Synthesis of Undecagold Cluster Molecules as Biochemical Labeling Reagents. 1. Monoacyl and Mono[*N*-(succinimidooxy)succinyl] Undecagold Clusters†

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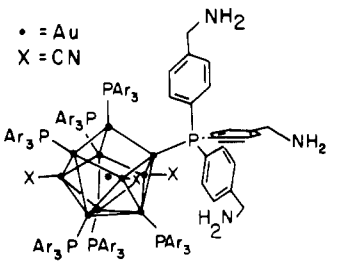
ABSTRACT: This paper describes the synthesis and characterization of succinyl, phthalyl, and *N*-(succinimidooxy)-succinyl derivatives of the undecagold cluster complex tricyanoheptakis[4,4′,4″-phosphinidynetris(benzenemethanamine)]undecagold, **1**, molecular formula $Au_{11}(CN)_3[P(C_6H_4CH_2NH_2)_3]_7$. These are useful as electron-dense reagents for labeling biological structures in preparation for electron microscopic analysis. Limited reaction of **1** with succinic or phthalic anhydrides produces a mixture of mono-, bis-, etc. (*N*-succinyl)-**1** or (*N*-phthalyl)-**1**, which can be separated by anion-exchange chromatography at pH 11.5. Yields of monoacylated derivatives can be maximized by controlling the ratio of succinic or phthalic anhydride to **1**. The remaining 20 primary amino groups can be dialkylated or acetylated, blocking their participation in further chemical modifications of the carboxylic functional group introduced in the succinylation or phthalylation of **1**. These carboxyl groups can be activated as *N*-hydroxysuccinimido esters, which are acylating derivatives of **1**. An example is mono[*N*-(succinimidooxy)-succinyl]icosa(*N*,*N*-dimethyl)-**1** whose synthesis is described. Bis- and tris(*N*-succinyl) and -(*N*-phthalyl) derivatives of **1** are also produced and isolated in usable quantities.

A fundamental problem in the interpretation of electron photomicrographs of biochemical preparations such as multienzyme complexes is the difficulty of distinguishing one type of protein from another of similar size and shape. A means by which individual proteins or other macromolecules in complex biochemical structures could be specifically or selectively marked with labels that can be detected in photomicrographs would simplify the electron microscopic analysis of their geometric arrangements. Useful chemical reagents for labeling a macromolecule within a complex structure would be more electron dense but smaller than typical proteins; owing to high electron density such labels should be identifiable by their characteristic appearance in photomicrographs. The reagents should react specifically or selectively with one or a few components of a complex and be either covalently bonded or strongly absorbed.

The methods of preparation, properties, and X-ray crystal structures of gold cluster compounds containing 8, 9, 11, and 13 gold atoms have been reported (Cariati & Naldini, 1971; Bellon et al., 1972; Steggerda et al., 1982; Vollenbroek et al., 1978). These compounds contain a compact core of gold atoms surrounded by triarylphosphine ligands and in some cases coordinating counterions. Tricyanoheptakis[4,4′,4″-phosphinidynetris(benzenemethanamine)]undecagold,[1] **1**, appears to be particularly well suited for use as an electron-dense labeling reagent (Bartlett et al., 1978). In addition to the high electron density in the core of this molecule, the 21 peripheral amino groups provide both high water solubility and functional groups for derivatization to produce labeling reagents. The recent use of a multibiotinylated derivative to locate the biotin binding sites on avidin has demonstrated the potential of this cluster for use as an electron-dense labeling reagent (Safer et al., 1982).



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In order to make **1** a reagent of general application for electron microscopic studies, derivatives containing a single attached biological ligand or reactive functional group are needed. We report here the synthesis of monosuccinyl and monophthalyl derivatives of **1**. We also describe chemical modifications of remaining amino groups to produce labeling reagents of variable net charge containing a single carboxyl

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[1] Abbreviations: **1**, tricyanoheptakis[4,4′,4″-phosphinidynetris(benzenemethanamine)]undecagold; henicosa(*N*,*N*-dimethyl)-**1**, **1** with all amino groups dimethylated; henicosa(*N*-acetyl)-**1**, **1** with all amino groups acetylated; henicosa(*N*-succinyl)-**1**, **1** with all amino groups succinylated; henicosa[*N*-(carboxymethyl)]-**1**, **1** with 21 *N*-carboxymethyl groups; henicosa[*N*-methyl-*N*-(carboxymethyl)]-**1**, **1** with 21 *N*-methyl and 21 *N*-carboxymethyl groups; mono(*N*-succinyl)-**1**, **1** with a single *N*-succinyl group; mono(*N*-succinyl)icosa(*N*,*N*-dimethyl)-**1**, **1** with a single *N*-succinyl group and 20 *N*,*N*-dimethyl groups; mono(*N*-succinyl)icosa(*N*-acetyl)-**1**, **1** with a single *N*-succinyl group and 20 *N*-acetyl groups; mono(*N*-phthalyl)icosa[*N*-methyl-*N*-(carboxymethyl)]-**1**, **1** with a single *N*-phthalyl group and 20 *N*-methyl and 20 *N*-carboxymethyl groups; mono(*N*-phthalyl)icosa(*N*-acetyl)-**1**, **1** with a single *N*-phthalyl group and 20 *N*-acetyl groups; DMF, dimethylformamide; THF, tetrahydrofuran; Me_4Si, tetramethylsilane; Me_2SO, dimethyl sulfoxide; HMPA, hexamethylphosphoramide, NMR, nuclear magnetic resonance; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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or *N*-hydroxysuccinimide ester group for use as acylating reagents.

Experimental Procedures

Chemicals. Succinic anhydride purchased from Fisher Scientific was recrystallized from acetic anhydride; phthalic anhydride from Fisher Scientific was purified by sublimation. Freshly precipitated gold cyanide was purchased from SPEX Industries in light-proof bottles. Methylene chloride, THF, dioxane, and acetonitrile from Fisher Scientific were dried over calcium hydride by stirring at room temperature for 24 h or by heating under reflux for 2–4 h. The solvents were then distilled into a flask containing 4-Å molecular sieves. The cyclic ethers were protected from light during storage. Pyridine from Fisher Scientific was dried over calcium hydride and distilled into a flask containing barium oxide. DMF from Fisher was dried over 4-Å molecular sieves and distilled at reduced pressure ($T < 25$ °C) into a flask containing barium oxide. *N*-Hydroxysuccinimido acetate was synthesized as described by Santi & Cunnion (1974).

4,4′,4″-Phosphinidynetris[(phenylmethyl)ammonium]tris-(4-methylbenzenesulfonate) was synthesized as described by Bartlett et al. (1978). This procedure was repeatable in our hands when precautions were taken to prevent air oxidation (Reardon, 1982).

