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CHARACTERIZATION OF AN ANTIGEN OF

NEISSERIA GONORRHOEAE

by

Eric C. B. Milner

A.B., University of California, 1972

Presented in partial fulfillment of the requirements
for the degree of

Master of Science

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1976

Approved by:

Chairman, Board of Examiners

Dean, Graduate School

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Characterization of an Antigen of Neisseria gonorrhoeae (97 pp.)

Director: Jon A. Rudbach

The increasing incidence of gonococcal infection in the United States is due in large part to the reservoir formed by the large number of female and male asymptomatic carriers. Control of the disease would be facilitated by a rapid diagnostic test suitable for screening the general population for asymptomatic carriers.

A radioimmunoassay which employs an antigen specific for Neisseria gonorrhoeae contained in an ethanol precipitate of a surfactant extract of T1 phase gonococci recently has been developed for the detection of antibodies specific for the gonococcus in human serum. In the present project, the chemical, physical, and biological properties of the gonococcal specific antigen were studied. In a major portion of this work an inhibition assay was employed. Treatments designed to destroy or alter specifically various components were performed on unlabeled antigen. This treated antigen was then tested for capacitiy to inhibit the specific binding of labeled antigen by human anti-gonococcal serum. It was found that gonococcal specific antigenic activity was destroyed by boiling for 5 min, by digestion with proteolytic enzymes, and by periodate oxidation. Antigenic activity was not destroyed by heating at 56 C for 1 hr, by treatment with 0.1 N acid or alkali, by saponification, or by digestion with ribonuclease or deoxyribonuclease. It was found that the specific antigen was not contained in T3 phase gonococci or in an endotoxin preparation of T1 phase gonococci. A fraction containing specific antigenic activity was eluted in the void volume from Sephadex G-200. Fractions containing specific antigenic activity were found in the regions of 40% and 10% sucrose following untracentrifugation on a sucrose gradient.

A model for the structure of the gonococcal specific antigen was proposed which could account for these findings.

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C	degrees Celsius
$CELD_{50}$	50% chick embryo lethal dose
CFA	complete Freund adjuvant
cpm	counts per minute
DDW	deionized distilled water
FI_{40}	fever index of 40 cm^2
g	grams
g	gravity
GCE	gonococcal extract
GCE*	^{125}I labeled GCE
gcLPS	gonococcal lipopolysaccharide
h	hour
IA_{50}	50% inhibitory amount
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
LPS	lipopolysaccharide
nm	nanometer
PBS	phosphate buffered saline
PPT	precipitate
SAGR	specific anti-gonococcal reaction
SF	supernatant fluid
Tris	0.1 M Tris-HCl Buffer, pH 8
T3-GCE	extract of T3 phase gonococci
µg	microgram

CHAPTER I

INTRODUCTION

Gonorrhea is the number one reportable infectious disease in the United States today (9, 49). While treatment of the disease is relatively simple, diagnosis can be difficult (63). In females, even with acute infection, symptoms may be absent or go unnoticed, so that many women go untreated and become chronic, asymptomatic carriers (35). In males, although symptoms are more often noticed and treatment sought, the rate of asymptomatic carriers is surprisingly high (21). It has been reported that as many as 68% of infected males may be asymptomatic (21). Present diagnostic procedures which include culturing (32, 62), gram staining smears and fluorescent antibody techniques (14, 18, 32, 64, 66, cf. 55), are expensive and time consuming. This renders them impractical for screening large numbers of people (63). These procedures are usually not performed unless gonorrhea is suspected, either from overt symptoms or a history of sexual contact with an individual known to be infected. Because the great number of asymptomatic carriers form a reservoir from which the disease is disseminated, eradication will not be achieved until a method is available which is suitable for screening the general population for carriers (57). A blood test, because of the ease of sample collection and social acceptability, would be ideal.

Numerous investigators have studied the gonococcus with the aim of finding an antigenic component which would provide a reagent suitable for detecting humoral antibodies specific for the gonococcus. This would be the first step toward development of a serodiagnostic test for gonorrhea. Among the antigens studied have been whole cells (14, 37), protoplasm (2, 3, 11, 13, 29, 30, 51, 54, 58), pili (8, 52), endotoxin (17, 38-46, 61), and ribosomes (26). Whole cells (64, 66) and protoplasmic extracts (2, 3, 11, 13, 30, 51, 54, 58) have been used in complement fixation and gel diffusion tests. Antibodies in the serum of patients have been found which reacted with these preparations, but no assay has been clinically acceptable. Major problems encountered with seroassays have been high rates of false positive reactions (3, 14, 30, 33, 37, 53), high rates of false negative reactions (3, 23, 33, 37), and lack of sensitivity (23, 54, cf. 55). Also, antisera to species of Neisseria other than gonococci cross react extensively with these antigens (33).

Two antigenic fractions of gonococcal endotoxin have been characterized which react in complement fixation and gel diffusion tests (38). One fraction, "β", cross reacted extensively with other Neisseris (39, 42). The other fraction, "α", demonstrated some specificity for the gonococcus (39, 42). Fraction "α" has not, however, been shown to be any more reliable than the whole cell or protoplasmic antigens for use in diagnostic assays for gonorrhea.

Gonococcal pili have been used in a radioimmunoassay by Buchanon *et al.* (8) with some success. Other investigators (50) have shown that pili are highly strain specific, a finding which, if confirmed, would limit the usefulness of pili in a diagnostic test.

Gonococcal ribosomes have been used successfully in delayed hypersensitivity (DH) type skin tests with animals immunized with N. gonorrhoeae (26). Usefulness of delayed skin tests in diagnosis of gonococcal infection in human patients has not been shown.

More recently a disrupted cell extract has been used in a radioimmunoassay for the detection of specific antibodies against the gonococcus in human sera (36). A high degree of specificity has been obtained with this antigen. In a study of sera from 382 patients, 90% were correctly diagnosed, 5% were incorrectly diagnosed and 5% could not be diagnosed as either positive or negative (36). These results were equal to or superior to results reported for the serological diagnosis of gonorrhea employing pili antigens (8).

The objective of this research project was to characterize the specific antigen contained in the disrupted cell extract and, if possible, to purify the antigen from the crude extract. The goal of characterization was to elucidate the chemical and physical nature of the gonococcal specific antigen, to measure its biological properties and to test the stability of the antigen to various treatments. It was hoped that such a study would increase the knowledge of specific antigens on the gonococcus and would lead to procedures for purification of the antigen. Purification of the antigen would increase the specificity of the assay for the diagnosis of gonococcal infection.

Several properties of the model system employed in this study rendered efforts to characterize the antigen more difficult. Perhaps the greatest hindrance was that all work was performed with human sera rather than antisera obtained from artificially immunized animals. The

human serum model required that the assay system had to be capable of detecting very low antibody levels. This eliminated gel precipitation or flocculation assays as adjuncts to characterization procedures. Also, because of the low antibody titers and the extreme sensitivity required of the assay, non-specific reactions in some cases overshadowed specific reactions. This created problems of interpretation of assay results. However, because humans are the only species infected naturally by the gonococcus (34) and because results from previous work with animal models of gonococcal infection have not been shown to apply to the human system, it was felt that despite the difficulties, a study of the human system was amply justified.

Another property of the system which was to be studied was the complex nature of the antigenic extract. The specific antigen probably was only a fraction of the crude extract. Non-specific antigens in the extract could react with antibodies in the human sera and, thus, affect the assay results. If such non-specific reactions occurred it would be difficult to know if an experimentally determined characteristic was a property of the specific gonococcal fraction or was a property of other material contained in the extract. A possible way to overcome this problem would have been to purify the specific gonococcal antigen before attempting to characterize it. This approach was not taken, however, because it was felt that attempts to purify an unknown entity could easily turn out to be a fruitless, time-consuming endeavor. Therefore, an inhibition assay, which allowed study of the specific antigen in the presence of other materials, was employed for most determinations of antigenic activity. In some cases a direct assay of antigenic activity

also was employed to add confidence to interpretation of results obtained with the inhibition assay.

In order to characterize the gonococcal specific antigen it was necessary to determine the chemical, physical, and biological properties of the antigenic extract. Also, it was necessary to study the relationship of these properties to gonococcal specific antigenic activity. Finally, it was hoped that the experimentally determined characteristics could be used to propose a model for the structure of the gonococcal specific antigen.

CHAPTER II

MATERIALS AND METHODS

Gonococcal strains

Neisseria gonorrhoeae strain F62 was obtained from Dr. D. S. Kellogg, Jr., Center for Disease Control, Atlanta, GA. In addition, a strain freshly isolated from an infected patient was obtained from the Infectious Disease Center, Missoula, MT, and designated strain IDC.

Maintenance and cultivation of organisms

Strain F62 organisms were stored in lyophilized form. Both strain F62 and strain IDC were stored for short periods (up to 4 weeks) at -20°C in a special freezing medium containing Eagle minimum essential medium plus 20% glycerol, pH 7.0.

During periods of frequent use, the cultures were maintained on GC Medium Agar Base (Difco, Detroit, MI.) supplemented with 1% IsoVitalex (Bioquest, Cockeysville, MD.). T1 phase colonies of strain F62 and T3 phase colonies of strain IDC were selected and transferred daily. Plates were incubated 18 h at 37°C in a candle extinction jar.

For extraction, cultures were grown in modified Thayer-Martin broth medium (62) containing 15 g proteose peptone #3 (Difco), 4 g K_2HPO_4, 0.1 g cornstarch, and 5 g NaCl in 1 l deionized distilled water (DDW). The pH was adjusted to 7.2 with HCl. The medium was supplemented with

sterile calf serum to 5% (v/v) prior to inoculation. Scrapings from an 18 h plate culture were inoculated into one 1 broth in a wide bottom two l flask. Cultures were gassed with a mixture of 83% N_2, 7% O_2, and 10% CO_2 and incubated 24 or 48 h at 37°C with gentle rotation. After incubation, each broth culture routinely was checked for purity by Gram staining and plating. Extraction procedures were initiated if no contamination was revealed by the Gram stain. Plates were examined the following day as a check on purity, and to determine the distribution of gonococcal colony types.

Preparation of antigenic extracts

Cells from a 24 or 48 h broth culture were harvested by centrifugation and washed with 0.1 M Tris-HCl buffer, pH 8.0 (Tris). The washed cells from one 1 of medium were suspended in 25 ml of 2% sodium deoxycholate in Tris and allowed to stand at room temperature for 1.5 h. The disrupted cells were then centrifuged for 30 min at 27,000 X g and 25°C. The antigen was precipitated by decanting the supernatant fluid (SF) into six volumes of 95% ethanol and allowing the precipitate to develop overnight at room temperature. Strands of nucleic acid were removed by winding onto a wooden stick. Additional nucleic acid floccules were removed from the precipitate by filtration onto gauze. The filtrate was centrifuged at 1500 X g for 30 min to separate out an insoluble fraction. This pellet was suspended in 15 ml distilled water and recentrifuged at 27,000 X g for 30 min to sediment a particulate fraction. The supernatant fluid from the final centrifugation (containing the soluble antigen) was transferred to dialysis tubing and dialyzed for 5 days against

daily changes of distilled water at 4°C. After dialysis, the antigen was lyophilized. The product of this extraction procedure was designated GCE.

Labeling of antigens

Several batches of GCE were labeled with ^{125}I (Irvine, CA) by the method of Gruber and Wright (20). Briefly, in this technique, a modified Conway microdiffusion cell was made by cutting the top from a 1 dram vial and placing the bottom half into a 125 ml Erlenmeyer flask; this formed a separate inner chamber. The antigen solution to be labeled was pipetted into the outer chamber. Then 0.2 ml of 0.002 M KI and 500 μ Ci of ^{125}I were added to the inner chamber. The flask was sealed with a skirt-type vaccine stopper. Two-tenths ml of a 1/20 dilution of 0.27 M $Na_2Cr_2O_7$ in 36 N H_2SO_4 was added to the inner chamber with a syringe and four inch needle. The flask was allowed to sit at room temperature for 1 to 3 h with occasional gentle shaking. The labeled antigen (GCE*) was separated from the free ^{125}I by dialysis against four daily changes of one 1 PBS. All work was performed behind lead shielding.

Human serum source

Two human serum pools were employed. One pool was prepared by mixing, in varying proportions, sera obtained from a local blood bank so that when it was reacted with the gonococcal antigen in the radioimmunoassay, it gave an activity ratio similar to that obtained with serum samples from anatomically virginal females; it was designated V1.

A positive serum pool was obtained from blood drawn from a 24 year old Caucasian female. This individual had been culturally positive for N. gonorrhoeae 3 times and had been treated for gonorrhea two additional times. The patient was in average general health. This pool of serum was designated R-65.

Preparation of anti-immunoglobulin

Antiserum to human gamma globulin was prepared by injecting New Zealand white rabbits on day 1 with 4 mg of Cohn Fraction II of pooled human sera (Cutter Laboratories, Berkeley, CA), as follows: 1 mg intravenously suspended in saline, 1 mg intraperitoneally suspended in saline, and 2 mg intramuscularly suspended in an equal volume Complete Freund Adjuvant (Difco, Detroit, MI). The rabbits were then injected intramuscularly at weekly intervals with 2 mg protein in Complete Freund Adjuvant. Rabbits were bled by cardiac puncture at the end of the fifth week. Following the initial bleeding, the rabbits were alternately bled and boosted at weekly intervals.

Radioimmunoassay

The reaction mixture for assaying the specific antigonococcal reaction (SAGR) of the serum samples consisted of 0.5 ml of phosphate buffered saline (0.15 M NaCl, 0.0033 M PO_4; pH 7.2) (PBS), 20 µl human serum, and 10 µl labeled antigen solution containing 1 µg GCE* in a 12 ml conical centrifuge tube. Following an initial incubation, precipitation was induced by the addition of anti-human immunoglobulin. Eppendorf microliter pipets (Brinkman Instruments, Inc., Westbury, NY) were employed

to disperse the serum and the antigen solutions. The assay procedure is outlined in Figure 1.

Inhibition assay

The inhibition assay was used to measure the effects of various treatments on GCE. Each inhibition assay included nine reaction mixtures: An uninhibited control, four standards containing untreated GCE, and four experimental tubes containing treated GCE. The assay was set up according to the protocol outlined in Figure 2 and assayed as described in Figure 3. The activity ratios were calculated for each reaction mixture. If the unlabeled GCE inhibited the binding of GCE* there would be a decrease in the value of the activity ratio. Inhibition curves were obtained from a plot of the values of the activity ratios against the log of the amounts of inhibitor added.

In order to compare inhibition curves from different assays, the inhibition curves were normalized. This normalization was accomplished by designating the activity ratio of the uninhibited control as 100% of control binding and designating the lowest activity ratio obtained by inhibition with untreated GCE as 0% of control binding. The values of the remaining activity ratios were graphed proportionally as percentage values. The experimental curve, thus, was plotted relative to the standard curve. This procedure always resulted in a standard curve showing a decrease in percent of control binding from 100% to 0% as the amount of untreated GCE inhibitor was increased incrementally from 0.0 µg to 10.0 µg. The amount of inhibitor added which resulted in a 50% inhibition of control binding was determined by visual inspection of the graph

Figure 1. Specific antigonococcal reaction (SAGR)

10 µl GCE* (^{125}I labeled GCE) added to 20 µl human serum in 0.5 ml PBS and mixed gently

↓

Incubate 30 min, 37°C

↓

0.25 ml rabbit anti-human immunoglobulin added

↓

Incubate 30 min, 37°C and 18 h, 4°C

↓

Separate precipitate (PPT) from supernatant fluid (SF) by centrifugation at 1000 X g, 30 min, 4°C

↓

PPT washed 3X in ice-cold PBS; SF decanted and 100 µl saved for analysis

↓

PPT and 100 µl SF counted in a manual crystal scintillation unit at a voltage in the Geiger-Müller region (1064 volts).

↓

The ratio of bound (precipitated) to a total of bound plus unbound antigen was determined form the following formula:

$$\text{Activity ratio} = \frac{\text{cpm PPT - Background (BKG)}}{\text{cpm PPT - cpm BKG + cpm 100 µl SF - cpm BKG}}$$

Figure 2. Protocol for inhibition assays.

	Standards[a]				Experimental[b]				Control
Tube #	1	2	3	4	5	6	7	8	9
Human serum, µl	20	20	20	20	20	20	20	20	20
Untreated GCE, µg	0.2	1.0	5.0	10.0	--	--	--	--	--
Treated GCE, µg	--	--	--	--	0.2	1.0	5.0	10.0	--
PBS	Volume of all tubes adjusted to 0.52 ml								
	Assayed as outlined in Figure 3.								

[a]Untreated GCE employed in preparation of standard curve

[b]Treated GCE

Figure 3. Assay procedure for inhibition of specific antigonococcal reaction.

Inhibitor pre-incubated 30 min, 37°C with 20 ml human serum

↓

10 µl GCE* (^{125}I labelled GCE) added

↓

Incubate 30 min at 37°C

↓

0.25 ml rabbit anti-human immunoglobulin added

↓

Incubate 30 min at 37°C and 18 h at 4°C

↓

Separate PPT from SF by centrifugation at 1000 X g for 30 min at 4°C.

