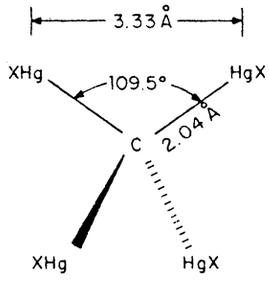
Visualization of Polymercurimethane-Labeled fd Bacteriophage in the Scanning Transmission Electron Microscope

Abstract. *Each of the 2700 coat proteins of fd bacteriophage was labeled with tetrakis(acetoxymercuri)methane (TAMM) or aquoglycylmethionineplatinum(II). The TAMM-labeled specimens reveal striking bright spots in the scanning transmission electron microscope which arise from clustering. Measurements of mass show increases consistent with the addition of four mercury atoms or one platinum atom, respectively, to each coat protein.*

Tetrakis(acetoxymercuri)methane, $C(HgO_2CCH_3)_4$ (TAMM), is an electron-dense reagent that binds sulfur in nucleic acids (*1*) and hemoglobin (*2*). We report that TAMM can be quantitatively attached to the 2700 copies of the B coat protein of fd bacteriophage. At high total



TAMM, X = CH_3CO_2

electron doses, clusters of TAMM form as bright spots ranging from 5 to 20 Å in diameter as observed in the scanning transmission electron microscope (STEM). Clustering of TAMM can be chemically induced by use of 2-aminoethanethiol (AET) hydrochloride as the solubilizing ligand (*1*). Clustering may be avoided through use of a sterically hindered solubilizing thiol ligand such as *d,l*-penicillamine. Mass measurements in the STEM (*3*) of the latter sample and of fd bacteriophage labeled with aquoglycylmethionineplatinum(II) reveal the presence of four and one heavy atoms, respectively, attached to each coat protein. Mass loss measurements as a function of total electron dose are also described.

The 2700 copies of B coat protein of bacteriophage fd (*4*) have a single lysine residue, which can be converted into a thiol residue (*5*) by treatment with *S*-acetylmercaptosuccinic anhydride at *p*H 7, in 0.2*M* phosphate followed by hydroxylamine (*6*). Titration with *p*-chloromercuribenzoate at *p*H 8 (*7*) established the presence of 1.00 ± 0.06 thiol groups per modified coat protein copy. The freshly thiolated bacteriophage (~ 1 mg/ml) was labeled with heavy atoms through stoichiometric addition of either aquoglycylmethionineplatinum(II) (*8*) or TAMM · L_3 (*1*), where L = AET or *d,l*-penicillamine, and diluted to a final protein concentration of ~ 30 μg/ml. Control experiments in the STEM showed no heavy atom labeling of tobacco mosaic virus (TMV) or unmodified bacteriophage fd under the conditions described for staining the thiolated bacteriophage with either the mercury or the platinum reagent. The TAMM was dissolved to $10^{-3}M$ in 25 ml of freshly distilled dimethyl sulfoxide containing a few drops of acetic acid and added to three volumes of L at $10^{-3}M$ in water. The solution was then diluted with water to the desired concentration for staining. Alternatively, TAMM, a threefold excess of L, and a drop of acetic acid were dissolved to 100 ml in water and diluted to the final staining concentration. Preparation of the sample and the details of STEM microscopy have been described (*3*). All microscopy measurements were made at −130°C on a cold stage.

Figure 1a displays a STEM photomicrograph of fd bacteriophage labeled with one equivalent of TAMM · 3AET per modified coat protein prior to spreading of the sample. The striking bright spots range from 5 to 20 Å in diameter. We attribute this result to chemically induced clustering of TAMM molecules in solution and beam-induced clustering in the STEM. Chemical clustering may be accomplished through the formation