The procedure described by Bartlett et al. (1978) for synthesis of **1** by sodium borohydride reduction of a complex formed between 4,4′,4″-phosphinidynetris(benzenemethanamine) and AuCN was used with minor modifications (Reardon, 1982).

[1,4-^{14}C]Succinic anhydride and [*carbonyl*-^{14}C]phthalic anhydride were purchased from Amersham Chemical Co. [*carbonyl*-^{14}C]Phthalic anhydride was diluted with sublimed carrier phthalic anhydride in THF and the specific radioactivity measured. The THF was removed under a slow stream of nitrogen gas, and the solid residue was sublimed at 50 °C under reduced pressure. The sublimed anhydride was weighed, and the specific radioactivity was again determined. Sublimation of the material a second time did not change the specific radioactivity. A sample was weighed, dissolved in THF, and stored at −70 °C until further use. The remaining solid was stored at −20 °C. [1,4-^{14}C]Succinic anhydride was diluted with carrier succinic anhydride and used without additional purification. All other materials, of highest available purity, were purchased from Aldrich Chemical Co., Eastman Kodak Chemical Co., Fisher Scientific, Bio-Rad Laboratories, or Sigma Chemical Co. and used as supplied.

Analytical Procedures. Proton NMR spectra were obtained on Varian EM390, Bruker WP-200, and Bruker WH-270 NMR spectrometers field frequency locked on the deuterium resonance of 99.8 atom % D_2O, $CDCl_3$, or Me_2SO-d_6. The chemical shifts were referenced to a 0.1% Me_4Si internal standard when the Varian EM390 spectrometer was used. A 0.1% Me_4Si external standard was used to reference chemical shifts from spectra obtained on the Bruker WP-200 or Bruker WH-270 instruments.

Phosphorus-31 NMR spectra were obtained on Bruker HX-90, Bruker WP 200, or Nicolet NT-200WB instruments operating at 36.43, 80.10, and 80.98 MHz, respectively. The spectrometer was field frequency locked on the deuterium resonance of 50% D_2O, 50% $CDCl_3$, or 50% Me_2SO-d_6. Chemical shifts were referenced to a 85% D_3PO_4 external standard.

Liquid scintillation counting was done by adding the sample to a glass scintillation vial in a total volume of 1.0 mL of H_2O. To this was added 15.0 mL of Aquasol scintillation fluid, and the samples were counted in a Beckman LS-100C liquid scintillation spectrometer.

The procedure used for the detection of amino groups with ninhydrin is essentially that described by Moore & Stein (1954). The ninhydrin solution was freshly prepared each time and contained 1.0 g of ninhydrin (spectrophotometric grade), 40 mg of $SnCl_2 \cdot 2H_2O$, 25 mL of methyl-Cellosolve, and 25 mL of 0.40 M sodium citrate at pH 5.0. Aliquots of sample (20–50 μL) were diluted to 1.0 mL with the ninhydrin solution and heated to 100 °C for 20 min. The samples were then cooled for 15 min prior to reading the absorbance at 570 nm.

Solutions of undecagold clusters were assayed spectrophotometrically by measuring their A_{415} and calculating their concentrations assuming the extinction coefficient 2.95×10^4 cm^{-1} M^{-1}. This extinction coefficient was determined as described below.

Titrations were performed on a Radiometer Model 26 pH meter equipped with a Corning glass hydrogen electrode. The electrode was standardized with 0.050 M potassium biphthalate (pH 4.00), 0.050 M potassium phosphate (pH 7.00), and 0.050 M potassium borate–potassium carbonate–potassium hydroxide (pH 10.00) before and during the titrations. The samples for titration were heated to 60 °C, purged with nitrogen gas for 60 min, stoppered, and cooled to room temperature. Nitrogen gas was passed over the solution during the titration to prevent CO_2 uptake. Standard solutions of NaOH and HCl for titration were prepared and standardized with 1.000 N HCl or 1.000 N NaOH.

Ion-Exchange Chromatography of **1**. The hydrochloride salt of **1** (109 A_{415} units) was dissolved in water to a concentration of about 0.25 mM. The pH was adjusted to 7.0 and the sample applied to a 2.0 × 16 cm column of SP-Sephadex C-25 in Cl^- form. The column was eluted with a linear gradient of sodium chloride increasing in concentration from 1.00 to 3.00 M and formed from 350 mL of each component. Fractions of 8.2 mL were collected at a flow rate of 2 mL/min, and the A_{415} of each fraction was measured.

Undecagold cluster, **1**, as the hydrochloride salt (780 A_{415} units) was dissolved in deionized water to a concentration of about 0.25 mM. The pH was adjusted to 7.0 and the sample applied to a 1.5 × 30 cm column of SP-Sephadex C-25. The column was eluted with a linear gradient of triethylammonium bicarbonate increasing in concentration from 0.10 to 0.30 M and formed from 450 mL of each component. Fractions of 7.5 mL were collected at a flow rate of 1.5 mL/min, and the A_{415} of each fraction was measured.

The bicarbonate salt of **1** (89 A_{415} units) was dissolved in deionized water to a concentration of about 0.17 mM. The sample was applied to a 1.5 × 12 cm column of QAE-Sephadex Q-25 in the HCO_3^- form and eluted with a linear gradient of triethylammonium bicarbonate increasing in concentration from 0.10 to 1.00 M (pH 7.8) and formed from 300 mL of each component. Fractions of 4.5 mL were collected at a flow rate of 1.5 mL/min. The absorbances of the fractions were measured at 415 nm.

Conversion of Counteranions. The undecagold cluster, **1**, was generally prepared as the hydrochloride salt for use in neutral aqueous solutions. In order to increase the solubility of the cluster in aprotic solvents, the counteranion was changed to *p*-toluenesulfonate. This was accomplished by passing a solution of **1** as the hydrochloride salt (0.34–0.68 mM) through a column of QAE-Sephadex Q-25–*p*-toluenesulfonate. The ratio of exchangeable sites on the column to the number of counteranions present in the gold complex sample was 30–50

to 1. The sample was concentrated by rotary evaporation in vacuo, shell frozen, and lyophilized. The solid was then dissolved in the desired solvent. **1** in the tosylate form is soluble in DMF, Me_2SO, and HMPA. Conversion of the gold complex back to the hydrochloride salt was accomplished by the reverse of the procedure above.

Concentration and Desalting of **1** *and Derivatives.* Large volumes of **1** or derivatives in dilute salt solutions were routinely concentrated by ultrafiltration (pH 7–8) in an Amicon Model 202 ultrafiltration cell employing a UM05 membrane (62 mm). The membrane was hydrated by soaking in 20% glycerol for 30–60 min prior to use. The samples were concentrated at room temperature or at 4 °C at 50–70 psi. Retention of gold complex was always >99%. The concentrate was diluted with water and reconcentrated. This procedure was repeated until the salt concentration was less than 0.03 M. The material was removed from the ultrafiltration cell, concentrated by rotary evaporation in vacuo, and desalted by passage through Sephadex G-10. The desalted samples were stored at −70 °C until further use.