↓

PPT washed 3X in ice-cold PBS; SF decanted and 100 µl saved for analysis.

↓

PPT and 100 µl SF counted in a manual crystal scintillation unit at a voltage in the Geiger-Müller region (1060 volts).

↓

The ratio of bound (precipitated) to a total of bound plus unbound antigen was determined from the following formula:

$$\text{Activity ratio} = \frac{\text{cpm in PPT - Background (BKG)}}{\text{cpm in PPT - cpm BKG + cpm in SF - cpm BKG}}$$

and designated the IA_{50}. By the nature of the normalization procedure the value of the IA_{50} of the standard curves was approximately 1.0 µg. The value of the IA_{50} of the experimental curves varied widely depending on the treatment performed on GCE. The capacity of treated GCE to inhibit SAGR was expressed as a percent of the capacity of untreated GCE to inhibit SAGR according to the following formula:

$$\% \text{ of standard inhibition} = 100 \times \left(\frac{IA_{50} \text{ untreated GCE}}{IA_{50} \text{ treated GCE}}\right).$$

The percentage value obtained from this formula was a measure of the effect of the treatment on GCE.

Nitrogen determination

Organic nitrogen was converted to inorganic nitrogen by Kjeldahl digestion (cf. 27). Inorganic nitrogen was estimated by the Nessler reaction as modified by Johnson (25a). Ammonium sulfate nitrogen standards and reagent blanks were carried through the entire procedure. Protein content was calculated assuming 16% nitrogen.

Protein determination

Protein content of GCE was estimated by the method of Lowry *et al.* cf. 27). Solutions of 2% Na_2CO_3 in 0.10 N NaOH and 0.5% $CuSO_4$-$5H_2O$ in 1% sodium tartrate were prepared. Subsequently, 1 ml of the copper solution was added to 50 ml of the Na_2CO_3-NaOH just before the reagent was to be used. Then 2.5 ml of this reagent was added to 0.5 ml protein (10-100 µg). After 10 min at room temperature, 0.25 ml of Folin reagent diluted to 1 N was added with vigorous mixing. Reagent blanks and bovine

serum albumin standards were treated similarly. After 30 min at room temperature the solutions were read against a reagent blank on a Beckman DU-2 spectrophotometer at a wavelength of 650 nm.

DNA determination

DNA content of GCE was estimated by the diphenylamine reaction (cf. 27). Diphenylamine reagent was prepared by dissolving 1.5 g diphenylamine in 100 ml glacial acetic acid followed by the addition of 1.5 ml concentrated H_2SO_4. Then 0.10 ml aqueous acetaldehyde (16 mg/ml) was added per 20 ml of the above solution. Two ml of this latter reagent was mixed with 1 ml DNA (10-200 µg) and the tubes were heated for 10 min in a boiling water bath. Blanks and DNA standards (calf thymus DNA, highly polymerized, Sigma Chemical Company, St. Louis MO) were treated similarly. Tubes were cooled and read at 600 nm on a Beckman DU-2 spectrophotometer against a reagent blank. DNA content was determined by interpolation from a standard curve prepared by plotting absorbancies obtained with varying amounts of a standard DNA solution.

RNA determination

RNA content of GCE was estimated by the orcinol reaction for pentose determination (cf. 27). The orcinol reagent was prepared by mixing 1 volume of a solution of 1.6 g orcinol in 100 ml H_2O with 7.5 volumes of 21.6 N H_2SO_4. Two ml of this orcinol reagent was mixed with 1 ml RNA (10-100 µg). The tubes were heated for 20 min in a boiling water bath. Reagent blanks and RNA standards (yeast RNA, Schwartz/Mann, Orangeburg, NY) were treated similarly. The tubes were cooled and read at 640 nm

against a reagent blank on a Beckman DU-2 spectrophotometer. RNA content was determined by interpolation from a standard curve prepared by plotting absorbancies obtained with varying amounts of a standard RNA solution.

Ultraviolet absorption spectra

The absorbance of 100 µg/ml samples of GCE were measured in a Beckman DU-2 spectrophotometer at wavelengths of 220 nm to 320 nm. The 260/280 ratio was used to estimate protein content and nucleic acid content of the antigen extractions according to the absorbance relationships determined by Warburg and Christian (65).

Sephadex chromatography

Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) was suspended in PBS and allowed to swell overnight. The slurry was dearated by vacuum and then packed in a 30 cm x 1 cm column. A 0.25 ml sample (containing 25 µg GCE labeled with ^{125}I in PBS) was layered on the column and eluted with PBS, pH 7.2. One ml fractions were collected at a rate of 0.5 ml/mg and analyzed for total radioactivity. Fractions containing 2000 cpm/ml or over were assayed for reactivity with the standard positive serum (R65).

Sucrose velocity gradient ultracentrifugation

Sucrose velocity gradient ultracentrifugation was used in an attempt to separate components of the crude antigen extract. A 10% to 50% discontinuous sucrose gradient was prepared in 13 ml polyallomer tubes.

Then 0.4 ml GCE* in 10% sucrose was layered on the top of the gradient. Following ultracentrifugation in a Beckman L2-65B ultracentrifuge at 100,000 X g for 16 h at 4°C in a Spinco type 65 rotor, 12-drop fractions were collected from the bottom of the tubes and these fractions were analyzed for total radioactivity. Fractions containing 2000 cpm/ml or over were dialyzed against PBS for 4 days and assayed for reactivity with the standard positive serum. Samples from each fraction were analyzed for sucrose concentration by the anthrone reaction (cf. 27).

Physical and chemical treatments

In experiments where the effects of the treatment on GCE were measured by the inhibition assay treatments were performed on 20 µg GCE. Following the treatment the volume was adjusted to 1 ml and divided into samples containing 10, 5, 1, and 0.2 µg GCE. Each sample was adjusted to a final volume of 0.5 ml with PBS and assayed by the previously described inhibition assay (cf. Figure 2).

In some experiments treatments were performed on labeled GCE (GCE*). In these experiments 20 µg GCE* was treated. Following treatment the volume was adjusted to 1.0 ml and 0.5 ml aliquots were assayed directly for reactivity with the positive human serum.

Heat stability. Twenty µg GCE in 1 ml PBS was heated in a boiling water for 2 min or 1 h. Other samples were heated at 56°C for 1 h and assayed as described above (cf. Figure 2).

pH stability. Twenty µg GCE in 20 µl PBS was incubated for 1 h at 23-25°C with 0.25 ml 0.1 N HCl. Following incubation the reaction mixture

was neutralized with 25 μ eq NaOH and the volume was adjusted to 1.0 ml with PBS. The effects of the treatment were determined by the inhibition assay (cf. Figure 2). Stability to treatment with base was determined similarly, incubating with 0.1 N NaOH and neutralizing with HCl.

Trypsin digestion. Twenty μg GCE and 100 μg trypsin, Nutritional Biochemicals Corporation, Cleveland, OH, (2X crystalline, 50% $MgSO_4$), in 1 ml PBS were incubated overnight at 37°C. The reaction was stopped by boiling the samples for 2 min and assaying them as above (cf. Figure 2). When the treatment was performed on labeled GCE (GCE*), the following modifications were employed. Twenty μg GCE* and 20 μg trypsin in 0.14 ml PBS were incubated 3.5 h at 37°C. The volume was adjusted to 1.0 ml and the reaction was stopped by boiling for 2 min. The effects of the treatment were measured by assaying 0.5 ml aliquots for reactivity with the standard human serum.

Pronase digestion. Pronase (B grade, Calbiochem, Los Angeles, CA) treatment was performed in exactly the same manner as the trypsin digestion described above (cf. Figure 2).

Periodate oxidation. Twenty μg GCE was incubated overnight in the dark at room temperature with 46 μg sodium meta periodate in 1 ml PBS. The reaction was stopped by the addition of 20 μg glycerol and assayed as above (cf. Figure 2).

Deoxyribonuclease digestion. Twenty μg GCE was incubated 2 h at 37°C with 20 μg DNase (Deoxyribonuclease 1, Crude, Sigma Chemical Company, St. Louis, MO) in 0.25 ml PBS pH 5.5. The pH was then adjusted to 7.2

and the volume adjusted to 1 ml with 0.1 m tris buffer. Activity was assayed as above (cf. Figure 2).

When the treatment was performed on labeled GCE the following modifications were employed. Twenty µg GCE* and 20 µg DNase in 0.15 ml PBS were incubated 3.5 h at 37°C. The volume was then adjusted to 0.5 ml with PBS pH 7.2. Activity was assayed by reactivity with the standard positive human serum.

Ribonuclease digestion. Twenty µg GCE was incubated 2 h at 37°C with 20 µg RNase (Ribonuclease-A, Sigma Chemical Company, St. Louis, MO) in 0.25 ml PBS pH 5.5. The volume was adjusted to 1 ml and the pH was adjusted to 7.2 with 0.1 m tris HCl buffer. Activity was assayed as above (cf. Figure 2).

When the treatment was performed on labeled GCE the same modifications as used in the DNase digestion were employed.

Saponification. Twenty µg GCE in 20 µl PBS was added to 0.23 ml absolute methanol. To this was added 0.25 ml methanol containing 0.2 M KOH. The mixture was incubated 1 h at 23-25°C followed by neutralization with 0.5 m eq Dowex 50. The mixture was mixed 15 seconds on a vortex mixer the the Dowex beads were allowed to settle. The antigen solution was removed with a capillary pipet. Saponified lipids were then extracted by the addition of 0.5 ml ether and 0.5 ml water. The tube was mixed 15 seconds on a vortex mixer and the phases separated by centrifugation at 1000 X g for 10 min at 4°C. The ether phase was discarded and the aqueous phase was dialyzed against twice daily changes of PBS for 2 days at 4°C. The effects of the treatment were measured by the inhibition assay (cf. Figure 2).

Electron microscopy

Heavy suspensions of whole cells of T1 phase F62 and T3 phase IDC were obtained by washing the colonies from an 18-24 h plate culture with distilled deionized water. Samples of the cell suspensions were mixed with an equal volume 3% (w/v) phosphotungstic acid pH 7.2. A drop of this mixture was placed on a formvar coated grid. After allowing the bacteria to settle on the grid, excess moisture was removed with a capillary pipet. The grids were allowed to air dry and then observed and photographed with a Zeiss EM9-S2 electron microscope (Carl Zeiss, West Germany).

Biological tests

The pyrogenic responses of rabbits to intravenously administered GCE and gcLPS were determined by standard procedures leading to determinations of FI_{40} (dose producing a fever index of 40 cm^2) (47, 67). To obtain the FI_{40}, groups of 4 rabbits were inoculated with graded doses of pyrogen and the temperatures were recorded at intervals for a period of 7 h. The individual curves were plotted with 1°C and 1 h each allotted one inch. For each animal the area included between the temperature curve and the baseline was obtained in square centimeters by computer. This area was called the fever index (FI). The average FI, at each dose level, was then plotted against the logarithm of the dose, and the FI_{40}, or that dose expected to give a FI of 40 cm^2, was read from the graph.

Lethality for chick embryos was estimated as described previously (47). Embryos, after 11 days of incubation, were inoculated intravenously with doses contained in 0.1 ml. In each test, ten embryos were

inoculated with each of five graded doses, differing by five-fold. The results, after an additional 24 h of incubation, were used to calculate the median lethal dose for chick embryos ($CELD_{50}$) as described by Irwin and Cheeseman (25).

Extraction of endotoxin from N. gonorrhoeae

A 48 h flask culture of N. gonorrhoeae was harvested by centrifugation at 1800 X g for 30 min at 4°C and washed once in cold PBS. The cells were resuspended in 50 ml DDW and an equal volume of 88% liquid phenol (U.S.P., J. T. Baker Chemical Company, Phillipsburg, NJ) was added. The mixture was placed in a 65°C water bath and stirred until the temperature reached 65°C and the phenol and aqueous phases homogenized. Stirring was continued for another 30 min, and the solution was distributed to glass centrifuge tubes, which were then chilled in an ice bath overnight to allow the phases to separate. The tubes were centrifuged at 4°C for 60 min at 1800 X g. The aqueous phases, after removal from the inter- and phenol-phases, were combined. An equal volume of water was added to the combined phenol and inter-phases and the extraction was repeated; the second aqueous phase was combined with the first, and the phenol residues were discarded. The aqueous phases were dialyzed against running tap water at about 6°C for seven days and against three daily changes of distilled water at 4°C. Sufficient sodium acetate was added to the aqueous phases to bring the concentration to 0.15 M: the solution was cooled and 95% ethanol was added at 4°C, dropwise with stirring, to bring the alcohol concentration to 68%. After allowing the suspension to stand overnight at 4°C, the precipitate was

collected by centrifugation at 1800 X g for 30 min. The precipitate was dissolved in distilled water and lyophilized. The endotoxin produced by this method was designated gcLPS. The above procedure was a modification of the method of Westphal *et al.* (cf. 56).

CHAPTER III

RESULTS

Chemical and biological analyses of GCE

A radioimmunoassay for the diagnosis of gonorrhea has been developed recently (36). The antigen used in this assay was a crude extract of whole cells of Neisseria gonorrhoeae strain F62; this extract contained at lease one antigenic fraction specific for the gonococcus. The next step in refining the assay was to characterize the antigen in the extract with the ultimate aim of purifying the antigenic fraction. Also this could increase both understanding of the antigenicity of the gonococcus and specificity of the seroassay.

As a first step in the characterization, a limited series of chemical analyses were performed on various preparations. The analyses were, of necessity, limited; the yield from a 1 liter culture was only 1 to 2 mg GCE. It was decided that a 100 µg sample was the maximum amount which would be used for any given assay in order to carry out the maximal number of tests on a single preparation. If a given substance constituted less than 1% of the preparation, then the assay would have to be sensitive to amounts of less than 1 µg in order to detect the substance.

As a working hypothesis, based on the extraction procedure and some preliminary results (Rudbach, unpublished data), it was felt that

the gonococcal specific antigen might be a protoplasmic polypeptide, glycoprotein, or nucleoprotein. However, the possibilities existed that gonococcal endotoxin or pili could have contributed to the specific antigenic reactivity.

The first analytical data obtained were UV absorption spectra (from 220 nm to 320 nm) on several GCE preparations (Figure 4). Protein and nucleic acid were estimated from a nomograph based on the relationships described by Warburg and Christian (65). It was found that GCE contained from 80% to 90% protein with substantial nucleic acid contamination (Table 1). Several preparations were analyzed for total nitogen (cf. 27) and protein was estimated assuming 16% nitrogen (Table 1). By this method it was found that GCE contained from 57% to 87% protein. More specific assays were then employed for the estimation of protein and nucleic acid. The results are summarized in Table 1. Protein was estimated by the Lowry method (cf. 27) to be from 40% to 68%. RNA content was estimated to be from 6.4% to 37% by the orcinol reaction (cf. 27). DNA content was estimated from the diphenylamine reaction (cf. 27) to be less than 1.0% in all preparations.

