Bergström, S., Carlson, L. A., and Weeks, J. R. (1968), *Pharmacol. Rev. 20*, 1.
Bergström, S., Danielsson, H., and Samuelsson, B. (1964), *Biochim. Biophys. Acta 60*, 207.
Granström, E., Lands, W. E. M., and Samuelsson, B. (1968), *J. Biol. Chem. 243*, 4104.
Gréen, K., and Samuelsson, B. (1964), *J. Lipid Res. 5*, 117.
Hamberg, M., and Samuelsson, B. (1967), *J. Biol. Chem. 242*, 5336.
Kupiecki, F. P. (1965), *Life Sci. 4*, 1811.
Lands, W. E. M., and Samuelsson, B. (1968), *Biochim. Biophys. Acta 164*, 426.
Lapidus, M., Grant, N. H., and Alburn, H. E. (1968), *J. Lipid Res. 9*, 371.
May, H. E., and McCay, P. B. (1968), *J. Biol. Chem. 243*, 2288.
Pace-Asciak, C., and Wolfe, L. S. (1970a), *Biochim. Biophys. Acta 218*, 539.
Pace-Asciak, C., and Wolfe, L. S. (1970b), *Chem. Commun.*, 1234.
Pace-Asciak, C., and Wolfe, L. S. (1970c), *Chem. Commun.*, 1235.
Pace-Asciak, C., and Wolfe, L. S. (1971), *Biochemistry 10*, 3657.
Ramwell, P. W., Shaw, J. E., Clarke, G. B., Grostic, M. F., Kaiser, D. G. and Pike, J. E. (1968), *Progr. Chem. Fats Other Lipids IX*, 231.
Sih, C., Takeguchi, C., and Foss, P. (1970), *J. Amer. Chem. Soc. 92*, 6670.
Struijk, C. B., Beerthuis, R. K., Pabon, H. J. J., and Van Dorp. D. A. (1966), *Recl. Trav. Chim. Pay-Bas 85*, 1233.
Vonkeman, H., and Van Dorp, D. A. (1968), *Biochim. Biophys. Acta 164*, 430.
Wallach, D. P. (1965), *Life Sci. 4*, 361.

Gold(III) Complexes of Adenine Nucleotides*

Dean W. Gibson,† Michael Beer, and Russell J. Barrnett

ABSTRACT: Adenine, adenosine, and adenine nucleotides (AMP, dAMP, 3′AMP, cAMP, ADP, and ATP) were allowed to react with equilibrium systems of hydroxochloroaurate ions, at several pH + pCl. Potassium salts of gold–adenine nucleotide adducts, Au·A (K), were isolated by ethanol precipitation, and analyzed for gold and phosphate contents. In mixtures containing 4 mM A and 12 mM Au ions at pH 5–8, the adducts contained 2 Au/A after 4 hr, and 3 Au/A after 24 hr. The adducts appear to be polymerized to some extent, possibly because of N → Au ← N bridges. The Au·Ade and Au·Ado adducts are very insoluble in aqueous media, whereas potassium salts of gold–adenine nucleotides are soluble to greater than 10 mM [A]. Magnesium, calcium, and manganous salts of Au·cAMP are very water soluble, whereas the corresponding salts of Au·AMP, Au·ADP, and Au·ATP are relatively insoluble, to degrees that depend on the number of phosphates and the divalent cation. The Au·A (K) adducts were allowed to react further with amino acids, related amines, and with biological stains containing primary and heterocyclic amines. The resulting amine·Au·A adducts were water soluble. In contrast, hydroxochloroaurate ions were reduced to Au(0) by amino acids, and gave insoluble amine·Au adducts with most other amines. These differences between Au·A adducts and hydroxochloroaurate ions, in reactivities toward amines and amino acids, are evidence that gold(III) is tightly bound to adenine nucleotides. Amine·Au·A adducts may contain N → Au ← N bridges between amino or heterocyclic nitrogens in the amine and adenine residues. The binding of gold(III) to adenine residues has potential applications for electron microscope studies of base sequences within isolated nucleic acids. The coupling of Au·A adducts to tissue amines, or to biological stains, has potential applications for electron microscope cytochemistry of nucleoproteins within tissue. Solubility differences between Au·Ado and potassium and divalent cation salts of gold–adenine nucleotide adducts suggest methods for electron microscope cytochemical localizations of nucleotide-dependent enzymes within tissue.

Many metals are known to bind ionically to nucleotide phosphates; others bind covalently to sugar hydroxyl groups, or coordinate with nitrogens in the base moieties (*e.g.*, Eichhorn *et al.*, 1970). Such interactions affect the secondary and tertiary structures of RNA and DNA, and the activities of polymerases, nucleases, esterases, kinases, and other nucleotide- or polynucleotide-dependent enzymes. Heavy metal interactions with nucleotides and polynucleotides are also potentially useful in physical techniques such as X-ray diffraction and electron microscopy. Beer and coworkers have studied reactions of several heavy metals with base moieties of nucleotides and nucleic acids, with a view toward determining base sequences by electron microscopy (Beer and Moudrianakis, 1962; Moudriankis and Beer, 1965; Beer *et al.*, 1966, 1970). Heavy atom derivatives of nucleotides are potentially useful for cytochemical localizations of enzymes by electron microscopy, if they can be converted by tissue enzymes

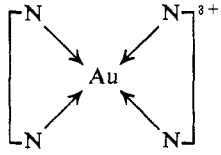
* From Yale University School of Medicine, New Haven, Connecticut, and The Johns Hopkins University, Baltimore, Maryland. *Received March 9, 1971.* This work was supported by the National Institute of General Medical Sciences (GM-18840 and GM-08968), and by the National Cancer Institute (TICA-05055). This is the first in a series of papers on gold(III) reactions with nucleotides and polynucleotides, based in part on the Ph.D. Dissertation of D. W. G. (Gibson, 1969). Preliminary reports were presented in Beer *et al.* (1963, 1970) and Gibson and Beer (1968).

† Present address: Virology Branch, Armed Forces Institute of Pathology, Washington, D. C. 20305; to whom correspondence should be addressed.

from soluble substrate analogs into insoluble products (Barrnett, 1964).

This work is concerned with reactions of gold(III) with adenine, adenosine, and adenine nucleotides. Subsequent papers will describe analogous reactions with guanine, hypoxanthine, cytidine, uracil, and thymine nucleotides, NAD(H), polynucleotides, RNA, and DNA.

Gold(III) does *not* occur as Au^{3+} in aqueous solutions, but rather occurs in square-planar complexes; ligands include halide, hydroxide and cyanide ions, NH_3, primary and heterocyclic amines, and sulfhydryl compounds (Sidgwick, 1949; Gmelin, 1954). With ethylenediamine, En,[1] a chelated complex $Au(En)_2^{3+}$ is formed, from which one proton dissociates with pK_b of 6.5 (*e.g.*, Gangopadhayay and Chakravorty, 1961).



Chloroauric acid, $HAuCl_4 \cdot 3H_2O$, is a common commercial form of gold(III). It is water soluble to greater than 20 M [Au], and is extensively ionized in dilute solutions, as $H^+ + AuCl_4^-$, (Gmelin, 1954). The $AuCl_4^-$ undergoes stepwise hydrolysis to equilibrium systems of hydroxochloroaurate ions or Au ions, according to eq 1 and 2 (Bjerrum, 1948; Bardet and Bontoux,

$$AuCl_{5-n}(OH)_{n-1}^- + H_2O \overset{K_n}{\rightleftharpoons} AuCl_{4-n}(OH)_n^- + H^+ + Cl^- \quad (1)$$

$$K_n = \frac{[AuCl_{4-n}(OH)_n^-][H^+][Cl^-]}{[AuCl_{5-n}(OH)_{n-1}^-]} f^2; \text{ for } n = 1, 2, 3, \text{ and } 4 \quad (2)$$

1962), where f is an activity coefficient approximated by $-\log f = pf = (I)^{1/2}/(1 + (I)^{1/2})$, for ionic strength I. From titrometric studies, these authors reported: $pK_1 = 6.1$, $pK_2 = 7.1$, $pK_3 = 8.1$, and $pK_4 = 8.5$–9.3. The first and second hydrolyses are complete in about 1 min and 1 hr, respectively, whereas the third and fourth take several hours.

At equilibrium the gold mole fraction in the form of each species $AuCl_{4-n}(OH)_n^-$ is a function of pH + pCl + pf^2; at low ionic strengths, pf^2 is negligible. The predominant species are: (a) $AuCl_3(OH)^-$, $AuCl_2(OH)_2^-$, and $AuCl(OH)_3^-$, respectively, in aqueous solutions of 12 mM [Au] at pH 4, 5.5, and 7, as in reaction mixtures A-3; (b) $AuCl_4^-$, $AuCl_3(OH)^-$, and $AuCl_2(OH)_2^-$, respectively, in aqueous 0.2 M KCl at pH 4, 5.5, and 7, as for ethanol precipitation of Au·A (K) adducts (Experimental Procedures 5). Higher pH + pCl were avoided, since $Au(OH)_4^-$ forms orange colloidal suspensions, and decomposes to some extent to insoluble $Au(OH)_3^0$ and OH^- above 10 mM [Au] (Bjerrum, 1948).

Below pH + pCl + $pf^2 = 3$, where $AuCl_4^-$ is the only Au ion species present, there is a band at 313 nm, with $\epsilon_{Au}(313) = 5.5 \times 10^3$ OD M^{-1} cm^{-1}; this is used in gold determinations (Experimental Procedures 7). This band has been attributed to a $p \rightarrow d_{z^2-y^2}$ (chloride to gold) charge transfer (Gangopadhayay and Chakravorty, 1961). As the pH + pCl + pf^2 increases (as OH^- replaces Cl^- in Au ions), the absorbance of the band decreases, and there is a blue shift in the position of the band. The aurate ion, $Au(OH)_4^-$, has no band in the near-uv region. A solution of 0.1 mM $HAuCl_4$ in 10% En at pH 12.3 yields the dissociated form of $Au(En)_2^{3+}$ (above). This solution has a broad band near 300 nm, with $\epsilon_{Au}(300) = 2.8 \times 10^3$, attributed to a (nitrogen to gold) charge transfer (Gangopadhayay and Chakravorty, 1961).

Reactions of $AuCl_4^-$ with pyridine, quinoline, and their derivatives, in several alcoholic solvents, have been described by Cattalini *et al.*, 1968 (and references cited therein). They found the major pathway in amine–chloroaurate reactions to be a bimolecular nucleophilic substitution (S_N2) of amine for chloride.

