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Bacterial receptor sites for uptake of transforming DNA

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BACTERIAL RECEPTOR SITES FOR UPTAKE OF TRANSFORMING DNA

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INTRODUCTION

Bacterial transformation is defined as a mechanism of genetic exchange whereby a population of bacteria can obtain genetic information as a result of cellular uptake and integration of extracellular deoxyribonucleic acid released from other bacteria by natural or induced lysis. In order for a transformable strain of bacteria to take up DNA and to undergo transformation, the cells must be in a physiological state called competence. A cell is referred to as competent if it has the ability to bind extracellular DNA irreversibly and subsequently to integrate and express the DNA.

The early experiments in transformation laid the foundation that showed DNA to be the hereditary material. Today, transformation is a valuable research tool which gives us a method of correlating the effects of physical and chemical alterations in the DNA molecule with its biological activity. Transformation is an important genetic mechanism allowing gene mixing in populations of asexual haploid cells. It is one of the known ways by which a continual gene flow is maintained in a species of bacteria.

The details of transformation or, more specifically, the competent state *vs.* non-competent state of a cell is poorly understood (Schaeffer, 1964), but competence is thought to involve the development of active DNA uptake sites located on or near the cell surface (Hotchkiss, 1954; Barnhart and Herriott, 1963). Justification of the research presented in this thesis is based on the lack of present-day knowledge

surrounding the existence and nature of such DNA uptake sites on *Haemophilus influenzae*.

There were two objectives to this research. First, to demonstrate the existence on competent *H. influenzae* of specific antigenic sites that stimulate the production of antibody inhibitory to the irreversible binding of DNA and transformation. Second, if such sites were demonstrated, they could be fractionated by centrifugation and column chromatography to identify their cellular location. The objectives of this research were achieved. Competent *H. influenzae* cells did stimulate production of antibody that was inhibitory both to the irreversible binding of transforming DNA and to drug-resistant transformation. Using antibody absorption as the assay, the antigens specific for anti-DNA binding serum were found in the soluble portion of disrupted cells, while the antigens specific for anti-transforming serum remained attached to cellular fragments.

The results and conclusions of the study are specifically applicable to *H. influenzae* since it was the transformation system investigated; however, some conclusions and interpretations can be generalized to shed new light on the phenomenon of genetic transformation. The antigenic materials isolated from these bacteria are related to the binding and uptake of DNA insofar as they block antibody inhibitory to binding and transformation. While these antigens are apparently involved in the uptake of DNA for transformation, they may only be a part of the uptake site or represent part of a functional component involved in transporting DNA into the cell. Elucidation of all the components of DNA uptake is left to future research.

LITERATURE REVIEW

In 1928 Griffith unknowingly observed bacterial transformation while doing experiments with *Diplococcus pneumonia*. Since Griffith's primary interests were in epidemiology, he did not pursue transformation. In 1931 Dawson and Sia, working along the same lines as Griffith, extended transformation experiments from mice to the test tube. Two years later Alloway (1933) used cell-free extracts of pneumococcus as the transforming principle to transform recipient cells. Due to lack of interest and understanding, the problem was ignored until 1944 when Avery, MacLeod, and McCarty, at the Rockefeller Institute, isolated the transforming principle and identified it as deoxyribonucleic acid.

Transformation is not unique to pneumococcus. It has been reported in *H. influenzae* (Alexander and Leidy, 1951), in species of *Neisseria* (Alexander and Redman, 1953; Catlin and Cunningham, 1961), in *Bacillus subtilis* (Spizizen, 1958), and in many other species (Ravin, 1961). It soon became evident to investigators that the original system of transformation was not the system of choice. There were many pitfalls to getting reproducible transformation with pneumococci, and it was very unlikely that the frequency of capsular transformation could be determined with quantitative precision (Hotchkiss, 1957). To improve the basis of selection, investigators turned to drug-resistance (Hotchkiss, 1951; Hotchkiss and Evans, 1958), sugar fermentation (Austrian and Colowick, 1953), enzyme synthesis, sporulation, antibiotic dependence, specific protein synthesis, and some other genetic markers

(Ravin, 1961).

It became desirable to get a high percentage of cells to transform in a culture. Investigators soon found that the level of transformation obtainable was dependent on the frequency of competent cells in the culture. Even though competence was somehow under genetic control (Young and Spizizen, 1961), it was also a physiological condition and could be induced or suppressed by controlling the physiological state of the cell culture. By using various incubation conditions and defined culture media, the percentage of competent cells in a transformation culture could be enhanced (Spizizen, 1958; Spencer and Herriott, 1965).

The development of the competent state was found to be sensitive to metabolic inhibitors and specifically to inhibitors of protein synthesis (Fox and Hotchkiss, 1957; Stuy, 1962; Spencer and Herriott, 1965). This, along with DNA competition experiments (Hotchkiss, 1954) and experiments showing that binding of radioactive DNA is sensitive to pH, divalent cations, and ionic strength (Barnhart and Herriott, 1963), suggested that specific receptor sites might be produced by competent cells for the uptake of DNA. Extracellular competence factors of cellular origin were found to be necessary in pneumococci, streptococci, and *H. influenzae* for these cells to develop subsequent functional DNA uptake mechanisms (Tomasz and Hotchkiss, 1964; Pakula and Hauschild, 1965; Barnhart, 1967). Further evidence of specific receptor sites came from finding specific antigens on competent pneumococci, streptococci, and *H. influenzae* that evoked production of antisera inhibitory to irreversible DNA binding and transformation (Nava,

Galis, and Beiser, 1963; Pakula, 1965 and 1967; Tomasz and Beiser, 1965; Bingham and Barnhart, 1970). The production of inhibiting antibody was unique to the competent state, since no inhibition was detected from antisera prepared against non-competent cells.

Proteins involved in active transport across the cell membrane have been isolated [e.g., proteins binding β-galactoside, sulfate, calcium, galactose, and neutral amino acids (Pardee, 1968)]. These transport systems share three characteristics. First, each is specific for a particular molecule. Second, the initial rate of substrate entry depends on substrate concentration which indicates a limited number of uptake sites. Third, the transport is dependent on the cell's energy production (Stein, 1967). Likewise, the hypothesis of active transport sites for DNA uptake meets the criteria established by the above-mentioned mediated transport systems. First, uptake is specific for native DNA (Lerman and Tolmach, 1957 and 1959; Barnhart, 1965) and size of the DNA (Litt et al., 1958; Barnhart and Herriott, 1963; Cato and Guild, 1968), and in *Haemophilus* the specificity extends to homologous or closely related DNA (Schaeffer, 1964). Second, there appears to be a limited number of uptake sites on competent bacteria (Fox and Hotchkiss, 1957; Barnhart and Herriott, 1963). Third, the DNA uptake depends on the cell's energy production (Fox and Hotchkiss, 1957; Barnhart and Herriott, 1963; Young and Spizizen, 1963). It is deemed theoretically possible, therefore, to find specific molecules on competent bacteria mediating DNA uptake.

MATERIALS AND METHODS

Terms having special meanings in the field of bacterial transformation are defined in Section A of the Appendix.

Experimental Organisms

Haemophilus influenzae type Rd was originally isolated by Alexander and Leidy (1953). The Rd used in this laboratory was originally obtained from Dr. Roger M. Herriott at Johns Hopkins University. It is a gram negative rod (1.0-1.5 μm x 0.3 μm) of the rough (R) variety and serological type d. It is a facultative anaerobe which requires hemin and nicotinamide adenine dinucleotide (NAD) for aerobic growth in addition to a number of nutritional factors. *Haemophilus influenzae* BC200 is an ultraviolet- (UV) resistant mutant of Rd. It was originally isolated by Barnhart and Cox (1968) and has the same general characteristics and nutritional requirements as described for Rd.

Culture Techniques and Stock Preparation

Rd cells were incubated aerobically at 37°C in 50 ml of HI medium: 2.5% Difco heart-infusion broth supplemented with 2 μg/ml of NAD and 10 μg/ml of hemin. The culture was contained in a 500-ml nephelo flask fitted with a 22-mm glass tube side arm (Bellco) to allow spectrophotometric measurements without transferring the culture from the flask. The cells were grown to an optical density of 1.0 (2 x 10^9 viable centers/ml) as measured at a wavelength of 650 nanometers

(nm) on a Bausch and Lomb Spectronic 20 spectrophotometer. Glycerin was added to the culture (17.5%, v/v), and the cells were stored in vials (1.5 ml/vial) at -86°C.

BC200 was grown the same way as Rd except 25 ml of Ca-BHI medium (3.0% Difco brain-heart infusion broth supplemented with 2 µg/ml of NAD, 10 µg/ml of hemin, and 1 mM of $CaCl_2$) was used in place of 50 ml of HI medium. The BC200 stock was stored the same way as the Rd stock only in Ca-BHI medium rather than in HI medium.

Preparation of Competent Bacteria

Spencer's technique. One and four-tenths ml of thawed *H. influenzae* stock culture were added to 50 ml of HI medium contained in a 500-ml nephelo flask. The culture was grown aerobically (aerating at 100 1" strokes/minute on a Research Specialties Co. reciprocal shaking water bath) at 37°C. The optical density of the growing culture was measured at 30-minute intervals in a Bausch and Lomb Spectronic 20 spectrophotometer set at a wavelength of 650 nm. When the optical density reached 0.5 (1×10^9 viable centers/ml), the mixture was centrifuged for 10 minutes at 1000 x gravity (*g*) in an IEC clinical centrifuge fitted with an angle head. The cells were resuspended in 55 ml of M-II medium (Spencer and Herriott, 1965) and incubated at 37°C while aerating at 80 1" strokes/minute. After incubating 2 hours in M-II medium the cells were competent and remained so for about 3 hours (Spencer and Herriott, 1965).

Competence in Ca-BHI. BC200 was usually made competent using

Goodgal and Herriott's (1961) technique as modified by Barnhart and Herriott (1963). One ml of BC200 overnight culture was added to 25 ml of Ca-BHI medium contained in a 500-ml nephelo flask. The culture was grown aerobically (with shaking) to an optical density of 0.5, incubated anaerobically (without shaking) for 1 hour, and finally grown aerobically for 0.5 hour. The cells were considered competent and used for transformation or transfection. Cultures made competent using this method had one-fifth to one-tenth the frequency of transformation compared to those made competent using Spencer and Herriott's (1965) technique.

Preparation of Non-Competent Cells

The same procedure was used to prepare non-competent cells as was used to prepare competent cells, except a final concentration of 10 µg/ml of L-valine was added to the M-II medium (Spencer and Herriott, 1965). Non-competent cells prepared in this way had a frequency of transformation of 100- to 1000-fold less than competent cells.

Preparation of Transforming DNA

Nb_1-DNA. Transforming DNA bearing the Nb_1 marker (resistance to 2.5 µg/ml of novobiocin) was prepared by a procedure similar to the one used by Goodgal and Herriott (1961).

Novobiocin-resistant (2.5 µg/ml) *H. influenzae* type Rd were grown to 2×10^9 viable centers/ml in 2 liters of BHI medium supplemented with 2.4 µg/ml of novobiocin. The cells were centrifuged out of

the broth at 2065 x *g* for 50 minutes on a Mistral 6L centrifuge, resuspended in 20 ml of 0.05 M Na_3 citrate and 0.15 M NaCl (citrate-saline), and incubated at 37°C for 10 minutes. The bacteria were lysed by adding a 2:1 mixture of 1 M NH_4OH and 1 M NaOH to the suspension until the pH was 11.0. The solution was immediately neutralized with 1 M KH_2PO_4, and NaCl was added (1.17 g/10 ml of lysed cells) until the solution was 2 M.

The lysed cell suspension were deproteinized by the "Sevag" procedure (Sevag *et al.*, 1938). An equal volume of chloroform and octanol in a 9:1 ratio was added to the lysed cells, and the mixture was shaken on a wrist-action shaker for 15 minutes and centrifuged at 1000 x *g* in a clinical centrifuge for 15 minutes. The aqueous portion was removed and saved. The protein-chloroform gel was shaken with 20 ml of 2 M NaCl for 15 minutes and centrifuged, and the aqueous portion was saved and added to the first aqueous extraction. The combined aqueous supernatant fluids were mixed with an equal volume of the chloroform-octanol mixture, shaken for 15 minutes, and centrifuged. The aqueous phase containing the nucleic acids was saved.

The DNA and RNA from the aqueous phase were precipitated by slowly adding a two-thirds volume of 95% cold ethanol while stirring and collecting the precipitating nucleic acids on a stirring rod. The precipitated DNA and RNA were quickly resuspended in saline containing 10 µg/ml of pancreatic ribonuclease [RNase (Worthington)] and allowed to incubate overnight at 37°C.

The following day NaCl was added to the mixture to a final concentration of 2 M, and the solution was centrifuged at 7700 x *g* on

a RC2-B Sorval centrifuge to remove any insoluble material from the solution. The supernatant fluid was shaken with an equal volume of chloroform-octanol mixture and centrifuged to remove residual protein from the solution. This "Sevag" procedure was repeated until all the visible protein-chloroform gel had disappeared. The DNA was precipitated from the aqueous phase with a two-thirds volume of 95% cold ethanol, collected on a stirring rod, and resuspended in saline.

The optical density of the DNA solution was measured at 230, 260, and 280 nm wavelengths on a Beckman DU-2 spectrophotometer. The ratios of optical densities should be the following: $\frac{260}{230} = 2.4$ and $\frac{260}{280} = 1.8$. If these ratios were off by more than 0.2-0.3, it indicated the presence of RNA and protein, and the "Sevag" procedure was then repeated.

^{14}C-Labeled Nb_1-DNA. The ^{14}C-labeled transforming DNA bearing the Nb_1 marker was prepared from novobiocin-resistant (2.5 µg/ml) *H. influenzae* grown in M-Ic medium (Herriott *et al.*, 1970) containing 2.4 µg/ml of novobiocin and 0.25 µc/ml of thymidine-2-^{14}C. The DNA was isolated the same way as was described for Nb_1-DNA (Goodgal and Herriott, 1961). The ^{14}C-Nb_1-DNA was subsequently dialyzed for 48 hours against 250 volumes of 0.05 M citrate-saline to remove small ^{14}C-labeled material.

In the second preparation the ^{14}C-labeled Nb_1-DNA was prepared the same way except the cells were initially grown in BHI medium containing 2.4 µg/ml of novobiocin, 0.3 µc/ml of thymidine-2-^{14}C, and 2000 µg/ml of inosine (Carmody and Herriott, 1970).

Transformation Assay

Competent cells were treated with 10 μg/ml of L-valine to stop further development of competence (Spencer and Herriott, 1965). Transformations were done by adding 0.9 ml of L-valine treated competent cells to 0.1 ml of 20 μg/ml Nb_1-DNA or ^{14}C-Nb_1-DNA and gently shaking the mixture for 30 minutes at 36°C. One-tenth ml of 1 mg/ml pancreatic deoxyribonuclease [DNase (Worthington)] in 0.1 M $MgCl_2$ was added and incubated with the cell-DNA mixture for 5 minutes. The culture was diluted to 10^{-5} to measure transformed cells and to 10^{-7} to measure viable centers. One-ml samples from each dilution were added to duplicate Petri plates. Fifteen ml of molten Difco brain-heart infusion agar supplemented with 2 μg/ml of NAD and 10 μg/ml of hemin (BHI agar) were added to those plates used to score viable centers, and 10 ml of BHI agar were added to plates where Nb_1-transformed cells were scored. All plates were incubated in a 37°C Enviratech warm room for 2 hours, after which those plates used for scoring Nb_1-transformed cells were overlayed with 10 ml of BHI agar containing 4.8 μg/ml of novobiocin. The plates were incubated at 37°C for 18-20 hours before counting the colonies.