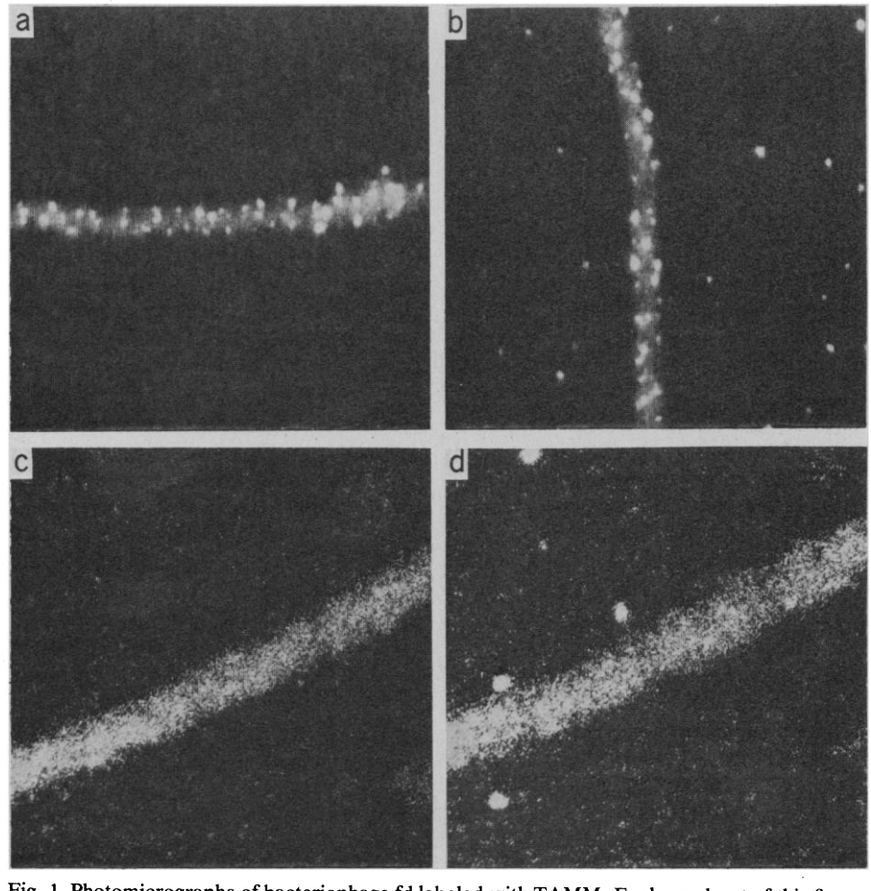


Fig. 1. Photomicrographs of bacteriophage fd labeled with TAMM. Each quadrant of this figure displays only one-quarter of the original image. (a) STEM image (1300 by 1300 Å) of fd bacteriophage labeled with TAMM · 3AET in solution containing dimethyl sulfoxide prior to sample spreading on the EM grid. The size of the clusters varies from 5 to 20 Å. There is very little heavy metal on the background. The total electron dose is 180 e $Å^{-2}$. (b) STEM image (1300 by 1300 Å) of fd bacteriophage labeled by floating an EM grid with attached fd bacteriophage for 7 minutes on a $10^{-5}M$ solution (1 percent dimethyl sulfoxide) of TAMM · 3AET, followed by immediate freeze-drying. The micrograph resembles (a), except for the appearance of spots on the background. Total electron dose is 310 e $Å^{-2}$. (c and d) STEM images (650 by 650 Å) of the same sample as in (b) except at one-half the beam current; (c) is at a total electron dose of 150 e $Å^{-2}$, while (d) is the same area after a total of 600 e $Å^{-2}$. The spots have reached their maximum intensity in (d) and are beginning to disappear. Disappearance to half of maximum intensity occurs at about 10,000 e $Å^{-2}$ (not illustrated).

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of thiolate-bridged oligomers of the kind $[(RSHg)_3C\text{-}Hg\text{-}S(R)\text{-}Hg\text{-}C(HgSR)_3]$ where R is the 2-aminoethyl moiety. Owing to the clustering with this reagent, it is not yet possible to trace the coat protein structure or to obtain accurate measurements of mass per unit length on the TAMM · 3AET-labeled bacteriophage. For comparison purposes, in a specimen in which freshly thiolated fd bacteriophage was first deposited on the electron microscope (EM) grid and then labeled with an excess of TAMM · 3AET, bright spots now appear on the background as well as on the bacteriophage (Fig. 1b). This result demonstrates that in Fig. 1a there was no heavy metal on the background and that stoichiometric addition corresponds to binding of essentially all the TAMM · 3AET to the thiolated fd bacteriophage. Figure 1, c and d, illustrates the changes induced by increasing the total electron dose. Clustering weakly visible in Fig. 1c, increases with total electron dose (Fig. 1d), then later disappears with even higher doses (not illustrated). The overall loss of intensity is due to beam damage.

Clustering of TAMM in solution was eliminated through the use of *d,l*-penicillamine $[HSC(CH_3)_2CH(CO_2^-)NH_3^+]$ as the solubilizing ligand. The two methyl groups adjacent to the sulfur atom presumably inhibit the formation of -Hg-S(R')-Hg linkages. When fd bacteriophage was labeled with this reagent it exhibited uniform spots in the STEM of correspondingly lower intensity (Fig. 2a) than when labeled with TAMM · 3AET (Fig. 1, a to d). Atom migration and mass loss eventually occurred with increased total electron dose (Fig. 2b). Thus even with *d,l*-penicillamine, repeated scans at low beam currents or at higher magnifications produce aggregation. Although ultimately the heavy metals and the sample disappear as a result of beam damage, their visibility even at 600 e Å^{-2} (Fig. 2a) demonstrates the advantages of the low temperature stage in minimizing atom migration and evaporation.

