INTERACTION OF MERCURY LABELED ANTIBODY WITH THE GLUCOSE DEHYDROGENASE OF BACILLUS CEREUS

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ABSTRACT

INTERACTION OF MERCURY LABELED ANTIBODY WITH THE GLUCOSE DEHYDROGENASE OF *BACILLUS CEREUS*

By Helen L. Engelbrecht

Stains for the detection of antigens by electron microscopic techniques can be produced by coupling heavy metals to specific gamma globulin. Mercury was coupled to anti-glucose dehydrogenase and the immunological properties of the modified globulin were investigated.

The enzyme specific globulin was produced in rabbits, carboxy methylated and coupled at pH 8.6 to the diazonium derivative of para-aminophenylmercuric acetate. The extent of labeling achieved ranged from seven to twenty-one atoms of mercury per molecule of antibody. The mercurated globulin was separated from the reaction products by gel filtration with Sephadex G-25. The antienzyme activity of untreated, carboxy methylated, and mercurated antibody (14 atoms mercury per molecule) and mercurated normal gamma globulin were compared. The enzyme specific globulin lost 80% of its original activity on acetylation but 50% of its activity was recovered on mercuration. Mercurated normal gamma globulin did not precipitate glucose dehydrogenase. Spores of *Bacillus cereus*, which had been fixed in formalin and partially disrupted, were reacted with mercurated specific globulin , enzyme specific globulin, and normal gamma globulin. Only mercurated specific globulin

produced discrete staining of the spore cytoplasm on electron microscopic examination.

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
LITERATURE REVIEW	4
Fractionation of Serum Proteins	4
Preparation of Protein Derivatives	5
Mercurated Antibody as an Electron Dense Stain	7
MATERIALS AND METHODS	9
Preparation and Assay of Glucose Dehydrogenase	9
Preparation of Medium	9
Preparation of Inoculum and Spore Crops	10
Assay of Glucose Dehydrogenase	11
Purification of Glucose Dehydrogenase	12
Estimation of Protein	13
Preparation of Specific Gamma Globulin	14
Assay for Mercury	15
Synthesis of P-aminophenylmercuric Acetate	16
Diazotization and Coupling of Mercurated Aniline to Gamma Globulin	16
Immunochemical Methods	17
Modification of the Procedure of Cohn and Torriani	17
Agar-gel Diffusion Studies	18
Electrophoresis of Gamma Globulin Fractions	19
EXPERIMENTAL RESULTS	20
Production and Assay of Antibody	20
Mercuration of Gamma Globulin	20
Determination of Changes in Properties of Gamma Globulin by Mercuration	26
Spectrophotometric Studies	26
Electrophoretic Mobility	26
Enzyme Inhibition	29
Electron Density	31
DISCUSSION	39
SUMMARY	45
REFERENCES	47

LIST OF TABLES

Table — Page

1. Titration of Glucose Dehydrogenase by Rabbit Anti-Glucose Dehydrogenase 21

2. Titration of Antibody and Antigen in Two-dimensional Diffusion (72 hours) 22

3. Effect of PAPMA Concentration on the Mercuration of Gamma Globulin. 27

4. Electrophoretic Mobility 30

5. Box Titration of Untreated and Mercurated Anti-enzyme vs. Glucose Dehydrogenase in Ouchterlony Double Diffusion Plates. 34

LIST OF FIGURES

Figure		Page
1.	Effect of Various Concentrations of PAPMA on Antienzyme Activity	24
2.	Effect of the Concentration of PAPMA on the Mercury Content and Immunochemical Properties of Gamma Globulin.	25
3.	Spectral Analysis of Gamma Globulin	28
4.	Effect of Treated and Untreated Normal Gamma Globulin and Antienzyme on Glucose Dehydrogenase Activity.	32
5.	Titration of Antienzyme Gamma Globulin by 2-Dimensional Immunodiffusion	33
6.	Electron Micrographs of Aggregates of Enzyme-Antienzyme Globulin which had been Reacted One Minute and Dried on Formvar-Coated, Electron Microscope Grids.	36
7.	Electron Micrographs of Spores which had been Disrupted in a Nossal Shaker, Formaldehyde-Fixed and Treated with Antienzyme Gamma Globulins . .	38

INTRODUCTION

Microbiology like many other sciences has been dependent for its development on advances in instrumentation. Leewoenhoek's superior ability in lens grinding permitted him to develop a microscope which allowed him to visualize the "animacules", some of which are now known as bacteria. The development of stain technology was a consequence of the need to distinguish microorganisms from their environment. The Gram stain, used to differentiate bacterial cells from tissue was a major contribution in this field. The application of modern cytological techniques to the study of microorganisms with the light microscope was exploited for example, by Bisset (1950), Knaysi (1951), and Robinow (1960, 1960a). Their contributions consisted of the standardization of fixation techniques in order to minimize morphological distortion and the development of staining techniques for the maximal visualization of the fine structure.

With the widespread use and improvement of electron microscopic techniques, stress has been placed on the development of specific stains for use with this instrument. These stains are utilized for the identification of organelles within the cells. Electron micrographic investigations have supplemented information already acquired by metabolic studies from which one can obtain an understanding of the integrated processes of the cell.

Singer, (1959, 1960) borrowing from the fluorescent antibody technique of Coons (1942), coupled an electron dense

moiety to specific antibody and established a means for localizing specific proteins. He coupled ferritin to specific gamma globulins producing electron dense antibody and reacted the conjugate with cells. Antigen-antibody complexes were formed in which the specific sites of the antigen could be seen. It is likely that the conjugation of any heavy metal to antibody could be used as an electron dense stain if (a) the atomic number of the metal is sufficiently high and (b) the antigen-antibody aggregate is of sufficient size.

The molecular weight of the ferritin molecule is approximately four times that of gamma globulin. The high molecular weight complex might limit the resolution and could also affect the specificity of the antigen-antibody reaction. The objectives of current research in staining technique for electron microscopy are to achieve lower resolution and higher specificity of the stain, yet not alter the reactivity or specificity of the conjugated antibody.

Kulberg and Azadova (1964) stained viral particles with virus-specific gamma globulin which had been coupled to the diazonium salt of p-aminophenylmercuric acetate (PAPMA). This compound is of considerable interest for it is possible to couple several of these molecules to the protein and still maintain its immunochemical properties.

Immunochemical techniques have been widely applied to the study of cell differentiation. The success of the procedure lies in the fact that in the course of differentiation, uniquely different proteins are synthesized within the cells

and these can be readily detected. Sporulation is a differentiation process which occurs in the Bacillaceae and the physiology of the process should be ammenable to study by immunochemical procedures. The following criteria should be met in order to apply immunochemistry to the sporulation process. The proteins investigated must be either spore-specific or vegetative cell specific. They should be antigenic in suitable laboratory animals, be readily assayed and be capable of purification so that highly specific antibody against them can be prepared. Glucose dehydrogenase from spores of Bacillus cereus was selected as the antigen in this study because it is a spore specific protein. The enzyme is stable on storage and a sensitive, yet simple assay is available. Furthermore, highly purified preparations of glucose dehydrogenase can be prepared with relative ease.

The object of the work discussed in this thesis is twofold: (a) to establish the conditions for the preparation of highly mercurated, specific antibody to glucose dehydrogenase; (b) to investigate the feasibility of its use as an electron dense stain.

LITERATURE REVIEW

One of the objects of the present study was to modify the structure of specific gamma globulin by coupling to it mercurated diazonium salts. In order to determine the properties of the resulting conjugate, it was essential that a separation of the modified protein from the native protein be achieved.

Fractionation of Serum Proteins

Various systems of fractionation of serum proteins have been used. Landsteiner (1914) and Nichols et. al. (1948) described methods utilizing alcohol precipitation. These procedures were conducted at 0 to 5 C. Alcohol precipitation above these temperatures frequently results in protein denaturation. Marenzi and Barrow (1960) investigated the fractionation of serum proteins using 2.13 to 2.5 M ammonium sulfate solution as precipitating agents. They found that there was no influence on the total globulin precipitation by either time or temperature and that the globulins recovered were native when examined electrophoretically.

A chromatographic method of separation of proteins was developed by Peterson and Sober (1956) using substituted celluloses. The substituent groups were either anionic or cationic substances. The proteins were eluted by change of pH or ionic strength of the eluant. Humphrey and Porter (1957) separated proteins from human serum on diethyl amino ethyl cellulose columns using a sodium phosphate concentration gradient for

elution. They obtained one principal fraction, gamma globulin, and three smaller fractions.

Human serums have also been fractionated by gel filtration. Fireman et. al. (1964) used Sephadex G-200 to study the elution characteristics of nineteen known serum proteins. They obtained three major peaks which eluted in order dependent on their molecular size with the larger proteins eluting first. Smaller molecules appeared in later effluent fractions depending on their penetration of the gel and their reaction with the gel.

Zone electrophoresis has been used for separation of blood proteins. Cellulose acetate films were used by Kohn (1958) who separated the serum into twelve distinct protein bands. Similar experiments on paper yielded a maximum of five proteins. Only very small quantities of protein can be separated by zone electrophoresis. That is, it would be difficult to utilize this procedure as a preparative method, but it is useful for the identification of proteins on a semi-micro scale.