Assay for Irreversibly Bound ^{14}C-Labeled DNA

After DNase treatment of a 1.0-ml transformation culture, a 0.1-ml sample of cells was assayed for the number of cells transformed to the Nb_1 marker. The remainder of the culture was centrifuged and washed three times with 1.0 ml of saline and resuspended after the last

wash in 0.5 ml of saline. A 0.1-ml sample was dried and counted on a filter paper disc using 10 ml of toluene scintillation fluid [32 g of 3,5-diphenyloxazole; 0.8 g of 1,4-bis-2(5-phenyloxazolyl)-benzene; and 16 pints of toluene]. Each sample was counted for 10 minutes in a Packard Model 3320 Tri-Carb scintillation spectrometer. The ^{14}C-Nb_1-DNA had a specific activity of 5000 counts/minute/μg of DNA.

In the second preparation, the ^{14}C-Nb_1-DNA had a specific activity of 6800 counts/minute/μg of DNA. In experiments using the second ^{14}C-Nb_1-DNA preparation, the transformed cells were resuspended in 1.0 ml of distilled water after the last saline wash. A 0.5-ml sample was taken and counted in Aquasol (New England Nuclear). The counting efficiency was about the same for both systems.

Preparation of HP1C1 Phage Stock

The HP1C1 phage was originally obtained from Harm and Rupert (1963). BC200 cells were grown to 4 x 10^8 viable centers/ml in Ca-BHI medium. One ml of the culture was added to 5 ml of HP1C1 phage (10^8 pfu/ml) suspended in the same medium. After incubating 15 minutes at 37°C, the culture was diluted 10-fold in Ca-BHI medium and grown at 37°C for 150 minutes. The whole cells were centrifuged out of suspension, and the supernatant fluid was centrifuged at 10,000 x *g*. The supernatant solution from the last centrifugation was stored at 2°C and used as HP1C1 phage stock.

Phage Titration

Solutions to be titrated for HP1C1 phage were diluted, and 0.2 ml of each dilution was added to 1.8 ml of Ca-BHI medium containing 2×10^8 BC200 cells/ml. The mixtures were incubated for 30 minutes at 37°C, and 1.0 ml of each mixture was mixed with 3.0 ml of soft melted Ca-BHI agar. The soft agar tubes containing the phage-infected cells were poured into Petri plates containing 10 ml of solidified Ca-BHI agar. The plates were incubated at 37°C for 18 hours. The plaques were counted and scored as plaque-forming units (pfu) of HP1C1 phage.

Preparation of HP1C1 Phage DNA

DNA was isolated from the HP1C1 phage using the technique of Mandell and Hershey (1960) as applied to HP1C1 phage by Harm and Rupert (1963). Fifty ml of BC200 cells (2×10^9 viable centers/ml) grown in Ca-BHI medium were added to 100 ml of stock HP1C1 phage (6×10^8 pfu/ml) and incubated at 37°C for 10 minutes. One liter of Ca-BHI medium was added to the infected bacteria, and the culture was incubated for 150 minutes at 37°C under gentle aeration. The culture was centrifuged to remove the whole cells and debris. Two mg of DNase and RNase were added to the phage culture and incubated for 1 hour at 37°C. The phage were centrifuged out of the suspension at 23,000 x *g* for 4 hours and resuspended in 8.0 ml of 0.01 M phosphate-buffered saline, pH 7.0, containing 0.01 M ethylenediaminetetraacetic acid (EDTA). The phage suspension was chilled, added to cold buffer-saturated phenol, and shaken for 2 minutes. The aqueous fraction was saved and washed five times

with 8.0 ml of cold ether to remove the phenol. The remaining ether was removed by bubbling air through the solution. The phage DNA was assayed for transfection and stored at 2°C.

Transfection Assay

One ml of cells made competent in Ca-BHI medium was added to 0.2 ml of HP1C1 DNA stock in 0.8 ml of calcium-supplemented (1 mM $CaCl_2$) eugonbroth. The cells were incubated with the phage DNA at 36°C for 30 minutes (1 hour when samples were not plated) and diluted, and 0.1 ml was added to 2.5 ml of soft Ca-BHI agar containing 2 x 10^8 indicator cells. The transfected cells were mixed into the agar and poured into Petri plates containing 10 ml of hard Ca-BHI agar. The plates were incubated at 37°C for 18 hours, and the plaques were counted and scored as transfected cells.

Isolation of HP1C1 Phage-Resistant Mutants

BC200 was used to isolate a phage-resistant mutant because of its UV-resistant properties. The BC200 strain was grown to 10^9 viable centers/ml and treated for 10 minutes with 15 µg/ml of the mutagen N-methyl-N'-nitro-N-nitrosoguanidine [NG (Aldrich Chemical Co.), Adelberg, Mandel, and Chen, 1965]. The cells were washed, resuspended in Ca-BHI medium, grown for 90 minutes, and infected with a multiplicity of 1-2 pfu/cell of HP1C1 phage. The infected bacteria were incubated for 65 minutes, centrifuged, resuspended in UV buffer (Setlow *et al.*, 1968), diluted 10-fold in UV buffer, and UV-irradiated with 100 ergs/mm^2 to induce lysogenic cells. The irradiated bacteria were diluted and

surface-plated for viable centers. After growing for 16 hours at 37°C, the 10^{-4} and 10^{-5} dilution plates were replica-plated onto Ca-BHI agar plates spread with 8.9 x 10^{9} pfu of HP1C1 phage/plate. The replica plates were incubated for 2 hours at 37°C and UV-irradiated with 250 ergs/mm^2 to induce lysogenic cells. The plates were put back in the warm room (37°C) for 18 hours. Colonies were picked from the replica plates the next day and assayed for phage infectivity. Twenty-six clones of BC200 were isolated that were resistant to phage infection. Mutant number 1 was tested and found to be non-lysogenic and suspectible to transfection. It was subsequently used for isolation of low-transformation frequency mutants.

Selection of Low-Transforming Mutants

Mutant number 1 of the phage-resistant mutants was grown to 10^{9} viable centers/ml. Three ml were treated for 10 minutes with 15 µg/ml of NG, washed, and resuspended in Ca-BHI medium. These cells were made competent, and 1.0 ml was transfected with HP1C1 DNA. The transfected culture was again made competent in Ca-BHI medium. One ml was transfected, centrifuged, resuspended in UV buffer, and UV-irradiated with 250 ergs/mm^2. The selection procedure above was repeated to enrich the frequency of low-transforming mutants. After the last UV irradiation the culture was streaked onto Ca-BHI agar Petri plates, and the next day 50 isolated colonies were picked. Each isolated colony was made competent in 2.0 ml of Ca-BHI medium, transformed with Nb_1-DNA for 30 minutes, diluted, plated, and checked for frequency of transformation.

Four of the isolated colonies had low frequencies of transformation: numbers 27, 33, 37, and 47. Number 27 was grown in Ca-BHI medium, treated with NG, washed, made competent in Ca-BHI medium, and transfected. The culture was streaked onto Ca-BHI agar and incubated at 37°C for 18 hours. The next day 77 isolated colonies were picked, made competent, and transformed with Nb_1-DNA. From the 77 isolates, three mutants were selected that had lower frequencies of transformation than mutant number 27.

Preparation of Antisera

Equal volumes of 0.4% formalized saline (0.15 M NaCl) were added to both competent and non-competent cultures of *H. influenzae* type Rd prepared in M-II medium. The formalin-treated cultures were incubated overnight at room temperature, centrifuged, resuspended, concentrated 4-fold in 0.1% formalized saline, and stored at 4°C until used for injections.

Twelve-week-old New Zealand White male rabbits were injected intravenously with 2.0 ml of formalin-treated cells three times a week for 6 weeks or until the antibody titers were equal to or greater than 2500. The rabbits were bled 6 days after the last injection by cardiac puncture, and the sera were separated and stored at -20°C. Before using the antisera in transformation experiments, the gamma globulin fractions were isolated by the technique described by Strauss *et al.* (1960):

> The serum pools were diluted with equal volumes of sterile 0.85% NaCl, and to each 10 ml of serum saline mixture 2.04 g of anhydrous sodium sulfate were added. After standing at room temperature for 2 hours or more, the suspensions were centrifuged

> and precipitates dissolved in volumes of distilled water equal to two-thirds of the initial volumes of the diluted serum pools. The globulins were reprecipitated by adding 1.93 g of sodium sulfate for each 10 ml of distilled water added. The suspensions were again centrifuged, and a third precipitation was carried out in the same manner. Final precipitates were dissolved in a volume of water equal to the initial volume of serum and dialyzed against running cold tap water until the first appearance of reprecipitation; then dialysis was continued against 0.9% NaCl with mechanical stirring overnight at 4-6°C.

The resulting globulin solutions were then filtered through a 0.25-μm Millipore filter and stored at 2°C until used.

Antiserum Assay

Only the gamma globulin fractions were used in experiments where antiserum effects were studied. When testing the effect of antiserum on transformation and binding of transforming DNA, 0.9 ml of competent cells (made competent in M-II medium) was added to 0.1 ml of the desired antiserum dilution and incubated for 30 minutes. After treating the competent cells with antiserum 2 μg of Nb_1-DNA or ^{14}C-Nb_1-DNA were added, and the mixture was incubated for another 30 minutes followed by a 5-minute exposure to 0.1 ml of 1 mg/ml DNase in 0.1 M $MgCl_2$. The transformation culture was tested for the number of Nb_1-transformed cells and for irreversibly-bound ^{14}C-DNA if labeled DNA was used. Saline was used in place of the gamma globulin for the controls. This method of testing the antiserum was used in all experiments except the kinetic studies. In the kinetic experiments the cells were exposed to the antiserum in the same way but for varying lengths

of time, after which 0.1 ml of each culture was added to 0.9 ml of M-II medium containing 2 μg/ml of Nb_1-DNA and 10 μg/ml of L-valine. The culture was incubated 30 minutes, and 0.1 ml of 1.0 mg/ml DNase was added. After 5 minutes the cells were diluted and plated for Nb_1-transformed cells and viable centers.

Heat Treatment of the Antiserum

Antiserum prepared against competent Rd cells was heated for 30 minutes at 56°C and tested for its ability to inhibit transformation as compared to unheated antiserum. Another sample of antiserum was heated to 92°C (boiling at 7300 feet above sea level) for 10 minutes and tested for its ability to inhibit transformation as compared to an unheated sample.

Enzymatic Digestion of Antiserum

Trypsin digestion. Antiserum against competent Rd cells was digested by adding an equal volume of 100 μg/ml trypsin (Worthington TRL508-8JA) to the antiserum for 30 minutes at 37°C. The digested antiserum was assayed on competent cells both with and without the prior addition of soybean trypsin inhibitor (Worthington) to stop the trypsin activity.

Papain digestion. Antiserum against competent Rd cells was digested for 3 hours with concentrations varying from 5-500 μg/ml of papain (Worthington PAP 9KA) diluted in activating buffer [0.1 M sodium phosphate (pH 7.0), 0.01 M cysteine, and 0.001 M EDTA (Porter, 1958)].

The digested antiserum was tested for cell agglutination and inhibition of transformation.

Bacterial Disruption Techniques

Disruption by sonication. Rd cells (25 ml of 3 x 10^9 viable centers/ml) suspended in M-II medium were chilled and sonicated for five 1-minute intervals on a Sonifier Cell Disruptor Model W 185 C (Heat Systems Co.). The cells were measured for viability and examined under a microscope to check the efficiency of disruption.

Disruption by rapid decompression. Chilled Rd cells (55 ml of 1.5 x 10^9 viable centers/ml) suspended in M-II medium were placed in a Parr Cell Disruption Bomb set in crushed ice. The cell suspension was placed under 1500 psi pressure from a nitrogen gas cylinder for 30 minutes. The cells were removed from the bomb to allow rapid decompression. The suspension was again placed in the bomb at 1500 psi pressure for a second rapid decompression. After a 30-minute equilibration time, the cells were released, checked for viability, and examined under a microscope to determine if any disruption had taken place (Fraser, 1951).

Disruption by grinding with glass beads. One liter (1.5 x 10^9 viable centers/ml) of Rd cells was centrifuged at 5100 x *g* for 10 minutes in a Sorval RC-3 centrifuge and resuspended in 15 ml of chilled 0.01 M potassium phosphate-buffered saline, pH 7.2. All subsequent manipulations were done at 2°C. The cells were added to 15 ml of glass beads chilled in a 50-ml cup attachment for a Sorval Omni-Mixer. The cup containing the cell suspension and glass beads was set in an ice bath, and the Omni-Mixer was attached. The cells were stirred with

the glass beads at 5500 rpm for 7.5 minutes and at 6500 rpm for 2.5 minutes. The viable centers were measured, and the cells were examined under a microscope to determine the extent of cell lysis. The glass beads were separated from the lysed cell suspension by centrifuging at 1500 rpm for 1 minute. The disrupted cell suspension was centrifuged for 8 minutes at 3000 x *g* to remove any whole cells. The resulting supernatant fluid was centrifuged for 20 minutes at 17,000 x *g* to sediment the fragments from the disrupted cells (Neihof and Echols, 1968). The supernatant fluid and debris from the lysed cells were used directly for experimentation or else frozen and saved.

Osmotic Shock of Rd Cells

Osmotic shock with high molar salt solution. Fifty-five ml of Rd cells were centrifuged for 15 minutes in a clinical centrifuge and resuspended in 15 ml of 0.01 M potassium phosphate-buffered 0.5 M NaCl, pH 7.2. The cells were incubated for 10 minutes at 36°C and centrifuged for 15 minutes. The supernatant fluid was tested on Ouchterlony (1953) plates for soluble antigens, and the cells were tested for any loss in transformability.

EDTA-Tris treatment. Fifty-five ml of Rd cells were centrifuged for 15 minutes at 1000 x *g* in a clinical centrifuge and resuspended in 10 ml of chilled (pH 7.4) 0.001 M EDTA, 0.01 M Tris-(hydroxymethyl)-amino methane, and 20% sucrose solution. After 5 minutes incubation at 2°C, the cells were centrifuged (2°C) for 8 minutes at 3000 x *g* and resuspended in 10 ml of 2°C distilled water for 5 minutes. The cells were again centrifuged, added back to M-II medium, and tested

for loss in transformability. The supernatant fluid from the EDTA-Tris and distilled water treatments were assayed for antigenic activity with the antibody absorption assay as described in a later section (see page 22).

Detergent Extraction of Cellular Fragments

After whole cells had been removed from the glass bead-disrupted cells, the suspension of fragments was divided into five equal volumes and centrifuged at 17,000 x *g* for 20 minutes. Each pellet of disrupted cell fragments was resuspended in 2.0 ml of one of four 1% detergent solutions: sodium lauryl sulfate (Sigma Chemical Co.), deoxycholate (Matheson, Coleman, and Bell), Triton X-100 (Mann Laboratories), and Nonidet P-40 (Shell Chemical Co.). The fifth pellet of fragments was suspended in phosphate-buffered saline (2 ml, pH 7.2) and used as a control to measure the antibody absorption capacity of the disrupted cell fragments before detergent extraction. The cellular fragments were incubated with the detergent solutions for 20 minutes at 36°C and stored overnight at 2°C. The next day each detergent extraction solution was centrifuged at 17,000 x *g* for 20 minutes to remove cellular fragments, and the supernatant fluid was assayed for absorption with anti-DNA binding serum and anti-transformation serum (described on page 22).

