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The Ohio State University

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SYNTHESIS OF ALKYLATING AND ACYLATING UNDECAGOLD CLUSTERS:
APPLICATION TO BIOCHEMICAL STRUCTURAL ANALYSIS

DISSERTATION
Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By
John E. Reardon, B.A.

The Ohio State University
1982

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Approved by
Professor Perry A. Frey
Advisor
Department of Chemistry
To my parents for instilling the belief that
an education is invaluable
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ABBREVIATIONS

The terms undecagold, undecagold cluster and undecagold complex all refer specifically to tricyano-heptakis[4,4',4''-phosphinidynetri(benzenemethanamine)]-undecagold. Derivatives of undecagold are denoted by a prefix. The terms eicosi and heneicosi are used to specify twenty and twenty-one, respectively. The term mono-functional undecagold refers to derivatives of the cluster in which one of the twenty-one amino groups has been derivatized. The remaining amino groups may be underivatized or all derivatized in the same manner. The term gold complex is a general term referring to all derivatives of the undecagold cluster.

All other abbreviations are standard.
CHAPTER I
INTRODUCTION

1.1 General Background on Electron Microscopy

Image formation involves the scattering of incident radiation by an object followed by the focusing of the scattered radiation to form the image. Upon magnification, the resolution of object detail in the image is dependent upon the wavelength of the incident radiation. The wavelength of radiation used must be smaller than the dimensions of the detail to be resolved. X-rays possess a wavelength range suitable for studies at the atomic level. Although X-rays cannot be focused, information about an object that has scattered X-rays may be obtained by data interpretation. In this sense, the Fourier transform is substituted for an optical system in the formation of an image.

Electrons of moderate energies are also of short enough wavelength to provide information at the molecular and atomic level. The diffraction of electrons by the electrostatic potential of atomic nuclei and orbital electrons can be treated similarly to X-ray diffraction data to reveal the potential distribution within the object. In addition, since electrons are charged they
may be focused by a suitable electromagnetic lens to directly form an image of the object. A diagram of a conventional transmission electron microscope (CTEM) is shown in Diagram 1. The hot filament electron source emits electrons at controlled energies. The electron beam is collimated by a condenser lens and aperture and then impinges on the sample. The scattered electrons are focused through the objective aperture onto the photographic plate by the objective lens.

When exposed to the electron beam, areas of the sample of higher electron density scatter more electrons than areas of lower electron density. Some of the scattered electrons do not participate in formation of the final image because they are not properly focused and are stopped by the objective aperture. Other electrons are inelastically scattered and thus are not focused in the image plane. As a result, the image on the photographic plate appears darker in areas corresponding to greater electron density. This type of contrast is termed amplitude contrast and is the only contrast desired in the photomicrograph.

In a thin specimen for CTEM the scattered electrons possess information concerning the three-dimensional mass distribution of the sample. Due to the limited objective aperture, the depth of field of the electron microscope

- Source
- Condenser lens
- Specimen
- Objective lens
- Back focal plane
- Selected area aperture
- Intermediate lens
- Projector lens
- Final image
is several hundred angstroms. As a result, all levels of the specimen are superimposed and focused on the two-dimensional image plane. For this reason, interpretation of electron photomicrographs can be very complex. Only by reviewing samples of the object at many orientations relative to the incident electron beam is one able to construct a three-dimensional interpretation of the two-dimensional image.

As stated above, information about the object examined exists in the image as a result of the interaction of electrons in the incident beam with the object. If the magnitude of the interaction is insufficient to produce observable contrast, the object will be invisible. The problem of insufficient amplitude contrast is frequently encountered in bright phase CTEM of biological molecules. Under conditions commonly used for observation of proteins and protein aggregates, the minimum thickness of unstained protein which will give significant contrast is about 100 Å (1). With this resolution features of large structures are not resolved and most single protein molecules are invisible. The inability of bright phase CTEM to produce sufficient contrast for the imaging of unstained biological molecules at low resolution is largely due to the inefficient collection of scattered electrons. For
this reason, methods have been developed to enhance contrast in the image.

One technique for improving contrast using a CTEM is dark phase microscopy. This method involves blocking the main electron beam after it has transversed the specimen, thus forming the image using only electrons that have been scattered by the object into the objective aperture. The most common method for blocking the main electron beam is by tilting it so that it impinges on the rim of the objective aperture. Although this is a relatively inefficient method for detecting scattered electrons, dark phase CTEM has been used in the imaging of single atoms and relatively small biological molecules.

The second method most commonly used to improve the amplitude contrast in CTEM photomicrographs involves staining the specimen preparation with a heavy atom stain. These methods are discussed in more detail in section 1.2.1.

The thin films of amorphous carbon commonly used as specimen supports are generally about 100 Å thick and show apparent granularity which is of minimum size at focus. The size of this grain, termed phase grain or phase contrast, is determined at a given accelerating
potential by the degree of defocus. These effects are caused by structural irregularities in the carbon film. These irregularities are not contrasted in bright field microscopy but are readily observed in dark field micrographs. This spurious contrast has led to many misinterpretations of substructure in the dark phase images of biological macromolecules.

A diagrammatic representation of a scanning transmission electron microscope (STEM) is shown in Diagram 2. The field emission electron gun produces an extremely narrow, finely focused, beam of electrons. This beam of electrons moves from one point in the specimen to the next to form a raster pattern as in television imaging. The electrons scattered by the sample are then collected by an array of annular detectors. In addition, the state of the art STEM has an energy dispersive X-ray spectrometer above the specimen, an energy loss spectrometer below the specimen, detectors for back-scattered electrons and deflection coils above and below the specimen. This array of detection systems allows for analysis of the energy lost by electrons upon collision with the atoms of the sample. Scattered electrons which have not lost energy may also be more efficiently detected. X-rays and secondary electrons emitted from the sample may also be analyzed.
Diagram 2. Scanning Transmission Electron Microscope

- Electron gun
- Condenser lens system
- Prespecimen deflection coil
- Backscattered electron detector
- Secondary electron detector
- Photomultiplier tube
- Objective lens
- Intermediate lens system
- Projector lens
- Postspecimen deflection coils
- Viewing screen and camera
- Electron energy-loss spectrometer
- X-ray energy-dispersive spectrometer
- Objective aperture
- Intermediate aperture
- Condenser aperture
The development of the electron gun and the sophisticated array of detection systems have made STEM the most powerful instrument for electron microscopic analysis of biological molecules at high resolution. The imaging of single heavy atoms by the STEM (2-5) has opened a new frontier in the imaging of selectively stained and unstained biological specimens.

The concept of using fluorescent labels on biological molecules for light microscopic visualization of their interaction within biological systems was extended in the late 1950's to include the use of electron-dense labels for electron microscopic studies. The early work involved relatively large labels, described in more detail in section 1.3. As instrumentation improved, the need arose for smaller electron-dense labels to use in studies of biological molecules. The present day technology in STEM and dark phase CTEM instrumentation has made feasible the resolution of very small electron-dense labels. This ability has vastly increased the number and types of biochemical questions that may be addressed with electron microscopy. Recent advances in this rapidly expanding area are discussed in section 1.4.
1.2 **Electron Microscopy of Stained Biological Macromolecules**

The positive stain method involves specific combination of an electron dense reagent with the biological specimen. In the electron microscope the stained object appears electron opaque (dark) against a light background. The required characteristics of a positive stain are that it is electron dense, it specifically binds to or reacts with organic matter, and unbound stain can be washed away from the specimen support. A variety of positive stains have been found to interact with specific components (protein, carbohydrate, nucleic acid) of biological specimens.

The use of positive staining techniques in the electron microscopic imaging of proteins and protein aggregates suffers several disadvantages. For a spherical protein with a molecular weight of 20,000 and diameter of 25 Å to be visualized under ordinary conditions with a CTEM, its density must be increased three to four times (1). Extensive staining is required to achieve this; and such procedures usually distort or denature the specimen.

Negative staining, embedding the specimen of interest in a matrix of electron-dense material, results
in the opposite type of contrast enhancement. The specimen appears as an electron transparent area in an electron opaque field. In a typical negative staining procedure, a small aliquot of a dilute solution containing the specimen is placed on a thin carbon film supported by a copper mesh grid. After about thirty seconds most of the liquid is removed and a drop of a dilute (1-2%) stain solution is applied. After a short interval of time most of the negative stain solution is removed. As the water evaporates the heavy metal salts concentrate and form a glassy solid covering the specimen and support and penetrating into the interstitial spaces of the macromolecule. The stain is more dense on the carbon support where there are no specimen molecules and most dense in a halo around the specimen due to surface tension. For this reason the background appears darkest immediately around the image. A list of commonly used negative stains is presented in Table 1.

The use of negative staining techniques in the imaging of macromolecules by CTEM has several advantages. When the macromolecules (proteins) are dried within a matrix of negative stain, surface tension is dissipated against the stain bed, resulting in minimal distortion of the specimen. In addition, as the specimen dries,
<table>
<thead>
<tr>
<th>Stain</th>
<th>Density (g/cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Phosphotungstate</td>
<td>3.8</td>
</tr>
<tr>
<td>Sodium Tungstate</td>
<td>4.2</td>
</tr>
<tr>
<td>Uranyl Nitrate</td>
<td>3.7</td>
</tr>
<tr>
<td>Cadmium Iodide</td>
<td>5.7</td>
</tr>
<tr>
<td>Silver Nitrate</td>
<td>4.4</td>
</tr>
<tr>
<td>Thallium Fluoride</td>
<td>8.6</td>
</tr>
</tbody>
</table>
the enveloping stain penetrates into the clefts and hydrated regions of the specimen. Ideally, this process of engulfing the specimen in stain is complete and results in enhanced contrast of substructure in the image. Non-uniform stain thickness and density and the incomplete penetration of stain around the specimen can lead to spurious contrast and is easily misinterpreted.

Using negative staining procedures, resolution to about 25 Å can be routinely achieved. For these reasons, the majority of bright phase CTEM studies of proteins and protein aggregates have been done by this method.

The use of negative staining in the CTEM imaging of multienzyme complexes has provided valuable information concerning the subunit organization and symmetry of these protein aggregates. Furthermore, the comparison of this EM data with X-ray data on these multienzyme complexes has substantiated the power of CTEM in the analysis of biological macromolecules.

**E. coli Pyruvate Dehydrogenase Complex**

The *E. coli* pyruvate dehydrogenase complex (PDH) is a multienzyme complex catalyzing the oxidative decarboxylation of pyruvate to form acetyl Coenzyme A (acetyl CoA) by the sequence of reactions shown below.
The complex is composed of three enzymes. Pyruvate dehydrogenase \((E_1)\) is the thiamine pyrophosphate (TPP) requiring subunit which catalyzes the decarboxylation of pyruvate to form hydroxyethyl-TPP. Dihydrolipoyl-transacetylase \((E_2)\) contains lipoic acid covalently bonded via the \(\varepsilon\) amino group of a lysine residue in the polypeptide chain. In a poorly understood reaction the lipoyl group is reduced with concomitant oxidation of hydroxyethyl-TPP to acetyl-TPP.

\[
\begin{align*}
H^+ + E_1\cdotTPP + CH_3\text{COCO}_2^- & \rightarrow CO_2 + E_1\text{ hydroxyethyl-TPP} \\ 
E_1\text{ hydroxyethyl-TPP} + \text{lipoyl-}E_2 & \rightarrow E_1\cdotTPP + S\text{-acetyl-dihydrolipoyl-}E_2 \\
S\text{-acetyl-dihydrolipoyl-}E_2 + \text{CoA} & \not\rightarrow \text{AcetylCoA} + \\
dihydrolipoyl-\text{E}_2 & \\
dihydrolipoyl-\text{E}_2 + E_3\cdot\text{FAD} & \not\rightarrow \text{lipoyl-}E_2 + \text{dihydro-}E_3\cdot\text{FAD} \\
dihydro-\text{E}_3\cdot\text{FAD} + \text{NAD}^+ & \not\rightarrow E_3\cdot\text{FAD} + \text{NADH} + H^+ \\
\end{align*}
\]

The acetyl group is transferred to the dihydrolipoyl group to form acetyl-dihydrolipoic acid and regenerated TPP. The acetyl group is then transferred from the dihydrolipoyl group to CoA forming acetylCoA. This
reaction is believed to occur in an active site on E₂. Finally, the dihydrolipoyl group is reoxidized via transfer of the electrons from the dihydrolipoyl group to nicotinamide adenine dinucleotide (NAD) by the third enzyme, dihydrolipoyl dehydrogenase (E₃). The dehydrogenase contains a redox active disulfide and a molecule of flavin adenine dinucleotide (FAD).

The PDH complex molecular weight, subunit stoichiometry and active site stoichiometry have been debated since the early 1960's. Koike et al. (6) purified the PDH complex and reported a molecular weight of 4.8 x 10⁶. Resolution of the complex into the component enzymes showed E₁ to be a dimer with a MW of 183,000. E₃ was also isolated as dimers of MW 112,000 containing two moles of FAD per dimer. E₂ was isolated as an aggregate of MW 1.6 x 10⁶. The lipoic acid content was determined to be eleven nmoles per mg of intact complex. These findings were later modified by Reed and Oliver (7). The molecular weight of the complex was determined to be 3.9 x 10⁶. The MW of E₂ was determined to be 36,000. The subunit stoichiometry proposed by these workers was 24E₁:24E₂:24E₃. This model was however not consistent with the earlier reported lipoic acid content assuming one lipoic acid per E₂ polypeptide.
Eley et al. (8) reported the PDH complex molecular weight and $E_2$ molecular weight at $4.6 \times 10^6$ and 70,000, respectively. The lipoic acid content was reported to be 5.2-5.7 nmol per mg of PDH complex. This corresponds to a ratio of 24 $E_1$ chains, 24 $E_2$ chains and 12 $E_3$ chains. This model is further supported by Moe et al. (9) who measured the binding of 22.4 moles of TPP per mole of PDH complex and by Speckard et al. (10) who found 2.5 nmol of FAD per mg of PDH complex. This corresponds to 12 moles of FAD per mole of PDH complex. Speckard et al. (11) found 9.6-10.1 nmol per mg protein of acetylation sites associated with $E_2$ and proposed that at least 48 lipoyl moieties were attached to $E_2$. This was confirmed by Hale et al. (12). *E. coli* were grown in media containing $[^{35}S]$-lipoic acid. The PDH complex isolated contained more than 2 moles of $[^{35}S]$-lipoic acid per mole of $E_2$. These findings are consistent with a stoichiometry of 24 $E_1$-TPP sites, 24 $E_2$ chains with at least 48 lipoic acid moieties and 12 $E_3$-FAD sites. Although other models have been proposed (13) this appears to be the most accurate model for the PDH complex subunit and cofactor stoichiometries.

The use of electron microscopy, as a tool for investigating the structure of the PDH complex, has proven a valuable method for distinguishing which of
the proposed stoichiometries is most likely correct. Electron photomicrographs of the PDH complex (14) stained with 1% phosphotungstate showed polyhedral clusters 300-400 Å in diameter. In the central portion tetrads with sides of 130-150 Å are well defined. Surrounding the tetrad is an array of subunits 60-90 Å in diameter. Resolution of the PDH complex into its component enzymes followed by reconstitution of the complex yielded electron photomicrograph images identical to the native complex. This finding supports earlier biochemical evidence that the native and reconstituted enzyme complexes are very similar.

Further studies on the isolated transacetylase "core" (7,14) show that the morphological subunits of the transacetylase are situated at the eight vertices of a cube and that the cube possesses (432) symmetry. This symmetry supports a model with 24 identical E₂ chains. Stereoscopic pairs of electron photomicrographs of the transacetylase core have the appearance of cubes and aggregates of the transacetylase particles appear as face to face polymers. The finding that the PDH complex consists of twelve E₁ dimers and six E₃ dimers suggests that the E₁ dimers are located on the twelve edges and the E₃ dimers on the six faces of the
cube. This model is consistent with reconstitution studies (6,15,16) showing that $E_1$ and $E_3$ do not combine with each other but do combine with $E_2$ independent of one another. The crystallization of *E. coli* dihydrolipoyltransacetylase-dihydrolipoyl dehydrogenase ($E_2E_3$) subcomplex by Fuller et al. (17) and subsequent X-ray diffraction analysis of the crystals substantiates the (432) symmetry of the transacetylase core. Crystal density measurements, however, revealed that the dihydrolipoyltransacetylase polypeptides in the crystal were of about one-half the mass generally reported for the intact $E_2$ core. Biochemical studies on the intact core of PDH complex from *E. coli* grown in $[2-^{3}H]$-lipoic acid demonstrated that the transacetylase polypeptide exists as two distinct domains. Fragment D is the compact subunit binding domain that confers the quaternary structure to the $E_2$ aggregate and possesses the $E_1$ and $E_3$ binding domains. Fragment A is a trypsin sensitive flexible extension containing the lipoyl groups (18). Electron photomicrographs of the trypsin modified transacetylase show the familiar cube-like core. Higher resolution electron micrographs of the native transacetylase show a faint fuzz around the cube core. Glutaraldehyde fixation of the native core,
to crosslink the lipoyl domains, thereby reducing motion, yielded an interpretive model in which the lipoyl domains extend from the core (18).

**Mammalian Pyruvate Dehydrogenase Complex**

In addition to the three component enzymes of the *E. coli* PDH complex, mammalian PDH complex also has a kinase and a phosphatase responsible for catalyzing the phosphorylation and dephosphorylation of one of the three proteins in the complex, thereby regulating the catalytic activity. Negatively stained samples of mammalian transacetylase core are quite different from the *E. coli* enzyme; its morphological subunits appear to be situated at the twenty vertices of a pentagonal dodecahedron (19,20). The dimensions of the core are 210 Å-240 Å (7,21) and it appears to possess (532) symmetry. The native and reconstituted PDH complex has a diameter of 400 Å-450 Å (7). *E₁*, *E₃* and the regulatory subunits appear to pack around the transacetylase core giving the overall polyhedral appearance. Electron micrographs of the *E₁E₂* subcomplex and *E₂E₃* subcomplex are very similar in appearance and show various aspects of a polyhedron structure similar to that of the dihydro-lipoyltransacetylase core (22). The minimum MW for the mammalian PDH complex is 7.6 x 10⁶ (22). The
transacetylase core consists of sixty polypeptide chains of MW 52,000 and each contains one molecule of lipoic acid (22). The isolated pyruvate dehydrogenase (E₁) has a MW of 154,000 and consists of two subunits of MW 41,000 and two subunits MW 36,000 (α₂β₂) (23). The complex probably contains sixty αβ dimers (24). The flavoprotein (E₃) has a MW of 110,000 and consists of two identical subunits, each of which contains one molecule of FAD (25). The purified complex contains variable amounts of flavoprotein (26). The regulatory kinase (MW 100,000) is relatively strongly bound to the transacetylase. The phosphatase (MW 100,000) exists in association dissociation equilibrium with the transacetylase (26).

In the purified complex there are only a few molecules of each regulatory enzyme; however, many may be lost in purification (24). As was seen with the transacetylase from E. coli, the core enzyme consists of a subunit binding domain and a trypsin sensitive lipoyl domain. The subsequent electron microscopic analysis of native and trypsin modified PDH complex yielded a similar interpretive model to that for the complex from E. coli (19).
**E. coli α-Ketoglutarate Dehydrogenase Complex**

α-Ketoglutarate dehydrogenase complex catalyzes the oxidative decarboxylation of α-ketoglutarate to form succinyl-CoenzymeA. The three component enzymes of the α-KGDH complex catalyze the same class of reactions as the PDH complex. The molecular weight of the complex has been determined to be $2.3 \times 10^6$ and a subunit stoichiometry of $24E_1:24E_2:12E_3$ has been proposed (27,28). In contrast to the *E. coli* PDH complex, there is only one mole of lipoic acid per dihydrolipoyltranssuccinylase polypeptide. Electron photomicrographic images of negatively stained *E. coli* α-KGDH complex show the familiar polyhedral structure. The transsuccinylase core has a MW near $1 \times 10^6$ and appears to consist of eight morphological subunits situated at the vertices of a cube possessing (432) symmetry (7).

As with the PDH complex, the molecules of α-ketoglutarate dehydrogenase ($E_1$) and the dihydrolipoyl dehydrogenase flavoprotein ($E_3$), appear to be distributed in a regular manner on the core surface. The dimensions of the core have been estimated at 150 Å (7).

The α-KGDH complex from pig heart (MW $2.7 \times 10^6$) (29-31) and beef kidney (29) have the same appearance.
in the electron microscope as the *E. coli* PDH and α-KGDH complexes. Crystallization of the *E. coli* lipoyltrunsuccinylase core by DeRosier et al. (32) and X-ray analysis (33) once again confirms the (432) symmetry seen with the *E. coli* PDH complex. While the subunits are not resolved at 12 Å resolution, the electron density map shows 24 peaks at a radius of about 64 Å situated at the vertices of a truncated cube (33). The dimensions of the core as measured from the crystals was 157 Å (32) which is in good agreement with the EM value reported earlier. Unlike the crystallized E₂E₃ subcomplex of the *E. coli* PDH complex, the crystallized transacetylase core appears to be intact (32).

There is a striking similarity between the *E. coli* PDH complex, *E. coli* α-KGDH complex and the mammalian α-KGDH complex as visualized by negative stain transmission electron microscopy. These findings have been confirmed by biochemical and physiochemical studies. The apparent structural uniqueness of the mammalian PDH complex undoubtably arises from the more complex regulatory features of this system.

From the preceding discussion it can be seen that bright phase negative stain transmission electron
microscopy has been of considerable value in understanding the structural organization of the component enzymes in α-keto acid dehydrogenase complexes. Although much has been learned from the EM images of these complexes, several important questions remain unsolved. 1) Although suggested from stoichiometry arguments, no direct demonstration of the relative orientations of the E1 and E3 components around the E2 core has been achieved. 2) The demonstration of a lipoyl domain in the tertiary and quaternary structure of the transacetylase core by a combination of biochemical and electron microscopic methods is not surprising, considering the "swinging arm" mechanism proposed for the enzyme. The electron microscopic demonstration of the lipoyl group interaction with both E1 and E3 and the symmetry of these components with respect to the lipoic acid groups around the E2 core has also not been accomplished.

Electron microscopic images of the E. coli PDH complex and E. coli α-KGDH complex appear very similar. The PDH complex, however, has twice the lipoic acid content of the α-KGDH complex. A large number of biochemical studies have been performed in an attempt to better understand the mechanistic significance of this difference. High resolution STEM analysis of these
complexes after selective labeling of specific components with an electron-dense labeling reagent may prove to be an effective method for gaining further insight into these questions.

**Yeast Fatty Acid Synthetase**

The biosynthesis of long chain fatty acids from acetylCoA and malonylCoA is shown in Scheme 1. In most eucaryotes this series of enzymatic reactions is catalyzed by a multienzyme complex termed fatty acid synthetase. Lynen et al. (34,35) have purified and crystallized the enzyme from bakers yeast. It has a MW of about $2.3 \times 10^6$. Although the yeast synthetase has not been separated into enzymically active components, biochemical evidence suggests that the synthetase complex contains three identical functionally distinct sub-complexes containing seven proteins. One of these proteins is the acyl group carrier protein onto which the growing fatty acid is linked via the 4'-phosphopantetheine prosthetic group which is bound in a phosphodiester linkage to a serine residue in the polypeptide chain. As with the PDH complex, a "swinging arm" model has been proposed to account for the ability of the growing fatty acid chain on the pantetheine arm to interact with the different active sites within the complex.
Scheme I. Fatty Acid Synthesis
Images of the yeast fatty acid synthetase, from CTEM visualization of negatively stained specimens, show oval particles with a diameter of 210-250 \( \text{Å} \) surrounded by an equatorial ring. Substructure is clearly visible in the images. The relative orientations of the three acyl group carrier proteins within the complex has not been determined. Also, the organization of the remaining six proteins around the acyl group carrier protein has not been addressed using electron microscopic techniques.

**Transcarboxylase**

Perhaps the most elegant demonstration of the power of negative stain CTEM as a tool for studying the subunit organization of multienzyme complexes is the work of Green et al. (36) on the enzyme transcarboxylase. Transcarboxylase [methylmalonylCoA:pyruvate carboxytransferase (EC 2.1.3.1)] catalyzes the reaction shown in Scheme 2. The enzyme has been found only in propionic acid bacteria (37). Like all biotin containing enzymes, transcarboxylase contains a biotinyl group covalently attached via the \( \varepsilon \) amino group of a lysine residue in the protein. This "flexible arm" then is presumably able to move back and forth from the two catalytic sites on the enzyme. For a schematic
Scheme 2. Transcarboxylase Reaction

\[
\begin{align*}
\text{CH}_3\text{CH}(\text{CO}_2^-)\text{COSCoA} & \quad \text{CoASHCH}_2\text{CH}_3 \\
\text{E-Biotin} & \quad \text{E-Biotin-CO}_2^- \\
-\text{O}_2\text{CCH}_2\text{COCO}_2^- & \quad \text{CH}_3\text{COCO}_2^- \\
\end{align*}
\]

Diagram:

○~pyruvate  □~biotin  ■~propionyl CoA
○~C\text{O}_2~ oxaloacetate  -O\text{C}_2~ □~methylmalony CoA
representation, see Scheme 2. The enzyme complex (MW 792,000) appears to dissociate in a complex fashion as shown in Scheme 3 (36)

Negative stain images of intact transcarboxylase (18s) show a cylindrical "head" with three ears. The preparations also contained substantial amounts of the 16s subcomplex. These molecules have the famous "Mickey Mouse" appearance given by the cylindrical head with two ears. Some 14s complex (head with one ear) is also present. The ears then represent the 6\textsubscript{E} subcomplex containing the biotin, Co\textsuperscript{2+}, and Zn\textsuperscript{2+} binding sites. The 12s\textsubscript{H} subunit showed two profiles, one rectangular (80 x 100 Å) and one polygonal (100 Å diameter) which appear to represent side and end on views of an approximately cylindrical structure (37). The 12s head shows apparent two fold and three fold axis of symmetry. The asymmetric attachment of the ears to only one face of the cylindrical head would indicate C\textsubscript{6} symmetry; however, a parallel arrangement of the six 2.5s\textsubscript{H} subunits conflicts with two observations. First, when the 12s\textsubscript{H} subunit dissociates it gives a stable dimer (6s\textsubscript{H}). This would be consistent with a dihedral (D\textsubscript{3}) symmetry. Secondly, the penetration of stain along the long axis of the rectangular form of the 12s\textsubscript{H} subunit suggests a double ring of subunits. This
SCHEME 3: SUBUNIT STRUCTURE OF TRANSCARBOXYLASE

18s (790K)

16s (650K) 6s_E (144K)

14s (500K) 6s_E (144K)

12s_H (360K) 6s_E (144K)

(3) 6s_H (120K) (3) 5s_E (120K) (6) 1.3s_E (12K)

(6) 2.5s_H (60K) (6) 2.5s_E (60K)
apparent cleft around the head structure could also be explained by selective penetration of the stain within all six $2.5s_H$ subunits (36). When viewed down the threefold axis, the head appears to have a central hole. It has been proposed that the three $6s_H$ subunits pack in a cylindrical arrangement leaving the hole in the center. The $6s_E$ (ear) subunits show a cylindrical/ellipsoid structure with dimensions of 55 Å x 90 Å. Reconstitution of the complex from the $12s_H$ and $6s_E$ subunits yields a mixture of relatively intact "Mickey Mouse" type images along with smaller particles.

It has also been proposed, based on the EM micrographs, that the link between the head and ear is flexible, unlike the classic model of rigid intersubunit binding in multisubunit enzymes. This theory is based on the observable difference in position of the ears in the population of 18s molecules. In the various two dimensional profiles from the photomicrographs, the distance between ears varies from 20 Å-80 Å and the distance between the ears and head vary from 0 Å-30 Å.

The position of the biotin groups on the $1.3s_E$ subunit within the 18s complex was determined by incubating the 18s complex with avidin prior to staining and EM analysis. Avidin (MW 65,000; 55 Å x 55 Å x 41 Å)
has four subunits arranged with two-fold \( (D_2) \) symmetry (38). The four biotin binding sites \( (K_d \ 10^{-15} \ M^{-1}) \) are grouped in two pairs at opposite ends of the short axis of the tetramer. Photomicrographs of the conjugate reveal avidin molecules around the \( 12s_H \) subunit intercalated between the \( 6s_E \) subunits giving the appearance of a flower with six petals. This use of avidin as a marker for the position of biotin within the \( 18s \) transcarboxylase complex is a clear example of the power of combined negative staining and biospecific labeling with a macromolecule to obtain structural information about a multienzyme complex. In the next section this concept is discussed in more detail with specific reference to high electron density biospecific markers.

1.3 Large Electron-Dense Labels for Electron Microscopic Studies

The first example of an electron-dense label for use in the electron microscopic analysis of biological systems was the preparation of ferritin-antibody conjugates by Singer (39,40). Ferritin is an iron storage protein of MW 460,000 containing up to 25% iron in ferrichydroxide-phosphate micelles (41). The ferritin molecule has a spherical appearance in the CTEM with a diameter of about 120 Å (42). Conjugates of ferritin
with antibodies against bovine serum albumin and ribonuclease were prepared using m-xylene diisocyanate, to achieve about 33% labeling of the antibody. These conjugates retained their antigen specificity and were specifically precipitated from an antibody mixture (39). Since the report of this methodology, ferritin has been used as a site specific marker in a variety of electron microscopic studies. A representative list of ferritin conjugates which have been prepared is shown in Table 2.

The more recent development of an iron dextran particle (Imposil) has provided a second large electron dense reagent that may be covalently coupled to biological molecules. The Imposil particle has an ellipsoid appearance in a CTEM with dimensions of about 120 Å x 210 Å (43). The combined use of ferritin and Imposil conjugates has provided a method for performing double label EM experiments.

The use of these conjugates as electron-dense regents for EM studies has been largely confined to studies involving large biological structures (cells, cell organelles, membrane preparations, etc.). The resolution obtainable is limited by the size of the electron-dense label. The dimensions of ferritin limit the resolution to about 200 Å (40). In a biological sense, the limit of resolution is determined by the
TABLE 2
Ferritin Conjugates for Electron Microscopic Localization of Specific Biological Structures

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Target Structure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin-Concavalin A</td>
<td>Human erythrocyte membranes</td>
<td>44</td>
</tr>
<tr>
<td>Ferritin-Avidin</td>
<td>Biotinylated membrane ghosts</td>
<td>45</td>
</tr>
<tr>
<td>Ferritin-Avidin</td>
<td>Biotinyl-Insulin</td>
<td>46,48</td>
</tr>
<tr>
<td>Ferritin-Insulin</td>
<td>Adipocyte membranes</td>
<td>47,49</td>
</tr>
<tr>
<td>Ferritin-Avidin</td>
<td>Biotinyl-phosphotidyl ethanolamine</td>
<td>50</td>
</tr>
<tr>
<td>Cationized Ferritin</td>
<td>Erythrocyte membranes</td>
<td>51</td>
</tr>
<tr>
<td>Ferritin-Hydrazide</td>
<td>Erythrocyte membranes</td>
<td>52</td>
</tr>
<tr>
<td>Imposil-Antibody</td>
<td>Erythrocyte membranes</td>
<td>53</td>
</tr>
</tbody>
</table>
distance of the electron-dense reagent from the site of labeling. The resolution of detail in samples which have been treated with ferritin-antibody or ferritin-avidin conjugates is limited by this distance. This level of resolution is not sufficient to allow for the analysis of structure and structure-function relationships in smaller macromolecular aggregates such as the multienzyme complexes discussed earlier.

1.4 Recent Developments in the Visualization of Single Electron-Dense Atoms and Small Molecules

Both dark phase CTEM and STEM have been used to visualize small biological molecules and single heavy atoms (2-4,54,55). This capability is a result of improvements in specimen preparation and rapid improvements in instrumentation. The computer interfaced STEM and CTEM have made possible the collection of electron scatter data which may be optically averaged and filtered before the image is reconstructed. The annular detectors on the STEM make it the most efficient instrument in capturing scattered electrons. This is especially useful in dark phase operation. For example, with identical thin carbon specimen supports, the electron collection efficiency of dark field STEM is five to six times greater than a CTEM using the dark
field beam inclination technique (56). Because of this enhanced efficiency the specimen preparation in dark field STEM may be illuminated with a less intense electron beam, resulting in reduced radiation damage. The problem of radiation damage is greatest in the unstained preparations used in dark-phase microscopy. In addition, when selective heavy atom stains are employed, the STEM instrumentation allows for a multiple scan sequence to be used. In the first scan of the specimen an electron beam of less than two square angstroms is employed with a density of one to ten electrons per square angstrom. This scan is done to obtain a general outline of the specimen. The position of any heavy atom labels is then determined by rescanning with a higher electron dose, usually about 100 electrons per square angstrom.

With the advent of the sophisticated instrumentation and techniques discussed, the use of EM in the analysis of the substructure and organization of biological molecules is now possible. One of the first biological systems to which selective heavy atom labeling and STEM analysis was applied was in the attempt to develop a method to sequence DNA by EM (57). Several approaches to this problem have been tried. Derivatives of the four bases with mercury in the 5
position of the pyrimidine ring and in the 7 position of 7-deazapurines have been synthesized (58). Although the modified nucleotides are acceptable substrates for DNA polymerase, the polynucleotides formed are not very stable, decomposing to form dimers with loss of mercury (59). The use of nucleotide phosphotioates as substrates for DNA synthesis offered a second method for selective marking of the base sequence in a DNA strand. The binding of an organomercury compound (60) and inorganic platinum complexes (61) to the thio groups has been examined and appears to be a feasible method. The reagent most heavily investigated for use as a nucleotide selective heavy atom label is osmium tetroxide-bipyridine (OsO₄/bipyr) (62-64). This reagent reacts specifically with the 5-6 double bond in cytidine, uridine and thymidine. If the cytosine bases are first blocked with O-methylhydroxylamine, OsO₄/bipyr becomes a thymine/uracil specific label. Finally, if the DNA is pretreated with chloroacetaldehyde to form etheno-adenosine, OsO₄/bipyr can be used to label adenine as well (71). In addition to this reaction, exchange reactions of Os₂O₆/bipy₄⁺ or OsO₄/bipyr with vicinal diols has been useful for labeling the ribose moieties in polynucleotides (65) and vicinal diols in
glycoproteins (66). Although not yet reported, the ligand exchange of these osmium complexes with the vicinal diol created by reacting arginine with 1,2-diones should prove to be a useful method for EM imaging of specific arginine residues in protein specimens. In addition, this method would allow for X-ray analysis on crystals which are selectively impregnated with a heavy atom. The biggest problem in the area of selective labeling of polynucleotides with heavy atoms is now atomic motion (65,67), however some very promising results are being obtained. Present day DNA sequencing techniques are capable of obtaining DNA sequences rapidly and accurately, however the idea of sequencing DNA by visual inspection is still aesthetically appealing. In any event, the concepts and techniques developed in this work have been of tremendous value when applied to other biological systems.