Extinction Coefficients of **1**. The hydrochloride salt of **1** in aqueous solution at pH 7.0 (approximately 1000 A_{415} units) was placed in a 250-mL tared round-bottom flask. The sample volume was reduced to approximately 15 mL by rotary evaporation in vacuo. The concentrate was shell frozen and lyophilized (0.05 torr, 12 h). After the flask was weighed, the weighed sample was dissolved in H_2O and diluted to 100.0 mL. This solution (3.44×10^{-4} M) was then used to prepare a series of solutions from 3.44×10^{-5} to 3.44×10^{-6} M. The absorbances of these solutions were measured at 305 and 415 nm. From the slopes of plots of the absorbances at 305 and 415 vs. concentration by weight, the extinction coefficients were calculated to be 9.93×10^4 and 2.95×10^4 M^{-1} cm^{-1}, respectively, at the two wavelengths.

Undecagold cluster **1** (3800 A_{415} units, 12960 A_{305} units), as the hydrochloride salt, desalted by passage through Sephadex G-10 (10% ethanol–water), was concentrated to dryness by rotary evaporation in vacuo. The solid was dissolved in 25.0 mL of freshly boiled, deionized water. The pH was adjusted to 5.0 with 1.0 N HCl and the solution purged with nitrogen gas for 120 min at 65 °C. The flask was sealed, cooled to room temperature, and titrated with 0.490 M NaOH. This titration provided a measure of the concentration of amino groups in solution. The concentration of **1** was calculated on the assumption that each molecule contains 21 amino groups; from this concentration and the measured absorbances at 305 and 415 nm, the extinction coefficients were calculated to be 9.6×10^4 and 2.8×10^4 M^{-1} cm^{-1}, respectively, in agreement with those measured above and the published values of 1×10^5 and 3.0×10^4 M^{-1} cm^{-1} for $Au_{11}(CN)_3[P(p\text{-}C_6H_4CH_3)_3]_2$ (Bartlett et al., 1978).

Henicosa(N,N-dimethyl)-**1**. The procedure used for reductive methylation of **1** is a modification of a general procedure for reductive methylation of amino groups in proteins (Means & Feeney, 1968). Undecagold cluster, **1** (560 A_{415} units, 18.98 μmol), was dissolved in 10.0 mL of H_2O. Formaldehyde (100 μL of 37%, 1.00 mmol) was added and the pH adjusted to 7.5 with 2.0 N HCl. Sodium borohydride (100 mg, 2.67 mmol) was added to initiate the reaction and again after 10 and 20 min. Formaldehyde (100 μL of 37%, 1.00 mmol) was added every 60 s. The reaction mixture was stirred in an ice–water bath to maintain the temperature below 10 °C, and 2.0 N HCl was added to maintain the pH between 8.0 and 10.0. Aliquots (20 μL) of the reaction mixture were removed at 2- or 5-min intervals and quenched with 50 μL of acetone for use in determining the rate of disappearance of primary amino groups using a ninhydrin assay. After 30 min the reaction mixture was concentrated to approximately 5 mL and applied to a Sephadex G-10 column (1.5 × 45 cm). The material was eluted with 10% ethanol–water, concentrated to dryness by rotary evaporation in vacuo, and stored in deionized H_2O at −70 °C: yield 93%; ^1H NMR (270 MHz, D_2O) δ 7.63 (2 H), 6.65 (2 H), 3.17 (2 H), 2.00 (6 H); ^{31}P NMR (36.4 MHz, D_2O, proton decoupled) δ 52.50 (S).

Henicosa(N-acetyl)-**1**. Undecagold cluster, **1** (159.5 A_{415} units, 5.41 μmol), was dissolved in 25.0 mL of water and the pH adjusted to 5.0. The solution was heated to 60 °C and maintained at that temperature for 60 min while purging with nitrogen gas. After the solution was cooled to room temperature and the pH adjusted to 8.0, acetic anhydride (20 μL, 0.21 mmol) was added and the pH of the reaction mixture maintained between 7 and 8 by addition of 2.0 M NaOH. When the pH became constant, it was readjusted to 8.0 and the addition of acetic anhydride repeated. A total of five 20-μL aliquots of acetic anhydride were added. Before each addition a 25-μL sample was removed from the reaction mixture, 1.0 mL of ninhydrin solution was added, and the samples were assayed as described previously. The product was concentrated to approximately 12 mL by rotary evaporation in vacuo. One milliliter of 95% ethanol was added and the sample applied to a 2.5 × 45 cm column of Sephadex G-10 and eluted with 10% ethanol–water. The desalted henicosa(*N*-acetyl)-**1** peak was concentrated to dryness by rotary evaporation in vacuo, dissolved in 15 mL of water, and stored at −70 °C until further use (yield 97%). Titration of the acetylated **1** showed no detectable ionizable groups in the pH range from 3 to 11.

This was also prepared by using *N*-succinimidyl acetate as the acetylating agent. Undecagold cluster, **1** (89.6 A_{415} units, 3.04 μmol), was dissolved in 5.0 mL of degassed 0.20 M Pipes buffer (pH 7.00). *N*-Succinimidyl acetate (95.4 mg, 608 μmol) was dissolved in 1.5 mL of THF. The ester/THF solution was added to the solution of **1** over a 30-s period. Aliquots of the reaction mixture were removed and used for the ninhydrin assay. After 30 min, the reaction mixture was concentrated to 3 mL by rotary evaporation in vacuo and applied to a 1.5 × 45 cm column of Sephadex G-10. The product, henicosa(*N*-acetyl)-**1**, was eluted with 10% ethanol–water to give 84.1 A_{415} units (94% yield): ^1H NMR (270 MHz, D_2O) δ 7.33 (2 H), 6.66 (2 H), 4.07 (2 H), 1.91 (3 H).

Henicosa(N-succinyl)-**1**. Undecagold cluster, **1** (190 A_{415} units, 6.44 μmol, 135 μequiv of amino groups), was dissolved in 10.0 mL of water. The pH was adjusted to 5.0 with 1.0 N HCl and the solution purged with nitrogen gas for 60 min at 60 °C. After the solution was cooled to room temperature, the pH was readjusted to 8.0. Succinic anhydride (260 mg, 2.60 mmol), dissolved in 1.50 mL of acetonitrile, was added in 100-μL aliquots. The pH was maintained between 7.5 and 8.0 with 2.0 N NaOH. Aliquots of the reaction mixture (50 μL) were removed prior to each addition of succinic anhydride and used to monitor the rate of disappearance of the primary amines in the sample. The product was concentrated to approximately 4 mL and applied to a column of Sephadex G-10 (1.5 × 45 cm). The column was eluted with 10% ethanol–water, and the desalted cluster was concentrated to dryness by rotary evaporation in vacuo, dissolved in 5.0 mL of water, and stored at −70 °C until further use (yield 96%): ^1H NMR (270 MHz, D_2O) δ 7.15 (2 H), 6.65 (2 H), 4.07 (2 H), 2.40 (4 H).