Antiserum Absorption with Whole Cells

Five ml (1.5×10^9 viable centers/ml) of competent or non-competent Rd cells were centrifuged for 15 minutes at 1000 x *g* in a

clinical centrifuge and resuspended in 0.5 ml of M-II medium. Two-tenths ml of antiserum against competent bacteria was mixed with 0.2 ml of cells and incubated for 1 hour in a 36°C water bath and stored overnight at 2°C. The next day the cells were centrifuged out of suspension, and the supernatant fluid was assayed for antiserum activity as previously described (see page 17).

Antiserum Absorption with Cell Fragments and Soluble Antigens

Antiserum against competent Rd cells (0.075 ml) was added to a 6 x 50-mm tube. An equal volume of material being tested for antiserum absorption was added to the antiserum. The mixture was corked, mixed, incubated at 37°C for 2 hours, and stored overnight in the cold room. The next day the tube was centrifuged to remove any precipitate, and the supernatant fluid was assayed for any change in antiserum activity as previously described (see page 17).

Gel and Resin Preparation for Chromatography

The methods for preparing and pouring the gel filtration columns were taken from the Sephadex handbook describing gel filtration techniques (Beckman Hansson AB, 1966). Sephadex G-100 and G-200 dextrans were swollen for 3 days at room temperature in 0.01 M potassium phosphate-buffered saline (pH 7.2) and 0.2% sodium azide. The fine particles were removed periodically during the swelling process. The G-100 dextran was chilled after swelling and poured into a column 1.9 cm in diameter with a final length of 83 cm. The G-200 dextran was chilled and poured in a column 1.5 cm in diameter and 81 cm in length.

All chromatography was done in an Enviratech cold room (2°C). The sodium azide was washed from the columns with azide-free buffer before applying a sample for chromatography. Sodium azide was added back to the buffer after each run and equilibrated on the column to prevent bacterial contamination. Ion exchange chromatography was done using Cellex E (ECTEOLA) anion exchange resin (see Section B of the Appendix).

Chromatography of Soluble Antigens

After removing the cellular fragments by centrifugation, the supernatant fluid of the glass bead-disrupted cells was fractionated by gel filtration on G-100 and G-200 Sephadex. The eluent was 0.01 M potassium phosphate-buffered saline at pH 7.2. The Triton X-100 extract of glass bead-disrupted cell fragments was fractionated on Cellex E anion exchange resin (see Section B of the Appendix).

Analysis of the Chromatographic Fractions

Fractions were collected in 3-4 ml volumes, and the optical density of each fraction was measured at 260 and 280 nm wavelengths on a Beckman DU-2 spectrophotometer. A 0.2-ml sample of each fraction was placed on an Ouchterlony plate with antiserum against competent Rd bacteria, incubated for 1 week at room temperature, and read for precipitation lines (Ouchterlony, 1953). The remainder of each fraction was lyophilized and resuspended in 0.5 ml of distilled water. The lyophilized samples were assayed for soluble antigens against anti-DNA binding serum and anti-transformation serum (see page 22).

RESULTS

Low-Transforming Mutants

Genetic mutants with low frequencies of transformation have been isolated from highly transformable strains of *B. subtilis*, *D. pneumoniae*, and Streptococcus group H strain Challis (Young and Spizizen, 1961; Ephrate-Elizur, 1965; Siroknak, Lunt, and Hutchison, 1963; Perry, 1968). Low-frequency transformation mutants of *H. influenzae* were reported by Caster *et al.* (1969) shortly after the mutants described in Table 1 were isolated.

Table 1. Generation times, frequencies of transformation, and binding of DNA in low-transforming mutants of *H. influenzae* BC200.

Assay	Mutant strain		
	74	40	67
Log phase generation[+] time in HI medium (minutes)	32	48	57
Percent of control[*] bound DNA	23.5	30	2
Percent of control Nb_1 transformations[†]	0.18	1.5	0.06
Cell morphology	normal	short filaments	long filaments

[+]BC200 had a generation time of 30 minutes.

[*]BC200 was run simultaneously as the control.

[†]Resistance to 2.4 μg novobiocin/ml.

The mutants of BC200 in Table 1 require the same nutritional supplements as Rd and BC200. Mutant numbers 40 and 67 have a slower rate of growth in HI medium as compared to BC200. They also have altered morphologies in that they tend to form filaments, especially mutant number 67. The low frequency of transformation in mutant numbers 74 and 40 seems to be partly due to a lack in their ability to get the irreversibly bound DNA integrated and expressed.

The isolation of mutants deficient in binding DNA and undergoing transformation showed that competence is under genetic control as well as physiological control and that the production of specific materials or structures is necessary for a *H. influenzae* cell to become competent. Low-transforming mutants were isolated in hope that mutants lacking all or part of the components for competence would provide a means by which the physical and chemical differences between competent and non-competent cells could be elucidated. However, this research only progressed to the point where these mutants were to become useful in the study of competence.

DNA Uptake Sites on Competent Cells

The isolation of genetic mutants of transformation showed that the competent state requires special components to take up transforming DNA. This raised the question: Are DNA uptake sites on the cell surface part of the difference between competent and non-competent cells? If so, one should be able to demonstrate their existence by chemical and immunological means.

DNA protection of DNA-uptake sites on competent Rd cells. The technique used by Fox and Kennedy (1965) to isolate the M protein component of the β-galactoside transport system in *E. coli* was modified to demonstrate DNA-uptake sites on competent cells. Transforming DNA was used as the substrate to block the action of N-ethylmaleimide [NEM (Gregory, 1955; Smyth, Nagamatsu, and Fruton, 1960)] on parts of the cell surface (see Section C of the Appendix). The results were affirmative but were not complete or convincing regarding the uniqueness or specificity of the DNA-uptake sites to competent cells.

Because the NEM experiment was not conclusive, the question of DNA uptake sites was asked with immunological assays. Competent and non-competent *H. influenzae* cells were checked immunologically as had been done for pneumococci and Streptococci (Nava, Galis, and Beiser, 1963; Pakula, 1965; Tomasz and Beiser, 1965) to see if there were unique antigenic sites on competent bacteria that provoked antibody production inhibitory to the uptake of transforming DNA.

The effect of anti-*H. influenzae* serum on competent cells. Antiserum prepared against competent Rd and BC200 cells (anti-CRd serum and anti-CBC200 serum) inhibited irreversible binding of transforming ^{14}C-Nb_1-DNA by 4- to 5-fold and reduced the frequency of transformation to novobiocin resistance by 10- to 20-fold (Table 2). Antisera against non-competent Rd and BC200 cells (anti-NCRd serum and anti-NCBC200 serum) had little effect on the binding of transforming DNA or on reducing the level of transformation. The inhibitory activity of each antiserum was of the same magnitude whether assayed on competent Rd or BC-200 cells.

Table 2. Transformation and DNA binding in competent *H. influenzae* cells pretreated with antisera.

30 minutes preincubation of 0.9 ml competent cells with:	Nb_1 transformations/ml	cpm of irreversibly bound DNA/ml
0.1 ml of saline	1.74×10^7	2704
0.1 ml of anti-NCRd serum	1.16×10^7	2352
0.1 ml of anti-CRd serum	9.60×10^5	542
0.1 ml of anti-NCBC200 serum	1.24×10^7	2926
0.1 ml of anti-CBC200 serum	2.01×10^6	649

The effect of antisera on transforming DNA. Antisera prepared against competent and non-competent cells were incubated with transforming DNA to determine if inhibition of transformation was due to antisera-DNA interactions such as serum nuclease inactivation of the nucleic acid. Each antiserum was incubated separately with Nb_1-DNA for 30 minutes and diluted to an ineffective antibody titer (1:1000). The DNA was tested for any reduction in transforming activity as compared to a saline control. The results in Table 3 show no significant changes in the transforming activity of Nb_1-DNA treated with antisera. It was concluded from these experiments that inhibition of DNA binding and transformation was a unique property of antisera prepared against competent cells and that the action of the antisera was on the cell and not on the transforming DNA.

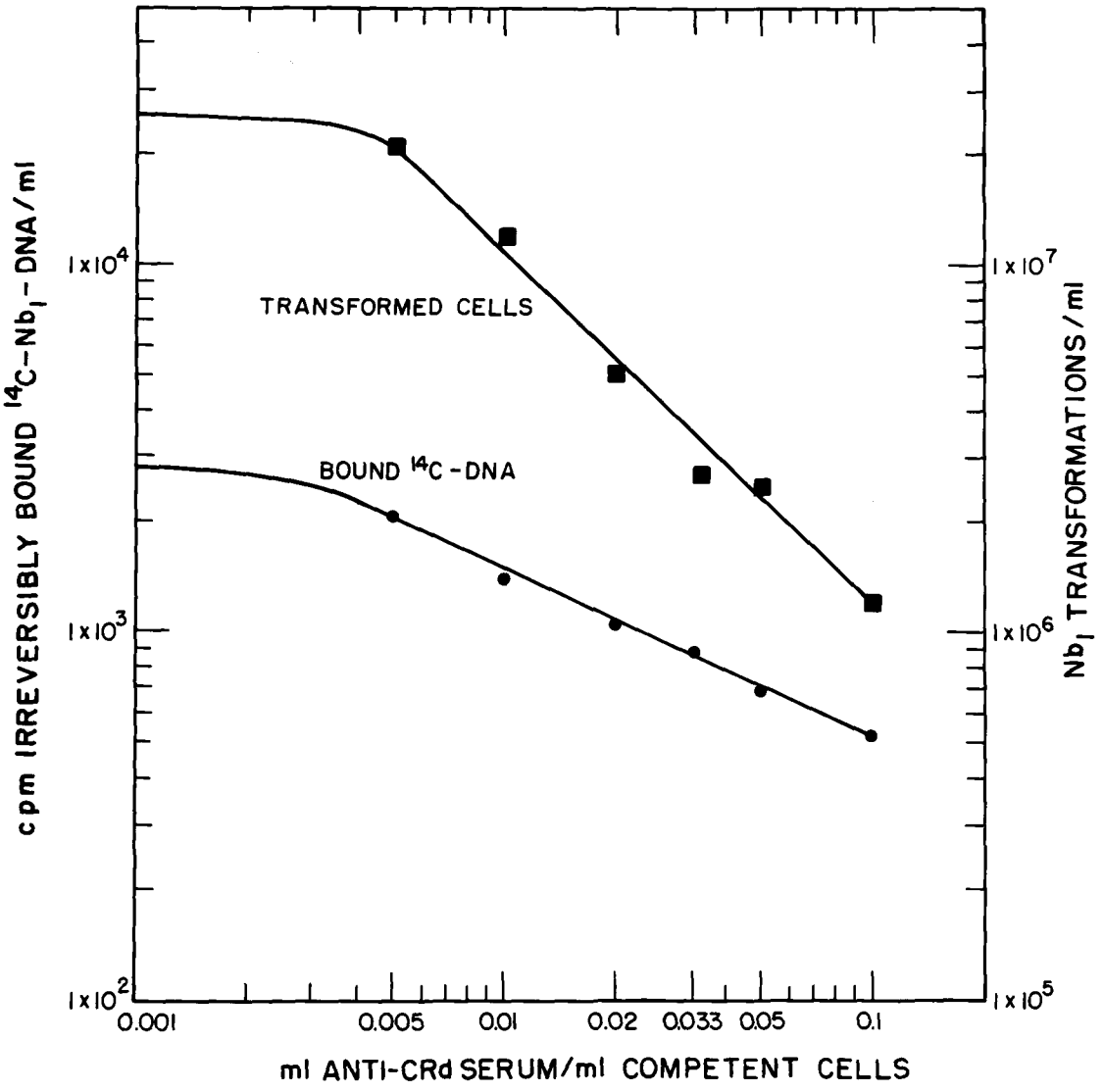
Table 3. The effect of pretreating Nb_1-DNA with antisera prior to assaying the Nb_1-DNA for transforming activity on competent Rd cells.

30 minutes preincubation of 0.05 ml (1 µg) Nb_1-DNA with:	Nb_1 transformations/ml	Viable centers/ml
0.05 ml saline	1.29×10^5	1.58×10^9
0.05 ml preinjection serum	1.24×10^5	1.43×10^9
0.05 ml anti-NCRd serum	1.17×10^5	1.57×10^9
0.05 ml anti-CRd serum	1.22×10^5	1.53×10^9
0.05 ml anti-CBC200 serum	1.15×10^5	1.49×10^9

Characterization of the Anti-*H. influenzae* Serum

The possibility was recognized of using the antisera as an assay for determining the presence of antigens involved in DNA uptake. Before using the antisera in absorption experiments, it was necessary to show that the materials inhibiting DNA binding and transformation behaved like antibodies.

Kinetics of inhibition by antisera. Various concentrations of antisera were tested as to their effect on binding of transforming DNA and transformation to novobiocin resistance. In Figure 1 the dose-response curve for anti-CRd serum was linear from the 1:10 dilution to the 1:200 dilution for both binding of ^{14}C-Nb_1-DNA and transformation to novobiocin resistance (i.e., Nb_1 transformants). However, the slopes of the two curves were different, indicating a possible variation in

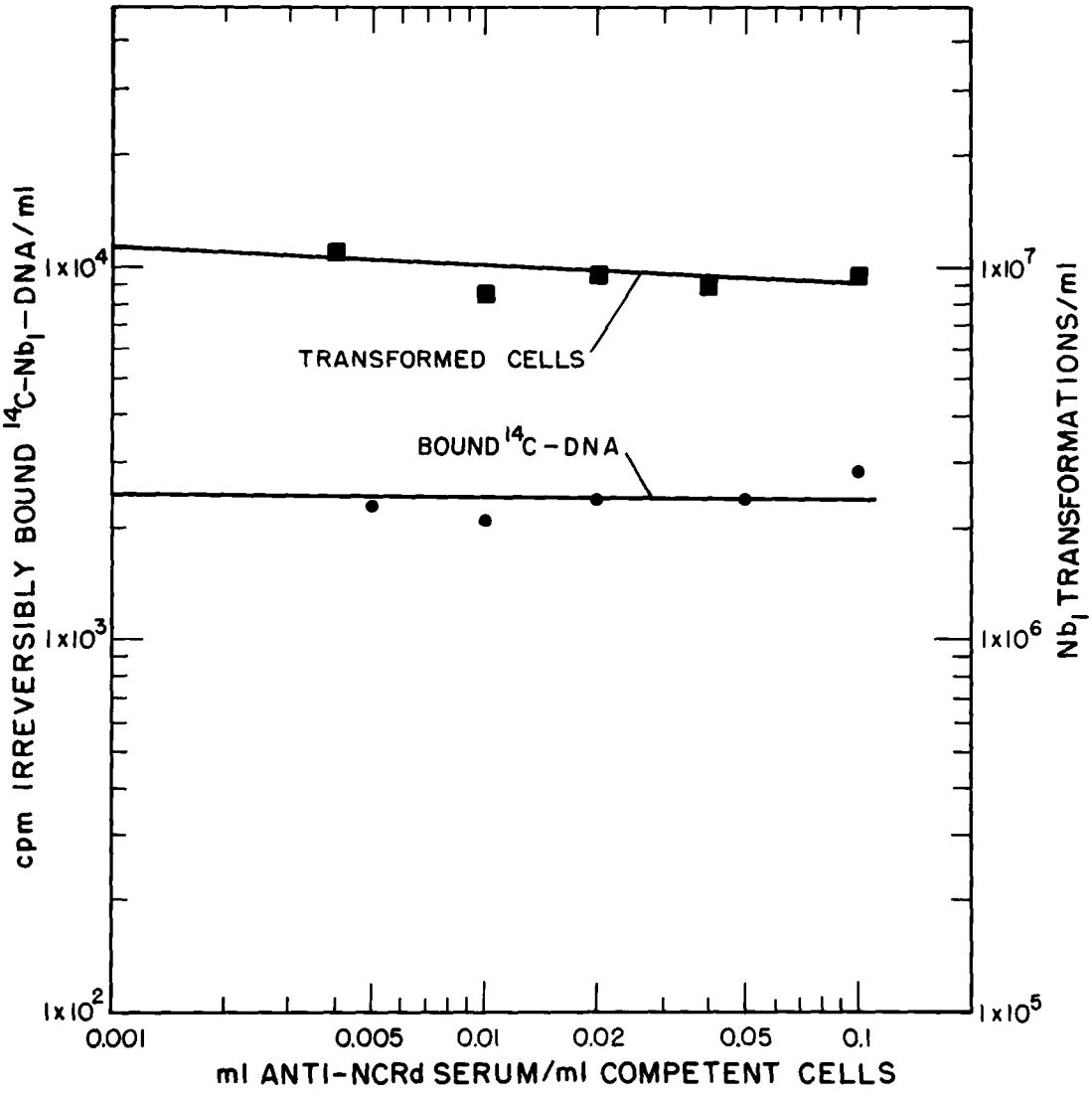

cpm IRREVERSIBLY BOUND ^{14}C-Nb_1-DNA/ml
Nb_1 TRANSFORMATIONS/ml
TRANSFORMED CELLS
BOUND ^{14}C-DNA
1×10^4
1×10^3
1×10^2
1×10^7
1×10^6
1×10^5
0.001
0.005
0.01
0.02
0.033
0.05
0.1
ml ANTI-CRd SERUM/ml COMPETENT CELLS