The imaging of proteins specifically labeled with single heavy atoms is the area now receiving increased interest. The visualization of cadmium-metallothionein using dark phase CTEM is a good example of the results obtainable by CTEM. Approximately one-third of the amino acid residues in this protein are cysteine. The native form of the protein has a reported molecular
weight of near 10,000 (68,69). A minimum molecular weight of 6000 has been reported by Vallee et al. (70) with six to seven atoms of cadmium per monomer. This corresponds to one cadmium per three cysteine which is in agreement with the coordination properties of cadmium with sulfhydryl groups (71). The dark field images of Cd-metallothionein show the presence of both monomers and dimers. Three-dimensional reconstruction of the images indicates that the dimer has a rectangular structure with dimensions of 36 Å x 25 Å x 12 Å (55). In the monomer there are six or seven strong electron scattering centers stacked as opposed pairs with a separation of about 9 Å. These stacks are also visible in the dimer. From the three-dimensional reconstruction of the image at a resolution of 6-9 Å, a model for the folding of the polypeptide backbone around the cadmium atoms was proposed (55).

The use of selective heavy atom labeling of histones in nucleosomes and subsequent STEM visualization, to determine the location of the different histone proteins within the aggregate, is a project currently in progress. Intact nucleosomes can be treated with methyl(methyl-thio)acetimidate and then the aggregate is dissociated. The modified histones are separated
into pairs (H_{2A}, H_{2B} and H_{3H_4}) by gel electrophoresis. The modified pairs are then mixed with the unmodified other pair and the nucleosomes are reconstituted (71). These nucleosomes are then treated with platinum glycyl-L-methionine chloride which binds specifically to the sulfur atoms (72). In micrographs of these labeled complexes, the Pt atoms are visible. Work on this system is still in progress.

The use of organic mercurials as labels for the various sulfhydryl groups in the α-keto acid dehydrogenase complexes would appear to be a method of unraveling the symmetry and structure-function relationships in these systems; however, attempts in this area have not proven encouraging. The relatively large size of these complexes and the multiplicity of identical sites limit the useful resolution. For this reason, somewhat more electron-dense labels will probably be required.

From the preceding discussion it can be seen that present day instrumentation and data manipulation capabilities have made it possible to gather high resolution (5-10 Å) data on the structure of selectively heavy atom labeled biological molecules. In order to exploit this capability, small electron dense reagents capable of being attached to specific sites in
biological molecules are needed. Single heavy atom labeling reagents should prove useful in the analysis of smaller biological systems where the highest possible resolution is desired. In order to investigate the symmetry relationships between subunits in larger biological aggregates such as the α-keto acid dehydrogenase complexes, a somewhat larger label is required. A resolution of 10-20 Å should prove sufficient for systems of this size. A small reagent containing several heavy atoms would provide the necessary electron scattering. Any electron-dense reagent prepared for this purpose must be capable of being covalently attached to specific functional groups in the biological macromolecule or contain an attached ligand for binding to specific sites within the protein.

The synthesis of tricyanoheptakis[phosphinidyne-tri(benzenemethanamine)]undecagold by Bartlett et al. (73) appears to be the most exciting prospect in the search for a molecule with the characteristics outlined above (Scheme 4). This organometallic cluster consists of an eleven gold atom core surrounded by seven triaryl phosphine and three cyanide ligands. A variety of these undecagold clusters have been prepared differing in the nature of the coordinating counterion and the substituent present in the para-positions of the
SCHEME 4

Tricyanoheptakis [4,4',4'''-phosphinidynetri (benzenemethanamine)]
undecagold
triaryl phosphine ligands (73-75). The X-ray crystal structure of tri-iodoheptakis(tri-p-fluorophenyl-phosphine)undecagold has been determined (75). Ten of the gold atoms in the core are arranged in an incomplete icosahedron in which one of the triangular faces has been substituted by a single gold atom. The remaining gold atom is situated at the center of this cage.

The core of gold possesses $C_{3v}$ symmetry. The eleven metal atoms fall into five classes exhibiting different coordination patterns and coordination numbers. The central gold atom is ten-coordinate and appears to be in the +3 oxidation state. Ten one-electron donors would give this central gold the noble gas configuration. The apical gold atom, occupying the missing face of the icosahedron, is five-coordinate. Three gold atoms with phosphine ligands and the three with cyanide ligands form an equatorial chair-like hexagon and are six-coordinate. Finally, there are three seven-coordinate gold atoms possessing phosphine ligands (75). This complexity of the core structure may result from steric crowding of the phosphine ligands. That steric crowding is a factor is clear when one considers that, in the absence of coordinating counterions (e.g., SCN$^-$, CN$^-$, I$^-$) the cluster formed is $\text{Au}_9[\text{P}(\text{PhX})_3]_8$. The radius of the core is 4.1 Å and the radius of the entire
molecule is 10.5-11.0 Å. The stability and high water solubility of tricyanoheptakis[4,4',4'"-phosphinidynetri-(benzenemethanamine)]undecagold (undecagold cluster) make it a suitable candidate for potential use as an electron-dense labeling reagent.

The exterior of this molecule is composed of twenty-one benzylammonium ions grouped in the seven phosphine ligands. The $S^W_{20}$ was determined from sedimentation velocity measurements to be 2.4s. For a molecule of molecular weight 5000, this leads to a calculated specific volume of 0.54 and a Stokes radius of 10 Å (73). STEM photomicrographs of the gold complex at 3 Å resolution show substructure within the core. At 20 Å resolution the gold complex particles appear spherical and are easily visible past 50 Å resolution.

The first demonstration of the usefulness of this organometallic cluster in the STEM analysis of biological molecules was in the recent work of Safer et al. (76). The gold complex was treated with an excess of N-hydroxysuccinimidobiotin to form a multibiotinyl gold complex mixture. After removal of unreacted biotin by gel filtration, the biotin-gold complex conjugate was added to a solution of avidin and then passed through a gel filtration column. The earliest eluting fractions were examined by CTEM.
Negatively stained images showed linear chains of avidin molecules. Similar structures have been seen upon addition of multi-biotinyl compounds to avidin (38). Examination of the unstained specimens by dark phase STEM revealed the same short polymers of avidin. The molecules of avidin were usually linked by pairs of gold clusters appearing 10 Å in diameter and spaced 20 Å apart. Adjacent pairs of clusters were seen to be separated by 45 Å. The gold complex did not appear to move significantly under the irradiation conditions employed, although significant damage slowly appeared after multiple scans. This study clearly demonstrated the potential use of the undecagold cluster in the electron microscopic analysis of structure and structure-function relationships in biological systems.

The future use of the undecagold cluster as a site specific electron-dense label, in the study of a wide range of biological systems, depends on the development of procedures for preparing a variety of undecagold derivatives containing a biological ligand or reactive functional group.

In the study of Safer et al. (76) discussed above, a multi-biotinyl undecagold mixture was used in a system in which trivalent and monovalent species did not interfere. For more precise stoichiometry and
structure-function studies on complex biological aggregates, an undecagold cluster derivative containing a single attached ligand or reactive functional group is required. The α-keto acid dehydrogenase complexes are an example of such aggregates. In order to determine the structural organization of the lipoic acid moieties and the $E_1$ and $E_3$ subunits arranged about the $E_2$ core, procedures are required for specifically linking a single gold complex derivative to a single lipoyn group, $E_1$ or $E_3$ subunit.

The synthesis of undecagold derivatives containing a single biological ligand can be viewed much the same as the preparation of an affinity chromatography column. If one considers the gold complex as the resin bead, the successful attachment of the biological ligand to the cluster while retaining its biological activity requires an understanding of the precise binding interactions between the ligand and the macromolecule of interest. The site on the ligand used for attachment to the undecagold cluster should be in a region of the molecule which is minimally involved in these binding interactions. In certain cases, a spacer arm may be required to separate the bulky undecagold cluster from the ligand. If the molecule to be attached to the undecagold complex contains a reactive functional
group for use as an amino acid selective modification reagent or affinity label, many of the considerations mentioned above apply.

The application of the gold complex of immediate interest in this laboratory is to the study of symmetry and structure-function relationships in α-keto acid dehydrogenase complexes. The pyruvate dehydrogenase complex and α-KGDH complex from *E. coli* have been studied in this laboratory for several years. The selective modification of specific classes of sulfhydryl groups in these multienzyme complexes has been extensively investigated. The dihydrolipoyl groups have been selectively modified with α-haloacetamide, α-haloacetic acid and maleimide derivatives (77-79). The redox active disulfide present in dihydrolipoyl dehydrogenase (E₃) can be selectively modified with α-haloacetic acid and α-haloacetamide compounds (80). Finally, there is a sulfhydryl group in the TPP binding site on pyruvate dehydrogenase (E₁) which may also be selectively modified by these reagents (81). The attachment of derivatives of these reagents to the undecagold cluster would provide a method for studying the structural arrangement and symmetry relationships between these sites within the multienzyme complex.
The major objectives of this research program have been to develop procedures for synthesizing monofunctional derivatives of tricyanoheptakis[4,4',4"-phosphindynetri(benzenemethanamine)]undecagold and to synthesize monofunctional alkylating derivatives using these methods. In developing these procedures, emphasis has been placed on the preparation of monofunctional undecagold derivatives having wide application. Derivatives containing a sulfhydryl selective alkylating reagent have been prepared specifically for application to studies on the α-keto acid dehydrogenase multi-enzyme complexes. A monofunctional undecagold derivative containing an amino group acylating reagent has also been prepared.

The use of these reagents in preliminary studies on the E. coli pyruvate dehydrogenase complex, rat liver mitochondria and cytochrome oxidase membrane preparations is discussed in Chapter VI.
CHAPTER II
MATERIALS AND METHODS

2.1 Materials

Borane-THF (1.0 M) and n-butyl lithium (1.6 M in hexane) were purchased from Aldrich Chemical Co.
Phosphorus trichloride was purchased from Aldrich Chemical Co., distilled from calcium hydride and stored in sealed ampules at 4°C. Bromoacetic acid was purchased from Aldrich Chemical Co., recrystallized from hexane and stored at -20°C. Triethylorthoformate was purchased from Eastman Kodak Chemical Co. and stored over 4 Å molecular sieves. Succinic anhydride, purchased from Fisher Chemical Co., was recrystallized from acetic anhydride. Phthalic anhydride was purchased from Fisher Chemical Co. and purified by sublimation. Freshly precipitated gold cyanide was purchased from SPEX Industries in light-proof bottles.

Methylenechloride, tetrahydrofuran, dioxane and acetonitrile, purchased from Fisher Chemical Co., were dried over calcium hydride by stirring at room temperature for 24 hr or by heating under reflux for 2-4 hr. The solvents were then distilled into a flask.
containing 4 Å molecular sieves. The cyclic ethers were protected from light during storage. Pyridine, purchased from Fisher Chemical Co., was dried over calcium hydride and distilled into a flask containing barium oxide. Dimethylformamide was purchased from Fisher Chemical Co., dried over 4 Å molecular sieves and distilled at reduced pressure (T < 25°C) into a flask containing barium oxide. Further drying of these solvents for specific purposes is detailed elsewhere.

[1,4-$^{14}$C]Succinic anhydride, [carbonyl-$^{14}$C]phthalic anhydride, [1-$^{14}$C]-bromoacetic acid and [U-$^{14}$C]-cysteine hydrochloride were purchased from Amersham Chemical Co. [1,2-$^{14}$C]Sodium acetate was purchased from New England Nuclear Co. All other materials were purchased from Aldrich Chemical Co., Eastman Kodak Chemical Co., Fisher Chemical Co., Bio-Rad Laboratories, or Sigma Chemical Co. and used without purification.

2.2 Methods

2.2.1 Ultraviolet-visible Spectrophotometry

Absorbance readings and spectra were obtained either on a Unicam SP-1800 spectrophotometer equipped with a Unicam AR-25 linear recorder, or on a Cary 118C UV-visible spectrophotometer.
2.2.2 NMR Spectrometry

Proton NMR spectra were obtained on either a Varian EM390, Bruker WP-200 or Bruker WH-270 NMR spectrometer, field frequency locked on the deuterium resonance of 99.8 atom % D$_2$O, CDCl$_3$ or DMSO-d$_6$. The chemical shifts were referenced to a 0.1% tetramethyisilane (TMS) internal standard when using the Varian EM390 spectrometer. A 0.1% TMS external standard was used to reference chemical shifts from spectra obtained on the Bruker WP-200 or Bruker WH-270 instruments.

Carbon-13 NMR spectra were obtained on the Bruker WP-200 spectrometer operating at 60.28 MHz. The instrument was field frequency locked on the deuterium resonance of 99.8 atom % D$_2$O. The chemical shifts were referenced to a 0.1% TMS external standard.

Fluorine-19 NMR spectra were obtained on a Nicolet NT-200WB instrument field frequency locked on the deuterium resonance of 99.8 atom % D$_2$O. Chemical shifts were referenced to the spectrometer frequency (188.236661 MHz).

Phosphorus-31 NMR spectra were obtained either on the Bruker HX-90, Bruker WP-200 or Nicolet NT-200WB instrument operating at 36.43 MHz, 80.10 MHz and 80.98 MHz, respectively. The spectrometer was field frequency locked on the deuterium resonance of 50% D$_2$O,
50% CDCl₃ or 50% DMSO-d₆. Chemical shifts were referenced to a 85% D₃PO₄ external standard.

2.2.3 Titrations

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. Samples for titration were heated to 60°C and 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 CO₂ uptake. Standard solutions of NaOH and HCl for titration were prepared and standardized with 1.000 N HCl or 1.000 N NaOH.

2.2.4 Radiochemical Analysis

Liquid scintillation counting was done by adding the sample to a glass scintillation vial in a total volume of 1.0 ml H₂O. To this, 15.0 ml Aquasol scintillation fluid was added and the samples were counted in a Beckman LS-100C liquid scintillation spectrometer. The quenching caused by increasing concentrations of the undecagold cluster was measured as follows. A
series of vials containing a small sample (25 μl) of 14C-succinic anhydride and 15.0 ml Aquasol were counted to 0.2% error. 1.0 ml of a solution of gold complex (0.000-0.600 A415 units/ml) in H2O was placed in each vial and the samples were again counted to 0.2% error. The % quench vs. concentration of gold complex was determined for each vial. The data shown in Table 3 were obtained.

2.2.5 Chromatography

QAE Sephadex Q-25, chloride form, was swelled in H2O for 24 hr prior to use. For chromographic separation of succinyl and phthalyl gold complex derivatives, the poured columns were washed with several column volumes of distilled water followed by two column volumes of water at pH 11.5. The pH adjustments were made with 1.0 N NaOH or 4.0 N NaOH. QAE Sephadex Q-25, chloride form, was converted to the p-toluene sulfonate form by passing 3-5 column volumes of 1.0 M sodium p-toluene sulfonate at pH 5.0 through the column. The column packing was then removed, washed with 20-40 volumes of H2O prior to repouring the column. The column was then washed with several column volumes of distilled H2O prior to use. QAE Sephadex Q-25, chloride form, was converted to the bicarbonate
TABLE 3
Quench of $^{14}$C-Radioactivity by Undecagold Cluster$^a$

<table>
<thead>
<tr>
<th>Sample</th>
<th>$A_{415}$ units</th>
<th>% Quench</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.000</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>0.048</td>
<td>0.12</td>
</tr>
<tr>
<td>3</td>
<td>0.119</td>
<td>0.51</td>
</tr>
<tr>
<td>4</td>
<td>0.151</td>
<td>0.63</td>
</tr>
<tr>
<td>5</td>
<td>0.190</td>
<td>0.71</td>
</tr>
<tr>
<td>6</td>
<td>0.259</td>
<td>0.96</td>
</tr>
<tr>
<td>7</td>
<td>0.322</td>
<td>1.21</td>
</tr>
<tr>
<td>8</td>
<td>0.376</td>
<td>1.44</td>
</tr>
<tr>
<td>9</td>
<td>0.439</td>
<td>1.70</td>
</tr>
<tr>
<td>10</td>
<td>0.506</td>
<td>1.91</td>
</tr>
</tbody>
</table>

$^a$The % quench of $^{14}$C-cpm's was determined for increasing concentrations of undecagold cluster as described in the text. Quenching by water was subtracted from the data.
form by passing 10-15 column volumes of 1.0 M NaHCO₃ through the column. The resin was removed from the column, washed with water and the column was repoured. The column was then equilibrated with 0.10 M triethylammoniumbicarbonate (TEAB) by passing 2-3 column volumes of the buffer through the column prior to sample application. QAE Sephadex Q-25, chloride form, was converted to the carbonate form by the same procedure using sodium carbonate.

SP Sephadex C-25, sodium form, was swelled in water for 24 hr prior to use. The resin was converted to the triethylammonium form by passing 10-15 volumes of triethylammoniumchloride through the column. The column was then washed with several column volumes of 0.10 M TEAB prior to loading the sample.

Dowex 1, hydrogen form, was swelled in 10% ethanol-1.0 N HCl. After pouring, the column was washed with 8-10 volumes of the same solution. The resin was then washed with 5-7 column volumes of water prior to loading the sample.

1.0 M TEAB was prepared as follows. A flask containing a 1.0 M solution of triethylamine was placed in an ice-water bath. A gas dispersion frit, connected to a stoppered side arm flask half full of dry ice
chunks, was inserted into the triethylamine solution. The solution was stirred while CO$_2$ was bubbled through it until the pH reached 7.8. The 1.0 M TEAB buffer was stored tightly stoppered at room temperature or at 4°C.

When salt gradients were run, a Pharmacia peristaltic pump, Model P3, was used to maintain a constant flow rate.

Silica gel (35-60 mesh) was mixed with the elution solvent system and poured at room temperature. Solvents were used as purchased, without further purification. Silica gel columns were routinely monitored by spotting column fractions on thin-layer silica gel plates. The plates were developed with the same solvent system used for the column to give a qualitative elution profile.

BioGel P-6 (100-200 mesh) was allowed to swell in 0.10 M sodium chloride or 0.10 M TEAB for 6 hr. The material was degassed under vacuum for 2-3 hr. After the column was poured, 2-3 column volumes of the same buffer were passed through at the flow rate to be used. Samples of gold complex were then applied and eluted with the same buffer solution. BioGel P-2 (100-200 mesh) was swelled in water and degassed as described above. The columns were equilibrated and eluted with water or 10% ethanol-water.
Sephadex G-25 and G-50 were swelled in 0.10 M sodium chloride or 0.10 M ammonium chloride. Columns were equilibrated and developed with the same buffer. A peristaltic pump was used to maintain a constant flow rate. Sephadex G-10 was allowed to swell in water for 24 hr prior to use. After pouring, the column was equilibrated with 10% ethanol-water. The same solution was used for elution of the column.

2.2.6 Concentration and Desalting of Undecagold Complex and Derivatives

Large volumes of undecagold cluster 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.
2.2.7 Melting Points

Melting points were obtained on a Hoover capillary melting point apparatus employing a heated oil bath for sample immersion.

2.2.8 Chemical Analyses

Chemical analyses were performed by Galbraith Laboratories.

2.2.9 Ninhydrin Assay

The procedure used for the detection of amino groups with ninhydrin is essentially as described by Moore et al. (82). The ninhydrin solution was freshly prepared each time and contained 1.0 g ninhydrin (spectrophotometric grade), 40 mg SnCl₂·2H₂O, 25 ml 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.

2.2.10 Conversion of Counterions on the Gold Complex

Chloride ion was generally used as the counterion for the gold complex in aqueous solution. In order to increase the solubility of the cluster in aprotic
solvents, the counterion was changed to p-toluene sulfonate. This was accomplished by passing a solution of the cluster in the chloride form (10-20 A415 units/ml) through a column of QAE Sephadex Q-25 in the tosylate form. The ratio of exchangeable sites on the column to the number of counterions 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. The undecagold cluster in the tosylate form is soluble in DMF, DMSO and HMPA. Conversion of the gold complex from the tosylate to the chloride form was accomplished by the reverse of the procedure above.

2.2.11 Preparation of Radiochemicals

[Carbonyl-14C]-phthalic anhydride was diluted with sublimed carrier phthalic anhydride in tetrahydrofuran and the specific radioactivity measured. The THF was removed under a slow stream of nitrogen gas. The solid residue was sublimed at 50°C under reduced pressure. The sublimed anhydride was weighed and the specific radioactivity was again determined. The material was sublimed a second time. This treatment did not change the specific radioactivity of the sample. A sample was weighed, dissolved in THF and stored at -70°C until
further use. The remaining solid was stored at -20°C.

[1-\textsuperscript{14}C]-Bromoacetic acid was diluted with carrier and used without purification. N-hydroxysuccinimido-[1-\textsuperscript{14}C]\(\alpha\)-bromoacetate was synthesized by the same procedure used to synthesize the unlabeled compound. This procedure is described in Chapter V.

[1,2-\textsuperscript{14}C]-Sodium acetate was purchased in ethanol. The ethanol was removed under a stream of nitrogen gas. The solid was further dried under vacuum for several hours. The dry [1,2-\textsuperscript{14}C]-sodium acetate was diluted with acetic anhydride. This solution was used without additional purification.

[U-\textsuperscript{14}C]-Cysteine hydrochloride was diluted with carrier cysteine hydrochloride and recrystallized from absolute ethanol. The crystals were collected by suction filtration and dried under vacuum for 12 hr. The mother liquor was stored at -70°C for future use. The crystals were weighed in a small vial. The vial was covered with a septum and gently purged with argon gas. Deoxygenated water was added to dissolve the sample and the specific radioactivity was measured. The specific radioactivity was also determined by measuring the concentration of sulfhydryl groups in the sample. This was done using a standard dithiodinitrobenzoate
(DTNB) assay. 1.15 mM DTNB in 10 mM Tris buffer, pH 8.0, was used in the assay. The sample was stored at -70°C until further use.

[1,4-\textsuperscript{14}C]-Succinic anhydride was diluted with carrier succinic anhydride and used without additional purification.
3.1 Introduction

The first step in the preparation of undecagold derivatives suitable for use in STEM analysis of biological samples was the synthesis of the native undecagold cluster. Bartlett et al. (73) have published a procedure for synthesis of the undecagold cluster by sodium borohydride reduction of the 4,4',4"-phosphinidynetri(benzenemethanamine) gold (I) cyanide complex (74). This method has been used, with minor modifications, in this laboratory and is presented in detail in the following section. The procedure described by Bartlett et al. (73) for synthesis of 4,4',4"-phosphinidynetri-(benzenemethanammonium)tris(4-methylbenzenesulfonate) was unrepeatable in our hands. A detailed description of the procedure used in this laboratory for the synthesis of large quantities of this phosphine ligand is provided in Chapter V.

After the synthesis of the undecagold cluster had been achieved, a more extensive characterization of its
physical and chemical properties was necessary. These studies provided necessary information concerning the physical properties of this molecule for use in subsequent work.

The undecagold cluster has three absorbance bands in the UV-visible region. The twenty-one benzene rings in the molecule produce an intense absorbance band centered at about 256 nm. The extinction coefficient for this band has not been measured accurately. The gold complex also has absorbance bands at 305 nm and 415 nm. The extinction coefficients for these bands were measured by two independent methods. The most accurate method used for measuring the extinction coefficient was by mass-spectral correlation. A sample of desalted, lyophilized undecagold cluster was weighed. A series of solutions of known concentration were prepared, and the absorbances of these solutions were measured at 305 nm and 415 nm. The extinction coefficients were then calculated from the slopes of plots of absorbance vs. concentration of undecagold cluster. The second method used to determine the extinction coefficient of the undecagold complex was by titration of the amino groups. The total absorbance of a solution of undecagold cluster was measured at 305 nm and 415 nm. The sample was then titrated with
a standardized solution of NaOH. The extinction coefficient was calculated assuming the gold complex contains twenty-one primary amines.

The chromatographic properties of the undecagold complex were evaluated using both ion-exchange and gel filtration resins. The behavior of the undecagold complex on both crosslinked polyacrylamide and poly-dextran gel filtration resins was evaluated using several elution buffers. The information obtained from these studies has been used to determine the best method for purification and desalting of the undecagold complex derivatives discussed in the following sections. The undecagold cluster was also chromatographed on both cation and anion exchange resins as a further characterization of its physical properties. The position of elution of the undecagold cluster from these resins was examined using several ionic strength gradient buffer systems. The results from these studies were also useful in determining the best method for purification of the mono-functional undecagold derivatives discussed in Chapter IV.

The successful use of the undecagold cluster as an electron-dense label for electron microscopic studies required the development of procedures for the preparation of mono-functional derivatives of the
complex. In the preparation of the mono-functional derivative, the remaining twenty amino groups on the cluster must be rendered non-nucleophilic to prevent them from interfering in later chemical reactions. The unique functional group introduced may then be used as the site for specific linkage of a molecule of interest. The problems inherent in the preparation of mono-functional derivatives of a compound containing twenty-one functionally identical groups and the methods developed to overcome these problems are discussed in the next chapter.

The problem of reducing the nucleophilic reactivity of the remaining amino groups in the mono-functional undecagold derivative has been approached in several ways. There are a variety of reagents that are very reactive toward amino groups. The use of some of these to modify the amino groups in proteins and other biological macromolecules exemplifies the selectivity of these reagents and the mildness of the reaction conditions required (83,84). Any reagent chosen to modify the amino groups in a mono-functional undecagold derivative must have the following characteristics: 1) It must be capable of quantitatively reacting with all of the amino groups present; 2) the reaction conditions should be as mild as possible; 3) the reagent
must not react in an irreversible manner with the unique functional group present in the mono-functional undeca-gold derivative; 4) the reagent should provide an easy method for radiochemical labeling of the cluster; and 5) the product of the reaction must be soluble in buffered aqueous solutions. In most cases, an amino group-selective reagent can be added in sufficient excess to insure quantitative reaction of all amino groups present. Due to the limited solubility of the undecagold cluster in most aprotic solvents, reagents that can be used in aqueous solutions are the most attractive candidates for modification of the amino groups. In addition, these reagents generally require the mildest reaction conditions. The methods for preventing these reagents from reacting with the unique functional group present in the mono-functional undecagold derivative are discussed in the following chapter.

The solubility properties of the gold complex after modification of the amino groups is, of course, dependent upon the nature of the modification reaction. Reagents that maintain the positive charge on the amino group after modification, or change the charge from positive to negative, should produce clusters with high solubility in aqueous solutions. The only undecagold complex derivatives of potentially limited solubility
in aqueous solutions are those in which the amino group modification results in loss of all ionizable functional groups. The use of amino group modification reagents to change the amount and type of charge in the mono-functional undecagold derivatives provides a method for producing the mono-functional derivative most suited for the biological system under study. For example, in a study on polynucleotides, the use of a mono-functional reagent prepared with preservation of the high positive charge present in the native cluster could lead to non-specific interactions between the electron-dense label and the sugar-phosphate backbone. In a study of this type, a derivative of the gold complex in which the high positive charge has been reduced or abolished would probably yield the best results.

In order to develop the procedures for quantitative modification of the amino groups in mono-functional undecagold derivatives, the reagents chosen and the reaction conditions required were first characterized using model systems. The best model amine for these studies is the amino group present in the native undecagold cluster. These model studies also provide means for further characterization of the structure and reactivity of the undecagold cluster. When a model
system for the undecagold cluster was required, benzylamine provided a reasonably good small molecule model.

The first method chosen for quantitatively blocking the primary amino groups in the undecagold cluster was reductive methylation. The formation of dimethyl tertiary amines by treatment of alkyl or aryl amines with formaldehyde and sodium borohydride is well documented (85,86). This reaction may be performed in aqueous solution at neutral to mildly alkaline pH. Because the rate of hydrolysis of sodium borohydride is pH dependent, the maximal utilization of reagents occurs at pH 9.0. This method has been used to reductively methylate the ε amino groups in the lysine residues of ribonuclease without effecting reduction of the disulfide bonds (85). This study illustrates the mildness of the conditions required for the reductive methylation of amino groups.

The mild reaction conditions make this an attractive method for blocking the amino groups in a mono-functional undecagold cluster. Reductive methylation of the amino groups in the cluster would reduce the nucleophilic reactivity of these groups while retaining the positive charge. Also, [3H]-sodium borohydride could be used in the reductive methylation to label the undecagold derivatives with a radioisotope. For these reasons, a procedure was developed for the
reductive methylation of the amino groups in the undecagold cluster. This procedure was then used in the preparation of mono-functional undecagold derivatives.

The second method used for blocking the primary amino groups in the undecagold cluster is a combination of carboxymethylation with bromoacetate and the reductive methylation reaction described above. Bromoacetate is another reagent that can be used to alkylate amino groups under mild conditions (83). The reaction may be performed in aqueous solution at mildly alkaline pH. The rate of the carboxymethylation reaction increases with increasing pH up to about one unit above the $pK_a$ of the amino group. Above this pH the rate of carboxymethylation becomes independent of pH. Bromoacetate is stable in aqueous solution at pH values up to 11, hence, the carboxymethylation of amino groups with bromoacetate is generally done between pH 8-10. After treatment with bromoacetate, reductive methylation of the carboxymethylamines by formaldehyde and sodium borohydride results in the formation of N-carboxymethyl,N-methyl amines. The N-methyl,N-carboxymethylamines produced should have a significantly reduced nucleophilic reactivity. The undecagold cluster, when derivatized in this manner, will be zwitterionic in character. In fact, careful control of the carboxymethylation reaction provides a
means for production of undecagold cluster with any number of charges between +21 and -21, at neutral pH. In addition, radioisotope labeling of the undecagold cluster can be achieved with $[^{14}\text{C}]$-bromoacetate, $[^{3}\text{H}]$-bromoacetate, or $[^{3}\text{H}]$-sodium borohydride. The versatility of this procedure for derivatizing the amino groups in the undecagold cluster to produce derivatives of variable net charge provides a simple method for producing mono-functional undecagold derivatives well suited for particular biological applications.

Anhydrides are a third type of reagent that may be used to modify amino groups under mild conditions. The acylation of amino groups with acetic anhydride or succinic anhydride can be performed in aqueous solution under mild conditions. Once again the pseudo first order rate of the acylation reaction increases with increasing pH; however, the rapid hydrolysis of anhydrides by hydroxide ions limits the useful pH range for these reactions. Use of the reagent is generally optimal between pH 7 and 8. The use of acetic anhydride to acylate the amino groups in the undecagold cluster results in production of an undecagold cluster with no ionizable groups between pH 1-14. This modification also abolishes the nucleophilic reactivity of the amino groups in the cluster. Acylation of the undecagold
cluster with succinic anhydride also abolishes the nucleophilic reactivity of the amino groups; however, this modification changes the charge from positive to negative. Also, \(^3\text{H}\) and \(^{14}\text{C}\) labeled acetic anhydride and succinic anhydride are available for radiochemical labeling of the mono-functional undecagold derivatives when necessary.

The gold complex was also acetylated by treatment with N-hydroxysuccinimidoacetate. N-hydroxysuccinimidoacetate is less susceptible to hydrolysis than acetic anhydride. In addition, the ester is significantly more selective towards primary amino groups. The difference in reactivity between these reagents is not important in the preparation of heneicosi(N-acetyl)undecagold; however, in the synthesis of mono-functional undecagold derivatives, the selectivity of the N-hydroxysuccinimide ester becomes useful. For this reason, procedures were developed to quantitatively acetylate the cluster with both reagents.

The time course for the amino group modification reactions was monitored by several methods. The reductive methylation conditions were first developed using benzylamine as a model for the amino groups in the undecagold cluster. The extent of reaction was
first measured as a function of the amount of reagent added using a ninhydrin assay. These results were confirmed by following the time course of the reaction by proton NMR spectroscopy. The conditions required for quantitative reductive methylation of benzylamine established by these studies were then applied to the reductive methylation of the undecagold cluster. The time course for the reductive methylation of the undecagold cluster was also determined by the ninhydrin assay method. The product was then analyzed by proton and phosphorus-31 NMR spectroscopy. The same methods for monitoring the extent of reaction were used to follow acylation of the undecagold cluster with acetic anhydride, succinic anhydride and N-hydroxysuccinimidoacetate.

The time course of the carboxymethylation reaction was monitored by measuring the amount of HBr produced (87). This method provides a very accurate measure of the extent of carboxymethylation. An accurate method for routinely following the time course of this reaction is necessary to allow for precise control of the resultant net charge on the undecagold derivative. The extent of reaction was also monitored by the chromatographic behavior of the cluster on SP Sephadex C-25 and
QAE Sephadex Q-25. Finally, the product was analyzed by proton NMR spectroscopy.
3.2 Experimental Procedures

3.2.1 Synthesis of Undecagold Cluster

4,4',4"-Phosphinidynetri(benzenemethanammonium)tris (4-methylbenzene sulfonate) (1.24 g, 1.44 mmol) and gold (I) cyanide (320 mg, 1.44 mmol) were placed in a 100 ml round bottom flask. The flask was sealed with a septum and purged with nitrogen gas. To this, 50 ml of deoxygenated 95% ethanol was added by syringe, and the suspension was stirred until homogeneous and pale yellow (90-120 min). Potassium hydroxide (363.6 mg, 6.48 mmol), dissolved in 4 ml of deoxygenated 50% ethanol, was added by syringe. Sodium borohydride (60 mg, 1.54 mmol) was dissolved in 4 ml of deoxygenated 50% ethanol and added in eight 0.50 ml aliquots over two min. The dark red-brown solution was stirred at room temperature for 60 min and then filtered by vacuum filtration. The filtrate was diluted with 50 ml H₂O and the pH adjusted to 5.0.