Henicosa[N-(carboxymethyl)]-**1**. Undecagold cluster, **1** (1727 A_{415} units, 58.55 μmol, 1230 μequiv of amino groups),

was dissolved in boiled water to a total volume of 25 mL. The pH was adjusted to 5.0 with 1.0 N HCl and the solution purged with nitrogen gas for 60 min at 60 °C. The flask was stoppered and the solution cooled to room temperature. The sample was always handled under a nitrogen atmosphere. The pH was adjusted to 9.10 with 1.0 and 0.50 N NaOH. Bromoacetic acid (57.0 mg, 410 μmol) in 1.0 mL of H_2O (pH 9.1) was then added and this addition repeated after 12 and 36 h at 25 °C. The total reaction time was 72 h. The pH was maintained between 8 and 9 during the reaction.

The extent of reaction was monitored by titration of the protons produced using as the titrant 0.490 M NaOH and was stopped when 1200 μmol of HBr had been produced (corresponding to 98% conversion) by adjusting the pH to 4.5 with 1.0 N HCl. The reaction mixture was then concentrated to approximately 10 mL by rotary evaporation in vacuo, applied to a column of Sephadex G-10 (2.5 × 45 cm) and eluted with 10% ethanol–water at a flow rate of 1.5 mL/min. The desalted, (carboxymethyl)undecagold was then concentrated to dryness by rotary evaporation in vacuo and dissolved in 25 mL of boiled deionized water, and 1000 A_{415} units (33.90 μmol) was placed in 80.0 mL of H_2O. The pH was adjusted to 5.5 with 1.0 N HCl and the solution purged with nitrogen gas for 60 min at 60 °C. After cooling to room temperature, the sample was divided into halves which were diluted to 4.0 A_{415} units/mL and the pH readjusted to 5.5. These samples were applied to columns of SP-Sephadex C-25 and QAE-Sephadex Q-25. The material not retained by each of these columns was pooled separately and stored at −70 °C for later use. The columns were eluted with 400-mL linear NaCl gradients increasing from 0.00 to 0.40 M (pH 5.5). The material not retained on the QAE-Sephadex Q-25 column above was loaded on SP-Sephadex C-25, sodium form (1.5 × 10 cm). The material not retained on the SP-Sephadex C-25 column was applied to QAE-Sephadex Q-25, chloride form (1.5 × 10 cm). These columns were eluted with the same gradient described above. The peaks from the ion-exchange columns above corresponding to carboxymethylated undecagold species of different net charge were pooled separately and stored at −70 °C. The gold complex not retained by either SP-Sephadex or QAE-Sephadex represents henicosa(*N*-carboxymethyl)-**1** (22.5%). This material was concentrated to about 5 mL by rotary evaporation in vacuo, desalted by passage through Sephadex G-10 (1.5 × 45 cm, 10% ethanol–water), and stored at −70 °C until further use: ^{1}H NMR (270 MHz, D_2O) δ 7.57 (2H), 6.69 (2 H), 3.98 (2 H), 3.65 (2 H).

Henicosa[*N-methyl-N-*(*carboxymethyl*)]-**1**. Henicosa[*N*-(carboxymethyl)]-**1** (79.2 A_{415} units, 2.68 μmol) was dissolved in 7 mL of water and the pH adjusted to 5.0. The solution was heated to 60 °C and maintained at that temperature for 60 min while purging with nitrogen gas. After the solution was cooled to room temperature, the pH was adjusted to 7.5. This material was reductively methylated according to the procedure above for formation of henicosa(*N,N*-dimethyl)-**1**. The product was desalted on Sephadex G-10 to give 76.6 A_{415} units (97% yield): ^{1}H NMR (270 MHz, D_2O) δ 7.55 (2 H), 6.68 (2 H), 3.75 (2 H), 3.54 (2 H), 2.22 (3 H).

Mono(*N-succinyl*)-**1**. Undecagold (564 A_{415} units, 19.1 μmol) was dissolved in 5.0 mL of H_2O and the pH adjusted to 7.5 with 1.0 N NaOH. Succinic anhydride (28.6 mg, 286 μmol) was dissolved in 1.00 mL of acetonitrile, and 100 μL of this solution was added to **1** in two 50-μL aliquots. After 10 min, 20 mL of H_2O was added and the pH of the solution adjusted to 5.0. The solution was purged with nitrogen gas for 60 min at 60 °C and cooled to room temperature, and the pH was adjusted to 11.5. The volume was then increased to 125 mL with boiled water at pH 11.5. The sample was chromatographed at pH 11.5 through a 1.5 × 28 cm column of QAE-Sephadex Q-25 in the chloride form, eluted with a 800-mL linear NaCl gradient increasing from 0.00 to 0.40 M. The fractions containing mono(*N*-succinyl)-**1** were pooled and adjusted to pH 7.0. The combined fractions were concentrated to about 15 mL by ultrafiltration, further concentrated to approximately 5 mL by rotary evaporation in vacuo, and desalted by passage through a 1.5 × 45 cm column of Sephadex G-10 (10% ethanol–water). The eluted material was concentrated to dryness by rotary evaporation in vacuo and redissolved in 20 mL of H_2O. The pH was adjusted to 5.0 and the solution again purged with nitrogen for 60 min at 60 °C. The sample was diluted with 30 mL of boiled water (pH 11.5) and the pH adjusted to 11.5 with 1.0 N NaOH. The solution was then applied to QAE-Sephadex Q-25, chloride form (1.5 × 5 cm). After the sample was absorbed, the column was washed with 2–3 volumes of water (pH 11.5). Mono(*N*-succinyl)-**1** was then eluted isocratically with 0.12 M NaCl (pH 11.5). The eluted mono(*N*-succinyl)-**1** was concentrated and desalted by ultrafiltration to approximately 10 mL. This material was further concentrated to about 5 mL by rotary evaporation in vacuo and applied to a column of Sephadex G-10 (1.5 × 45 cm). The eluted mono(*N*-succinyl)-**1** was concentrated to dryness by rotary evaporation in vacuo and diluted with 10 mL of water, and the pH was adjusted to 7.5. This material was stored at −70 °C until use (yield 5–10%).