Since endotoxin (LPS) is a major component in cell walls of gram negative bacteria, it was necessary to determine LPS content of GCE. The easiest and most sensitive way to do this was through a comparison of quantitative bioassays for endotoxin performed on GCE and on LPS extracted from the gonococcus. A phenol-water extract of gonococci was prepared by a modification of the method of Westphal *et al.* (cf. 56). This endotoxin extract was designated gcLPS. Both GCE and gcLPS were assayed for endotoxic activities by the quantitative capacity to cause

Figure 4. UV absorption curves of two GCE preparations

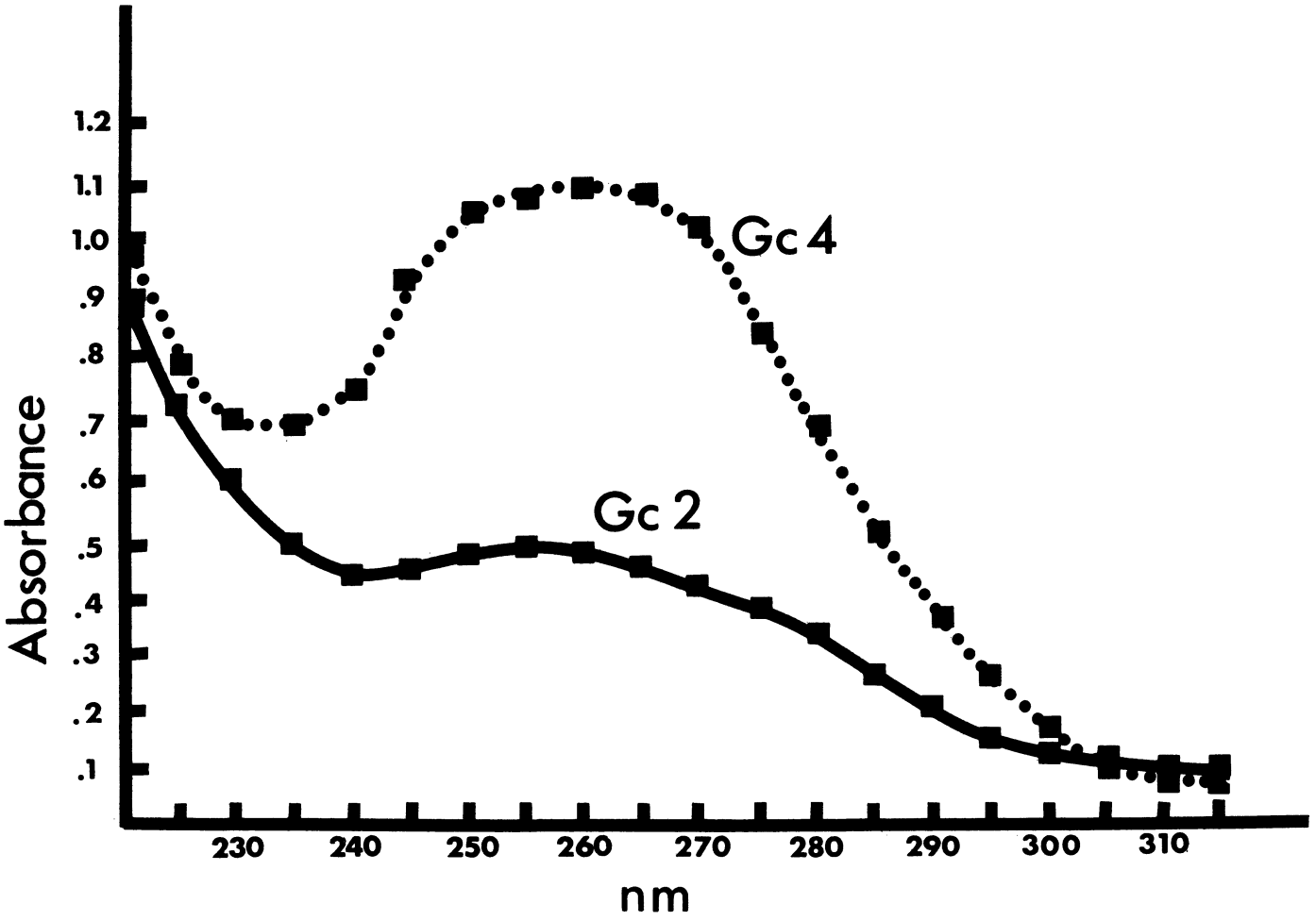

Absorbance
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
Gc 4
Gc 2
230
240
250
260
270
280
290
300
310
nm

Table 1. Chemical analyses of various GCE preparations

GCE	Protein %	RNA %	DNA %	Total Nucleic Acid %
gc 2	120.0[a] 57[c]	nd[b]	nd	18.0[a]
gc 4	90.0[a] 86.9[c]	nd	nd	51.0[a]
gc 11	70.0[a]	nd	nd	55.0[a]
gc 22	68.0[d]	6.4[e]	<1.0[f]	6.4[g]
gc 26	40.0[d]	26.0[e]	<1.0[f]	26.0[g]
gc 31	45.2[d]	37.8[e]	<1.0[f]	37.8[g]

[a]Protein and nucleic acid content estimated by UV absorption.

[b]nd = not done.

[c]Protein estimated from nitrogen content determined by Nessler Nitrogen Analysis.

[d]Protein estimated by Lowry method.

[e]RNA estimated by reaction with orcinol reagent.

[f]DNA estimated by reaction with diphenylamine.

[g]Nucleic acid as RNA.

fever in rabbits and to kill chick embryos (47). The biological activities are tabulated in Table 2. Both preparations demonstrated endotoxic activity. However, gcLPS was 20 fold more potent in the $CELD_{50}$ and nearly 10 fold more potent in the FI40 than GCE when compared on a weight basis. From these results it can be estimated that GCE contained no more than 15% LPS.

Taken as a whole, the results of chemical and biological analyses of GCE indicated several things. GCE was mostly protein but there were substantial amounts of RNA in some preparations. GCE had endotoxic activity, but endotoxin was not the major constituent of GCE. These facts were taken into account in subsequent experiments which were designed to characterize the specific gonococcal antigenic fraction of GCE.

Ability of GCE to inhibit SAGR

Previous studies (W. R. Cross and J. A. Rudbach, unpublished data) have established that the concentrations of GCE* and anti-gonococcal human serum employed in SAGR fell in the equivalence zone or region of slight antigen excess. Under these conditions all available antibody combining sites are bound by antigen. Addition of non-radioactive GCE, therefore, would compete with radiolabeled GCE (GCE*) for antibody combining sites. The ratio of bound-GCE* to unbound-GCE* would be directly proportional to the amount of cold GCE added to the reaction mixture. It should be emphasized that conditions were established for SAGR so that all the human immunoglobulin was precipitated by the addition of rabbit anti-human immunoglobulin. It has been shown (19) that if all anitbody was precipitated, the fraction of labeled antigen precipiated

Table 2. Biological activities of GCE and gonococcal LPS.

	GCE	LPS	% LPS in GCE
FI_{40}[a]	3.5 µg	0.5 µg	14.3[c]
$CELD_{150}$[b]	0.62 µg	0.03 µg	4.8[d]

[a] FI_{40} = µg material required to produce 40 cm^2 under fever response curve. This was determined as described in Materials and Methods.

[b] Mean lethal dose (µg) for chick embryos.

[c] Calculated by:

$$\% \text{ LPS} = 100 \times \left(\frac{FI_{40} \text{ LPS}}{FI_{40} \text{ GCE}}\right)$$

[d] Calculated by:

$$\% \text{ LPS} = 100 \times \left(\frac{CELD_{50} \text{ LPS}}{CELD_{50} \text{ GCE}}\right)$$

would decrease in linear fashion if plotted against the log of the concentration of unlabeled inhibitor added.

Figure 5 shows a series of inhibition curves for a variety of GCE* and GCE preparations. In this figure the activity ratios, which are a measure of the fraction of GCE* precipitated, are plotted against the log of the amount GCE added. There is considerable scattering of points and variation from preparation to preparation and assay to assay. In order to compare results obtained with different GCE and GCE* preparations, some means of standardization was needed. The means selected was to consider each standard curve separately and express each activity ratio as a percent of the activity ratio obtained with the uninhibited control for that single experiment. The zero point was defined as the lowest activity ratio obtained by inhibition with GCE. This was usually, but not always, the activity ratio obtained with 10 μg GCE added as inhibitor. Figure 6 shows the same curves from Figure 5 plotted in this "normalized" manner.

The amount of GCE added which gave 50% inhibition of control binding was designated the 50% inhibition point, or the IA_{50}. The value of the IA_{50} of the standard curves was approximately 1 μg.

Once it was established that GCE could be used to inhibit SAGR and the results were standardized to allow comparisons, treatments could be performed on GCE and the effects of these treatments could be measured as a change in the capacity of the treated GCE to inhibit SAGR as compared to untreated GCE. In every experiment a standard curve was prepared by assaying untreated GCE. The experimental curve was plotted relative to the standard curve. The IA_{50} of each was determined from

Figure 5. Capacity of GCE to inhibit SAGR. Activity ratios are plotted against amount GCE added. Standard curves from 9 different experiments shown. Curves with the same symbol represent assays employing the same GCE and GCE* preparations. The same standard human positive serum was used throughout.

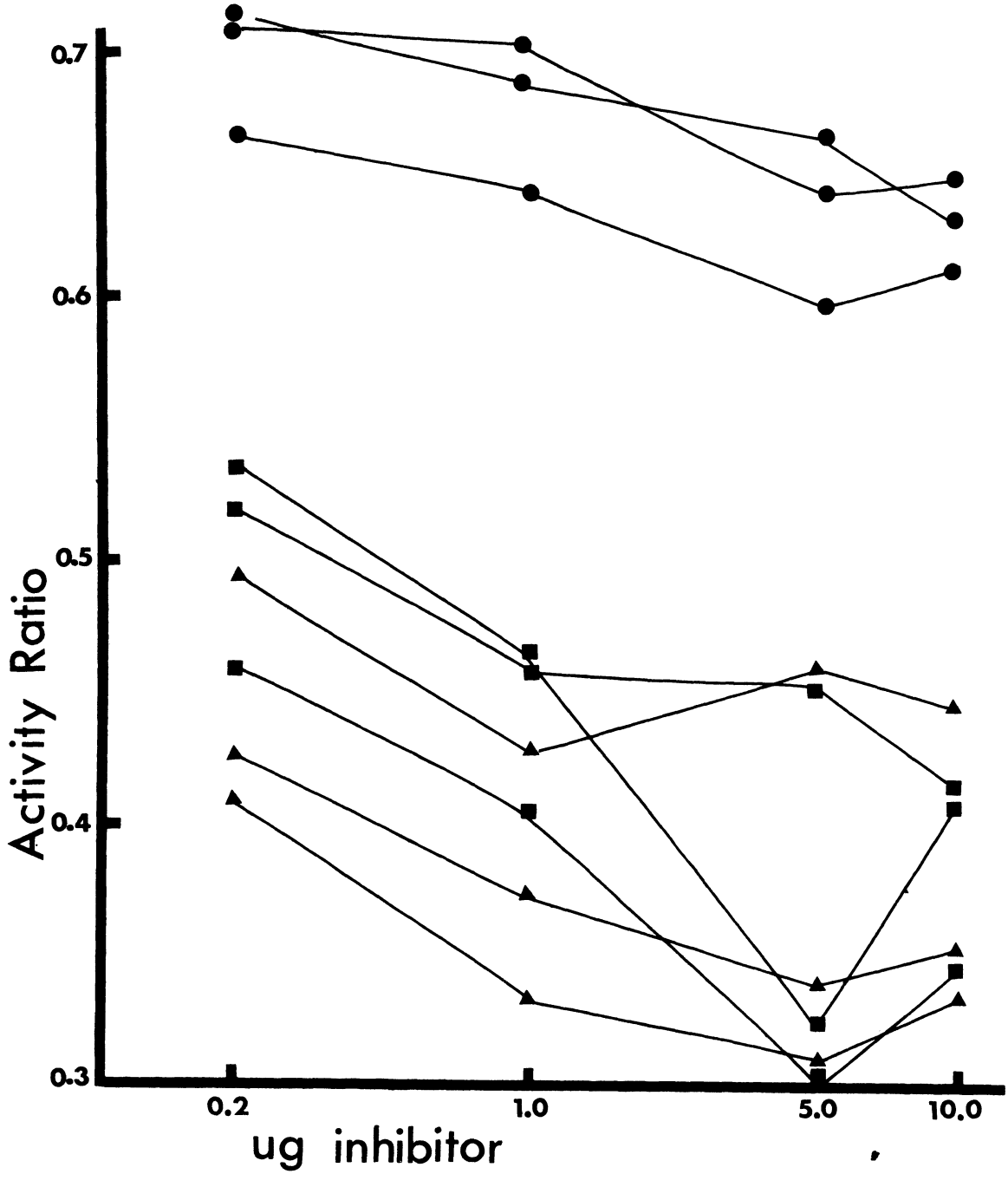
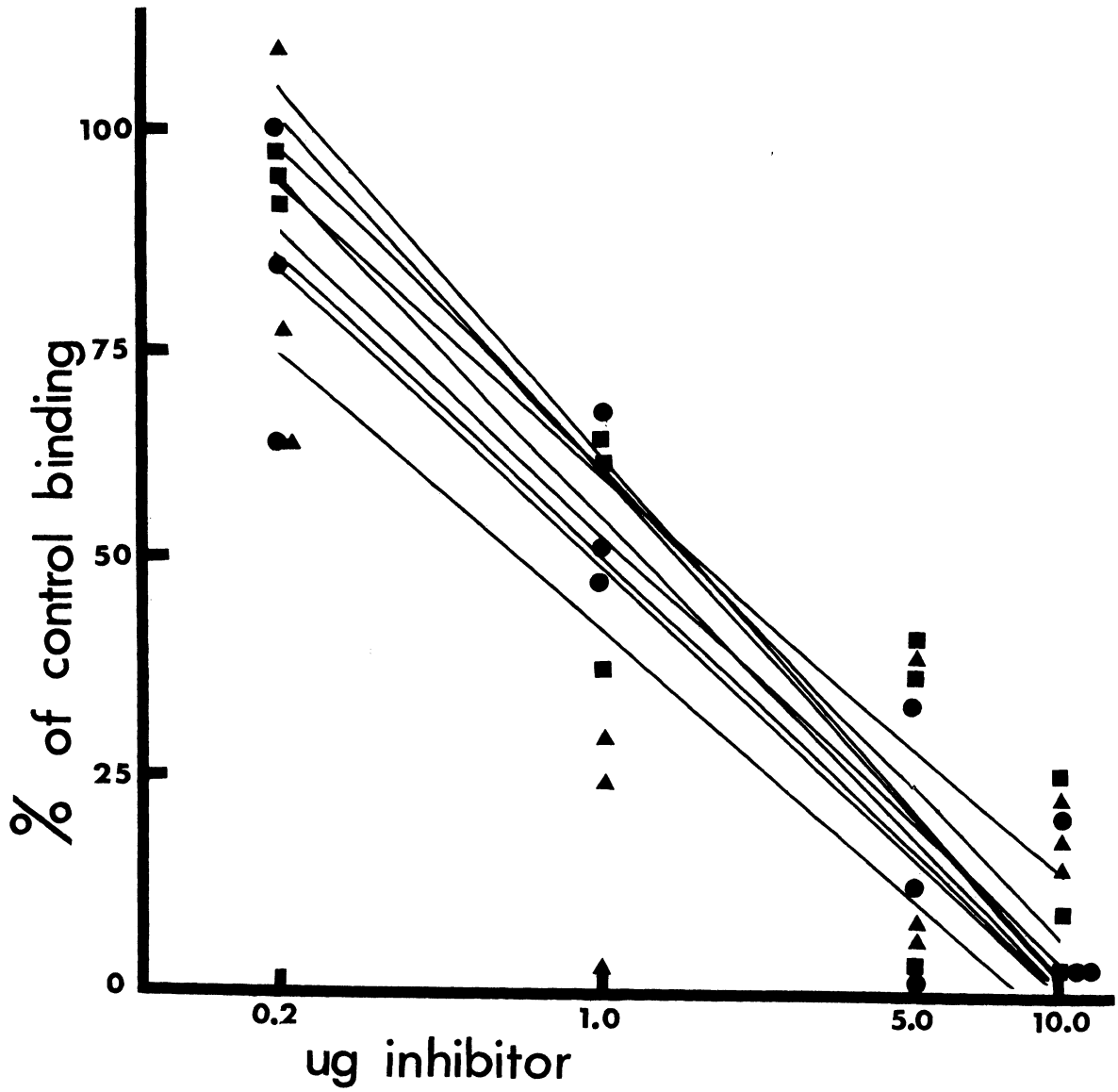

0.7
0.6
0.5
0.4
0.3
Activity Ratio
0.2
1.0
5.0
10.0
ug inhibitor

Figure 6. Standardization of inhibition curves. Nine inhibition curves plotted as a percentage of control binding. Standard curves from 9 different experiments are shown. Curves with the same symbol represent assays employing the same GCE and GCE* preparations. The same standard human positive serum was used throughout.


100
75
50
25
0
% of control binding
0.2
1.0
5.0
10.0
ug inhibitor

inspection and the activity of the treated GCE was then expressed as a percentage of the activity of the untreated GCE.

Effect of heat treatment on the capacity of GCE to inhibit SAGR

It is known that most protein antigens are denatured by heating at 56°C 30 min or by boiling (cf. 1), whereas other antigens such as lipopolysaccharides (cf. 48) and glycoproteins (cf. 22) are resistant to heat treatment. When GCE was heated at 56°C, 1 h or boiled 2 min there was a slight increase or no change in ability to inhibit SAGR. Longer heating in a boiling water bath resulted in substantial loss of ability to inhibit SAGR (Figures 7 and 8). The IA_{50} values of controls were 1.57 µg to 1.0 µg; after heating at 56°C for 1 h or boiling 2 min, the IA_{50} values were reduced to 0.2 to 0.7 µg respectively, an increase in activity of up to 785% of the control. If GCE was heated in a boiling water bath for longer than 2 min, the IA_{50} increased to 3.3 to 4.0 µg which represented a decrease in activity of 70% (Table 3).

The biphasic nature of the effect of heating was found to be reproducible. It could be that moderate heating caused an increase in solubility or caused some degree of disaggregation of subunits of GCE, therefore increasing activity. Prolonged heating at high temperatures might have caused denaturation of the antigen. These results were not inconsistent with the hypothesis that the specific fraction of GCE was a low molecular weight polypeptide.

Effect of acid and alkali treatment on capacity of GCE to inhibit SAGR

It was necessary to obtain knowledge of the stability of GCE under various conditions of acidity and alkalinity as background information

Figure 7. Effect of heat treatment on quantitative capacity of GCE to inhibit SAGR. Untreated GCE, A; GCE boiled 1 hr, B; GCE heated 56°C, 1 hr, C.