Wulff (1893) reported the reaction of $AuCl_4^-$ with adenine in dilute HCl to give a water-insoluble adduct containing 1 Au/Ade. Holtz and Müller (1924) produced a 2 Au/Ade adduct by a modified procedure. Water-insoluble adducts of guanine, xanthine, and hypoxanthine, containing 1 Au/base, were subsequently described by Hoppe-Seyler and Schmidt (1928). Hartman (1967) briefly cited changes in infrared spectra as evidence for formation of gold(III) complexes with AMP, GMP, cytidine, and uridine. Highton and Beer (1968) reported electron microscope studies of polyadenylic acid reacted with Au ions. Fitzsimons *et al.* (1970) described a method for cytochemical localization of acid phosphatase, using an Au·AMP (K) adduct as substrate.

Experimental Procedures

1. Adenine Nucleotides and Related Compounds. Ade, Ado, AMP, 3′AMP, cAMP, Na_2·ADP, and Na_2·ATP were from Schwarz BioResearch, Inc. (Orangeburg, N. Y.), and Na_2·dAMP, from Calbiochem Co. (Los Angeles, Calif.). Stock solutions of 10 mM A, in water, 0.2 M KCl, or 0.2 M potassium acetate buffer, were adjusted to the desired pH with KOH and HCl. Stock solutions of 10 mM ribose 5-phosphate (Schwarz BioResearch, Inc.) and deoxyribose 5-phosphate (Nutritional Biochemical Co., Cleveland, Ohio) were similarly prepared. The [A] were estimated from the Ab(259), and in some cases were confirmed by nucleotide phosphate analysis (Experimental Procedures 8). For ^{14}C studies, [8-^{14}C]dAMP (Schwarz BioResearch, Inc.) in 50% ethanol was evaporated to dryness, and dissolved in 10 mM dAMP, for a final activity of 0.035 Ci/mole.

2. Au Ions. Aqueous stock solutions of 25 mM $HAuCl_4$ were prepared from reagent grade $HAuCl_4 \cdot 3H_2O$ (Fisher Scientific Co., Pittsburgh, Pa.): 1/8-oz. bottle/400 ml (chloroauric acid is hygroscopic, and difficult to weigh accurately). The [Au] of stock solutions were determined from the Ab(313) of a 1:250 dilution in 0.1 N HCl (1-cm light path), using $\epsilon_{Au}(313)$

[1] Abbreviations not listed in *Biochemistry 5*, 1445 (1966), are: A, generic abbreviation for Ade, Ado, AMP, dAMP, 3′AMP, cAMP, ADP, and ATP, using abbreviations in *Biochemistry 9*, 4022 (1970), except 3′AMP = adenosine 3′-phosphate, and cAMP = adenosine 3′:5′-cyclic phosphate = cyclic AMP; [P], nucleotide phosphate concentration, of uncomplexed A, or Au·A adduct; [A], calculated concentration of adenine residues in A or Au·A adduct: [A] = (1/*n*) [P], where $n = 1$ for AMP, dAMP, 3′AMP, and cAMP; $n = 2$ for ADP; and $n = 3$ for ATP; Au ions, equilibrium system of hydroxochloroaurate ions, $\{AuCl_{4-n}(OH)_n^-, (n = 0, 1, \ldots 4)\}$, at specified pH + pCl; [Au], gold(III) concentration; En, ethylenediamine, $H_2N(CH_2)_2NH_2$; reaction mixtures A-1: 5 mM A + 5 mM Au ions, and A-3: 4 mM A + 12 mM Au ions, at initial pH, pH(t_0); Au·Ade, Au·Ado, gold adducts of adenine and adenosine; Au·A (K, M^{2+}, ...), potassium or M^{2+}, ... salts of gold adducts of adenine nucleotides A; amine·Au·A (K or Tl), potassium or thallium(I) salts of adducts of amines with Au·A, where "amine" includes amino acids (AA) and biological stains containing primary and heterocyclic amines; gold extinction coefficient $\epsilon_{Au}(\lambda) = Ab(\lambda)/[Au] \cdot l$, and adenine residue extinction coefficient $\epsilon_A(\lambda) = Ab(\lambda)/[A] \cdot l$, where $Ab(\lambda)$ = absorbance at wavelength λ (nm), in optical density units (ODU), and l = light path in centimeters.

$= 5.50 \times 10^3$ OD M^{-1} cm^{-1}. Equilibrium systems of 10 or 20 mM Au ions were prepared by titrating 25 mM $HAuCl_4$ with 6 N KOH to the desired pH, and equilibrating overnight.

3. All aqueous solutions were prepared with deionized distilled water, using reagent grade compounds. The TlOH and $TlNO_3$ were from K & K Labs, Inc. (Plainview, N. Y.). Thiocarbohydrazide, $CS(NHNH_2)_2$, was from Polysciences, Inc. (Warrington, Pa.). Stock solutions of 80 mM potassium glutamate, potassium aspartate, and thallium(I) aspartate at pH 7.0 were prepared by titration of slurries of L-glutamic acid or DL-aspartic acid with KOH or TlOH.

4. Reaction mixtures A-1 and A-3 were prepared by mixing appropriate aliquots of 10 mM A, and 10 or 20 mM Au ions (Experimental Procedures 1 and 2), each in water, 0.2 M KCl or 0.2 M acetate buffer, at the desired pH.

5. Isolation and Analysis of Au·A Adducts. The Au·Ade and Au·Ado adducts, which precipitated from the respective A-1 and A-3 mixtures (without ethanol addition), were centrifuged, dried, and analyzed for gold content as described below for Au·A (K) adducts. Water-soluble potassium salts of gold–adenine nucleotide adducts, Au·A (K), were isolated by ethanol precipitation: a 60-ml aliquot of A-1 or A-3 was brought to 0.2 M KCl (or 0.2 M potassium acetate), and then two to four volumes of cold ethanol was added. After centrifugation for 30 min at 600*g*, 2°, the supernatant was decanted, and the pellet dried, *in vacuo*, to yield the Au·A (K) adduct. Gold analysis was performed on 40–60 mg of adduct (Experimental Procedures 7). Another 12–25 mg of adduct was dissolved in 50 ml of water for uv spectrum (1-mm light path), and for phosphate analysis (Experimental Procedures 8). The results were expressed as: % Au; % P; mole of Au/mole of A; and $\epsilon_A(\lambda)$, for [A] = [P]/*n*. The saturation ratio, mole of *bound* Au/*total* mole of A, was estimated as follows for mixture A-3 at time *t*. The Ab(λ) of A-3 at time *t* was compared to a system of spectra generated by appropriate linear combinations of the Ab(λ) for A, Au ions, and Au·A (K) adducts having 1, 2, 3 Au per A. At the chosen λ (290 and 330 nm), the Ab(λ) of A was nil; and the Ab(λ) of the Au ions, much less than the Ab(λ) of the adducts (*cf.* Figure 1a,b).

6. Ultraviolet spectra between 220 and 370 nm were taken on a Bausch & Lomb Spectronic 505 double-beam spectrophotometer, using matched quartz cells with 1-mm or 1-cm light path.

7. Gold and Thallium Analysis. Gold, in any oxidation state, was converted to gold(III) by treatment with aqua regia (after Herschlag, 1941); $[AuCl_4^-]$ was then determined from the Ab(313) (after Vydra and Čelikovský, 1958): to sample (0–120 μmole of Au/10 ml, in a 250-ml round-bottomed flask) was added 7.5 ml of concentrated HCl and 2.5 ml of concentrated HNO_3. Flask was heated briefly in hood, until all of sample was in solution. When cool, 12.5 ml of 4–6% NaOCl (Fisher Scientific Co.) was added, causing effervescence of chlorine gas. After addition of 17.5 ml of water, solution was evaporated just to dryness in hood, avoiding overheating (gold reduction). Residue was dissolved volumetrically in 100 ml of 0.1 N HCl, and the Ab(313) measured (1-mm light path). The method was calibrated with purified powdered gold, (Fisher Scientific Co.), and the results fitted by the method of least squares. Representative results were μmoles of Au/100 = [Ab(313) − 0.090]/0.550; root-mean-square deviation = 0.4 μmole of Au. Nucleotides and inorganic phosphate did not interfere. Samples containing no gold had Ab(313) = 0.090, because of the shoulder of the nitrate band at 300 nm. (Repeated evaporation to dryness from concentrated HCl, to reduce the nitrate ion concentration (Herschlag, 1941), was considered unnecessary for this work.)

Thallium determinations were by EDTA titration, after aqua regia digestion to Tl(III) (Pribil *et al.*, 1961). The presence of Au(III) did not interfere with Tl determinations, and *vice versa*. The same aqua regia digest was used for both Au and Tl determinations on Asp·Au·dAMP (T1) adducts.

8. Organic (nucleotide) phosphate analysis was performed on 0.10-ml aliquots of nucleotide A, or Au·A (K) adduct (0.3–0.5 mM in [P]), by the method of Ames and Dubin (1960). The determinations were calibrated with nucleotide solutions having known Ab(259), or with dibasic potassium phosphate, in the presence of 0.0–2.0 mM Au ions. Calibrations over the range 0.0–0.5 mM [P] were fitted by the method of least squares to: [P] = [(Ab(820) − a_0)/a_1] (mM). Representative results, in the presence or absence of 0.8 mM Au, respectively, were: a_0 = 0.029 or 0.065 ODU; a_1 = 2.69 or 2.66 ODU/mM [P]; root-mean-square deviation = 0.008 or 0.014 mM [P]. Thus the presence of Au(III) increased the intercept a_0 slightly, but had a negligible effect on the slope a_1.

9. For thin-layer chromatography, 10-μl aliquots of sample were applied to Eastman Chromagram sheets Type K301R: silica gel, with fluorescent indicator (Distillation Products Industries, Rochester, N. Y.). Ascending chromatograms were developed for 1 hr with solvent 1 (Mangold, 1965): *n*-butyl alcohol + acetone + glacial acetic acid + 5% NH_3 + water (7 + 5 + 3 + 3 + 2). Chromatograms were dried, examined with a short-wavelength uv lamp, and (for ^{14}C studies) cut into 2.5-cm strips which were scanned with a Nuclear-Chicago Model C-11B Actigraph II scanner.