This solution was concentrated to approximately 12 ml and applied to a column of Sephadex G-25 (2.5 x 48 cm). The column was pre-equilibrated and eluted with 0.10 M NH₄Cl (Figure 1). The 415 nm absorbing fractions were pooled and concentrated to about 20 ml by ultrafiltration. The concentrate was further
Figure 1. Sephadex G-25 Elution Profile of Undecagold Cluster.

The undecagold cluster product mixture was applied in 12 ml to a 2.5 x 45 cm column of Sephadex G-25. The sample was eluted with 0.10 M ammonium chloride at a flow rate of 1.5 ml per minute. Fractions of 3.75 ml were collected and the absorbances measured at 415 nm (●-●-●-●) and at 260 nm (▲-▲-▲-▲). Fractions 25-32 (2085 A_{415} units) were pooled and treated as described in the text.
concentrated by rotary evaporation in vacuo to approximately 10 ml and passed through a column of Sephadex G-10 (2.5 x 45 cm). The column was pre-equilibrated and eluted with a solution of 10% ethanol-water (Figure 2), giving 1930 A$_{415}$ units (65.42 µmol) of the desired product, tricyanoheptakis[4,4',4"-phosphinidynetri(benzenemethanammonium)tris(chloride)]-undecagold (yield 50%). The material was stored at -70°C. Samples of undecagold cluster were prepared for proton, carbon-13 and phosphorus-31 NMR spectroscopy. The data from these NMR spectra are presented in Table 4 and are in accord with the structure of this complex. The UV-visible spectrum of the undecagold cluster shows major absorbance bands at 305 and 415 nm (Figure 3), in agreement with the published spectrum for this molecule (73).

3.2.2 Physical Properties of the Undecagold Cluster

3.2.2.1 Extinction Coefficients of Undecagold Cluster

The extinction coefficients of the undecagold cluster at 305 and 415 nm were determined by two methods. These methods differed by the way in which the concentrations of standard solutions prepared for spectral analysis were measured.
The concentrated undecagold cluster (2085 $A_{415}$ units, 10 ml) was applied to a 2.5 x 45 cm column of Sephadex G-10. The column was eluted with 10% ethanol-water at a flow rate of 1.0 ml per minute. Fractions of 4.0 ml were collected and the absorbance measured at 415 nm. The desalted gold complex (fractions 17-26, 1930 $A_{415}$ units) was stored at -70°C.
TABLE 4

NMR Analysis of Undecagold Cluster\textsuperscript{a}

\begin{center}
\begin{tabular}{lcc}

<table>
<thead>
<tr>
<th>Nucleus\textsuperscript{b}</th>
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<th>Integration</th>
<th>Assignment</th>
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<td>b</td>
</tr>
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<td>2</td>
<td>a</td>
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<td>c</td>
</tr>
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<td>d</td>
</tr>
<tr>
<td>(^{13}\text{C})</td>
<td>144.7</td>
<td>--</td>
<td>g</td>
</tr>
<tr>
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<td>f</td>
</tr>
<tr>
<td>(^{13}\text{C})</td>
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<td>--</td>
<td>e</td>
</tr>
<tr>
<td>(^{13}\text{C})</td>
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<td>--</td>
<td>h</td>
</tr>
<tr>
<td>(^{31}\text{P})</td>
<td>53.3</td>
<td>--</td>
<td>i</td>
</tr>
</tbody>
</table>
\end{tabular}
\end{center}

\textsuperscript{a}All spectra were recorded on the Bruker WP-200 instrument, field frequency locked on the deuterium resonance of D\textsubscript{2}O.

\textsuperscript{b}The carbon-13 and phosphorus-31 spectra were proton decoupled using a broadband decoupler centered at 200 MHz.
Figure 3. Ultraviolet-Visible Absorption Spectrum of Undecagold Cluster

A sample of desalted undecagold cluster was dissolved in water to give an absorbance of approximately 1.0 at 305 nm. The pH was adjusted to 7.0 and the spectrum of the sample was measured using a Cary 118C UV-visible spectrophotometer. A scan rate of 1.0 nm per second was used.
A plot of the absorbance of the cluster at 305 and 415 nm vs. concentration by weight is shown in Figure 4. The extinction coefficients calculated from the slopes are $9.93 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$ and $2.95 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$ for the 305 and 415 nm absorbance bands, respectively.

In a second determination a spectrophotometrically measured amount of undecagold cluster was titrated with standard base to measure the primary amino groups. The extinction coefficients at 305 and 415 nm were then calculated on the assumption that twenty-one amino groups were associated with the molecule. The titration data in Figure 5 were used to calculate $\varepsilon_{305} = 9.6 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$ and $\varepsilon_{415} = 2.8 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$.

3.2.2.2 Chromatographic Properties of the Undecagold Cluster

Samples of the undecagold cluster were chromatographed on BioGel P-6 and Sephadex G-25 in 0.10 M NH$_4$Cl, 0.10 M NaCl and 0.10 M TEAB. The elution profiles for these columns eluted with 0.10 M NH$_4$Cl are shown in Figures 1 and 6. The gold complex was also chromatographed on BioGel P-2 and Sephadex G-10 in 10% ethanol-water. The elution profiles for these columns are shown in Figures 7 and 2, respectively.
Undecagold cluster in the chloride salt form, pH 7.0 (approximately 1000 \(A_{415}\) units) was placed in a 250 ml tarred 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 hr). The flask was weighed and the sample transferred to a 100 ml volumetric flask. The stock solution (3.44 \(x\) 10\(^{-4}\) M) was then used to prepare a series of solutions from 3.44 x 10\(^{-5}\) M to 3.44 x 10\(^{-6}\) M. The absorbances of these solutions were measured at 305 nm (\(\bullet-\bullet-\bullet\bullet\)) and 415 nm (\(\Delta-\Delta-\Delta\Delta\)). From plots of \(A_{305}\) and \(A_{415}\) vs. concentration of gold complex, values of 9.93 x 10\(^4\) cm\(^{-1}\) M\(^{-1}\) and 2.95 x 10\(^4\) cm\(^{-1}\) M\(^{-1}\) were calculated for \(\varepsilon_{305}\) and \(\varepsilon_{415}\), respectively.
FIGURE 4

[Graph showing two linear relationships: one for [Undecagold Cluster] × 10^5 on the x-axis and A on the y-axis. The graph has two lines, one with circles and another with triangles.]
Undecagold cluster (3800 A415 units, 12,960 A305 units) in the chloride salt form, 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 sample was purged with nitrogen gas for 120 minutes at 65°C. The flask was sealed, cooled to room temperature and titrated as described in section 2.2.3. From the titration curve it was determined that 2.8 mmoles of amino groups had been titrated. This corresponded to 135 μmoles of undecagold cluster and calculated extinction coefficients of $9.6 \times 10^4$ cm$^{-1}$ M$^{-1}$ and $2.8 \times 10^4$ cm$^{-1}$ M$^{-1}$ for $\varepsilon_{305}$ and $\varepsilon_{415}$, respectively.
A sample of undecagold cluster (764 $A_{415}$ units) was applied in 6.0 ml to a 2.0 x 35 cm column of BioGel P-6 and eluted with 0.10 M ammonium chloride. Fractions of 2.0 ml were collected at a flow rate of 0.50 ml per minute. Fractions 25-33 (1630 $A_{415}$ units) were pooled and desalted as described in section 3.2.1.
Undecagold cluster (1137 A_{415} units) was applied in 5 ml to a 2.0 x 45 cm column of BioGel P-2 and eluted with 10% ethanol-water at a flow rate of 0.50 ml per minute. Fractions of 2.0 ml were collected and the absorbances measured at 415 nm.
The chromatographic properties of the cluster were examined on SP Sephadex C-25 and QAE Sephadex Q-25 using several ionic strength gradient elution systems. The elution of the undecagold cluster from SP Sephadex C-25 was evaluated using a linear NaCl gradient increasing from 1.0 M to 3.0 M and a linear TEAB gradient increasing from 0.10 M to 0.30 M. The elution profiles for the NaCl and TEAB gradients are shown in Figures 8 and 9. The cluster was also chromatographed on QAE Sephadex Q-25 using a linear TEAB gradient increasing from 0.10 M to 1.00 M and a linear Na₂CO₃ gradient increasing from 0.10 M to 1.00 M. The elution profile for the TEAB gradient is shown in Figure 10. The position of elution of the gold complex from these columns is presented in Table 5.

3.2.3 Reductive Methylation of Benzylamine

Benzylamine (32.8 μl, 300 μmol) was dissolved in 10.0 ml of 20% dioxane-water. Formaldehyde (20 μl of 37%, 200 μmol) was added and the pH adjusted to 7.5 with 1.0 N HCl. Sodium borohydride (45 mg, 1.2 mmol) was added to initiate the reaction. The addition was repeated at the 25 minute time point. Formaldehyde (20 μl, 200 μmol) was added every 60 seconds. Aliquots (20 μl) were removed at 5 minute intervals and quenched
Undecagold cluster in the chloride salt form (109 $A_{415}$ units) was dissolved in deionized water to give a concentration of about 7 $A_{415}$ units per ml. The pH was adjusted to 7.0 and the sample applied to a 2.0 x 16 cm column of SP Sephadex C-25. The sample was eluted with a 700 ml linear sodium chloride gradient increasing from 1.00 M to 3.00 M. Fractions of 8.2 ml were collected at a flow rate of 2 ml per minute, and the absorbance measured at 415 nm.
Undecagold cluster in the chloride salt form (780 $A_{415}$ units) was dissolved in deionized water to give a concentration of about 7 $A_{415}$ units per ml. The pH was adjusted to 7.0 and the sample applied to a 1.5 x 30 cm column of SP Sephadex C-25. The sample was eluted with a 900 ml linear TEAB gradient increasing from 0.10 M to 0.30 M. Fractions of 7.5 ml were collected at a flow rate of 1.5 ml per minute and the absorbances measured at 415 nm.
Undecagold cluster in the bicarbonate salt form (89 $A_{415}$ units) was dissolved in deionized water to give a concentration of about 5 $A_{415}$ units per ml. The sample was applied to a 1.5 x 12 cm column of QAE Sephadex Q-25 and eluted with 600 ml of a linear TEAB gradient increasing from 0.10 M to 1.00 M, pH 7.8. Fractions of 4.5 ml were collected at a flow rate of 1.5 ml per minute. The absorbances of the fractions were measured at 415 nm.
### TABLE 5

**Summary of Ion-Exchange Elution Data for Undecagold Cluster**

<table>
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<tr>
<th>Resin</th>
<th>Gradient</th>
<th>Eluting salt concentration (M)</th>
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<td>SP Sephadex C-25</td>
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<td>SP Sephadex C-25</td>
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<td>QAE Sephadex Q-25</td>
<td>Na₂CO₃</td>
<td>0.32</td>
</tr>
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</table>

*a* The tabulated values were obtained from the ion-exchange elution profiles of undecagold cluster described in section 3.2.2.2.
with 50 µl of acetone. These aliquots were used to measure the extent of reaction by the ninhydrin method (section 2.2.9). The time course for the reductive methylation of benzylamine is shown in Figure 11.

In order to determine whether the conditions for the reductive methylation of benzylamine described above are adequate to produce N,N-dimethylbenzylamine, the extent of reaction vs. time was measured by proton NMR spectroscopy. Six parallel reaction mixtures were prepared as follows. Benzylamine (100 µl, 915 µmol) was placed in 10.0 ml of 20% dioxane-water. Formaldehyde (100 µl of 37%, 1.0 mmol) was added and the pH was adjusted to 7.5 with 2.0 N HCl. Sodium borohydride (67.5 mg, 1.79 mmol) was added to initiate the reaction and the addition repeated at the 5, 10 and 15 minute time points. Formaldehyde (100 µl of 37%, 1.00 mmol) was added every 60 seconds. The reactions were stopped at the 3, 6, 9, 12, 15 and 20 minute time points by addition of 2.0 ml acetone. The pH of the reaction mixtures was then adjusted to 11.5 with 4.0 M NaOH and the samples were extracted with three 20 ml volumes of diethyl ether. The organic extracts were concentrated to an oil by rotary evaporation at reduced pressure. The samples were diluted with 3.0 ml H₂O and the pH adjusted to 7.0. These samples were then prepared for
Figure 11. Time Course for Reductive Methylation of Benzylamine

Twenty µl aliquots were removed from the reaction mixture and quenched with 50 µl of acetone as described in the text. These samples were diluted to 1.0 ml with the ninhydrin assay solution (section 2.2.9) and heated at 100°C for 10 min. The samples were then allowed to cool for 15 min. The absorbance was measured at 570 nm as follows. A pair of 1 ml spectrophotometric cells were filled with 0.90 ml of water, placed in the spectrophotometer, and the absorbance was set to zero. One hundred µl of a control assay solution containing no benzylamine was added to the reference cuvette. One hundred µl aliquots from the assay solutions were diluted with 900 µl of water and the absorbance was measured.
FIGURE 11

A\textsubscript{570} vs. Time (Min.)
proton NMR spectroscopy in D$_2$O. The methyl group peak integration, relative to a value of 5.00 for the aromatic protons, was plotted vs. reaction time and is shown in Figure 12.

3.2.4 Synthesis of Heneicosi(N,N-Dimethyl)-Undecagold

Undecagold cluster (560 $A_{415}$ units, 18.98 µmol, 399 µeq of amino groups) was placed in 10.0 ml H$_2$O. Formaldehyde (100 µl of 37%, 1.00 mmol) was added and the pH was adjusted to 7.5 with 2.0 N HCl. Sodium borohydride (100 mg, 2.67 mmol) was added to initiate the reaction and again at the 10 and 20 minute time points. Formaldehyde (100 µl at 37%, 1.00 mmol) was added every 60 seconds. The reaction mixture was stirred in an ice-water bath to maintain the temperature below 10°C, and 2.0 N HCl was used to maintain the pH between 8.0 and 10.0. Aliquots (20 µl) of the reaction mixture were removed at 2 minute or 5 minute intervals and quenched with 50 µl of acetone. These aliquots were then used for the ninhydrin assay as described in section 2.2.9. The rate of disappearance of the primary amino groups as measured by the ninhydrin assay was plotted vs. time and is shown in Figure 13. The reaction mixture was concentrated to approximately 6 ml.
Figure 12. Proton NMR Analysis of the Reductive Methylation of Benzylamine

The reductive methylation reaction mixtures were prepared for proton NMR analysis as described in section 3.2.3. The spectra were obtained using the Varian EM 390 spectrometer. The relative integration of the peaks in each spectrum was determined. These ratios were normalized to give a value of 5.00 for the peak corresponding to the aromatic protons. The relative integration of the peak corresponding to the N-methyl group was plotted vs. time of reaction in minutes.
FIGURE 12

Relative Integration vs. Time (Min.)
Figure 13. Time Course for the Reductive Methylation of Undecagold Cluster

Twenty μl aliquots were removed from the reaction mixture and quenched with 50 μl of acetone as described in the text. These samples were diluted with 1.0 ml of the ninhydrin assay solution and heated at 100°C for 10 min. The samples were then allowed to cool for 15 min. The absorbance was measured at 570 nm as described previously.
and applied to a BioGel P-2 column (1.5 x 45 cm). The column was run in 0.05 M TEAB, pH 7.8. The eluted gold complex was concentrated to dryness by rotary evaporation \textit{in vacuo} and prepared for proton and phosphorus-31 NMR spectroscopy in D$_2$O. The NMR data are presented in Table 6. The UV-visible spectrum was recorded before and after the reductive methylation reaction and was found to be unchanged.

3.2.5 Synthesis of Heneicosi(N-Carboxymethyl)-Undecagold

Undecagold cluster (1727 $A_{415}$ units, 58.55 $\mu$mol, 1230 $\mu$eq of amino groups) was placed in freshly boiled water to give a total volume of 25 ml. The pH was adjusted to 5.0 with 1.0 N HCl and the solution was purged with nitrogen gas for 60 min at 60°C. The flask was stoppered and the solution was cooled to room temperature. The sample was always handled under a nitrogen atmosphere. The pH was adjusted to 9.10 with 1.0 N and 0.50 N NaOH. Bromoacetic acid (57.0 mg, 410 $\mu$mol) in 1.0 ml of H$_2$O, pH 9.1, was then added, and this addition was repeated at the 12 hour and 36 hour time points. The total reaction time was 72 hours. The pH was maintained between 8–9 during the reaction.
### TABLE 6

NMR Analysis of Uniform Derivatives of Undecagold Cluster

#### Heneicosi(N,N-Dimethyl)Undecagold

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<thead>
<tr>
<th>Chemical shift (δ)</th>
<th>Integration</th>
<th>Assignment</th>
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<td>52.50</td>
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#### Heneicosi(N-Carboxymethyl)Undecagold

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<tr>
<td>3.65</td>
<td>2</td>
<td>d</td>
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</tbody>
</table>

Table 6 continued on following page.
Table 6 (continued)

| Heneicosi(\(\text{N-Carboxymethyl,}\text{N-Methyl}\))Undecagold |  |
|---|---|---|
| Chemical shift (\(\delta\)) | Integration | Assignment |
| 7.55 | 2 | b |
| 6.68 | 2 | a |
| 3.75 | 2 | c |
| 3.54 | 2 | d |
| 2.22 | 3 | e |

| Heneicosi(\(\text{N-Acetyl}\))Undecagold |  |
|---|---|---|
| Chemical shift (\(\delta\)) | Integration | Assignment |
| 7.33 | 2 | b |
| 6.66 | 2 | a |
| 4.07 | 2 | c |
| 1.91 | 3 | d |

Table 6 continued on following page.
Table 6 (continued)

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<thead>
<tr>
<th>Chemical shift (δ)</th>
<th>Integration</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.15</td>
<td>2</td>
<td>b</td>
</tr>
<tr>
<td>6.65</td>
<td>2</td>
<td>a</td>
</tr>
<tr>
<td>4.07</td>
<td>2</td>
<td>c</td>
</tr>
<tr>
<td>2.40</td>
<td>4</td>
<td>d</td>
</tr>
</tbody>
</table>

Heneicosi(N-Succinyl)Undecagold

[^1] H NMR spectra were recorded on the Bruker WH-270 instrument, field frequency locked on the deuterium resonance of D₂O.

[^b] The proton decoupled phosphorus-31 spectrum was recorded on the Bruker HX-90 spectrometer, field frequency locked on the deuterium resonance of 50% D₂O.

![Chemical structure](image)
The extent of reaction was monitored by titration of the protons produced in the reaction with 0.490 M NaOH. In a control reaction, containing no undecagold cluster, there was no detectable formation of HBr. The reaction 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 was also monitored by assessment of the amount of the gold complex which was retained by SP Sephadex C-25, sodium form and QAE Sephadex Q-25, chloride form, at pH 5.5. The difference in total absorbance of the solutions before and after passing through the columns provided a qualitative method for monitoring the extent of reaction. The μmol of HBr produced vs. time is shown in Figure 14. The % retention of the gold complex on SP Sephadex C-25 and QAE Sephadex Q-25 vs. time of reaction is given in Table 7.

The reaction mixture was concentrated to approximately 10 ml by rotary evaporation in vacuo. The sample was then applied to a column of Sephadex G-10 (2.5 x 45 cm). The column was eluted with 10% ethanol-water at a flow rate of 1.5 ml/minute. The desalted, carboxymethyl undecagold was then concentrated to dryness by rotary evaporation in vacuo. The sample
Figure 14. Time Course for Carboxymethylation of Undecagold Cluster

The undecagold cluster was incubated with bromoacetate as described in section 3.2.5. The pH of the reaction mixture was readjusted to 9.10 at the times indicated. The pH adjustments were made with 0.490 M NaOH. The volume of NaOH added was plotted vs. reaction time. Arrows indicate the time points of bromoacetate addition.
FIGURE 14

NaOH (mL)

Time (Hours)
### TABLE 7
Retention of the Carboxymethylation Reaction Mixture on Ion-exchange Resins

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>( A_{415} ) units before column</th>
<th>( A_{415} ) units after QAE</th>
<th>( A_{415} ) units after SP</th>
<th>% Retention on QAE</th>
<th>% Retention on SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>0.427</td>
<td>0.420</td>
<td>0.000</td>
<td>2.0</td>
<td>100</td>
</tr>
<tr>
<td>36</td>
<td>0.412</td>
<td>0.416</td>
<td>0.000</td>
<td>0.0</td>
<td>100</td>
</tr>
<tr>
<td>48</td>
<td>0.421</td>
<td>0.409</td>
<td>0.000</td>
<td>3.0</td>
<td>100</td>
</tr>
<tr>
<td>60</td>
<td>0.433</td>
<td>0.368</td>
<td>0.108</td>
<td>15.1</td>
<td>75.1</td>
</tr>
<tr>
<td>72</td>
<td>0.413</td>
<td>0.264</td>
<td>0.285</td>
<td>36.1</td>
<td>31.0</td>
</tr>
</tbody>
</table>

\( A_{415} \) units

\( A_{415} \) units

% Retention on QAE

% Retention on SP

*Small columns of QAE Sephadex Q-25 and SP Sephadex C-25 were made with disposable pipets. A small aliquot (50 \( \mu l \)) from the reaction mixture was diluted to 3.0 ml with H\(_2\)O and the pH was adjusted to 5.5. The absorbance of the solution at 415 nm was measured. The sample was split in half and loaded on the two columns. The columns were washed with one column volume of water after the addition was complete. The absorbances of the effluents were then measured at 415 nm.*
was dissolved in 25 ml of freshly boiled deionized water and stored at -70°C until further use.

A sample of the carboxymethylated undecagold cluster was titrated as described in section 2.2.3. The titration curve is shown in Figure 15. A sample was also prepared for proton NMR spectroscopy in D₂O. These data are presented in Table 6.

The distribution of carboxymethylated undecagold species present in the product mixture was investigated by ion-exchange chromatography. Carboxymethyl undecagold cluster (1000 A₄₁₅ units, 33.90 µmol) was placed in 80.0 ml of H₂O. The pH was adjusted to 5.5 with 1.0 N HCl and the solution was purged with nitrogen gas for 60 min at 60°C. After cooling to room temperature, the sample was split in half. The samples were diluted to 4.0 A₄₁₅ units per ml and the pH was readjusted to 5.5. These samples were applied to columns of SP Sephadex C-25 and QAE Sephadex Q-25. The material which was not retained by each of these columns was pooled separately and stored at -70°C for later use. The columns were eluted with the same linear ionic strength gradient increasing from 0.00 M to 0.40 M (pH 5.5). The elution profiles are shown in Figures 16 and 17. The material which was not retained on the QAE Sephadex Q-25 column
Heneicosi(N-carboxymethyl)undecagold (1130 A$_{415}$ units, 38.31 μmoles), desalted by passage through Sephadex G-10, was placed in 25.0 ml of freshly boiled, deionized water, and the pH adjusted to 5.0. The solution was purged with nitrogen gas for 60 min at 60°C. The flask was sealed, cooled to room temperature and titrated as described in section 2.2.3. A 0.490 M NaOH solution was used for the titration.
Figure 16. SP Sephadex C-25 Elution Profile of Carboxymethylated Undecagold Cluster

The carboxymethylated undecagold product mixture (500 $A_{415}$ units, 125 ml) was adjusted to pH 5.0 and purged with nitrogen gas for 60 min at 60°C. The sample was cooled to room temperature and applied to a 1.5 x 10 cm column of SP Sephadex C-25, sodium form. The column was eluted with a 350 ml linear sodium chloride gradient increasing from 0.00 M to 0.40 M at pH 5.5. Fractions of 4.0 ml were collected at a flow rate of 1.5 ml per minute and the absorbances measured at 415 nm. Fractions eluting at 0.09 M (fractions 14-24, 60.3 $A_{415}$ units), 0.16 M (fractions 30-38, 44.0 $A_{415}$ units), 0.22 M (fractions 41-50, 18.3 $A_{415}$ units) and at 0.26 M (fractions 52-57, 7.5 $A_{415}$ units) were pooled separately. These samples were concentrated by rotary evaporation in vacuo, desalted by passage through Sephadex G-10 (10% ethanol-water) and stored at -70°C.
Figure 17. QAE Sephadex Q-25 Elution Profile of Carboxymethylated Undecagold Cluster

The carboxymethylated undecagold product mixture (500 A\textsubscript{415} units, 125 ml) was adjusted to pH 5.0 and purged with nitrogen gas for 60 min at 60°C. The sample was cooled to room temperature and applied to a 1.5 x 10 cm column of QAE Sephadex Q-25, chloride form. The column was eluted with a 350 ml linear sodium chloride gradient increasing from 0.00 M to 0.40 M at pH 5.5. Fractions of 4.0 ml were collected at a flow rate of 1.5 ml per minute and the absorbances measured at 415 nm. Fractions eluting at 0.06 M (fractions 7-17, 81.0 A\textsubscript{415} units), 0.11 M (fractions 19-25, 70.1 A\textsubscript{415} units), 0.14 M (fractions 26-31, 46.7 A\textsubscript{415} units) and at 0.16 M (fractions 32-37, 25.7 A\textsubscript{415} units) were pooled separately. These samples were concentrated by rotary evaporation \textit{in vacuo}, desalted by passage through Sephadex G-10 (10% ethanol-water) and stored at -70°C.
above was loaded on SP Sephadex C-25, sodium form (1.5 x 10 cm). The material which was not retained on the SP Sephadex C-25 column was applied to QAE Sephadex Q-25, chloride form (1.5 x 10 cm). Once again, these columns were eluted with the gradient described above. The elution profiles are shown in Figures 18 and 19. The peaks corresponding to the carboxymethylated undeca-gold cluster species of different net charge present in the mixture were pooled separately. The percentage of each species with a net charge between +4 and -4 at neutral pH was determined and is shown in Table 8.

3.2.6 Synthesis of Heneicosi[(N-Carboxymethyl)-(N-Methyl)]Undecagold

Heneicosi(N-carboxymethyl)undecagold (79.2 Å415 units, 2.68 μmol) was dissolved in 7 ml of water and the pH was 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 cooling to room temperature the pH was adjusted to 7.5. This material was reductively methylated according to the procedure developed for formation of heneicosi(N,N-dimethyl)-undecagold. The product was desalted on Sephadex G-10 to give 76.6 Å415 units (97% yield). A sample was prepared for proton NMR spectroscopy (Table 6).
The carboxymethylated undecagold cluster not retained by QAE Sephadex Q-25 (Figure 17) was applied to a 1.5 x 10 cm column of SP Sephadex C-25 and eluted with a 350 ml linear sodium chloride gradient increasing from 0.00 M to 0.40 M at pH 5.5. Fractions of 4.0 ml were collected at a flow rate of 1.5 ml per minute and the absorbances measured at 415 nm. Fractions eluting at 0.09 M (fractions 13-24, 53.8 A415 units), 0.16 M (fractions 31-42, 37.7 A415 units), 0.23 M (fractions 45-53, 19.7 A415 units) and at 0.26 M (fractions 54-60, 6.0 A415 units) were pooled separately. The material which was not retained by the column (95.0 A415 units) was also pooled. These samples were concentrated by rotary evaporation in vacuo, desalted by passage through Sephadex G-10 (10% ethanol-water) and stored at -70°C.
Figure 19. QAE Sephadex Elution Profile of Carboxymethylated Undecagold-SP Sephadex Pre-gradient Effluent

The carboxymethylated undecagold cluster not retained by SP Sephadex C-25 (Figure 16) was applied to a 1.5 x 10 cm column of QAE Sephadex Q-25 and eluted with a 350 ml linear sodium chloride gradient increasing from 0.00 M to 0.40 M at pH 5.5. Fractions of 4.0 ml were collected at a flow rate of 1.5 ml per minute and the absorbances measured at 415 nm. Fractions eluting at 0.06 M (fractions 5-16, 63.2 A\textsubscript{415} units), 0.11 M (fractions 19-25, 66.0 A\textsubscript{415} units), 0.14 M (fractions 27-32, 46.7 A\textsubscript{415} units) and 0.16 M (fractions 34-38, 20.0 A\textsubscript{415} units) were pooled separately. The material which was not retained by the column (99.2 A\textsubscript{415} units) was also pooled. These samples were concentrated by rotary evaporation in vacuo, desalted by passage through Sephadex G-10 (10% ethanol-water) and stored at -70°C.
TABLE 8
Percentage of Different Charged Species in the Carboxymethylated Undecagold Product Mixture

<table>
<thead>
<tr>
<th>Net charge</th>
<th>A_{415} units from column</th>
<th>Total A_{415}</th>
<th>% of total in peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>+4</td>
<td>7.5 6.0</td>
<td>13.50</td>
<td>1.57</td>
</tr>
<tr>
<td>+3</td>
<td>18.29 19.69</td>
<td>37.98</td>
<td>4.41</td>
</tr>
<tr>
<td>+2</td>
<td>44.00 37.70</td>
<td>81.70</td>
<td>9.49</td>
</tr>
<tr>
<td>+1</td>
<td>60.28 53.75</td>
<td>114.03</td>
<td>13.25</td>
</tr>
<tr>
<td>0</td>
<td>103.72 90.31</td>
<td>194.03</td>
<td>22.54</td>
</tr>
<tr>
<td>-1</td>
<td>80.99 63.23</td>
<td>144.22</td>
<td>16.75</td>
</tr>
<tr>
<td>-2</td>
<td>70.13 66.06</td>
<td>136.19</td>
<td>15.82</td>
</tr>
<tr>
<td>-3</td>
<td>46.74 46.70</td>
<td>93.44</td>
<td>10.85</td>
</tr>
<tr>
<td>-4</td>
<td>25.70 20.04</td>
<td>45.74</td>
<td>5.31</td>
</tr>
</tbody>
</table>

The absorbances of the pooled fractions from Figures 16-19 were measured at 415 nm. The total absorbance for each pool is listed above. The combined total from both columns was then used to calculate the percentage of each net charge form present.
3.2.7 Synthesis of Heneicosi(N-Acetyl)Undecagold

3.2.7.1 Acetylation with Acetic Anhydride

Undecagold (159.5 Å units, 5.41 µmol, 113.6 µeq-NH₂ groups) was placed 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 cooling to room temperature and adjusting the pH to 8.0, acetic anhydride (50 µl, 0.53 mmol) was added and the pH of the reaction mixture maintained between 7 and 8 by addition of 4.0 M NaOH. When the pH became constant, it was readjusted to pH 8.0 and the addition of acetic anhydride was repeated. A total of 15, 50 µl aliquots of acetic anhydride were added. Before each addition, a 25 µl sample was removed from the reaction mixture. To these aliquots, 1.0 ml ninhydrin solution was added and the samples were assayed as described in section 2.2.9. These assays showed that acetylation was complete after the first addition of acetic anhydride. The reaction mixture was concentrated to approximately 12 ml by rotary evaporation in vacuo. One ml of 95% ethanol was added and the sample was applied to a 2.5 x 45 cm column of Sephadex G-10 and eluted with 10% ethanol-water. The desalted heneicosi(N-acetyl)undecagold peak was concentrated to dryness by rotary evaporation in vacuo, dissolved in
15 ml of water and stored at -70°C until further use. Titration of the acetylated undecagold cluster showed no detectable ionizable groups in the pH range from 3 to 11.

3.2.7.2 Acetylation with N-Hydroxysuccinimidoacetate

Undecagold cluster (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-hydroxysuccinimidoacetate (95.4 mg, 608 µmol) was dissolved in 1.5 ml of THF. The ester/THF solution was added to the undecagold cluster over a 30 second period. Aliquots of the reaction mixture were removed and used for the ninhydrin assay (Figure 20). After thirty minutes, the reaction mixture was concentrated to 3 ml by rotary evaporation in vacuo and applied to a 1.5 x 45 cm column of Sephadex G-10. The product, heneicosi(N-acetyl)undecagold, was eluted with 10% ethanol-water to give 84.1 $A_{415}$ units (94% yield).

Samples of the acetylated undecagold cluster were prepared for proton NMR spectroscopy in D$_2$O. This spectrum is shown in Figure 21. The spin-lattice relaxation times ($T_1$) for the protons in heneicosi-(N-acetyl)undecagold were measured by the standard inversion-recovery method. A stacked plot of the spectra
Figure 20. Time Course for Acetylation of Undecagold Cluster with N-hydroxysuccinimidoacetate

Fifty μl aliquots were removed from the acetylation reaction mixture and diluted immediately with 1.0 ml of the ninhydrin assay solution. The samples were incubated at 100°C for 10 min, cooled for 15 min and the absorbances measured at 570 nm as described previously.
A dried sample of heneicosi(N-acetyl)undecagold (33.7 A\textsubscript{415} units, 1.14 μmol), pH 7.0, was diluted with 0.50 ml of 99.8 atom % D\textsubscript{2}O and concentrated to dryness by rotary evaporation \textit{in vacuo}. This procedure was repeated three more times. The sample was then dissolved in 0.8 ml of 99.8 atom % D\textsubscript{2}O, placed in a 5 mm NMR tube and gently purged with nitrogen gas for 20 min. The cap was then placed on the tube and sealed with Teflon tape. Two hundred scans were collected using the Bruker WH-270 spectrometer. δ 7.34, 2H; δ 6.66, 2H; δ 4.07, 2H; δ 1.91, 3H.
is shown in Figure 22. The relaxation times (T₁) are listed in Table 9. The spin-spin relaxation times (T₂) were obtained using a modified Carr-Purcell pulse train. A stacked plot of the spectra is shown in Figure 23 and the relaxation times (T₂) are given in Table 9.