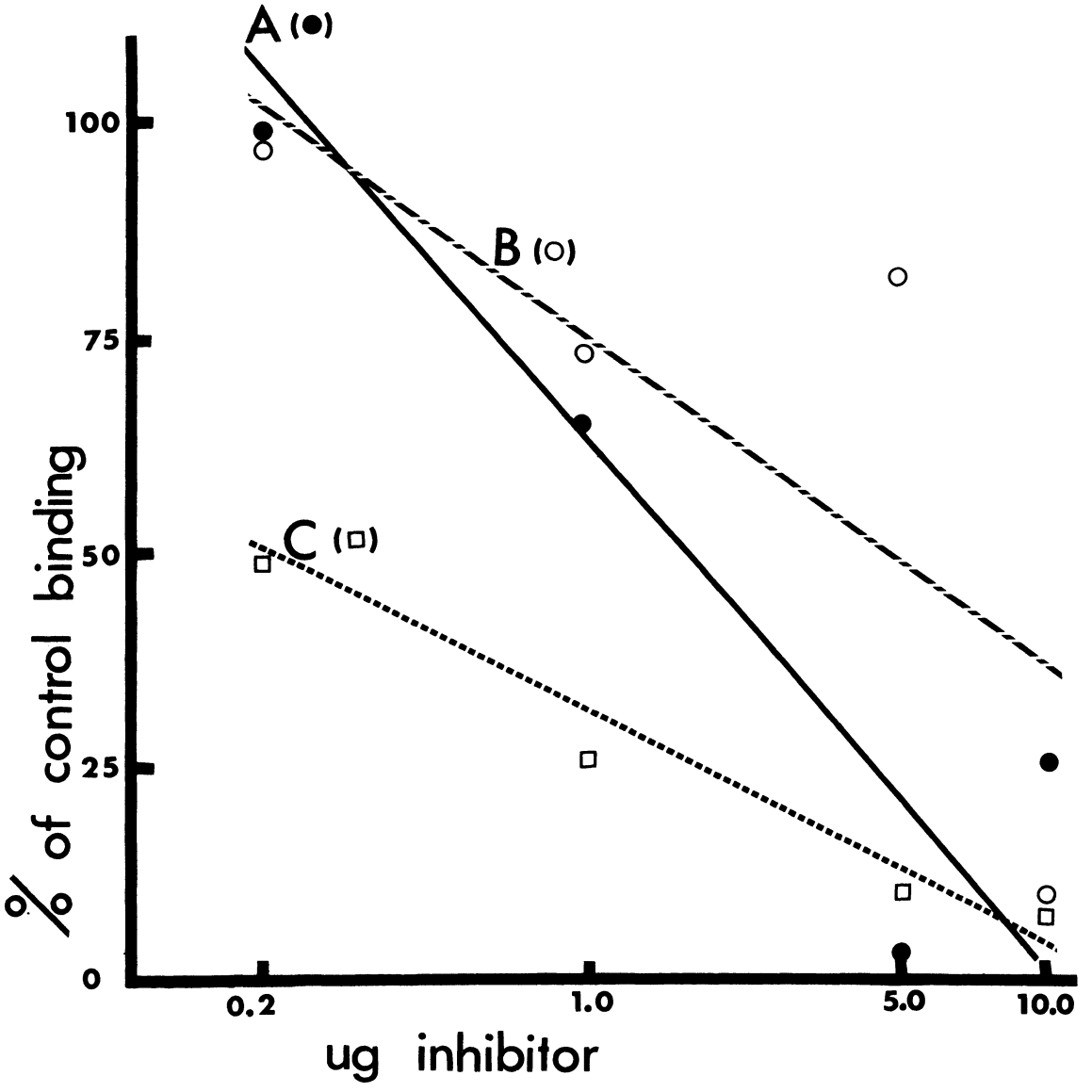
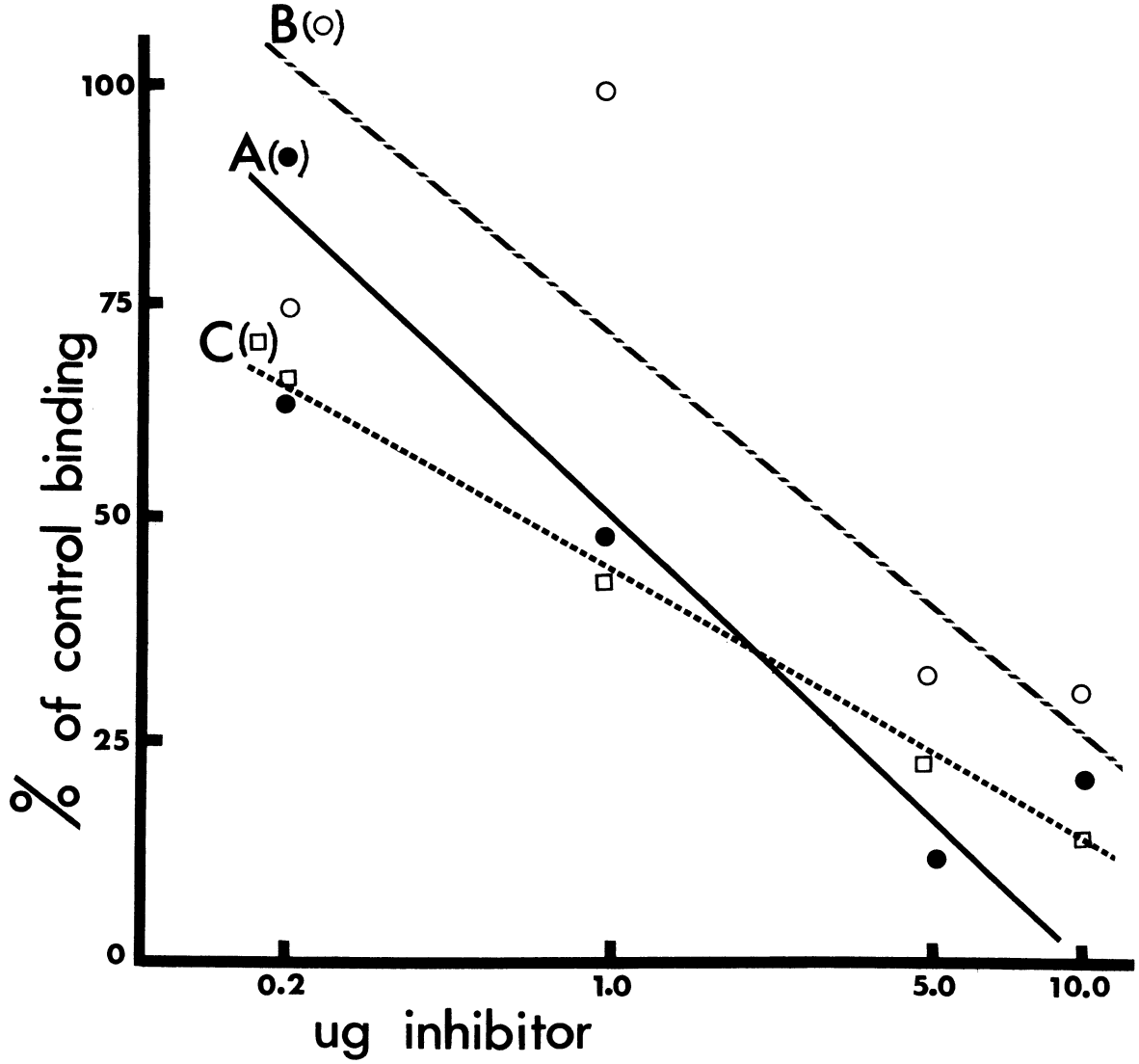

A (●)
B (○)
C (□)
100
75
50
25
0
% of control binding
0.2
1.0
5.0
10.0
ug inhibitor

Figure 8. Effect of boiling on quantitative capacity of GCE to inhibit SAGR. Untreated GCE, A; GCE boiled 5 min, B; GCE boiled 2 min, C.


B(○)
A(●)
C(□)
100
75
50
25
0
% of control binding
0.2
1.0
5.0
10.0
ug inhibitor

before designing other experiments. Because many of the subsequent procedures involved incubating GCE at varying pH, it was important to know what effects acid and alkali had on GCE so that these effects could be taken into account in interpreting other data.

When GCE was subjected to treatment with 0.1 N HCl for 1 h at 23°C, there was a moderate increase in activity (Figure 9), whereas treatment with 0.1 N NaOH had a negligible effect in either direction. The IA_{50} was reduced from 1.3 µg to 0.48 µg by acid treatment, which calculated as an increase in activity to 273% of the control.

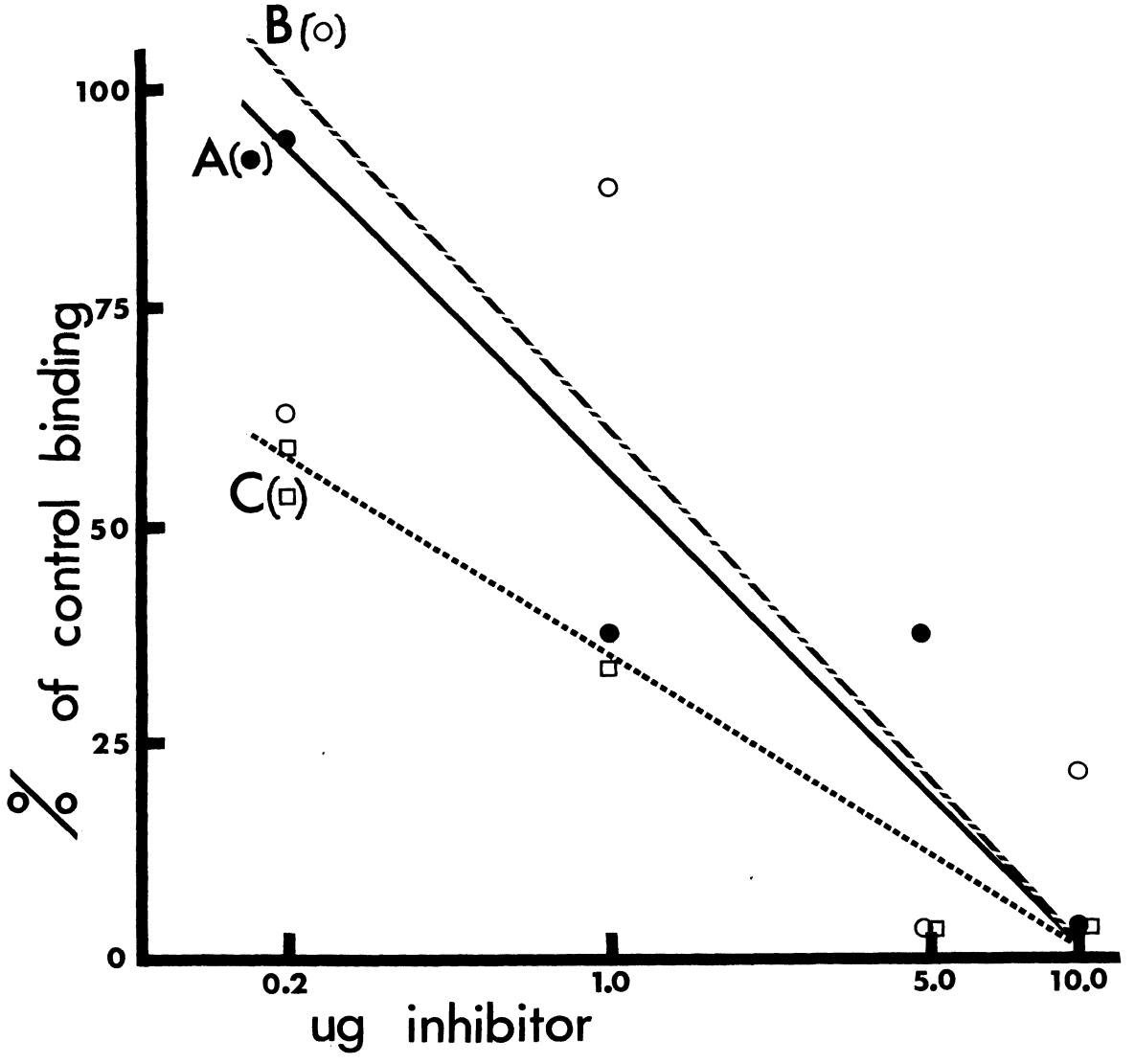
The increase in activity due to acid treatment might be interpreted as an indication that more sites had been made available for binding by specific antibody. The low pH achieved in this experiment was well below the isoelectric point of most proteins (31). At this pH each individual molecule has the same net charge. They would, therefore, repel each other preventing aggregation (31). Since the preparations were assayed immediately following neutralization, it is likely that a larger fraction of subunits of the treated GCE would exist in the disaggregrated form than would the subunits of untreated GCE.

The results of these experiments also showed that GCE was stable to treatment with 0.1 N acid or alkali.

Effect of periodate oxidation on capacity of GCE to inhibit SAGR

This experiment was designed to determine whether or not a carbohydrate determinant was important for the specific antigenic activity of GCE. Periodate is known to cleave sugars between adjacent hydroxyls (cf. 5).

Figure 9. Effect of pH on quantitative capacity of GCE to inhibit SAGR. Untreated GCE, A; 0.1 m NaOH treated GCE, B; 0.1 m HCl treated GCE, C. Treatments were for 1 h at 25°C.


B (○)
A (●)
C (□)
100
75
50
25
0
% of control binding
0.2
1.0
5.0
10.0
ug inhibitor

Oxidation with periodate reduced significantly the capacity of GCE to inhibit SAGR (Figure 10). The IA_{50} was increased from 1.5 µg for untreated GCE to 4.0 µg for periodate oxidized GCE (Table 3), a decrease in activity to 37.5% of the control. These results showed that GCE was sensitive to periodate oxidation. However, it is know that periodate can effect some changes in molecules other than sugars (cf. 5).

Effect of saponification on the capacity of GCE to inhibit SAGR

If a lipid moiety were important in the specific antigenic activity of GCE, this might be detected by a decrease in activity caused by saponification. KOH in methanol is known to hydrolyze the ester linkage of ester-bound fatty acids (22, 31). The fatty acids which are soluble in organic solvents would be removed by ether extraction. In this experiment the control was GCE treated with methanol alone and extracted with ether.

There was an increase in activity caused by saponification which is not easily explained (Figure 11). The effect was not due to the methanol of ether extraction, since the IA_{50} of the control was similar to the IA_{50} of other assays with untreated GCE (Table 3).

From these results it appeared, however, that there were no fatty acid esters essential to the antigenicity of GCE.

Effect of enzymatic digestion on the capacity of GCE to inhibit SAGR

The next series of experiments was designed to explore more fully the chemical nature of the moieties contributing to the antigenic activity of GCE. Enzymes specific for different classes of biological

Figure 10. Effect of periodate oxidation on quantitative capacity of GCE to inhibit SAGR. Untreated GCE, A; Periodate oxidized GCE, B.

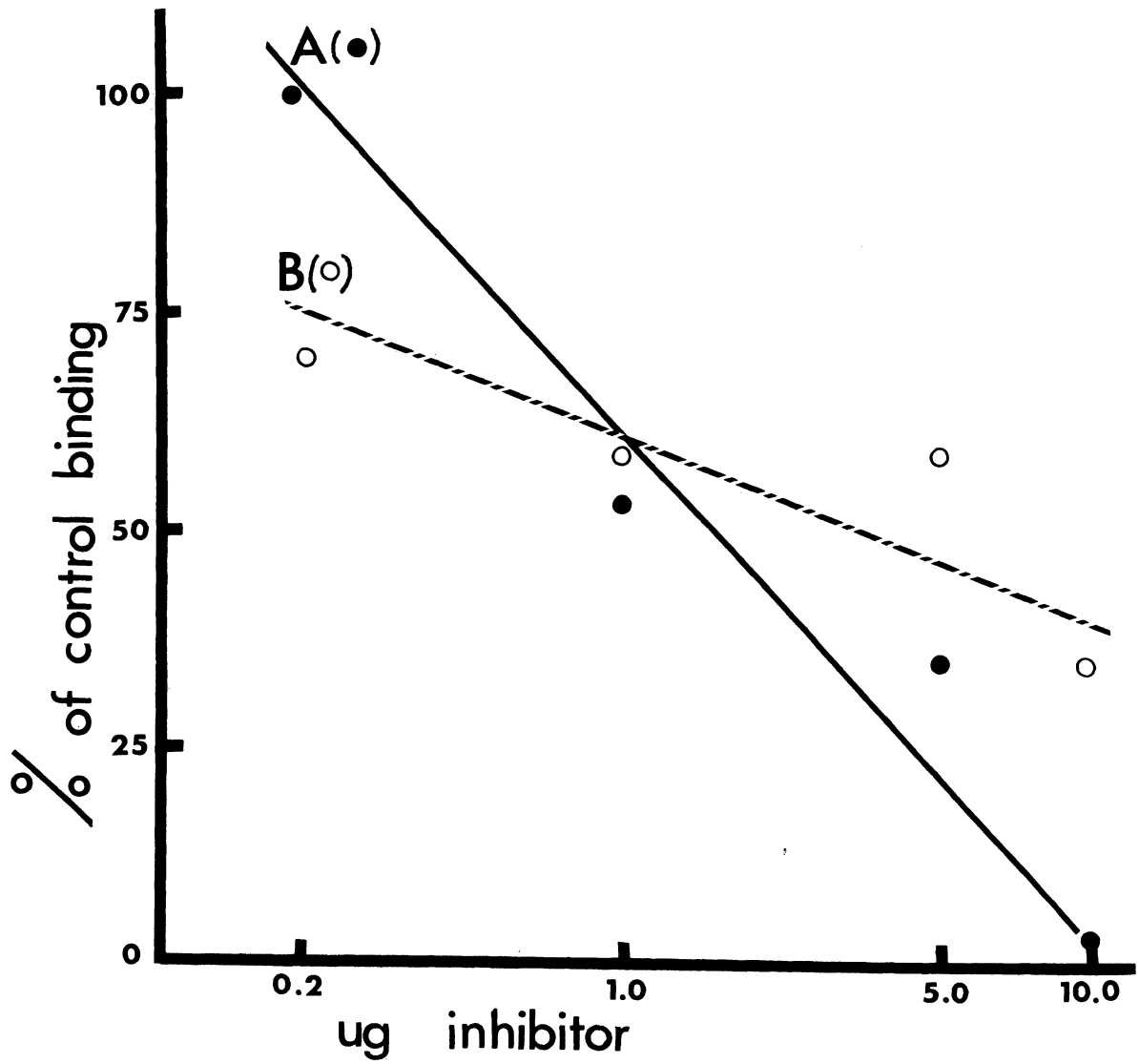

A(●)
B(○)
100
75
50
25
0
% of control binding
0.2
1.0
5.0
10.0
ug inhibitor

Figure 11. Effect of saponification on quantitative capacity of GCE to inhibit SAGR. GCE treated with 0.2 N KOH in methyl alcohol; cleaved fatty acids were extracted with ether. Control was GCE treated with methanol and extracted with ether. Control, A; Saponified GCE, B.