10. For cellulose acetate paper electrophoresis, 10-μl aliquots of sample were applied to Oxoid strips (Colab Labs, Chicago Heights, Ill.). (Gold(III) complexes were largely immobile during electrophoresis on Whatman No. 3MM paper.) Electrophoresis was run for 30 min at a regulated constant current of 13 mA/5 cm wide Oxoid strip, using either 0.1 M potassium acetate buffer at pH 5.5, or 0.1 M potassium phosphate buffer at pH 7.0. The strips were dried, examined with a uv lamp, and scanned for ^{14}C as in Experimental Procedures 9.

11. Column chromatography was performed in 18 mm diameter × 300 mm long columns of: Cellex-N, -D, -CM, and -SE (Bio-Rad Labs, Richmond, Calif.); Dowex resins AG50W-X8 and AG1-X8 (Bio-Rad Labs); and Sephadex G-10, G-25, and G-50 dextran gels (Pharmacia Fine Chemicals, Inc., Piscataway, N. Y.). Sephadex columns were eluted with 0.03 M potassium acetate buffer at pH 5.5. Ion-exchange columns were packed under pressure, and eluted with 0.01 M acetate buffer at an appropriate pH, with or without a 0.0–0.1 M KCl gradient. The applied sample was 1 ml of 4 mM nucleotide A, 4 mM Au·A (K), or 4 mM Au ions, in the elution buffer. Columns were eluted at a flow rate of 0.6 ml/min. Sephadex columns were calibrated with Blue Dextran 2000 (Pharmacia Fine Chemicals, Inc.), monitored by the Ab(625); and with KCl, monitored by AgCl precipitation upon addition of $AgNO_3$. Elution parameters for Sephadex chromatography are: $V_e(X)$ = effluent volume of X; $V_0 = V_e$ (Blue Dextran 2000); $V_i = V_e(KCl) - V_0$; $K_d(X) = (V_e(X) - V_0)/V_i$; thus K_d (Blue Dextran 2000) ≡ 0.00, and $K_d(KCl) \equiv 1.00$. The half-width of the X band is $\Delta K_d(X)/2$.

12. For ultracentrifugation, a 60 μM Au·A (K) solution was filtered through a 0.3 μ Millipore filter, and a 0.6-ml aliquot of the filtrate was then centrifuged at 25°, in a Spinco Model E analytical ultracentrifuge. Average sedimentation coefficients (without correction to 0° and infinite dilution) were determined from uv absorption photographs.

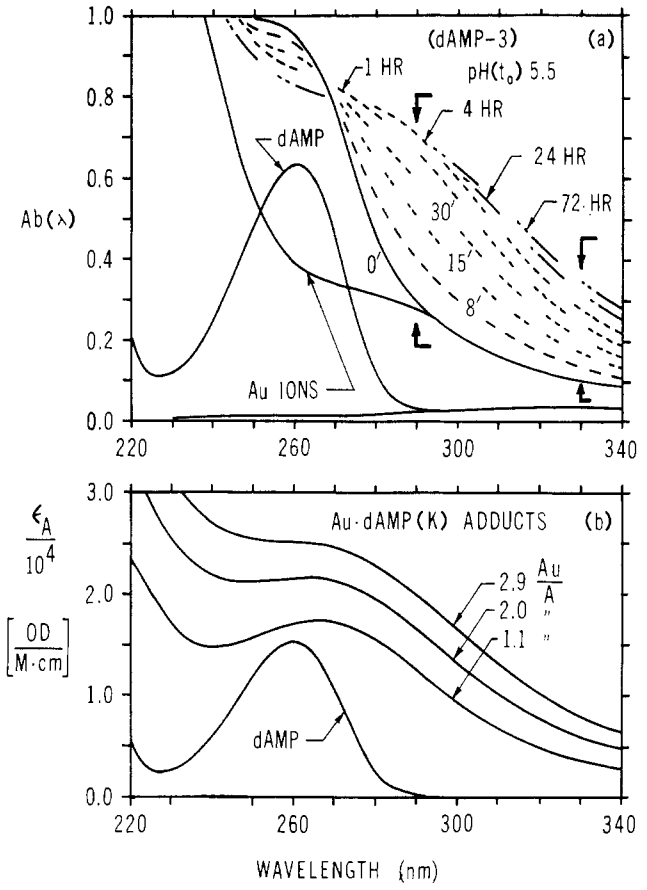


FIGURE 1: Uv spectra of reaction mixture and adducts. (a) Reaction mixture dAMP-3:4 mM dAMP + 12 mM Au ions, pH(t_0) 5.5, *ca.* 25°. Aliquots diluted 1:10 for spectra (1-mm light path). Heavy arrows: for plots of ΔAb(290) and ΔAb(330) *vs.* log *t*, see Figure 2a,b. (b) $\epsilon_A(\lambda)$ for dAMP and Au·dAMP (K) adducts at pH 5.5 (*cf.* Table IIa).

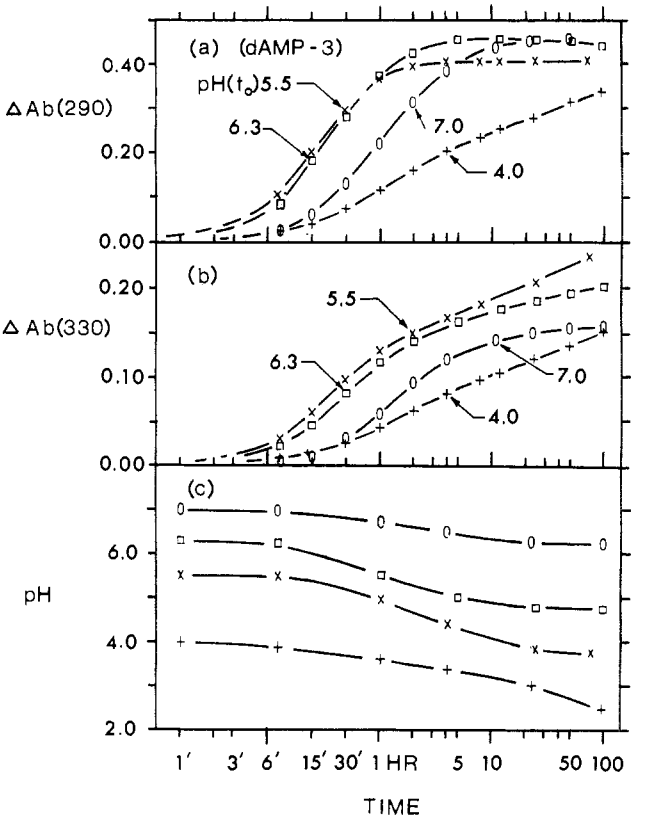


FIGURE 2: Changes in spectra and pH with time, for unbuffered reaction mixture dAMP-3 at several pH(t_0). Spectra as in Figure 1a. (a) ΔAb(290) *vs.* log *t*. Ab(290) at t_0 (due to Au ions only): 0.36, 0.27, 0.19, and 0.25, at pH(t_0) 4.0, 5.5, 6.3, and 7.0, respectively. (b) ΔAb(330) *vs.* log *t*. Ab(330) at t_0: 0.16, 0.07, 0.06, 0.12, at respective pH(t_0). (c) pH *vs.* log *t*.

Results

When aqueous solutions of adenine derivatives (A) were mixed with Au ions, changes were observed in the color, uv spectra, and pH of the mixtures, and in chromatographic and electrophoretic patterns. The results are discussed for adenine, adenosine, and adenine nucleotides. The principal reaction mixtures, A-3, contained 4 mM A and 12 mM Au ions at zero time, t_0. Equimolar mixtures, A-1, contained 5 mM A and 5 mM Au ions at t_0. The reactions were in water, KCl, or potassium acetate buffer, at several pH(t_0).

I. Changes in Color and Ultraviolet Spectra. Solutions of nucleotides and Au ions were colorless and yellow, respectively. Mixtures A-3 and A-1 changed from yellow to dark brown within 1 hr. Figure 1a shows changes in uv spectra for dAMP-3 at pH(t_0) 5.5; similar changes occurred for all adenine nucleotides. Figure 2a,b shows changes in Ab(290) and Ab(330), respectively, for dAMP-3 at pH(t_0) 4.0, 5.5, 6.3, and 7.0. The half-times, $\tau_{1/2}$, for the increase in Ab(290), are given in Table I for A-3 involving several adenine nucleotides in a variety of media.

In dAMP-3 at pH(t_0) 5.5, Ab(266) was constant ("isosbestic") between t_0 and 2 hr, whereas Ab(290) and Ab(330) increased markedly. The Ab(330) continued to increase between 4 and 72 hr, but the Ab(290) remained constant. The increases in Ab(290) and Ab(330) were more rapid at pH(t_0) 5.5 and 6.3 than at 4.0 and 7.0. Reactions at pH(t_0) 5.5 in water, 0.2 M KCl and 0.2 M potassium acetate buffer had similar $\tau_{1/2}$, though the distribution of Au ion species varies greatly with pH + pCl and ionic strength. This suggests that changes in $\tau_{1/2}$ with pH(t_0) are related more to dissociations of the adenine nucleotides, than to the hydroxochloroaurate hydrolyses (eq 1 and 2). Adenine nucleotides have a pK_b in the range 4.0–4.4, thought to be due to dissociation at N-1 (*e.g.*, Jardetsky and Jardetsky, 1960). The secondary phosphate pK_a is in the range 6.2–6.4. Competition between Au and proton for binding to N-1 would explain the slow rate at pH(t_0) 4.0. At pH(t_0) 7.0 the increased charge on the phosphate moiety would result in greater repulsion of the anionic Au ions.

An indication of the stoichiometry of the reactions was obtained by the method of continuous variations (Job, 1928). Figure 3a shows a plot of Ab(330) against gold mole fraction, for mixtures of dAMP and Au ions in acetate buffer (pH 5.5). At 4 hr the major break in the curve corresponds to 1.9 ± 0.4 Au/dAMP. The predominant break at 50 hr corresponds to 3.1 ± 0.8 Au/dAMP. A minor break corresponds to 1 Au/dAMP, when the amount of Au is limiting. In dAMP-3, which has gold mole fraction 0.75, dAMP appears to bind 2 and 3 Au at 4 and 50 hr, respectively, when the increases in Ab(290) and Ab(330) are nearly complete (Figure 2a,b). Similar results were obtained with mixtures of Au ions and AMP.