The ratio of mass to unit length of fd bacteriophage normalized to TMV, a convenient internal mass standard, is 0.131 (N = 71 virus particles, S.D. = 0.005, the standard deviation of mass measurements on individual virus particles) corrected to zero electron dose (Fig. 2, c and d). This ratio in the presence of a twofold excess (per modified B coat protein) of aquoglycylmethionineplatinum(II) was found to be 0.140 (N = 12, S.D. = 0.004), a 7 percent increase. Measurements (*9*) of heavy atom scattering in the STEM have shown the cross sections to be significantly lower than predicted theoretically (*10*). The average scattering intensity per heavy atom for uranyl, silicotungstate, and colloidal platinum specimens was measured to be 8.8 σ_C, where σ_C is the scattering cross section for carbon. Using this value for σ_{Pt}, the formula of aquoglycylmethionineplatinum(II), and the increase in scattering resulting from the addition of the thiolating reagent to the fd bacteriophage coat protein, we predict a 7.5 percent scattering increase (1.7 percent from platinum and 5.8 percent from nonmetallic matter) for the binding of one aquoglycylmethionineplatinum(II) molecule per modified coat protein subunit. This result compares quite well with the observed 7 percent scattering increase.

The ratio of mass per unit length found for fd bacteriophage stained with TAMM · L_3, where L = *d,l*-penicillamine, is shown in Fig. 2d as a function of electron dose. The average ratio of mass per unit length for stoichiometric addition of TAMM · L_3 is 0.160 (N = 16, S.D. = 0.007), and the value extrapolated to zero electron dose (Fig. 2d) is 0.163. Using the heavy atom scattering cross section described above, we compute that one equivalent of TAMM · L_3 bound to fd modified coat protein would result in a normalized mass per unit length ratio of 0.154. This value is somewhat less than the observed one, probably because TAMM [like colloidal platinum (*9*)] has a scattering cross section greater than that of a single heavy atom.