3.2.8 Synthesis of Heneicosi(N-Succinyl)Undecagold

Undecagold cluster (190 Å units, 6.44 μmol, 135 μeq of amino groups) was placed in 10.0 ml of water. The pH was adjusted to 5.0 with 1.0 N HCl and the solution was purged with nitrogen gas for 60 minutes at 60°C. After cooling 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. The amino groups were then detected using the ninhydrin method discussed in section 2.2.9 (Figure 24).

The product was concentrated to approximately 4 ml and applied to a column of Sephadex G-10 (1.5 x 45 cm). The column was eluted with 10% ethanol-water. The desalted cluster was concentrated to dryness by rotary evaporation in vacuo, dissolved in 5.0 ml of water and
The spin-lattice ($T_1$) relaxation time for heneicosi-(N-acetyl)undecagold was determined using the sample described in the legend to Figure 21. A standard inversion-recovery pulse program was used ($180^\circ$-$D_1$-$90^\circ$) where $D_1$ is a variable delay between pulses. $D_2$, a delay sufficiently long to allow for complete relaxation of all absorbing nuclei in the sample, was incorporated between pulse sequences. One hundred scans were collected for each delay time ($D_1$). The $180^\circ$ pulse length was determined to be 20.90 μsec (sweep width, 3003 Hz) using a progressive saturation pulse sequence. The first spectrum in the series was phased and scaled. The remaining spectra were phased and scaled using an automated sequence. Minor phase corrections were then made on the individual spectra before plotting. The spectra are displayed in order of increasing delay time ($D_1$). $D_1$ values: 5.0 msec, 10.0 msec, 20.0 msec, 30.0 msec, 40.0 msec, 50.0 msec, 60.0 msec, 70.0 msec, 80.0 msec, 90.0 msec, 100.0 msec, 200.0 msec, 300.0 msec, 400.0 msec, 500.0 msec, 600.0 msec, 900.0 msec, 1.00 sec, 2.00 sec, 3.00 sec; $D_2$: 10.0 sec.
Figure 23. Spin-Spin ($T_2$) Relaxation Spectra of Heneicosi(N-Acetyl)Undecagold

The spin-spin ($T_2$) relaxation time for heneicosi-(N-acetyl)undecagold was determined using a modified Carr-Purcell pulse train $[90^\circ-(D_1-180^\circ-D_1)2n]$ where $n = 2$ and $D_1$ is a variable length delay time. A second delay, $D_2$, was incorporated between successive pulse trains to allow for complete relaxation of all absorbing nuclei in the sample. One hundred scans were collected for each delay time ($D_1$). The spectra were phased, scaled and plotted as described in the legend to Figure 22. The spectra are displayed $180^\circ$ out of phase in order of increasing delay time ($D_1$).

$D_1$ values: 200.0 µsec, 300.0 µsec, 400.0 µsec, 500.0 µsec, 600.0 µsec, 700.0 µsec, 800.0 µsec, 900.0 µsec, 1.00 msec, 1.50 msec, 2.0 msec, 2.50 msec, 3.0 msec, 4.0 msec, 5.0 msec, 7.50 msec, 10.0 msec, 15.0 msec, 20.0 msec, 40.0 msec. $D_2$: 10.0 sec.
TABLE 9

Relaxation Rates of the Protons in Heneicosi(N-Acetyl)Undecagold\textsuperscript{a}

\begin{align*}
\begin{array}{cccccc}
\text{Nucleus} & T_1 (\text{sec}) & 1/T_1 (\text{sec}^{-1}) & T_2 (\text{sec}) & 1/T_2 (\text{sec}^{-1}) \\
\hline
a & 0.895 & 1.12 & 0.0059 & 169 \\
b & 1.20 & 0.835 & 0.0083 & 120 \\
c & 0.377 & 2.65 & 0.0096 & 104 \\
d & 0.612 & 1.63 & 0.057 & 17.5 \\
\end{array}
\end{align*}

\textsuperscript{a}The spectra were obtained as described in the legend to Figures 22 and 23. The relaxation times for each proton were determined, assuming Lorenzian line shape, by fitting the data to the equations below.

\begin{align*}
T_1 & \quad y = A[1-(1+w(1-e^{-K/T}))e^{-X/T}] \\
T_2 & \quad y = Ae^{-X/T}
\end{align*}

where, \( A \) = amplitude at \( x \gg T \)
\( T = T_1 \) relaxation time (sec)
\( K = D_2 \) in the pulse sequence
\( X = D_1 \) in the pulse sequence
\( W = -(\text{amplitude at } X=0)/A \)
\( \text{where } A = y\text{-intercept at } X=0 \)
\( T = T_2 \) relaxation time (sec)
\( X = 4(\text{echo}+1)D_1 \)
Figure 24. Time Course for Succinylation of Undecagold Cluster

Fifty µl aliquots were removed from the succinylation reaction mixture as described in section 3.2.8. To these samples, 1.0 ml of the ninhydrin assay solution was added. The samples were heated at 100°C for 10 min, cooled for 15 min and the absorbances measured at 570 nm as described previously.
stored at -70°C until further use. A sample of heneicosi-
(N-succinyl)undecagold (165 A_{415} units, 5.59 μmol) was
purged with nitrogen gas and titrated as described in
section 2.2.3 (Figure 25). A sample was also used for
analysis by proton NMR spectroscopy. These data are
shown in Table 6.
A sample of heneicosi(N-succinyl)undecagold (165 \text{ A}_{415} \text{ units}, 5.59 \mu\text{mol}) was dissolved in 10.0 ml of water and the pH adjusted to 5.0. The sample was purged with nitrogen and prepared for titration as described in section 2.2.3. The sample was titrated with 0.242 M HCl.
FIGURE 25

HCl (mL) vs. pH

HCl (mL)

pH
3.3 Results and Discussion

The procedure described for synthesis of tricyanoheptakis[4,4',4''-phosphinidynetri(benzenemethanamine)]-undecagold was adapted from the published procedure of Bartlett et al. (73). The percentage yield of this reaction was sensitive to reaction size. The most critical factor in determining the yield appears to be the effectiveness of dissolution of the gold cyanide to form 4,4',4''-phosphinidynetri(benzenemethanamine) gold (I) cyanide. Gold cyanide exists as a linear heteropolymer and is insoluble in 95% ethanol, the solvent used for the reaction. The phosphine slowly complexes gold cyanide, dissolving the precipitate. Reduction of the gold cyanide by prolonged exposure to light can result in a coating of elemental gold on the gold cyanide precipitate. This appears to greatly reduce the effectiveness of phosphine promoted dissolution of the precipitate and results in significantly lower yields. For this reason, freshly precipitated gold cyanide, stored in light-proof containers, was used exclusively.

Excess hydride in the reaction mixture was originally quenched with acetone in accordance with the procedure of Bartlett et al. (73). This has subsequently been found to cause reductive alkylation of the amino groups to form
trace amounts of N-isopropylamines. This contamination can potentially cause problems in the later chemical reactions. Excess hydride has subsequently been quenched by adjusting the pH to 5.0 before rotary evaporation in vacuo.

The NMR spectral data presented in Table 4 are consistent with the published structure of the undecagold complex. The phosphorus-31 chemical shift obtained from the proton-decoupled spectrum is 59.44 ppm downfield from the starting phosphine. This deshielding of the phosphorus nucleus suggests that a great deal of the electron density on the phosphine has been donated in formation of the gold-phosphine bond. The proton and carbon-13 NMR spectra are consistent with the structure of the phosphine ligand.

Perhaps the most striking feature of all three NMR spectra is the broadness of the peaks. The peak broadness appears to decrease as the distance between the core and the absorbing nuclei increases. That the relaxation rate (T_2) for all absorbing protons in the gold complex is greater than that for the phosphine free in solution was demonstrated by measuring the relaxation rates for the protons in a sample of heneicosi(N-acetyl)undecagold. These experiments are
discussed below. From the X-ray crystal data on a related cluster discussed earlier, small differences in the $^{31}$P NMR chemical shifts for the different classes of phosphine ligands might be predicted. If there are differences in the $^{31}$P NMR chemical shifts among the ligands, they are obscured by the increased relaxation rates for these nuclei. The weak quadrapole of the gold nucleus may also contribute to the breadth of the phosphorus-31 NMR signal.

The determination of the extinction coefficient of the undecagold cluster by the weight method was subject to considerably less volumetric error than the titration method. The values of $9.93 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and $2.95 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for the extinction coefficient of the 305 nm and 415 nm bands, respectively, are in close agreement with the values of $1.05 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ and $3.16 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for tricyanoheptakis[tris(4-methylphenyl)phosphine]undecagold (73). The values obtained from titration of the undecagold cluster ($\varepsilon_{305} = 9.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, $\varepsilon_{415} = 2.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) are also in good agreement.

The average $pK_a$ of the undecagold cluster was determined from the titration curve in Figure 5 to be 8.95. The breadth of the titration curve is a predictable consequence of the inherent statistical distribution
of $pK_a$ values in a compound containing twenty-one chemically identical ionizable functional groups. In fact, a distribution over 2.64 pH units would be predicted for a molecule containing twenty-one equivalent, non-interacting groups. The additional spread of the $pK_a$ range in the undecagold cluster indicates some degree of interaction between the amino groups. Largely because of the statistical phenomenon mentioned above, the undecagold cluster contains amino groups that are unprotonated at pH values below 8.0. This makes the undecagold cluster a very nucleophilic molecule at neutral pH.

The chromatographic properties of the undecagold cluster on Sephadex ion-exchange resins are highly dependent upon the elution system employed. Using SP Sephadex C-25, in the triethylammonium form, a linear TEAB gradient increasing from 0.10 M to 0.30 M resulted in elution of the cluster at a salt concentration of 0.18 M. When the undecagold cluster was chromatographed on SP Sephadex C-25 in the sodium form, using a linear NaCl gradient increasing from 1.00 M 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 the undecagold cluster has a charge of about +20. This high positive charge is reflected in the NaCl gradient elution profile. Elution of the undecagold cluster from SP Sephadex, using the TEAS gradient, at a salt concentration of 0.18 M indicates a net charge of +3 to +5 for the cluster under these conditions. This represents approximately 45% conversion of the primary amines on the undecagold cluster to the carbamate form.

Application of the undecagold cluster to QAE Sephadex Q-25 in the bicarbonate form resulted in 100% retention of the material by the column. The cluster was eluted from this column at a TEAB concentration of 0.50 M, reflecting a net charge of -5 to -7. This represents a 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 bond. Due to this increased stability, the equilibrium ratio of carbamates to primary amines increases and the undecagold cluster attains a net negative charge.

A further comparison of the chromatographic behavior of the cluster on these ion-exchange resins was made at pH 11.5 using a linear Na₂CO₃ gradient
increasing from 0.10 M to 1.00 M. Elution of the cluster from QAE Sephadex at 0.32 M Na₂CO₃ indicates that the equilibrium ratio of carbamate to free amine is greater at pH 7.8 than pH 11.5.

The behavior of the undecagold cluster on gel filtration resins was also dependent upon the elution buffer employed. In order to determine the best conditions for purification of the undecagold cluster by gel filtration chromatography, samples were applied to both Sephadex G-25 and BioGel P-6. These columns were eluted with 0.10 M TEAB, 0.10 M NaCl or 0.10 M NH₄Cl. From the elution profiles in Figures 1 and 6 the behavior of the cluster on these columns appears similar. The physical appearance of the columns after the chromatography is, however, considerably different. Non-specific interactions between the gold complex and the resin beads leads to retention of the cluster. This problem is greatly reduced when TEAB is used as the elution buffer. It was necessary, however, to avoid exposure of the cluster to carbon dioxide-containing buffers due to the problems caused by the presence of carbamates in subsequent chemical reactions. The crosslinked polyacrylamide resin appeared to interact more strongly with the cluster than the polydextran
resin. For these reasons, Sephadex gel filtration resins were used in this work.

The routine desalting of small amounts of gold complex derivatives has been achieved using Sephadex G-10. A 10% ethanol-water solution was used to elute these columns. Non-specific interactions between the cluster and BioGel P-2 were significant under these conditions. These interactions are reflected in the elution profile shown in Figure 7.

The procedure used for the reductive methylation of the undecagold cluster was adapted from a general method for the reductive methylation of amino groups in proteins (85). Sodium borate is generally used to buffer the pH during the reaction. Due to the limited solubility of benzylamine at pH 9, the model studies were performed using a mixed solvent system. 20% dioxane-water yielded a homogeneous solution suitable for use in the model studies. From the reaction profile in Figure 11, it appeared that the conditions employed were adequate to ensure complete reductive methylation of the primary amino groups present. In order to determine whether these conditions resulted in the quantitative formation of N,N-dimethylbenzylamine, the extent of reaction was measured by proton NMR spectroscopy. From the data presented in Figure 12, it was
concluded that the conditions employed were more than sufficient to convert benzylamine quantitatively to N,N-dimethylbenzylamine.

The reaction conditions developed from the model studies with benzylamine were employed in the reductive methylation of the undecagold cluster. The undecagold cluster is soluble in buffered aqueous solution, therefore, a mixed solvent system was not required. It was also decided that a buffer was not required to maintain the pH. The pH was maintained near 9 by titration of the borate produced in the reaction with 2.0 N HCl. Due to the buffering capacity of the undecagold cluster and the borate produced in the reaction, maintenance of pH presented no problem. From the reaction profile in Figure 13, it appeared that the conditions employed were sufficient to convert the undecagold cluster to heneicosi(N,N-dimethyl)undecagold. This was confirmed by proton NMR analysis of the product. A sample was also prepared for phosphorus-31 NMR spectroscopy. The data from these spectra are shown in Table 6.

The undecagold cluster was synthesized by sodium borohydride reduction of the 4,4',4"-phosphinidynetri-(benzenemethanamine) gold (I) cyanide complex. For this reason, there was concern that the further exposure
of the undecagold cluster to sodium borohydride might prove too harsh a treatment. This, however, does not appear to be the case. The phosphorus-31 chemical shift for heneicosi(N,N-dimethyl)undecagold of 52.5 ppm is only 0.8 ppm from the chemical shift for the native cluster. Further, the UV-visible spectrum of the cluster is virtually unchanged by the reductive methylation reaction.

The procedure for carboxymethylating the undecagold cluster was designed to allow for the preparation of mono-functional undecagold derivatives with any net charge between +20 and -20, at neutral pH. The reaction can be accurately monitored by titration of the protons produced. From the data in Table 7, it was apparent that the sample of heneicosi(N-carboxymethyl)undecagold prepared contained a distribution of species with different net charges at neutral pH. This was confirmed by ion-exchange chromatographic analysis of the product mixture. As can be seen from the elution profiles in Figures 16-19, the product mixture is predominantly composed of species with net charges between +4 and -4 at pH 5.5. The material not retained by these columns represents carboxymethylated undecagold cluster with a net charge of zero at pH 5.5. The percentage of each species present (Table 8) is
qualitatively consistent with the predicted distribution based on the titration of protons produced in the reaction. Due to the less effective separation of the cationic forms of the cluster on QAE Sephadex Q-25, the pooled fractions from these columns were cross-contaminated with species of lower net charge. For this reason, the calculated percentages of the +2, +3 and +4 species are greater than the percentages actually present. From these data, it was concluded that treatment of the undecagold cluster with twenty-one equivalents of bromoacetate results in the production of a distribution of carboxymethylated undecagold species with an average net charge of zero at pH 5.5.

The difference in chromatographic behavior of the carboxymethylated undecagold cluster on SP Sephadex and QAE Sephadex has consistently been observed with the various positively and negatively charged derivatives of the cluster that have been examined. The interaction of the quaternary ammonium groups on QAE Sephadex with carboxylate anions on the gold complex does not lead to as effective a separation of the negatively charged gold complex species as is observed for positively charged gold complex derivatives chromatographed on SP Sephadex.
The undecagold cluster amino groups were rapidly and quantitatively acetylated by acetic anhydride. Due to the anticipated rapid hydrolysis of acetic anhydride, the reagent was added in aliquots corresponding to a five-fold excess over the amino groups present in the reaction mixture. As shown by analytical data, the first five-fold excess of acetic anhydride appears to be sufficient to acetylate all of the amino groups in the cluster. The solubility of the acetylated undecagold cluster decreased as the ionic strength of the reaction mixture increased. Less excessive treatment with acetic anhydride greatly reduced this problem. The heneicosi(N-acetyl)undecagold produced contained no detectable ionizable functional groups between pH 1 and 13. The amino groups in the cluster were also rapidly acetylated by N-hydroxysuccinimidoacetate. A ten-fold molar excess of this reagent led to complete acetylation of the cluster in five to ten minutes as determined by ninhydrin assay of the reaction mixture.

The 270 MHz proton NMR spectrum of heneicosi-(N-acetyl)undecagold (Figure 21) displays the characteristic broad absorption signals seen with all gold complex derivatives. The breadth of these signals appears to decrease as the distance between the core and the absorbing nuclei increases. From the data in
Table 9 it appears that this peak broadening is caused by spin-spin relaxation of the nuclei. The spin-lattice relaxation times are not markedly different from the values expected for the phosphine free in solution. The large difference in the values of $T_1$ and $T_2$ for the aromatic protons can most easily be interpreted as an indication of a relatively large value for the correlation time $\tau_c$. This result is consistent with the structure of the gold complex. The steric bulk of the phosphine ligands in the gold complex reduces the mobility of the aromatic rings. Zero frequency (static) local magnetic fields within the cluster result in a dephasing of the NMR signal in the XY plane, thereby causing relaxation of the nuclei. Further, the relatively large size of the cluster and, hence, slowed tumbling of the molecule in solution result in a large value for the correlation time $\tau_c$. This reduces the contribution of spin-lattice relaxation to the observed relaxation. The anisotropic rotation of the acetyl methyl group about its symmetry axis causes an increase of the relaxation time for these protons. Further work in this area will involve measurement of the relaxation times at other magnetic field strengths. This will allow the determination of
the correlation time ($\tau_c$) for the molecule. In addition, measurement of $\tau_c$ from the ratio of $T_1/T_2$ at several frequencies and from the ratio of $T_2^{-1}$ at different frequencies should provide information concerning the relative rates of the anisotropic methyl group rotation and the isotropic tumbling of the entire molecule.

It has been suggested by Bellon et al. (75) that the central gold atom in the gold complex core is paramagnetic. Careful measurement of the $T_1$ relaxation times for the protons in the gold complex at several frequencies will provide useful data concerning this possibility. The spin-lattice relaxation time is proportional to $1/r^6$ where $r$ is the distance of the nuclei from the paramagnetic atom. The aromatic protons are 6-8 Å from the central gold atom, hence the effect of this central gold, if paramagnetic, will not be large. Measurement of the $^{31}P$ relaxation rates will probably be necessary to detect the paramagnetism, if present.

The final method chosen to modify the amino groups in the undecagold cluster was acylation with succinic anhydride. The product was insoluble in water below pH 4. The titration curve for heneicosi(N-succinyl)-undecagold also displays the broad $pK_a$ range seen in the previous titration data.
All of the methods for derivatization of the amino groups in the undecagold cluster discussed above appear to satisfy the necessary requirements for use in the preparation of mono-functional derivatives. The presence of the single unique functional group in the mono-functional undecagold derivative introduces problems not observed in these model studies. In the preparation of mono-functional undecagold derivatives, it has been found that there are advantages and disadvantages associated with each of the methods outlined above. The modifications of the above procedures for application to the synthesis of specific mono-functional derivatives are discussed in the next chapter.
CHAPTER IV
SYNTHESIS OF MONO-FUNCTIONAL ALKYLATING AND ACYLATING DERIVATIVES OF UNDECAGOLD CLUSTER

4.1 Introduction

The undecagold cluster is a promising molecule for use as an electron-dense reagent to label specific components of a complex biological aggregate. The work of Safer et al. (76) demonstrates the potential value of the undecagold cluster for this purpose. The methodology used in that work is, however, not applicable to a wide variety of biological systems. The successful application of the undecagold cluster as an electron-dense labeling reagent requires the development of mono-functional derivatives. The general use of multi-functional derivatives of the undecagold cluster for labeling specific biological sites suffers several disadvantages. The work of Safer et al. illustrates this fact. The multi-biotinylated undecagold cluster used in their work was very effective at cross-linking tetramers of avidin to produce polymers. Although desired in the study above, aggregation of the specimen under study upon addition of the undecagold cluster
derivative is not envisioned as a generally useful phenomenon. The undecagold cluster will have further use as a cross-linking agent; however, most applications will require the methodology for specific labeling of a single biological site of interest. Stoichiometric studies also require that the undecagold cluster contain a single reactive functional group or biological ligand.

In order to make the methodology for preparation of mono-functional undecagold derivatives of the widest possible application, a protocol was envisioned in which the undecagold cluster would first be derivatized with a reagent containing a unique functional group and the mono-functional derivative would be isolated. The remaining amino groups would then be blocked using one of the procedures outlined in the previous chapter, to prevent their interference during subsequent chemical manipulations. The unique functional group introduced could then be used to couple a wide variety of biological ligands or amino acid selective alkylating or acylating reagents to the cluster. In addition, this type of procedure would allow for the introduction of a variable length spacer arm between the cluster and attached ligand.

The successful synthesis of a mono-functional undecagold cluster capable of being coupled to a wide
range of molecules depends on the proper choice of reagents for formation of the mono-functional derivative. Any reagent chosen should be capable of reacting with amino groups in a specific manner. After reaction with the gold complex, this reagent must then provide a means for specific linkage of the cluster to a variety of biological ligands and alkylating or acylating reagents.

The undecagold cluster contains twenty-one functional groups of similar reactivity. This presents a statistical problem in the synthesis of a mono-functional undecagold derivative. For example, if the undecagold cluster were treated with several molar equivalents of acetic anhydride, a series of undecagold derivatives would be produced containing variable numbers of acetyl groups. The distribution of species in the product mixture and the relative amounts of each species present would depend solely on the amount of acetic anhydride added. Because of this, in order to prepare undecagold derivatives with a single unique functional group, a procedure is required to separate the derived clusters based on the number of these functional groups present. This separation could be performed at any stage of the synthesis; however, to make the methodology of the
widest possible application, separation of the initial mixture would be advantageous.

In any procedure for preparing mono-functional undecagold derivatives, the most difficult problem is the separation of the product mixture. The approach most likely to be successful is the separation of the product mixture by ion-exchange chromatography. In the example of acetic anhydride above, at neutral pH, the unreacted undecagold cluster would have a charge of +21. Mono-acetyl undecagold would have a charge of +20, di-acetyl undecagold would have a charge of +19, and so on. Provided the reagent used to modify the undecagold cluster results in a change in the net charge of the derivative produced, the product mixture can in principle be separated by ion-exchange chromatography. The percentage difference in charge of undecagold and mono-acetyl undecagold is about 5%. As will be discussed shortly, this difference is not sufficient to allow for purification of the mono-derivatized undecagold cluster by conventional cation exchange chromatography at neutral pH. One method for increasing the percentage difference in charge between the derivatized clusters is by raising the pH. At pH 9.0 approximately half of the amino groups are deprotonated.
The percentage difference in charge between undecagold cluster and mono-acetyl undecagold at pH 9.0 is approximately 10%. As the pH is raised to 10-11, this percentage difference in charge becomes large enough to allow for separation by cation exchange chromatography; however, the dynamic nature of protonic equilibria would make a clean separation virtually impossible.

Another method for increasing the difference in charge between the derivatized clusters is the use of a reagent that not only neutralizes the amino group positive charge but also introduces a negative charge. The increment in net charge between the derivatized clusters after treatment with a reagent of this type would be two instead of one. This would double the percentage difference in charge of the derivatized clusters at a given pH. Cyclic anhydrides are a good example of this type of reagent. For example, after treatment with succinic anhydride, the net charge of unreacted undecagold cluster would be +21 and mono-succinyl undecagold would have a charge of +19, at neutral pH. Although doubling the percentage difference in charge does not greatly improve the chances for separation by cation exchange chromatography, the negative charge provides a second potential method for
separation. At high pH's, all of the amino groups in the cluster are deprotonated. Under these conditions, the charge of unreacted undecagold cluster is zero, the mono-succinyl undecagold has a charge of -1, di-succinyl undecagold has a charge of -2, and so forth. These species should be separable by anion-exchange chromatography. As will be shown below, this appears to be the best method for preparing mono-derivatized undecagold cluster in pure form. In addition, the carboxyl group generated by the reaction is a convenient functional group to be used for coupling molecules of interest to the cluster.

Any molecule containing an alcohol or amino functional group could potentially be coupled to mono-succinyl undecagold through an ester or amide linkage with the lone carboxyl group. Finally, the succinyl group provides an arm of about 4 Å when fully extended. If a longer arm were necessary, extensions such as glycine, glycyl-glycine, etc. could be coupled to the succinyl carboxyl group, providing for extension of the arm while maintaining a carboxyl group at the terminal position. The rigidity in these molecules due to the resonance delocalization of electron density in the amide bonds could also be useful in maintaining the arm in an extended conformation.
An alkyl diamine such as 1,3-diaminopropane or 1,4-diamino butane could also be coupled to the succinyl group. Not only would this provide a longer arm, it would also change the terminal functional group to an amino group. This would allow for the coupling of molecules containing a carboxyl group to form an amide bond.

From the above discussion, it can be seen that succinic anhydride possesses all of the characteristics required for use in preparing mono-functional derivatives of the undecagold cluster. The procedures used to prepare mono-succinyl undecagold and related derivatives are discussed in sections 4.2.1-4.2.4 (Scheme 5).

When the undecagold cluster is visualized in the electron microscope, the core of eleven gold atoms causes most of the electron scattering. The amino groups on the periphery of the cluster are 8 Å from the core. Because of this, if the gold complex were coupled directly to a macromolecule through one of the amino groups, it would appear to be separated from the macromolecule by at least 8 Å when visualized in the proper orientation in an electron microscope. Addition of the succinyl group increases the apparent arm length to about 12 Å. For some applications this apparent separation of the electron-dense label from the site of
SCHEME 5

**= Au
X+CN

mono-, di-, tri-, tetra-, ...succinylation
undecagold cluster

chromatography

\[
\text{NH}_2
\]

\[
\text{NHAC}
\]

\[
\text{H}_2\text{CO/NaBH}_4
\]

\[
\text{N(CH}_3\text{)}_2
\]

\[
\text{DCC, N-OH/DMF}
\]

\[
\text{NHAc}
\]

\[
\text{N(CH}_3\text{)}_2
\]
attachment to the biological system may be too large to provide the desired information. An example of this potential problem is the $\alpha$-keto acid dehydrogenase lipoic acid groups. In this case, not only is there a separation of about 12 Å between the gold complex core and the succinyl carboxyl group, but the lipoyl group is extended up to 14 Å from its site of attachment to the polypeptide backbone. After addition of a sulfhydryl reactive group to the mono-succinyl undecagold carboxyl group to allow for alkylation of the lipoic acid moieties, the distance between the undecagold core and the lysine $\alpha$-carbon in the polypeptide backbone is up to 30-35 Å. This distance is too large to allow for accurate mapping of the positions of the lipoyl groups in these multienzyme complexes.

For studies such as these, a mono-functional undecagold cluster was needed with a minimum separation of the undecagold core from the sulfhydryl reactive group. Ideally, a procedure for direct coupling of the alkylating reagent to one of the undecagold cluster amino groups was desired. Once again, the major problem is the separation of the mono-derivatized form of the undecagold cluster from the other forms present in the mixture. One method for achieving this separation, in
general, is by the direct coupling of a reagent to the undecagold complex amino groups which not only possesses the desired reactivity or binding specificity but also has a negative charge. This methodology suffers several disadvantages. First the criteria for separation of the product mixture limit the potential range of molecules which can be coupled to the cluster. The method for separation of the mono-derivatized cluster from the product mixture would also depend on the stability of the unique functional groups introduced.

A second approach for preparing mono-functional derivatives with the molecule of interest coupled directly to an amino group is as follows. The cluster is first treated with an amino group protecting reagent. The remaining amino groups in the product mixture are then blocked by one of the procedures discussed in the preceding chapter. Finally, the amino protecting groups are removed. This procedure yields a mixture of species containing zero, one or more primary amino groups. The problem of separating the mono-primary amino derivative from the product mixture can potentially be solved in several ways. The exact method used will of course depend on the nature of the reagent(s) used to block the remaining amino groups in the cluster.
Although this procedure is of general application, it suffers one serious disadvantage. In the preparation of mono-succinyl derivatives of the undecagold cluster, separation of the mono-succinyl derivative is achieved prior to derivatization of the remaining amino groups. Undecagold cluster that did not react with succinic anhydride can therefore be isolated and recycled in future succinylation reactions. In the general procedure described above, the remaining amino groups in the product mixture are blocked prior to isolating the mono-functional derivative. Undecagold cluster that did not react with the amino group protecting reagent is completely derivatized with the amino group blocking reagent(s) and is not longer usable for further reactions. For this reason, the yield of mono-protected undecagold cluster must be optimized to make the procedure practically useful.

Trifluoroacetic anhydride is a convenient amino group blocking reagent that can be introduced and removed under mild conditions. Due to the rapid hydrolysis of the anhydride in aqueous solution, a quantitative trifluoroacetylation reaction is most easily performed in non-hydroxylic solvents. The trifluoroacetyl protecting group can be removed by any
of several methods. Trifluoroacetylated pancreatic ribonuclease is reported to be deacylated in 1.0 M piperidine at 0°C (88). The deacylation occurs more slowly in carbonate buffer, pH 10.7 (89). Direct hydrolysis of the amide by hydroxide ion can also be used to quantitatively deacylate more stable amines.

Because of the high reactivity of trifluoroacetic anhydride, its use to acetylate the undecagold cluster presents one problem. As was discussed earlier, the use of trifluoroacetyl protecting group in the synthesis of mono-amino undecagold derivatives requires that the remaining amino groups in the cluster be derivatized prior to separating mono-, di-, tri-...functional undecagold derivatives. Because of this, any undecagold cluster not trifluoroacetylated becomes totally derivatized by the reductive methylation or acetylation procedure. This material cannot be recycled for future reactions as was done with the unsuccinylated undecagold cluster. Because of this problem, it is critically important to maximize the amount of mono-trifluoroacetyl undecagold and minimize the amount of unreacted undecagold cluster in the trifluoroacetylation reaction.

Since no simple method was available for routinely monitoring the reaction to determine the degree of
trifluoroacetylation, a standard procedure was required for reproducibly preparing anhydrous undecagold cluster. In an anhydrous sample, the amount and distribution of trifluoroacetylated species produced in the reaction should be directly related to the amount of trifluoroacetic anhydride added. Trace amounts of water in the sample could greatly affect this relationship.

The gold complex can be treated with a few equivalents of trifluoroacetic anhydride, the remaining amino groups reductively methylated with formaldehyde and sodium borohydride and the trifluoroacetyl protecting groups removed. This mixture can then be exposed to a solution containing carbon dioxide, which should convert the primary amino groups to carbamates as discussed in Chapter III. At pH 11.5 the mixture of species should be separated by anion-exchange chromatography on the basis of the number of carbamate groups present. As will be shown below, this method is not capable of separating mono-primary amino eicosi(N,N-dimethyl)undecagold in pure form from the mixture.

A related procedure for preparing mono-amino eicosi(N-acetyl)undecagold is as follows. As before, the gold complex is first treated with trifluoroacetic anhydride. The remaining amino groups are acetylated
as described in Chapter III and the trifluoroacetyl protecting groups are removed. Undecagold cluster that did not react with trifluoroacetic anhydride is completely acetylated and has no charge. Eicosi(N-acetyl)undecagold has a charge of +1, nonadecyl(N-acetyl)undecagold has a charge of +2, and so on. This mixture can be separated by cation-exchange chromatography at neutral pH. These procedures discussed here lack the general application to include blocking the remaining amino groups by any of the procedures discussed in Chapter III.

The procedure of most general application which allows for blocking the remaining amino groups by any of the methods outlined in Chapter III relies on the use of phthalic anhydride as the amino protecting group (Scheme 6). After treatment of the gold complex with phthalic anhydride, the mixture can be separated by anion exchange chromatography as was done with the succinyl undecagold mixture. The remaining amino groups in the purified mono-phthalyl undecagold can then be derivatized by any of the methods in Chapter III. Finally the phthalyl blocking group is removed. Molecules of interest may then be coupled directly to the single remaining primary amino group.
SCHEME 6

mono-, di-, tri-, tetra-... phthalylated undecagold cluster

chromatography

\[ \text{AcOH} / \text{NaBH}_4 \]

\[ \text{pH 3.1, 48°C} \]
The mechanism of hydrolysis of phthalic acid monoesters and amides has been well characterized. The hydrolysis of these compounds occurs with anchimeric assistance from the neighboring carboxyl group. The initial product formed in the hydrolysis reaction is phthalic anhydride. This slow intramolecular cyclization reaction is followed by the rapid hydrolysis of the anhydride to form phthalic acid. The exact mechanism of the intramolecular anhydride bond formation step depends on the nature of the leaving group. For leaving groups with a $pK_a > 11$, the reaction rate increases with increasing pH up to about one pH unit above the $pK_a$ of the carboxyl group. Above this pH the reaction rate is constant (90,91). For leaving groups with a $pK_a < 11$, the relationship between reaction rate and pH is the opposite (92). The rate increases with decreasing pH to about 3.0. Below this pH the rate remains constant. At extremes of pH the direct hydrolysis by hydroxide ion or the specific acid catalyzed hydrolysis by hydrogen ion become the predominant mechanism. In the case of leaving groups with high $pK_a$'s, the neighboring carboxylate anion participates directly in nucleophilic attack at the amide carbonyl carbon. With leaving groups having lower $pK_a$'s, the protonated
carboxyl group participates both as a general acid catalyst and as the nucleophile in the displacement reaction. This is the predominant mechanism for hydrolysis of mono-phthalyl undecagold derivatives.