In Au–Ado mixtures having gold mole fractions 0.2–0.9, Au·Ado adducts precipitated within minutes (Figure 3b). The micromoles of Au in the precipitate was greatest for gold mole

TABLE I: Half-Times and pH Changes for A-3.[a]

A	Medium[b]	pH (t_0)	pH (t_1)	t_1 (hr)	$\tau_{1/2}$ (290)[c] (min)
dAMP	H_2O	4.0	2.5	72	120[d]
		5.5	4.4	5	15[d]
		6.3	5.2	5	21[d]
		7.0	6.3	24	63[d]
	KCl	5.5	4.6	5	20
	KAc	4.5	4.3	24	33
		5.5	5.3	5	22
AMP, 3′AMP	H_2O	5.5	4.7	5	21
	KAc	5.5	5.3	5	25
cAMP	KCl	7.5	7.2	5	40
ADP	KCl	7.5	7.2	5	55
ATP	KCl	7.5	7.2	5	43
	H_2O	5.5	4.7	5	33

[a] Reaction mixture A-3: 4 mM A + 12 mM Au ions, pH(t_0), room temperature (~25°). [b] Media: water, 0.2 M KCl, or 0.2 M potassium acetate (KAc). [c] Uv spectra were taken on 1:10 or 1:100 (ml/ml) dilutions, 1-mm or 1-cm light path, respectively; ΔAb(290) *vs.* log t was plotted; $\tau_{1/2}$(290) is time for 50% of total spectral increase at 290 nm. [d] See Figure 2a.

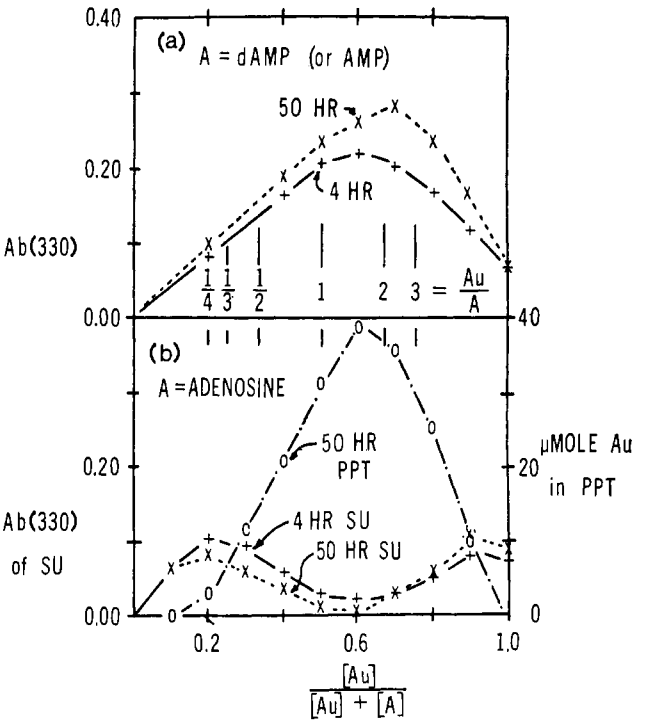


FIGURE 3: Stoichiometry, by continuous variations. Mixtures of Au ions and A in 0.2 M acetate buffer (pH 5.5); varying gold mole fractions; constant ([Au] + [A]). Spectra as in Figure 1a; Ab(330) plotted against gold mole fraction. Abscissa values indicated for 0.25, 0.33, 0.5, 1, 2, 3 Au per A. (a) A = dAMP (or AMP); [Au] + [A] = 16 mM. Au·dAMP (K) and Au·AMP (K) adducts remained in solution. (b) A = Ado; [Au] + [Ado] = 14.5 mM (limit of Ado solubility in acetate buffer); 5-ml mixture for each gold mole fraction. Au·Ado adducts precipitated within minutes, for gold mole fractions 0.2–0.9. At 4 and 50 hr, tubes were centrifuged at 500g for 15 min, and spectra of supernatants (SU) were taken. Gold contents of adducts precipitated by 50 hr are shown (Experimental Procedures 7).

fractions 0.6–0.7, corresponding to 2 Au/Ado. Dried Au·Ado adduct from Ado-3 at 4 hr contained 46.1% Au, in good agreement with the value 45.3% Au expected for Ado·$(AuCl_3)_2$ (Table IIb). The analogous Ade·$(AuCl_3)_2$ was reported by Holtz and Müller (1924). For Au–Ado mixtures having gold mole fractions 0.1–0.4, the increase in Ab(330) of the supernatant indicates that some water-*soluble* Au·Ado adducts having multiple Ado/A are formed, when Ado is in large excess.

II. Changes in pH, for Unbuffered Mixtures. Most of the reaction mixtures used were nominally unbuffered.[2] The pH changes in A-3, for various A, are given in Figure 2c and Table I. In water or KCl at pH(t_0) 5.5, 6.4, 7.0, and 7.5, there were decreases of 0.2–1.1 pH units, which accompanied increases in Ab(290) and Ab(330) (Figure 2a–c). The lower the pH(t_0), the greater the decrease in pH with time. In dAMP-3 at pH(t_0) 4.0, the pH decreased to 2.5 at 100 hr, which is consistent with displacement of proton from N-1 by Au: for 4 mM A, $-\log(4 \times 10^{-3}) = 2.4$.

III. Reduction of Au Ions by Sugars and Sugar-Phosphates. In mixtures of 12 mM Au ions and 4 mM ribose or deoxyribose 5-phosphate at pH 5.5, large amounts of metallic gold were formed by 50 hr. Reduction of Au ions to Au(0) occurred almost immediately in 4.5% sucrose or dextrose at pH 5.5 and 7.0. The reducing (aldehyde) end of the open form of the sugar is probably involved, as with reduction of other metals by sugars. The sugar moiety in nucleotides has no reducing end. No Au(0) was formed in any of the A-1 mixtures. In mixtures A-3, no Au(0) formed for A = dAMP, cAMP, poly(A), RNA, or DNA; but a small percentage of the Au(III) was reduced to Au(0) by 50–100 hr, for A = AMP, 3′AMP, ADP, and ATP. Isolated Au·A (K) adducts were stable in the presence of 4.5% sucrose or dextrose, in contrast to the reduction of Au ions by these sugars.

IV. Isolation and Analysis of Adducts. Gold–adenine nucleotide adducts remained soluble in aqueous media containing K^+ or Na^+ as the only cations, in contrast to the insolubility of Au·Ade and Au·Ado (see Figure 3a,b). When aliquots of A-1 and A-3 were made 0.2 M in KCl, followed by addition of cold ethanol, brown precipitates were formed. The potassium salts of gold–adenine nucleotide adducts thus isolated (Experimental Procedures 5) are denoted Au·A (K). The Au ions, A, and KCl were not precipitated by this procedure.

The Au·A (K) adducts were analyzed for gold and phosphate contents, and adenine residue contents were estimated by eq 3, where n = number of phosphates in nucleotide. Table

$$[A] = [P]/n \qquad (3)$$

IIa gives the % Au, % P, Au/A, and ϵ_A(270) for many Au·A (K); phosphate analyses are also given as grams of adduct/mole of A ("adenine residue equivalent weight") to facilitate calcu-

[2] Buffers other than acetate were avoided. The Au ions were found to react with ammonium salts, amino acids, Tris, cacodylate, and polycarboxylic acid buffers. Phosphate buffers would coprecipitate with Au·A (K), in ethanol precipitations of Experimental Procedures 5; [Au]/([P]/n) would not then serve as an index of the Au/A in Au·A (K) adducts. Mixtures A-1 and A-3 should be partially buffered by the hydroxochloroaurate equilibria (eq 1), and by adenine nucleotide dissociation having pK_b in the range 4.0–4.4, and pK_a in the range 6.2–6.4. The Au·A (K) adducts were soluble in the above buffers, and did not undergo the same reactions as observed between Au ions and buffers.

TABLE II

a. Observed Gold and Phosphorus Contents, and $\epsilon_A(270)$, for Au·A (K) Adducts

Reaction Mixture[a]	t_f[b] (hr)	Recov (%)[c]	Adduct	% Au[d]	% P[e]	g[f]/Mole of A	Mole of Au[g]/ Mole of A	$\epsilon_A(270)$[h]/ 10^3
A-1, H_2O, 5.5	4	68	Au·dAMP (K)	30.8	4.3	720 ± 20	1.1 ± 0.1	17.7
A-1, H_2O, 5.5	4	83	Au·AMP (K)	28.5	4.6	670	1.0	16.1
A-1, H_2O, 5.5	4	88	Au·3′-AMP (K)	26.8	4.4	700	1.0	17.0
A-1, KCl, 7.5	4	72	Au·AMP (K)	29.4	4.2	740	1.1	17.2
A-1, KCl, 7.5	5	78	Au·cAMP (K)	30.2	4.6	670	1.0	15.6
A-1, KCl, 7.5	6	76	Au·ADP (K)	21.2	7.3	850	0.9	17.5
A-1, KCl, 7.5	6	91	Au·ATP (K)	15.9	9.8	950 ± 20	0.8 ± 0.1	17.7
A-3, H_2O, 5.5	4	81	Au·dAMP (K)	39.0	3.0	1030 ± 30	2.0 ± 0.2	22.2
A-3, H_2O, 5.5	24	75	Au·dAMP (K)	41.1	2.2	1410 ± 40	2.9 ± 0.3	24.6
A-3, H_2O, 5.5	4	85	Au·AMP (K)	33.8	3.0	1030 ± 30	1.8 ±0.2	20.3
A-3, KCl, 7.5	4	88	Au·3′-AMP (K)	32.7	3.1	1000	1.7	20.4
A-3, KCl, 7.5	4	90	Au·AMP (K)	40.0	3.0	1030	2.1	21.2
A-3, KCl, 7.5	4	94	Au·cAMP (K)	39.1	3.3	940	1.9	18.8
A-3, KCl, 7.5	5	88	Au·ADP (K)	32.8	5.2	1190 ± 40	2.0	21.4
A-3, KCl, 7.5	5	98	Au·ATP (K)	27.9	7.6	1220 ± 40	1.7 ± 0.2	21.8

b. Expected Gold and Phosphorus Contents for $A\cdot(AuCl_3)_r$, r = 0–3.