Preparation of Protein Derivatives

Various modifications of purified proteins have been attempted. Changes in protein by addition of various chemical compounds have been investigated by Landsteiner and Gablons (1912, Landsteiner (1914), Landsteiner and Lampl (1914, 1915, 1917, and 1918), and by Landsteiner and Prasek (1916). The forementioned investigators found that the treatment of protein and with acetic anhydride changed their antigenicity

and reduced their species specificity. When serum proteins were treated with alcohol and H_2SO_4, alcohol and HCl and diazomethane, the derivatives obtained differed antigenically from the original proteins. They also found that the extent of acetylation varied with the proteins employed. They concluded that the acetate groups had attached to the nitrogen atoms. Landsteiner and Lampel (1918) coupled horse serum to diazo derivatives of aniline, sulfanilic acid, para-aminobenzoic acid and atoxyl. The conjugates were injected into rabbits and antibodies were produced whose specificities were directed at the aromatic ring structure. The various azoproteins were found to be distinguishable serologically. Landsteiner's studies have shown that injection of a modified antigen into suitable animals produces modified antibodies.

Marrack and Orlans (1954) showed that the precipitating character of various rabbit antibodies was destroyed by acetylation. However, the acetylated antibody still combined with the antigen since antigen-antibody aggregates were formed by coprecipitation. Karush (1957) showed that the disulfide bonds are essential in the maintainance of the specific configuration of the combining region of the antibody molecule. He also showed that antibody globulins which had been treated with an excess of monoiodoacetate did not change their serologic properties. Sulfhydral groups on antibody globulin do not appear to be involved in the primary antigen-antibody reaction.

Mercurated Antibody as an Electron Dense Stain

The use of mercury-antibody conjugates as immunochemical stains for electron microscopy was a modification of the work of Singer (1959, 1960). He utilized ferritin as an electron dense label for anti-bovine serum albumin, anti-bovine RNase, and anti-tobacco mosiac virus. Its size and iron content are the basis for its success as an electron dense stain.

Many attempts have been made to introduce organic mercurials into proteins and to examine the resulting changes in protein properties. Cook et. al. (1946) studied the effects of phenylmercuric nitrate on enzyme systems. They found that the activity of cytochrome oxidase, succinic oxidase, succinic, lactic, and glucose dehydrogenase as well as catalase were depressed due to the involvement of sulfhydral groups. Pepe (1961) used the diazonium salt of tetraacetoxymercuriarsanilic acid for conjugation to rabbit gamma-globulin. The resultant conjugate was electron dense, but less than six sites of conjugation were obtained with antibody gamma globulin.

Kochler and Dowben (1961) used p-aminophenylmercury acetate (PAPMA) to examine the interactions of a nonionic detergent with proteins using a bovine plasma albumin-azomercurial complex. The PAPMA was prepared according to the method of Dimroth (1902) and diazotized with sodium nitrite. The coupling was primarily to the tyrosine and tryptophan residues of the protein. This same azomercurial was used by Kulberg and Azadova (1964) for specific contrast in electron microscopy. They prepared an azo-mercurial

complex from virus-specific serum. The complex was more stable and greater yields of antibody were obtained than with the method of Pepe.

Rizok and Kallos (1965) introduced mercury into the active site of chymotrypsin by using p-chloromercuribenzene-sulfonyl fluoride. The reagent combined irreversibly with a serine residue and the enzyme was irreversibly inactivated.

Kendall (1965) labeled the amino groups of rabbit anti-serum (anti-PR 8 influenza virus) with N-acetyl-homocysteine thiolactone. He reacted the conjugate with 0.1% aqueous methyl mercuric hydroxide and produced an electron dense stain.

Koike et. al (1964) coupled diazotized anthranilic acid to tyrosine residues of anti-PR-8 influenza virus. The tyrosine-anthranilate complex was an effective chelating agent and treatment with heavy metals was used to produce electron dense stains.

MATERIALS AND METHODS

This thesis describes the mercuration of antibody to glucose dehydrogenase and the effects of antibody on the enzyme. The following is a description of those specialized techniques used in the preparation of antigen and antibody and in the measurement of the effects of mercuration.

Preparation and Assay of Glucose Dehydrogenase

Bacillus cereus is a member of the Bacillaceae and forms endospores upon completion of its exponential growth. The organism employed in this research was originally isolated at the University of Illinois and has been variously known as *Bacillus terminalis* (Stewart and Halvorson, 1953) and *Bacillus cereus* T (Church and Halvorson, 1955). This strain produces an enzyme which lyses the sporangia so that free spores are easily obtained.

Preparation of Medium

Sporulation levels of 95% or greater are achieved in semi-synthetic growth medium, G medium, described by Stewart and Halvorson, 1953 and Hashimoto et al., 1960. The modified 'G' medium contained the following materials per liter: K_2HPO_4, 1g; $(NH_4)_2SO_4$, 4g; $MnSO_4 \cdot H_2O$, 0.1g; $MgSO_4$, 0.8g; yeast extrast, 2g; glucose, 4g; $ZnSO_4$, 0.01g; $CuSO_4 \cdot 5H_2O$, 0.01g; $CaCl_2$, 0.1g; and $FeSO_4 \cdot 7H_2O$, 0.001g. Dow Corning antifoam AF was also added at the rate of 1 ml/liter. The organism grew and sporulated well in either large or small

quantities at 30 C. For convenience in extraction and preparation of enzyme, one pound batches of spores were produced.

Preparation of inoculum and Spore Crops

To obtain the levels of sporulation described, it was necessary to attain some level of synchrony in the culture. This was achieved by developing a schedule of transfers in order to prepare an active inoculum for one hundred liters of medium. The stock culture was maintained in the sporulated state at 4 C on nutrient agar.

The inoculation and transfer schedule was as follows: A fresh nutrient agar slant was inoculated from stock and incubated for eight hours. 'G' medium, 50 ml in a 500 ml Erlenmeyer flask was inoculated from the slant and shaken for two hours. Two similar flasks were inoculated with 10 ml each of the culture and shaken for two hours. These cultures were then used to inoculate six flasks. After two hours, the 300 milliliters of culture were introduced into 2700 milliliters of 'G' medium in a New Brunswick fermenter. (New Brunswick Scientific Co., New Brunswick, N. J.) An active inoculum for the 100 liter culture was attained after two hours of growth.

The culture prepared in the 100 liter stainless steel fermenter (Stainless Steel Products Co.) sporulated, and lysed to produce free spores in approximately nineteen hours. The medium was cooled, the spores harvested in a Sharples

centrifuge, Model A5-12, and stored as a frozen paste at -15 C.

Assay of Glucose Dehydrogenase

Church and Halvorson (1955) first reported the nucleotide-linked glucose dehydrogenase in extracts of Bacillus cereus spores. The enzyme was partially purified and characterized by Doi, Church, and Halvorson (1959). These formentioned authors were unaware of the manganese requirement for the enzyme because they had not achieved sufficiently high levels of purification. A spectrophotometric assay was used to measure the activity of glucose dehydrogenase. One molecule of nicotinamide adenine dinucleotide (NAD) is reduced per molecule of glucose oxidized, therefore, the rate or progress of the reaction may be followed by observing the light absorption by reduced NAD at 340 millimicrons. These assays were conducted in a Beckman DU spectrophotometer equipped with a Ledland log converter and a SR Sargent recorder. The sample compartment of the spectrophotometer was maintained at 25 C.

The assay cuvettes contained 0.5 milliliters 1 M tris buffer, pH 8, 0.2 milliliter 0.001 M Mn $SO_4 \cdot H_2O$, 0.1 milliliter 1 M glucose, 0.1 milliliter 0.02 uM/ml NAD, and 0.1 milliliter of enzyme preparation. One unit of enzyme catalyzed the reaction producing an optical absorbance change of 0.001 per minute. Specific activity was defined as the activity per milligram of protein. Pure enzyme has a specific activity of 500,000 (Bach, 1963).

Purification of Glucose Dehydrogenase

Glucose dehydrogenase was extracted from disrupted spores of Bacillus cereus. It was purified by successive ammonium sulfate fractionations and sequential gel filtrations. Five hundred grams of frozen spores were thawed and suspended in 1200 milliliters of 0.05 M acetate buffer, pH 5.0. The suspension was placed in an Eppenbach colloid mill (model MV-6-3 Gifford-Wood Co., Hudson, N. Y.) which was cooled by circulating alcohol at -20 C. The initial rotor-stator distance was 0.06 inches (wide separation). Four milliliters of antifoam were added to the suspension and the mill was brought to about 25% of the full speed. Seven hundred and forty grams of glass beads (No. 110 pavement marking beads, Minnesota Mining and Manufacturing Co.) were added slowly. The speed of the unit was increased to approximately 85 per cent of the full speed and the rotor-stator gap reduced to 0.03 inches. The spores were subjected to this treatment for 10 to 30 minutes and drained from the mill. Glucose dehydrogenase was present in the supernatant, obtained on centrifugation of the extract.

The enzyme precipitated in solutions of ammonium sulfate between 0.5 and 0.8 saturation. The precipitates were resuspended in 150 milliliters of cold 0.05 M acetate buffer, pH 5.0. On refractionation, the enzyme precipitated in the 0.35 - 0.60 saturation range. The precipitate was resuspended in 15 milliliters of cold buffer and then treated with one milliliter of 2% solution of protamine sulfate per 100

milligrams of protein to remove the nucleic acids.