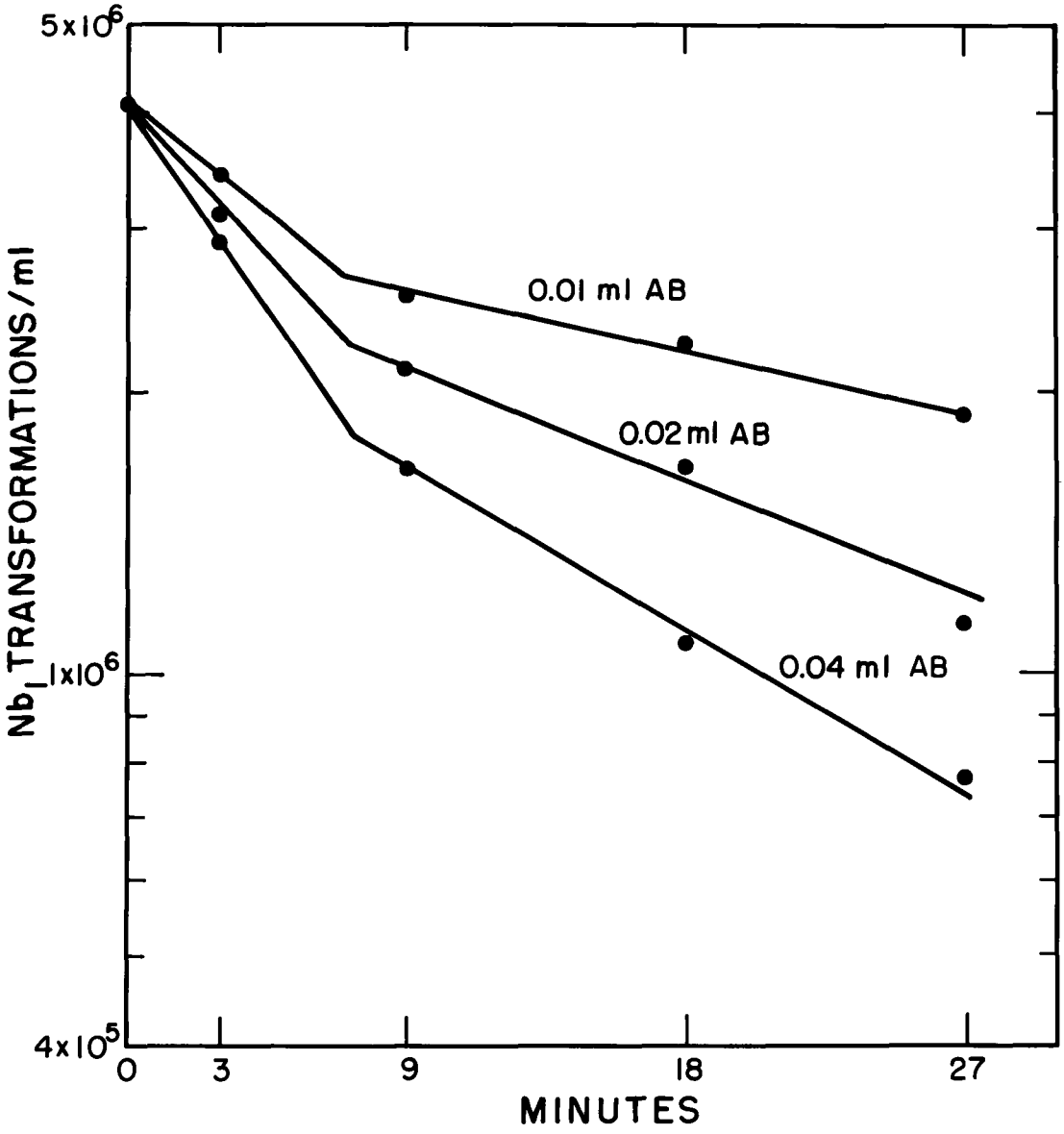
antibodies involved in the two processes. At dilutions of 1:1000 or more the inhibitory effects of the antisera were diluted out. Similar results were reported for antisera prepared against pneumococci by Tomasz and Beiser (1965). The titration of anti-NCRd serum is shown in Figure 2. The binding of transforming DNA was not altered by increasing the concentration of antiserum. Transformation to novobiocin resistance was inhibited slightly at higher concentrations of antiserum, but this may be expected since the non-competent cells against which the antiserum was prepared showed low levels of transformation.

Figure 3 shows the action of three different dilutions of anti-CRd serum as a function of time. During the 27-minute interval of this experiment, the control cells maintained the same level of transformation to novobiocin resistance. The two-phase character of these curves is not understood at this time, but it may be due to a multi-phase uptake mechanism with steps subsequent to the irreversible binding of transforming DNA.

The titrations of anti-CBC200 serum and anti-NCBC200 serum were very similar to antisera prepared against competent and non-competent Rd cells. The inhibition as a function of time of anti-CBC200 serum was also very similar to anti-CRd serum. Because of the close similarity, only the curves from anti-CRd serum were included in this thesis.

Sensitivity of anti-CRd serum to trypsin digestion. Anti-CRd serum was treated with 50 µg/ml of trypsin for 30 minutes at 36°C. The antiserum was assayed for inhibition of transformation on competent Rd cells. Antiserum treated with trypsin lost 5% of its inhibition


cpm IRREVERSIBLY BOUND ^{14}C-Nb_1-DNA/ml
Nb_1 TRANSFORMATIONS/ml
1x10^4
1x10^3
1x10^2
1x10^7
1x10^6
1x10^5
TRANSFORMED CELLS
BOUND ^{14}C-DNA
0.001
0.005
0.01
0.02
0.05
0.1
ml ANTI-NCRd SERUM/ml COMPETENT CELLS


5x10^6
1x10^6
4x10^5
Nb_1 TRANSFORMATIONS/ml
0.01 ml AB
0.02 ml AB
0.04 ml AB
0
3
9
18
27
MINUTES

to transformation as compared to a saline-treated control (Table 4). Trypsin was no more effective at inactivating anti-CRd serum than trypsin inactivated with soybean trypsin inhibitor. It is doubtful, therefore, that there was any digestion resulting in inactivation of the anti-CRd serum.

Antisera prepared against competent and non-competent cells reduced the viable centers of a transformation culture by about two-thirds that of the untreated control (Table 4). It was assumed that the reduction in viable centers was caused by cell agglutination rather than bacteriolysis, since all the complement had been removed from the antisera (Pfeiffer, 1894) by the gamma globulin isolation procedure (Strauss *et al.* (1960). This assumption was tested by treating the

Table 4. Nb_1 transformations in competent Rd cells pretreated with trypsin-digested anti-CRd serum.

Anti-CRd serum treatment	Viable centers/ml	Nb_1 transformations/ml
Saline control (no antiserum)	4.50×10^8	1.36×10^7
Trypsin control (no antiserum)	4.10×10^8	1.24×10^7
Saline for 30 minutes	1.53×10^8	8.80×10^5
50 µg of trypsin for 30 minutes	1.50×10^8	1.35×10^6
50 µg of inactivated-trypsin* for 30 minutes	1.50×10^8	1.37×10^6

*Trypsin treated with soybean trypsin inhibitor for 45 minutes.

antiserum with papain which has been shown to split divalent antibodies into non-agglutinating monovalent forms which still bind to specific antigens (Porter, 1958).

Sensitivity of anti-CRd serum to papain digestion. Anti-CRd serum was incubated with various concentrations of papain to determine the best conditions for digestion. The antiserum was diluted so that the percent decrease in the number of novobiocin-resistant transformants was about equal to the percent decrease in the number of viable centers. In experiment number 1 in Table 5, 420 µg/ml of papain digested anti-CRd serum to the point where it destroyed the antiserum's capacity to inhibit transformation and to agglutinate cells. Digesting the antiserum with 16 µg/ml of papain (experiment number 2 in Table 5) removed all the agglutinating activity but only reduced the capacity to inhibit transformation by 13%. These results supported the assumption that the decrease in transformation due to antiserum was not caused by the loss of viable centers.

Anti-CRd serum inactivation by heat. Anti-CRd serum that had been heated for 30 minutes at 56°C lost 11% of its capacity to inhibit transformation (Table 6). After boiling the antiserum for 10 minutes, 67% of the antiserum's capacity to inhibit transformation had been destroyed. These results agree with the expected behavior of antibody preparations.

Antiserum Absorption with Whole Cells

Anti-CRd serum was absorbed with competent and non-competent Rd cells in an attempt to absorb out some of the inhibitory capacity

Table 5. The effect of treating anti-CRd serum with 420 µg/ml and 16 µg/ml of papain for 3 hours at 36°C prior to exposure to competent Rd cells.

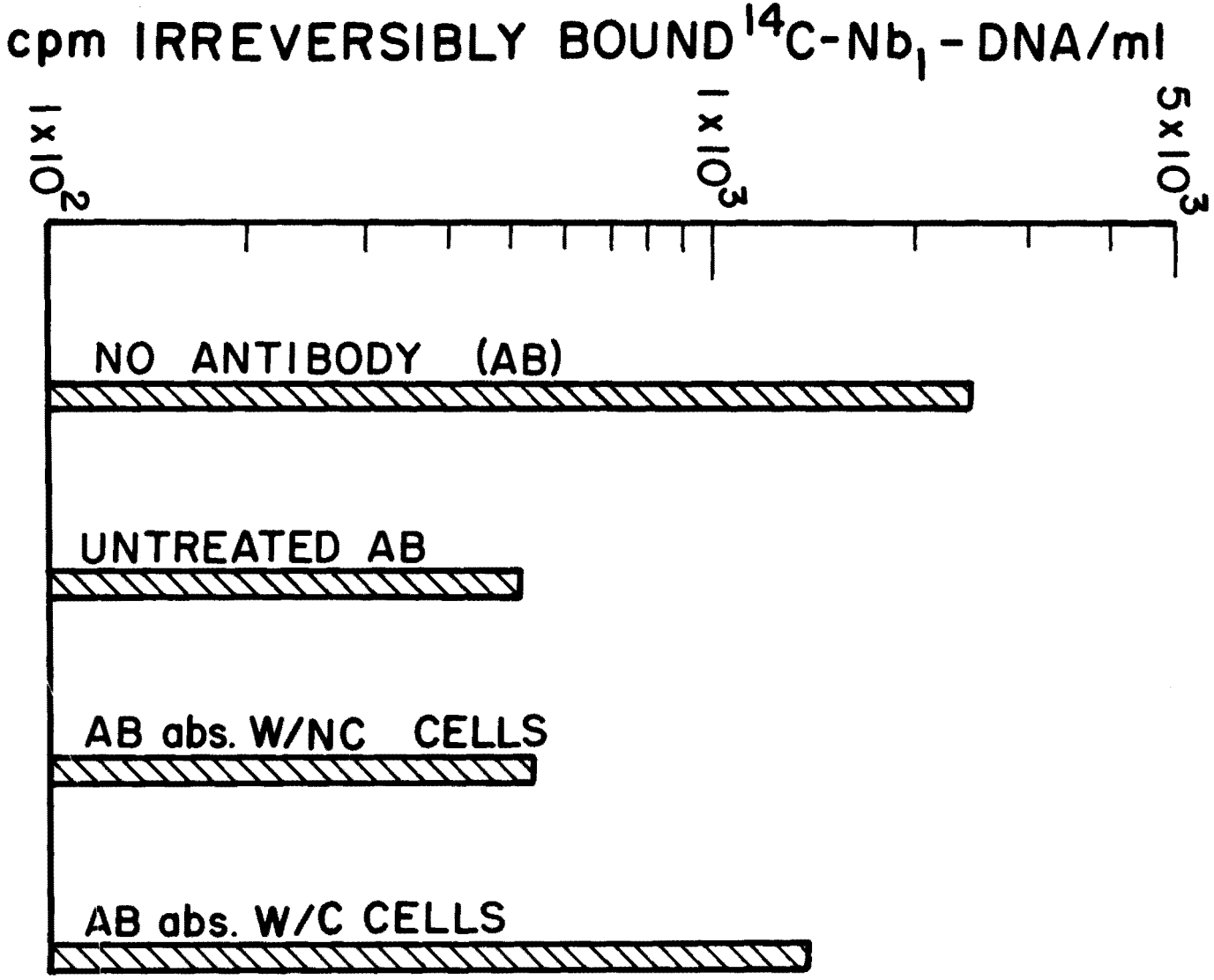
Anti-CRd serum treatment	Viable centers/ml	Nb_1 transformations/ml
Experiment Number 1		
Saline control (no antiserum)	1.48×10^8	3.42×10^6
Papain control (no antiserum)	1.56×10^8	3.47×10^6
Saline for 3 hours	5.70×10^7	9.30×10^5
420 µg papain for 3 hours	1.70×10^8	3.02×10^6
Experiment Number 2		
Saline control (no antiserum)	1.20×10^8	3.49×10^6
Papain control (no antiserum)	1.44×10^8	3.62×10^6
Saline for 3 hours	5.30×10^7	1.30×10^6
16 µg of papain for 3 hours	1.41×10^8	1.78×10^6