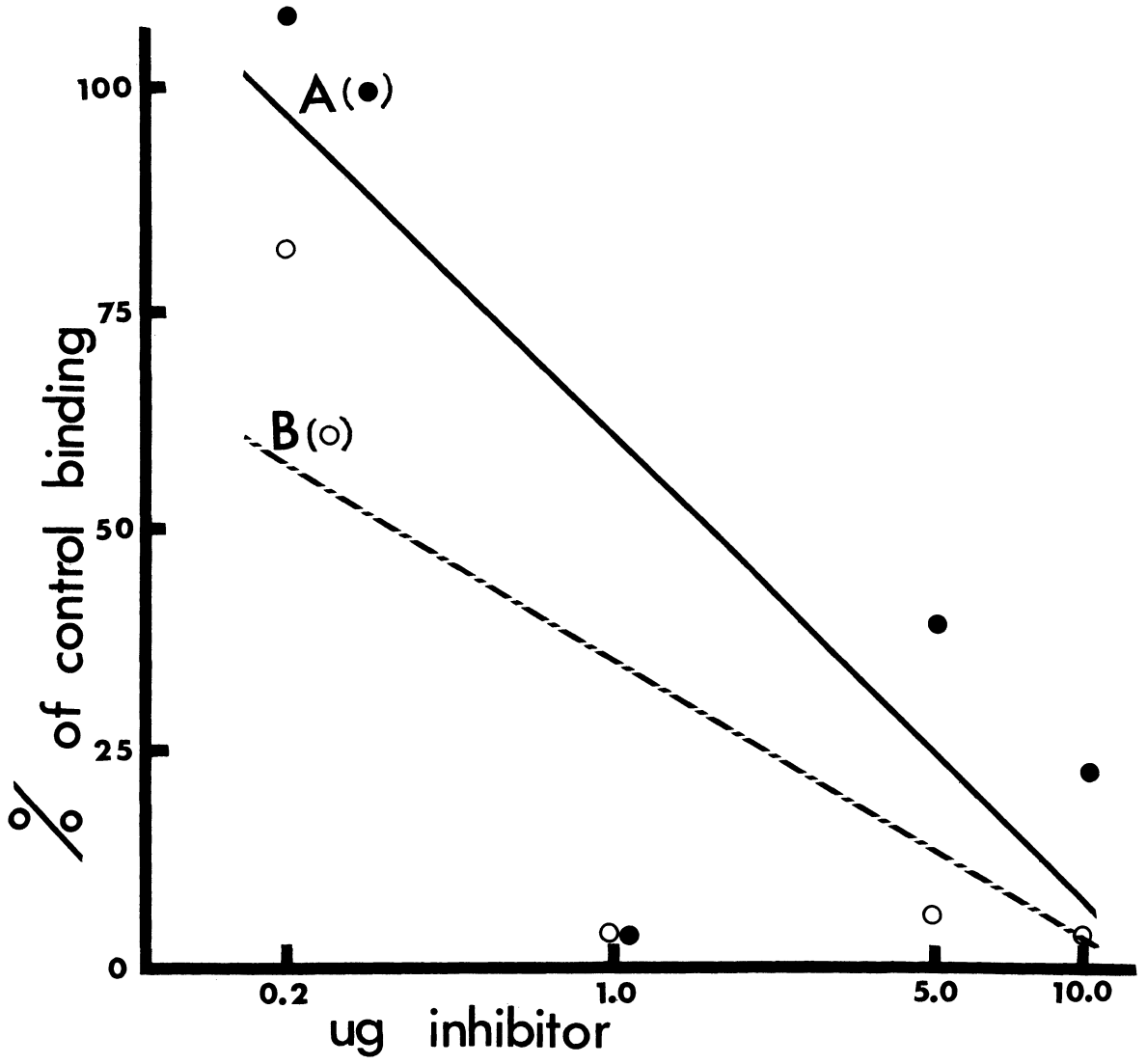

100
75
50
25
0
% of control binding
A (●)
B (○)
0.2
1.0
5.0
10.0
ug inhibitor

Table 3. Summary of effects of various physical and chemical treatments on quantitative capacity of GCE to inhibit SAGR.

Experiment #	Treatment	IA_{50} (μg)[a]		% of standard inhibition
		Treated	Untreated	
1	0.1 M HCl	0.48	1.30	270.8
2	0.1 M NaOH	1.40	1.30	92.8
3	0.2 M KOH-Methanol	0.42	1.15	273.8
4	Periodate	4.00	1.50	37.5
5	56°C, 1 h	0.20	1.57	785.0
6	100°C, 1 h	4.00	1.57	39.2
7	100°C, 2 min	0.70	1.00	142.9
8	100°C, 5 min	3.30	1.00	30.3

[a] IA_{50} = amount GCE required to achieve 50% inhibition of SAGR

[b] % of standard inhibition = $100X \left(\frac{IA_{50} \text{ untreated GCE}}{IA_{50} \text{ treated GCE}}\right)$

molecules were employed. A series of controls was carried through the entire procedure for each assay containing the enzyme, but no GCE. These controls (results not shown) indicated that the enzymes alone had no effects on SAGR. Proteolytic enzymes were inactivated by boiling for 2 min following digestion.

DNase and RNase digestion had little effect on the capacity of GCE to inhibit SAGR (Figure 12). However, if the DNase was not inactivated before addition of the preparation to the antibody, some inhibition was noted (Figure 13). It was felt that this effect might have been due to proteases contaminating the crude DNase preparation. It can be seen from the summary on Table 4 that the IA_{50} of both nuclease treated GCE and untreated GCE are similar in all cases but one.

When GCE was digested with a proteolytic enzyme, considerable inactivation was noted. Trypsin-treated GCE was much less able to inhibit SAGR than untreated GCE (Figure 14). The IA_{50} for trypsin treated GCE was 3 μg compared with an IA_{50} of 1.0 μg for untreated GCE, which calculated a decrease in activity of 67% (Table 4).

These results showed that GCE was resistant to ribonuclease digestion and deoxyribonuclease digestion, but was sensitive to trypsin digestion.

Effect of enzyme digestion of GCE* on SAGR

Next to be performed was a direct assay on the susceptibility of GCE to enzymatic digestion. It was felt that direct treatment of radiolabeled GCE (GCE*) might provide results more easily interpretable than results from the inhibition assays. An additional set of controls was

Figure 12. Effect of nuclease digestion on quantitative capacity of GCE to inhibit SAGR L. Untreated GCE, A; DNase digested GCE, B; RNase digested GCE, C. Enzymes were heat inactivated by boiling 2 min following digestion.

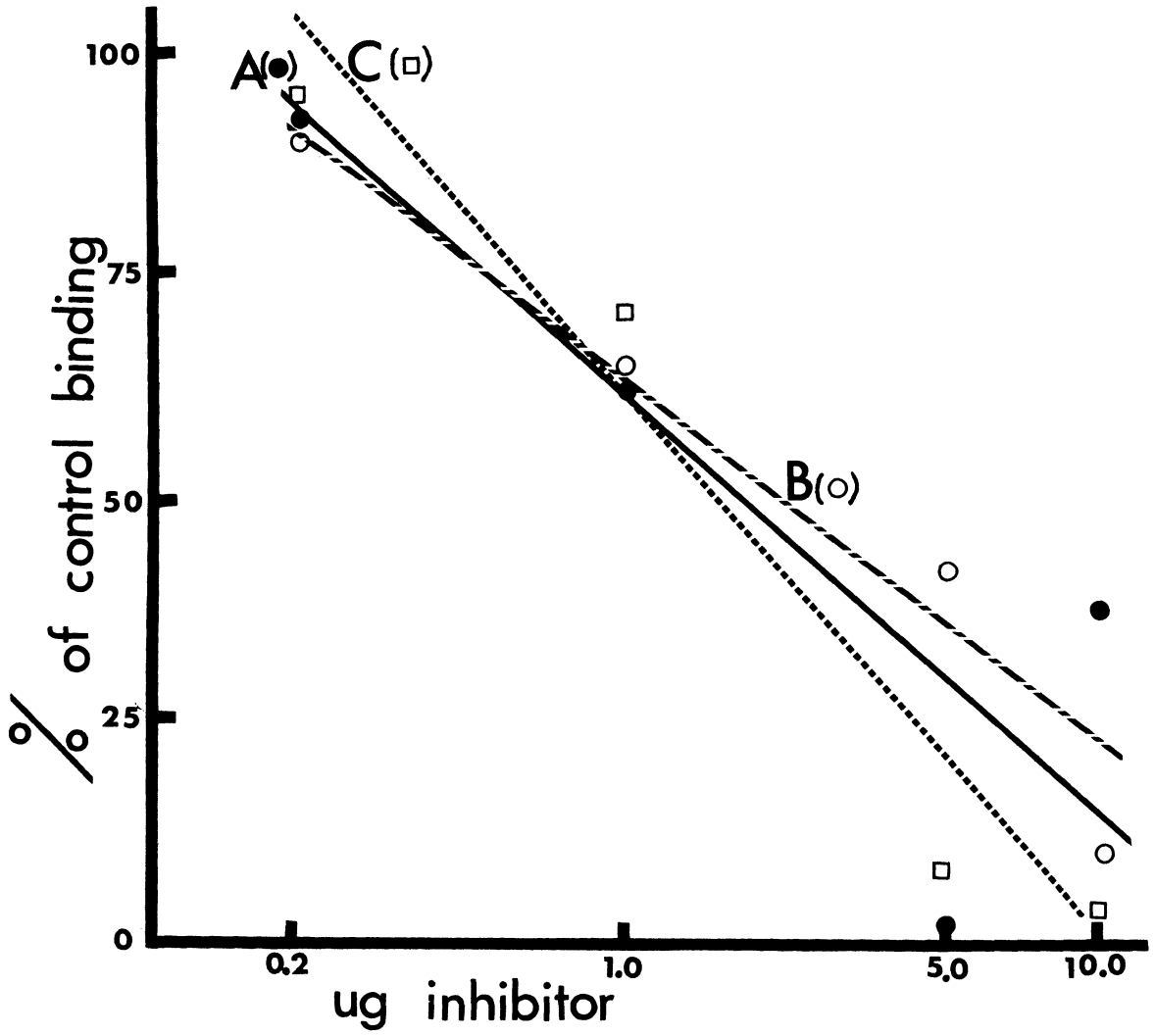

100
75
50
25
0
% of control binding
A(●)
C(□)
B(○)
0.2
1.0
5.0
10.0
ug inhibitor

Figure 13. Effect of nuclease digestion on quantitative capacity of GCE to inhibit SAGR II. Untreated GCE, A; DNase digested GCE, B; RNase digested GCE, C. Enzymes were not inactivated following digestion.

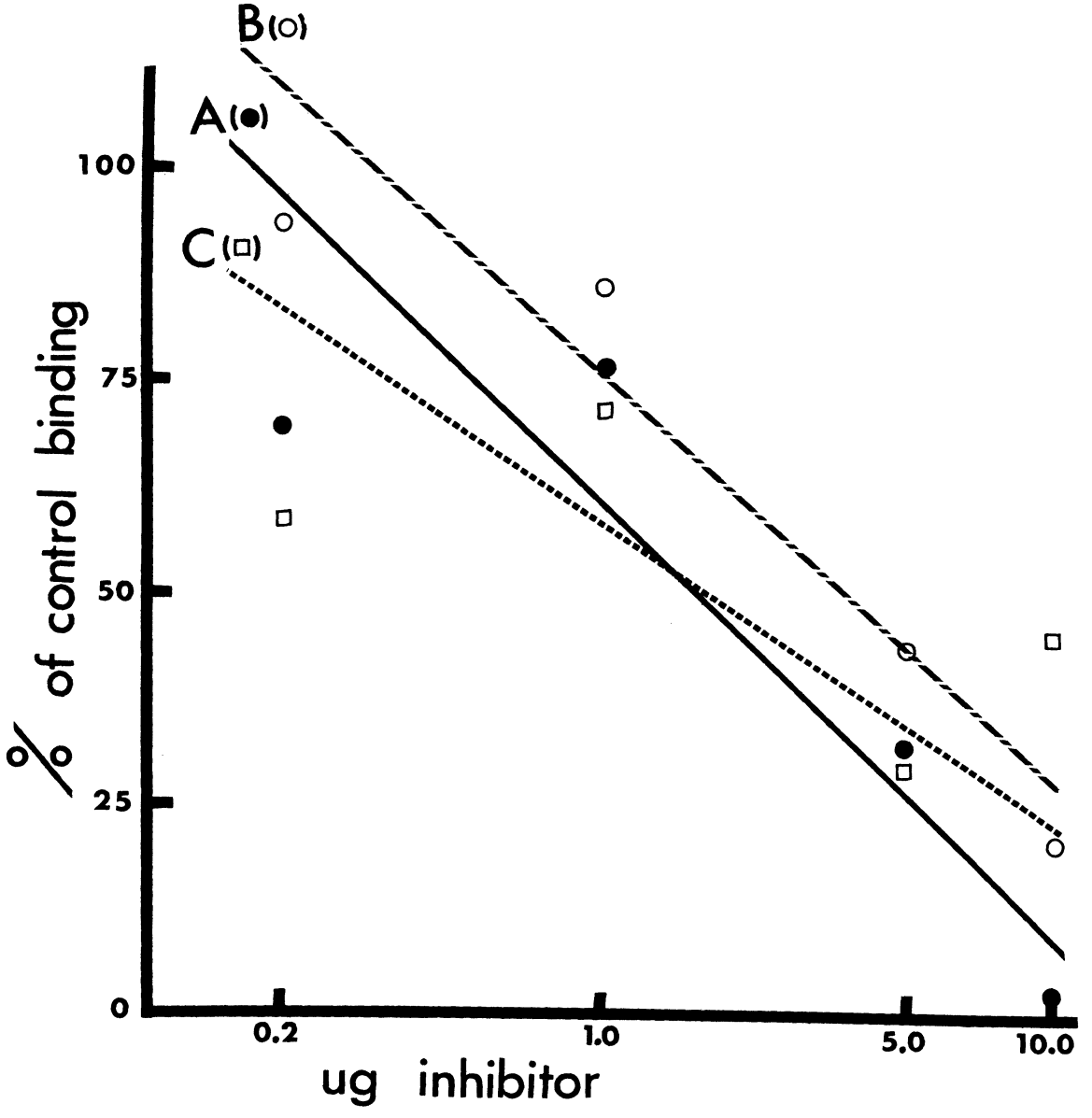

B(○)
A(●)
C(□)
100
75
50
25
0
% of control binding
0.2
1.0
5.0
10.0
ug inhibitor

Figure 14. Effect of trypsin digestion on quantitative capacity of GCE to inhibit SAGR. Untreated GCE, A; trypsin treated GCE, B.