Further purification of the enzyme was accomplished by gel filtration using Sephadex G-100 (Pharmacia). The columns were 4 x 45 centimeters. Fractions of 5 milliliter volume were collected at ten minute intervals. The tubes containing the highest specific activity were pooled and dialyzed against 0.05 M acetate buffer, pH 5.0. To obtain more highly purified enzyme, the dialysate was concentrated by lyophilization and re-chromatographed on a Sephadex G-200 column. By this procedure, enzyme of 60% purity in 50% yield was obtained (Sadoff, unpublished).

Estimation of Protein

The protein content of glucose dehydrogenase was estimated spectrophotometrically by the 280/260 ratio according to the method of Warburg and Christian (1942).

The estimation of the protein content of rabbit gamma-globulin preparations was performed by use of the biuret test according to Kabat and Meyer (1961). One half milliliter of the protein sample was added to .75 ml of the biuret reagent. The mixture was incubated at 37 C for one half hour and the optical absorbance read at 555Å in Beckman DU Spectrophotometer using one milliliter matched cuvettes. A standard curve for protein content was prepared by using normal, electrophoretically pure rabbit gamma globulin. The protein content was determined in terms of milligrams per milliliter and converted to micromoles per milliliter using 167,000 as the molecular weight of gamma globulin.

Preparation of Specific Gamma-Globulin

Ten Dutch belt rabbits were inoculated for the production of antibody to glucose dehydrogenase. Equal quantities of glucose dehydrogenase (specific activity 100,000) and complete Freund's adjuvant (Difco Laboratories) were emulsified in a 1 cubic centimeter syringe with a 23 gauge needle. One tenth milliliter of the enzyme-adjuvant was injected subcutaneously into each of ten sites in the regions of the inguinal and brachial nodes of each rabbit. This amounted to the injection of 0.02 - 0.025 milligrams of protein per rabbit. The pattern of inoculation was repeated after two weeks. After the second series of injections, twenty milliliters of blood were drawn by cardiac puncture. The third and subsequent injections were given in four or six sites. The enzyme, however, was emulsified in incomplete Freund adjuvant (Difco Laboratories). This was done to prevent severe reactions to the Mycobacterium lipoproteins which are in the complete adjuvant. After four bleedings, the animals could no longer sustain cardiac puncture and all subsequent bleedings were done from the marginal ear veins.

The blood samples were collected in centrifuge tubes, placed in a slanted position, and allowed to clot. The clots were freed from the sides of the tubes and stored for one hour at 37 C prior to 12-24 hours refrigeration at 4 C. All serums were decanted and both serums and clots were subjected to centrifugation at 2000xg in a Survall-SS-1 centrifuge. Additional serum was recovered from the clots by this procedure. The serum was stored at -15 C.

To obtain gamma globulin from the serum, .65 milliliters of saturated ammonium sulfate solution per milliliter of whole serum was added dropwise. The final concentration of ammonium sulfate was .39 saturation. The precipitate was allowed to stand in the cold for one half hour, then removed by centrifugation at 10,000 x g for twenty minutes. The supernant was decanted and the precipitate brought to the original serum volume with phosphate buffered saline, pH 7.2 (Merchant, 1964). The preparation was stable at either 4 C or at -15 C.

Assay for Mercury

The mercury assay was performed according to a modification of the method of Abadi and Wilcox (1960). The solvent for the mercurials including mercurated protein was a solution of five parts dimethyl formamide to one part glacial acetic acid. Diphenylthiocarbazone (Dithizone, Fisher Scientific, $C_6H_5NH\ NH{\cdot}CS{\cdot}N{:}NC_6H_5$) was used for the colorimetric titration. (1 milligram dithizone per 200 milliliters chloroform.) The mercury standard contained one macromole $HgCl_2$ per milliliter. Mercuric compounds appear intense orange with the titration procedure while mercuri compounds are lighter. At their respective end points, each mercuric ion is bound to two dithizone molecules as keto complexes and each mercuri compound to one dithizone as an enol complex.

The endpoint of the mercury titration is difficult to discern for an untrained technician. Cross-lighting with a microscope illuminator and a white background facilitated the titration.

Synthesis of P-aminophenylmercuric Acetate

Para-aminophenylmercuric acetate was prepared according to the method of Dimerot (1902). Mercuric acetate, 15.4 grams, was dissolved in 80 milliliters distilled water and stirred into 25 milliliters freshly distilled aniline (b. pt. 180°C) in 35 milliliters 90% ethyl alcohol. After forty-five minutes, crystals of product were formed. Five hours later, the crystals were filtered in a Buchner funnel with Whatman #1 filter paper, washed with distilled water, and air-dried overnight. The product was assayed for its mercury content and found to contain 57%. Assuming one atom of mercury per molecule, the product had a molecular weight of 352 which compares to 352 theoretical.

Diazotization and Coupling of Mercurated Aniline to Gamma Globulin

Fifty milligrams of para-aminophenylmercuric acetate were dissolved in 0.5 milliliters of 50% glacial acetic acid and placed in an ice bath. An excess of sodium nitrite was added slowly (approximately 10 milligrams) with constant stirring. Potassium iodide-starch test paper was used to ascertain that an excess of nitrite existed. The reaction proceded for one half hour.

The sulfhydral groups of rabbit gamma globulin were carboxy methylated in the following manner. Two milliliters of gamma globulin, 15-25 milligrams per milliliter, were incubated at room temperature for two hours with one milliliter of 0.1 M iodoacetic acid. The modified protein was dialyzed

overnight against 0.1 M Veronal buffer, pH 8.6.

Three milliliters of protein solution were coupled to a 1:50 dilution of the diazotized mercurial at pH 8.6 in a Sargent pH stat. Forty moles of diazonium were used per mole of protein. The temperature was maintained in the range of 12 - 15 C.

The reaction mixture was placed in an ice bath for thirty minutes and then applied to a Sephadex G-25 (coarse) column (1 x 20 centimeters). Upon elution with saline, the mercurated protein moved with the solvent front. Those fractions containing the maximal concentration of protein (biuret determination) were saved.

Immunochemical Methods

Modification of the Procedure of Cohn and Torriani

A modification of the method of Cohn and Torriani (1952) was used to test for antibody specific for glucose dehydrogenase. Two-fold serial dilutions of the enzyme, 15000 units per milliliter were prepared in 13 x 100 millimeter tubes. The final activity was 235 units per milliliter which was 1/64 of the initial enzyme activity. One tenth milliliter of undiluted anti-enzyme globulin was added to each tube. Normal gamma globulin was used as a control. Phosphate buffered saline, 0.5 milliliter was added to another set of tubes containing enzyme as an antigen control. The volume of all tubes was then adjusted to one milliliter with saline. The reaction mixtures were incubated 1-2 hours at room temperature, overnight at 4 C, and then clarified for twenty minutes (Servall

SS-1) at 5000 x g. The supernatant solutions were tested for the presence of glucose dehydrogenase.

Agar-Gel Diffusion Studies

A gel diffusion technique was developed by Ouchterlony (1948) based upon the single diffusion method of Oudin (1946). The Ouchterlony procedure involves double diffusion in two dimensions. A layer of agar was poured into petri dishes and holes punched in the agar. The reagents were added to the holes and diffused from the holes, toward each other.

Pre-cut Ouchterlony plates containing six center wells of five millimeters diameter and two outer rows of five wells of seven millimeters diameter were obtained from Dr. John G. Ray Jr. of Fort Detrick, Md. The gel, 12 milliliters per plate, contained 10 grams monobasic potassium phosphate, 9 grams sodium chloride, 10 grams Noble Agar (Difco), and 0.1% thimersal (E. Lilly) per liter of distilled water.

Dilutions of 1:1, 1:2, 1:4, 1:8, and 1:16 of enzyme, 69,000 units per milliliter and specific activity 150,000 were placed in the center wells of the five plates. Serial dilutions of untreated antibody gamma globulin was added to the top row of large wells. Serial dilutions of mercurated antibody gamma globulin were added to the lower rows. Similar control plates were prepared using normal serum gamma globulin. The plates were incubated at room temperature in a plastic bag, in a water-saturated atmosphere.

The plates were observed at 24, 48, and 72 hours and the precipitation lines were photographed.

Electrophoresis of Gamma Globulin Fractions

Electrophoretic studies were carried out on cellulose acetate strips to detect changes in the mobility of the protein due to acetylation and mercuration. (Sepraphore II and Sepraphore III, Gelman Instrument Co.) The proteins were stained on the strips with nigrosin and Ponceau S as described by Consden and Kohn (1959).

Ten microliters of the protein solution were applied to the strips. Owen buffer (1958) of the following composition was used:

5.0 grams Sodium diethyl barbitone
3.25 grams Sodium acetate (hydrated)
34.2 milliliters 0.1 N HCl
Distilled H_2O to one liter

The pH of this buffer was 8.6. The potential gradient of 27 volts per centimeter was maintained.

EXPERIMENTAL RESULTS

Production and Assay of Antibody

Antibodies specific for glucose dehydrogenase were elicited in rabbits as described in materials and methods. In the initial attempts where enzymes of 2% purity was used, no detectable antibody response was elicited. The rabbits which had been injected with this protein were removed from the test group. All subsequent antigens used were at least 20% pure. These rabbits produced high titer enzyme specific globulin according to the Cohn and Torriani assay (1952) (Refer Table 1) and the Ouchterlony plate method of Ray and Kadull (1964) (Refer Table 2).

The titration data show that one milligram of globulin protein, when suspended in phosphate buffered saline, will complex with 10,600 units of glucose dehydrogenase.