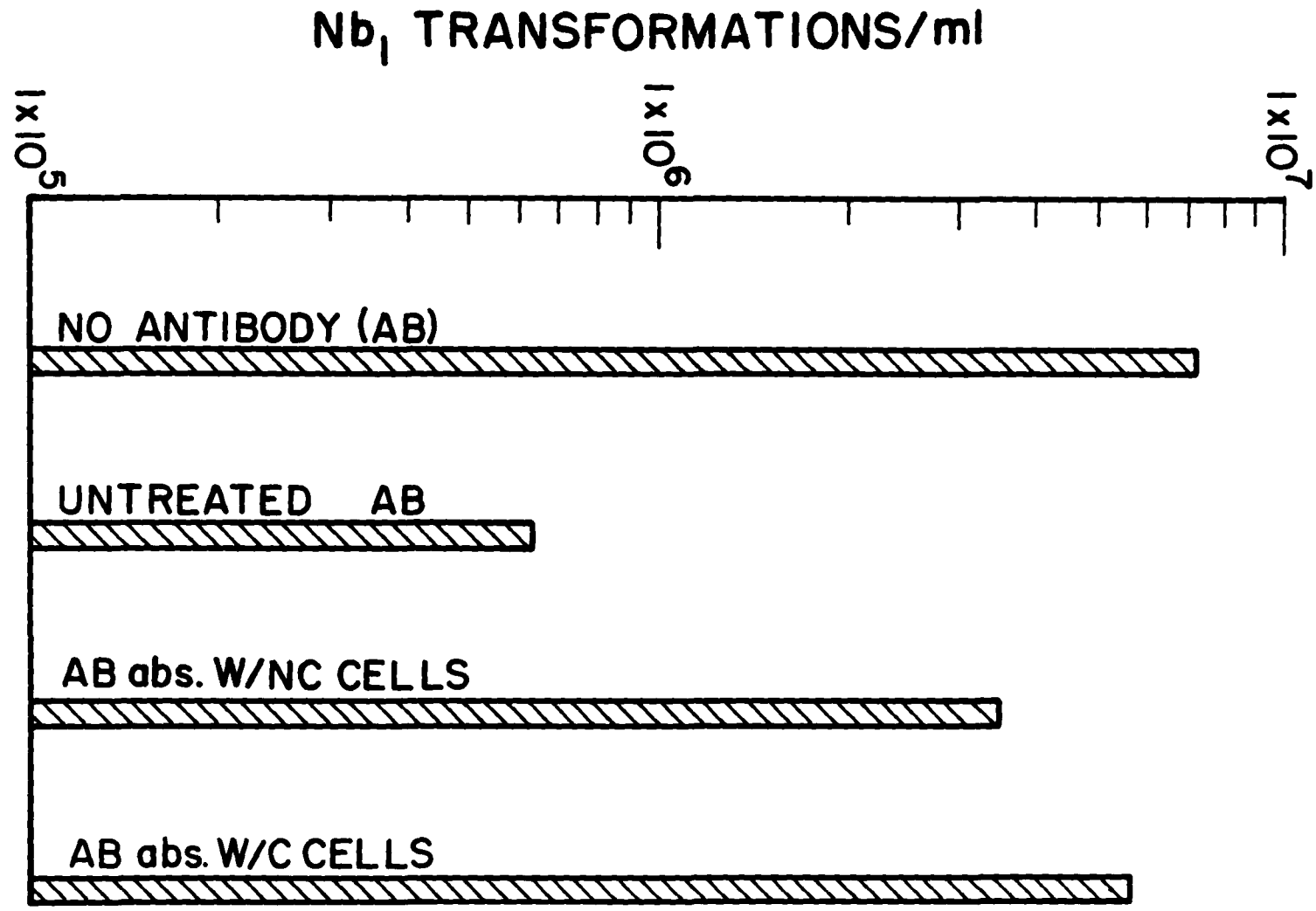
Table 6. Nb_1 transformations in competent Rd cells preincubated with heat-treated anti-CRd serum.

Anti-CRd serum treatment	Viable centers/ml	Nb_1 transformations/ml
No antiserum (control)	4.50×10^8	1.36×10^7
28°C for 30 minutes (control)	1.53×10^8	8.80×10^5
56°C for 30 minutes	2.50×10^8	2.30×10^6
92°C for 10 minutes	3.50×10^8	9.00×10^6

toward DNA binding and transformation. The experiment was done several times using different concentrations of cells. The competent cells were always effective at absorbing out part of the inhibitor of both DNA binding and transformation (Figures 4 and 5). The competent cells, however, were more effective at absorbing the inhibitors of transformation than the inhibitors of DNA binding. Anti-CRd serum absorbed with non-competent cells always lost part of its capacity to inhibit transformation but maintained its ability to inhibit DNA binding (Figures 4 and 5).

After reading the results on antiserum absorption with non-competent cells, one might ask how DNA binding could be inhibited while transformation is not if DNA is necessary for transformation. Such results are obtained when anti-CRd serum is absorbed with non-competent cells and can be explained on the grounds that irreversible DNA binding was only inhibited 7% in the antiserum control. Consequently, the inhibited cells still managed to bind about 25% of the control level or


cpm IRREVERSIBLY BOUND ^{14}C-Nb_1-DNA/ml
1×10^2
1×10^3
5×10^3
NO ANTIBODY (AB)
UNTREATED AB
AB abs. W/NC CELLS
AB abs. W/C CELLS


Nb1 TRANSFORMATIONS/ml
1x10^5
1x10^6
1x10^7
NO ANTIBODY (AB)
UNTREATED AB
AB abs. W/NC CELLS
AB abs. W/C CELLS

4 molecules of DNA per cell. Enough DNA was bound to theoretically maintain the control level of Nb_1 transformation assuming that the irreversibly bound DNA could subsequently find its way into the cell and be integrated.

Release of Antigenic Material from Rd Cells

The results of the foregoing experiments support the hypothesis of specific DNA-uptake sites from both a genetic and an immunological standpoint. The next objective was to isolate the DNA-uptake sites from the cell. The release of the material involved in DNA uptake was approached in two ways: (1) mechanically-disrupting the cells, and (2) osmotically-shocking the cells.

Mechanical disruption of competent and non-competent Rd cells. Three methods were used to disrupt competent and non-competent Rd cells: (1) sonication by ultrasonic sound, (2) rapid decompression in a nitrogen bomb, and (3) grinding with glass beads. The results of subjecting cells to the above mechanical disruption techniques are summarized in Table 7. Sonication of the cells increased the viable centers and was ineffective at releasing measurable amounts of soluble antigen from the cell. The increase in viable centers from sonication was probably due to breaking pairs and quadruplets of cells into singles. Rapid decompression of the cells did not cause loss of viability, cell breakage, or detectable release of soluble antigens. Disruption of the cells by grinding with glass beads resulted in a rapid decrease in viable centers, broken cells and debris, and the release of large amounts of soluble antigens into the supporting buffer

Table 7. Disruption of competent and non-competent Rd cells by mechanical techniques.

Disruption technique	Viable centers/ml	Whole cells[+]	Soluble antigens[*]
Sonication			
time = 0 minute	1.13×10^9	+	-
time = 5 minutes	3.30×10^9	+	-
Decompression			
time = 0 minute	1.69×10^9	+	-
time = 1 hour	1.44×10^9	+	-
Grinding			
time = 0 minute	1.06×10^{11}	+	-
time = 10 minutes	6.20×10^8	-	+

[+]Determined by microscopic examination.

[*]Determined by applying samples of supernatant fluid from disrupted cells on Ouchterlony plates with anti-CRd serum.

solution (Figure 6). Consequently, disruption by sonication and rapid decompression were discarded in favor of rupturing the cells by grinding with glass beads.

Cellular antigens released by osmotic shock. Rd cells were osmotically shocked (1) with high molar salt solution and (2) with EDTA-Tris treatment followed by cold distilled water. Table 8 summarizes the results of the two procedures. Competent cells maintained a high frequency of transformation, and no soluble antigens were detected after osmotically shocking the cells with 0.5 M NaCl. When competent cells were treated with EDTA-Tris and cold distilled water, the frequency of transformation dropped by about 20-fold but no soluble antigens were detected. Non-competent cells treated with EDTA-Tris and cold distilled water increased their frequency of transformation by 3000-fold. The increase in frequency of transformation by the non-competent cells is not understood at this time but may be worth pursuing at a later date.

There was no change in viability after incubating the cells with the EDTA-Tris solution in the osmotic shock sequence. However, the EDTA-Tris solution remained cloudy with cellular debris remaining in suspension after the whole cells had been removed by centrifugation at 3000 x *g* for 8 minutes. The cell fragments were removed from suspension by centrifuging the 3000 x *g* supernatant at 17,000 x *g* for 20 minutes. The pellet of cellular debris was resuspended in 0.1 ml of 0.01 M $CaCl_2$ and used to absorb anti-CRd serum. The results of absorbing the antiserum with competent and non-competent cell fragments are given in Figures 7 and 8.

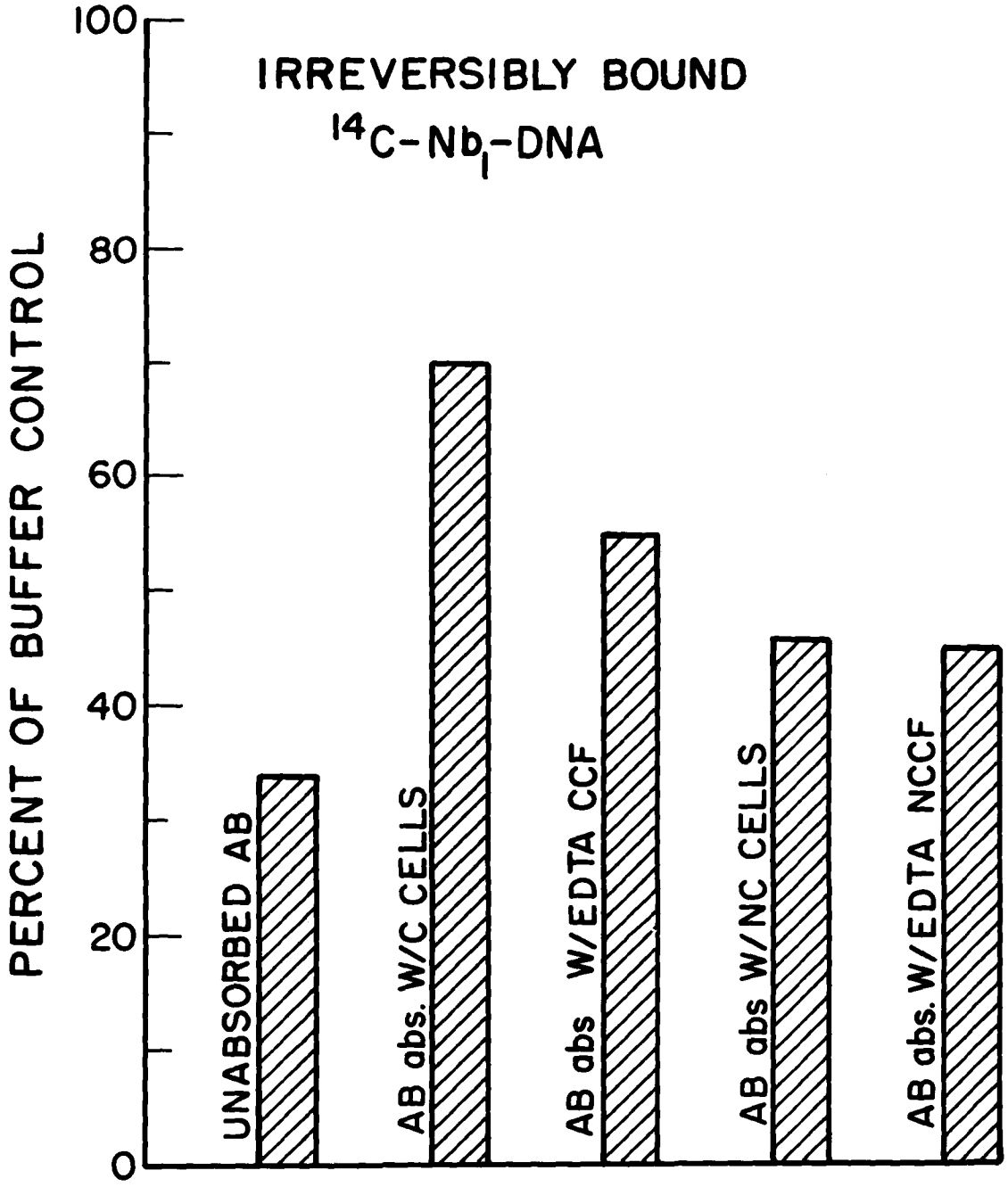

9L 9NC

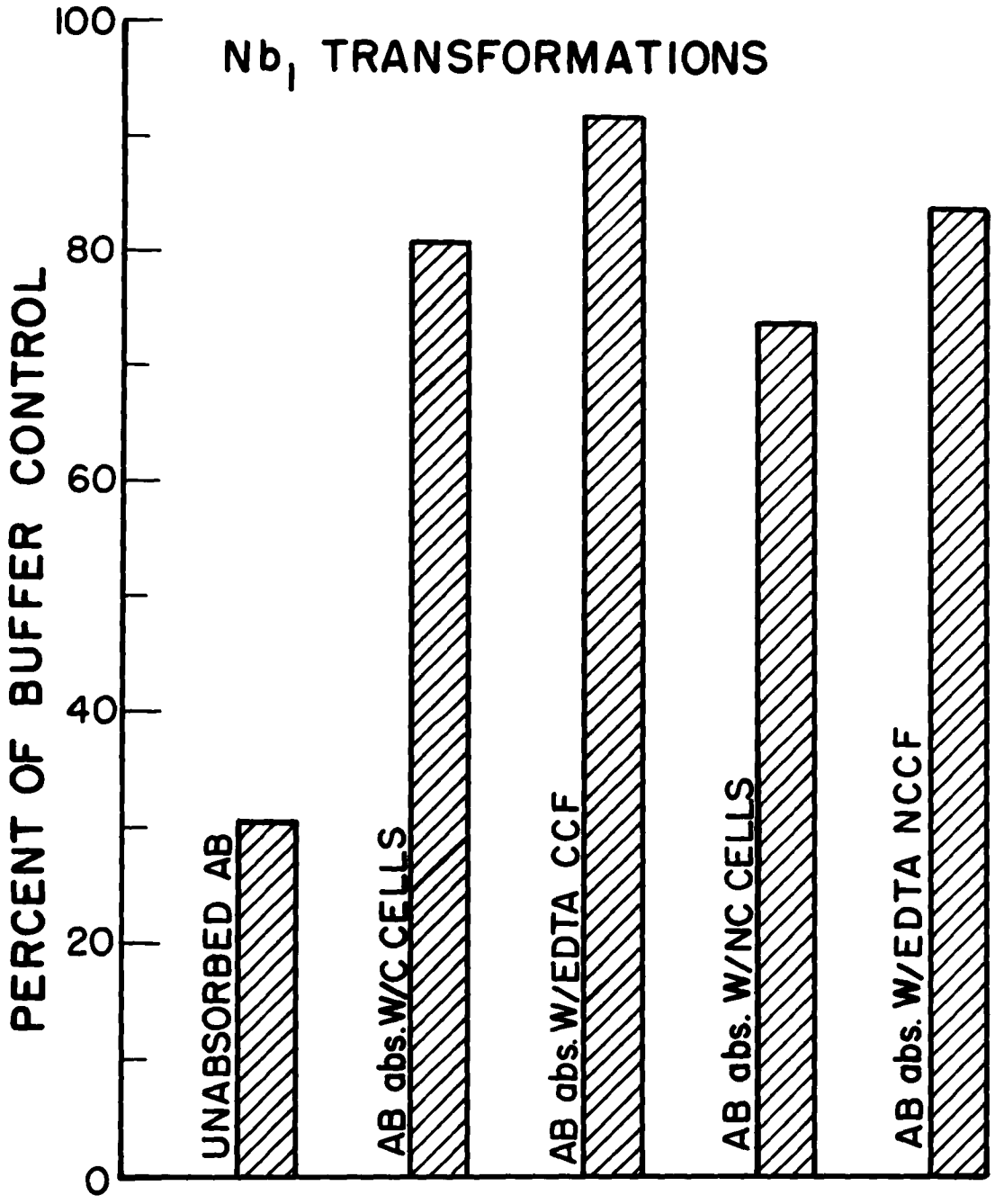
Table 8. The effect of osmotic shock on transformation and release of soluble antigens into the shock fluid.