Mono(*N-succinyl*)*icosa*(*N,N-dimethyl*)-**1**. Mono(*N*-succinyl)-**1** (28 A_{415} units, 0.95 μmol, 19.9 μequiv of amino groups) was dissolved in 5.0 mL of water. The pH was adjusted to 5.0 and the solution purged with nitrogen gas for 40 min at 60 °C. After the sample was cooled to room temperature, the pH was adjusted to 7.5 and the solution cooled by stirring in an ice bath. Formaldehyde (10 μL of 37%, 0.10 mmol) was added. Sodium borohydride (10.0 mg, 0.267 mmol) was added to initiate the reaction and the addition repeated after 10 and 20 min. Formaldehyde (10 μL of 37%, 0.100 mmol) was added every 60 s. The pH was maintained below 10.0 by addition of 1.0 M HCl when necessary. When the additions were complete, acetic anhydride (100 μL, 1.06 mmol) was added. The pH was maintained between 7 and 8 by addition of 1.0 M NaOH. After the pH stabilized, the acetic anhydride addition was repeated. When the pH became stable, it was adjusted to 7.5. The sample was concentrated to about 5.0 mL and desalted by gel filtration through Sephadex G-10 (1.5 × 48 cm). The desalted mono(*N*-succinyl)icosa(*N,N*-dimethyl)-**1** (25.5 A_{415} units, 0.864 μmol) was stored at −70 °C until further use (yield 91%).

Mono(*N-succinyl*)*icosa*(*N-acetyl*)-**1**. Mono(*N*-succinyl)-**1** was acetylated by reaction with either acetic anhydride or *N*-succinimidyl acetate was described above for the synthesis of henicosa(*N*-acetyl)-**1**. Acetylation by acetic anhydride produced a substantial percentage of cross-linked product, largely dimers. Acetylation by *N*-succinimidyl acetate produced exclusively monomeric mono(*N*-succinyl)icosa(*N*-acetyl)-**1**.

Mono(*N-phthalyl*)-**1**. Mono(*N*-phthalyl)-**1** was prepared by a procedure very similar to that used to prepare mono(*N*-succinyl)-**1**. The *p*-toluenesulfonic acid salt of **1** (490 A_{415} units, 16.64 μmol) was dissolved in 5.0 mL of 50% DMF–H_2O and the pH adjusted to 8.0. Phthalic anhydride (2.48 mg, 16.74 μmol) was dissolved in 0.20 mL of dry THF and added

to **1** in four 50-μL aliquots over a 2-min period. The pH was maintained at 8.0 with 1.0 N NaOH. After the additions were completed, the solution was stirred for an additional 10 min. The sample was then diluted to 15 mL with deionized water and the pH adjusted to 6.0. The sample was purged with nitrogen for 60 min at 60 °C. After the sample was cooled to room temperature, the pH was adjusted to 11.5 and the solution diluted to 80 mL with boiled water at pH 11.5. The product mixture was applied to a 1.5 × 12 cm column of QAE-Sephadex Q-25 and eluted with a 700-mL linear NaCl gradient increasing from 0.00 to 0.40 M (pH 11.5). The fractions containing mono(*N*-phthalyl)-**1** were pooled, and the pH was adjusted to 7.0. The sample was concentrated by ultrafiltration to 15–20 mL and then further concentrated by rotary evaporation in vacuo to about 5 mL. This material was desalted by passage through a column of Sephadex G-10 (1.5 × 45 cm), equilibrated and eluted with 10% ethanol–water. After the sample was evaporated to dryness by rotary evaporation in vacuo, the mono(*N*-phthalyl)-**1** was dissolved in 20 mL of water and the pH adjusted to 5.0. This solution was purged with nitrogen for 60 min at 40 °C. After the solution was cooled to room temperature, the pH was adjusted to 11.5 and the volume adjusted to 40 mL with boiled deionized water (pH 11.5). The solution was then applied to QAE-Sephadex Q-25, chloride form (1.5 × 5 cm). After the sample was absorbed, the column was washed with 1–2 column volumes of H_2O at pH 11.5. The mono(*N*-phthalyl)-**1** was then eluted with 0.09 M NaCl at pH 11.5. The eluted undecagold derivative was concentrated to approximately 6 mL by rotary evaporation in vacuo and desalted by passage through a column of Sephadex G-10 (1.5 × 48 cm), equilibrated and eluted with 10% ethanol–water. The desalted mono(*N*-phthalyl)-**1** was concentrated to dryness by rotary evaporation in vacuo, dissolved in 5 mL of water, and stored at −70 °C until further use (yield 5–10%).

*Mono(N-phthalyl)icosa[N-methyl-N-(carboxymethyl)]-***1**. Mono(*N*-phthalyl)-**1** (58.56 A_{415} units, 1.99 μmol) was placed in 5.0 mL of water and purged with nitrogen gas for 30 min at pH 6.0 and 45 °C. The solution was handled under a stream of nitrogen for the remainder of the procedure. The pH was adjusted to 9.15 with NaOH. Bromoacetic acid (28.97 mg, 0.208 mmol) was dissolved in 400 μL of water, the pH adjusted to 9.10 with NaOH, and the volume adjusted to 1.00 mL. A 100-μL aliquot of this solution was added to the gold complex solution and the pH adjusted to 9.16. After 12 h at room temperature, the HBr produced was titrated with 0.244 N NaOH. A second 100-μL aliquot of the bromoacetate solution was added and the reaction mixture left at room temperature for 15 h. The HBr was once again titrated. To this point, 40.0 μmol of HBr had been produced. The pH was adjusted to 6.5 and the solution concentrated to about 2 mL by rotary evaporation in vacuo. This material was desalted by passage through a 1.5 × 28 cm column of Sephadex G-10. The desalted mono(*N*-phthalyl)icosa[*N*-(carboxymethyl)]-**1** dissolved in 7.0 mL of water was purged with nitrogen gas for 20 min at pH 6.0 and 45 °C. This material was permethylated according to the procedure used to synthesize mono(*N*-succinyl)icosa(*N*,*N*-dimethyl)-**1** (overall yield 93%).

*Mono(N-phthalyl)icosa(N-acetyl)-***1** *and Mono(N-phthalyl)icosa(N,N-dimethyl)-***1**. Mono(*N*-phthalyl)icosa-(*N*,*N*-dimethyl)-**1** was synthesized according to the procedure used to prepare mono(*N*-succinyl)icosa(*N*,*N*-dimethyl)-**1**. Acetylation of mono(*N*-phthalyl)-**1** with *N*-succinimidyl acetate was performed by using the procedure developed to prepare henicosa(*N*-acetyl)-**1**. The samples were purged with nitrogen gas for 30 min at pH 6.0 and 45 °C prior to derivatization.