Mercuration of Gamma Globulin

The mercuration procedures of Kulberg and Azadova (1964) were unsatisfactory for preparation of labeled gamma globulin. These authors employed approximately two thousand fold excess of diazotized PAPMA with the result that excessive precipitation of the gamma globulin occurred.

A study was instituted to examine the effects of the concentration of PAPMA on the extent of mercuration of gamma globulin. The mercuration of the gamma globulin was performed with diazotized PAPMA in molar excesses of 200, 100, 67, 40, and 29 fold. The yellow azoproteins were separated from the

TABLE 1: TITRATION OF GLUCOSE DEHYDROGENASE BY RABBIT ANTI-GLUCOSE DEHYDROGENASE

	Total enzyme per milliliter	Fraction of enzyme remaining active after treatment						
		Antigen control	Normal gamma[1] globulin	Antienzyme gamma globulin[2]				
		No serum	1:10	1:2	1:10	1:20	1:100	1:200
Run #5 7/8/65	7,500		1.06		0.093		0.95	
	3,700		1.1		0.074		0.675	
	1,800		1.1		0		0.278	
	935		1.06		0		0.347	
	467		1.06		0		0.645	
	223		0.5		0		0	
Run #13 9/10/65	6,000	0.90	1.08	0		0.117		0.92
	3,000	0.90	1.07	0		0.133		0.87
	1,500	0.80	1.03	0		0		0.87
	750	1.06	1.06	0		0		0.93
	375	0.80	1.07	0		0		0.54
	167	1.07	1.07	0		0		0

1. Addition of normal gamma globulin increases the activity of the enzyme.

2. Pooled antienzyme gamma globulin from four rabbits with a protein concentration of twelve milligrams/ml.

TABLE 2: TITRATION OF ANTIBODY AND ANTIGEN IN TWO-DIMENSIONAL DIFFUSION (72 HOURS)

	Enzyme (Specific activity 100,000)				
Antienzyme gamma-fraction	1:1	1:2	1:4	1:8	1:16
1:1	+	+	+	+	+
1:2	+	+	+	+	+
1:4	+	+	+	+	+
1:8	+	+	-	-	-
1:16	-	-	-	-	-
Normal gamma globulin	-	-	-	-	-

$MRD_S^1 = 1:8$

$MRD_A^2 = 1:16$

1. MRD_S is defined as the minimal reacting dilution of serum.
2. MRD_A is defined as the minimal reacting dilution of antigen.

reaction mixtures on G-25 Sephadex columns. The mercury content of the protein was assayed with dithizone reagent.

Immunochemical properties of the gamma globulin were determined by the method of Cohn and Torriani (1952). Typical results are shown in figure 1. The controls were normal gamma globulin. The enzyme precipitating titer of the individual globulins can be obtained by extrapolating these curves to zero soluble enzyme. The titer is the point of intersection of the extrapolated line and the abcissa.

The effect of the concentration of PAPMA on the mercury content and the immunochemical properties of gamma globulin are shown in figure 2. It should be noted at forty-fold molar excess of PAPMA that an average of thirteen atoms of mercury are introduced into the gamma globulin molecule with concomitant reduction of precipitating activity of 60%. Increases of PAPMA to 200 molar excess increased the mercuration to twenty-four mercury atoms per gamma globulin with little change in precipitating activity.

The yield of mercurated protein was also dependent on the concentration of PAPMA. The optimal concentration of PAPMA for coupling is in the range of 40-67 molar excess. Table 3 presents data showing the yield of mercurated gamma globulin decreases as the molar excess of PAPMA increases. Furthermore, the highly mercurated gamma globulins are unstable and tend to precipitate on storage.

All studies requiring mercurated gamma globulin were therefore carried out with gamma globulin which had been

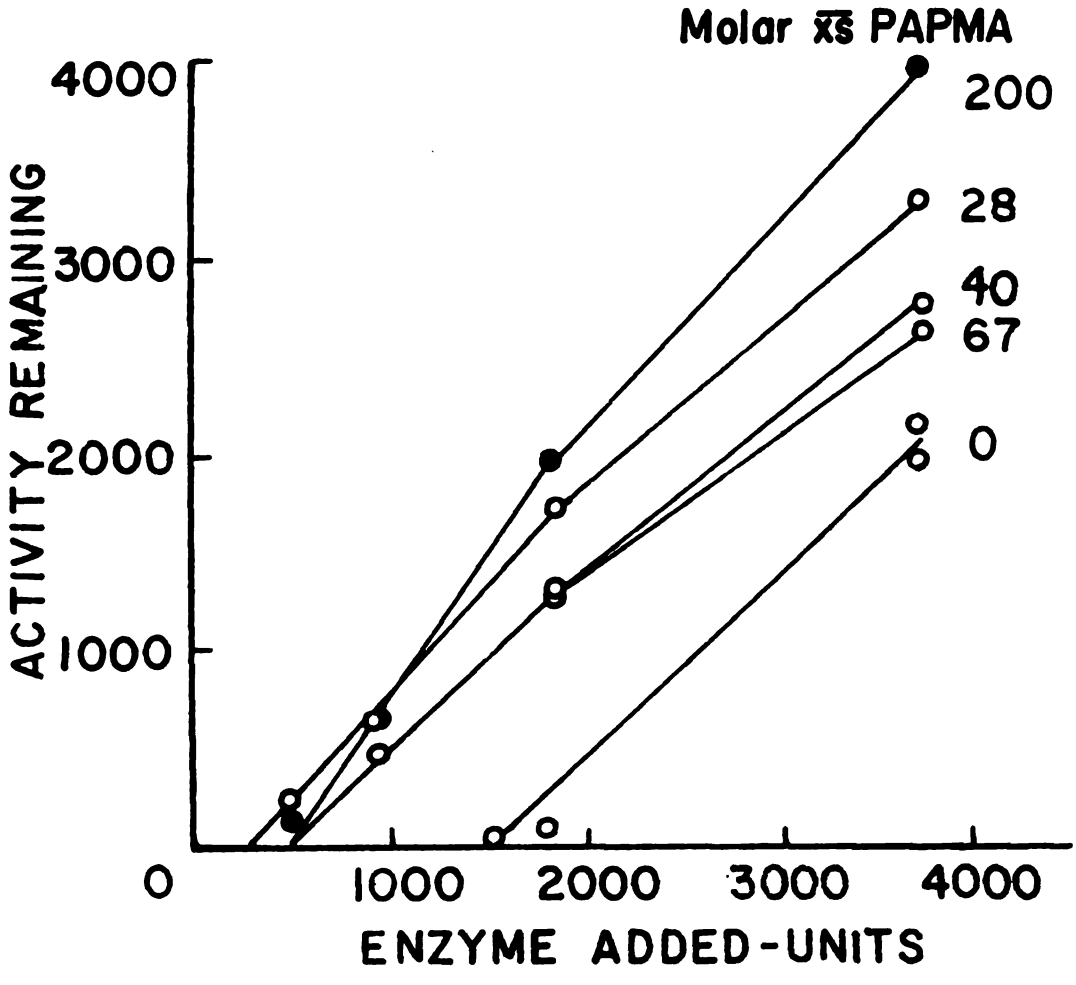

Figure 1: The effect of concentration of PAPMA on antienzyme activity.