Shock technique	Frequency of Nb_1 transformation (%)		Release of soluble antigens
	before	after	
0.5 M NaCl			
Competent cells	5.9	4.2	-[+]
EDTA-Tris and H_2O			
Competent cells	4.3	0.17	-[*]
Non-competent cells	0.0002	0.64	-[*]

[+]Determined by applying samples of shock fluid on Ouchterlony plates with anti-CRd serum.

[*]Insoluble antigenic material was released as assayed by anti-CRd serum absorption.


IRREVERSIBLY BOUND
^{14}C-Nb_1-DNA
PERCENT OF BUFFER CONTROL
100
80
60
40
20
0
UNABSORBED AB
AB abs. W/C CELLS
AB abs W/EDTA CCF
AB abs W/NC CELLS
AB abs. W/EDTA NCCF

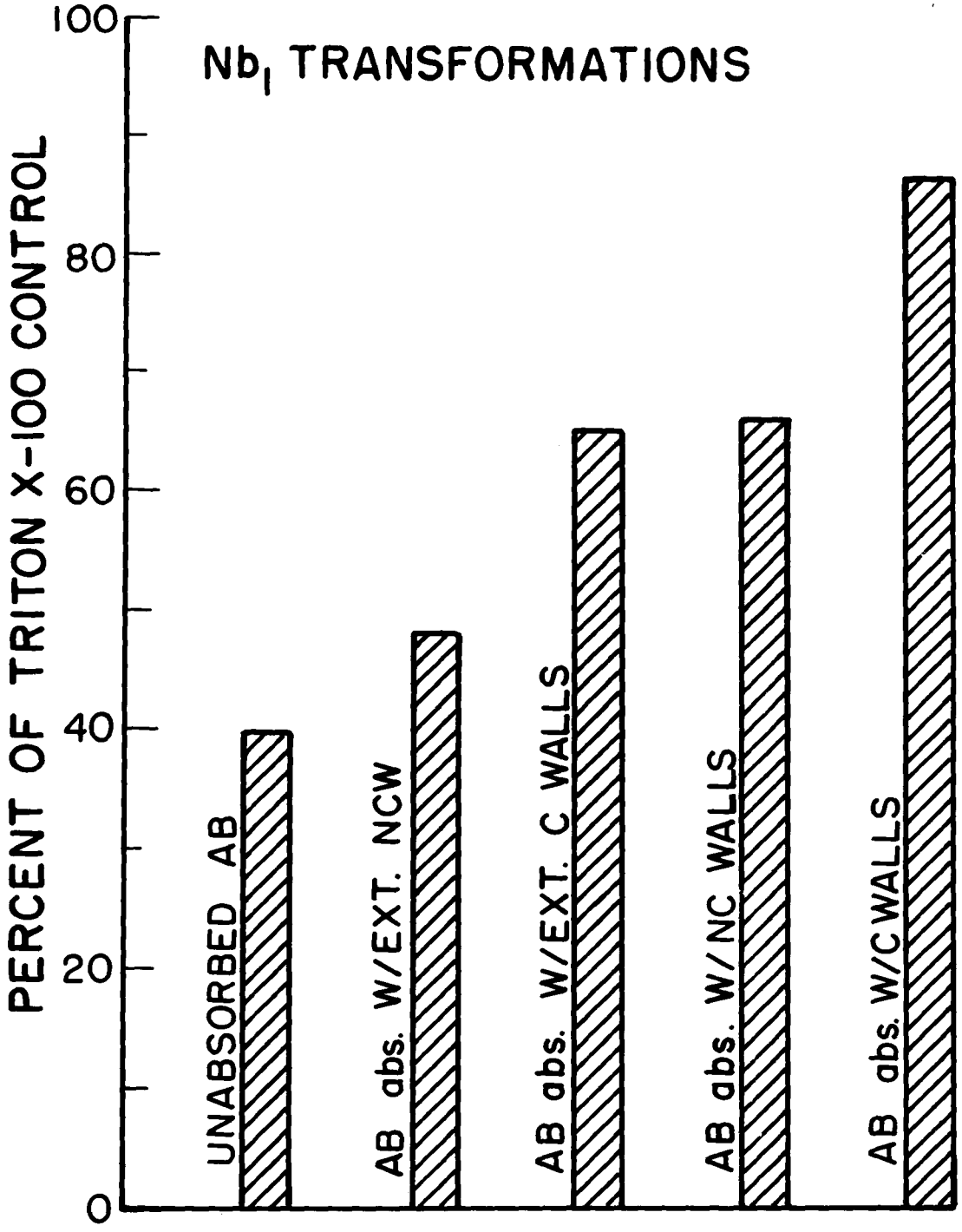

Nb1 TRANSFORMATIONS
PERCENT OF BUFFER CONTROL
100
80
60
40
20
0
UNABSORBED AB
AB abs.W/C CELLS
AB abs.W/EDTA CCF
AB abs. W/NC CELLS
AB abs. W/EDTA NCCF

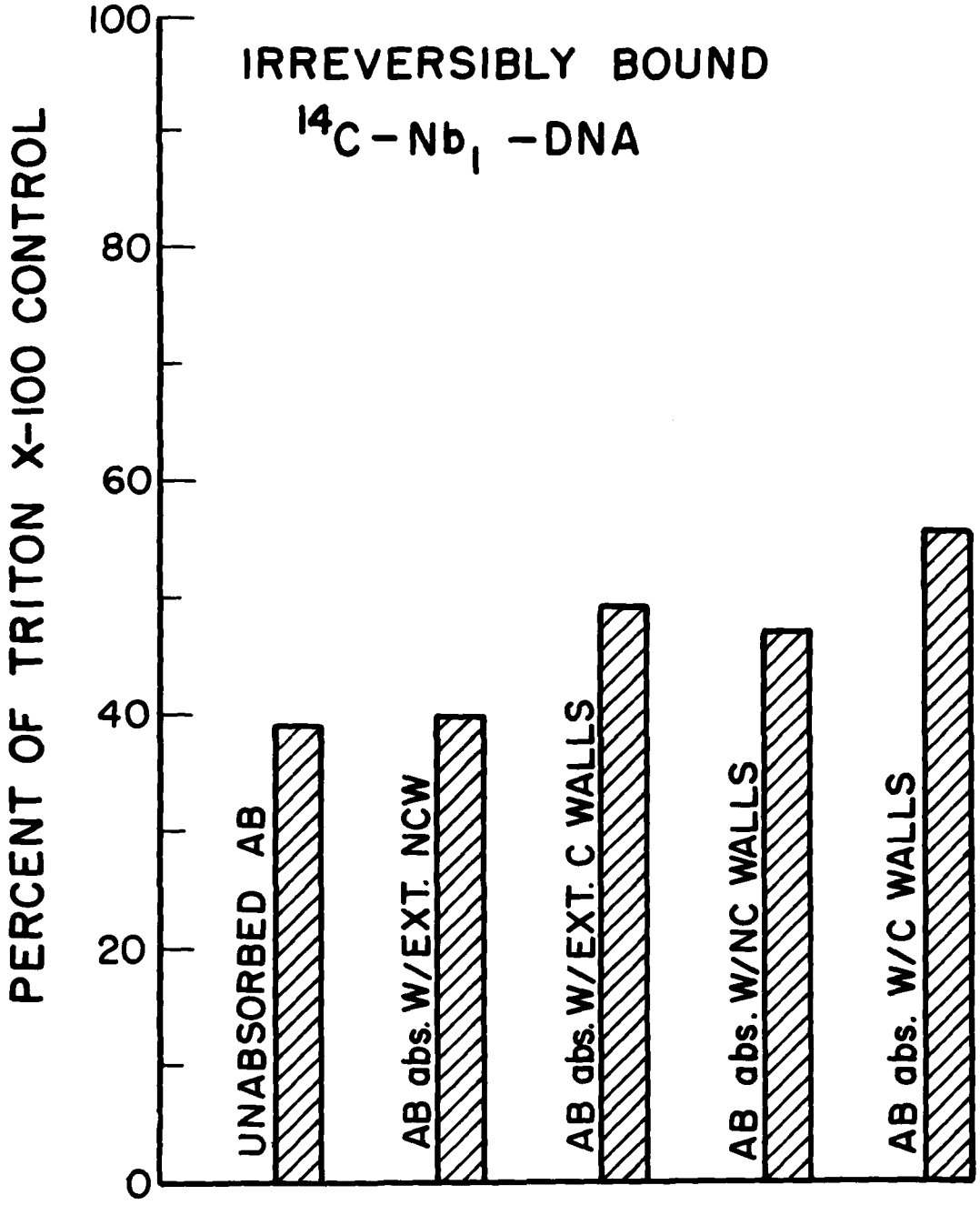
The cellular fragments from competent and non-competent cells were more effective at absorbing the anti-CRd serum's capacity to inhibit transformation than their whole-cell counterparts (Figure 8). However, competent whole cells were more effective than cell fragments at absorbing the anti-CRd serum's inhibition of DNA binding (Figure 7). The EDTA-Tris treatment of cells appeared to separate the antigens that absorb the anti-CRd serum's inhibition of transformation from antigens involved in absorbing the antiserum's inhibition of DNA binding.

Extraction of Antigenic Material from Cell Walls

After glass bead disruption of competent and non-competent cells, the cellular debris (less whole cells) was considered to be mostly of cell wall (wall-membrane) origin (Neihof and Echols, 1968). The cell wall material showed strong absorption of the anti-transforming activity of anti-CRd serum (Figure 9) and weak absorption of the capacity to inhibit irreversible DNA binding (Figure 10). These results indicate that, after cells are fractionated by disruption and centrifugation, the antigens involved in absorbing anti-transformation activity reside on the cell wall. This corresponds to the results described in the previous section for cell fragments released from cells by EDTA-Tris treatment.

To chromatograph the cell wall material, the cell walls were extracted with detergents (see the Materials and Methods section) to solubilize the materials responsible for absorbing the anti-CRd serum's inhibition of transformation. Four different detergents were used:


Nb1 TRANSFORMATIONS
PERCENT OF TRITON X-100 CONTROL
100
80
60
40
20
0
UNABSORBED AB
AB abs. W/EXT. NCW
AB abs. W/EXT. C WALLS
AB abs. W/NC WALLS
AB abs. W/C WALLS


IRREVERSIBLY BOUND
$^{14}C-Nb_1-DNA$
PERCENT OF TRITON X-100 CONTROL
100
80
60
40
20
0
UNABSORBED AB
AB abs. W/EXT. NCW
AB abs. W/EXT. C WALLS
AB abs. W/NC WALLS
AB abs. W/C WALLS

(1) sodium lauryl sulfate, (2) deoxycholate, (3) Triton X-100, and (4) Nonidet P-40. These detergents removed antigenic material from the cells (Figure 11); therefore, the detergent extracts were used to absorb anti-CRd serum. Sodium lauryl sulfate and deoxycholate interfered with transformation and DNA binding to the point that it was impossible to detect any decrease in the inhibiting activity of anti-CRd serum absorbed with cell wall extracts of these two anionic detergents. Triton X-100 and Nonidet P-40 did not interfere with transformation or DNA binding. Both of these non-ionic detergents behaved similarly; therefore, only the absorption results from Triton X-100 extracts are given (Figures 9 and 10).

The material solubilized by detergent extraction of competent cell walls did absorb a significant amount of the anti-CRd serum's capacity to inhibit transformation but absorbed little or none of the antiserum's ability to inhibit DNA binding (Figures 9 and 10). The technique was inefficient at removing all of the absorbing material from cell walls. When the small amount of material extracted by the non-ionic detergents was fractionated on Cellex E anion exchange resin (see Section B of the Appendix), the resulting fractions were too dilute to assay for antibody absorbing activity. An assay of the fractions from the anion exchange column against anti-CRd serum on Ouchterlony plates revealed that the chromatography procedure had separated at least three different antigens. The extraction of anti-transformation absorbing antigens will have to be improved before further study can be done towards understanding its function in competence.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Antiserum Absorption with Soluble Antigens

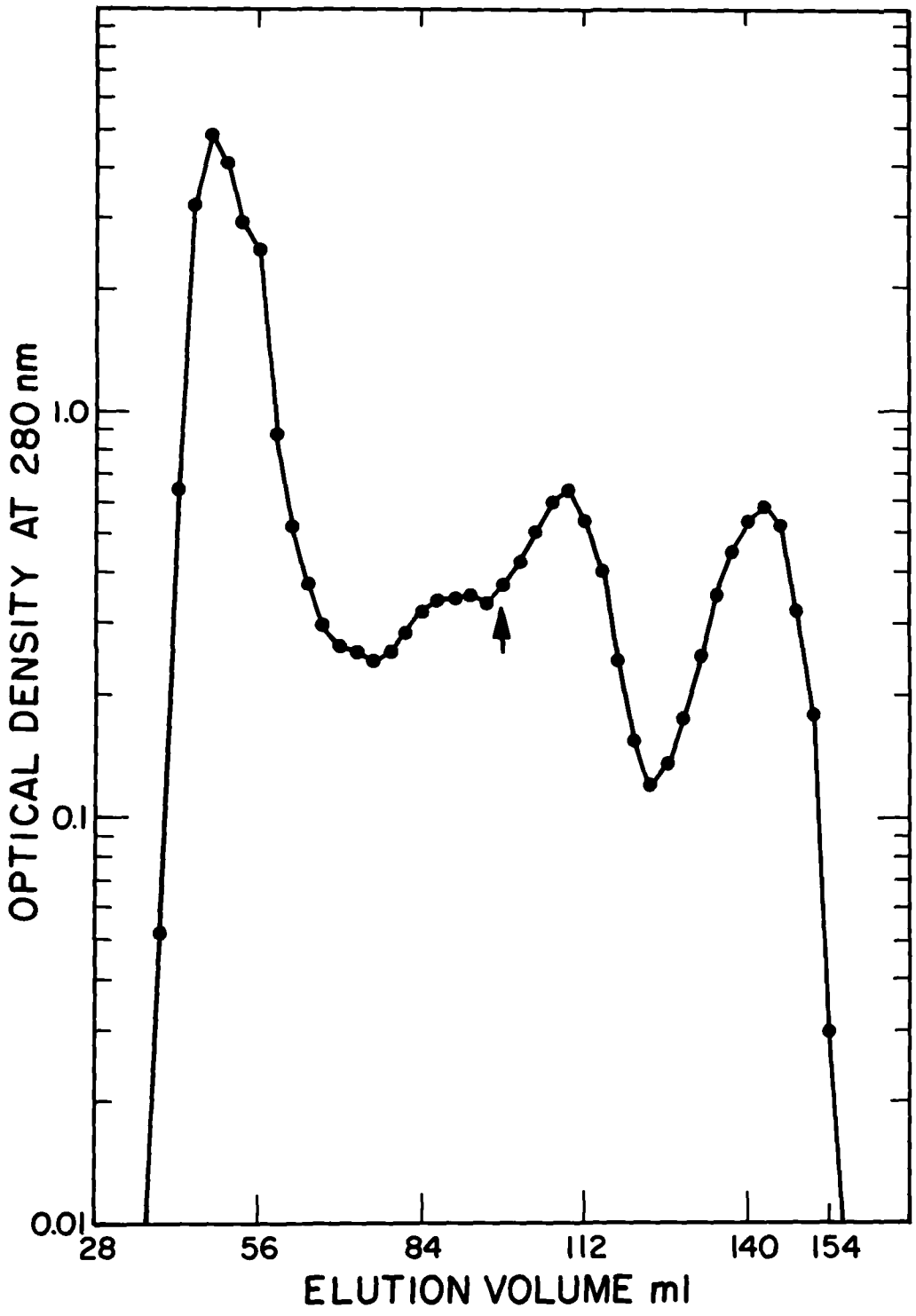
Absorption with supernatant fluid from glass bead-disrupted competent Rd cells. The particulate fraction of disrupted competent cells exhibited very little absorption of the anti-CRd serum's capacity to inhibit DNA binding. The fluid containing the cell cytoplasm and all of the soluble cell components were assayed for antibody absorption in hope of finding the antigens responsible for absorbing the inhibitor of DNA binding from anti-CRd serum. The results in Table 9 show that the fluid from disrupted cells was extremely inhibitory to DNA binding and transformation. This strong inhibition was assumed to be caused by the DNA from the ruptured cells competing for sites on the competent bacteria and preventing the transforming ^{14}C-Nb_1-DNA from binding and being integrated. In order to examine the fluid for antiserum-absorbing activity, the residual DNA had to be removed. This was done by column chromatography.