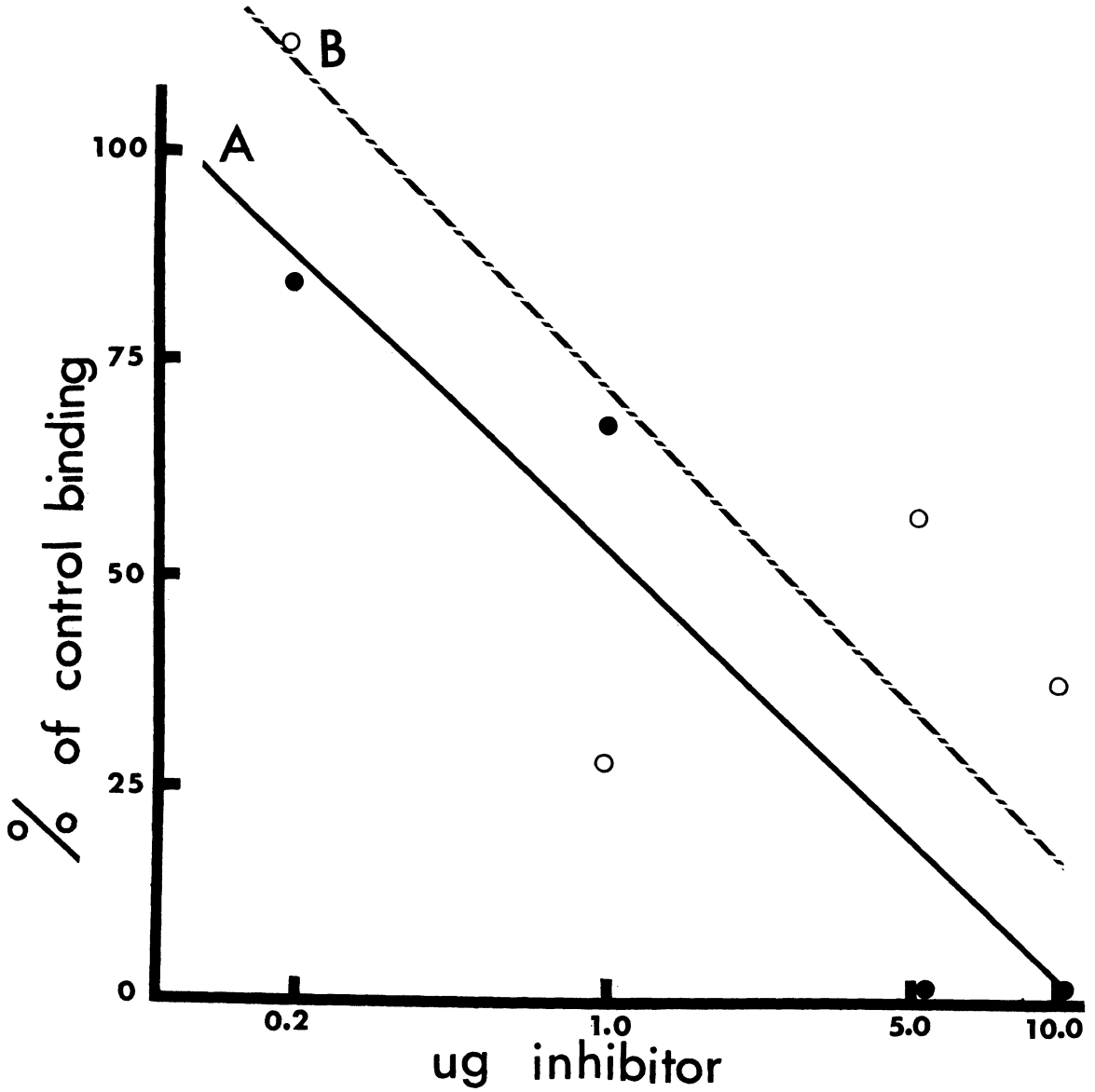

B
A
100
75
50
25
0
% of control binding
0.2
1.0
5.0
10.0
ug inhibitor

Table 4. Summary of effects of enzyme digestion on quantitative capacity of GCE to inhibit SAGR.

Experiment #	Treatment	IA_{50} (μg)[a]		% of standard inhibition[b]
		Treated	Untreated	
1	DNase	2.1	1.7	81.0
2	DNase	4.5	2.1	46.7
3	RNase	2.6	2.1	80.8
4	RNase	1.5	1.7	113.3
5	Trypsin	3.0	1.0	33.3
6	None	-	1.0	100.0

[a] IA_{50} = amount GCE required to achieve 50% inhibition of SAGR.

[b] % of standard inhibition = $100 \times \left(\frac{IA_{50} \text{ untreated GCE}}{IA_{50} \text{ treated GCE}} \right)$

included for the proteolytic enzymes in which the GCE* was not added until after heat inactivation of the enzyme.

When nuclease digestion was carried out on GCE* it was found that RNase had little effect, and DNase reduced the activity by 24% (to 76% of the control) (Table 5).

Trypsin and pronase were also employed in the digestion of GCE* (Table 5). The activity ratios were significantly reduced by trypsin and pronase digestion, to as low as 38.7% of the control. The enzyme controls indicated that the heat inactivated proteolytic enzymes retained some capacity to reduce the activity of GCE*. This effect was probably due to incomplete inactivation of the enzymes.

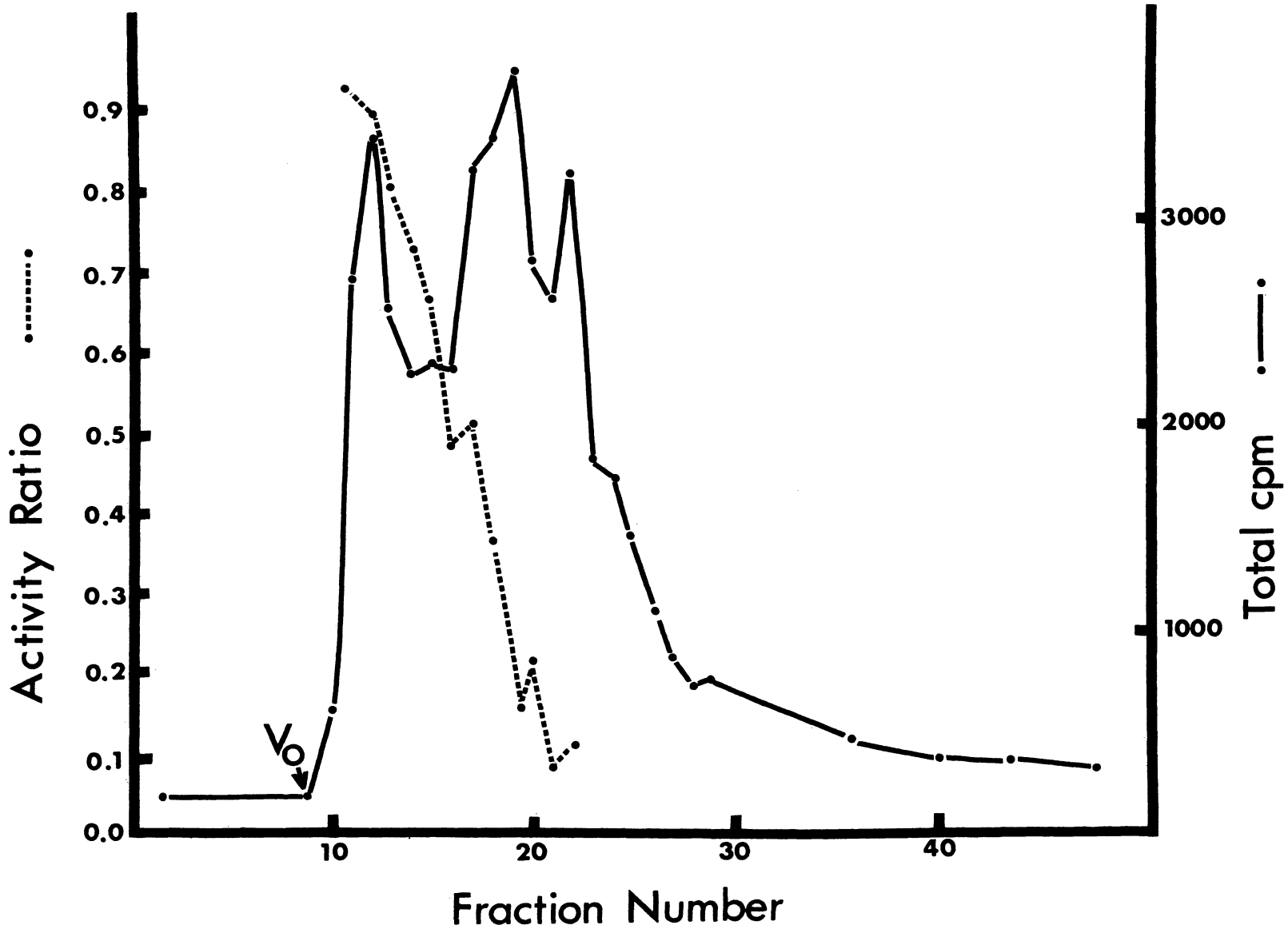
The results of these experiments again showed that GCE* was resistant to nuclease digestion but highly susceptible to digestion by proteolytic enzymes.

Separation of GCE* components by molecular sieving on Sephadex G-200

This experiment was designed to separate components of GCE on the basis of molecular volume. Sephadex G-200 excludes globular molecules of molecular weight greater than 800,000 daltons while molecules of lesser molecular weight are retained by the gel matrix and their flow through the column is impeded.

Figure 15 shows the results of molecular sieving of GCE* on Sephadex G-200. It can be seen from the total radioactivity profile that GCE* was separated into 3 major fractions. When the fractions were assayed directly in SAGR, it was found that there was a specific activity peak coinciding with the peak eluted with the void volume. This suggested

Figure 15. Separation of GCE components by molecular sieving on Sephadex G-200.


Activity Ratio
Total cpm
Fraction Number
V_O
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1000
2000
3000
10
20
30
40

that the active fraction of GCE existed in a high molecular weight form. It was also noted that approximately 30% of the total cpm added to the column could not be eluted.

Fractionation of GCE* by sucrose velocity gradient ultracentrifugation

Velocity gradient ultracentrifugation is another method of separating molecules on the basis of size and shape. High molecular weight, globular molecules sediment at a greater rate than do low molecular weight molecules. A 20 μg sample of GCE* was centrifuged at 100,000 x g for 16 h on a discontinuous gradient of 10% to 50% sucrose.

Figure 16 depicts the results of fractionation of GCE* by sucrose velocity gradient ultracentrifugation. Although little separation was detected in the total cpm profile, when the fractions were assayed by SAGR, two areas of high antigenic reactivity were found, one at each extreme of the gradient. This suggested either that the active fraction of GCE existed in an aggregated, high molecular weight form and as low molecular weight subunites, or that there were two distinct, active fractions, one of high molecular weight and one of low molecular weight.

Comparison of the capacities of GCE and gcLPS to inhibit SAGR

The biological studies previously described in this paper showed that GCE contained no more than 10% LPS. It was possible that this small LPS component was responsible for the specific activity of GCE. If this were the case then purified gonococcal LPS would be able to inhibit SAGR to a greater extent than could GCE. The following experiment was designed to test this possibility.

Figure 17 shows inhibition curves for GCE and gcLPS. The IA_{50} for each is shown in Table 6. The IA_{50} for the LPS preparation was 10 fold greater than the IA_{50} of GCE. If the major antigenic component of GCE were LPS, it would be expected that the LPS preparation would have approximately a 10 fold lower IA_{50} than GCE. Since the reverse was true, it could be assumed that the antigenic component specific for the gonococcus in GCE was not LPS.

Comparison of the capacities of "T1 phase" GCE and "T3 phase" GCE to inhibit SAGR

The following experiments were designed to investigate whether or not the specific fraction of GCE was unique to virulent phases of gonococci. Four colony types of N. gonorrhoeae have been described by Kellogg, *et al.* (28). Two types (T1 and T2) are virulent and two types (T3 and T4) are avirulent (28). The most obvious difference among the bacteria contained in these different types of colonies is that colony types T1 and T2 contain piliated organisms whereas colony types T3 and T4 contain nonpiliated organisms (25, 52, 60). These characteristics were confirmed by electron microscopy (Figure 18) for the organisms used in this study.

GCE from T1 phase gonococci and GCE from T3 phase gonococci (T3-GCE) were compared in their ability to inhibit SAGR. The results of inhibition studies are shown in Figure 19 and Table 6. The IA_{50} of T3-GCE was 10.0 µg compared to 0.46 µg for GCE. The activity of T3-GCE was only 5% of the activity of GCE. These results indicated that the specific component of GCE was not found in T3 phase gonococci and was, therefore, probably associated with virulent gonococci.

Figure 16. Separation of GCE components by sucrose density gradient ultracentrifugation.

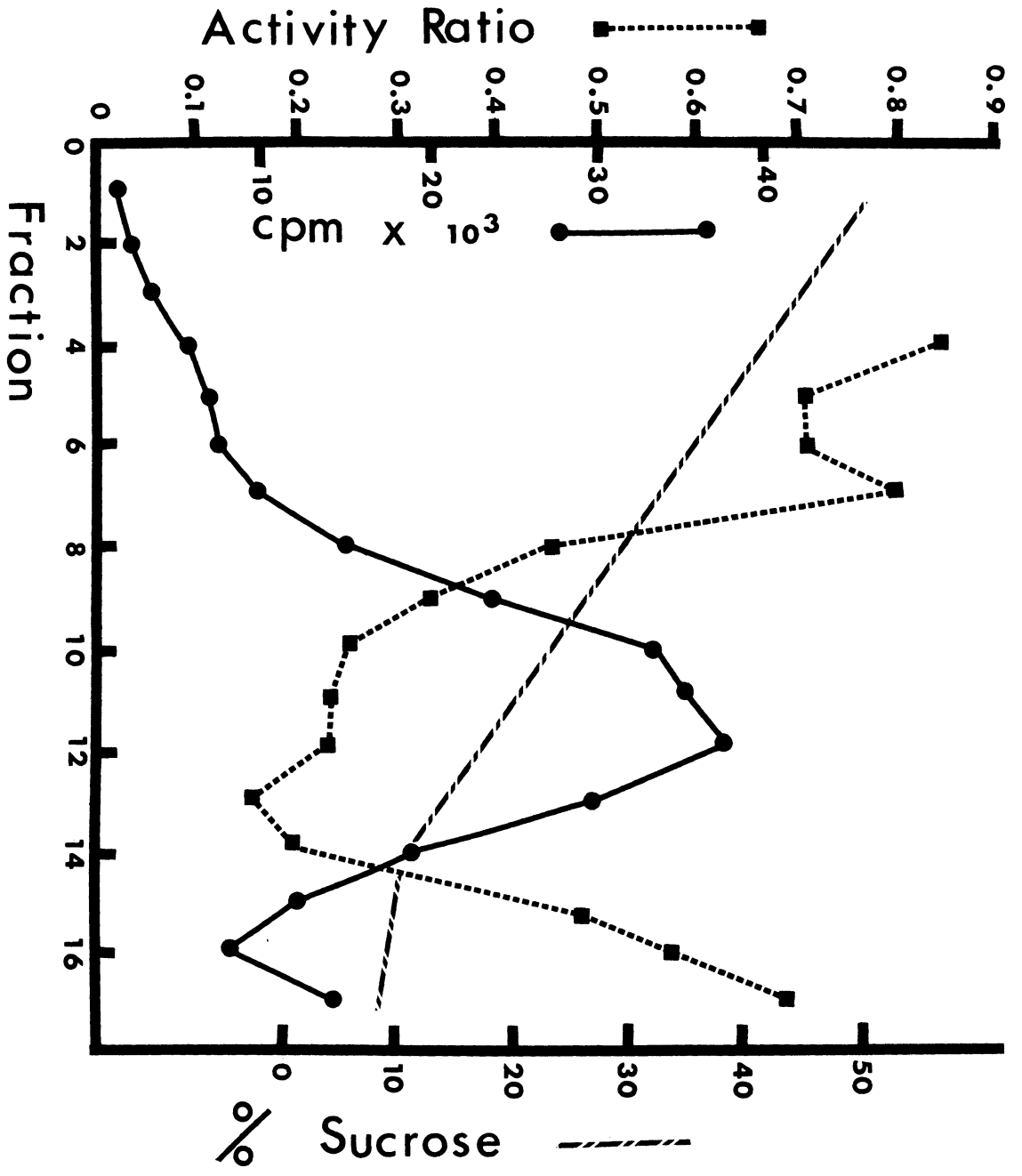

Activity Ratio
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
10
20
30
40
cpm x 10³
Fraction
0
2
4
6
8
10
12
14
16
0
10
20
30
40
50
% Sucrose

Figure 17. Comparison of capacities of GCE and gcLPS extract of T1 phase gonococci to quantitatively inhibit SAGR. GCE, A; gcLPS, B.