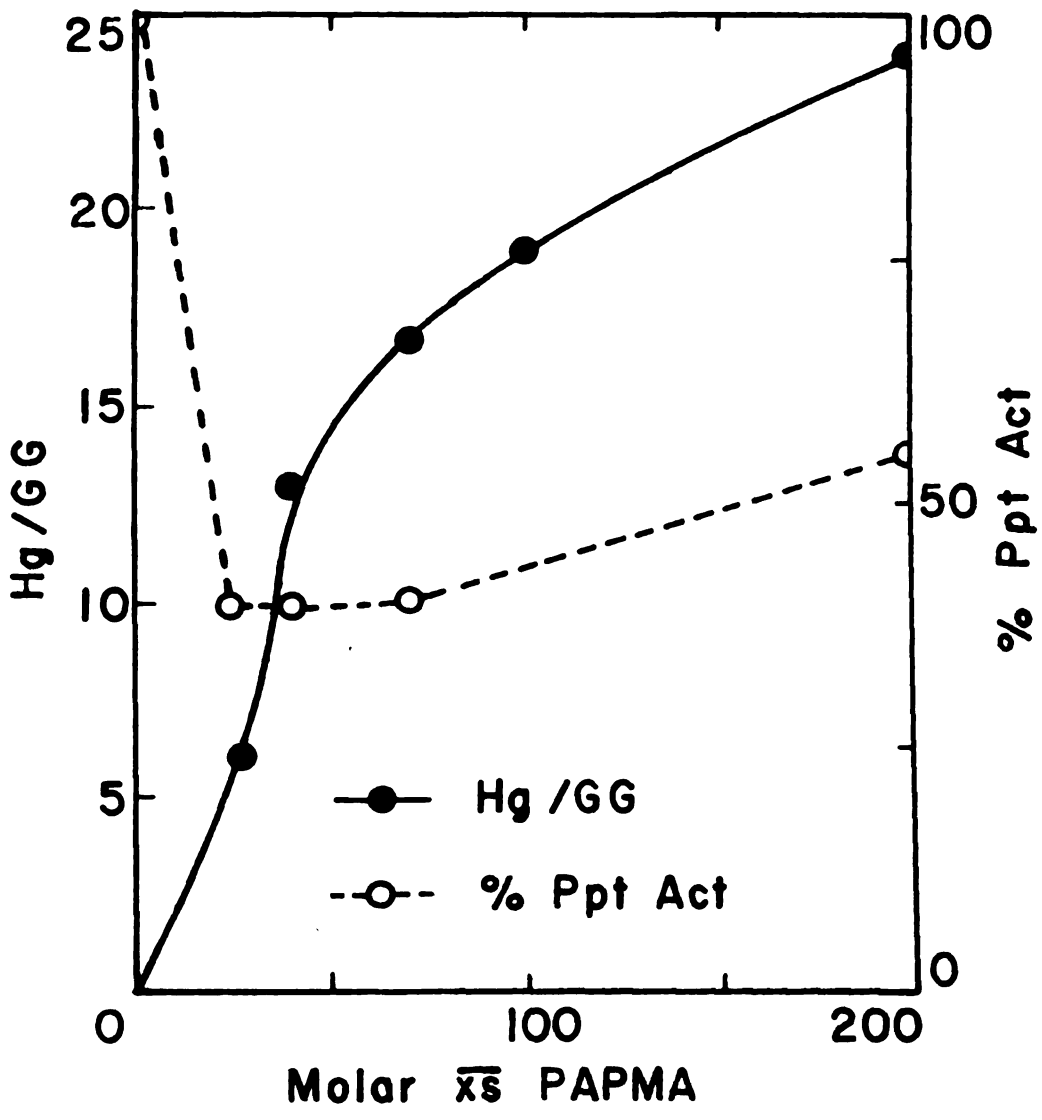

Figure 2: The effect of the concentration of PAPMA on the mercury content and immunochemical properties of gamma globulin.

treated with a forty-molar excess of diazotized PAPMA. The resultant protein remained in solution at least twenty-four hours at 4 C and for prolonged periods at -20 C.

Determination of Changes in Properties of Gamma Globulin by Mercuration

Spectrophotometric Studies

The absorption spectra of antienzyme globulin and its carboxy methylated and mercurated derivatives were examined in an effort to determine the effects of the experimental procedures on the protein. One milliliter aliquots containing 14.4 milligrams of gamma globulin were used. Absorbance values were read for each sample vs. a saline blank in a Beckman DU spectrophotometer at increments of 5 mu from 200-240 mu, of 2 mu from 240-310 mu, of 10 mu from 310-350 mu and of 25 mu from 350-600 mu.

It can be observed in figure 3 that iodoacetate treatment and mercuration produce specific spectral changes. A peak occurs at 235 mu on the carboxy methylated and mercurated materials. This represents a change from -SH to S-ether linkage by carboxy methylation or modification of histadyl side chains. An absorbance peak at 340 mu in the mercurated protein is due to the presence of the azomercurial.

Electrophoretic Mobility

Modifications in a protein cause changes in its net charge which can be seen as changes in its electrophoretic mobility. Mobility studies were carried out on cellulose acetate strips, Sepraphore III (Gelman Instrument Co.). At

TABLE 3: EFFECT OF PAPMA CONCENTRATION ON THE MERCURATION OF GAMMA GLOBULIN

Molar excess	Mercury per mole of gamma globulin	% Protein Recovery
29	6.5	95
40	13	95
66.7	17	90
100	19	50
200	23.8	65

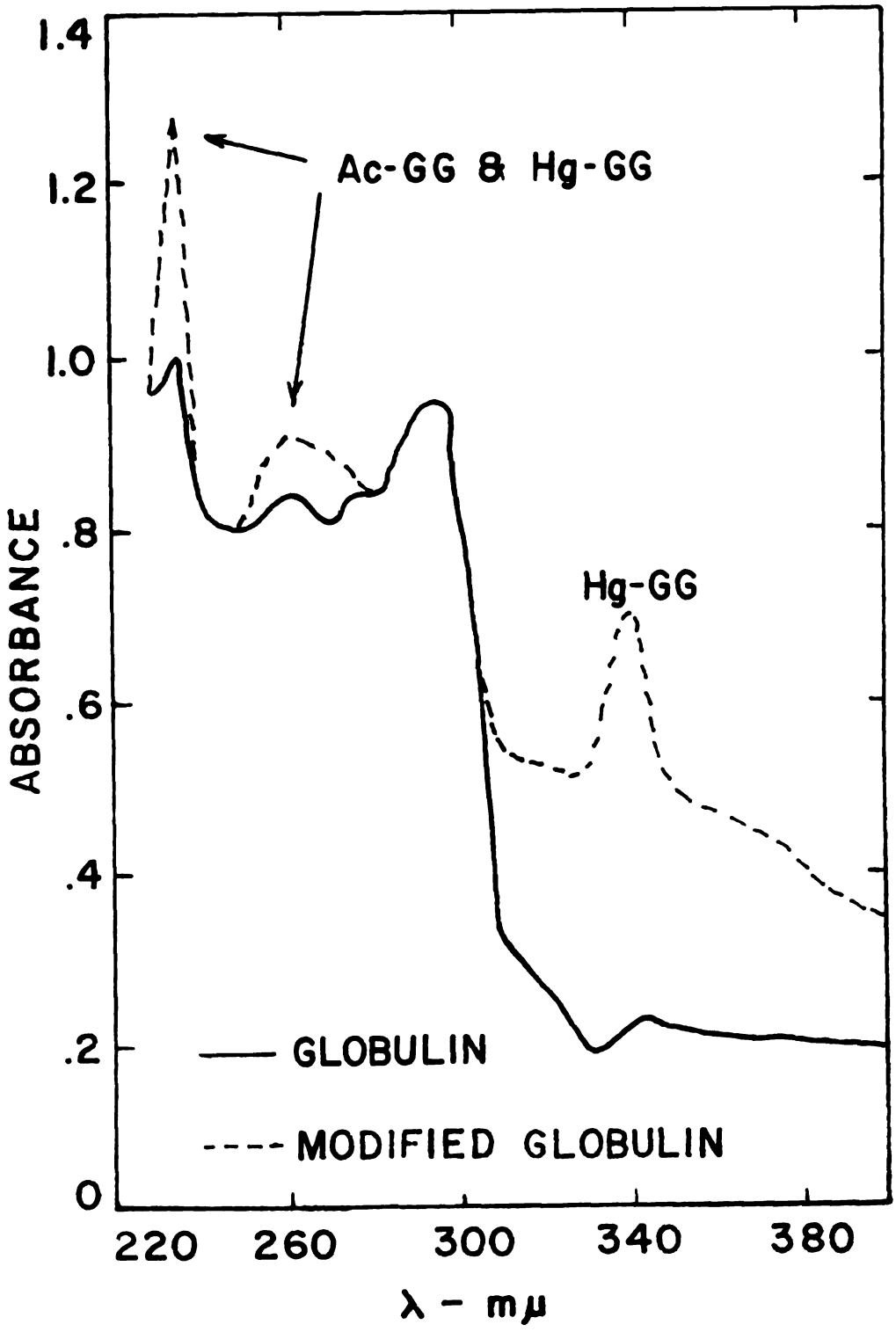

Figure 3: Spectral analysis of gamma globulin.

Key: AcGG-carb. meth. gamma globulin,
Hg-GG-mercurated gamma globulin.

pH 8.6, 27 volts per centimeter, it was noted that carboxy methylated gamma globulin moved at a greater rate than normal gamma globulin (Refer Table 4). The higher mobility is due to the ionization of the carboxyl groups. The introduction of mercury into the gamma globulin then reduced the electrophoretic mobility to a point approximately equivalent to normal gamma globulin. This was attributed to the positive charge of the mercury which balanced the negative charge from the carboxyl groups.

Enzyme Inhibition

Modified Cohn and Torriani (1952) tests were performed on normal and enzyme specific gamma globulins and on carboxy methylated and mercurated derivatives.

The data presented in Figure 4 show that the enzyme is not precipitated by normal gamma globulin. Neither carboxy methylated nor mercurated gamma globulin precipitate the enzyme. Enzyme specific globulin precipitated glucose dehydrogenase but the precipitate does not readily dissociate in an excess of antigen. The antienzyme in this experiment removed 8,600 units of enzyme per milligram of gamma globulin. Treatment with iodoacetate produced about a 75% reduction in the precipitating titer and the antigen-antibody complex solubilized in the presence of excess antigen. Mercuration appeared to compensate for the effect of acetylation by a two-fold increase in the precipitating ability of the anti-enzyme. The mercurated antibody-antigen complex did not become completely soluble in the presence of excess of antigen.

TABLE 4: ELECTROPHORETIC MOBILITY*

Sample	Average mobility $\frac{cm^2}{volt \cdot sec}$		
	270 v/2 hrs.	270 v/1 hr.	
Normal gamma globulin	1.13×10^{-5}	9.3×10^{-5}	
Normal, carboxy methylated gamma globulin		19.1×10^{-5}	
Normal, PAPMA-gamma globulin	$.82 \times 10^{-5}$	9.8×10^{-5}	
Antienzyme gamma globulin	$.82 \times 10^{-5}$	10.3×10^{-5}	11.9×10^{-5}
Carboxy methylated antienzyme gamma globulin		10.8×10^{-5}	13.7×110^{-5}
PAPMA-antienzyme gamma globulin	$.67 \times 10^{-5}$	9.3×10^{-5}	11.3×10^{-5}

*Not corrected for electro-endoosmotic flow.