*Mono[N-(succinimidooxy)succinyl]icosa(N,N-dimethyl)-***1**. Mono(*N*-succinyl)icosa(*N*,*N*-dimethyl)-**1** in the tosylate salt form (12.7 A_{415} units, 0.43 μmol) was placed in 150 μL of alumina-dried DMF. Dicyclohexylcarbodiimide (10 μL of a 1.00 M solution in DMF) and *N*-hydroxysuccinimide (10 μL of a 1.00 M solution in DMF) were added, and the reaction was stirred for 60 min at room temperature. The product mixture can be used without purification for coupling to amino groups in DMF. Alternatively, the sample should be passed through a 0.7 × 18 cm column of Sephadex G-10 immediately prior to use as an acylating reagent in aqueous solution. The presence of the *N*-hydroxysuccinimide ester was confirmed by its ability to form multimers of the cluster. Multimer formation was accomplished by addition of **1** to the product mixture; multimers were detected by gel permeation chromatography on Sephadex G-50.

Results

Chromatographic Properties of **1**. The chromatographic properties of **1** on Sephadex ion-exchange resins are highly dependent upon the elution system employed. By use of SP-Sephadex C-25 in the triethylammonium form, a linear gradient of triethylammonium bicarbonate from 0.10 to 0.30 M eluted the cluster at a salt concentration of 0.18 M. When **1** was chromatographed through SP-Sephadex C-25 in the sodium form using a linear NaCl gradient increasing from 1.00 to 3.00 M, the cluster was eluted at a salt concentration of 2.08 M. The large difference in salt concentration required to elute the gold complex from these columns reflects the propensity of the cluster to form carbamates in carbon dioxide containing buffers. At pH 7.8 **1** has a charge of about 20+, which is reflected in the high [NaCl] gradient required to elute it. Elution of **1** from SP-Sephadex by triethylammonium bicarbonate at a concentration of 0.18 M indicates a net charge of 3+ to 5+ under these conditions. This represents approximately 45% conversion of the primary ammonium groups to carbamates.

Application of **1** to QAE-Sephadex Q-25 in the bicarbonate form resulted in 100% retention of the material by the column. It was eluted from this column at 0.5 M triethylammonium bicarbonate, reflecting a net charge of 5– to 7–. This represents 65% conversion of the amino groups to carbamates and suggests that the ionic interaction of the carbamate oxygen anion with the quaternary ammonium groups on QAE-Sephadex has a stabilizing effect on the carbamate groups. This increased stability shifts the carbamoylation equilibrium toward carbamates, and **1** attains a net negative charge.

The chromatographic behavior of **1** with ion exchangers and triethylammonium bicarbonate exemplifies and highlights its tendency to react with CO_2. With 21 primary amino groups per molecule, this tendency is marked, even in solutions and buffers other than bicarbonates or carbonates that nontheless contain CO_2 and HCO_3^- owing to equilibration with atmospheric CO_2. For this reason any sample of **1** that has not been expressly freed of CO_2 will contain some carbamate groups and will, therefore, be absorbed by anion exchangers at high pH. These carbamates undermine chromatographic purifications of derivatives through ion-exchange media and also severely complicate chemical modifications of the amino groups. For these reasons, all chemical modification procedures must be preceded by purging the solution with N_2 at pH 5 and 45 °C to remove CO_2.

Synthesis of Monocarboxyl Derivatives. We have sought to develop efficient procedures for synthesizing **1** containing

a single reactive functional group in each molecule. In our procedure, a sample of **1** would be acylated with slightly more than 1 equiv of succinic or phthalic anhydride. The resulting mixture of mono-, di- and trisuccinyl-**1** or phthalyl-**1** and unreacted **1** would then be separated by anion-exchange chromatography at a high pH, where they would exist as uncharged, monoanionic, dianionic, etc., species. The remaining 20 primary amino groups in mono(*N*-succinyl)-**1** or mono(phthalyl)-**1** would then be chemically modified in such a way as to prevent them from interfering with subsequent chemical activations of the carboxyl group in preparation for reaction with biochemical specimens.

After samples of **1** were acylated with 1–2 equiv of succinic anhydride or phthalic anhydride, the product mixtures were separated by anion-exchange chromatography at high pH. The peaks eluted from the columns were identified as mono(*N*-phthalyl)-**1**, di(*N*-phthalyl)-**1**, etc., by carrying out the acylation with [*carbonyl*-^{14}C]phthalic anhydride (2.78×10^5 cpm μmol^{-1}). After acylating as described under Experimental Procedures, the sodium [*carbonyl*-^{14}C]phthalate produced as a hydrolytic side product was removed by passage of the diluted reaction mixture through a column of QAE-Sephadex Q-25 in the chloride form (1.5 × 10 cm) at pH 7.0. The effluent was then chromatographed through QAE-Sephadex at high pH (see Figure 1), and the specific radioactivities of the eluted peaks were measured. The flow through contained both cluster and ^{14}C, but only the well-defined peaks were analyzed. The specific radioactivities of the first four were 4.5×10^5, 6.0×10^5, 8.3×10^5, and 10×10^6 cpm μmol^{-1}, respectively, or 1.6, 2.2, 3.0, and 3.6 times that of the [*carbonyl*-^{14}C]phthalic anhydride used in the acylation. Nonintegral ratios of specific activities associated with the peaks relative to that of [*carbonyl*-^{14}C]phthalic anhydride were consistent with cross-contamination among the peaks, as was the failure to achieve base-line separations in the A_{415} elution profile. Pure mono(*N*-[^{14}C]phthalyl)-**1** was obtained by rechromatographing the first defined peak from Figure 1A. This elution profile revealed the contaminations as well as a major peak of mono(*N*-[^{14}C]phthalyl)-**1** with a specific radioactivity of 2.90×10^5 cpm μmol^{-1}, very near that of the phthalic anhydride. Similar results were obtained when **1** was treated with [^{14}C]succinic anhydride. The relative ratio of specific radioactivity for peaks in Figure 1B was 1.0:1.8:2.3:2.7, again deviating from the integral ratios indicative of clean separations. The ratio of 1.0:1.8 suggests that the mono(*N*-succinyl)-**1** is less contaminated by other undecagold derivatives than the mono(*N*-phthalyl)-**1** derivative. It is, nevertheless, necessary to rechromatograph mono(*N*-succinyl)-**1** to obtain the material in pure form.

Chemical Modifications of Primary Amino Groups. Before further chemical manipulations of the carboxyl groups in mono(*N*-succinyl)-**1** or mono(*N*-phthalyl)-**1** could profitably be pursued, the nucleophilic reactivity of the remaining amino groups had to be reduced. Several straightforward methods for chemically modifying the remaining primary amino groups were evaluated with satisfactory results.