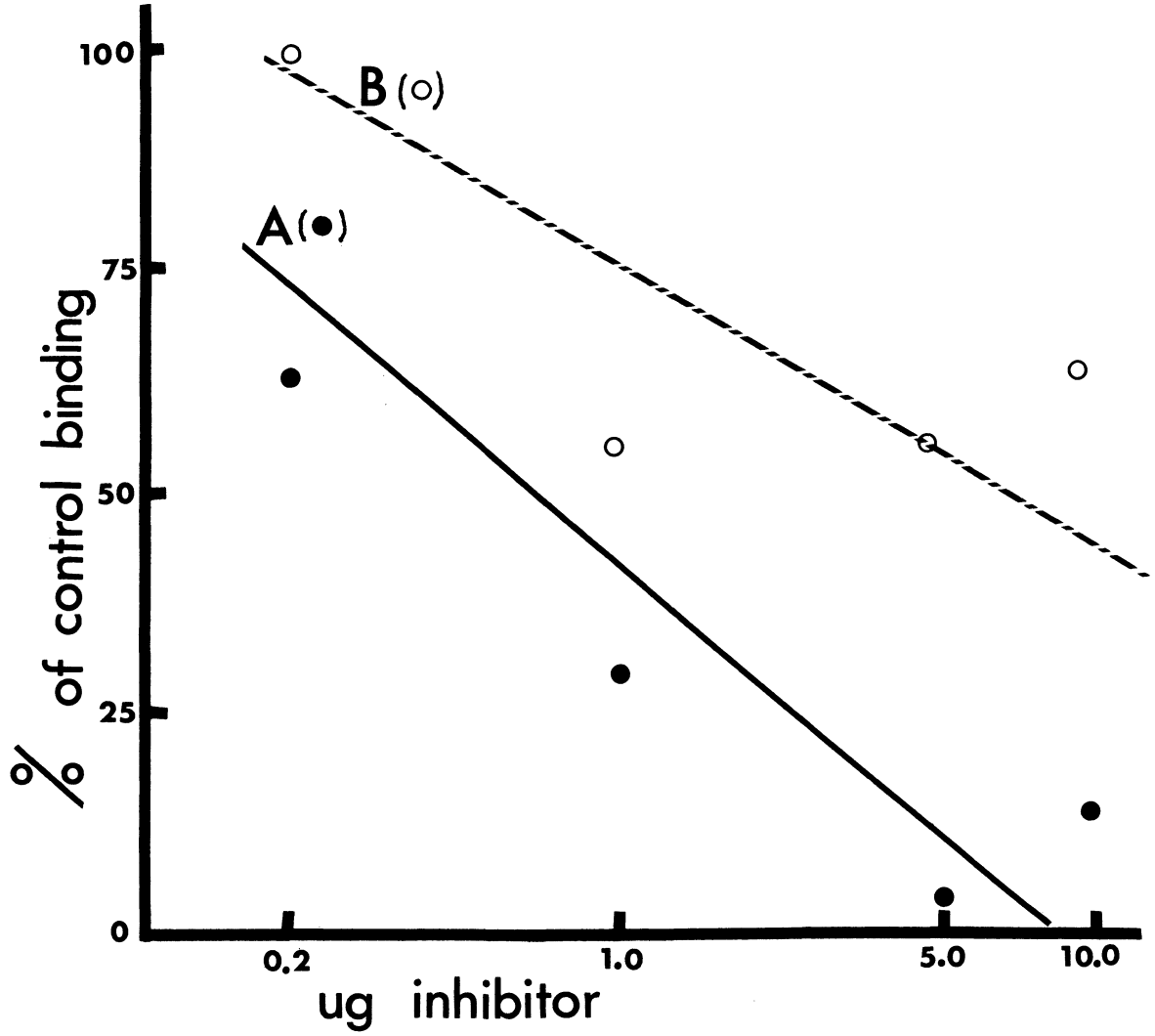

100
75
50
25
0
% of control binding
B (○)
A (●)
0.2
1.0
5.0
10.0
ug inhibitor

Figure 18. Electron micrographs of gonococci from T1 and T3 colony types. Plates A and B show gonococci from T1 colonies; note presence of pili. X 24,000. Plate C shows gonococci from a T3 colony; note absence of pili. X 24,000.

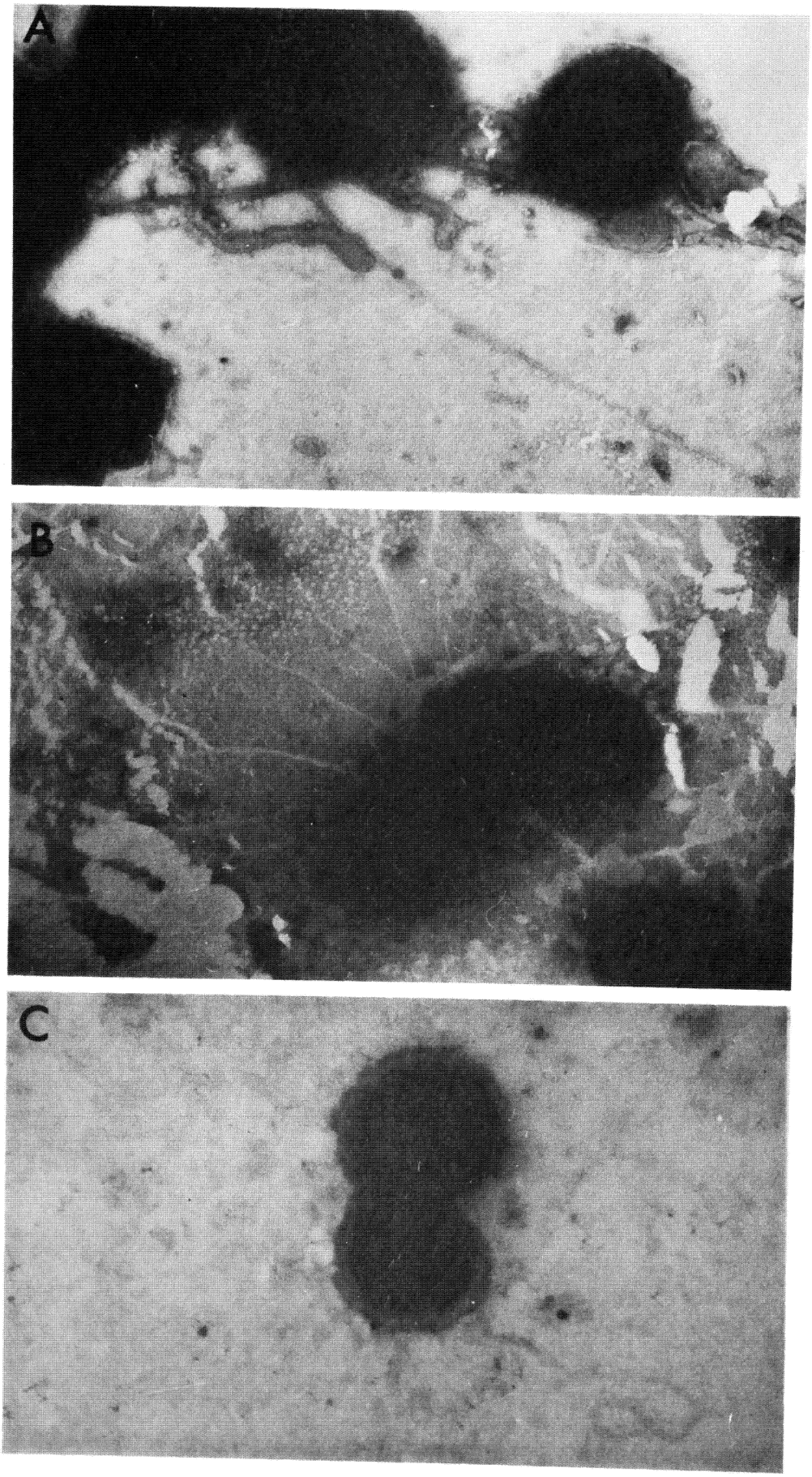

A
B
C

Figure 19. Comparison of T1 phase GCE with T3 phase GCE on quantitative capacity to inhibit SAGR. T1 phase GCE, A; T3 phase GCE, B.

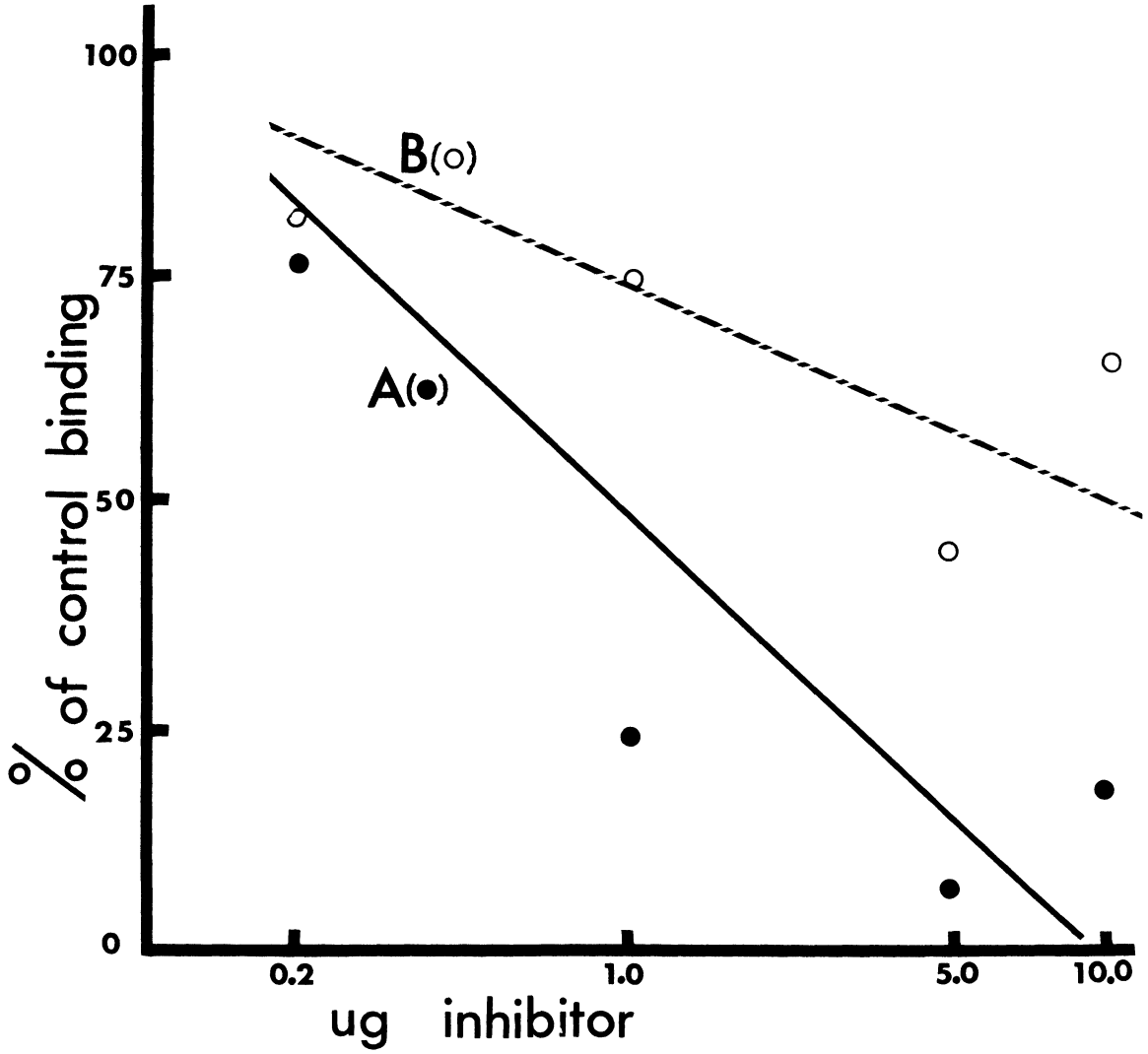

100
75
50
25
0
% of control binding
B(○)
A(●)
0.2
1.0
5.0
10.0
ug inhibitor

Table 5. Summary of effects of enzyme digestion of GCE*[a] on SAGR.

Experiment #	Treatment	Activity ratio[b]		% of control[c]
		Treated	Untreated	
1	DNase	0.633	0.831	76.2
2	DNase	0.633	0.819	77.3
3	RNase	0.792	0.762	103.9
4	RNase	0.789	0.880	89.7
5	Trypsin	9.318	0.822	38.7
6	Trypsin control[d]	0.628	0.822	76.4
7	Pronase	0.328	0.822	39.9
8	Pronase control[d]	0.656	0.822	79.8
9	None	--	0.822	100.0

[a] GCE* = ^{125}I labelled GCE.

[b] $$\text{Activity ratio} = \frac{\text{cpm in ppt - background (BKG) cpm}}{\text{cpm in ppt - cpm BKG + cpm in 100 µl SF - cpm BKG}}$$

[c] $$\% \text{ of control} = 100 \times \left\{\frac{\text{activity ratio treated GCE*}}{\text{activity ratio untreated GCE*}}\right\}$$

[d] Trypsin and pronase control: Enzymes incubated in saline followed by boiling 2 min prior to addition of GCE*.

Table 6. Comparative ability of GCE, T3 Phase GCE, and gcLPS extract of T1 Phase gonococci to quantitatively inhibit SAGR.

inhibitor tested	IA_{50} (μg)[a]	relative activity[b]
gcLPS	6.00	10.8
GCE	0.65	100.0
"T3 Phase" GCE	10.00	4.6
GCE	0.46	100.0

[a] IA_{50} - amount of extract required to achieve 50% inhibition of SAGR.

[b] Relative activity = $100 \times \left(\frac{IA_{50}\ GCE}{IA_{50}\ sample}\right)$

CHAPTER IV

DISCUSSION

In this study an antigen, specific for Neisseria gonorrhoeae which was contained in a crude gonococcal extract, was characterized by a variety of techniques. The crude extract was found to contain mostly protein and substantial amounts of RNA in some preparations. The extract was toxic for chick embryos and caused febrile responses in rabbits. These biological activities were typical of those of purified endotoxin preparations. However, on a weight to weight comparison, GCE had not more than 15% of the biological activity of purified gonococcal endotoxin. The specific gonococcal fraction of GCE was consistent with the properties of a low molecular weight glycoprotein. These low molecular weight molecules could have aggregated spontaneously to form large complexes with molecular weights of 800,000 daltons or more.

The original objectives of this study were to characterize a gonococcal specific antigen which was contained in a crude gonococcal extract. The crude extract, containing a mixture of antigens currently is employed in a radioimmunoassay for the quantification of anti-gonococcal activity of human serum (36). It was hypothesized, based on the extraction procedure and previous work (Rudbach, unpublished data), that the specific fraction of GCE was a protoplasmic polypeptide, glycoprotein, or nucleoprotein. The possibility existed, however, that other cell components,

such as endotoxin or pili, were important in the specific anti-gonococcal reaction (SAGR).

Two general approaches were used in characterizing the antigen. In the first approach chemical analyses were performed for protein, DNA, and RNA; endotoxic activity was determined, and molecular size was estimated. In the second approach GCE was treated by a variety of methods designed to destroy or alter specific materials. GCE so treated was then analyzed for capacity to inhibit SAGR. By this means it was hoped to characterize the specific gonococcal fraction of GCE with respect to activity in SAGR. Any treatment which denatured or destroyed the gonococcal specific fraction of GCE would result in an increase in the amount of antigen required to inhibit by 50% the binding of GCE* with the specific antibody. Conversely, any treatment that increased solubility or exposed more antigenic determinants would result in a decrease in the amount of antigen required for 50% inhibition, reflecting an increase in the capactiy of the treated GCE to inhibit SAGR.

Chemical and biological properties of GCE and their relationship to specific gonococcal antigenic activity

A first step in the process of characterizing the gonococcal specific antigen was to learn something of the overall chemical composition of GCE. This could indicate the direction subsequent investigation should take. By direct chemical analysis it was found that GCE was mostly protein, but that there were amounts of RNA of up to 34% in some preparations. DNA was found to account for less than 1% of the weight of GCE. These were the only chemical analyses performed because of the

small amounts of GCE available. The results of chemical analyses suggested that the gonococcal specific fraction of GCE could be a protein or a nucleoprotein. Although direct analyses for carbohydrate and ester-bound lipid content were not performed, the contribution of these materials to the gonococcal specific antigen was investigated in later experiments.

A variety of evidence suggested that the protein component of GCE was important for the gonococcal specific antigenicity of GCE. It was found that the capacity of GCE to inhibit SAGR was greatly reduced when GCE was digested with either pronase or trypsin. Becasue the capacity of GCE to inhibit SAGR was destroyed when the protein component of GCE was destroyed, it was concluded that a protein moiety was part of the gonococcal specific antigen. In order to add confidence to this interpretation, radio-labeled GCE (GCE*) was treated directly with proteolytic enzymes. When the protein component of labeled GCE was destroyed by digestion with proteolytic enzymes there was a great loss in antigenic activity as indicated by a marked reduction in its binding with antibodies in the standard anti-gonococcal human serum. From these results it was confirmed that a protein moiety was essential to the antigenicicy of GCE.

Because several GCE preparations contained appreciable amounts of RNA, it was of interest to investigate the possible role of nucleic acids in the gonococcal specific antigenic activity of GCE. It was found that GCE retained nearly full capacity to inhibit SAGR following incubation of GCE with RNase or DNase. These results suggested that nucleic acids were not essential for the gonococcal specific antigenic activity

of GCE. In order to add confidence to the interpretation of these results, as was done previously with proteolytic enzymes, radio-labeled GCE was itself incubated with nucleases. GCE* so treated retained most of its origianl specific gonococcal antigenic activity as shown by no decrease, or only a slight decrease, in binding activity. It was concluded on the basis of these results that a nucleic acid component was not essential to the gonococcal specific antigenic activity of GCE.