	A		$A\cdot AuCl_3$			$A\cdot(AuCl_3)_2$			$A\cdot(AuCl_3)_3$		
A	% P[i]	g[j]/Mole of A	% Au[i]	% P[i]	g[j]/Mole of A	% Au[i]	% P[i]	g[j]/Mole of A	% Au[i]	% P[i]	g[j]/Mole of A
(0)	0.0	(0)	65.0	0.0	(303)	65.0	0.0	(606)	65.0	0.0	(909)
Ade	0.0	(135)	44.8	0.0	440	53.3	0.0	740	56.8	0.0	1040
Ado	0.0	(267)	34.6	0.0	570	45.3	0.0	870	50.1	0.0	1180
dAMP (K_2)	7.6	410	27.8	4.4	710	38.6	3.0	1020	44.8	2.3	1320
AMP (K_2) / 3′AMP (K_2)	7.2	430	27.0	4.2	730	37.8	3.0	1040	44.1	2.3	1340
cAMP (K)	8.4	370	29.4	4.6	670	40.2	3.2	980	46.1	2.4	1280
ADP (K_3)	11.5	540	23.5	7.4	840	34.2	5.4	1150	40.7	4.3	1450
ATP (K_4)	14.1	660	20.5	9.7	960	31.0	7.3	1270	37.6	5.9	1570

[a] A-1 or A-3, H_2O, 5.5: reaction mixture at pH(t_0) 5.5; in water between times t_0 and t_f; at t_f, brought to 0.2 M KCl. A-1 or A-3, KCl, 7.5: reaction mixture at pH(t_0) 7.5; in 0.2 M KCl between t_0 and t_f. The 60-ml aliquots of A-1 contained 300 μmoles each of A and Au ions; 60 ml of A-3, 240 μmoles of A + 720 μmoles of Au ions. [b] At t_f, four volumes of cold ethanol added; centrifuged and dried, for Au·A (K) adduct (Experimental Procedures 5). [c] Recovery: % of A (% of [P]) of reaction mixture recovered in Au·A (K). Yields, 150–330 mg: (% of A/100)·(300 or 240 μmoles A)·(g/mole of A)·(mg·mole/10^3 g·μmole). [d] % Au (w/w): from gold analysis on 40–60 mg of adduct (Experimental Procedures 7). [e] % P (w/w): from [P] analysis on aqueous solution of 12–25 mg of adduct/50 ml (Experimental Procedures 8). [f] g/mole of A = $(30.975\cdot 100/\%\,P)\cdot n = (3.0975n/\%\,P)\cdot 10^3$, assuming [A] = $(1/n)$[P]. [g] Mole of Au/mole of A = $(\%\,Au/197)\cdot(30.975n/\%\,P) = 0.15723n(\%Au/\%\,P)$. [h] $\epsilon_A(270)$, (ODU·M^{-1} cm^{-1}): from Ab(270), 1-mm light path, of solution used for [P] analysis (footnote *e*). [i] % Au = $100\cdot 197r$/(g/mole of A); % P = $100\cdot 30{,}975n$/(g/mole of A) (see *j*). [j] g/mole of A: (formula weight of A) + $303r$, rounded to nearest 10 g—except for Ade, Ado, and $(AuCl_3)_r$; does not take into account water of hydration, or slight variations with salt form–(HK/K_2, HK_2/K_3, and HK_3/K_4), or replacement of Cl^- by OH^-, or polymerization.

lations of [A] in solutions of the adducts. Adducts isolated from A-1 or A-3 at a given time had Au/A and $\epsilon_A(270)$ which were essentially independent of "n," consistent with the expectation that anionic Au ions should not bind to nucleotide phosphates. No evidence for hydrolysis of nucleotide phosphates during Au–A reactions was observed. Recoveries of [P] in the adducts were relatively high: 70–90% of [P] from A-1, and 80–100% of [P] from A-3. The use of eq 3 therefore seems justified.

Adducts from A-1 at 4-6 hr contain 0.9 − 1.1 Au/A; the % Au and % P are consistent with values expected for $A\cdot AuCl_3$, with a small degree of hydration (Table IIa,b). Adducts from A-3 at 4–6 hr contain 1.7–2.1 Au/A, and % Au, % P consistent with $A\cdot(AuCl_3)_2$. An Au·dAMP (K) adduct from dAMP-3 at 24 hr contains 2.9 Au/A, and % Au, % P consistent with $dAMP\cdot(AuCl_3)_3$.

The Au·A (K) were soluble in water to greater than 10 mM [A]. The uv spectra of aqueous solutions of adducts were proportional to [A] over the range 4×10^{-6}–4×10^{-4} M (1-mm or 1-cm light path, as required),–*i.e.*, Beer's law was obeyed–, and varied little over the range pH 3 − 8. The uv spectra of the Au ions, in contrast, varied greatly with [Au] and pH, because of the hydroxochloroaurate equilibria (eq 1 and 2). Figure 1b shows the $\epsilon_A(\lambda)$ for Au·dAMP (K) adducts con-

taining 1.1, 2.0, and 2.9 Au per A. The spectra of all adducts having the same Au/A showed only slight differences, consistent with slight differences in spectra of the parent nucleotides. Adducts with 1 Au/A have a maximum near 270 nm, with $\epsilon_A(270) = 15.6\text{–}17.7 \times 10^3$ (Table IIa and Figure 1b). The 2 Au/A adducts have a spectral plateau near 270 nm, with $\epsilon_A(270) = 18.8\text{–}22.2 \times 10^3$.

V. Thin-Layer Chromatography and Electrophoresis. Reaction mixture dAMP-3 and isolated Au·dAMP (K), prepared with [8-^{14}C]dAMP, were subjected to thin-layer chromatography and cellulose acetate paper electrophoresis (Experimental Procedures 9 and 10). In ascending thin-layer chromatography for 1 hr with solvent 1, the solvent front moved 10 cm; the R_F were: Au ions, 0.82; dAMP 0.52; and Au·dAMP (K), 0.00. All ^{14}C was in the immobile adduct spot after 2-hr reaction, in dAMP-3. There was no evidence of dissociation of Au·dAMP (K) to Au ions and dAMP. After 30-min electrophoresis in pH 5.5 acetate buffer, the species had moved the following distances toward the anode: Au ions, 8.0 cm; dAMP, 6.3 cm; and Au·dAMP (K), 7.9 cm. The brown adduct spot contained all of the ^{14}C after 2-hr reaction, but had similar mobility to the purple Au ion spot.

VI. Column Chromatography. The Au·dAMP (K) were irreversibly adsorbed to anion-exchange columns such as DEAE-cellulose (Cellex-D) and Dowex 1 (AG1-X8). This might result from interaction of Au in the adduct with nitrogens in the media. These adducts were rapidly eluted from cation-exchange columns such as carboxymethyl- and sulfoethylcellulose (Cellex-CM, -SE) and Dowex 50 (AG50W-X8). The dAMP, in contrast, was retarded by these cation exchangers, possibly due to the basic nitrogen of the adenine residue (*e.g.*, Blattner and Erickson, 1967). Binding of Au to this nitrogen would eliminate the cation exchange, and result in the observed rapid elution.

The adducts were completely excluded from Sephadex G-10 and G-25 gels, except for portions irreversibly adsorbed. Elution patterns with Sephadex G-50 are shown in Figure 4. The Au ions reacted with the gel, giving a narrow, purple immobile band at the top of the column. The dAMP eluted as a narrow band having $K_d = 1.2$ and $\Delta K_d/2 = 0.05$. With the 1.1 Au/A adduct, in contrast, 74% of the Ab(270) eluted as a very broad band having $K_d = 0.6$ and $\Delta K_d/2 = 0.4$. The 2.0 Au/A adduct was mostly adsorbed to the gel; but 2% of the Ab(270) eluted as a narrow excluded band ($K_d = 0$), and 6% as a broad band having $K_d = 0.7$ and $\Delta K_d/2 = 0.5$. Only 3% of the Ab(270) of the 2.9 Au/A adduct was eluted, as a narrow excluded band. These results suggest that the adducts are oligomers with molecular weights of several thousands.

VII. Ultracentrifugation. The Au·dAMP (K) adduct with 2 Au/A sedimented as a polydisperse boundary with average sedimentation coefficient of about 7.5 S, consistent with a molecular weight of several thousands (Experimental Procedures 12).

VIII. Solubilities and Reactions in Salt and Buffer Solutions. The Au·Ade and Au·Ado adducts with 1 and 2 Au per A were water soluble to less than 0.02 mM [A], assuming $\epsilon_A(270) = 16\text{–}20 \times 10^3$ (as for Au·AMP (K), Table IIa). Gold–adenine nucleotide adducts, in contrast, were soluble to greater than 10 mM [A], in water and in aqueous 0.2 M LiCl, NaCl, KCl, RbCl, and CsCl. The divalent cation salts of these adducts, Au·A (M^{2+}), had varying solubilities, depending on the M^{2+} and the number and position of nucelotide phosphates. All Au·A (Mg^{2+}, Ca^{2+}, and Mn^{2+}), 2 mM in [A], were soluble in the respective 1 mM MCl_2. The Au·cAMP (M^{2+}) remained soluble in 10 mM MCl_2, whereas the other Au·A (M^{2+}) (A =

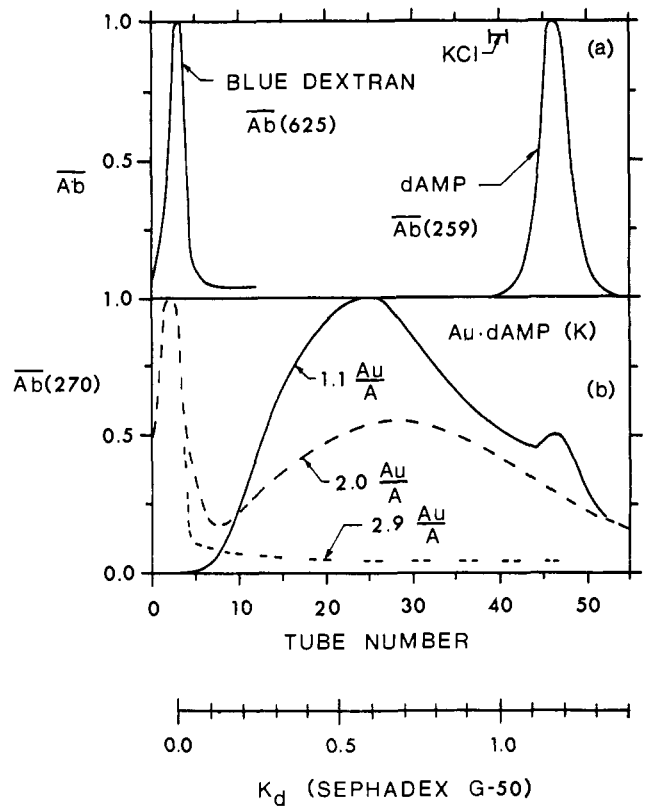


FIGURE 4: Chromatography on Sephadex G-50. Samples (1 ml) were eluted according to Experimental Procedures 11. Eluate spectra, Ab(λ), were normalized to $\overline{Ab}(\lambda) = 1.0$ at peak. Abscissa: tube number and derived K_d. (a) Calibration: (i) Blue Dextran 2000 (2×10^6 molecular weight), Ab(625) = 10 ODU units; (ii) 10 mM KCl, Ag precipitation; (iii) 4 mM dAMP, Ab(259) = 61 ODU. (b) 4 mM Au·dAMP (K) adducts: (i) 1.1 Au/A, Ab(270) = 70 ODU; 74% of Ab(270) eluted between $K_d = 0.0\text{–}1.5$; (ii) 2.0 Au/A, Ab(270) = 89 ODU; 8% of Ab(270) eluted; (iii) 2.9 Au/A, Ab(270) = 98 ODU; 3% of Ab(270) eluted. For 4–12 mM Au ions, top 5 mm of column turned purple; no Au ions were eluted.