The two types of chemical modifications most commonly used for primary amines are alkylation to tertiary amines and acylation to amides. Tertiary amines are less reactive as nucleophiles than primary amines due to steric hindrance, and amides are even less nucleophilic. By appropriate choice of reagents it is possible to arrange any desired electrostatic charge type in the chemically modified derivative. Parent compound **1** is a polycation in neutral solution, and this charge is retained when the primary amino groups are converted to

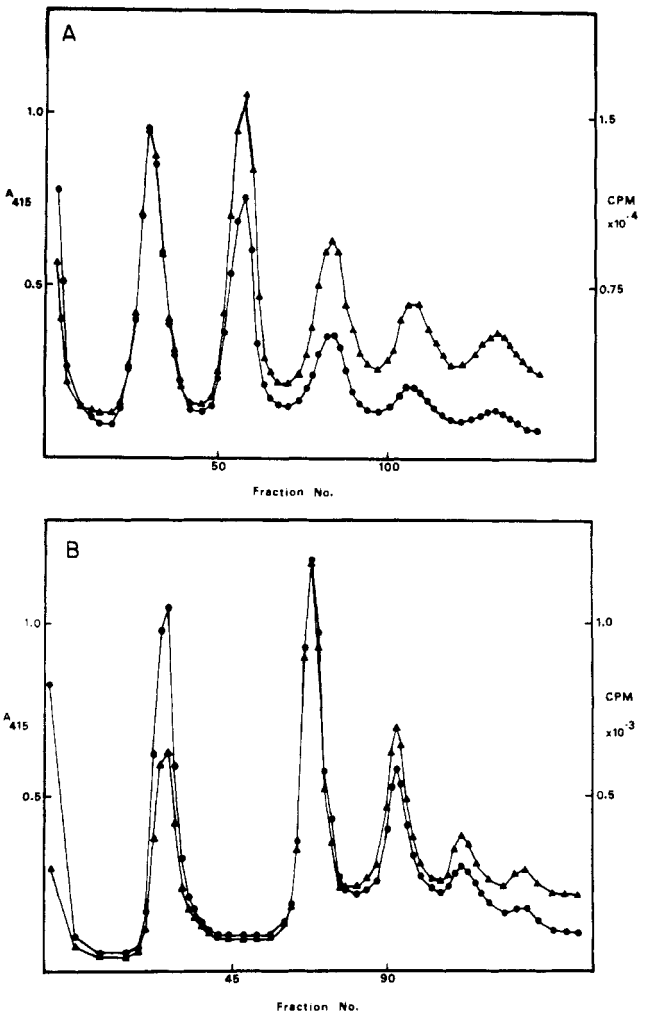
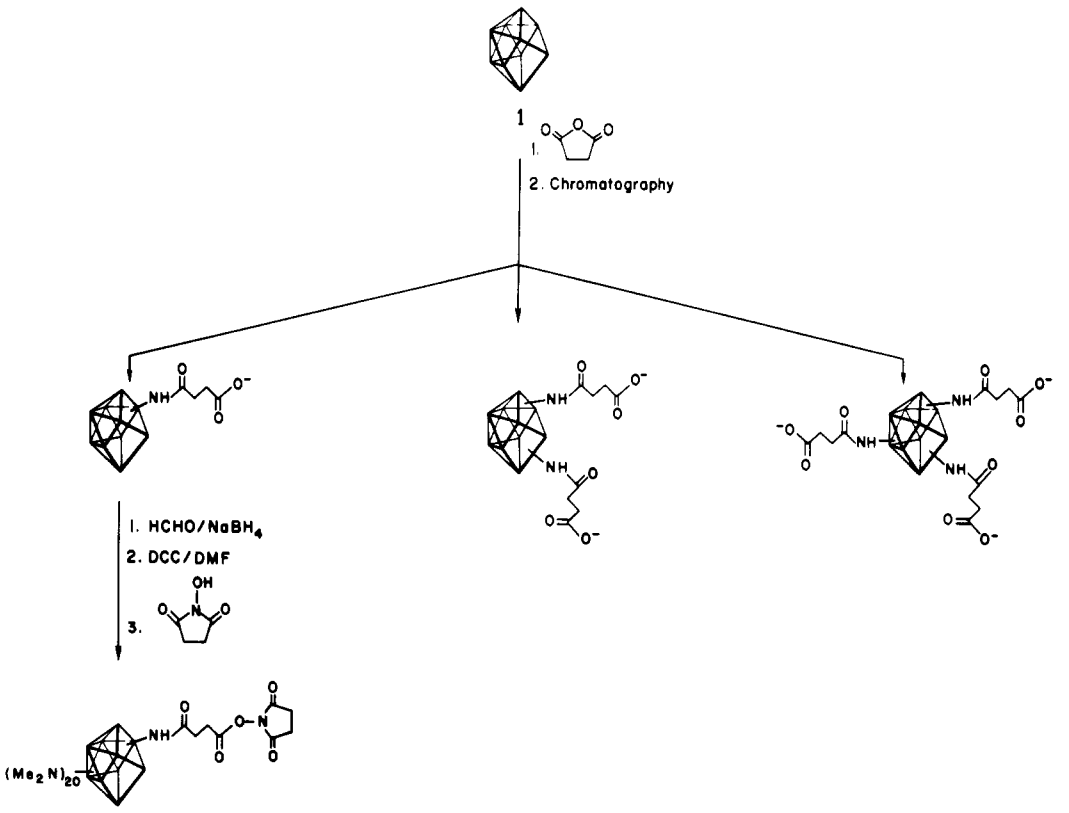


FIGURE 1: Chromatographic purification of monoacyl derivatives of **1**. Samples of ^{14}C-labeled phthalylated **1** and ^{14}C-labeled succinylated **1** prepared as described under Experimental Procedures were separated on QAE-Sephadex Q-25 at pH 11.5. (A) The phthalylation reaction mixture was applied to a 1.5 × 12 cm column of QAE-Sephadex Q-25 and eluted with a 700-mL linear NaCl gradient increasing from 0.00 to 0.40 M (pH 11.5). (B) The succinylation reaction mixture was applied to a 1.2 × 28 cm column of QAE-Sephadex Q-25 and eluted with an 800-mL linear NaCl gradient increasing from 0.00 to 0.40 M. Fractions were assayed for absorbance at 415 nm (●) and radioactivity (cpm) (▲).

dimethylamino groups by reductive methylation using formaldehyde and $NaBH_4$. The cluster can be made essentially zwitterionic by first alkylating it with 21 equiv of bromoacetate and then reductively methylating the product with formaldehyde and $NaBH_4$. This modification is not perfectly uniform because it cannot be perfectly controlled to produce exclusively *N*-methyl-*N*-carboxymethyl groups to the total exclusion of *N*,*N*-bis(carboxymethyl) and *N*,*N*-dimethyl groups. The clusters are, nevertheless, essentially zwitterionic in neutral solution as shown by the titration curve in Figure 2. Only small percentages of such samples are absorbed by anion- or cation-exchange resins from neutral solutions, confirming their essential neutrality. Polyanionic clusters may be prepared by reaction of the primary amino groups with succinic anhydride. The titration curves in Figure 2 illustrate the electrostatic differences among **1**, henicosa[*N*-methyl-*N*-(carboxymethyl)]-**1**, and henicosa(*N*-succinyl)-**1**. The titration curves are broad, typical of molecules undergoing multiple ionizations. Nevertheless, the transitions are well enough separated to verify the essential nature of these derivatives. The succinyl derivative behaves as a polycarboxylic