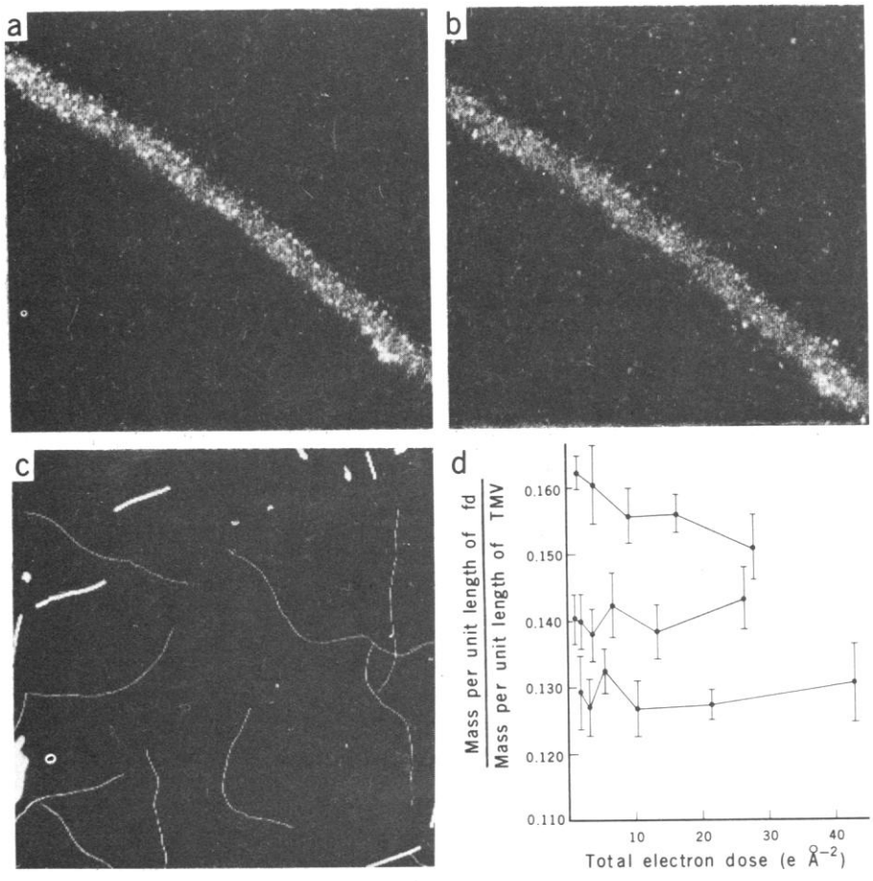

Fig. 2. (a) A STEM image (1300 by 1300 Å) of fd bacteriophage labeled with TAMM · L_3, L = penicillamine, in solution (containing dimethyl sulfoxide) prior to sample spreading on the EM grid. In contrast to Fig. 1a the sample exhibits uniformly sized spots of about 5 Å in diameter. The increased noise is due to use of a low electron beam current to minimize specimen damage. The total electron dose is 100 e Å^{-2}. (b) Same sample area as in (a), but the total electron dose was 600 e Å^{-2}. Atom migration and aggregation occur with repeated scanning as in Fig. 1, c and d, but aggregation is not as extensive. Atom loss is apparent. (c) Example of the type of micrograph data used for calculating mass per unit length and mass loss as a function of total electron dosage for fd bacteriophage and TMV. This sample has fd bacteriophage labeled with one equivalent of TAMM · L_3, no dimethyl sulfoxide, and unlabeled TMV at a total electron dose of 1.8 e Å^{-2} (first scan). The image displayed is 2.08 by 2.08 μm^2. No TAMM induced virus aggregation is observed. (d) Data of mass per unit length ratio as a function of total electron dose from micrograph in (c) and later scans (upper curve). The extrapolated value of mass per unit length for fd bacteriophage (normalized to TMV) at zero electron dose is 0.163. Also shown are fd labeled with glycylmethionineplatinum(II) (center curve) and fd control (bottom curve). The mass loss for TMV is 0.5 percent per e Å^{-2}. Error bars represent the standard deviation in measuring individual viruses on one micrograph.

Our results demonstrate the potential utility of polymercurimethanes as labels in conjunction with STEM microscopy for the study of biological structure at high resolution. The clustered TAMM · L_3 units are readily visualized and can be used to identify selectively stained components of macromolecular assemblies, such as the modified fd coat protein in our study.

JAMES J. LIPKA
STEPHEN J. LIPPARD
Department of Chemistry, Columbia University, New York 10027
JOSEPH S. WALL
Biology Department, Brookhaven National Laboratories, Upton, New York 11973

References and Notes

1. K. G. Strothkamp, J. Lehmann, S. J. Lippard, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1181 (1978).
2. J. J. Lipka, H. Bazari, C. Levinthal, S. J. Lippard, *Biophys. J.* **25**, 42a (1979).
3. J. S. Wall, *Scanning Electron Microscopy*, O. Johari, Ed. (Univ. of Chicago Press, Chicago, 1979), pp. 291–302.
4. D. A. Marvin and B. Hohn, *Bacteriol. Rev.* **33**, 172 (1969); J. Newman, H. L. Swinney, L. A. Day, *J. Mol. Biol.* **116**, 593 (1977).
5. L. Day, personal communication.
6. S. Huichi *et al.*, *Bull. Chem. Soc. Jpn.* **43**, 3849 (1970); I. M. Klotz and R. E. Heinig, *Arch. Biochem. Biophys.* **96**, 605 (1962).
7. J. F. Riordan and B. L. Vallee, *Methods Enzymol.* **25**, 449 (1972).
8. H. C. Freeman and M. L. Golomb, *Chem. Commun.* (1970), p. 1523; J. Lipka, unpublished results.
9. J. S. Wall, *Chim. Scripta*, in press.
10. J. D. Langmore, J. Wall, M. S. Isaacson, *Optik* **38**, 335 (1973).
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Spontaneous Diabetes Mellitus: Reversal and Prevention in the BB/W Rat with Antiserum to Rat Lymphocytes

Abstract. *Injections of rabbit antiserum to rat lymphocytes reversed hyperglycemia in 36 percent of spontaneously diabetic rats (Bio Breeding/Worcester) and prevented diabetes in susceptible nondiabetic controls. These findings strengthen the hypothesis that cell-mediated autoimmunity plays a role in the pathogenesis of diabetes in this animal model that mimics many morphologic and physiologic characteristics of human insulin-dependent diabetes mellitus.*

Diabetes mellitus occurs spontaneously in approximately 30 percent of a nonobese, outbred colony of Bio Breeding/Worcester (BB/W) (*1*) rats. The rapidly progressive syndrome is characterized by abrupt early onset (60 to 120 days), pronounced hyperglycemia, reduced concentrations of pancreatic and circulating insulin, hyperglucagonemia, and ketoacidosis. Without insulin replacement therapy, most animals succumb within 1 to 2 weeks of the detection of glycosuria (*2*). A unique feature of this model is the presence of profound insulitis prior to and early in the syndrome, with lymphocytes, macrophages, and occasionally eosinophils infiltrating the pancreatic islets. Late in the disease the islets are small and marked by the absence of insulin-synthesizing B (β) cells (*2*). The physiologic and morphologic characteristics of these animals closely resemble those of insulin-dependent humans with juvenile-onset diabetes. The demonstration that selective inbreeding of diabetic animals increases the frequency of diabetes, and the lymphocyte and macrophage nature of the insular infiltrate, suggests a cell-mediated autoimmune pathogenesis of the syndrome.