Mono(N-succinyl)eicosi(N-acetyl)undecagold and mono(N-succinyl)eicosi(N,N-dimethyl)undecagold are potentially capable of being covalently attached to either a hydroxyl group or an amino group to form an ester or amide bond, respectively. Amino acid alkylating reagents, affinity labels and biological ligands containing either a hydroxyl or amino group should therefore be capable of being attached to the gold complex via the succinyl carboxyl group to produce monofunctional derivatives of the undecagold cluster for a variety of biological applications. In addition, preparation of a derivative of the mono-succinyl clusters in which the succinyl carboxyl group has been "activated" toward nucleophilic attack would allow for the use of these clusters directly as amino acid acylating reagents.

Historically, a variety of methods have been employed in the formation of ester and amide bonds. Dialkylcarbodiimides have proven to be mild condensing agents for use in formation of both ester and amide bonds.
The mechanism for the carbodiimide coupling reaction is shown in Scheme 7 (93). Due to competition between alcohols and water for the O-acyl urea intermediate, ester bond formation must be done in a non-hydroxylic solvent. The \( pK_a \) values for dialkylocarbodiimides are around 5.0. For this reason, the pH of an aqueous reaction mixture is generally buffered between pH 4 and 6. The presence of the dimethylamino propyl group in the reagent ethyl dimethylamino propyl carbodiimide (EDC) results in the rearrangement shown in Scheme 8. The equilibrium distribution at pH 7 is 93% in the ring form (94). The \( pK_a \) for the ring closed form is 3.1 (95). For this reason, the effective use of EDC as a condensing agent requires a reaction pH of 4-5. At this pH, most typical alkyl amines are completely protonated, so that a large excess of an amine is needed to provide a reasonable rate for the second half reaction. With amines of lower \( pK_a \) such as aniline or glycine ethyl ester, this problem is significantly reduced.

The limitations of the carbodiimide coupling procedure discussed above make difficult the development of a general procedure for coupling a wide range of amino groups to the succinyl carboxyl group.

Another mild method for forming amide bonds in aqueous and non-aqueous solutions is via the
SCHEME 7. MECHANISM OF CARBODIIMIDE CONDENSATION REACTION

\[ R_1N=C=NR_2 \overset{H^+}{\leftrightarrow} R_1N=\overset{+}{C}NHR_2 \]

\[ R_1NH-C-NHR_2 \overset{O}{\leftrightarrow} R_1N=C-NHR_2 \]

\[ R_4NHCR_3 \]

SCHEME 8. TAUTOMERIZATION OF EDC

\[ CH_3CH_2N=C-N \overset{(CH_3)_2-N^+}{\leftrightarrow} CH_3CH_2NH \overset{(CH_3)_2-N^+}{\leftrightarrow} \]

\[ \overset{(CH_3)_2-N^+}{\rightarrow} \]

\[ \overset{(CH_3)_2-N^+}{\rightarrow} \]
N-hydroxysuccinimide ester (96,97). N-hydroxysuccinimide esters can be formed in high yield from a carboxylic acid and N-hydroxysuccinimide by the use of dicyclohexylcarbodiimide in aprotic solvents. This ester can be used for formation of amide bonds by reaction with amines in either protic or aprotic solvents. In aqueous solution, at pH 7, N-hydroxysuccinimide esters are quite selective for amino groups. Formation of the N-hydroxysuccinimide ester of the succinyl group should result in the preparation of a mono-functional undecagold derivative capable of being coupled to a wide variety of amino groups. In addition, this derivative of the cluster is suitable for direct use as an amino group acylating reagent to label amino groups in biological preparations.

Eicosi(N-acetyl)undecagold, eicosi(N,N-dimethyl)-undecagold and eicosi(N-carboxymethyl,N-methyl)-undecagold are derivatives suitable for coupling the cluster to molecules of interest which contain a free carboxyl group. Once again, the reagent most likely to be of general use for this coupling reaction is the N-hydroxysuccinimide ester. Addition of the N-hydroxysuccinimide ester of a molecule of interest containing a carboxyl group to a mono-primary amino cluster should
provide a convenient one-step method for formation of the amide bond.

The biological systems of immediate interest in this laboratory can be most easily studied using monofunctional derivatives of the undecagold cluster that contain a sulfhydryl selective alkylating reagent. For this reason our studies have been directed in this area.

The most common sulfhydryl selective alkylating reagents for protein modification studies are the \( \alpha \)-haloacetyl and N-aryl or N-alkyl maleimide groups. These reagents have been widely used in the analysis of protein structure and function. A large body of information is available concerning the reaction of these reagents with specific sulfhydryl, amino, imidazole and phenolic groups in biological systems for which the gold complex may be of use in further characterization of structure and/or structure-function relationships.
4.2 Experimental Procedures

4.2.1 Synthesis of Mono(N-Succinyl)Undecagold

Undecagold (564 A415 units, 19.1 µmol) was dissolved in 5.0 ml H2O. The pH was 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 the undecagold complex in two 50 µl aliquots. After 10 min, 20 ml H2O was added and the pH of the solution was adjusted to 5.0. The solution was purged with nitrogen gas for 60 min at 60°C. After cooling to room temperature, the pH was adjusted to 11.5. The volume was then adjusted to 125 ml with freshly boiled H2O at pH 11.5. The sample was then chromatographed at pH 11.5 through a column of QAE Sephadex Q-25, in the chloride form, eluted with a linear NaCl gradient as described in the legend to Figure 26. The fractions containing mono-succinyl undecagold were pooled and adjusted to pH 7.0. The combined fractions were concentrated to about 15 ml by ultrafiltration. The concentrate was further concentrated to approximately 5 ml by rotary evaporation in vacuo. This sample was then desalted by passage through a 1.5 x 45 cm column of Sephadex G-10 (10% ethanol-water). The eluted material was concentrated
The succinylation reaction mixture described in section 4.2.1 was purged with nitrogen gas and prepared for chromatography as described in the text. The sample was applied to a 1.5 x 28 cm column of QAE Sephadex Q-25 that had been prewashed with 3.2 mM NaOH. The sample was eluted with a 800 ml linear sodium chloride gradient increasing from 0.00 M to 0.40 M at pH 11.5. Fractions of 4.5 ml were collected at a flow rate of 1.5 ml per minute and the absorbances measured at 415 nm. Fractions eluting at 0.05 M (fractions 16-32, 47.5 A₄₁₅ units), 0.15 M (fractions 61-74, 34.2 A₄₁₅ units), 0.21 M (fractions 88-100, 23.2 A₄₁₅ units) and at 0.24 M (fractions 106-116, 15.9 A₄₁₅ units) were pooled separately. The samples were adjusted to pH 7.5, concentrated by ultrafiltration, desalted by passage through Sephadex G-10 (10% ethanol-water) and stored at -70°C.
to dryness by rotary evaporation in vacuo and redissolved in 20 ml H₂O. The pH was adjusted to 5.0 and the solution was again purged with nitrogen for 60 min at 60°C. The sample was diluted with 30 ml of freshly boiled H₂O, pH 11.5, and the pH was adjusted to 11.5 with 1.0 N NaOH. The solution was then applied to QAE Sephadex Q-25, chloride form (1.5 x 5 cm). After the sample was adsorbed, the column was washed with 2-3 volumes of water, pH 11.5. The mono-succinyl undecagold was then eluted isocratically with 0.12 M NaCl, pH 11.5. The eluted mono-succinyl undecagold 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 x 45 cm). The eluted mono-succinyl undecagold was concentrated to dryness by rotary evaporation in vacuo, diluted with 10 ml of water, and the pH adjusted to 7.5. This material was stored at -70°C until further use (yield 5-10%).

4.2.2 Characterization of the Succinylation Reaction

4.2.2.1 Optimization of the Mono-succinyl Derivative

Three identical succinylation reaction mixtures were
prepared. Undecagold cluster (75 A_{415} units, 2.54 µmol), in 1.0 ml of water, was placed in 5.0 ml flasks. Succinic anhydride (25 µl, 50 µl and 100 µl of a 0.10 M solution in acetonitrile) was added to the reaction vessels. The mixtures were stirred for 60 min at room temperature. The samples were then diluted with 25 ml of water and the pH was adjusted to 11.5 with 2.0 N NaOH. The samples were applied to columns of QAE Sephadex Q-25 and eluted with a linear salt gradient (Figure 27).

4.2.2.2 Analysis with [1,4-^{14}C] Succinic Anhydride

The succinylation reaction was also performed with [1,4-^{14}C] succinic anhydride. A sample of commercial [1,4-^{14}C] succinic anhydride was diluted with recrystallized carrier succinic anhydride and used without further purification. This material was used to succinylate the undecagold cluster according to the procedure introduced earlier. Two equivalents of the anhydride were added. After the succinylation reaction, the solution was diluted to 25 ml with water and the pH adjusted to 7.5. This solution was then passed through a 1.5 x 6 cm column of QAE Sephadex Q-25, chloride form, to remove the [^{14}C]-succinate produced by water
The partially succinylated reaction mixtures described in section 4.2.2 were applied to 0.70 x 18 cm columns of QAE Sephadex Q-25 at pH 11.5. The columns were prewashed with two column volumes of 3.2 mM NaOH. The samples were eluted with a 400 ml linear sodium chloride gradient increasing from 0.00 M to 0.40 M at pH 11.5. Fractions of 4.1 ml were collected at a flow rate of 1.0 ml per minute and the absorbances measured at 415 nm.
FIGURE 27

Fraction No.

A₄₁₅

25 50

Fraction No.
hydrolysis. The succinylated undecagold cluster was collected and prepared for ion-exchange chromatography as previously described. The elution profile for this material chromatographed on QAE Sephadex Q-25, pH 11.5, is shown in Figure 28.

4.2.3 Synthesis of Mono(N-Succinyl)Eicosi(N,N-Dimethyl)Undecagold

Mono-succinyl undecagold (28 A\textsubscript{415} units, 0.95 μmol, 19.9 μeq of amino groups) was dissolved in 5.0 ml of water. The pH was adjusted to 5.0 and the solution was purged with nitrogen gas for 40 min at 60°C. After cooling to room temperature, the pH was adjusted to 7.5 and the solution was 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 at the 10 and 20 minute time periods. Formaldehyde (10 μl of 37%, 0.100 mmol) was added every 60 seconds. 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 and
Figure 28. QAE Sephadex Q-25 Elution Profile of Partially \[^{14}\text{C}\] -Succinylated Undecagold Cluster

The \[^{1,4}\text{C}\] -succinyl undecagold product mixture was prepared for chromatography as described in section 4.2.2.2. The sample was applied to a 1.5 x 28 cm column of QAE Sephadex Q-25 at pH 11.5 that had been prewashed with 3.2 mM NaOH. The material was eluted with a 800 ml linear sodium chloride gradient increasing from 0.00 M to 0.40 M at pH 11.5. Fractions of 4.5 ml were collected at a flow rate of 1.5 ml per minute. One hundred \(\mu\)l aliquots were used to measure the absorbance at 415 nm (---), and for liquid scintillation counting (\(\uparrow\rightarrow\downarrow\downarrow\downarrow\)) as described in section 2.2.4. Fractions eluting at 0.05 M (fractions 20-36, 38.1 \(A_{415}\) units, \(1.65 \times 10^4\) cpm per \(\mu\)mol), 0.15 M (fractions 60-76, 44.9 \(A_{415}\) units, \(2.91 \times 10^4\) cpm per \(\mu\)mol), 0.21 M (fractions 86-100, 26.1 \(A_{415}\) units, \(3.68 \times 10^4\) cpm per \(\mu\)mol) and at 0.24 M (fractions 108-120, 15.1 \(A_{415}\) units, \(4.21 \times 10^4\) cpm per \(\mu\)mol) were pooled separately. These samples were adjusted to pH 7.5, concentrated by rotary evaporation in \(\text{vacuo}\), desalted by passage through Sephadex G-10 (10% ethanol-water) and stored at \(-70\text{°C}\).
FIGURE 28

A_{415}

CPM \times 10^{-3}

Fraction No.
was adjusted to 7.5, the sample was concentrated to about 5.0 ml and applied to a column of Sephadex G-10 (1.5 x 48 cm). The desalted mono(N-succinyl)eicosi-(N,N-dimethyl)undecagold (25.5 A\textsubscript{415} units, 0.864 \mu mol) was stored at -70°C until further use (yield 91%).

4.2.4 Synthesis of Mono(N-Succinyl)Eicosi(N-Acetyl)Undecagold

Mono-succinyl undecagold (47.5 A\textsubscript{415} units, 1.61 \mu mol, 33.8 meq of amino groups) was dissolved in 10.0 ml of water. The pH was adjusted to 5.0 and the solution was purged with nitrogen gas for 45 min at 60°C. After cooling to room temperature, the pH was adjusted to 7.5 and the solution was cooled by stirring in an ice bath. Acetic anhydride (25 \mu l, 0.27 mmol) was added and the pH was maintained between 7 and 8 by addition of 1.0 N NaOH. When the pH became constant and was readjusted to 7.5, the acetic anhydride addition was repeated. A total of six 25 \mu l aliquots of acetic anhydride were added in this manner. The reaction mixture was then concentrated to about 5 ml by rotary evaporation in vacuo and applied to a column of Sephadex G-10 (1.5 x 45 cm). The column was eluted with 10% ethanol-water and the desalted mono-(N-succinyl)eicosi(N-acetyl)undecagold (43.6 A\textsubscript{415} units, 1.48 \mu mol) was stored at -70°C until further use (yield 92%).
4.2.5 **Synthesis of Trifluoroacetylated Undecagold Cluster**

Undecagold cluster (550 A415 units, 18.64 μmol) in the tosylate salt form was dissolved in 10.0 ml of water and the pH adjusted to 7.8. The sample was concentrated by rotary evaporation *in vacuo* to about 3 ml, shell frozen, and lyophilized to dryness. When the flask reached room temperature and the sample appeared dry, lyophilization was continued for an additional 3 hr to ensure removal of as much water as possible. The flask containing the dry undecagold cluster was sealed with a septum and all additions were made by syringe. A sample of DMF dried and stored over BaO was further dried by passage through a column of Alumina, Basic Grade I. The dry DMF was collected in a dry septum-covered flask. 4.7 ml of this was transferred to the flask containing the dry undecagold cluster. Triethylamine (14.6 μl, 10.6 mg, 105 μmol) was added by syringe. Finally, trifluoroacetic anhydride (29.4 mg, 18.8 λ, 140 μmol) was diluted with 200 μl of dry DMF. This solution was added to the reaction mixture in five aliquots over a 2 minute period. The reaction mixture was stirred at room temperature for 60 min. Approximately 15% of this material was removed and prepared for NMR analysis as follows. The sample was diluted
with an equal volume of water while cooling in dry ice-isopropanol. This material was passed through a 1.5 x 7 cm column of QAE Sephadex Q-25 in the chloride form at pH 7.0 to remove trifluoroacetate from the sample and to exchange the tosylate counterion for chloride. The eluted gold complex was concentrated by rotary evaporation in vacuo to about 2 ml and applied to a 1.5 x 18 cm column of Sephadex G-10. The column was eluted with 10% ethanol-water and the desalted trifluoroacylated undecagold cluster was concentrated to dryness by rotary evaporation in vacuo. Samples of this material were then used for proton and fluorine-19 NMR analysis in D$_2$O (Table 10). The remainder of the reaction mixture was stored at -70°C until further use.

4.2.6 Characterization of the Trifluoroacetylation Reaction

In order to determine the degree of trifluoroacetylation of the undecagold cluster as a function of the amount of trifluoroacetic anhydride added under the conditions to be routinely used in this reaction, a series of reactions were carried out with increasing amounts of trifluoroacetic anhydride added, followed by $^{19}$F NMR analysis of the products. Undecagold cluster (500 A$_{415}$ units, 16.94 μmol) was prepared for
TABLE 10
Fluorine-19 NMR Analysis of Trifluoroacetylated Undecagold Derivatives

<table>
<thead>
<tr>
<th>Sample</th>
<th>$^{19}\text{F}$ Chemical shift (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFGC</td>
<td>18869.71</td>
</tr>
<tr>
<td>TFDMGC</td>
<td>18874.45</td>
</tr>
<tr>
<td>DMGC</td>
<td>--</td>
</tr>
</tbody>
</table>

$^a$Samples were prepared for NMR analysis as described in the text. Spectra were recorded on the Nicolet NT200WB spectrometer.

$^b$TFGC = trifluoroacetylated undecagold, TFDMGC = trifluoroacetylated, permethylated undecagold, DMGC = methylated undecagold (de-trifluoroacetylated).

$^c$Chemical shifts are referenced to the spectrometer frequency (188.236661 MHz).
trifluoroacetylation as described previously. The dry undecagold cluster was dissolved in 3.7 ml of dry DMF. One ml aliquots of this solution were transferred to four dry septum covered vials. A sample of trifluoroacetic anhydride was then added to each of the vials (5.0 µl, 35 µmol, 8.3 eq; 10.0 µl, 70 µmol, 16.6 eq; 15.0 µl, 105 µmol, 24.9 eq; 20 µl, 140 µmol, 33.2 eq). The reaction mixtures were left at room temperature for 60 min.

The reaction mixtures containing 8.3 equivalents and 16.6 equivalents of added trifluoroacetic anhydride were prepared for fluorine-19 NMR as follows. The sample was diluted with 0.4 ml of water while cooling in a dry ice-isopropanol bath. This sample was passed through QAE Sephadex Q-25, chloride form (1.5 x 18 cm) to remove the trifluoroacetate produced in the reaction and exchange the tosylate counterion for chloride ion. The first third of the band eluted from the column was collected for preparation of the NMR sample. The reaction mixtures containing 24.9 eq and 33.2 eq of TFAA were diluted with 3 ml of water while cooling in dry ice isopropanol. The precipitated gold cluster was collected by centrifugation, dissolved in 2 ml of 95% ethanol and concentrated to dryness by rotary evaporation
in vacuo. These four samples were dissolved in DMSO-\textsubscript{d\textregistered}. After recording their fluorine-19 NMR spectra, the total absorbance of each sample was measured at 415 nm. The fluorine-19 NMR spectra are shown in Figure 29.

4.2.7 Synthesis of Trifluoroacetylated, Per­
methylated Undecagold

Trifluoroacetylated undecagold (470 \textsubscript{A\textregistered} \textsubscript{415}, 15.9 \textsubscript{\mu}mol) in 4.3 ml of DMF was diluted to 25.0 ml with 0.20 M sodium borate at pH 8.0 while cooling the solution in a dry ice-isopropanol bath. This material was methylated with formaldehyde and sodium borohydride essentially as described in section 3.2.4. The sample was not purged with nitrogen gas prior to reductive methylation. Glacial acetic acid was used to maintain the pH below 9.0. Approximately 15\% of the product solution was prepared for proton and fluorine-19 NMR analysis in D\textsubscript{2}O as described for the trifluoroacetylated derivative above (Table 10). The remaining trifluoroacetylated, permethylated undecagold cluster was concentrated by rotary evaporation in vacuo to approximately 10 ml and applied to a 2.5 x 40 cm column of Sephadex G-10. The column was eluted with 10\% ethanol-water. The desalted gold complex was stored at -70°C until further use.
The samples of trifluoroacetylated undecagold were prepared for $^{19}$F NMR analysis as described in section 4.2.6. The spectra were obtained on the Nicolet NT200WB instrument and were plotted using a constant y-scaling factor to allow direct comparison of peak areas.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$A_{415}$ units</th>
<th>Normalized integration</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>44.8</td>
<td>2.12</td>
</tr>
<tr>
<td>II</td>
<td>47.2</td>
<td>7.76</td>
</tr>
<tr>
<td>III</td>
<td>40.0</td>
<td>17.1</td>
</tr>
<tr>
<td>IV</td>
<td>47.2</td>
<td>21.0</td>
</tr>
</tbody>
</table>
4.2.8 Preparation of Eicosi(N,N-Dimethyl)Undecagold

Trifluoroacetylated, permethylated undecagold (368 $A_{415}$ units, 12.5 $\mu$mol) in 40 ml of 10% ethanol-water was concentrated to dryness by rotary evaporation in vacuo, dissolved in 15 ml of water and the pH adjusted to 12.0 with 4.0 N NaOH. This solution was stirred at room temperature for 12 hr. The pH was then adjusted to 7.0 and the sample volume reduced to about 5 ml by rotary evaporation in vacuo. This material was desalted by passage through a 1.5 x 48 cm column of Sephadex G-10. An aliquot of the desalted gold complex was prepared for proton and fluorine NMR analysis in $D_2O$. The remaining material (307 $A_{415}$ units) was concentrated to dryness by rotary evaporation in vacuo and then chromatographed through a column of QAE Sephadex Q-25 at a high pH in the presence of $CO_3^{2-}$ (Figure 30).

4.2.9 Synthesis of Trifluoroacetylated, Peracetylated Undecagold

Trifluoroacetyl undecagold (280 $A_{415}$ units, 9.49 $\mu$mol), prepared by addition of sixteen molar equivalents of trifluoroacetic anhydride in 5.0 ml of DMF, was diluted with 30 ml of water and the pH was adjusted to 7.5. This sample was acetylated according to the procedure described for the mono-succinyl derivative.
The permethylated cluster from which trifluoroacetyl groups had been removed (307 $A_{415}$ units) was dissolved in 10.0 ml of 0.50 M TEAB, pH 7.8, and concentrated to dryness by rotary evaporation in vacuo. The solid was dissolved in 10 ml of 0.35 M NaHCO$_3$ and the pH raised to 11.5 with NaOH. The volume was increased to 100 ml with 3.2 mM NaOH and the sample was immediately applied to a 1.5 x 19 cm column of QAE Sephadex Q-25 in the carbonate form at pH 11.5. The sample was eluted with a 400 ml linear sodium carbonate gradient increasing from 0.01 M to 0.40 M at pH 11.5. Fractions of 2.5 ml were collected at a flow rate of 1.5 ml per minute and the absorbances measured at 415 nm. Fractions eluting at 0.05 M (fractions 10-27, 47.2 $A_{415}$ units), and at 0.10 (fractions 34-48, 18.2 $A_{415}$ units) were pooled separately and stored at -70°C.
The product was concentrated to about 10 ml by ultrafiltration and desalted by passage through a 2.5 x 45 cm column of Sephadex G-10. The eluted gold complex was stored at -70°C until further use.

4.2.10 Preparation of Eicosi(N-Acetyl)Undecagold

Trifluoroacetyl, acetyl undecagold (252 Å_{415} units, 8.54 µmol) was concentrated to dryness by rotary evaporation in vacuo. The sample was dissolved in 10 ml of water and the pH adjusted to 12.0 with 4.0 N NaOH. The reaction mixture was stirred at room temperature for 12 hr. The pH was then adjusted to 7.0 and the volume was reduced to about 5 ml by rotary evaporation in vacuo. This sample was passed through a 1.5 x 48 cm column of Sephadex G-10. The desalted gold complex was concentrated to dryness by rotary evaporation in vacuo and dissolved in 25 ml of water. A small aliquot (100 µl) was removed and diluted to 1.0 ml with water, pH 6.0. This sample was applied to a small column of SP Sephadex C-25. 100% of the material was retained by the column. The remaining material was treated with acetic anhydride to partially acetylate the free amines in the sample. A 1.0 M solution of acetic anhydride in acetonitrile was prepared and 17 µl of this was added to the gold complex. When the pH stabilized, an aliquot was again tested for
retention on SP Sephadex C-25. This procedure was repeated three more times to obtain a sample of the cluster that exhibited 70% retention on SP Sephadex C-25. The data from these small columns are presented in Table 11. The remaining stock solution was then chromatographed through a column of SP Sephadex C-25 as described in Figure 31.

4.2.11 Synthesis of Mono(N-Phthalyl)Undecagold

Mono-phthalyl undecagold was prepared by a procedure very similar to that used to prepare mono-succinyl undecagold. Undecagold in the tosylate form (490 $A_{415}$ units, 16.64 µmol) was dissolved in 5.0 ml of 50% DMF-H$_2$O 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 the undecagold cluster in four 50 µl aliquots over a 2 minute 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 cooling to room temperature, the pH was adjusted to 11.5 and the solution diluted to 80 ml with freshly boiled water at pH 11.5. The partially phthalylated cluster
### TABLE 11
Retention of Acetylated Undecagold Cluster on SP Sephadex C-25

<table>
<thead>
<tr>
<th>Sample</th>
<th>$A_{415}$ units before column</th>
<th>$A_{415}$ units after column</th>
<th>% retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.915</td>
<td>0.000</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>0.906</td>
<td>0.000</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>0.910</td>
<td>0.041</td>
<td>95.5</td>
</tr>
<tr>
<td>4</td>
<td>0.896</td>
<td>0.162</td>
<td>81.9</td>
</tr>
<tr>
<td>5</td>
<td>0.889</td>
<td>0.279</td>
<td>68.6</td>
</tr>
</tbody>
</table>

The de-trifluoroacetylated cluster was treated with acetic anhydride as described in the text. Test columns of SP Sephadex C-25, sodium form, were prepared in dispo pipets. The columns were washed with two column volumes of $H_2O$ prior to sample application. The samples were then applied and the columns washed with 1.0 ml of water. The total absorbance of the samples was measured at 415 nm before being passed through the columns. The total absorbances of the column effluents were also measured at 415 nm, and the differences between the $A_{415}$ before and after chromatography were used to calculate the percent retention.
The reaction solution remaining from the analysis in Table 11 was applied to a 1.5 x 7 cm column of SP Sephadex C-25 in the sodium form. The column was eluted with a 300 ml linear sodium chloride gradient increasing from 0.00 M to 0.40 M at pH 5.5. Fractions of 3.0 ml were collected at a flow rate of 1.5 ml per minute and the absorbances measured at 415 nm. Fractions eluting at 0.70 M (fractions 12-22, 50.2 $A_{415}$ units), 0.14 M (fractions 30-38, 37.2 $A_{415}$ units), 0.19 M (fractions 43-51, 26.6 $A_{415}$ units) and at 0.24 M (fractions 55-62, 16.0 $A_{415}$ units) were pooled separately. The samples were concentrated by rotary evaporation in vacuo, desalted by passage through a column of Sephadex G-10 (10% ethanol-water) and stored at -70°C.
was purified by anion exchange chromatography as described in Figure 32. The fractions containing monophthalyl undecagold were pooled and the pH 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 x 45 cm) equilibrated and eluted with 10% ethanol-water. After concentrating to dryness by rotary evaporation in vacuo, the monophthalyl undecagold 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 cooling to room temperature, the pH was adjusted to 11.5 and the volume was adjusted to 40 ml with freshly boiled deionized water, pH 11.5. The solution was then applied to QAE Sephadex Q-25, chloride form (1.5 x 5 cm). After the sample was adsorbed, the column was washed with 1-2 column volumes of H₂O at pH 11.5. The monophthalyl undecagold 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 x 48 cm) equilibrated and eluted with 10% ethanol-water. The
Figure 32. QAE Sephadex Q-25 Elution Profile of Partially Phthalylated Undecagold Cluster

The phthalylation reaction mixture was prepared for chromatography as described in section 4.2.11. The mixture was applied to a 1.5 x 12 cm column of QAE Sephadex Q-25 that had been prewashed with 3.2 mM NaOH. The material was eluted with a 700 ml linear sodium chloride gradient increasing from 0.00 M to 0.40 M at pH 11.5. Fractions of 4.0 ml were collected at a flow rate of 1.5 ml per minute and the absorbances measured at 415 nm. Fractions eluting at 0.06 M (fractions 21-40, 41.2 $A_{415}$ units), 0.10 M (fractions 49-65, 33.1 $A_{415}$ units), 0.16 M (fractions 75-93, 19.4 $A_{415}$ units) and at 0.20 M (fractions 100-117, 10.9 $A_{415}$ units) were pooled separately. These samples were adjusted to pH 7.5, concentrated by ultrafiltration, desalted by passage through Sephadex G-10 (10% ethanol-water) and stored at -70°C. The crude, mono(N-succinyl)undecagold was prepared for rechromatography as described in the text.
desalted mono-phthalyl undecagold 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%).

4.2.12 Characterization of the Phthalylolation Reaction

The identification of the first peak in Figure 32 as mono-phthalyl undecagold, the second peak as di-phthalyl undecagold, etc. was achieved by carrying out the reaction with [carbonyl-\(1^4\)C]phthalic anhydride. A sample of [carbonyl-\(1^4\)C]phthalic anhydride was prepared with a specific radioactivity of \(2.78 \times 10^5\) cpm/\(\mu\)mol. This sample was used to acylate the gold complex as described previously. The disodium phthalate, produced by hydrolysis, was removed by passage of the diluted reaction mixture through QAE Sephadex Q-25 in the chloride form (1.5 x 10 cm) at pH 7.0. This treatment also exchanges the tosylate counterion for chloride. This sample was then chromatographed through QAE Sephadex Q-25 as described above. The elution profile is shown in Figure 33.

The crude mono-phthalyl undecagold (the first peak in Figure 33) was desalted and prepared for rechromatography on QAE Sephadex Q-25 as described above to
Figure 33. QAE Sephadex Q-25 Elution Profile of Partially \(^{14}\text{C}\)-Phthalylated Undecagold Cluster

The \(^{14}\text{C}\)-phthaly1 undecagold product mixture was applied to a 1.5 x 12 cm column of QAE Sephadex Q-25 that had been prewashed with 3.2 mM NaOH. The mixture was eluted with a 700 ml linear sodium chloride gradient increasing from 0.00 M to 0.40 M at pH 11.5. Fractions of 4.0 ml were collected at a flow rate of 1.5 ml per minute. One hundred \(\mu\)l aliquots were used to measure the absorbance at 415 nm (•••••) and for liquid scintillation counting (▲▲▲▲) as described in section 2.2.4. Fractions eluting at 0.06 M (fractions 20-39, 37.4 \(A_{415}\) units, 4.45 \(x\) \(10^5\) cpm per \(\mu\)mol), 0.10 M (fractions 49-64, 30.2 \(A_{415}\) units, 6.12 \(x\) \(10^5\) cpm per \(\mu\)mol), 0.16 M (fractions 74-91, 18.2 \(A_{415}\) units, 8.34 \(x\) \(10^5\) cpm per \(\mu\)mol) and at 0.20 M (fractions 100-115, 9.7 \(A_{415}\) units, 1.00 \(x\) \(10^6\) cpm per \(\mu\)mol) were pooled separately. The material which was not retained by the column was also pooled. These samples were adjusted to pH 7.5, concentrated by rotary evaporation in vacuo, desalted by passage through Sephadex G-10 (10% ethanol-water) and stored at \(-70^\circ\text{C}\). The sample of material which was not retained by the column and the crude mono(N-phthalyl)undecagold were prepared for rechromatography as described in the text.
determine whether this material was contaminated with di-phthalyl undecagold. The fraction of the phthalyla-
tion reaction mixture which was not retained by
QAE Sephadex Q-25 at pH 11.5 was concentrated by rotary
evaporation in vacuo and desalted by passage through a
column of Sephadex G-10. This material was also
reapplied to QAE Sephadex Q-25, pH 11.5, and eluted with
a linear NaCl gradient. The elution profiles for these
columns are shown in Figures 34 and 35.

4.2.13 Synthesis of Mono(N-Phthalyl)Eicosi(N-
Methyl,N-Carboxymethyl)Undecagold

Mono-phthalyl undecagold (58.56 A415 units, 1.99
μmol, 41.69 μeq of amino groups) 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 placed in 400 μl of water.
The pH was adjusted to 9.10 with NaOH and the volume was
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 hr at room temperature, the
HBr produced was titrated with 0.244 N NaOH. A second
100 μl aliquot of the bromoacetate solution was added
The crude mono(N-[14C]-phthalyl)undecagold was prepared for rechromatography as described in the text. The sample was applied to a 1.5 x 5 cm column of QAE Sephadex Q-25 that had been prewashed with 3.2 mM NaOH. The material was eluted with a 200 ml nitrogen purged, linear sodium chloride gradient increasing from 0.00 M to 0.40 M at pH 11.5. Fractions of 4 ml were collected at a flow rate of 1.5 ml per minute. The absorbance at 415 nm and radioactivity of each fraction was measured as previously described. Fractions 9-16, 12.5 A415 units, 2.90 x 10^5 cpm/µmol, were pooled, concentrated by rotary evaporation in vacuo, desalted by passage through Sephadex G-10 (10% ethanol-water) and stored at -70°C.
The partially $^{14}\text{C}$-phthalylated undecagold cluster which was not retained by QAE Sephadex Q-25 (Figure 33) was desalted and prepared for rechromatography as described in section 4.2.11 for the crude mono(N-$^{14}\text{C}$-phthalyl)undecagold. The sample was applied to a 1.5 x 5 cm column of QAE Sephadex Q-25 and eluted as described in the legend to Figure 34. Fractions 9-16, 13.5 $A_{415}$ units, $2.70 \times 10^5$ cpm/µmol were pooled, desalted and stored at -70°C as described in the legend to Figure 34.
and the reaction mixture was left at room temperature for 15 hr. The HBr was once again titrated. To this point 40.02 µmol of HBr had been produced. The pH was adjusted to 6.5 and the solution was concentrated to about 2 ml by rotary evaporation in vacuo. This material was desalted by passage through a 1.5 x 28 cm column of Sephadex G-10. The desalted mono(N-phthalyl)eicosi(N-carboxymethyl)undecagold was placed in 7.0 ml of water and the sample 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)eicosi(N,N-dimethyl)undecagold (overall yield, 93%).