Table 9. Transformations and irreversibly-bound DNA in competent cells treated with fluid from glass bead-disrupted competent Rd cells.

Preincubation (30 minutes) of 0.9 ml competent cells with:	Nb_1 transformations/ml	cpm of irreversibly bound DNA/ml
Saline (control)	1.20×10^7	2055
Fluid from glass bead-disrupted competent Rd cells	3.50×10^5	73

Absorption with fractions from column chromatography. By fractionating the fluid from the disrupted cells on Sephadex G-200 or G-100 the soluble material in the fluid could be separated into fractions of decreasing molecular weight. The Sephadex G-200 elution profiles of fluid from glass bead-disrupted competent and non-competent cells are shown in Figures 12 and 13. These elution profiles did not exhibit any discernible differences between the soluble components of competent and non-competent cells. The Sephadex G-100 elution profile of fluid from glass bead-disrupted competent cells is shown in Figure 14.

Each fraction from the column chromatography was assayed for antigens against anti-CRd serum on Ouchterlony plates. Those fractions showing positive reactions (Figure 15) were assayed for antibody absorption with anti-CRd serum, but the results were not conclusive. All antigen-containing fractions were subsequently lyophilized and resuspended in 0.5 ml of distilled water to effect a 6- to 8-fold concentration. The concentrated fractions were assayed for absorption of the capacity to inhibit DNA binding and transformation from anti-CRd serum. Figures 16 and 17 show the results for competent cell fluid fractionated on G-200. There was no significant absorption of the capacity to inhibit transformation from anti-CRd serum (Figure 16). However, Figure 17 shows a strong peak of anti-DNA binding absorption. The peak activity of anti-DNA binding absorption is indicated by the arrow on the elution profile (Figure 12) and corresponds to a molecular weight of around 50,000 (see Section D of the Appendix for calculations)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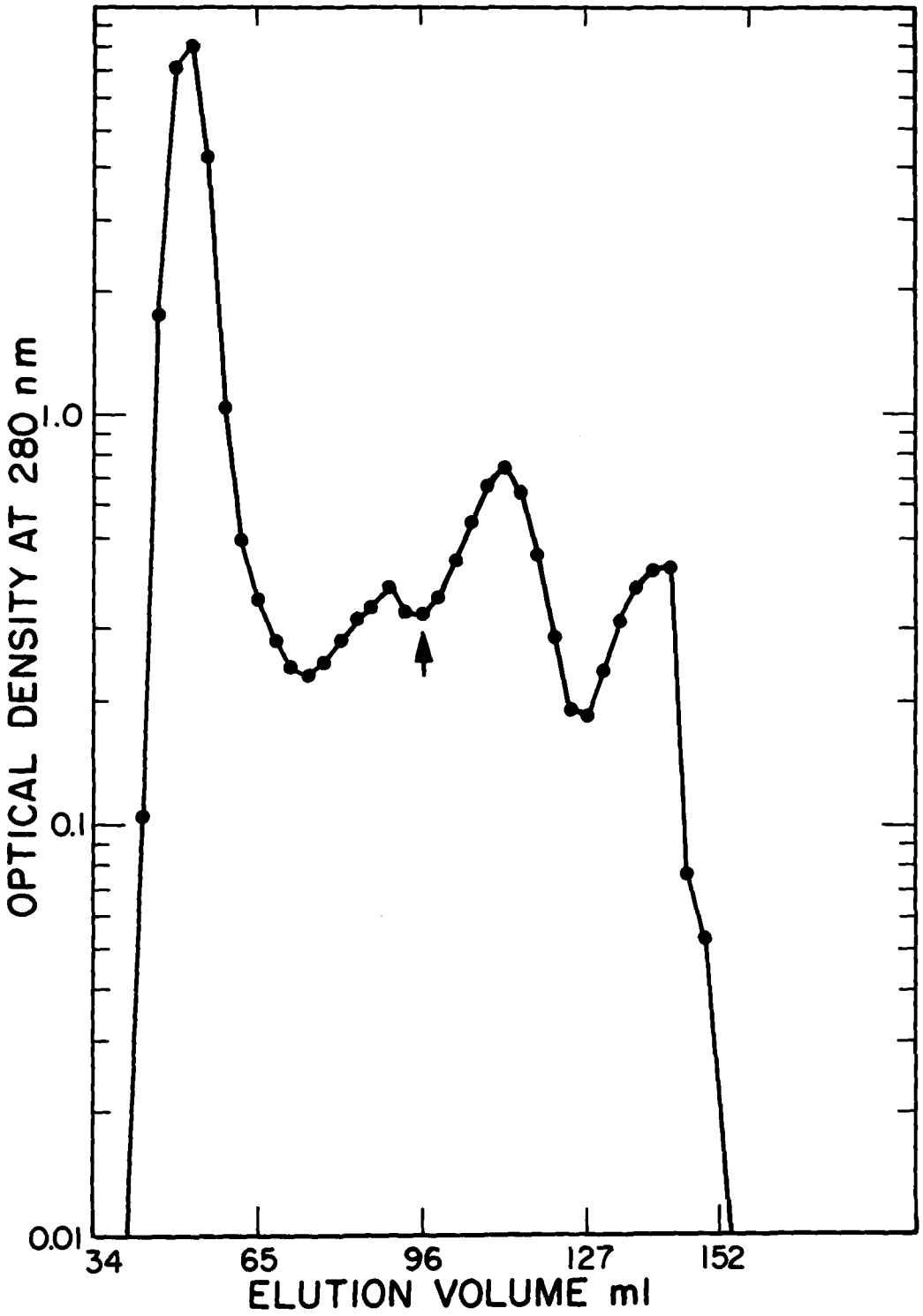
0.1
0.01
28
56
84
112
140
154
ELUTION VOLUME ml


OPTICAL DENSITY AT 280 nm
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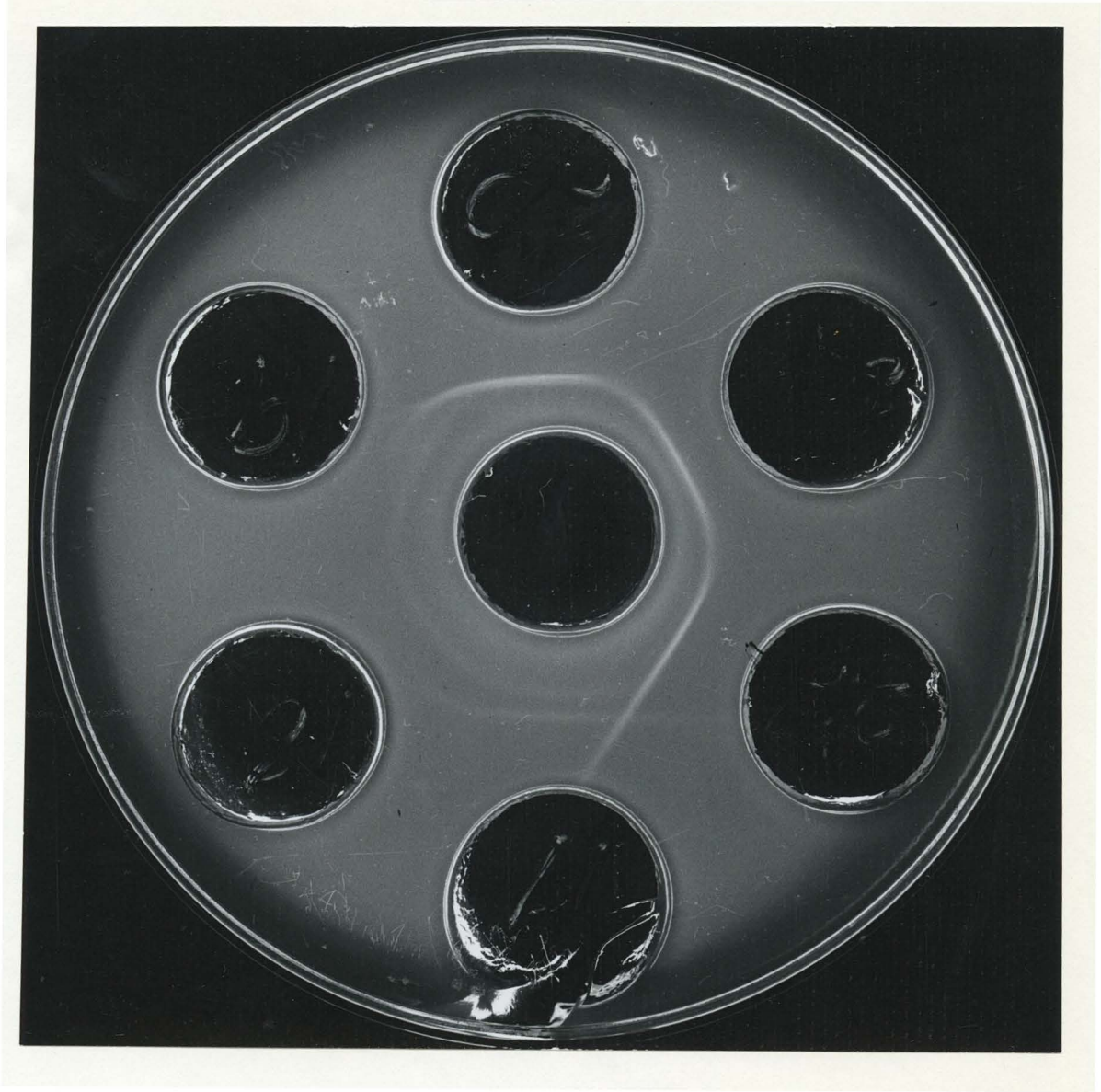
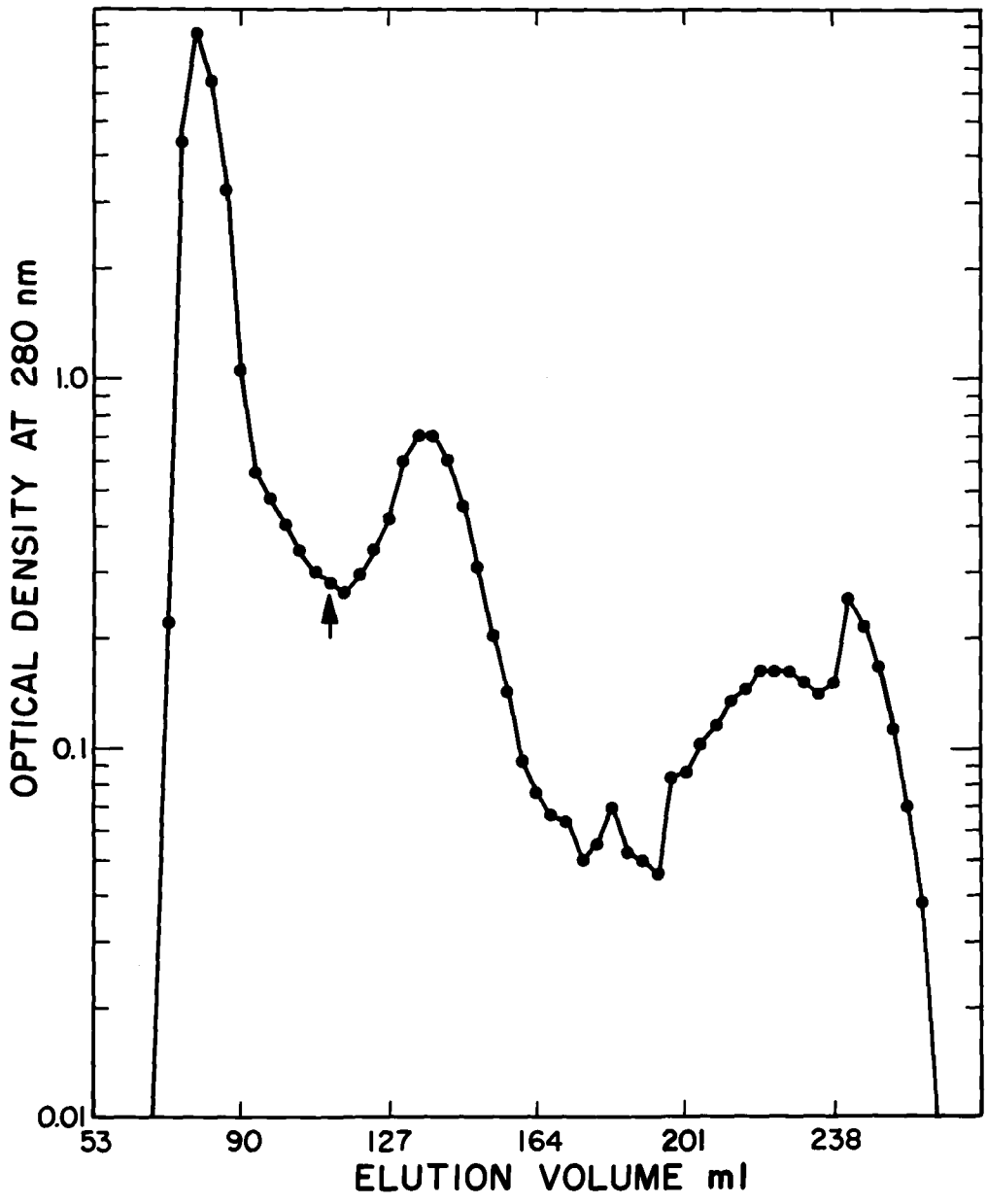
0.1
0.01
34
65
96
127
152
ELUTION VOLUME ml