We present evidence here that the administration of rabbit antiserum to rat lymphocytes (ALS) normalized the concentration of plasma glucose (PG) in 36 percent of acutely diabetic rats and prevented the occurrence of diabetes in susceptible nondiabetic littermates. In contrast, hyperglycemia persisted in rats that were untreated or were injected with normal rabbit serum (NRS). Furthermore, untreated and NRS-injected euglycemic littermates became diabetic with the expected frequency. The effectiveness of ALS in the treatment and prevention of diabetes supports an autoimmune pathogenesis of the syndrome.

The BB/W rats in the susceptible age range (60 to 120 days) were tested for glycosuria three times weekly. Rats were defined as diabetic if their urine glucose indicated 2+ with Testape (*3*) and if they had PG concentrations greater than 180 mg/dl. The disease was detected at a mean age of 101 days. The PG concentration in most animals exceeded 300 mg/dl. Diabetics (N = 93) and nondiabetic

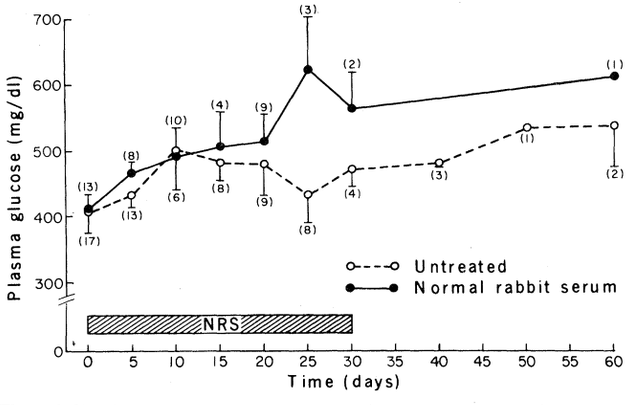

Fig. 1 (left). Plasma glucose concentrations in untreated and NRS-injected BB/W rats studied from the first detection of glycosuria (day 0). The concentrations for both groups of animals remained elevated for the duration of the experiment. Four rats survived beyond 30 days. The others died or were killed at earlier times when moribund. In one untreated rat the concentration spontaneously returned to normal (data not shown). Vertical bars indicate standard errors of the means; numbers of animals are shown in parentheses.

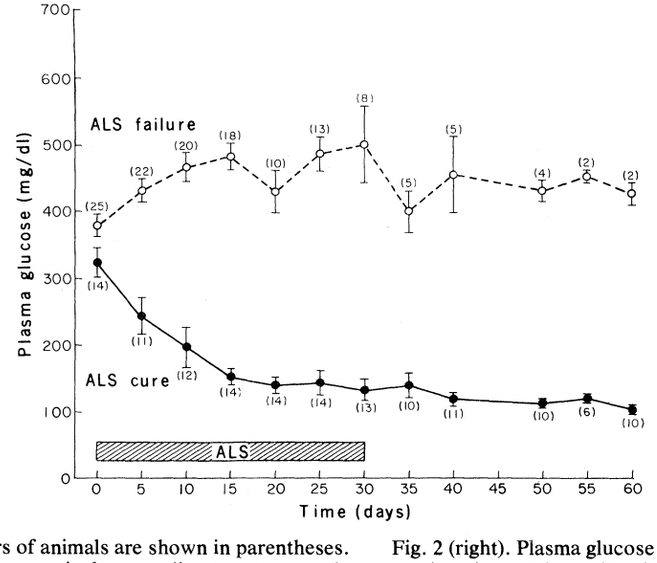

Fig. 2 (right). Plasma glucose concentrations (means ± S.E.) of ALS-injected BB/W rats, plotted retrospectively according to outcome. Concentrations in "ALS cure" animals returned to normal 10 to 15 days after the first injection and remained normal for the duration of the experiment. Concentrations in "ALS failure" rats resemble those of NRS-injected and untreated animals (Fig. 1). In ALS cure rats at day 0 the PG concentrations were significantly lower than in the ALS failure rats ($P < .05$).

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