Scheme I



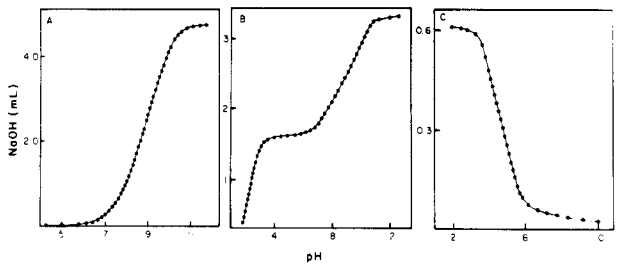


FIGURE 2: Acid–base titration of **1**. Shown are the titration curves for **1** (126 μmol) (part A), henicosa[*N*-(carboxymethyl)-*N*-methyl]-**1** (38.3 μmol) (part B), and henicosa(*N*-succinyl)-**1** (5.6 μmol) (part C). Titrations were performed as described under Experimental Procedures with 0.490 M NaOH or 0.242 M HCl.

acid and **1** as a polyamine; henicosa[*N*-methyl-*N*-(carboxymethyl)]-**1** titrates as a polyamine between pH 10 and 7 and as a polycarboxylic acid below pH 4. In the range between 4 and 7 it is electrostatically neutral.

A fourth method leading to electrostatically neutral acetamido groups is the reaction of **1** with acetylating agents such as acetic anhydride or *N*-succinimidyl acetate. The latter is perferred for acetylating mono(*N*-succinyl)-**1** or mono(*N*-phthalyl)-**1** since acetic anhydride tends to promote cross-linking, a process that may involve the transient formation of mixed anhydrides.

The experimental conditions for these chemical modifications are described under Experimental Procedures. All can be monitored by measuring the disappearance of primary amino groups. Those modifications involving the introduction of carboxyl groups are not compatible with the further chemical activation of mono(*N*-succinyl)-**1**.

Chemical modifications of the primary amino groups in mono(*N*-succinyl)-**1** to dimethylamino groups by reductive methylation or acetamido groups by acetylation produced mono(*N*-succinyl)icosa(*N*,*N*-dimethyl)-**1** or mono(*N*-succinyl)icosa(*N*-acetyl)-**1**, both of which contained a single carboxyl group per molecule. This group could be activated and used to acylate amino groups in proteins.

Acylating **1**. The synthesis of mono[*N*-(succinimidooxy)-succinyl]icosa(*N*,*N*-dimethyl)-**1** is described under Experimental Procedures and outlined in Scheme I. Partial succinylation of **1** produces a mixture of mono-, bis-, tris-, etc., (*N*-succinyl)-**1** which is separated by ion-exchange chromatography. The remaining primary amino groups in mono(*N*-succinyl)-**1** are alkylated to dimethylamino groups, and the succinyl group is activated by reaction, first with DCC in DMF and then with *N*-hydroxysuccinimide.

Reaction of this activated derivative with **1** itself produces a mixture of multimers that can be detected by gel filtration chromatography. Similar reaction of amino groups in proteins could be controlled by pH and the ratio of mono[*N*-(succinimidooxy)succinyl]icosa(*N*,*N*-dimethyl)-**1** to protein. Acylation of proteins by this reagent can be monitored and characterized by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, since the molecular weights are increased in increments of 5300 for each **1** covalently bonded to the protein. This technique has been used to identify the protein modified in cytochrome oxidase by a similar reagent.

Discussion

It is possible to synthesize derivatives of **1** with single reactive functional groups by extending and varying Scheme I. The activated acylating cluster in Scheme I is polycationic at pHs below 10. If the underivatized primary amino groups in mono(*N*-succinyl)-**1** were acetylated by reaction with *N*-hydroxysuccinimido acetate instead of being alkylated, the product would be uncharged. The corresponding mono(*N*-phthalyl)-**1** can be alkylated to polycationic or zwitterionic derivatives or acetylated to the icosa(*N*-acetyl) derivative. The phthalyl group can be removed by the procedure described in the following paper (Yang et al., 1984), unmasking a single primary amino group. The exposed amino group then can be further modified in a variety of ways, making it possible to

introduce alkylating or acylating functional groups. Two such derivatives are described in the following paper (Yang et al., 1984).

Bis- and tris(*N*-succinyl)-**1** and -(*N*-phthalyl)-**1** are produced and isolated in usable quantities as byproducts of the procedures described in this paper (see Figure 1 and Scheme I). These derivatives are potentially useful for specialized applications requiring two or three functional groups per cluster. For example, the polymerization of avidin described in the work of Safer et al. (1982) depends upon the presence of at least two biotinyl moieties bonded to each molecule of cluster. Bis- and tris(*N*-succinyl)-**1** can potentially also be used to prepare defined multimers of **1** for specialized applications. Such multimers could also be prepared from di- and triamino clusters resulting from removal of phthalyl groups from bis- and tris(*N*-phthalyl)icosa(*N*-acetyl)-**1**.

The mono(*N*-succinyl) and mono(*N*-phthalyl) derivatives of **1** described in this paper are homogeneous with reference to their succinyl and phthalyl contents. These preparations do not, however, consist of structurally identical species. This is because the primary amino groups in **1** are not structurally identical but are essentially equivalent in chemical reactivity. Therefore, monoacylation of **1** cannot be specific and must lead to a mixture of monoacyl species. This slight structural diversity will not undermine the usefulness of the compounds in the applications for which they are intended.

Other electron-dense labeling reagents have been used in electron microscopic analysis of the structural organization of biological systems. The most widely used is the iron storage protein ferritin covalently coupled to a biological ligand (Singer, 1959). These ferritin conjugates are then used to localize the positions of the complementary biological molecules, to which the coupled ligand binds, within the biological preparation under study. This technique has been successfully applied to analysis of the structural organization of large biological structures (membrane ghosts, cell organelles, etc.). The relatively large size of the ferritin molecule (120 Å o.d.) and the distance of separation of the label from the labeled site, determined by the size of the attached ligand, limit the meaningful resolution to about 200 Å. In order to examine the symmetry and structural organization of smaller biological aggregates (e.g., histones, ribosomes, and multienzyme complexes), a considerably smaller electron-dense label is required to obtain the necessary resolution of 10–20 Å. Methods have been developed to label nucleic acids, single protein molecules, and small protein aggregates with compounds containing a single heavy atom (Beer & Ottensmeyer, 1979; Cole et al., 1977); however, visualization of these labels has been difficult. To facilitate this type of analysis, by both conventional and scanning transmission electron microscopy, a molecule containing several closely packed heavy atoms is necessary. The **1** molecules described here may be applicable to such analyses.

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