The antienzyme in this experiment removed 8,600 units of enzyme per milligram of gamma globulin. Treatment with iodoacetate produced about a 75% reduction in the precipitating titer and the antigen-antibody complex solubilized in the presence of excess antigen. Mercuration appeared to compensate for the effect of acetylation by a two-fold increase in the precipitating ability of the antienzyme. The mercurated antibody-antigen complex did not become completely soluble in the presence of excess of antigen.

A second examination of the effects of mercuration on the enzyme specific rabbit gamma globulin was made using the Ouchterlony immunodiffusion technique. Figure 5 and Table 5 indicate the results of these studies. No apparent antigen-antibody reaction occurred in plates containing normal serum and enzyme. An examination of figure 5 showed both anti-enzyme and mercurated antienzyme did produce precipitation lines in the agar. It should be noted that the serum fraction contains at least two precipitating antibodies of different concentrations. The minor component disappears on dilution of the serum. The data in Table 5 show that the precipitating ability of mercurated and non-mercurated antienzyme are approximately the same.

Electron Density

In order for the mercurated enzyme specific globulin to be effective as a stain for electron microscopy, the product of its antigen-antibody reaction must be electron dense. This property was tested in the following manner.

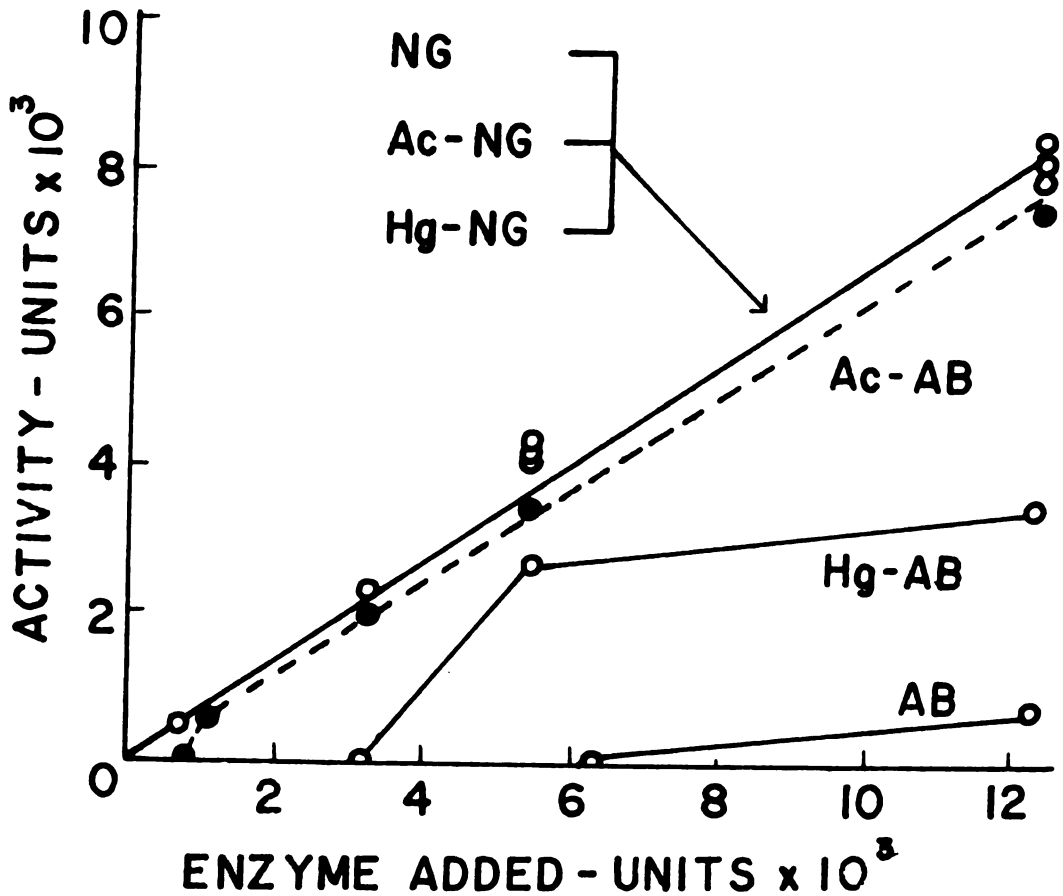

Figure 4: Effects of treated and untreated normal gamma globulin and antienzyme on glucose dehydrogenase activity.

Key: NG-normal gamma globulin, Ac-NG-carb. meth. normal gamma globulin, HG-NG-mercurated normal gamma globulin, AB-normal antienzyme Ac-AB-carb. meth . anti-enzyme, and Hg-Ab-mercurated antienzyme.

Plate 1. Glucose dehydrogenase in center wells, 69,000 units/ml.

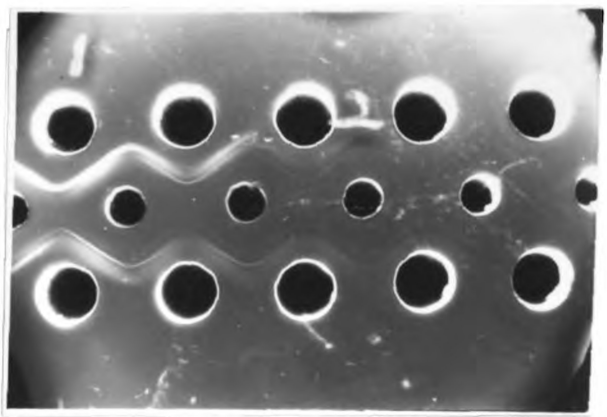

Plate 2. Glucose dehydrogenase in center wells, 34,500 units/ml.

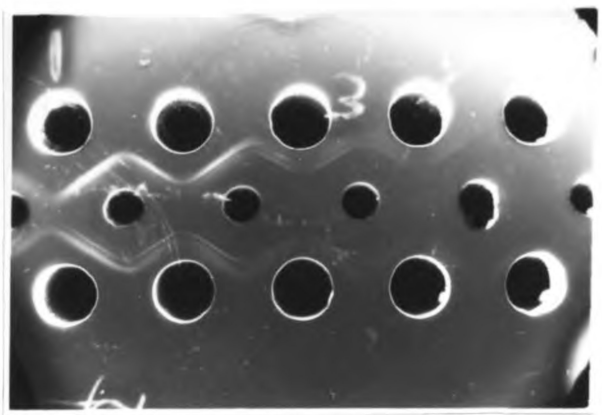

Plate 3. Glucose dehydrogenase in center wells, 17,250 units/ml.

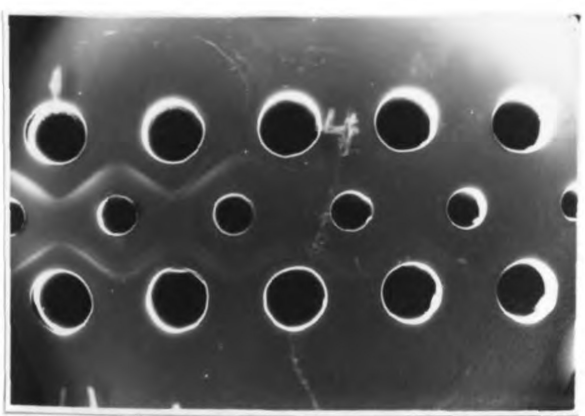

Plate 4. Glucose dehydrogenase in center wells, 8,625 units/ml.

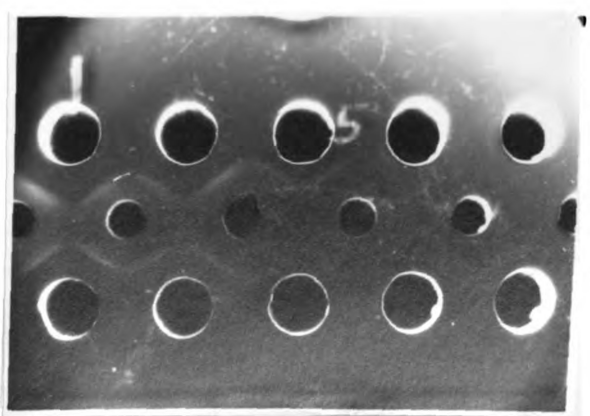

Plate 5. Glucose dehydrogenase in center wells, 4,312 units/ml.

Figure 5. Titration of antienzyme gamma-globulin by 2- dimensional immunodiffusion. Top rows contain (l to r) rabbit antienzyme gamma-globulin diluted 1:1, 1:2, 1:4, 1:8, and 1:16. Bottom rows contain (l to r) mercurated rabbit antienzyme gamma-globulin diluted 1:1, 1:2, 1:4, 1:8, and 1:16.