AMP, ADP and ATP) were partially soluble in 10 mM $MgCl_2$, less soluble in 10 mM $CaCl_2$, and quite insoluble in 10 mM $MnCl_2$. For each MCl_2, solubilities decreased in the orders cAMP ≫ AMP > ADP ≥ ATP, and 1 Au/A ≥ 2 Au/A. Solubility differences between Au·cAMP (Mg^{2+} and Mn^{2+}) and Au·AMP (Mg^{2+} and Mn^{2+}) are illustrated in Figure 5. Similar differences were observed with $SrCl_2$, $BaCl_2$, $ZnCl_2$, and $CdCl_2$.

All Au·A (Fe^{2+}, Fe^{3+}, UO_2^{2+}, and Pb^{2+}) were insoluble in 10 mM $FeSO_4$, $Fe_2(SO_4)_3$, uranyl acetate, and lead acetate, respectively, for A = cAMP, AMP, ADP, and ATP. The Au·A (Fe^{2+}, Fe^{3+}, Mg^{2+}, and Mn^{2+}) with 1 Au/A were similar light brown precipitates. But for 2 Au/A adducts, the Au·A (Fe^{2+}) were gray-black, and Au·A (Mn^{2+}) were much darker brown than the Au·A (Mg^{2+} and Fe^{3+}). The second bound gold appears more susceptible than the first to further (redox?) reactions with Fe^{2+} and Mn^{2+}. The adducts remained soluble in $K_3Fe(CN)_6$, (ferricyanide), but gave cream-brown precipitates in $K_4Fe(CN)_6$, (ferrocyanide). The adducts also remained soluble in $TlNO_3$, $HgCl_2$, and $AgNO_3$.

The Au ions were soluble in the above MCl_2 (except $MnCl_2$), and in $Fe_2(SO_4)_3$, uranyl acetate, and $K_3Fe(CN)_6$. The Au ions reacted to give a black precipitate with $MnCl_2$; an orange precipitate with lead(II) acetate; and a light orange precipitate with $K_4Fe(CN)_6$. The Au ions were reduced to Au(0) by

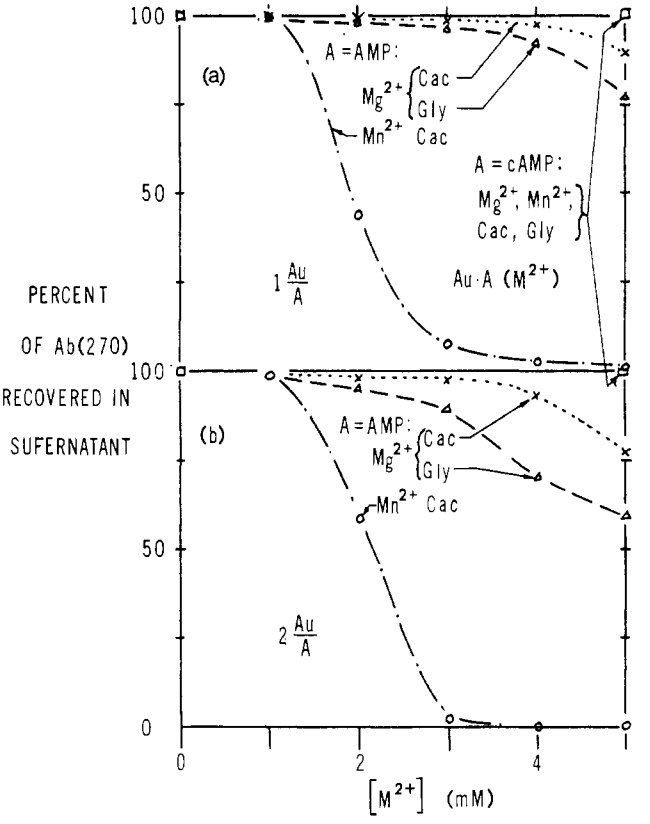


FIGURE 5: Differential solubilities of Au·cAMP (Mg^{2+} and Mn^{2+}) and Au·AMP (Mg^{2+} and Mn^{2+}). Aliquots of aqueous 4 mM Au·cAMP (K) and Au·AMP (K) diluted to 2 mM [A] in Gly or Cac buffers containing $MgCl_2$ or $MnCl_2$. Final concentrations: 0–5 mM [M^{2+}]; 40 mM glycine–KOH buffer (Gly), pH 8.0; 40 mM sodium cacodylate·HCl buffer (Cac), pH 7.8. Samples centrifuged at 500*g* for 15 min; aliquots of SU diluted 1:5 with water for uv spectra (1-mm light path). Percentage of original Ab(270) remaining in supernatant is plotted against [M^{2+}]. Au·cAMP (M^{2+}) were completely soluble, for all [M^{2+}]; Au·AMP (M^{2+}) precipitated for [M^{2+}] $\geq$2mM. (a) 1 Au/A adducts. (b) 2 Au/A adducts.

$FeSO_4$, and by $TlNO_3$. Such differences in solubilities and reactivities of Au·A adducts and Au ions, in the presence of the various salts, are evidence that Au binds very tightly to the adenine nucleotides.

The Au·A (K) adducts were soluble in a wide variety of buffers, including acetate (pH 3.5–5.5), dimethyl glutarate (pH 4.5), cacodylate·HCl (pH 7), phosphate (pH 7), Tris-HCl (pH 7), *s*-collidine (pH 7), and carbonate (pH 9). The Au ions were reduced to Au(0), gave colored precipitates, or otherwise reacted with most of these buffers.

IX. Reactions with Amino Acids, Amines, and Biological Stains. When 4 mM Au·dAMP (K) adducts were mixed at pH 7 with 40 mM Glu (K) or Asp (K or Tl), changes occurred within 1 hr in electrophoresis patterns, as well as small changes in Ab(270) and Ab(300). Addition of 1.5 volumes of cold ethanol precipitated red-brown AA·Au·dAMP (K or Tl) adducts, for AA = Glu or Asp. The Glu (K) and Asp (K or Tl) alone gave no ethanol precipitates. The AA·Au·dAMP (K or Tl) had the same Au/A as the parent Au·dAMP (K), within experimental error, and greater mobility toward the anode in cellulose acetate paper electrophoresis using 0.1 M phosphate buffer (pH 7). The Asp·Au·dAMP (Tl) adducts with 1–3 Au/A had 3–6 Tl/A (Experimental Procedures 7).

Similar mixtures were prepared containing a series of amino acids and related amines, and Au·A (K) adducts, for A = dAMP, AMP, and cAMP. Spectral changes occurred but the adducts remained soluble, in the presence of NH_4Cl, Gly, Ala, Asp, Glu, Gln, Lys, His, Trp, thiamine, urea, ornithine, and guanidine. Colored precipitates were formed in the presence of Arg and tryptamine. Mixtures of Au ions with NH_4Cl, Gln, urea, or guanidine turned brown, but no precipitation occurred within 2 hr. Mixtures of Au ions with the other amino acids above yielded Au(0) and/or other insoluble products; and insoluble amine·Au adducts with the other amines above. Binding of Au to adenine nucleotides thus prevents its reduction by amino acids; but amine·Au·A adducts are formed with amino acids and other amines.

The Au·A (K) adducts were also mixed in pH 5.5 acetate buffer with several biological stains containing amino and heterocyclic nitrogens: basic fuchsin, pyronine Y, methyl green, azures A and B, toluidine blue O, cresyl violet, proflavine, acriflavine, acridine orange, quinacrine, and quinacrine mustard. Large changes were observed in the visible spectra, with shifts in the λ_{max} of the chromophoric group of the stain. Mixtures of stain and 4 mM Au·A (K) in 0.2 M acetate buffer (pH 5.5) gave soluble adducts when [A]/[stain] $\geq$ 2. As the [stain] was increased, the stain·Au·A adducts precipitated, for some of the stains (*e.g.*, acridine and quinacrine derivatives). This might result from binding of excess stain-nitrogens to nucleotide phosphates. In cases where the adducts remained water soluble, addition of two volumes of cold ethanol precipitated the highly colored stain·Au·A (K) adducts. The stains alone gave no ethanol precipitates. The stain·Au·A (K) had smaller mobilities toward the anode than the parent Au·A (K), in cellulose acetate paper electrophoresis with pH 5.5 acetate buffer. Mixtures of Au ions with the stains, for all [Au]/[stain], gave colored water-insoluble stain·Au adducts.

X. Reactions with Sulfur-Containing Compounds. The Au·A (K) adducts remained soluble in $(NH_4)_2S$, whereas Au ions gave a brown-black precipitate, presumably Au_2S_3. White precipitates were formed when either Au·A (K) or Au ions were mixed with thiourea and cysteine; and black precipitates with thiocarbohydrazide, $CS(NHNH_2)_2$. Thiourea, cysteine, and thiocarbohydrazide reacted most rapidly with Au ions, and more rapidly with 2 Au/A adducts than with 1 Au/A adducts.

Discussion

The Au ions at several pH + pCl were found to undergo similar reactions with AMP, dAMP, 3′AMP, cAMP, ADP, and ATP. The method of continuous variations suggests adducts with 1 and 2 Au per A at short times, and 3 Au/A at longer times (Figure 3). Brown, water-*soluble* Au·A (K) adducts, isolated by ethanol precipitation from mixtures A-1 and A-3, were found to contain 1, 2, and 3 Au per A independent of the number of nucleotide phosphates "*n*," where [A] = [P]/*n* (Table IIa). The % Au and % P were consistent with simple adducts $A\cdot(AuCl_3)_r\cdot wH_2O$, where r = 1, 2, 3, and w is small (Table IIa,b). However, Sephadex G-50 chromatography (Figure 4) and ultracentrifugation (Section VII) suggest the adducts are oligomers with molecular weights of several thousands.