4.2.14 Synthesis of Mono(N-Phthalyl)Eicosi(N-Acetyl)Undecagold and Mono(N-Phthalyl)-Eicosi(N,N-Dimethyl)Undecagold

Mono(N-phthalyl)eicosi(N,N-dimethyl)undecagold was synthesized according to the procedure used to prepare mono(N-succinyl)eicosi(N,N-dimethyl)undecagold. Acetylation of mono(N-phthalyl)undecagold with acetic anhydride to prepare mono(N-phthalyl)eicosi(N-acetyl)undecagold was carried out using the procedure developed for formation of mono(N-succinyl)eicosi(N-acetyl)undecagold. Acetylation of mono(N-phthalyl)undecagold with N-hydroxy-
succinimidoacetate was performed using the procedure developed for formation of heneicosi(N-acetyl)undecagold. The samples were purged with nitrogen gas for 30 min at pH 6.0 and 45°C. This less rigorous treatment of mono(N-phthalyl)undecagold was necessary to prevent loss of the phthalyl protecting group.

4.2.15 Synthesis of Eicosi(N-Acetyl)Undecagold

Mono(N-phthalyl)eicosi(N-acetyl)undecagold (29.5 A₄₁₅ units, 1.00 µmol) was dissolved in 5.0 ml of water and the pH adjusted to 5.0. The sample was concentrated to dryness by rotary evaporation in vacuo. A solution of 5% acetic acid (v/v) was prepared and the pH adjusted to 3.1. A 1.0 ml aliquot of this solution was transferred to the flask containing the dried gold complex. After the reaction mixture became homogeneous, it was adjusted to pH 3.20 and placed at 45°C for 72 hr. At the end of this period, the sample was diluted with 1.0 ml of 10% ethanol-water and gel filtered through a 1.5 x 48 cm column of Sephadex G-10, eluted with 10% ethanol-water. The desalted eicosi(N-acetyl)undecagold (22.1 A₄₁₅ units) was concentrated to dryness by rotary evaporation in vacuo, diluted with 1.5 ml of water, and stored at -70°C until further use (yield 75%).
Mono(N-phthalyl)eicosi(N,N-dimethyl)undecagold and mono(N-phthalyl)eicosi(N-methyl,N-carboxymethyl)undecagold were dephthalylated by the procedure described above for the acetylated derivative.

4.2.16 Characterization of the Dephtalylation Reaction

Mono(N-[^14]C)phthalyl)eicosi(N-acetyl)undecagold (29.5 A^15 units, 1.00 μmol, 7.53 x 10^4 cpm/μmol), prepared with acetic anhydride, was dephthalylated under the conditions outlined above. The pH of the reaction mixture was 3.20. After 48 hr, the absorbance of the reaction mixture was measured and about one-third of the material was removed. This aliquot was diluted with an equal volume of 10% ethanol-water and gel filtered through a column of Sephadex G-10. After 72 hr the remaining material was treated in the same manner. The elution profile for this material chromatographed on Sephadex G-10 is shown in Figure 36. After 48 hr the specific radioactivity of the gold complex was 1.89 x 10^4 cpm/μmol. After 72 hr the specific radioactivity was 1.79 x 10^4 cpm/μmol. The pH dependence of the dephthalylation reaction was characterized using mono(N-[^14]C)phthalyl)eicosi(N-acetyl)undecagold. The dephthalylation of mono(N-[^14]C)phthalyl)eicosi(N-acetyl)-
The dephthalation product mixture, after 72 hr at pH 3.20 (section 4.2.16) was applied in 1.0 ml to a 1.5 x 19 cm column of Sephadex G-10. The sample was eluted with 10% ethanol-water at a flow rate of 0.5 ml per minute. Fractions of 1.0 ml were collected and 100 µl aliquots used to measure the absorbances at 415 nm (●●●●) and for liquid scintillation counting (▲▲▲▲) as described in section 2.2.4. The product (fractions 15-20, 16.54 A_{415} units) was pooled and stored at -70°C.
FIGURE 36

![Graph showing CPM on the y-axis and Fraction No. on the x-axis, with data points at 415 and 1000 CPM]
undecagold, synthesized with \( N \)-hydroxysuccinimidoacetate, and \( N-[^{14}C] \)phthalal\( eicosi(\text{N,N-dimethyl}) \)undecagold were also characterized using the procedure described above. The results are presented in Table 12.

4.2.17 Synthesis of \( N-\alpha-\text{Bromoacetyl}\)Eicosi-(\( N-\text{Acetyl}\))Undecagold

Eicosi(\( N-\text{acetyl}\))undecagold (14.1 \( A_{415} \) units, 0.478 \( \mu \text{mol} \)) was dissolved in 5 \( \text{ml} \) of \( H_2O \). The \( \text{pH} \) was adjusted to 5.5 and the solution was purged with nitrogen gas for 30 min at 50-60°C. After cooling to room temperature, the \( \text{pH} \) was adjusted to 7.5 and the sample was concentrated to dryness by rotary evaporation in vacuo. The gold complex was dissolved in 300 \( \mu \text{l} \) of DMF. \( N \)-hydroxysuccinimide (36 \( \mu \text{l} \) of a 0.100 \( M \) solution in DMF) was added to the reaction and the solution was left at room temperature in the dark for 6 hr. The reaction mixture was diluted with 300 \( \mu \text{l} \) of 50 mM sodium acetate, \( \text{pH} \) 4.5, and applied to a column of Sephadex G-10. The column was eluted with 10% ethanol-water, \( \text{pH} \) 4.5. The desalted mono(\( N-\alpha-\text{bromoacetyl}\))eicosi-(\( N-\text{acetyl}\))undecagold was stored in 10% ethanol-water, \( \text{pH} \) 4.5, at -70°C until further use (yield 96%).
TABLE 12

Depthalylation of Mono(N-(\(^{14}\)C)-Phthalyl)Eicosi(N-Acetyl)Undecagold
and Mono(N-(\(^{14}\)C)-Phthalyl)Eicosi(N,N-Dimethyl)Undecagold\(^a\)

<table>
<thead>
<tr>
<th>Sample(^b)</th>
<th>Compound(^c)</th>
<th>Initial specific radioactivity (cpm/(\mu)mol)</th>
<th>Reaction (pH)</th>
<th>Reaction time (hr)</th>
<th>Final specific radioactivity (cpm/(\mu)mol)</th>
<th>% Yield</th>
<th>% Decomposition(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MPACGC</td>
<td>(7.53 \times 10^4)</td>
<td>3.20</td>
<td>48</td>
<td>(1.89 \times 10^4)</td>
<td>74.9</td>
<td>17.1</td>
</tr>
<tr>
<td>1</td>
<td>MPACGC</td>
<td>(7.53 \times 10^4)</td>
<td>3.20</td>
<td>72</td>
<td>(1.79 \times 10^4)</td>
<td>76.2</td>
<td>20.3</td>
</tr>
<tr>
<td>2</td>
<td>MPACGC</td>
<td>(6.19 \times 10^4)</td>
<td>2.85</td>
<td>48</td>
<td>(4.26 \times 10^4)</td>
<td>31.2</td>
<td>58.0</td>
</tr>
<tr>
<td>2</td>
<td>MPACGC</td>
<td>(6.19 \times 10^4)</td>
<td>2.85</td>
<td>96</td>
<td>(4.90 \times 10^4)</td>
<td>20.6</td>
<td>81.5</td>
</tr>
<tr>
<td>3</td>
<td>MPACGC</td>
<td>(9.73 \times 10^4)</td>
<td>3.25</td>
<td>72</td>
<td>(6.71 \times 10^3)</td>
<td>93.1</td>
<td>12.1</td>
</tr>
<tr>
<td>4</td>
<td>MPDMGC</td>
<td>(7.16 \times 10^4)</td>
<td>3.22</td>
<td>48</td>
<td>(5.73 \times 10^3)</td>
<td>92.0</td>
<td>12.3</td>
</tr>
<tr>
<td>4</td>
<td>MPDMGC</td>
<td>(7.16 \times 10^4)</td>
<td>3.22</td>
<td>72</td>
<td>(3.72 \times 10^3)</td>
<td>94.8</td>
<td>14.1</td>
</tr>
</tbody>
</table>

\(^a\) Reaction conditions are as described in the text.

\(^b\) Samples 1 and 2 were prepared with acetic anhydride. Sample 3 was prepared with N-hydroxysuccinimidoacetate.

\(^c\) MPACGC = mono(N-phthalyl)eicosi(N-acetyl)undecagold. MPDMGC = mono(N-phthalyl)eicosi(N,N-dimethyl)undecagold.

\(^d\) The % decomposition was determined from the difference in \(A_{415}\) units before and after the reaction.
4.2.18 **Synthesis of Mono[N(p-Maleimido)Benzoyl]-Eicosi(N-Acetyl)Undecagold**

Eicosi(N-acetyl)undecagold (8.60 A₄₁₅ units, 0.292 µmol) was purged with nitrogen and concentrated to dryness as described in the preceding procedure. The sample was dissolved in 250 µl of DMF and 22 µl of a 0.10 M solution of N(4-carboxy)phenylmaleimide N-hydroxysuccinimide ester in DMF was added. The solution was left for 6 hr at room temperature, in the dark. The sample was then purified by gel filtration chromatography as described for the mono(N-α-bromoacetyl) derivative. The desalted material was stored at -70°C until further use (yield 93%).

4.2.19 **Characterization of the Coupling Reaction With N-hydroxysuccinimido[1-¹⁴C]α-bromoacetate**

A sample of mono(N-[1-¹⁴C]α-bromoacetyl)eicosi-(N-acetyl)undecagold was synthesized according to the procedures used in the synthesis of the unlabeled compound. The specific radioactivity of the N-hydroxysuccinimido[1-¹⁴C] -bromoacetate was 4.0 x 10⁴ cpm/µmol. The specific radioactivity of the desalted mono(N-[1-¹⁴C]α-bromoacetyl)eicosi(N-acetyl)undecagold was 3.98 x 10⁴ cpm/µmol. Mono(N-phthalyl)eicosi(N-acetyl)-undecagold and mono(N-phthalyl)eicosi(N,N-dimethyl)
undecagold were also treated with the $^{14}\text{C}$-labeled
N-hydroxysuccinimide ester to determine whether the
erster reacts with other functional groups in the
cluster. A sample of mono(N-phthalyl)eicosi(N,N-
dimethyl)undecagold was also treated with methyltri-
fluoromethanesulfonate prior to exposure to the $^{14}\text{C}$-
labeled ester as follows. Mono(N-phthalyl)eicosi-(N,N-dimethyl)undecagold, tosylate form (27.2 Å$^4$15
units, 0.922 μmol) was dissolved in 600 μl of DMF.
Methyltrifluoromethanesulfonate (100 μl, 0.885 mmol)
was added. After 2 min, triethylamine (48 μl, 330 μmol) was added and the reaction mixture was left
at room temperature for 40 min. The sample was applied
to a 1.5 x 45 cm column of Sephadex G-10 and eluted
with 10% ethanol-water. The desalted gold complex was
converted to the tosylate form and dried as previously
described.

These control reactions with N-hydroxysuccinimido-
[1-$^{14}\text{C}$]α-bromoacetate were carried out using the same
procedures used in the synthesis of mono(N-[1-$^{14}\text{C}$]α-
bromoacetyl)eicosi(N-acetyl)undecagold. A fifteen fold
excess of the N-hydroxysuccinimide ester was used in
these reactions. The results are presented in Table 13.
## TABLE 13

Reactivity of Gold Complex Derivatives Towards N-Hydroxysuccinimido[1-$^{14}$C]a-Bromoacetate$^a$

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific radioactivity (cpm/μmol)</th>
<th>moles of bromoacetyl groups mole of gold complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPDMGC</td>
<td>$3.06 \times 10^5$</td>
<td>7.67</td>
</tr>
<tr>
<td>MPTMGC</td>
<td>$6.72 \times 10^4$</td>
<td>1.68</td>
</tr>
<tr>
<td>MPACGC</td>
<td>$7.89 \times 10^3$</td>
<td>0.20</td>
</tr>
<tr>
<td>ACGC</td>
<td>$3.98 \times 10^4$</td>
<td>0.99</td>
</tr>
</tbody>
</table>

$^a$Reaction conditions are described in section 4.2.17. A fifteen-fold excess of the N-hydroxysuccinimide ester was used.

$^b$MPDMGC = mono(N-phthalyl)eicosi(N,N-dimethyl)undecagold. MPTMGC = methyltrifluoromethanesulfonate-treated MPDMGC. MPACGC = mono(N-phthalyl)eicosi(N-acetyl)-undecagold. ACGC = eicosi(N-acetyl)undecagold. The acetylated derivatives were prepared with acetic anhydride.
4.2.20 Spectrophotometric Assay of Sulfhydryl Reactivity

Pipes buffer, 0.10 M, pH 7.0, was deoxygenated by purging with nitrogen gas for 30 min. To 1.0 ml spectrophotometric cells, pipes buffer, glucose oxidase (20 units), catalase (500 units), glucose (4 mM final concentration) and the sample of gold complex (0.15 A415 units) were added to give a final volume of 0.965 ml. The cells were covered with parafilm and Teflon tape and incubated at room temperature for 30 min. Cysteine (10 or 20 µl of a 1.00 mM solution) was added, the samples were covered and again incubated at room temperature for 90 min. The absorbance of the sample was measured at 412 nm. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) (25 µl of a 12.0 mM solution) was added and the absorbance was measured immediately at 412 nm. Alternatively, 4,4'-dithiopyridine (DTP) was used in the assay. Absorbance measurements were then made at 324 nm. Control incubations were run to measure the amount of cysteine which was oxidized during the incubation. Bromoacetamide and N-ethylmaleimide were also used in control incubations to measure the extent of reaction of cysteine with these reagents under the reaction conditions described above. The results of
these assays are presented in Table 14. The time course for the reaction of DTP and DTNB with the remaining cysteine was followed at 324 nm and 412 nm, respectively.

4.2.21 Radiochemical Assay of Sulphydryl Reactivity

Mono(N-α-bromoacetyl)eicosi(N-acetyl)undecagold (1.0 \(A_{415}\) units, 33.9 nmol) in 150 \(\mu l\) of water was placed in a small vial. 50 \(\mu l\) of pipes buffer (0.10 M, pH 7.0) was added and the vial was covered with a septum. The sample was purged with argon gas for 60 min at room temperature. [U\(^{14}\)C]-Cysteine·HCl (5 \(\mu l\) of a 0.105 M solution, 7.06 \(x\) 10\(^4\) cpm/mol) was added and the sample was incubated under an argon atmosphere for 60 min at room temperature. The sample was then applied to a column of Sephadex G-10 and eluted with 10% ethanol-water. The specific radioactivity of the eluted gold complex was then measured. This same procedure was used to assay the maleimide derivatives. The results are presented in Table 15.

4.2.22 Synthesis of Mono[N-(N'-Hydroxysuccinimido-succinyl)]Eicosi(N,N-Dimethyl)Undecagold

Mono(N-succinyl)eicosi(N,N-dimethyl)undecagold in the tosylate salt form (12.7 \(A_{415}\) units, 0.43 \(\mu mol\)) was
### TABLE 14

**Spectrophotometric Assay of the Reactivity of Gold Complex Derivatives with Cysteine**

<table>
<thead>
<tr>
<th>Sample</th>
<th>nmol</th>
<th>nmol of cysteine added(^c)</th>
<th>nmol of cysteine remaining(^d)</th>
<th>moles of cysteine reactive groups mole of gold complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>--</td>
<td>--</td>
<td>20.1</td>
<td>20.1</td>
<td>--</td>
</tr>
<tr>
<td>Bromoacetamide</td>
<td>10.0</td>
<td>20.1</td>
<td>10.3</td>
<td>--</td>
</tr>
<tr>
<td>BAACGC</td>
<td>8.47</td>
<td>20.1</td>
<td>13.9</td>
<td>0.732</td>
</tr>
<tr>
<td>MIACGC</td>
<td>8.52</td>
<td>20.1</td>
<td>14.8</td>
<td>0.622</td>
</tr>
<tr>
<td>MPACGC</td>
<td>10.6</td>
<td>20.1</td>
<td>19.2</td>
<td>0.085</td>
</tr>
</tbody>
</table>

\(^a\) Assay conditions are described in the text. DTP was used to obtain the data above.

\(^b\) BAACGC = *mono*(N-\(\alpha\)-bromoacetyl)eicosi(N-acetyl)undecagold. MIACGC = *mono*(N-(p-maleimido)benzoyl-eicosi(N-acetyl)undecagold. MPACGC = *mono*(N-phthalyl)-eicosi(N-acetyl)undecagold.

\(^c\) The concentration of cysteine in the stock solution was determined by weight and confirmed by assaying an aliquot with DTP.

\(^d\) The cysteine remaining after 60 min was calculated from the difference between the absorbance of the sample before and after addition of DTP. The value was corrected for absorbance due to the added DTP and for cysteine oxidation using data from control incubations.
TABLE 15

[^14]C-Cysteine Assay of Gold Complex Derivatives\(^a\)

<table>
<thead>
<tr>
<th>Sample(^b)</th>
<th>Compound(^c)</th>
<th>Specific radioactivity (cpm/mol)</th>
<th>moles of cysteine mole of gold complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BAACGC</td>
<td>(4.11 \times 10^4)</td>
<td>0.581</td>
</tr>
<tr>
<td>1</td>
<td>BAACGC</td>
<td>(3.90 \times 10^4)</td>
<td>0.551</td>
</tr>
<tr>
<td>2</td>
<td>BAACGC</td>
<td>(4.69 \times 10^4)</td>
<td>0.662</td>
</tr>
<tr>
<td>2</td>
<td>BAACGC</td>
<td>(4.72 \times 10^4)</td>
<td>0.667</td>
</tr>
<tr>
<td>3</td>
<td>MIACGC</td>
<td>(4.89 \times 10^4)</td>
<td>0.691</td>
</tr>
<tr>
<td>3</td>
<td>MIACGC</td>
<td>(4.93 \times 10^4)</td>
<td>0.696</td>
</tr>
<tr>
<td>4</td>
<td>MIACGC</td>
<td>(5.52 \times 10^4)</td>
<td>0.779</td>
</tr>
<tr>
<td>4</td>
<td>MIACGC</td>
<td>(5.50 \times 10^4)</td>
<td>0.777</td>
</tr>
<tr>
<td>5</td>
<td>MPACGC</td>
<td>(3.06 \times 10^3)</td>
<td>0.043</td>
</tr>
<tr>
<td>5</td>
<td>MPACGC</td>
<td>(3.17 \times 10^3)</td>
<td>0.045</td>
</tr>
</tbody>
</table>

\(^a\)Assay conditions are described in the text.

\(^b\)Samples 1 and 3 were from the same dephthalylolation reaction mixtures. Samples 2 and 4 were also from a single dephthalylolation reaction mixture. The acetylated derivatives were prepared with acetic anhydride.

\(^c\)BAACGC = mono(N-\(\alpha\)-bromoacetyl)eicosi(N-acetyl)undecagold. MIACGC = mono(N-(p-maleimido)benzoyl)eicosi(N-acetyl)undecagold. MPACGC = mono(N-phthalyl)-eicosi(N-acetyl)undecagold.
placed in 150 µl of alumina dried DMF. Dicyclohexylcarbodiimide (DCC) (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 was used without further purification for coupling the succinyl carboxyl group to amino groups in DMF.

4.2.23 Condensation of Mono[N-(N'-Hydroxysuccinimido-succinyl)]Eicosi(N,N-Dimethyl)Undecagold with Amino Groups

A sample of once chromatographed, tri(N-succinyl)-octadecyl(N,N-dimethyl)undecagold in the tosylate salt form (15.3 A_{415} units, 0.52 µmol) was placed in 0.50 ml of alumina dried DMF. Dicyclohexylcarbodiimide (2.06 mg, 10.0 µmol) and N-hydroxysuccinimide (1.15 mg, 10 µmol) were dissolved in 150 µl DMF and added to the gold complex/DMF solution. The reaction was stirred at room temperature for 60 min. Undecagold cluster, tosylate form (140 A_{415} units, 4.75 µmol) was dissolved in 0.30 ml DMF and added to the reaction. The reaction mixture was stirred at room temperature for 18 hr. One-third of this material was removed, diluted to 0.80 ml with deionized H_{2}O and applied to a 1.5 x 28 cm column of Sephadex G-50. The elution profile is shown in Figure 37.
Figure 37. Chromatography of Multimeric Undecagold Cluster on Sephadex G-50

The product mixture was applied in 0.80 ml to a 1.5 x 28 cm column of Sephadex G-50 and eluted with 0.10 M ammonium chloride. Fractions of 0.50 ml were collected at a flow rate of 0.50 ml per minute and the absorbances measured at 415 nm (○-○-○). A sample of undecagold cluster was chromatographed on the same column, under identical conditions, to determine the position of elution of the monomeric species (▲-▲-▲).
Mono[N-(N'-hydroxysuccinimidosuccinyl)]eicosi(N,N-dimethyl)undecagold (15.2 $A_{415}$ units, 0.52 μmol) was synthesized as previously described. N-(3-aminopropyl)-bromoacetamide trichloroacetate (4.1 mg, 10.0 μmol) was dissolved in 100 μl of DMF and added to the reaction mixture. The solution was left standing at room temperature for 18 hr. At the end of the reaction time, the solution is pale yellow in color. The UV-visible spectrum shows no absorbance bands at 305 nm and 415 nm.

A series of control reactions were run to better characterize the decomposition reaction. Incubation of heneicosi(N,N-dimethyl)undecagold with a 20-fold excess of either bromoacetamide or iodoacetamide results in decomposition over 24 hr. The potassium salts of bromide and iodide do not cause the decomposition. N-ethylmaleimide and N-phenylmaleimide also have no effect. Phosphorus-31 NMR analysis of the decomposition reaction mixture shows a single absorption signal at -26.2 ppm.
4.3 Results and Discussion

The synthesis of mono(N-succinyl)undecagold and mono(N-phthalyl)undecagold in pure form has proven to be the best method for preparing mono-functional derivatives of the undecagold cluster. As expected, the most difficult aspect of the procedure was the chromatographic separation of the mono-functional cluster from the product mixture. The preparation of these derivatives was achieved only after several unexpected problems were solved.

The first step in development of a general procedure for preparing mono(N-succinyl)undecagold was to determine the stoichiometry of reagents that lead to optimizing the yield of the mono-succinyl derivative in the product mixture. Succinic anhydride is a relatively stable anhydride in aqueous solution. In addition, the undecagold cluster contains amino groups with $pK_a$ values near the neutral pH range. For this reason, relatively little hydrolysis of the anhydride was expected during the reaction period. The optimal conditions were determined by preparing several reaction mixtures containing increasing amounts of succinic anhydride. The product mixtures were then separated by anion-exchange chromatography as shown in Figure 27. From the
elution profiles for these reaction mixtures it is apparent that addition of between 1 and 2 molar equivalents of succinic anhydride results in maximization of the first peak in the chromatogram. From this study it was concluded that the undecagold cluster could be easily succinylated at pH 7.5 in aqueous solution and that the optimal molar ratio of succinic anhydride to undecagold cluster required for maximal yield of the mono-succinyl derivative is 1.5 to 1.

Minor modifications in the procedure were required to achieve effective conditions for phthalylating the undecagold cluster. The poor solubility of phthalic anhydride in water dictated the use of a mixed solvent system. A 50% DMF-water mixture was found to allow for repeatable preparation of mono(N-phthalyl)undecagold in optimal yield. From empirical observation, it has been found that the hydrolysis of phthalic anhydride under the reaction conditions described above is somewhat slower than the hydrolysis of succinic anhydride under the conditions used in the succinylation reaction. The optimal yield of mono(N-phthalyl)undecagold has been achieved with a ratio of phthalic anhydride to undecagold cluster of 1:1.

From the elution profiles shown in Figures 26 and 32 it would appear that the product mixture can be
easily separated using a linear ionic strength gradient at pH 11.5. There are, however, several problems with this procedure. These problems were discovered when performing the succinylation and phthalation reactions with $^{14}$C-labeled anhydrides. These reactions were originally performed in order to determine whether the first peak in the elution profiles was indeed the monofunctional undecagold derivative. The data dealing with phthalation of the undecagold cluster with $^{14}$C-phthalic anhydride presented in Figures 33-35 unmasked several problems. First, all peaks in the elution profile shown in Figure 33 were of a higher specific radioactivity than the $^{14}$C-phthalic anhydride used in the reaction. The ratio of specific radioactivity of the first four peaks in Figure 33 is 1.6:2.2:3.0:3.6, based on the value of 1.0 for the specific radioactivity of the $^{14}$C-phthalic anhydride. From this ratio it was obvious that the peaks in this elution profile did not contain pure samples of the mono-, di-, tri- and tetra-phthalylated undecagold derivatives. This fact was clearly demonstrated by rechromatography of the first peak (peak I) in the elution profile. From the data presented in Figure 34 it was concluded that peak I contained undecagold cluster as well as mono-, di- and tri-(N-phthalyl)undecagold. The specific
radioactivity of the rechromatographed mono(N-phthalyl)-undecagold was $2.90 \times 10^5$ cpm/µmol, in good agreement with the specific radioactivity of $2.78 \times 10^5$ cpm/µmol for the $^{14}$C-phthalic anhydride used in the reaction. The data were obtained using samples that had been thoroughly purged with nitrogen gas to remove contaminating carbamates prior to the chromatographic separation. When this precaution was not taken, considerably more contamination of peak I with undecagold cluster was observed.

Much the same result was obtained when the undecagold cluster was treated with $^{14}$C-succinic anhydride. The ratios of specific radioactivities for the peaks in Figure 28 were 1.0:1.8:2.3:2.7. Once again, deviation from a 1:2:3:4 ratio was observed. The ratio of 1.0:1.8 would, however, suggest that the mono(N-succinyl)-undecagold is less contaminated by other undecagold derivatives than the mono(N-phthalyl)undecagold derivative discussed above. It has nevertheless been found to be necessary to rechromatograph mono(N-succinyl)-undecagold in order to obtain the material in pure form. The removal of carbamates from the sample prior to rechromatography is critical to the successful preparation of mono-primary amino derivatives of the cluster.
from mono(N-phthalyl)undecagold. In general, purging the samples with nitrogen gas for 60 min at 60°C has proven more than adequate to ensure the removal of carbamates from the preparation. Due to the acid lability of the phthalyl group, somewhat less rigorous conditions have been used with the mono(N-phthalyl)-undecagold derivatives to avoid premature dephthalyla-
tion of the mono-derivatized cluster.

From the elution profiles in Figures 28 and 33, another problem is apparent. Upon loading the QAE Sephadex Q-25 column with the product mixture, some 14C-labeled undecagold passes through the column. Reapplication of this material to a column of QAE Sephadex Q-25 results in retention of some material on the resin. The elution profile for this column in Figure 35 shows that both mono- and di(N-phthalyl)-undecagold are present. This problem of leakage of the derivatized undecagold cluster from QAE Sephadex Q-25 at pH 11.5 appears to be more significant with the phthalylated cluster than with the succinylated cluster. The presence of DMF in the phthalylation reaction mixture may be in part responsible for this leakage. The sample concentration has also been observed to affect the amount of leakage of mono- and di(N-phthalyl)-undecagold derivatives during the column loading process.
Sample concentrations of 3-4 $A_4_{15}$ units per ml have been found to result in improved retention of the monofunctional derivatives on the column, however, some leakage still occurs.

There are two additional factors which are undoubtedly, in part, responsible for less than quantitative retention of the mono- and di-functional derivatives on QAE Sephadex Q-25 at pH 11.5. The titration curve for the undecagold cluster discussed in Chapter III indicates that the cluster is unprotonated at pH 11.5. In actuality, the undecagold cluster is probably 95-99% unprotonated. The dynamic nature of this residual protonic equilibrium could lead to the premature elution of a significant amount of the mono(N-phthalyl) and mono(N-succinyl) derivatives as zwitterionic species. If this were in fact the major cause of leakage, raising the pH to 12.0 should improve the retention; however, no significant improvement in retention could be seen at pH 12.0. The improved retention of the mono-functional derivatives at pH 12.0 due to a decreased concentration of the zwitterionic species is probably counteracted by the increased ionic strength at this pH. Examination of the ionic strength required to elute the mono-, di-, tri- and tetra-functional undecagold species from QAE Sephadex Q-25,
at pH 11.5, provides clear evidence that the undecagold cluster derivatives do not interact strongly with the quaternary ammonium groups attached to the resin. This appears to be especially true of the phthalylated species. The sterically hindered carboxyl group in the mono-phthalyl undecagold derivative may not interact with the resin as effectively as the more exposed carboxyl group on the mono-succinyl derivative. This would explain the greater leakage of the mono-phthalyl derivative. The actual cause of the less than quantitative retention of the mono- and di-functional derivatives on QAE Sephadex Q-25 is probably a combination of the various factors discussed above.

The procedures described in section 4.2 for preparing mono(N-succinyl)undecagold and mono(N-phthalyl)-undecagold have been designed to overcome the problems discussed above. The material not retained on the first QAE Sephadex Q-25 column was routinely desalted and rechromatographed. The amount of mono(N-phthalyl)-undecagold recovered by this procedure was significant enough to warrant incorporation of this process in the overall procedure.

The di(N-phthalyl)undecagold and tri(N-succinyl)-undecagold are also potentially useful compounds for preparation of multimeric derivatives of the cluster.
These compounds would be extremely useful in performing double label experiments with complex biological aggregates. The synthesis of multimeric forms of the undecagold cluster is a project being pursued by others in this laboratory.

The phthalyl blocking group has proven to be the amino protecting group best suited for use in the preparation of mono-primary amino derivatives of the undecagold cluster. The trifluoroacetyl group was also investigated as a potential amino group protecting group for this purpose. Fluorine-19 NMR was used in attempts to optimize the conditions for formation of mono(N-trifluoroacetyl)undecagold. As has been discussed previously, it is most important that the mono-trifluoroacetyl form of the cluster be optimized due to the order of steps in the procedure. From the data in Figure 29, it was apparent that the repeatable preparation of anhydrous undecagold cluster would be difficult. The normalized peak areas for the NMR spectra shown in Figure 29 are 21.0:17.1:7.76:2.12. These ratios have obviously been formulated assuming that treatment of the undecagold cluster with 33.2 equivalents of trifluoroacetic anhydride results in quantitative acylation of all 21 amino groups in the cluster. That this assumption
appears to be valid can be seen from the following discussion. Assuming that the normalized ratio above is a direct reflection of the number of amino groups acylated, then the difference between the number of amino groups acylated and the number of equivalents of trifluoroacetic anhydride added represents the amount of trifluoroacetic anhydride that has been hydrolyzed by residual water and that which remains unreacted after 60 min. This value should be constant for the three samples in which the gold complex was not completely acylated, provided that all water present in the sample reacts with the trifluoroacetic anhydride. The ratio of equivalents of trifluoroacetic anhydride which did not react with the undecagold cluster is 12.2:7.84:8.84:6.18. The constant value for the three incompletely acylated samples is consistent with the interpretation of the data presented above. The amount of residual water present in the sample can be expected to vary slightly from one preparation to the next. This makes optimization of the mono-trifluoroacetyl derivative difficult.

The separation of eicosi(N,N-dimethyl)undecagold from the mixture of methylated species by anion exchange chromatography of the carbamates formed upon exposure
to a carbon dioxide based buffer does not appear to be a useful method for preparation of the mono-functional derivative in pure form. From the elution profile shown in Figure 30, several observations can be made. First, the separation between the different charged forms on the column was poor. This is largely due to the equilibrium nature of the carbamate bond. As was discussed in section 3.2.2.2, the undecagold cluster is heavily carbamated upon exposure to CO₂ containing buffers. After reductive methylation of the majority of the amino groups in the cluster, however, the equilibrium between the free amine and the carbamate form is not favorable enough to permit chromatography of the carbamates at pH 11.5. The first peak in Figure 30 undoubtedly consists of eicosi(N,N-dimethyl)-undecagold, nonadecyl(N,N-dimethyl)undecagold and octadecyl(N,N-dimethyl)undecagold, all present in the mono-carbamate form.

The use of trifluoroacetic anhydride as an amino group protecting reagent for the formation of eicosi-(N-acetyl)undecagold provides a more reliable method for formation of a mono-primary amino derivative of the cluster. The problem of optimization of the mono-amino derivative is more easily achieved using this method.
When the undecagold cluster was treated with 16 equivalents of trifluoroacetic anhydride followed by acetylation of the remaining amino groups with acetic anhydride and the trifluoroacetyl protecting groups were removed, 100% of the material was retained by SP Sephadex C-25. The mixture was then treated with two molar equivalents of acetic anhydride and the % retention by SP Sephadex C-25 was again determined. This procedure was repeated until approximately 30% of the material was not retained by the ion-exchange resin. This preparation was then separated by cation exchange chromatography on SP Sephadex C-25 at pH 6.0 as shown in Figure 31. This method is satisfactory for preparation of eicosi(N,N-dimethyl)undecagold; however, it lacks the versatility required to prepare the other mono-amino derivatives of the cluster.

The limited success of the trifluoroacetyl group as an amino protecting group is due to the fact that the mono-functional derivative is separated from the product mixture after reacting all of the remaining amino groups in the sample with one of the reagent(s) discussed in Chapter III. As has been mentioned before, this sequence of procedures is disadvantageous for several reasons. The procedures required to use phthalic
anhydride as the amino group protecting reagent were developed to circumvent these problems.

The remaining amino groups in mono(N-succinyl)-undecagold and mono(N-phthalyl)undecagold have been derivatized using the procedures discussed in Chapter III. Due to the unique functional group present in these compounds, minor modifications and additional precautions were necessary. The permanent presence of the carboxyl group in the mono(N-succinyl) derivatives makes it imperative that the method used to derivatize the remaining amino groups does not affect the lone carboxyl functional group. The carboxyl group present in mono-(N-phthalyl)undecagold is required for the dephthalylation reaction, hence the same arguments apply to this derivative as well.