TABLE 5: BOX TITRATION OF UNTREATED AND MERCURATED ANTIENZYME VS GLUCOSE DEHYDROGENASE IN OUCHTERLONY DOUBLE DIFFUSION PLATES

		Enzyme				
Untreated antienzyme		1:1	1:2	1:4	1:8	1:16
	1:1	+	+	+	+	+
	1:2	+	+	+	+	+
	1:4	+	+	+	+	+
	1:8	+	+	+	-	-
	1:16	-	-	-	-	-
Mercurated antienzyme						
	1:1	+	+	+	+	+
	1:2	+	+	+	+	+
	1:4	+	+	+	+	+
	1:8	+	+	+	+	-
	1:16	-	-	-	-	-

Key: + = precipitation band

- = no precipitation

One tenth milliliter of PAPMA-labeled antienzyme gamma globulin was mixed in a test tube with one half milliliter of glucose dehydrogenase (5,000 units per milliliter) and droplets were immediately placed on formvar-coated electron microscope grids (Ernest F. Fullam, Inc.). The films were dried about thirty minutes, and inverted on distilled water for ten minutes. Following washing, the grids were dried for two to five hours. A similar treatment was made using non-mercurated antibody gamma globulin. Figure 6, Plate A shows an electron micrograph of enzyme-antienzyme complex using unlabeled antienzyme. Figure 6, Plate B shows an electron micrograph of antigen-antibody complex using mercury-labeled antienzyme. There are considerable electron dense areas, especially noticeable toward the borders of the complex which are not seen in Plate A.

Bacillus cereus spores were fixed in 5% formaldehyde in 0.1 M phosphate buffer, pH 7.4 for one hour. The spores were washed in buffer, shaken with Super-Brite beads in a Nossal shaker, and treated with mercurated and non-mercurated antibody. The partially disrupted spore-antibody complexes were washed in saline and then in distilled water. They were placed on grids and treated in the same manner as previously described.

Figure 7, Plate A, is an electron micrograph of a spore of Bacillus cereus which had been fixed, ruptured, and treated with unlabeled antienzyme. Extruded spore "cytoplasm" appears amorphous and ill-defined. Figure 7, Plate B is an electron

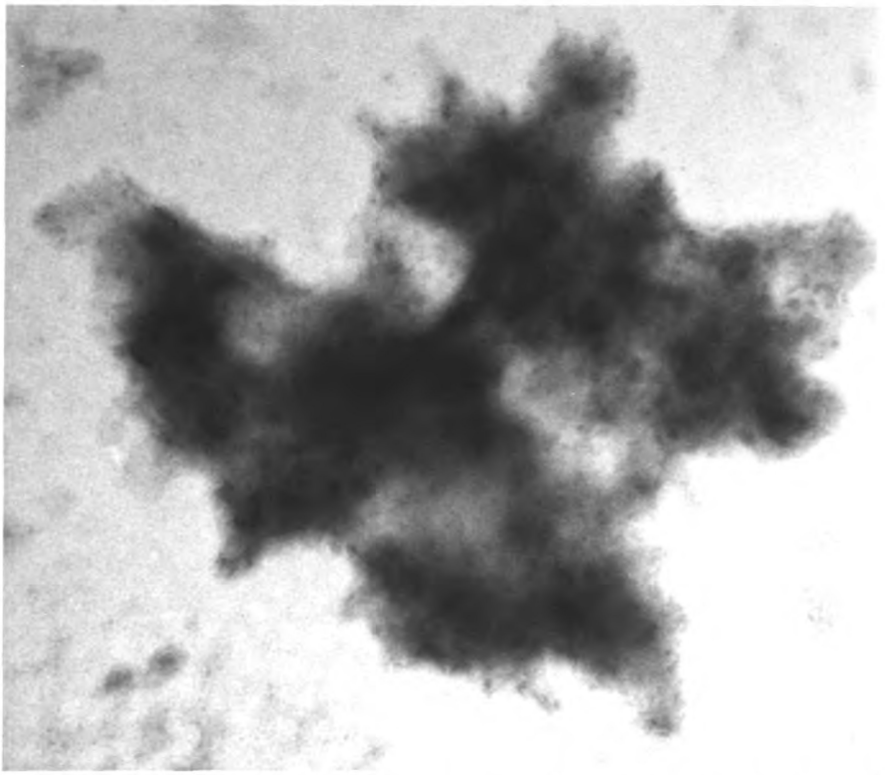

Plate A. Enzyme-antienzyme complex. (32,000 x)

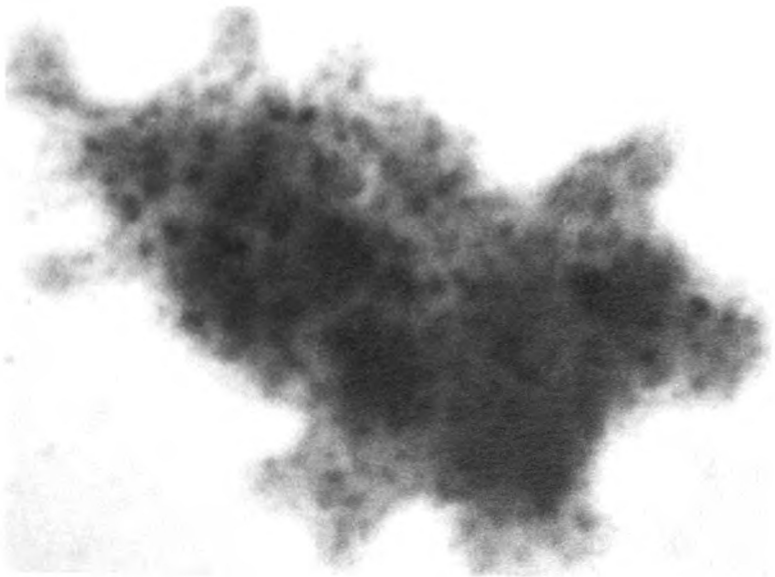

Plate B. Enzyme-mercurated antienzyme complex. (38,750 x)

Figure 6. Electron micrographs of aggregates of enzyme-antienzyme globulin which had been reacted one minute and dried on formvar-coated, electron microscope grids.

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micrograph of the extruded "cytoplasm" from a Bacillus cereus spore that was treated with mercurated antienzyme. The boundary of the spore material was well-defined (electron dense). Electron dense areas of 200Å diameter occurred in the "cytoplasm". If these represent the interaction of antibody and intraspore glucose dehydrogenase, each aggregate contains approximately 25 molecules of antibody or 425 atoms of mercury.

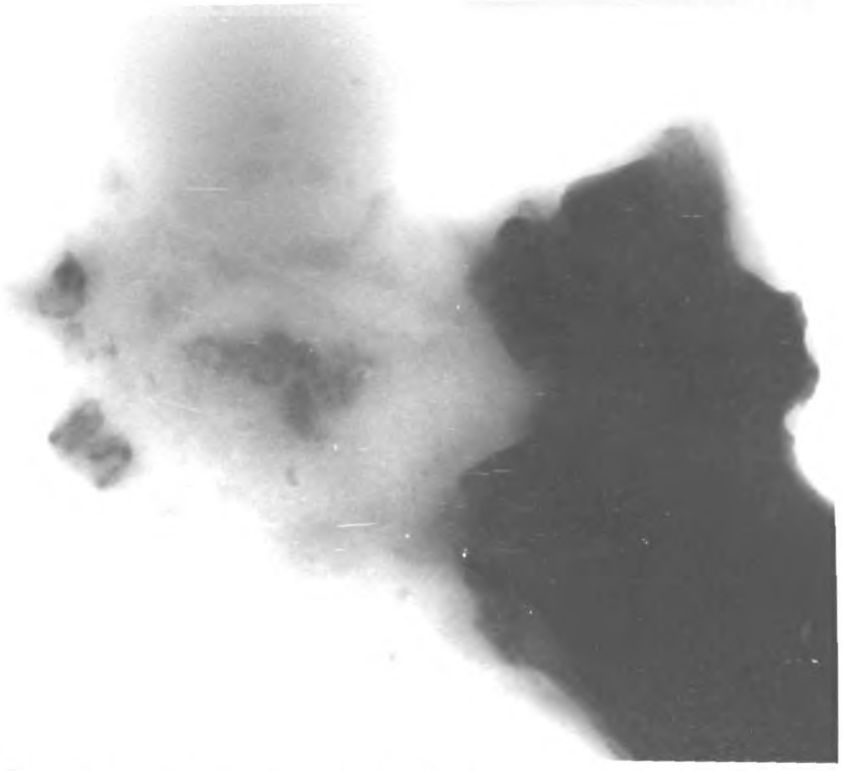

Plate A. Disrupted Bacillus cereus spore, treated with antienzyme gamma-globulin. (63,500 x)

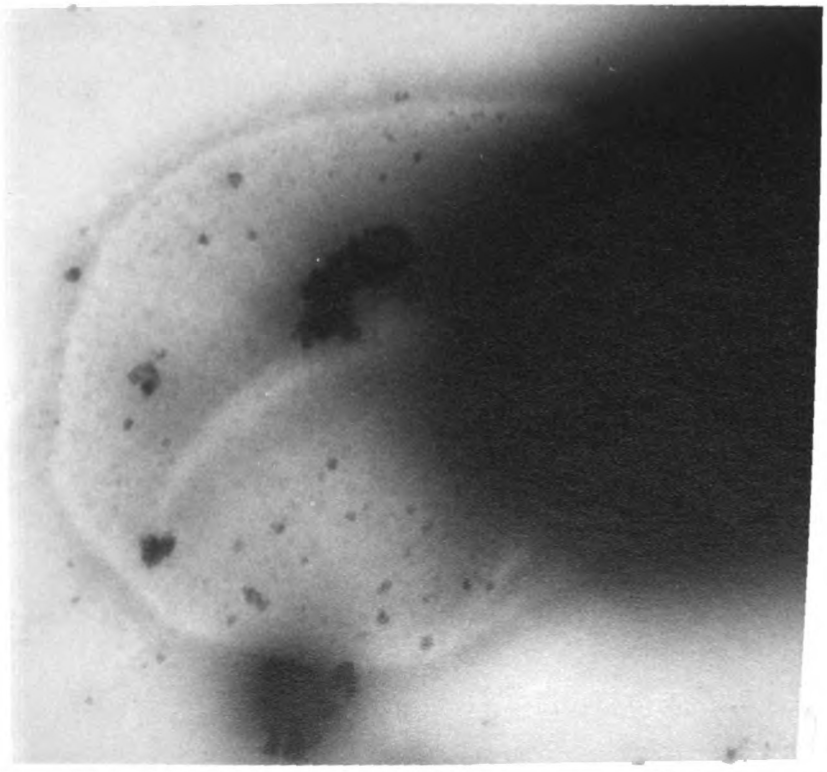

Plate B. Disrupted Bacillus cereus spore, treated with mercurated antienzyme gamma-globulin. (63,500 x)

Figure 7. Electron micrographs of spores which had been disrupted in a Nossal shaker, formaldehyde-fixed, and treated with antienzyme gamma-globulins.