The Au·Ado reported here and the Au·Ade reported by Wulff (1893) and by Holtz and Müller (1924) contain 1 and 2 Au per A, but are water *insoluble*. Nucleotide phosphates are not expected to bind anionic Au ions, but evidently solubilize the Au·A (K) adducts. The sugar moieties of adenosine and adenine nucleotides do *not* reduce Au ions to Au(0) in

mixtures A-1, as do ribose and deoxyribose 5-phosphate, sucrose, and dextrose (section III). Gold(III) is known to coordinate with primary and heterocyclic amines. It is therefore reasonable to propose that gold(III) coordinates with amino and ring nitrogens of the adenine residues. Figure 6 shows a hypothetical structure for an oligomeric Au·A adduct in which gold(III) is chelated between N-7 and N^6, and coordinated to N-1, with N → Au ← N bridges between adenine residues.

The proposed chelation involves a five-membered ring like the two in gold(III)–ethylenediamine complexes. The difference spectra, [Au·dAMP (K) − dAMP], derived from Figure 1b, show a band between 270 and 340 nm which is reminiscent of the band at 300 nmf or Au·En complexes. The greater reactivities of 2 Au/A adducts than 1 Au/A adducts toward Fe^{2+}, Mn^{2+}, thiourea, cysteine, and thiocarbohydrazide (sections VIII and X), suggest that the *first* bound gold might be the one which is chelated.

The *second* bound gold would perhaps be coordinated to the basic nitrogen of the adenine residue, thought to be ring nitrogen N-1 (Jardetsky and Jardetsky, 1960). The slow rate for dAMP-3 at pH(t_0) 4.0, and the decrease in pH with time (Figure 2a–c) are consistent with competition between Au and proton for binding to the basic nitrogen (pK_b = 4.4). The fast elution of Au·dAMP (K) from cation-exchange columns *vs.* retardation of dAMP (section VI) is likewise consistent with binding of Au to the basic nitrogen. The *third* gold binds much more slowly, possibly to N-3. More evidence is needed for definite assignment of binding sites.

In mixtures of 30 mM AMP and 60 mM Au ions in D_2O at pD 6–8, the infrared bands of AMP at 1626 and 1640 cm^{-1} disappeared, and a broad band at 1600 cm^{-1} appeared (Hartman, 1967). Miles (1961) has attributed these AMP ir bands to ring vibrations in the adenine residue. Perturbations in these ir bands in the presence of Au ions are consistent with coordination of gold(III) to ring nitrogens of the adenine residue. The broadness of the new band might result from the several types of N → Au coordinate bonds and N → Au ← N bridges shown in Figure 6. Nuclear magnetic resonance studies on the Au·A (K) adducts remain to be performed, but might provide information on the binding sites. The slightly paramagnetic susceptibility of $AuCl_3$ may interfere with analysis by nmr.

Ethanol precipitation from 0.2 M KCl solutions, used to isolate Au·A (K) adducts, also precipitates poly(A), RNA, and DNA, but *not* nucleotides A, or Au ions. Precipitation of the adducts may be related in part to their apparent polymerization. As reported elsewhere, Au·G (K) and Au·C (K) adducts give ethanol precipitates, whereas Au·T (K) and Au·U (K) do not. (However Au·TMP (Ba) and Au·UMP (Ba) are isolated from 0.1 M $BaCl_2$ by ethanol precipitation.) The Au·A, Au·G, and Au·C adducts appear to be polymerized, from Sephadex G-50 chromatography and ultracentrifugation, but Au·T and Au·U adducts do not. The AMP, GMP, and CMP bind 2–3 Au/base residue, whereas TMP and UMP bind only 1 Au/base residue. When a nucleotide has at least two binding sites for gold(III), each base residue might bridge two Au, and *vice versa*, resulting in polymerization (Figure 6).

Many observations indicate that gold is *irreversibly* bound to the adenine nucleotides. Thus, Au·A (K) adducts can be isolated from Au ions and A, move as single spots in electrophoresis, and are immobile in thin-layer chromatography, with no indication of dissociation to Au ions and A (section V). In dAMP-3 prepared with [8-^{14}C]dAMP, all ^{14}C appears in the adduct spot; that is, the reaction goes to completion (depletion of dAMP). The Au·A (K) remain soluble in the presence of sugars and thallium(I), and form new AA·Au·A (K) and stain·Au·A (K) adducts. The Au ions, in contrast, are reduced to Au(0) by sugars, thallium(I), and amino acids, and give insoluble stain·Au adducts. The AA·Au·A and stain·Au·A adducts might have N → Au ← N coordination bridges between amino or heterocyclic nitrogens in the AA or stain, and adenine residues.

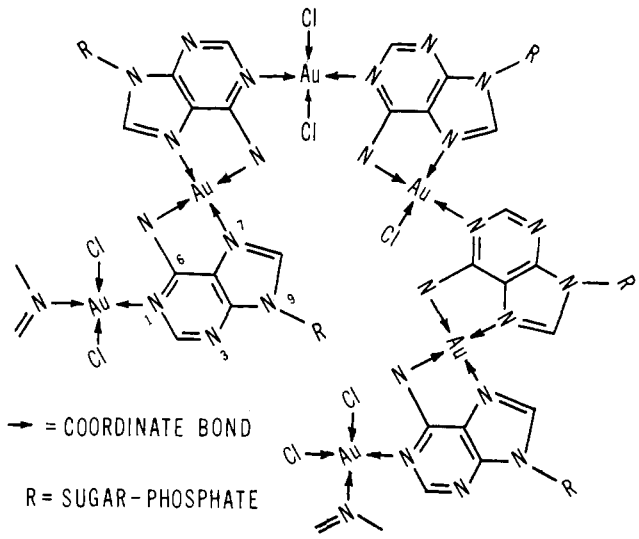


FIGURE 6: Hypothetical structure for oligomeric Au·A adducts.

A simplified kinetic analysis of dAMP-3 was given in Gibson (1969). The second-order constants for binding of Au to each site (k_1, k_2, and k_3) were assumed not to vary with the state of binding at the other sites ("independent sites"). Saturation curves [bound Au/total A *vs.* log ([A]·k_1t)] were derived for varying ratios k_2/k_1, k_3/k_1, and for [Au]/[A] = 3, in reaction mixture. The spectra for dAMP-3 were compared to appropriate linear combinations of spectra for dAMP, Au ions and Au·dAMP adducts with 1, 2, and 3 Au per A (Figure 1a,b). Thus values of (bound Au/total A) were estimated for the several reaction times. Plots of these (bound Au/total A) *vs.* log t were compared to the derived plots of (bound Au/total A) *vs.* log ([A]·k_1t) for several k_2/k_1 and k_3/k_1. Rate constants for dAMP-3 at pH(t_0) 5.5 were estimated in this way to be: k_1 = 500 hr^{-1} M^{-1}, k_2 = 100 hr^{-1} M^{-1}, and k_3 = 6 hr^{-1} M^{-1}. This analysis ignores the apparent polymerization; the assumed "independence of sites" has no proof or disproof from our data. However, the values of the k_i are useful for comparison to k_i similarly calculated for dGMP-3, dCMP-3, TMP-3, and UMP-3, to be presented elsewhere.

The gold–adenine nucleotide reactions reported here have several potential applications in cytochemistry at the level of the electron microscope, which will be briefly discussed.

A. Electron Staining of Isolated Polynucleotides and Nucleic Acids, by Au Ions. Gold(III) coordination to base residues of polynucleotides should enhance their contrast in electron micrographs. Poly(A) stained *on the grid* with Au ions had 1.5% contrast above background, along stained strands, under conditions in which 2 Au/A are bound *in solution* (Highton and Beer, 1968). Individual Au atoms were not resolved. Poly(A) stained *in solution*, before deposition on the grid, showed extensive aggregation, presumably because of intra- and interstrand N → Au ← N bridges between adenine residues. To amplify the "electron staining" of polynucleotides

on the grid, Asp (T1) might be coupled to bound Au (section IX).

B. Electron Staining of Nucleoproteins and Nucleohistones, in Tissue Sections, by Au·A (K) Adducts. The Au·A (K) were found to couple to amino acids, related amines, and biological stains containing amino and heterocyclic nitrogens (section IX). The coupling of Au·A (K) to tissue amines, or to biological stains, might give new staining methods, to supplement the common electron microscope stains such as OsO_4, uranyl acetate, lead(II) citrate, and phosphotungstic acid. The Au·AMP (K) adducts were found to give positive staining of chromatin, nucleoli, and ribosomes in glutaraldehyde-fixed hepatocytes, without the coarse granules (Au(0)?) observed in staining with Au ions. The apparent reduction of Au ions to Au(0) by tissue components might be analogous to the reduction of Au ions (but not Au·A adducts) by sugars and amino acids, (sections III and IX). Cell membranes were not stained by either Au ions or Au·A adducts.

The coupling of Au·A (K) adducts to stains could be used in two types of cytochemical procedure: (1) treat tissue with stain alone, followed by Au·A (K) alone; and (2) treat tissue with the stain·Au·A (K) adduct. The free stains are cationic, whereas Au·A and stain·Au·A adducts are anionic, as confirmed by cellulose acetate paper electrophoresis (section IX). For stains which interact ionically with tissue groups, procedures 1 and 2 are expected to give different results. However, acridine dyes (*e.g.*, proflavine, acriflavine, acridine orange, quinacrine, and quinacrine mustard) are thought to intercalate between base residues of polynucleotides; procedures 1 and 2 might then give similar staining patterns.

C. Contrast Enhancement with Thiocarbohydrazide. Orange-brown insoluble Au·Ade and Au·Ado adducts, and soluble Au·A (K) adducts are all converted into black, very insoluble products in the presence of thiocarbohydrazide (section X). Aldehyde-fixed tissue is stained light brown by Au·A (K), but the contrast is poor in light microscopy. Treatment with thiocarbohydrazide greatly increases the contrast for light microscopy, and could serve to bridge other heavy atom groups such as OsO_4, UO_2^{2+}, or additional Au(III), for amplified electron staining (D. W. Gibson and R. J. Barrnett, unpublished data).