In addition to the experiments in which enzymatic treatments were used to analyze the contributions of protein and nucleic acid components to the gonococcal specific antigenic activity of GCE, other treatments of GCE were performed to investigate contributions made by putative carbohydrate or lipid components of GCE. The possibility that carbohydrate was part of the specific immunodeterminant group of GCE was investigated by subjecting GCE to oxidation by periodate. It was found that GCE so treated lost much of its capacity to inhibit SAGR. Although other reactions might have taken place, these data were interpreted as indicating that destruction of carbohydrate component reduced the gonococcal specific antigenic activity of GCE.

The possible role of an ester-bound lipid component was investigated by subjecting GCE to saponification with KOH in methanol. It was found that following saponification, the capacity of GCE to inhibit SAGR increased slightly. These results showed that no ester-bound lipid was essential to the gonococcal specific antigenic activity of GCE.

The experiments discussed to this point were designed to investigate contributions to the gonococcal specific antigenic activity of GCE made

by specific classes of biological molecules. It was hypothesized that neither a lipid nor a nucleic acid component was essential. Of the classes of bio-molecules studied only a protein and a carbohydrate component were found to be important to the gonococcal specific antigenic activity of GCE. These results suggested that the gonococcal specific antigenic fraction of GCE was a glycoprotein.

The following experiments represented a change in direction of the approach to characterization of the gonococcal specific fraction of GCE. In these experiments the relationship between GCE and two known gonococcal components was studied. Gonococcal endotoxin (LPS) and pili have received considerable attention from other researchers (8, 17, 38-46, 52, 61) investigating the antigenicity of the gonococcus, and it was necessary to determine the relationship of GCE to these components.

Endotoxin, which is a cell wall component of gram negative bacteria, has been shown to be defined best by its biological activities (48). Two biological properties of endotoxin are its toxicity for chick embryos (15, 16, 59) and its abiltiy to produce a febrile response in rabbits (47, 67). GCE was compared to a preparation of purified gonococcal LPS by these two assays. It was found that on a weight to weight comparison, GCE had not more than 15% of the biological activity of the purified endotoxin. These results were interpreted to indicate that endotoxin represented 15% by weight of the total composition of GCE. If this endotoxic component were in fact the gonococcal specific fraction of GCE then purified gonococcal endotoxin would be expected to have nearly a 10 fold greater capacity than GCE to inhibit SAGR. However, when GCE and gonococcal endotoxin were compared in their capacities to inhibit SAGR, it was found

that endotoxin had only one tenth of the capacity of GCE to inhibit SAGR. It was, therefore, concluded that the specific fraction of GCE was not represented by an antigenic determinant of gonococcal endotoxin.

It has been shown that colonial morphology of cultures of N. gonorrhoeae could be correlated with virulence (28). One physical characteristic that differentiates cells from virulent colony types from cells from avirulent colony types is that virulent cells possess pili (25, 60). In addition, a number of cytoplasmic factors have been described in cells from other colony types (11, 51). It was of interest to investigate the relationship between these "virulence factors" and the gonococcal specific fraction of GCE.

In order to determine if the gonococcal specific antigen was found only in virulent cells, an extract of cells from a colony type representative of avirulent gonococci was compared with GCE (which was prepared from virulent cells). The avirulent cells were shown by electron-microscopy to lack pili. The extract of these cells was designated T3GCE. It was found that T3GCE had less of a capacity to inhibit SAGR than did GCE. This suggested that the gonococcal specific antigen might be virulence associated.

The fact that the gonococcal specific antigen was associated with virulent cells raised the possibility that the antigen was in fact pili or the subunit, pilin. The only evidence obtained relevant to this point was ultra violet absorption data for GCE. GCE was found to have a UV absorption peak only at 260 nm. It is not known if gonococcal pili have UV absorption spectra identical to E. coli type I pili from Escherielia coli; however, Brinton (6, 7) has shown that the latter have UV absorption

peaks at 280 and 285 nm. The difference in absorption spectra between GCE and E. coli pili was considered evidence that pili or pilin were not a major component of GCE.

Physical properties of GCE and their relationship to specific gonococcal antigenic activity

From the experiments discussed previously it was shown that the gonococcal specific fraction of GCE was probably a glycoprotein associated with cells of virulent colony types. The experiments discussed in this section were designed to investigate the molecular size and shape, the state of aggregation or disaggregation, and other physical properties of the gonococcal specific antigenic fraction of GCE.

The heat stability of GCE was one of the first properties investigated. It was found that the capacity of GCE to inhibit SAGR was not reduced by heat treatments of 56°C for 30 min or boiling for 2 min. In several instances, in fact, the capacity of GCE to inhibit SAGR was increased by these treatments. However, if GCE was boiled for longer than 2 min, the capacity to inhibit SAGR was reduced greatly. Denaturation of protein antigens is thought to destroy antigenic specificity by causing unfolding of the peptide chain (cf. 1). The moderate heat stability of GCE was, therefore, considered evidence that the gonococcal specific antigen, already thought to be a glycoprotein, was a small, linear or loosely folded molecule, the antigenic specificity of which would not be altered by further unfolding. If aggregates of the specific antigen formed, moderate heating could have disrupted such aggregates, thereby exposing more antigenic determinants. This could explain the increased

capacity of GCE to inhibit SAGR after GCE was subjected to moderate heating. The fact that prolonged boiling of GCE reduced its capacity to inhibit SAGR was consistent with the notion that the gonococcal specific antigen had an essential protein moiety. Prolonged boiling would be expected to cause coagulation and loss of solubility of the antigen (cf. 1) thereby decreasing the capacity of GCE to inhibit SAGR.

GCE was also tested for stability to treatment with acid and alkali. When GCE was treated with 0.1 N NaOH for 1 h there was no loss of capacity to inhibit SAGR. Treatment with 0.1 N HCl resulted in a slight increase in the capacity of GCE to inhibit SAGR. Acid conditions have been shown to increase the solubility of many proteins (cf. 31). Acid treatment could have, again, disrupted aggregates of a small glycoprotein by increasing the solubility of the subunits. More antigenic determinants could have become available for antibody binding thereby increasing the capacity of a given amount of GCE to inhibit SAGR. The results of these experiments are consistent with the hypothesis that the gonococcal specific antigen was a small glycoprotein.

More direct evidence of the size and shape of the gonococcal specific antigen was obtained when GCE was subjected to fractionation by gel filtration and density gradient ultracentrifugation. GCE was separated into 3 major fractions by molecular sieving on Sephadex G-200. The gonococcal specific portion of GCE was eluted in the void volume. Sephadex G-200 gel excludes molecules of molecular weight greater than 800,000 daltons. It was concluded, therefore, that the specific fraction of GCE exists in a form with a molecular weight equal to or greater than 800,000. It was noted that approximately 30% of the total radioactivity

added to the column was not eluted which suggested that some fraction of GCE binds Sephadex or was otherwise trapped in the gel matrix.

When GCE was fractionated by ultracentrifugation on a gradient of 10% to 50% sucrose, two peaks of high activity were found, one at each extreme of the gradient. This suggested either that the specific fraction of GCE existed in an aggregated, high molecular weight form and as low molecular weight subunits, or that there were two distinct, specific fractions, one of high molecular weight and one of low molecular weight. It was thought that the portion of GCE not eluted from the Sephadex column might correspond to the low molecular weight fraction from the sucrose gradient. In velocity gradient ultracentrifugation molecules sediment at a rate proportional to their size (31). However, a molecule, even of macromolecular dimensions, that had a density less than that of 10% sucrose would not penetrate the gradient. Lipids are the most likely class of bio-molecules to have such low density. It was possible, therefore, that the non-sedimenting fraction of high gonococcal specific activity represented a lipid moiety. However, the fact that the activity of GCE was not reduced by saponification showed that an esterified lipid was not essential for the specific antigenicity of GCE. Therefore, the non-sedimenting fraction of high gonococcal specific antigenic activity most probably represented a low molecular weight moiety.

A model for the structure or GCE

This study has shown that GCE is a heterogeneous mixture of cellular components containing at least one moiety that is antigenically specific for the gonococcus. Fractionation by molecular sieving and

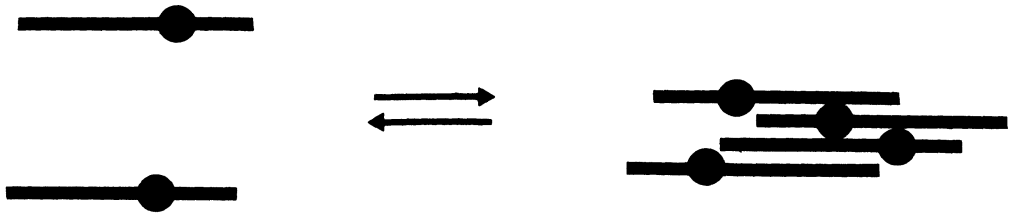
velocity gradient untracentrifugation showed that while GCE contained a wide range of molecular sized, the specific activity was found in only a small portion of the total. Many of the problems encountered in establishing the inhibition assay and in interpreting results may have been attributable to effects of these antigenically non-specific materials.

Because much of the specific antigenic activity of GCE was destroyed by digestion with proteolytic enzymes and by oxidation with periodate, it was felt that the gonococcal specific antigen was a glycoprotein. It was thought that the specific antigen was a large molecule because the high activity fraction of GCE was eluted in the exclusion volume from Sephadex G-200. Other evidence, such as heat stability and position on sucrose velocity gradient untracentrifugation, however, suggested that the gonococcal specific antigen could exist as a small molecule and that the high molecular weight fraction represented an aggregation of these small glycopeptides.

Moderate heat treatment, treatment with mild acid, and saponification are treatments that would be expected to disrupt aggregates of glycopeptides. One effect of disrupting an aggregate of antigenic molecules would be to expose more antigenic determinants which would result in an increase in reactivity of the antigen with antibodies. That these treatments did, in fact, increase this activity of GCE indicated that the gonococcal specific antigen formed large aggregates spontaneously. This view was supported by the demonstration of a fraction with high gonococcal specific antigenic activity in the upper layers of the sucrose velocity gradient untracentrifuge tube.

A schematic representation of the hypothetical structure of the gonococcal specific antigen is shown in Figure 20.

Figure 20. Schematic model of the structure of the gonococcal specific antigen. The polypeptide component of the antigen is indicated by solid black lines; the carbohydrate component is indicated by solid black circles. Integral to this model is the ability of the monomers to spontaneously aggregate into micellar bundles.



Monomers

Aggregate

Relationship of GCE to other known gonococcal antigens

Because there already was much known about antigens of the gonococus it was of interest to compare the properties of GCE with the properties of antigens reported by others. Antigens reported by others include cell wall extracts (17, 38-46, 61), pili (8, 52), and protoplasmic antigens (2, 3, 11, 13, 29, 30, 51, 54, 58). GCE was found to share some characteristics with some of these antigens. However, because characterization of these antigens and GCE has been incomplete, the exact relationship between GCE and other reported antigens could not be determined precisely.

Maeland (39-41) has extracted gonococcal endotoxin with phenol-water and aqueous ether. He found two antigenic determinants designated α and β. Determinant α was found to be a polysaccharide reactive in complement fixation tests (39). This antigen showed some degree of specificity for N. gonorrhoeae. The β determinant was proteinaceous in nature and also reacted in complement fixation tests but was found to cross react extensively with other Neisseria species (39). The β determinant has been characterized further as an acidic glycoprotein (3). Because endotoxin was found to have no importance in the specificy of GCE, there is probably little relationship between these antigens and GCE.

Buchanan *et al.* (8) have worked with gonococcal pili in a radioimmunoassay for the detection of antibodies against pili antigens in sera of human patients. They have demonstrated considerable serological specificity with this assay. Recently, Novotny and Turner (50) have shown that the antigens of gonococcal pili are highly strain (sic) specific. They investigated 31 strains of N. gonorrhoeae by gel diffusion

against antisera raised against pili of 3 strains. They found few reactions of identity between strains. These results cast some doubt on the usefulness of gonococcal pili as diagnostic antigens.

Although the presence of pili on the cell surface has been shown to be the major morphological feature distinguishing virulent gonococci from avirulent gonococci (25, 60), pili are not the only distinguishing feature. Cheng *et al.* (11) have isolated from protoplasm of cells from colony type T1 a toxic, heat labile polypeptide, designated B, that was not found in protoplasm of cells from colony type T3 of the homolgous isolate. Also, Pierce *et al.* (51) isolated a polypeptide unique to virulent cells that was shown by gel diffusion to be distinct from LPS, pili, and the B antigen described by Cheng. This polypeptide was designated Ag-1. Both the B antigen and Ag-1 were eluted in the exclusion volume from Sephadex G-200, and both were destroyed by proteolytic enzymes (11, 51). The active fraction of GCE appears to be similar to Ag-1 and the B antigen in as much as all are eluted in the exclusion volume from Sephadex G-200, all are destroyed by proteolytic anzymes, and all are associated with cells from T1 colony types. Also, GCE possessed toxicity for chick embryos similar to that of the B antigen. Toxicity of Ag-1 was not determined (51). However, the active fraction of GCE could not be identified completely with Ag-1 or the B antigen at this time.

Another antigen that may bear some relation to GCE was characterized by Danielsson *et al.* (13). They passed gonococcal protoplasm through Sephadex G-200 and found a component in the first half of the first elution peak that reacted in complement fixation tests with sera of 20% of males and 73% of females who were culturally positive for N. gonorrhoeae.

No positive reactions were found in negative controls (54). Because this antigen was not characterized biochemically its relationship to the specific fraction of GCE cannot be determined.

Gonococcal ribosomes have been used with promising results as skin test antigens in guinea pigs (26). However, studies employing hyperimmunized animals have been of questionable value when applied to the human system because of the minimal immunological response elicited by the natural infection in humans. However, in other systems (cf. 26) ribosomes have proved to provide highly specific antigens. Also, some preparations of GCE did have a high RNA content. Therefore, the possibility that gonococcal ribosomes had a role in the antigenicity of GCE was considered. It was found that treatment with RNase had no effect on the specific activity of GCE which suggested that intact ribosomes were not essential for the antigenicity of GCE. However, the possibility that the specific gonococcal fraction of GCE was a ribosomal protein could not be excluded.

Much more work remains to be done before the specific antigens of the gonococcus will be fully characterized. This study has contributed to that process and information was obtained which will help to refine an assay for the detection of gonococcal specific antibodies in human sera.

CHAPTER V

SUMMARY

An antigen specific for Neisseria gonorrhoeae contained in an ethanol precipitate of a surfactant extract of T1 phase gonococci was characterized by a variety of techniques. A limited chemical analysis indicated that the crude extract (GCE) was mostly protein but that some preparations contained up to 37% RNA. The extract was biologically active, in that it had 1/20 and 1/7 the potency of purified gonococcal LPS in assays of chick embryo lethality and pyrogenicity in rabbits, respectively.

The major portion of the project was devoted to investigating the chemical, physical, and biological properties of GCE and their relation to specific gonococcal antigenic activity. The model system employed human sera from patients naturally infected with N. gonorrhoeae. Gonococcal specific antibodies in the human serum were detactable with a radioimmunoassay employing ^{125}I labeled GCE (GCE*). This specific antigonococcal reaction (SAGR) could be inhibited by the addition of cold GCE. Many properties of the gonococcal specific antigen were investigated by subjecting cold GCE to various treatments and then assaying this treated GCE for capacity to inhibit SAGR. It was found that gonococcal specific antigenic activity was increased by the following treatments: 1) heating at 56°C, 1 h; 2) treatment with 0.1 N HCl, 30 min, 25°C; 3) saponification by 0.1 N KOH in methanol. Gonococcal specific antigenic activity was decreased by the following treatments: 1) heating 5 min in a boiling water

bath; 2) oxidation by periodate; 3) enzymatic digestion with trypsin or pronase. It was found that gonococcal specific antigenic activity was unaffected by the following treatmens: 1) heating 2 min in a boiling water bath; 2) treatment with 0.1 N NaOH, 30 min, 25°C; 3) enzymatic digestion with DNase or RNase. It was found that a GCE preparation from non-piliated gonococci of T3 phase colony types lacked the gonococcal specific antigenic activity exhibited by GCE preparations from piliated bacteria of T1 phase colonies. It was also found that gonococcal LPS lacked the gonococcal specific antigenic activity exhibited by GCE.

The molecular size of the gonococcal specific antigen was investigated by fractionating labeled GCE by molecular sieving on Sephadex G-200 and by velocity gradient ultracentrifugation. Results of both fractionation methods revealed that gonococcal specific antigenic activity was contained in a high molecular weight fraction. In addition, a low molecular weight fraction exhibiting gonococcal specific antigenic activity was recovered from the top of the sucrose gradient.

A model for the structure of the gonococcal specific antigen was proposed to account for the above findings. It was hypothesized that the gonococcal specific antigen was a low molecular weight, linear or loosely folded, glycoprotein. Large, apparently stable, aggregates appeared to occur spontaneously.

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