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DISCUSSION

The purpose of the research described in this thesis was to develop an electron dense, highly specific stain which could be used to identify intracellular antigens; specifically glucose dehydrogenase of Bacillus cereus. An attempt was made to couple para-aminophenylmercuric acetate to glucose dehydrogenase-specific globulin in order to achieve these objectives. The serologic properties of the normal and mercurated antibodies were investigated to determine if mercuration changed the properties of the antienzyme. The ability to combine with specific antigens, nonspecific binding caused by mercuration, and changes in physical-chemical properties of the mercurated antienzyme were investigated.

The method of Cohn and Torriani (1952) was used as an assay because the Ouchterlony plates showed that more than one precipitating antibody existed in the serums. The advantage of the Cohn and Torriani assay was that it selected for only one antigen-antibody system. Furthermore, it was quantitative and the results were not affected by other precipitating systems.

Reduced nicotinamide adenine dinucleotide (NADH) oxidase was present in rabbit serum. This reacted with the NADH generated in the course of the enzyme assay and prevented accurate antibody assays. The antibody globulin and NADH oxidase were separated by precipitation of the antibody gamma globulin with 39% $(NH_4)_2SO_4$. This is not a specific procedure.

Other proteins were precipitated by the salt and could account in part for the low specific activity of the gamma globulin.

The Cohn and Torriani method was applied to determine the specificity of mercurated antibody. A comparison was made of the effects on the enzyme of untreated and mercurated normal gamma globulin and antienzyme and mercurated antienzyme. Neither mercurated nor untreated normal gamma globulin reacted with glucose dehydrogenase. The enzyme has been shown to be insensitive to -SH reagents and the mercury coupled to the protein therefore, did not produce a non-specific effect. Mercuration reduced the antibody titer and neither mercurated nor untreated antienzyme globulin reacted like normal rabbit antibody. That is, the insoluble rabbit antibody-antigen complex solubilizes in the presence of excess antigen. In the experiments performed, this did not appear to be the case because the antigen-antibody complex did not solubilize in the presence of excess enzyme. Either the enzyme was denatured during the solubilization or the soluble antigen-antibody complex was inactive. One reason for the inactivity of the antigen-antibody complex could be that the antibody was coupled near the active center of the enzyme.

The changes caused by mercuration of the antibody could be caused by steric effects due to the addition of ten mercurated diazonium molecules to the gamma globulin. According to the lattice theory most precipitating antibodies must have at least two sites for combination with their respective antigens. The mercury atoms or aromatic rings could prevent combination by shielding one or both of these sites.

Alternatively, this decrease on mercuration could be the result of a conformational change. There are three orders of structural integrity in protein molecules. The tertiary structure probably is the level of involvment of this reaction. This is maintained co-operatively by covalent disulfide linkages, salt bonds, hydrogen bonding and hydrophobic bonds. The carboxy methylation covers free sulfhydral groups which could cause a change in the configuration. Attachment of aromatic mercurials to the tyrosine and tryptophan residues could change the tertiary structure by modification of hydrophobic and ionic bonding at the active sites of the antibody.

Spectral analyses of the untreated, carboxy methylated, and mercurated antienzyme protein was performed to verify the mercuration of the protein by changes in light absorbancy. Three modifications in the spectrum of the carboxy methylated and mercurated protein were observed. Increased absorbancy at 235 mu and 260 mu were observed in both the carboxy methylated and mercurated proteins and at 340 mu in the mercurated protein. The 340 mu peak was attributable to the presence of the azomercurial. The 235 mu peak was the result of iodoacetate treatment of the protein and the 260 mu peak was enhanced by carboxy methylation. Though iodoacetate treatment was performed under alkaline conditions and the reaction with the sulfhydral groups was rapid, it is possible that other groups such as tyrosine were carboxy methylated. These spectral shifts prevented the use of a simple photometric assay for estimation of protein.

Attempts were made to fractionate mercurated antibody by column chromatography using DEAE cellulose and carboxymethyl cellulose. The proteins could not be removed from the DEAE column by change of ionic strength or pH of the eluant. This could have been due to the negative charges of the additional carboxyl groups. The cationic exchanger, carboxymethyl cellulose was also ineffective. This could probably by attributed to the mercury binding to the column. Therefore, another method for separation of mercurated and non-mercurated gamma globulin was sought.

The electrophoretic mobility of gamma globulin and its derivatives were examined on cellulose acetate strips. Mercurated and non-mercurated gamma globulins have approximately identical mobility. However, the negatively charged carboxy methylated protein had 1.3 - 2 times the mobility of the untreated gamma globulin. On the basis of this data, it was concluded that electrophoresis could not be readily used to separate normal and mercurated gamma globulin or globulins mercurated at various levels. The average number of mercury atoms per molecule of gamma globulin was used as the criterion of degree of mercuration for all gamma globulin preparations.

The primary question to be resolved for electron microscopy is the number of heavy atoms necessary to be visible under the electron microscope. Beer and Moudrianakes (1962) labeled the guanosine moiety of deoxyribonucleic acid (DNA) with uranyl ions. They were able to resolve individual uranyl ions in electron microscopic studies. They found that one

uranyl per 100 $\mathring{A}^2$ could be detected with ultra-high resolution electron microscopy. These results then formed the basis for the following considerations:

Bethe's Law states that the rate of energy loss in the scattering of beta-particles is proportional to the atomic number of the atom on which the electron impinges. Therefore, had Beer and Moudrianakis used mercury rather than uranium, they would have required

$$(1)\ \frac{92}{80} \times 1 = 1.15 \text{ Hg atoms per } 100\ \mathring{A}^2$$

In Figure 7, Plate B, small uniform electron dense areas of 200 $\mathring{A}$ diameter were observed. They possess a projected area of $3.14 \times 10^4\ \mathring{A}^2$ and a volume of $4.2 \times 10^6\ \mathring{A}^3$. In this volume, with 66% packing efficiency, 25 mercurated-antibody molecules could be fitted. This calculation is based on the assumption that gamma globulin molecules are 60 $\mathring{A}$ diameter. These aggregates therefore contain an average of 425 mercury atoms because the gamma globulin contained an average of seventeen mercury atoms per molecule. The average number of mercury atoms per 100 $\mathring{A}^2$ would therefore be:

$$(2)\ \frac{425}{3.14 \times 10^2} = 1.35 \text{ Hg atoms/}100\ \mathring{A}^2$$

This is within the range of concentration necessary for visualization in the electron microscope.

The very sharp definition of the edge of the cytoplasm of those spores treated with mercurated antibody appears to support this calculation.

Beer and Moudrianakis employed photographic amplifications in printing technique to resolve individual atoms. The long-range objectives of this research could be fulfilled by the resolution of aggregates of mercurated antibody-antigen complex.

SUMMARY

The serologic properties of mercurated glucose dehydrogenase were investigated. Enzyme specific globulins were produced in rabbits. One milligram of globulin protein, resuspended in a serum volume of phosphate buffered saline complexes with 10,600 units of glucose dehydrogenase. These were carboxy methylated and coupled to the diazonium derivative of para-aminophenyl-mercuric acetate. It was observed that the optimal concentration of diazonium was forty to sixty-seven molar excess. Increasing levels of diazonium caused increasing levels of mercuration. However high levels of mercuration decreased the specificity and yield of antienzyme protein. A range of seven to twenty-one atoms of mercury could be attached to each molecule.

The antienzyme activities of untreated, carboxy methylated, and mercurated antibody were compared. Normal gamma globulin treated in the same manner served as controls. The antienzyme globulin lost 80% of its original activity on iodoacetate treatment, but 50% of the activity was recovered on mercuration. Neither the normal gamma globulin nor its derivatives precipitated glucose dehydrogenase.

Chromatographic and electrophoretic methods were attempted to separate antienzyme on the basis of degree of mercuration. These methods were unsuccessful. An average number of mercury atoms per molecule of gamma globulin was, therefore, used as the basis for estimations of degree of mercuration.

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Antigen-antibody aggregates produced from mercurated antibody and glucose dehydrogenase were shown to be electron dense when compared to non-mercurated controls.

Spores of Bacillus cereus which had been formalin-fixed and ruptured were reacted with mercurated specific globulin, untreated specific globulin, and mercurated normal gamma globulin. Only mercurated enzyme specific globulin produced discrete staining of the spore cytoplasm on electron microscopic examination.

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