The remaining amino groups in mono(N-phthalyl)-undecagold can be derivatized using any of the reagent(s) discussed in Chapter III. Mono(N-succinyl)-undecagold has been derivatized by the acetylation and reductive methylation procedures. Derivatization of mono(N-succinyl)undecagold by the carboxymethylation-reductive methylation or succinylation procedures would destroy the mono-functional nature of the succinyl carboxyl group required for subsequent coupling reactions.
In the preparation of mono(N-succinyl)eicosi(N,N-dimethyl)undecagold and mono(N-phthalyl)eicosi(N,N-dimethyl)undecagold, one modification of the procedure developed in Chapter III has been made. After the reductive methylation procedure is complete, the product was treated with acetic anhydride to ensure complete derivatization of any remaining primary or secondary amines. One precaution must be taken when treating the mono-succinyl and mono-phthalyl derivatives with acetic anhydride, since addition of acetic anhydride to these derivatives results in the formation of significant amounts of mixed anhydrides. Any nucleophile present in the solution can react with these, derivatizing the phthalyl or succinyl carboxyl group. For this reason, precautions must be taken to remove all ethanol present in the sample from previous Sephadex G-10 desalting procedures prior to the reductive methylation reaction. The same precaution must be taken when treating the mono-phthalyl and mono-succinyl derivatives with acetic anhydride to form mono(N-phthalyl)eicosi(N-acetyl)-undecagold and mono(N-succinyl)eicosi(N-acetyl)undecagold.

When forming the acetylated derivatives with acetic anhydride, the amino groups initially present in the sample are also capable of reacting with the mixed
anhydride to potentially crosslink the cluster through
the phthalyl or succinyl group. This has, in fact, been
observed in SDS polyacrylamide gels of the compounds
synthesized by this method. Further evidence of this
cross-linking reaction is seen in the data for dephthalyl-
lation of \( \text{mono}(\text{N}-^{14}\text{C})-\text{phthalyl})\text{eicosi}(\text{N-acetyl})\text{undecagold.} \)

Synthesis of \( \text{mono}(\text{N-phthalyl})\text{eicosi}(\text{N-acetyl})\text{undecagold} \)
and \( \text{mono}(\text{N-succinyl})\text{eicosi}(\text{N-acetyl})\text{undecagold with} \)
\( \text{N-hydroxysuccinimidoacetate has been found to be necessary} \)
to circumvent the cross-linking reaction. Acetic
anhydride should, however, prove to be a useful reagent
for formation of multimeric forms of the undecagold
cluster.

The conditions required for the dephthalylation
reaction were examined using \( \text{mono}(\text{N}-^{14}\text{C})-\text{phthalyl})\text{eicosi-}
(N,N-dimethyl)undecagold and \( \text{mono}(\text{N}-^{14}\text{C})-\text{phthalyl})\text{eicosi-}
(N-acetyl)undecagold. \) The elution profile in Figure 36
shows that the dephthalylation of \( \text{mono}(\text{N-phthalyl})\text{eicosi-}
(N-acetyl)undecagold, synthesized with acetic anhydride, \)
is 76% complete after 72 hr. The yield was not improved
by longer reaction times. The incomplete dephthalyla-
tion of this material is a reflection of the cross-
linking that occurs during the acetylation reaction.
The dephthalylation of \( \text{mono}(\text{N-phthalyl})\text{eicosi}(\text{N-acetyl})-\)
undecagold, synthesized with \( \text{N-hydroxysuccinimidoacetate,} \)
is 93% complete after 72 hr (Table 12). The conditions for dephthalylation have been observed to be extremely sensitive to the pH used for the reaction. The conditions for dephthalylation described in section 4.2.16 are designed to provide minimal decomposition of the gold complex during the reaction. At pH 3.22 the mono-phthalyl derivatives can be completely dephthaylated in 72 hr. About 15-20% decomposition of the sample occurs at this pH. At pH 2.85, 80% decomposition of the reaction occurs over a 72 hr reaction period.

N-hydroxysuccinimide esters have proven to be the best reagents for specific attachment of molecules of interest to the mono-primary amino derivatives of the cluster. Incubation of eicosi(N-acetyl)undecagold with either N-hydroxysuccinimido α-bromoacetate or N-hydroxysuccinimido N'-(4-carboxy)phenylmaleimide results in formation of the amide bond in high yield. In general, N-hydroxysuccinimide coupling reactions can be performed in either aqueous or non-aqueous solutions. Due to the electrophilicity of the carbonyl group in N-hydroxysuccinimido α-bromoacetate, this reagent hydrolyzes rapidly in aqueous solution. For this reason, methods were developed to allow for the coupling reaction to be performed in an aprotic solvent.
The formation of mono(N-α-bromoacetyl)eicosi-
(N-acetyl)undecagold was characterized using N-hydroxy-
succinimido-[1-1^4C]α-bromoacetate. From the data in
Table 13, several points can be made. First, the mono-
(N-[1-1^4C]α-bromoacetyl)eicosi(N-acetyl)undecagold
formed contains 0.99 moles of α-bromoacetyl groups per
mole of gold complex. A value of 0.75/1.0 would be
expected, based on the % yield for the dephthalation
reaction. The difference must represent a second
reaction occurring between the 1^4C-labeled N-hydroxy-
succinimide ester and the gold complex. To further
investigate this possibility, N-hydroxysuccinimido-
[1-1^4C]α-bromoacetate was incubated with mono(N-phthalyl)-
eicosi(N-acetyl)undecagold and mono(N-phthalyl)eicosi-
(N,N-dimethyl)undecagold. From the results of this
study (Table 13), it was apparent that a secondary
reaction does indeed occur. The mono(N-phthalyl)eicosi-
(N-acetyl)undecagold reacts only slightly with the 1^4C-
labeled ester, however, the mono(N-phthalyl)eicosi(N,N-
dimethyl)undecagold derivative is very reactive. That
this unexpected reactivity of the N,N-dimethyl deriva-
tive is due to an unexpectedly high reactivity of the
tertiary amino groups was demonstrated by treating
mono(N-phthalyl)eicosi(N,N-dimethyl)undecagold with
methyltrifluoromethanesulfonate prior to incubation
with the $^{14}$C-labeled ester. This treatment, which converts the dimethyl tertiary amines to trimethyl quaternary amines results in a dramatic reduction of the reactivity of the cluster towards N-hydroxysuccinimido[1-$^{14}$C]$\alpha$-bromoacetate.

The products, mono(N-$\alpha$-bromoacetyl)eicosi(N-acetyl)-undecagold and mono(N-(4-maleimido)benzoyl)eicosi(N-acetyl)undecagold were further characterized by examining the reactivity of these components toward the cysteine sulfhydryl group. In developing the spectrophotometric assay, several problems were encountered. First, to prevent the rapid oxidation of cysteine by molecular oxygen, anaerobic conditions were required. Most of the oxygen was removed by purging the assay solutions with nitrogen gas. Residual oxygen was removed enzymatically with glucose oxidase and catalase. Under these conditions, oxidation of cysteine was reduced to about 20% over a 90 min period. The second problem encountered was the apparent secondary reaction of DTNB and DTP with the gold complex. The rate of the secondary reaction was considerably slower than the reaction of DTNB or DTP with cysteine. Despite this difference in rate, the existence of a secondary reaction has made difficult the accurate determination of the stoichiometry of sulfhydryl reactive
groups on the cluster. The secondary reaction appears to cause a significant error in the measurement when the amino groups are reductively methylated. The acetylated derivatives also show a side reaction, however, scatter in the data is significantly reduced.

A radiochemical assay for sulfhydryl reactivity was developed to circumvent these problems. Incubation of the mono-functional alkylating clusters with $^{14}$C-cysteine followed by gel filtration of the reaction mixture and determination of the specific radioactivity of the gold complex has proven to be a reliable method for obtaining accurate data. From the data in Table 15 it was concluded that the incubation of eicosi(N-acetyl)undecagold with N-hydroxysuccinimide esters of bromoacetic acid and N-(4-carboxy)phenylmaleimide result in near quantitative incorporation of the sulfhydryl alkylating groups onto the cluster. The repeatable difference in the amount of bromoacetyl groups vs. maleimide groups incorporated was attributed to the bi-functional reactivity of N-hydroxysuccinimido $\alpha$-bromoacetate towards the single primary amino group present on the cluster. These data indicated that approximately 13% of the $\alpha$-bromoacetate ester that reacted alkylated amino groups and 83% acylated amino
groups. This problem was reduced when dealing with the maleimide reagent, due to the decreased reactivity of amino groups toward the maleimide ring.

The limitations of the different mono-primary amino derivatives of the undecagold cluster that have been cited in discussing the preparation of mono(N-α-bromoacetyl) derivatives are not problems of a general nature. The mono-succinyl and mono-primary amino derivatives of the cluster described in this chapter are all capable of being coupled to biological ligands. The addition of amino acid alkylating reagents to the cluster presents problems that would not be encountered when coupling less reactive molecules. In fact, the α-bromoacetyl group is probably one of the most difficult molecules to couple successfully. It is therefore anticipated that, for the majority of applications, all of the mono-functional derivatives that have been synthesized will prove to be useful.

Preparation of the N-hydroxysuccinimide ester of mono(N-succinyl)eicosi(N-acetyl)undecagold and the N,N-dimethyl derivative has also proven to be the best way to activate the succinyl carboxyl group for condensation with amino or hydroxyl groups. The ability of the N-hydroxysuccinimide ester to serve as
an acylating reagent was demonstrated by formation of multimers of the gold complex (Figure 37). Attempts to couple N-α-bromoacetyl-1,3-diaminopropane to mono[N-(N'-hydroxysuccinimidosuccinyl)]eicosi(N,N-dimethyl)undecagold were, however, unsuccessful. The decomposition of the gold complex that was observed is difficult to explain. Further characterization of the decomposed material would be required to fully understand the reaction. Further attempts to couple N-α-bromoacetyl-1,3-diaminopropane to mono[N-(N'-hydroxysuccinimidosuccinyl)]eicosi(N-acetyl)undecagold have not yet been attempted. The acetylated derivative may be more resistant to the decomposition reaction. The successful attachment of sulfhydryl alkylating groups to eicosi(N-acetyl)undecagold has reduced the demand for derivatives of mono(N-succinyl)undecagold containing these reactive groups. N-hydroxysuccinimide esters of the mono-succinyl derivatives will, however, be very useful as amino group acylating reagents.
CHAPTER V

SYNTHESIS OF 4,4',4"-PHOSPHINIDYNETRI(BENZENEMETHANAMMONIUM)TRIS(4-METHYLBENZENESULFONATE) AND BI-FUNCTIONAL ALKYLATING REAGENTS

5.1 Introduction

The preparation of usable quantities of the various undecagold derivatives discussed in the preceding chapters required the large scale synthesis of 4,4',4"-phosphinidynetri(benzenemethanamine)Tris(4-methylbenzenesulfonate). The synthesis of this compound was accomplished by a modification of the published procedure (73) using the sequence of chemical reactions shown in Scheme 9. In the procedure described by Bartlett et al. (73), anaerobic conditions were employed only in the formation of Tris[4-((diethoxymethyl)phenyl)phosphine (II) and 4,4',4"-phosphinidynetri(benzene-methanamine) (V). This was presumably to protect against the oxygen sensitivity of n-butyllithium and borane-THF used in these reactions. When this procedure was repeated in this laboratory, complete oxidation of the phosphate by molecular oxygen occurred during the
SCHEME 9

\[
\text{Br-} \begin{array}{c}
\text{OCH}_2\text{CH}_3 \\
\text{OCH}_2\text{CH}_3
\end{array}
\]

95% yield

1) nBuLi
2) \(\text{PCl}_3\)

\[
\text{P-} \begin{array}{c}
\text{OCH}_2\text{CH}_3 \\
\text{OCH}_2\text{CH}_3
\end{array}
\]

90% yield

2N HCl/THF

\[
\text{P-} \begin{array}{c}
\text{C} \\
\text{H}_2 \text{C} \\
\text{H}_2
\end{array}
\]

95% yield

\[
\text{P-} \begin{array}{c}
\text{CH}_3\text{ONH}_2\cdot\text{HCl/Pyridine}
\end{array}
\]

90% yield

1) \(\text{BH}_3/\text{THF}\)
2) \(\text{OH}^-, \text{H}^+\)
3) \(\text{TsOH/H}_2\text{O}\)

\[
\text{P-} \begin{array}{c}
\text{NH}_3^+ \\
\text{TsO}^-
\end{array}
\]

85% yield
periods of exposure to the atmosphere. The successful
synthesis of 4,4',4"'-phosphinidynetri(benzenemethan-
ammonium)Tris(4-methylbenzenesulfonate) was achieved
only after stringent measures were taken to prevent
oxidation. The synthetic route used here is otherwise
essentially the same as that described by Bartlett
et al. (73) and is given in section 7.2.2. The
procedural details, however, are considerably different.
A detailed discussion of the methodology used in this
laboratory to prevent phosphine oxidation during the
synthesis of 4,4',4"'-phosphinidynetri(benzenemethan-
ammonium)Tris(4-methylbenzenesulfonate) is provided in
section 7.2.1. This is done in the hope that the
future synthesis of this compound by other workers can
be achieved without undue difficulty.

The successful coupling of amino acid alkylating
reagents to eicosi(N-acetyl)undecagold was achieved
using the N-hydroxysuccinimide esters of α-bromoacetic
acid and N-(4-carboxy)phenylmaleimide. These compounds
were synthesized using standard procedures (98,99) and
the details are presented here. Numerous attempts were
made to synthesize a short chain N-alkylmaleimide
containing a terminal carboxyl group. N-carboxymethyl-
maleamic acid, N-carboxyethylmaleamic acid and N-3-
carboxypropylmaleamic acid were prepared by condensation
of maleic anhydride with glycine, β-alanine and 3-aminopropionic acid, respectively. Several methods were then tried to effect ring closure. Refluxing in toluene in the presence of [2.2.2]Diazobicyclooctane, heating in acetic anhydride, dicyclohexylcarbodiimide coupling and direct fusion resulted in decomposition of the sample.

α-Bromoacetyl and maleimide derivatives were also desired for coupling reactions with the N-hydroxy-succinimide ester of the mono(N-succinyl) derivatives of the cluster. N-(α-bromoacetyl)-1,3-diaminopropane was synthesized for this purpose. Once again, numerous attempts were made to prepare an N-alkylmaleimide derivative suitable for coupling to this form of the cluster. Shafer et al. (100) reported the synthesis of N-(6-aminohexyl)maleimide in 10% yield by refluxing the monohydrochloride salt of 1,6-diaminohexane and maleic anhydride in acetic acid. In our hands, this procedure gave a 17% yield of the desired compound. The method did not, however, succeed in the attempted syntheses of N-(2-amino)ethylmaleimide or N-(3-amino)propylmaleimide. The other methods previously mentioned also proved to be unsuccessful. The preparation of an N-aryl maleimide derivative was also difficult. N-(4-Formyl)phenylmaleimide was synthesized as described below. Initial
attempts to isolate the reductively aminated derivative of this compound have been unsuccessful. Silica gel TLC analysis of the reaction indicates that the reaction does occur, however, purification of the final product without destruction of the maleimide ring has not been possible. Other synthetic routes to this type of compound are possible if future attempts to isolate N-(4-methylamino)maleimide from the reductive amination reaction mixture are unsuccessful. In addition, the synthesis of these compounds containing a terminal hydroxyl group has not been investigated. The successful synthesis of mono(N-α-bromoacetyl)eicosi(N-acetyl)-undecagold and mono[N-(p-maleimido)benzoyl]eicosi-(N-acetyl)undecagold has reduced the demand for mono-succinyl derivatives containing an attached sulfhydryl alkylating reagent.
5.2 Experimental Procedures

5.2.1 Apparatus for Anaerobic Synthesis

In order to allow for the synthesis of 4,4',4''-phosphinidynetri(benzenemethanamine) in large quantities, procedures were used to maintain an anaerobic environment within the reaction vessel. This allowed for the reactions to be carried out in a conventional fume hood. Prepurified nitrogen gas was found to be suitably free of oxygen and exclusively used to maintain anaerobic conditions. The nitrogen gas delivery system used is depicted by the drawing in Diagram 3. This system allowed for simultaneous use of the nitrogen gas for several purposes.

All solvents and solutions used were deoxygenated by purging with nitrogen gas using the deoxygenation chamber illustrated in Diagram 3. When anhydrous solvent was required, the deoxygenation chamber was dried overnight at 120°C. The chamber was connected to the nitrogen outlet while hot and purged with nitrogen gas while cooled. During seasons of high humidity, additional flame drying of the apparatus was required. Solvent was then added to the chamber and purged with nitrogen gas through the gas dispersion frit for 15-20 min per 200 ml.
Diagram 3. Nitrogen Delivery and Solvent Deoxygenation System

Diagram:

- $N_2$
- Drying Agent
- Tygon tubing
- Stopcock
- Septum
- Deoxygenation chamber
- Gas dispersion frit
- Oil bubbler
The deoxygenated solvent was routinely transferred to a reaction vessel by syringe or double-ended needle. All syringes and needles were dried for 12 hr at 120°C. When the transfer was made by syringe, the needle was inserted into the septum covered solvent outlet tube and solvent was drawn into the syringe. The first syringe-full of solvent was always discarded. Transfer was made by double-ended needle as follows. The double-ended needle was inserted into a septum on the reaction vessel and purged with nitrogen. When the other end of the needle was inserted into the septum covered solvent outlet tube on the deoxygenation chamber, nitrogen gas flowed from the reaction vessel into the deoxygenation chamber. The nitrogen gas inlet into the reaction vessel was then closed. The transfer of solvent was accomplished by closing the nitrogen outlet on the deoxygenation chamber. The positive pressure generated above the solvent in the deoxygenation chamber is sufficient to cause the transfer of solvent through the double-ended needle to the reaction vessel. When the solvent transfer had been completed, the positive pressure in the deoxygenation chamber was dissipated by opening the nitrogen outlet tube. The double-ended needle was removed first from the reaction vessel and then from the deoxygenation chamber.
The preparation of anhydrous THF for the borane-THF reduction of the Tris-O-methyloxime (III) was accomplished using the apparatus illustrated in Diagram 4. The apparatus was dried overnight at 120°C, assembled while hot, and flushed thoroughly with nitrogen until cool. The solvent was then transferred from the deoxygenation chamber to the column of alumina by double-ended needle using the procedure for transfer described above. When the space above the alumina chamber was half-filled with solvent, the nitrogen outlet above the column was removed and the nitrogen outlet on the deoxygenation chamber opened. Provided the nitrogen outlet below the alumina column is below the solvent deoxygenation chamber, the rate of solvent flow will be controlled by the alumina column flow rate. Minimal modulation of this process is required. The anhydrous, deoxygenated THF was transferred from the collection flask below the alumina column to the reaction vessel by double-ended needle. A nitrogen inlet was inserted into the collection flask to provide the necessary positive pressure for the transfer.

The apparatus used as the reaction vessel for the preparation of Tris[4-(diethoxymethyl)phenyl]phosphine and 4,4',4"-phosphinidynetri(benzenemethanamine) is
illustrated in Diagram 5. This apparatus was dried overnight at 120°C, assembled while hot and flushed thoroughly with dry nitrogen gas until cool. The apparatus was then dried by heating from bottom to top with an open flame (caution: deoxygenation of ether and THF should not be in progress during this process). The nitrogen purging is continued while the apparatus once again cools to room temperature. All transfers to and from this apparatus were made by syringe or double-ended needle through the septum in the neck of the flask or at the top of the dropping funnel. A needle was attached to the end of the nitrogen delivery tube and could be inserted through either septum to purge the apparatus and maintain an oxygen-free environment during the reaction.

A two-neck round bottom flask equipped with a magnetic stirring bar, septum and septum covered reflux condenser was used in the synthesis of 4,4',4"-phosphinidynetri(benzaldehyde) (III). The apparatus was purged thoroughly with nitrogen gas and was maintained anaerobic throughout the reaction. All transfers to or from the apparatus were made through the septum in the neck of the flask.

A round bottom flask equipped with a magnetic stirring bar and septum was used as the reaction vessel
DIAGRAM 5.

General Purpose Adaptor

Sintered Glass Funnel

double ended needle

Dropping funnel

Water cooled condenser

N2

Aspirator

Filtration adaptor

Septum

H2O

N2
in the preparation of 4,4',4''-phosphinidynetri(benzaldehyde)Tris(O-methyl)oxime. The flask was dried for 6 hr at 120°C, covered with a septum while hot and flushed thoroughly with nitrogen until cool. The nitrogen atmosphere was maintained throughout the reaction. All transfers to and from the flask were made by syringe or double-ended needle. This same apparatus was used to convert 4,4',4''-phosphinidynetri(benzene-methanamine) to its tosylate salt (V).

The apparatus used to filter the lithium chloride salt from Tris[4-(diethoxymethyl)phenyl]phosphine (II) is shown in Diagram 5. This apparatus was assembled and flushed thoroughly with nitrogen. A double-ended needle was inserted between the reaction vessel and the septum covering the fritted funnel. The nitrogen outlet tube on the reaction vessel was closed and the transfer of the product mixture initiated. When the area above the fritted disc was half full, the nitrogen outlet above the fritted disc was removed and a slow suction filtration started. As the pressure below the fritted disc decreased, the nitrogen outlet on the reaction vessel was reopened and the rate of flow through the double-ended needle was controlled with the vacuum stopcock. As the end of the transfer neared, the control of liquid
flow was gradually returned to positive pressure in the reaction vessel. This was done to prevent excessive drain on the nitrogen delivery system once the filtration was complete. The filtrate was then prepared for solvent evaporation as described below.

The apparatus used for drying the aldehyde (III), oxime (IV) and amine (V) derivatives and for solvent evaporation is shown in Diagram 6. The apparatus was assembled and purged with nitrogen gas. The product solution was transferred to the drying apparatus through a double-ended needle. After drying, the solution was drained through the drying agent into the receiving flask. Fresh deoxygenated solvent was then transferred to the drying chamber to wash the residual product from the drying agent. The filtered solution was then prepared for solvent evaporation.

This was achieved by removing the separatory funnel from the vacuum filtration adapter and rapidly inserting an adapter into the opening. A vacuum was immediately introduced and the solvent evaporated with stirring in a warm water bath. When the solvent had evaporated, the vacuum stopcock was closed and nitrogen gas was introduced through a needle inserted through the septum in the vacuum adapter. When required,
a vacuum pump (<0.05 torr) was used to remove trace solvent from the syrup. The flask, magnetic stirring bar and septum were preweighed so that the yield of phosphine syrup could be determined. The syrup was transferred to the next reaction vessel by syringe after dilution with the appropriate deoxygenated solvent.

5.2.2 Synthesis of 4,4',4"-Phosphinidylenetri-(Benzenemethanamine)Tris(4-Methylbenzene-sulfonate)

5.2.2.1 Synthesis of Tris[4-((Diethoxymethyl)-phenyl]Phosphine

p-Bromobenzaldehyde diethylacetal (21.75 g, 83.9 mmol) was weighed in a syringe and transferred to the reaction vessel (Diagram 5). Deoxygenated, anhydrous diethyl ether (125 ml) was then transferred to the reaction flask. The first portion was transferred from the deoxygenation chamber with the syringe used to weigh the acetal. The remainder was transferred by double-ended needle as described previously. The ether/acetal solution was stirred with the reaction vessel immersed in an ice-water bath for 10-15 min to cool the mixture to about 5°C. n-Butyllithium/hexane (1.6 M) (53 ml, 85 mmol) was then added via syringe over a 10 min period.
The pale yellow reaction mixture was stirred with the vessel in a water bath at 22-24°C for 45 min. The solution turned deep yellow during this period.

The determination of when to cool the lithioaryl anion solution is very important. Premature cooling of the reaction mixture leads to incomplete formation of the lithio anion, resulting in poor stoichiometry and multiple side products. Prolonged incubation of the reaction mixture leads to polymerization of the intermediate and low yields. Phosphorus trichloride (2.49 ml, 27.97 mmol) and 60 ml of deoxygenated, anhydrous diethyl ether were transferred to the dropping funnel. The deep yellow lithioaryl anion/ether solution was cooled to 0-5°C by stirring with the vessel in an ice-water bath for 15 min. The PCl$_3$/ether solution was then added dropwise over a 2 hr period, while the mixture was stirred at less than 5°C. After the addition was complete, the reaction mixture was stirred for an additional 60 min at room temperature. Eighty ml of deoxygenated, anhydrous diethyl ether was added to the dropping funnel. The lithium chloride was filtered as described earlier, and the ether in the dropping funnel used to rinse the reaction vessel and transfer apparatus. The ether was removed at reduced
pressure to give 14.31 g (90% yield) of Tris[4-(diethoxy-
 methyl)phenyl]phosphine as a deep orange syrup. This
material was used without further purification. \(^1\text{H} \text{NMR}
(90 \text{ MHz, CDCl}_3) \delta 7.41, \text{ multiplet, 4H; } \delta 5.49, \text{ singlet,}
1\text{H; } \delta 3.56, \text{ quartet, 4H; } \delta 1.23, \text{ triplet, 6H; } ^{31}\text{P NMR}
(80.1 \text{ MHz, CDCl}_3), \text{ proton decoupled, } \delta 6.7, \text{ singlet.}

5.2.2.2 Synthesis of 4,4',4"-Phosphinidyne-
tri(Benzaldehyde)

Tris[4-(diethoxymethyl)phenyl]phosphine (16.0 g,
28.1 mmol) was transferred to the reaction vessel. A
deoxygenated solution of 2.0 N HCl/THF, 1:4 (200 ml)
was added and the resulting solution stirred while
refluxing for 60 min. The mixture was cooled to room
temperature. One-hundred ml of deoxygenated H\(_2\)O and
60-80 ml of deoxygenated diethyl ether were then added
and the biphasic solution was vortexed. After the
layers had separated the organic layer was transferred
to a flask containing MgSO\(_4\). The ether extraction was
repeated three more times. The combined ether extracts
were dried for 2-3 hr. After filtering the dry
phosphinetrialdehyde, the ether/THF was removed under
vacuum to give 9.25 g (95% yield) of 4,4',4"-phosphini-
dynetri(benzaldehyde) as an orange-yellow syrup. \(^1\text{H}
\text{NMR} (90 \text{ MHz, CDCl}_3), \delta 10.0, \text{ singlet, 1H; } \delta 7.83,
doublet of doublets (J = 1.9 and 7.5 Hz), 2H; \( \delta \) 7.44,
triplet (J = 7.5 Hz), 2H; \(^{31}\)P NMR (80.1 MHz, CDCl\(_3\)),
proton decoupled, \( \delta \) 3.8, singlet.

5.2.2.3 Synthesis of 4,4',4"-Phosphinidyne-tri(Benzaldehyde)Tris(O-Methyloxime)

O-Methylhydroxylamine hydrochloride (4.51 g, 54 mmol) was placed in a flask that was then covered with a septum and purged with nitrogen gas. 4,4',4"-Phosphinidyne-tri(benzaldehyde) (12.47 g, 36 mmol) and 100 ml of deoxygenated dry pyridine were added and the reaction mixture was stirred for 3 hr with the flask immersed in an ice-water bath. The mixture was diluted at room temperature with 250 ml of deoxygenated diethyl ether. This solution was repeatedly extracted with deoxygenated 2.0 N HCl (8 x 100 ml) to remove the pyridine. Additional deoxygenated diethyl ether (150 ml) was added during the extraction procedure to maintain the volume of the organic layer at greater than 200 ml. The ether-phosphine solution was then dried over K\(_2\)CO\(_3\) for 8-10 hr. The solution was filtered and concentrated at reduced pressure to give 14.04 g (90% yield) of 4,4',4"-phosphinidyne-tri(benzaldehyde)tris(O-methyloxime) as an orange syrup. \(^1\)H NMR (90 MHz, CDCl\(_3\)), \( \delta \) 8.05, singlet, 1H;
6  7.40, multiplet, 4H; δ 3.95, singlet, 3H; $^{31}$P NMR (80.1 MHz, CDCl$_3$), proton decoupled, δ 5.6, singlet.

5.2.2.4 Synthesis of 4,4',4"-Phosphinidyne-tri(Benzenemethanammonium)Tris(4-Methylbenzenesulfonate)

4,4',4"-Phosphinidynetri(benzaldehyde)Tris(O-methyl-oxime) (18.0 g, 41.5 mmol) and alumina dried deoxygenated THF (80 ml) were transferred to the reaction vessel. Trace amounts of water in the deoxygenated THF markedly reduce the yield in this reaction. The solution was stirred with the flask immersed in an ice-salt water bath for 10-15 min (T <5°C). Borane·THF (1.0 M, 336 ml) was added to the dropping funnel in three aliquots to minimize the time of exposure of borane·THF to ambient temperature. The borane·THF solution was added to the reaction vessel dropwise over a 2.5-3 hr period while maintaining the temperature below 5°C. When the addition was complete, the reaction mixture was allowed to warm slowly to room temperature. The reaction mixture was then refluxed for 3 hr. When the solution warmed to room temperature and was heated to reflux, it changed from yellow to colorless. After refluxing, the temperature of the reaction mixture was reduced to less than 5°C. Deoxygenated distilled water
(120 ml) was transferred to the dropping funnel and added DROPWISE, SLOWLY. Vigorous foaming and hydrogen gas evolution occurred. Deoxygenated 20% NaOH (120 ml) was transferred to the dropping funnel, and added to the reaction mixture. This solution was refluxed for 60-90 min. The reaction mixture was cooled to room temperature and 4.05 M HCl (240 ml) was added dropwise. The reaction mixture was again refluxed for 90 min. After cooling to room temperature the product mixture was extracted with two 200 ml portions of deoxygenated diethyl ether. The aqueous layer was transferred to a separatory funnel and neutralized by addition of a deoxygenated NaOH solution (72.0 g in 250 ml H₂O). The phosphinetriamine was then extracted with four 200 ml portions of methylene chloride. The combined organic layer was dried over K₂CO₃ for 2-3 hr, filtered and the solvent removed under reduced pressure to give 12.32 g, 85% yield of 4,4′,4″-phosphinidynetri(benzenemethanamine) as an orange syrup. ¹H NMR (90 MHz, CDCl₃), δ 7.28, multiplet, 4H; δ 3.85, singlet, 2H; δ 1.55, singlet, 2H; ³¹P NMR (80.1 MHz, CDCl₃), proton decoupled, δ 8.6, singlet.

p-Toluene sulfonic acid (18.94 g, 110 mmol) was placed in a flask and purged with nitrogen. Deoxygenated
water (175 ml) was added and the solution heated to boiling. The hot solution was transferred rapidly to a flask containing the phosphinetriamine (12.32 g, 35.3 mmol). The homogeneous solution was allowed to cool slowly to room temperature and then placed in an ice bath. The product, 4,4',4''-phosphinidynetri(benzene-methanammonium)Tris(4-methylbenzenesulfonate) was collected by vacuum filtration and dried in a vacuum desiccator for 12-16 hr. The crude product was recrystallized in deoxygenated 95% ethanol to give 29.04 g (95% yield) of the tosylate in two crops as fine white needles. The needles were stored under vacuum and protected from light. $^1$H NMR (270 MHz, DMSO-$d_6$) $\delta$ 8.32, singlet (broad), 3H; $\delta$ 7.68, multiplet, 4H; $\delta$ 7.55, doublet, 2H; $\delta$ 7.16, doublet, 2H; $\delta$ 4.15, singlet, 2H; $\delta$ 2.32, singlet, 3H. $^{31}$P NMR (80.1 MHz, DMSO-$d_6$), proton decoupled, $\delta$ 6.1, singlet.

5.2.3 Synthesis of 4-Bromobenzaldehyde Diethyl-acetal

4-Bromobenzaldehyde (21.2 g, 114 mmol) and triethyl-orthoformate (34.0 ml, 200 mmol) were combined in 100 ml of absolute ethanol. Dowex 50 (H$^+$) (1.0 g, dried with ethanol and ether) was added and the suspension refluxed for 3 hr. After cooling to room temperature, the
ion-exchange resin was collected by suction filtration and the filtrate concentrated to a syrup by rotary evaporation in vacuo. The product was purified by vacuum distillation (78°C, 0.1 torr) to give 26.89 g (91%) of the acetal as a colorless liquid. $^1$H NMR (90 MHz, CDCl$_3$), $\delta$ 7.37, multiplet, 4H; $\delta$ 5.42, singlet, 1H; $\delta$ 3.50, quartet, 4H; $\delta$ 1.20, triplet, 6H.

5.2.4 Synthesis of N-(α-Bromoacetyl)-1,3-Diaminopropane

1,3-Diaminopropane (4.17 ml, 50 mmol) was added to 40 ml of methanol. The solution was cooled in an ice-water bath and stirred while the pH was adjusted to a reading of 8.0 with trifluoroacetic acid. The pH meter was calibrated with 50 mM potassium phosphate buffer at pH 7.0. Dicyclohexylcarbodiimide (11.33 g, 55 mmol) was dissolved in 20 ml of methanol and added to the solution. Finally, bromoacetic acid (7.65 g, 55 mmol) was dissolved in 15 ml of methanol and added to the reaction mixture dropwise over a 2 minute period. The reaction mixture was stirred in the ice-water bath for 30 min and at room temperature for an additional 60 min. The dicyclohexylurea was filtered by suction filtration and the filtrate was concentrated to a syrup by rotary evaporation in vacuo.
The syrup was diluted with 250 ml of water and extracted three times with 200 ml portions of low boiling petroleum ether. The aqueous layer was then extracted with two 200 ml of diethyl ether. The aqueous layer was diluted to 1.5 liters and applied to a 2.5 x 40 cm column of Dowex 50 in the hydrogen ion form. The column was eluted with a 2.0 liter linear trichloroacetic acid gradient increasing from 0.00 M to 0.50 M. Fractions of 20 ml were collected at a flow rate of 1.0 ml per minute. The fractions were spotted on Whatman 3M paper and the paper was sprayed with a 2% solution of ninhydrin in ethanol. The spots were developed by heating the paper in an oven (110°C) for 5-10 min. The fractions containing the product (purple spot) and those containing 1,3-diaminopropane (brown spot) were then identified by visualization. The fractions containing N-(α-bromoacetyl)-1,3-diaminopropane were combined and concentrated to one-half volume. This solution was extracted repeatedly with 200 ml portions of diethyl ether until the pH was less than 5.0. If emulsions formed, they were broken by addition of low boiling pet ether. The aqueous layer was concentrated to about 50 ml by rotary evaporation in vacuo and again extracted with diethyl ether until the pH was less than 6.0. The aqueous layer was finally concentrated to a
syrup by rotary evaporation in vacuo to give 7.71 g
(43% yield) of N-(α-bromoacetyl)-1,3-diaminopropane,
trichloroacetate as a colorless syrup. \(^1\)H NMR (270 MHz, D\(_2\)O), δ 3.89, singlet, 2H; δ 3.32, triplet, 2H; δ 3.01, triplet, 2H; δ 1.90, quintet, 2H.

