cdot 2\ H_2O$, the dihydrate formula which fits the elemental analysis. The ratios of the values of ε at the three characteristic principal absorption maxima of 526, 324, and 252 nm were $\varepsilon_{526}/\varepsilon_{324}$ = 1.73 and $\varepsilon_{526}/\varepsilon_{252}$ = 0.83. ^{13}C NMR (D_2O): δ 39.51, 51.07, 122.69, 122.91, 153.95, 162.00, 162.98.ppm.

Anal. Calcd. for $C_{12}H_{10}N_5O_{12}S_2Na_3 \cdot 2H_2O$: C, 24.62; H, 2.41; N, 11.96

Found: C, 24.74; H, 2.29; N, 12.01

Preparation of 1,1'-*bis*(2-Sulfoethyl)purpurate (BISEP) Tripotassium Salt (6 K) *via* the Potassium Salt of 1-(2-Sulfoethyl)violuric acid (4 K).- The sodium salt of SEVA (**4 Na**) (0.7 g, 2.429 mmol) was dissolved in water (10 ml) and run through an Amberlite IRA-118H column (2x10 cm) which had previously been treated with excess aqueous potassium hydroxide and washed with deionized water to remove the excess base. The potassium salt was eluted with deionized water. Fractions containing the SEVA potassium salt **4 K** showed a light purple color. They were combined and taken to dryness under vacuum on a rotary evaporator with the aid of several additions of absolute ethanol. The yield of SEVA potassium salt **4 K** was 0.66 g (2.18 mmol, 92%), ε = 15,000 at 312 nm (water, pH >10), mp. 230-260° dec, with shrinking from 195°. The salt was dissolved in 5 ml of deionized water and placed in a side-arm test tube containing a magnetic stirring bar. Hydrogen sulfide was passed into the stirred solution from a narrow inlet tube reaching nearly to the bottom of the tube, and the side arm was connected to system for absorbing unconsumed gas. A thick light-yellow precipitate was formed. Treatment with hydrogen sulfide was continued until precipitate formation seemed complete and the color of the mixture had faded to pale yellow or nearly white. When the gas was bubbled through in a rather rapid stream, 8 or 10 minutes sufficed to complete this change. The precipitate was collected and dried in a vacuum desiccator to yield 0.515 g of a mixture of 1-(2-sulfoethyl)uramil (SEU) potassium salt (**5 K**) and free sulfur. The UV spectrum of this material was that expected for a uramil, with the major absorption at 256 nm (water). Correction for the expected sulfur content indicated that 0.422 g (1.5 mmol) of **5 K** was present in this mixed product. Potassium bicarbonate (0.09 g, 0.9 mmol), sufficient for the conversion of this quantity of SEU to the purpurate (1.2 times the calculated required amount) was added in 5 ml of water, and air was bubbled through the solution until the absorption at 526 nm appeared to have reached a maximum (*ca.*1.5 hrs required). The mixture was filtered through a fine sintered-glass crucible to remove the sulfur. The filtrate was then diluted with 4 volumes of absolute ethanol to precipitate the potassium purpurate **6 K**, which was collected by centrifugation, washed in the centrifuge tube first with 15 ml of methanol then with 25 ml of ethyl acetate, and dried in a vacuum desiccator. The crude product was purified by three reprecipitations in which the dye was dissolved in water (30-40 mg/ml) and the solutions were diluted with 4 volumes of methanol to cause precipitation. The purified product (0.127 g, 17.5% yield) showed an absorbance in water at 524 nm which would correspond to a value for ε of 14,500 for $C_{12}H_{10}N_5O_{12}S_2K_3$, the anhydrous molecular formula, or 15,000 for $C_{12}H_{10}N_5O_{12}S_2K_3 \cdot 1.5\ H_2O$, the sesquihydrate formula which fits the elemental analysis. The ratios of the values of ε at the three characteristic principal absorption maxima of 524, 324, and 250 nm were $\varepsilon_{524}/\varepsilon_{324} = 1.74$ and $\varepsilon_{524}/\varepsilon_{250} = 0.83$. ^{1}H NMR ($D_2O$): δ 2.90 (t, 2 H, J = 7.5 Hz, $NCH_2\underline{CH_2}SO_3^-$); 4.24 ppm (t, 2 H, J = 7.5 Hz, $NC\underline{H_2}CH_2SO_3^-$).

Anal. Calcd. for $C_{12}H_{10}N_5O_{12}S_2K_3 \cdot 1.5\ H_2O$: C, 23.07; H, 2.10; N, 11.21
Found: C, 22.99; H, 2.20; N, 11.09

Preparation *via* 1-(2-Sulfoethyl)violuric Acid (SEVA Free Acid) (4 H).- SEVA as the free acid (**4**

H) was prepared from the sodium salt by treatment with the acidified Amberlite ion exchange resin as described above. A 0.457 g (1.73 mmol) quantity was dissolved in 4 ml of water, placed in a side-arm test tube and reduced to the uramil (SEU) with hydrogen sulfide as in the previous procedure. Potassium bicarbonate (0.26 g, 2.59 mmol) was then added, and the mixture was stirred while excess hydrogen sulfide was removed and autooxidation carried out by evacuating the system with an aspirator while drawing air through the solution from a narrow inlet tube. After a few minutes the color of the purpurate dye appeared. After 3 hrs, when the formation of the dye seemed to be complete (no further increase of absorbance at 526 nm), the mixture was filtered to remove sulfur and the filtrate was diluted with 4 volumes of absolute ethanol. The resulting dark red-purple precipitate was washed with methanol (15 ml) and with acetone (25 ml) and dried in a vacuum desiccator to yield 0.26 g (0.436 mmol, 25.2 %) of a crude product, ε_{524} 10,000 (water). Following 3 reprecipitations of this product from water-methanol mixtures as in the procedure given above 0.19 g (18.5%) of BISEP tripotassium salt sesquihydrate was obtained, ε_{524} 14,700 (water) ($\varepsilon_{524}/\varepsilon_{324} = 1.70$; $\varepsilon_{524}/\varepsilon_{250} = 0.83$).

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