D. Enzyme Cytochemistry, Using Au·A Adducts as Substrate Analogs. Electron microscopic localization of enzymes in general involves the conversion, by enzymes within a tissue block, of a soluble substrate into a product which is (or is made to be) insoluble and electron dense (*e.g.*, Barrnett, 1964). Classic examples include the localizations of phosphomonoesterases and ATPases by "lead(II) capture" of liberated phosphate, P_i (*e.g.*, Goldfischer *et al.*, 1964). Diffusion of P_i before combining with Pb(II), and diffusion of individual (soluble!) molecules of lead phosphate, before "microcrystallization," can limit the resolution of such localizations. If soluble Au·AMP (K) were hydrolyzed by a phosphomonoesterase, one might expect insoluble Au·Ado to precipitate near the site of the enzyme. The 1 Au/AMP adduct (but not the 2 Au/AMP adduct) was found to be hydrolyzed *in vitro* about 30% as rapidly as AMP, by acid phosphatase in pH 4.5 dimethyl glutarate buffer (Fitzsimons *et al.*, 1970). Electron microscopy of glutaraldehyde-fixed rat liver and kidney, which had been incubated in the 1 Au/AMP adduct, showed apparent localization of acid phosphatase in lysosomes (as by "lead-(II) capture method").

Differential solubilities of Au·A (M^{2+}) adducts might be used for studying other nucleotide-dependent enzymes, such as phosphodiesterases, phosphorylases, kinases, and polymerases (section VIII). For example, hydrolysis of soluble Au·cAMP (Mn^{2+}) by phosphodiesterases would be expected to yield insoluble Au·AMP (Mn^{2+}) (Figure 5). It remains to be determined which enzymes will actually use gold–adenine nucleotide adducts as substrate analogs, and which will be inhibited by the adducts—(as with many nucleotide analogs).

Acknowledgments

Table IIa,b and Figures 1b, 2a,b, 3a, and 6 are adapted from Tables 6 and 7 and Figures 7b–d, 8, and 5, respectively, of Gibson (1969) by permission of author. Figures 1a and 4a,b are adapted from Figures 7-2 and 7-4a–c respectively, of Beer *et al.* (1970), by permission of authors and Charles C. Thomas, Publisher.

References

Ames, B. N., and Dubin, D. T. (1960), *J. Biol. Chem. 235*, 769.

Bardet, L., and Bontoux, J. (1962), *Trav. Soc. Pharm. Montpellier 21*, 111.

Barrnett, R. J. (1964), *J. Roy. Microsc. Soc. 83*, 143.

Beer, M., Gibson, D. W., and Koller, T. (1970), *in* Effects of Metals on Cells, Subcellular Elements, and Macromolecules, Maniloff, J., Coleman, J. R., and Miller, M. W., Ed., Springfield, Ill., C. C. Thomas, p 131.

Beer, M., Gibson, D. W., and Mohlhenrich, K. H. (1963), *Biophys. Soc. Abstr. MB1.*

Beer, M., and Moudrianakis, E. N. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 409.

Beer, M., Stern, S., Carmault, D., and Mohlhenrich, K. H. (1966), *Biochemistry 5*, 2283.

Bjerrum, N. (1948), *Bull. Soc. Chim. Belg. 57*, 432.

Blattner, F., and Erickson, E. P. (1967), *Anal. Biochem. 18*, 220.

Cattalini, L., Ricevuto, V., Orio, A., and Tobe, M. L. (1968), *Inorg. Chem. 7*, 51 (see other references cited therein).

Eichhorn, G. L., Butzow, J. J., Clark, P., and Shin, Y. A. (1970), *in* Effects of Metals on Cells, Subcellular Elements, and Macromolecules, Maniloff, J., Coleman, J. R., and Miller, M. W., Ed., Springfield, Ill., C. C. Thomas, p 77.

Fitzsimons, J. T. R., Gibson, D. W., and Barrnett, R. J. (1970), *J. Histochem. Cytochem. 18*, 673.

Gangopadhayay, A. K., and Chakravorty, A. (1961), *J. Chem. Phys. 35*, 2206.

Gibson, D. W. (1969), Ph.D. Dissertation, The Johns Hopkins University, Baltimore, Md.; *Diss. Abstr. 30*, 513-B.

Gibson, D. W., and Beer, M. (1968), *Biophys. Soc. Abstr. A175.*

Gmelin, L. (1954), Gmelin's Handbuch der Anorganische Chemie, Vol. 62, Weinheim/Bergstrass, Verlag Chemie, p 697.

Goldfischer, S., Essner, E., and Novikoff, A. B. (1964), *J. Histochem. Cytochem. 12*, 72.

Hartman, Jr., K. A. (1967), *Biochim. Biophys. Acta 138*, 192.

Herschlag, V. E. (1941), *Ind. Eng. Chem. Anal. Ed. 13*, 561.

Highton, P. J., and Beer, M. (1968), *J. Roy. Microsc. Soc. 88*, 23.

Holtz, H., and Müller, H. (1924), *Arch. Exp. Pathol. Pharm. 105*, 30.

Hoppe-Seyler, F. W., and Schmidt, W. (1928), *Z. Physiol. Chem. 175*, 304.

Jardetsky, D. C., and Jardetsky, O. (1960), *J. Amer. Chem. Soc. 82*, 222.

Job, P. (1928), *Ann. Chim. 9*, 113.

Mangold, H. K. (1965), *in* Thin-layer Chromatography, Stahl, E., Ed., Berlin, Springer-Verlag, p 451.

Miles, H. T. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 791.
Moudrianakis, E. N., and Beer, M. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 564.
Pribil, R., Veselý, V., and Kratochuil, K. (1961), *Talanta 8*, 52.
Sidgwick, N. V. (1950), The Chemical Elements and Their Compounds, Vol. 1, Oxford, Clarendon Press, p 177.
Vydra, R., and Čelikovský, J. (1958), *Collect. Czech. Chem. Commun. 23*, 539.
Wulff, C. (1893), *Z. Physiol. Chem. 17*, 468, 507.

Ca^{2+}-Binding Activity of Protein Isolated from Sarcotubular Membranes*

H. A. Bertrand,† E. J. Masoro, T. Ohnishi, and B. P. Yu

ABSTRACT: The Ca^{2+}-binding activity of the protein fraction of rat skeletal muscle sarcotubular membranes, termed fraction 2, and accounting for 90% of the membrane protein has been investigated. Using a dual-wavelength spectrophotometer and murexide, a metallochromic indicator sensitive to Ca^{2+}, fraction 2 has been shown to have binding sites of both a high and low affinity for Ca^{2+}. The sites of fraction 2 with a high affinity for Ca^{2+} are characterized by an association constant greater than 10^5 M^{-1} and the low-affinity sites by an association constant of 10^4 M^{-1}. The high affinity of fraction 2 for Ca^{2+} is not markedly affected by protein or murexide concentrations nor by the temperature of the assay system. However, the high Ca^{2+} affinity of the membrane protein is affected by both pH and buffer concentration. Further, the Ca^{2+}-binding activity of fraction 2 is inhibited noncompetitively by the monovalent cations, Na^+ and K^+, and competitively by the divalent cation, Mg^{2+}. The Ca^{2+}-binding activity of fraction 2 is not enhanced in the presence of ATP. The similarity of Ca^{2+}-binding characteristics of fraction 2 and fragmented sarcoplasmic reticulum is discussed.

Sarcoplasmic reticulum, a complex tubular intracellular membrane system, is believed to play an important part in both the excitation–contraction coupling and the relaxation activities of skeletal muscle (Ebashi and Endo, 1968). The release of Ca^{2+} from its membranous structure or tubular lumen to the myoplasm (Winegrad, 1968, 1970), in response to excitation of the muscle, is the way the sarcoplasmic reticulum is envisioned to function in excitation–contraction coupling. This Ca^{2+} interacts with troponin (Fuchs and Briggs, 1968) to unfasten the troponin–tropomyosin molecular lock thus enabling the contractile interaction of actin with myosin to occur. The sequestering of Ca^{2+} by the sarcoplasmic reticulum either within its membranous structure or tubular lumen or both from the myoplasm depletes myoplasm and troponin of Ca^{2+} thus returning the muscle to the relaxed state

Muscle microsomes prepared from skeletal muscle homogenates by differential centrifugation contain membranous fragments of sarcoplasmic reticulum (which will be called sarcotubular membranes) along with other cellular structures (Hasselbach, 1964). The sarcotublar membranes are present in the form of closed vesicular structures (Hasselbach, 1964) which exhibit several biochemical activities believed to be related to their *in vivo* function such as the ATP-dependent, oxalate-enhanced active transport of large amounts of Ca^{2+} from the external medium into the aqueous spaces of the vesicular structure (Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1961), the $(Ca^{2+} + Mg^{2+})$-ATPase activity believed to energize this Ca^{2+} transport (Martonosi and Feretos, 1964), the capacity to bind Ca^{2+} to their membranous structure in the absence of ATP (ATP-independent Ca^{2+} binding) and the enhancement of this binding by ATP (ATP-dependent Ca^{2+} binding) (Carvalho and Leo, 1967).

Mommaerts (1967) suggested that sarcotubular membranes are highly specialized for the task of ejecting and resorbing massive amounts of Ca^{2+} and that they contain little protein not involved in this transport function. It is this high specialization which led us to postulate that these membranes might be of a simple enough molecular nature to provide experimental material ideally suited for the study of the molecular aspects of membrane structure and function.

A method was developed for the preparation of a highly purified population of sarcotubular membranes (Yu *et al.*, 1968a). This preparation as modified by Yu and Masoro (1970) provides sarcotubular membranes that appear to be almost entirely free of other cellular components (Deamer and Baskin, 1969; Yu and Masoro, 1970).

Solubilization of this purified sarbotubular membrane preparation (which we call SF_1) by treatment with sodium dodecyl sulfate permits more than 90% of the sarcotubular membrane protein to be recovered as a single fraction from Sepharose 4B column chromatography (Yu and Masoro, 1970). This fraction called fraction 2 is lipid poor, sodium dodecyl sulfate free, and composed of high molecular weight aggregates of low molecular weight species of polypeptide chains.

Of course, it is of interest to know if fraction 2 has any of the functional activities of the intact sarcotubular membranes. Obviously soluble proteins cannot transport Ca^{2+} across a membranous barrier. Moreover on the basis of other studies

* From the Department of Physiology and Biophysics, The Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129. *Received May 12, 1971.* This work was supported in part by Grant GB-12145 from the National Science Foundation and Grant AM-09458 from the National Institute of Arthritis and Metabolic Diseases. A preliminary report has been presented (Bertrand *et al.*, 1971).

† Postdoctoral Fellow on National Institutes of Health Training Grant GM-02008.