5.2.5 Synthesis of N-Hydroxysuccinimido(4-Carboxy)-
phenylmaleimide

Maleic anhydride (0.98 g, 10.0 mmol) was dissolved
in 10.0 ml of dry acetonitrile. p-Aminobenzoic acid
(1.37 g, 10.0 mmol) in 25.0 ml acetonitrile was added
dropwise over a 15 min period. After the addition was
complete, the reaction mixture was stirred in an ice-
water bath for 30 min. The product, N-(4-carboxyphenyl)-
maleamic acid, was collected by vacuum filtration and
dried overnight in a vacuum desiccator to give 2.17 g
(94% yield) of a yellow powder, mp 219-220°C. \(^1\)H NMR
(270 MHz, DMSO-d\(_6\)), δ 10.61, singlet, 1H; δ 7.94,
doublet, 2H; δ 7.73, doublet, 2H; δ 6.41, doublet of
doublets (AB, J = 12 and 46 Hz), 2H.

N-(4-Carboxyphenyl)maleamic acid (1.00 g, 42.5 mmol)
was placed in 9.0 ml acetic anhydride. Sodium acetate
(0.90 g) was added and the solution stirred while the
temperature was increased to 100°C. After the solution
became homogeneous, the reaction mixture was stirred for
an additional 15 min at 100°C. The solution was cooled to room temperature and poured into a separatory funnel containing 30.0 ml of water. The aqueous mixture was extracted two times with 20.0 ml portions of chloroform. The organic extracts were dried over MgSO₄ and concentrated by rotary evaporation in vacuo under reduced pressure. The residue was dissolved in 5.0 ml acetonitrile and added dropwise to 150 ml of 50% acetonitrile-water. The pH was maintained at 6.0 by addition of 0.50 M sodium bicarbonate. When the solution became homogeneous and stable at pH 6.0, the pH was adjusted to 3.0 with 1.0 N HCl. The solution was extracted twice with 20.0 ml portions of CHCl₃. The CHCl₃ extracts were combined and extracted twice with 20.0 ml portions of H₂O. The CHCl₃ layer was then dried over MgSO₄ and concentrated to a solid by rotary evaporation under reduced pressure. The crude N-(4-carboxyphenyl)-maleimide was recrystallized from ethanol to give 735 mg (80% yield) of pale yellow needles (mp 233-236°C).

¹H NMR (270 MHz, DMSO-d₆, δ 10.64, singlet, 1H; δ 8.05, doublet, 2H; δ 7.50, doublet, 2H; δ 7.22, singlet, 2H.

N-(4-Carboxyphenyl)maleimide (651 mg, 3.0 mmol) and N-hydroxysuccinimide (345 mg, 3.0 mmol) were placed in 25.0 ml acetonitrile. The mixture was stirred in an oil
bath at 50°C to dissolve the reactants. Dicyclohexyl-
carbodiimide (618 mg, 3.0 mmol), dissolved in 5.0 ml
acetonitrile was added to the solution. The reaction
mixture was removed from the oil bath and stirred for
60 min. The dicyclohexylurea was removed by vacuum
filtration and the filtrate concentrated to a syrup
by rotary evaporation in vacuo. The syrup crystallized
upon standing. The solid was recrystallized from
cyclohexane-ethyl acetate to give 810 mg (86\% yield)
of long pale yellow-white needles (mp 192-194°C). $^1$H
NMR (270 MHz, DMSO-$d_6$), $\delta$ 8.23, doublet, 2H; $\delta$ 7.70,
doublet, 2H; $\delta$ 7.25, singlet, 2H; $\delta$ 2.91, singlet, 4H.
Anal. Calcd for C$_{15}$H$_{10}$N$_2$O$_6$: C, 57.33; H, 3.21; N,
8.91; O, 30.55. Found: C, 56.93; H, 3.55; N, 8.83;
O, 30.77.

5.2.6 Synthesis of N-(4-Formylphenyl)Maleimide
p-Nitrobenzaldehyde (22.65 g, 0.15 mole) and
triethylorthoformate (42.0 ml, 0.25 mole) were placed
in a round bottom flask. Dowex 50·H$^+$ (1.5 g), washed
with 1.0 N HCl, ethanol and diethyl ether, was added
to the flask. Finally 125 ml of absolute ethanol was
added and the mixture refluxed for 4 hr. After cooling
to room temperature the reaction mixture was filtered
and the filtrate concentrated by rotary evaporation
in vacuo at <35°C. The syrup was vacuum distilled to give 30.4 g (90% yield) of 4-nitrobenzaldehyde diethylacetal as a colorless liquid. The distillation was performed at 0.015 torr, 90°C. \(^1\)H NMR (270 MHz, DMSO-\(d_6\)), \(\delta\) 8.19, doublet, 2H; \(\delta\) 7.63, doublet, 2H; \(\delta\) 5.59, singlet, 1H; \(\delta\) 3.53, quartet, 4H; \(\delta\) 1.14, triplet, 6H.

p-Nitrobenzaldehyde diethylacetal (22.51 g, 0.10 mole) was placed in a glass hydrogenation bottle. One-hundred ml of 95% ethanol were added and the bottle was placed on the hydrogenation apparatus. After the bottle had been evacuated with shaking for 2 min, it was filled to atmospheric pressure with nitrogen gas. The bottle was removed and platinum oxide (250 mg) added. The bottle was placed back on the apparatus and evacuated. Hydrogen gas was introduced to the reaction mixture at 44 psi (3 atm). The shaker was started and consumption of hydrogen monitored with the hydrogen pressure gauge. The pressure remained constant after 6 to 8 min. The reaction was allowed to remain at constant pressure for 3 min before the shaker was stopped and the hydrogen supply valve was closed. The remaining hydrogen pressure was released and the bottle was evacuated and refilled with nitrogen.
gas five to ten times. Finally, the bottle was removed from the apparatus and stoppered. The product solution was decanted from the catalyst through a scinttered glass funnel. The filtrate was concentrated and the product, 4-aminobenzaldehyde diethylacetal (19.11 g, 98% yield), was used without further purification. $^1$H NMR (270 MHz, DMSO-d$_6$), $\delta$ 7.03, doublet, 2H; $\delta$ 6.54, doublet, 2H; $\delta$ 5.29, singlet, 1H; $\delta$ 3.41, quartet, 4H; $\delta$ 1.12, triplet, 6H.

p-Aminobenzaldehyde diethylacetal (9.75 g, 0.05 mole) was diluted with 50 ml of diethylether and placed in a dropping funnel. Maleic anhydride (4.9 g, 0.05 mole) was dissolved in 50 ml of diethyl ether. The amine/ether solution was added dropwise to the anhydride/ether solution over a 30 min period. The suspension was stirred for an additional 30 min in an ice-water bath. N-(Maleyl)-4-aminobenzaldehyde diethylacetal was collected by suction filtration and dried in a vacuum desiccator to give 12.78 g (92% yield). $^1$H NMR (270 MHz, DMSO-d$_6$), $\delta$ 7.63, doublet, 2H; $\delta$ 7.35, doublet, 2H; $\delta$ 6.41, doublet of doublets (AB) ($J$ = 11.5 and 73 Hz), 2H; $\delta$ 5.45, singlet, 1H; $\delta$ 4.46, quartet, 4H; $\delta$ 1.14, triplet, 6H.
5.2.7 Synthesis of N-Hydroxysuccinimido α-Bromoacetate

The N-hydroxysuccinimide ester of bromoacetic acid was synthesized essentially as described by Santi et al. (98). N-hydroxysuccinimide (1.15 g, 10 mmol) and bromoacetic acid (1.39 g, 10 mmol) were dissolved in 40 ml of acetonitrile. Dicyclohexylcarbodiimide (2.06 g, 10 mmol), dissolved in 10 ml of acetonitrile, was added dropwise over a 5 min period. The reaction mixture was immersed in an ice-water bath and stirred for 30 min. The ice-water bath was removed and the stirring continued for an additional 30 min. The dicyclohexylurea was collected by suction filtration and the filtrate concentrated by rotary evaporation in vacuo to a colorless syrup. The syrup crystallized upon standing at room temperature for 10 min. The product was recrystallized from isopropanol to give 2.19 g (93% yield) of white needles (mp 114-115°C). $^1$H NMR (90 MHz, DMSO-d$_6$), δ 4.60, singlet, 2H; δ 2.80, singlet, 4H. Anal. Calcd for C$_6$H$_6$BrNO$_4$: C, 30.53; H, 2.56; Br, 33.86; N, 5.93; O, 27.12. Found: C, 30.62; H, 2.67; Br, 33.68; N, 6.04; O, 27.27.
5.3 Discussion

The procedure outlined above for the conversion of p-bromobenzaldehyde diethylacetal to 4,4',4"'-phosphinidynetri(benzenemethanammonium)Tris(4-methylbenzenesulfonate) was designed to generate the product with a minimum of oxidation. The solid tosylate is substantially less susceptible to air oxidation than the precursors, hence this sequence of reactions was routinely performed in less than 48 hr. The recrystallized tosylate salt is stable for months when stored under vacuum.

The reactions have been performed starting with 5-100 g of p-bromobenzaldehyde diethylacetal. The percentage yield of Tris[4-(diethoxymethyl)phenyl]-phosphine was the most sensitive to the scale of reaction. Optimal yield was obtained when starting with 15-30 g of p-bromobenzaldehyde diethylacetal. When performed on this scale, the overall yield of pure 4,4',4"'-phosphinidynetri(benzenemethanammonium)Tris-(4-methylbenzenesulfonate) was 50-60%. The borane-THF reduction of the oxime was never run above 30 g due to the large reaction volume generated in this reaction.

The published procedures (73) for purification of the intermediate phosphine products on Florosil caused oxidation of the phosphine, even when the column was run in a glove box under an inert atmosphere. These
purification procedures were, however, found to be unnecessary. In the preparation of Tris[4-(diethoxy-
methyl)phenyl]phosphine, the only major contaminant is the starting material, p-bromobenzaldehyde diethyl-
acetal. Both the phosphine and para-bromo compound can be quantitatively converted to the oxime form. After
the borane-THF reduction of the oxime mixture, the only compounds soluble in water under acidic conditions are
p-bromobenzylamine, 4,4',4"-phosphindinidetri(benzene-
methanamine) and the phosphine oxide. Other impurities are removed by ether extraction. The p-bromobenzyl-
amine and phosphine oxide are then removed by fractional crystallization of the tosylate salt from 95% ethanol.

In the synthesis of Tris(diethoxymethyl)phenyl-
phosphine, the yield is very sensitive to the exact procedure used for formation of the lithioaryl anion. After addition of the N-butyllithium is complete, the temperature of the reaction mixture is allowed to slowly rise to 22-24°C. The length of time the solution must be stirred at this temperature is dependent upon the reaction scale. When starting with 15-30 g of p-bromobenzaldehyde diethylacetal, the reaction mixture must be stirred for 45-60 min to achieve maximum production of the lithioaryl anion. If the mixture is
allowed to stir at this temperature for excessive periods of time, decomposition occurs. The exact length of time to stir the mixture at room temperature is best determined from experience.

The reduction of the Tris-0-methyloxime is also sensitive to the reaction conditions employed. Drying the solvent, THF, over alumina immediately prior to use has been found to be necessary for repeatable success of the reaction. The reaction is extremely sensitive to trace amounts of water. For this reason, during seasons of high humidity, extreme precautions must be taken to ensure that the reaction vessel and THF are anhydrous.

Fractional crystallization of the tosylate salt of 4,4',4"'-phosphinidynetri(benzenemethanamine) provides a convenient method for isolating the product from contaminating p-bromobenzylamine and phosphine oxide. Generally, the first two to three crops of crystals were pure as judged by proton and phosphorus-31 NMR spectroscopy.
CHAPTER VI
PRELIMINARY BIOLOGICAL APPLICATIONS

6.1 Introduction
The derivatives of undecagold cluster described in Chapters III and IV have been developed for use in electron microscopic analysis of complex biological aggregates. The uniform derivatives discussed in Chapter III are potentially useful as non-specific, electron dense stains, while the mono-functional alkylating and acylating derivatives (Chapter IV) have been designed for chemical modification of specific sites within biological macromolecular aggregates. Mono\([N-(p\text{-maleimido})benzoyl]eicosi(N\text{-acetyl})undecagold\) contains a single maleimide ring and should be useful for selectively alkylating sulfhydryl groups. Mono\((N\text{-}\alpha\text{-bromoacetyl})eicosi(N\text{-acetyl})undecagold\) contains a single \(\alpha\text{-bromoacetamido}\) functional group. This derivative should also be useful as a sulfhydryl selective alkylating reagent. \(\alpha\text{-Bromoacetamides are also reactive toward amino groups and in some cases hydroxyl groups. Mono}[N-(N'\text{-hydroxysuccinimidosuccinyl})]eicosi(N,N\text{-dimethyl})undecagold\) and the eicosi(N-acetyl)
derivative are capable of acylating amino groups. Application of these reagents to the EM analysis of biological aggregates will be largely confined to use as protein modification reagents. For this reason the present discussion of potential problems involved in the use of these reagents will be confined to this area.

Successful electron microscopic analysis of protein aggregates labeled with any of the reagents described above depends upon several factors. In a large aggregate containing one or two labels, localization of the clusters within the structure can be difficult. Although the clusters are electron-dense, the ability to identify their positions within a large aggregate depends upon the orientation of the specimen with respect to the electron beam. With specimens that exhibit a reasonably random distribution of relative orientations, a sufficient number of species should be present in orientations permitting identification of the label. Once located, the position of the label must be determined relative to a second identifiable feature within the structure in order to map the labeled site. The ability to do this depends upon the gross structural features of the aggregate. For example, after labeling ribosomes with an undecagold cluster derivative, the position of the label might be
referenced to some specific aspect of the characteristic bi-lobed appearance of the aggregate.

In order to obtain high-resolution data on aggregates labeled with an undecagold cluster, computer averaging is sometimes necessary for enhancement of image detail. With structures exhibiting random orientations, this process is difficult. With aggregates which possess a regular repeating structure such as polymers of tubulin, the process of locating the labeled site and obtaining high-resolution data is analogous to the X-ray analysis of crystals. For this reason, systems of this type are particularly attractive candidates for labeling with undecagold cluster derivatives and subsequent EM analysis.

Chemical modification of amino acid functional groups by the undecagold cluster alkylating and acylating derivatives can be viewed as much the same as any other protein modification study. Such use of these clusters will best be applied to the analysis of proteins that have previously been studied with model amino acid selective modifying reagents, thus simplifying analysis of the gold complex modification reaction. Incorporation into proteins of undecagold cluster derivatives can be conveniently monitored using derivatives that have been radiochemically
labeled, as discussed in Chapter III. Identification of the subunits of an aggregate that have been labeled with an undecagold cluster derivative is also, in some cases, simplified by the relatively high molecular weight of the labeling reagent. For example, covalent binding of a single undecagold cluster derivative to a subunit of MW 30,000 results in a 15% increase in its molecular weight. This change is easily detected by polyacrylamide gel electrophoresis in SDS.

The derivatives of undecagold cluster described above are presently being employed in the chemical modification and electron microscopic analysis of a variety of biological preparations. Applications of these reagents to the analysis of bovine heart and rat liver mitochondria, *E. coli* PDH complex and bovine heart cytochrome oxidase are described in this section.
6.2 Experimental Procedures

6.2.1 Characterization of the Effects of Uniform Derivatives of Undecagold Cluster on the Respiration of Rat Liver Mitochondria

Liver mitochondria from a two-month old male Wistar rat were prepared according to standard procedures (101). The rate of oxygen consumption was monitored using succinate as the substrate. Rotenone was included to uncouple site 1, ensuring that all oxygen consumption originated from site 2 in the respiratory chain. ADP and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) were added to achieve state 3 respiration and to uncouple respiration, respectively. The effects of undecagold cluster and uniform derivatives on the respiration rate were characterized under the respiratory states described above. The results are presented in Table 16.

6.2.2 CTEM Analysis of Bovine Heart Mitochondria Treated with Undecagold Cluster and Heneicosi(N,N-Dimethyl)Undecagold

Respiring mitochondria were exposed to undecagold cluster during fixation with 50% glutaraldehyde. After about 5 min the mitochondria had completely floculated and >95% of the cluster had been adsorbed. A control
<table>
<thead>
<tr>
<th>Sample(^b)</th>
<th>Respiratory state(^c)</th>
<th>Relative rate of O₂ consumption(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>II</td>
<td>1.00</td>
</tr>
<tr>
<td>control</td>
<td>III</td>
<td>1.66</td>
</tr>
<tr>
<td>control</td>
<td>uncoupled</td>
<td>2.28</td>
</tr>
<tr>
<td>GC</td>
<td>II</td>
<td>1.0→2.14(^e)</td>
</tr>
<tr>
<td>GC</td>
<td>III</td>
<td>1.66</td>
</tr>
<tr>
<td>GC</td>
<td>uncoupled</td>
<td>2.28</td>
</tr>
<tr>
<td>DMGC</td>
<td>II</td>
<td>0.00</td>
</tr>
<tr>
<td>DMGC</td>
<td>uncoupled</td>
<td>0.00</td>
</tr>
<tr>
<td>ACGC</td>
<td>II</td>
<td>1.00</td>
</tr>
<tr>
<td>CMGC</td>
<td>II</td>
<td>1.00</td>
</tr>
<tr>
<td>PSGC</td>
<td>II</td>
<td>1.00</td>
</tr>
</tbody>
</table>

\(^a\) A 70 μl sample of rat liver mitochondria (20 mg/ml) was placed in 0.25 M mannitol, 70 mM sucrose, 1 mM potassium phosphate, 5 mM MgCl₂, 10 mM HEPES-triethylamine (pH 7.4), 10 mM succinate and 4 μM rotenone in the sample compartment of the oxygen electrode in a total volume of 2.6 ml. ADP (0.60 mM), FCCP (1.0 μM) and gold complex (4 μM) were added when needed. The rate of oxygen consumption was measured 1 min after the final addition.

\(^b\) The abbreviations are: GC, undecagold cluster; DMGC, heneicosi(N,N-dimethyl)undecagold; ACGC, heneicosi-(N-acetyl)undecagold; CMGC, heneicosi(N-carboxymethyl)-undecagold and PSGC, heneicosi(N-acetyl)undecagold.

Table 16 continued on following page.
Table 16 (continued)

State II is the rate of respiration from succinate. State III is achieved by addition of ADP to mitochondria in state II respiration. Uncoupling of respiration is achieved by addition of FCCP.

The oxygen electrode was calibrated using a sample of oxygen saturated buffer and dithionite. The measurement of respiratory rates was made 1 min after the final addition.

After the addition of undecagold cluster, the rate of respiration shows a time dependent increase. After 4 min the relative rate of 2.14 was observed.
incubation without undecagold cluster remained suspended for several hours. The binding of undecagold cluster was not dependent upon the presence of a substrate for respiration or upon the addition of the fixative, glutaraldehyde. Addition of heneicosi(N,N-dimethyl)-undecagold resulted in the same phenomenon, however, the mitochondria were completely settled in less than 1 min.

Thinly sliced samples were prepared for electron microscopy either unstained or mildly stained with uranyl acetate and lead citrate to enhance detail in the membranes. Samples were also prepared using osmium tetroxide as both a fixative and stain. The results of these preliminary studies are discussed in section 6.3.

Some of these specimens were also analyzed by STEM X-ray fluorescence microprobe analysis. In an analysis of this type selected regions of a specimen are irradiated (~100 KeV). The irradiated sample emits X-ray photons whose wavelengths are characteristic of the elements being irradiated. The instrument collects the emitted photons and measures their energies in a multi-channel analyzer. The number of counts in a given channel is proportional to the number of photons of a defined energy range. These results are also discussed in section 6.3.
6.2.3 Labeling of *E. coli* PDH Complex

*E. coli* PDH complex (40 μmol NADH/min/mg protein), prepared according to published procedures (101,102) was incubated with samples of α-bromoacetylated (N,N-dimethyl)-undecagold and with the corresponding maleimide derivative. These alkylating clusters were prepared from eicosi(N,N-dimethyl)undecagold; however, as discussed in Chapter IV, they contain significantly more than the expected number of alkylating groups per cluster. These derivatives were used for initial studies. Labeling of the sulfhydryl group in E₁ of the intact PDH complex was achieved directly by incubating the enzyme with the undecagold derivatives. Labeling E₂ was achieved by the preliminary addition of pyruvate, TPP and Mg²⁺ to reductively acetylate the lipoic acid moieties and protect the E₁ sulfhydryl group prior to addition of the gold complex. A sample of mono(N-phthalyl)eicosi-(N,N-dimethyl)undecagold was used for control incubations. The results of these studies are presented in Table 17. Samples of the labeled PDH complex were prepared for EM analysis with and without glutaraldehyde fixation. Samples were negatively stained with uranyl acetate for bright phase CTEM and unstained samples were prepared for dark phase CTEM. The results of electron microscopic analysis are discussed in section 6.3.
### TABLE 17
Modification of *E. coli* PDH Complex with Mono-functional Gold Complex Alkylation Reagents

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pyruvate, TPP, Mg(^{2+})</th>
<th>μmol NADH/min/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPDMGC</td>
<td>+</td>
<td>30.2</td>
</tr>
<tr>
<td>BADMGC</td>
<td>-</td>
<td>31.9</td>
</tr>
<tr>
<td>Bromoacetamide</td>
<td>-</td>
<td>15.4</td>
</tr>
<tr>
<td>BADMGC</td>
<td>+</td>
<td>23.3</td>
</tr>
<tr>
<td>MIDMGC</td>
<td>+</td>
<td>11.1</td>
</tr>
<tr>
<td>Bromoacetamide</td>
<td>+</td>
<td>15.1</td>
</tr>
<tr>
<td>MPDMGC</td>
<td>+</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>34.0</td>
</tr>
</tbody>
</table>

*E. coli* PDH complex (2.08 μM, 40 μmol NADH/min/mg) was placed in 10 mM Pipes buffer, pH 7.0. Alkylation reagents (0.10 mM) were added directly to label E₁ sulfhydryl groups. A 1 min pre-incubation in 5.0 mM pyruvate, 0.20 mM TPP and 1.0 mM MgSO\(_4\) was used for labeling of the lipoic acid moieties. The samples were incubated for 18 hr at 4°C.

The abbreviations are: MPDMGC, mono(N-phthalyl)eicosi-(N,N-dimethyl)undecagold; BADMGC, N-a-bromoacetyl(N,N-dimethyl)undecagold and MIDMGC, N-(p-maleimido)benzoyl-(N,N-dimethyl)undecagold.

+ refers to the E₂ labeling conditions while - refers to the E₁ labeling conditions.

The amount of NADH produced per minute was measured at 340 nm on the Unicam SP-1800 spectrophotometer in 5.0 mM sodium pyruvate, 0.20 mM TPP, 1.0 mM MgSO₄, 0.10 mM CoenzymeA, 3.0 mM cysteine, 2.33 mM NAD⁺ and 0.05 M potassium phosphate, pH 8.1.
6.2.4 **Labeling of Cytochrome Oxidase Membrane Vesicles**

Suspensions of cytochrome oxidase membrane vesicles were prepared from beef heart mitochondria according to established procedures (103,104). The vesicles were incubated with a 5-10 molar equivalent of either mono-(N-α-bromoacetyl)eicosi(N-acetyl)undecagold or the maleimide derivative. A control reaction was run with no gold complex added. After a 4-1/2 hour incubation, aliquots were removed and reacted with a 5-fold excess of N-iodoacetyl-N'-(5-sulfo-1-naphthyl)ethylenediamine (I-AEDANS) in the dark for 2 hr. The remaining material was frozen in a dry ice-acetone bath and stored at -70°C for subsequent CTEM, STEM and X-ray fluorescence microprobe analysis. The samples treated with I-AEDANS were heated at 50°C for 15 min in 20% SDS, 6 M urea, 20% glycerol, Tris-HCl, pH 6.8, to dissociate the vesicles. These samples were applied to a 13.5% polyacrylamide slab gel cast in electrophoresis buffer containing SDS. The electrophoresis was performed using Laemmli/Kadenbach focusing and running buffers (105,106). The gel was examined under a fluorescent lamp to locate bands containing the fluorescent label I-AEDANS. The slab gel was then fixed in 10% TCA-50%
methanol for 2 hr, stained with 0.25% Coomassie Blue, 10% acetic acid-50% methanol for 3 hr and destained overnight in 10% acetic acid-10% methanol. The destained gel was photographed (Figure 38), sliced into individual lanes, and scanned at 620 nm. The gel scans for the control and the labeled samples are shown in Figure 40. The frozen stock samples were examined by X-ray fluorescence microprobe analysis and an example of the data obtained is shown in Figure 39.
Figure 38. SDS Polyacrylamide Disc Gel Electrophoresis of BAACGC and MIACGC Treated Cytochrome Oxidase

Cytochrome oxidase vesicles were incubated with mono(N-α-bromoacetyl)eicosi(N-acetyl)undecagold (BAACGC), mono[N-(p-maleimido)benzoyl]eicosi(N-acetyl)-undecagold (MIACGC) and I-AEDANS followed by gel electrophoresis as described in the text. The gel was stained with Coomassie Blue also as described in the text.

Figure 39. X-ray Fluorescence Microprobe Analysis of MIACGC Treated Cytochrome Oxidase Vesicles
Figure 40. 620 nm Gel Scans of BAACGC and MIACGC Treated Cytochrome Oxidase

The slab gel (Figure 38) was sliced into individual lanes and scanned in an ISCO Model 1310 gel scanner at 620 nm at a scan rate of 30 cm/min.
FIGURE 40

BAACGC

MIACGC

Control

Band I II III IV V VI

Distance (cm)

A

620
6.3 Results and Discussion

The studies on isolated rat liver and bovine heart mitochondria treated with uniform derivatives of the undecagold cluster have provided insight into the binding interactions that may occur when performing labeling experiments using the mono-functional alkylating and acylating derivatives. Both undecagold cluster and heneicosi(N,N-dimethyl)undecagold influence the respiration of rat liver mitochondria while heneicosi(N-acetyl)undecagold, heneicosi(N-carboxymethyl)undecagold and heneicosi(N-succinyl)undecagold do not. The reason for a time dependent increase in the rate of respiration in samples treated with undecagold cluster compared to abrupt cessation of respiration upon exposure to heneicosi(N,N-dimethyl)undecagold is not clear.

The results of the respiration studies were paralleled by measurement of the amount of the various uniform derivatives absorbed by bovine heart mitochondria. Once again, only the positively charged derivatives appeared to interact with the mitochondria and were rapidly and nearly quantitatively adsorbed.

Negatively stained samples of the undecagold cluster treated mitochondria appear similar to the negatively stained control sample. The most striking difference
in the appearance of the gold complex treated mitochondria is the presence of heavily stained ring structures within the matrix. Unstained specimens also show these ring structures as well as some weak staining of the membranous structures in the mitochondria. Samples of gold complex treated mitochondria fixed and stained with osmium tetroxide are similar to the negatively stained specimens; however, the ring-like structures are considerably more heavily stained. This "osmiophilicity" has been observed with other amine reagents such as diaminobenzidine. X-ray fluorescence microprobe analysis of the negatively stained samples confirmed the presence of gold in the ring structures.

Both mono(N-α-bromoacetyl)eicosi(N,N-dimethyl)-undecagold and mono(N-(p-maleimido)benzoyl)eicosi-(N,N,-dimethyl)undecagold are capable of inactivating the \textit{E. coli} PDH complex. Using a 2:1 ratio of gold complex to targeted sulfhydryl groups, 40-50% inhibition of the overall enzymatic activity was observed. The interacting network of \(E_1\) active sites and \(E_2\) lipoyl groups in this complex (107-109) make the total inactivation of the cluster possible only after near quantitative modification of either the TPP protected \(E_1\) sulfhydryl group or \(E_2\) lipoyl moieties. For this
reason, the number of groups modified is not proportional to the % activity of the cluster after labeling.

The samples of labeled complex prepared for EM analysis all showed significant aggregation. This aggregation makes EM analysis difficult if not impossible. Dark phase CTEM photomicrographs showed many dark spots on the aggregated protein. These spots may be the images of aggregates of the gold complex. This problem of aggregation of the protein sample was also observed when the N,N-dimethyl derivatives were incubated with glutamine synthetase and cytochrome oxidase vesicles. Attempts to use the undecagold cluster and heneicosi-(N,N-dimethyl)undecagold to dope crystalline preparations of glutamate dehydrogenase and fumarase led to serious disruption of the regular crystal packing arrangement. From the studies described above it was concluded that the highly positively charged derivatives of the cluster may be of limited use in labeling protein aggregates for EM analysis.

The extent of specimen aggregation upon exposure to the positively charged undecagold derivatives is probably, to some degree, dependent upon the isoelectric point for the particular protein aggregate. The labeling of the biotin binding site in avidin by Safer et al. (76) was successfully achieved with minimal non-specific
aggregation using a positively charged gold complex. Some success has also been achieved in preliminary labeling studies with the highly positively charged histone proteins using a N,N-dimethyl undecagold derivative.

From the preceding discussion, the importance of preparing mono-functional alkylating and acylating clusters with variable net charges is clear. The form of gold complex best suited for use in a labeling study will undoubtedly vary depending on the properties of the targeted structure.

The symmetrical structure and multiplicity of identical sulfhydryl groups in the α-keto acid dehydrogenase complexes will make the analysis of electron microscopic data on samples selectively labeled with gold complex derivatives very difficult. It will most likely be necessary to resolve the complex into its component enzymes, label the desired subunit and then prepare reconstituted sub-complexes in order to unravel the symmetry relationships in these complexes.

The most promising data on the labeling of specific sites within a complex biological aggregate using mono-functional alkylating clusters has been obtained with preparations of cytochrome oxidase vesicles. The cytochrome oxidase complex consists of at least seven
different polypeptide chains (110-112). There is still some dispute concerning the number of low molecular weight (<5000) subunits associated with the complex. The major components have been designated subunits I-VII in order of decreasing molecular weight. Subunits II (MW 28,000) and III (MW 23,000) contain exposed sulfhydryl groups that may be chemically modified using sulfhydryl selective reagents. This was confirmed in the control incubation described in section 6.2.4 in which the membrane vesicles were treated with the fluorescent reagent I-AEDANS. Examination of the slab gel showed incorporation of fluorescent label into both subunits II and III.

Labeling of cytochrome oxidase with mono(N-α-bromoacetyl)eicosi(N-acetyl)undecagold and mono[N-(p-maleimido)benzoyl)eicosi(N-acetyl)undecagold was characterized by investigating both the ability of these reagents to prevent incorporation of the fluorescent label I-AEDANS and by observed changes in mobility of the labeled enzyme on polyacrylamide gels. Examination of the unstained gels under a UV lamp showed weak fluorescence in bands II and III of the sample labeled with the bromoacetyl derivative of the cluster and virtually no fluorescence in bands II and III of the
sample treated with the maleimide derivative. From the photograph of the Coomassie Blue stained gel (Figure 38), movement of band II to a position of slightly slower migration is clearly visible in the sample treated with mono(N-α-bromoacetyl)eicosi(N-acetyl)-undecagold. A considerable decrease in the intensity of band III was also seen. Much the same result was obtained with the sample treated with the maleimide derivative. The movement of band II is clearly illustrated in the gel scan shown in Figure 40.

The samples of mono-functional alkylating clusters used in this study were acetylated by the acetic anhydride procedure. As discussed previously, this treatment results in the formation of dimers and trimers of the cluster. Labeling of the enzyme by these multimeric forms of the cluster may in part explain the diffuse appearance of band III after labeling. Labeling of band III may cause a shift in position of the subunit to a position coincidental with that of unlabeled band II which would also explain some of the apparent loss of band III.

X-ray fluorescence microprobe analysis of the labeled samples of cytochrome oxidase show the presence of a significant amount of gold in the sample. The enzyme contains two atoms of iron per complex. As can
be seen from the spectrum shown in Figure 39, considerably more gold appears to be present. Accurate calibration of the instrument with a suitable standard will be necessary before quantitative information can be obtained from this type of analysis, however, it seems clear that gold is present in the sample.

The characterization of cytochrome oxidase membrane vesicles treated with the mono-functional alkylating derivatives of the undecagold cluster discussed above strongly suggests that the alkylating derivatives of the undecagold cluster selectively react with subunits II and III of the multi-subunit complex. The use of acetylated derivatives of the cluster appears to have greatly reduced the problem of aggregation of the sample.

Whether the uncharged, acetylated clusters are adsorbed into the vesicle membranes is a question being addressed in conjunction with the CTEM and STEM analysis of the labeled samples discussed above. The zwitterionic N-carboxylmethyl,N-methyl derivatives may be useful in preventing this problem if it is indeed occurring. The labeling of cytochrome oxidase discussed above has provided encouraging signs that the mono-functional alkylating derivatives of tricyanoheptakis-[4,4',4"-phosphinidynetri(benzenemethanamine)]undecagold
described here may indeed be useful as electron-dense reagents in the selective modification of specific sites within a complex biological aggregate. Subsequent electron microscopic analysis of structure and structure-function relationships may thereby be significantly facilitated.
REFERENCES


76. D. Safer, J. Hainfeld, J. S. Wall and J. E. Reardon, Science, in press.


78. M. Apfel, unpublished data.