OPTICAL DENSITY AT 280 nm
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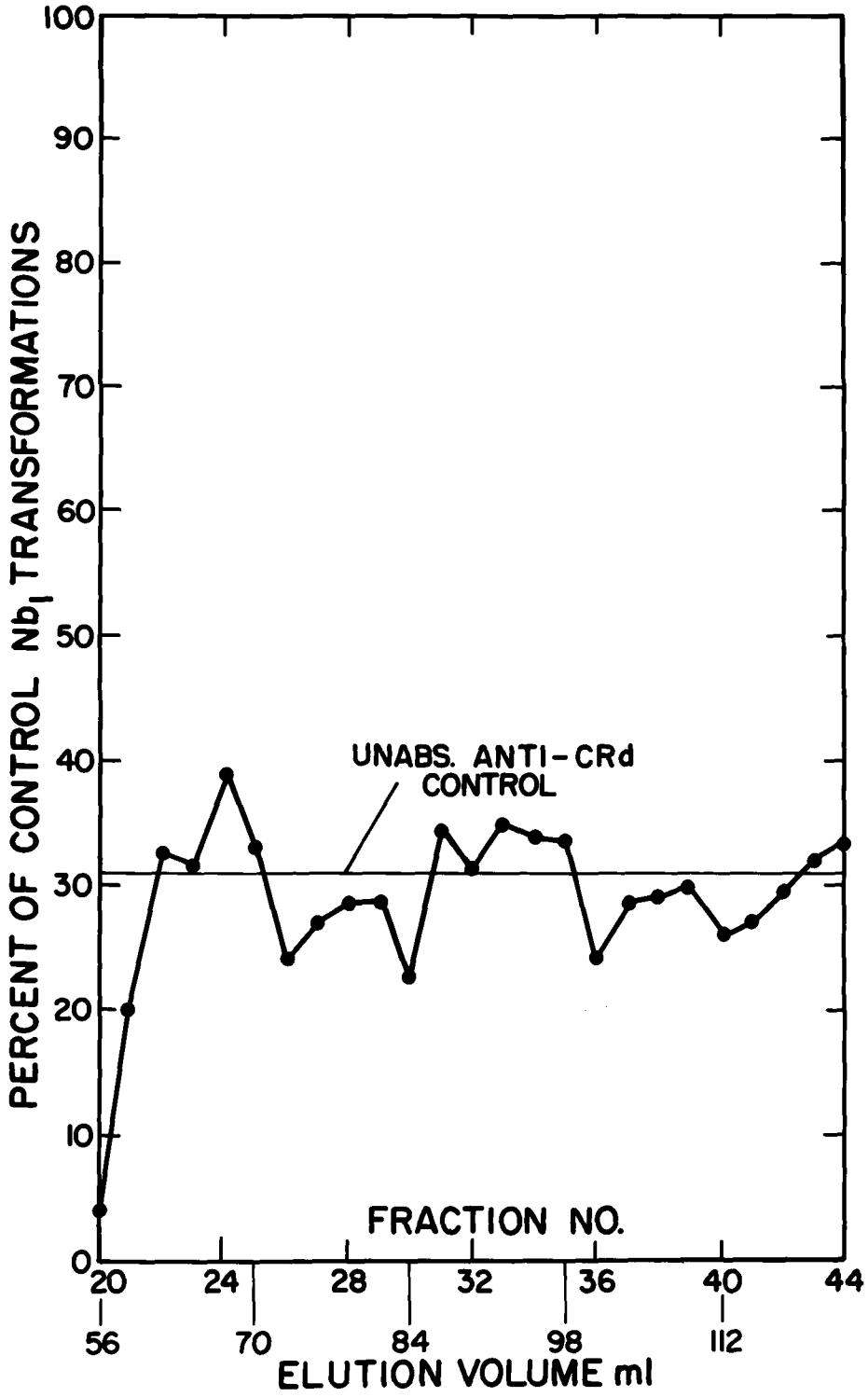
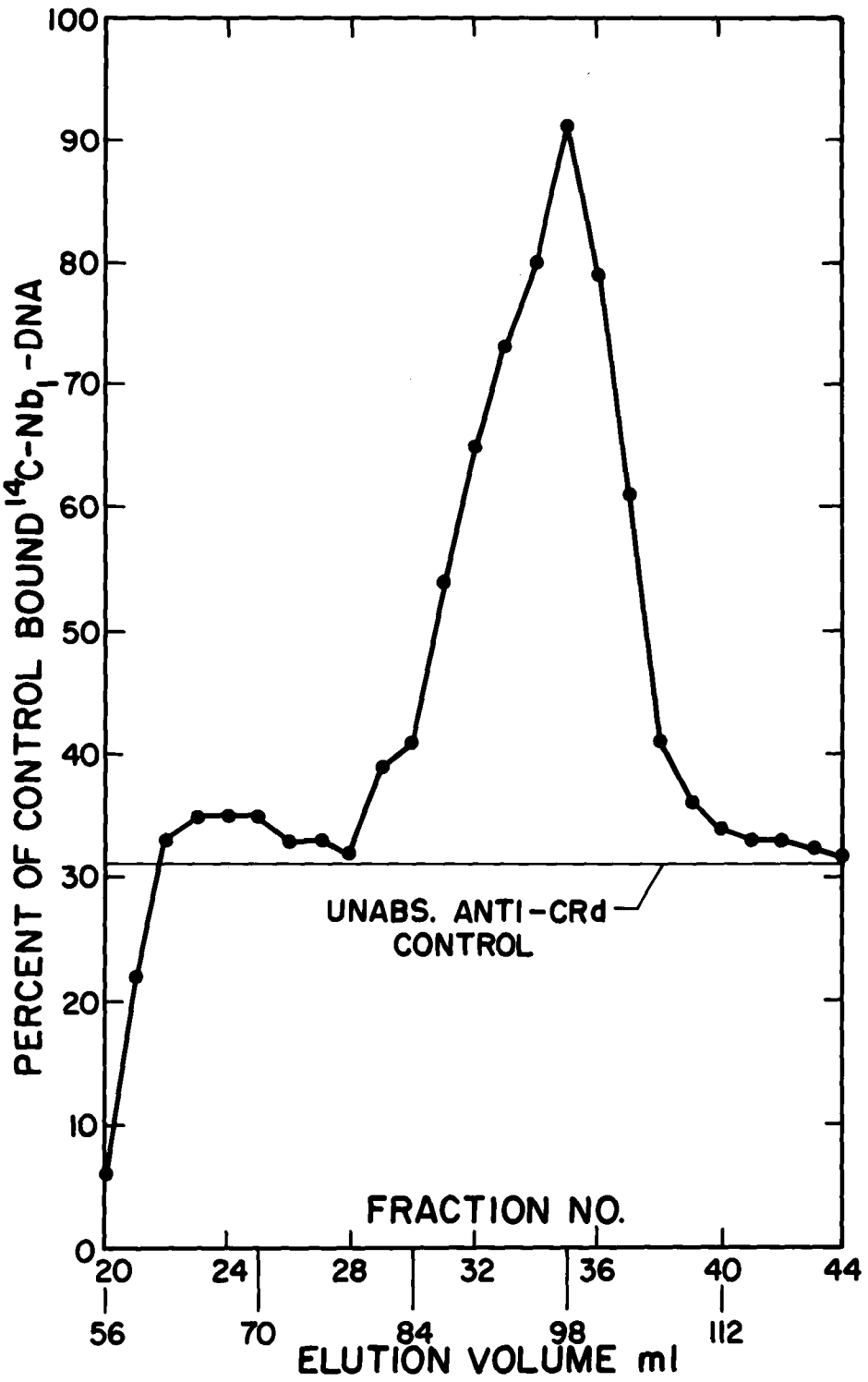
0.1
0.01
53
90
127
164
201
238
ELUTION VOLUME ml

Figure 16. Absorption of anti-transformation activity fron anti-CRd serum by concentrated Sephadex G-200 fractions of glass bead-disrupted competent Rd cells. The absorbed antiserum was assayed on competent Rd cells for its capacity to inhibit transformation to novobiocin resistance (Nb_1). The results are expressed as a percent of the (no antibody) control.


100
90
80
70
60
50
40
30
20
10
0
PERCENT OF CONTROL Nb1 TRANSFORMATIONS
UNABS. ANTI-CRd
CONTROL
FRACTION NO.
20
24
28
32
36
40
44
56
70
84
98
112
ELUTION VOLUME ml

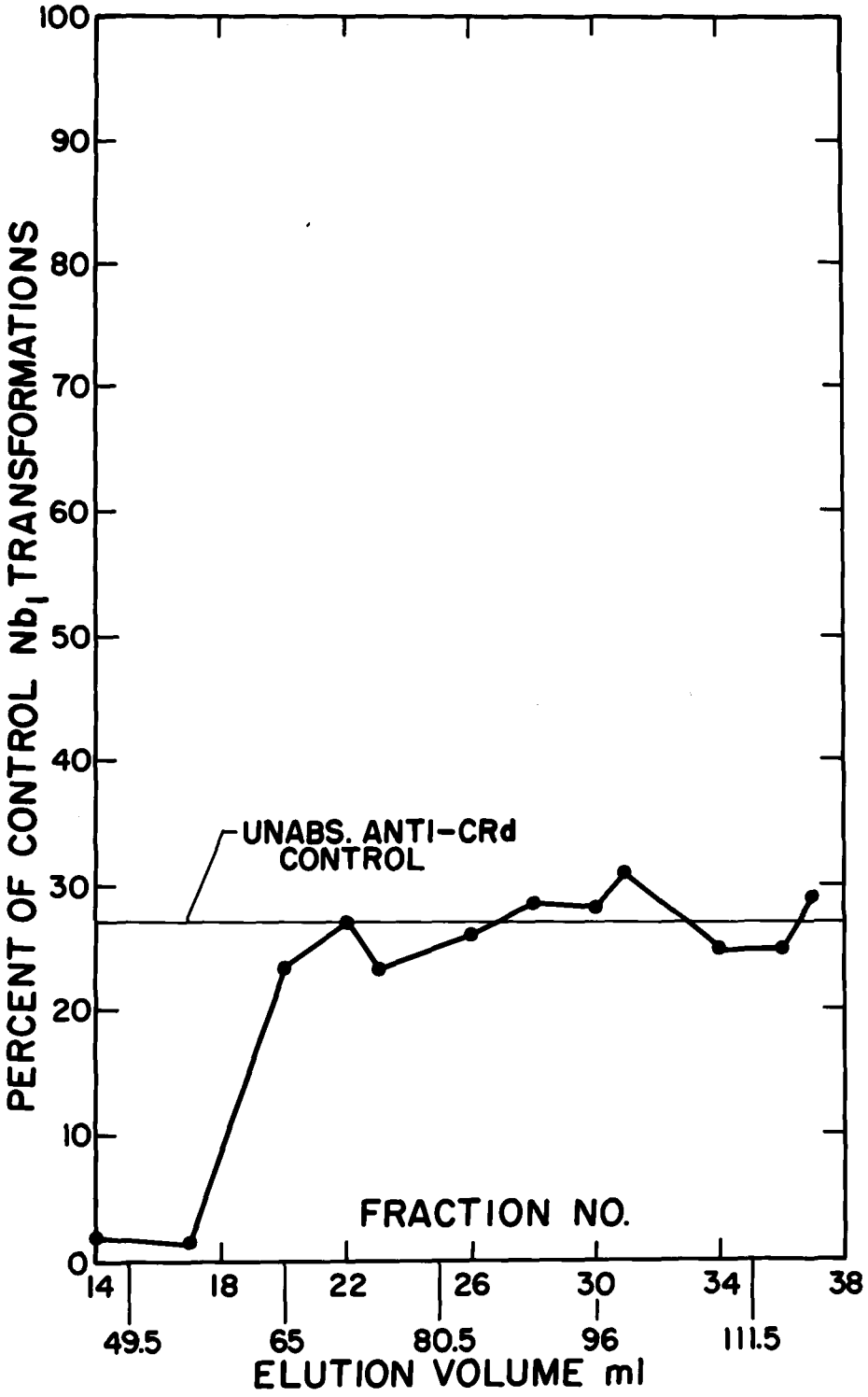

100
90
80
70
60
50
40
30
20
10
0
PERCENT OF CONTROL BOUND ^{14}C-Nb$_1$-DNA
UNABS. ANTI-CRd
CONTROL
FRACTION NO.
20
24
28
32
36
40
44
56
70
84
98
112
ELUTION VOLUME ml

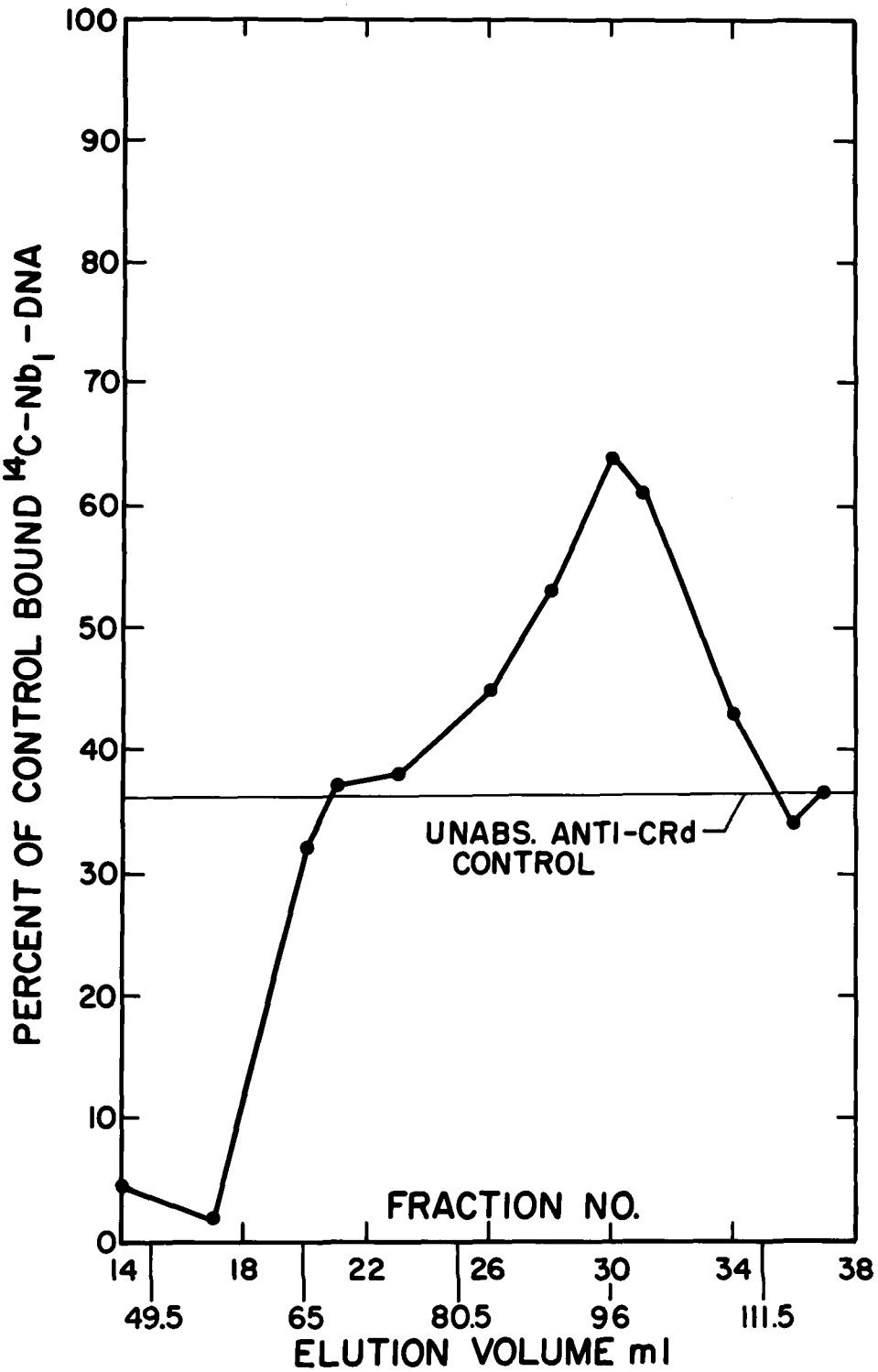
as determined by the procedure outlined in the Sephadex handbook (Beckman Hansson AB, 1966).

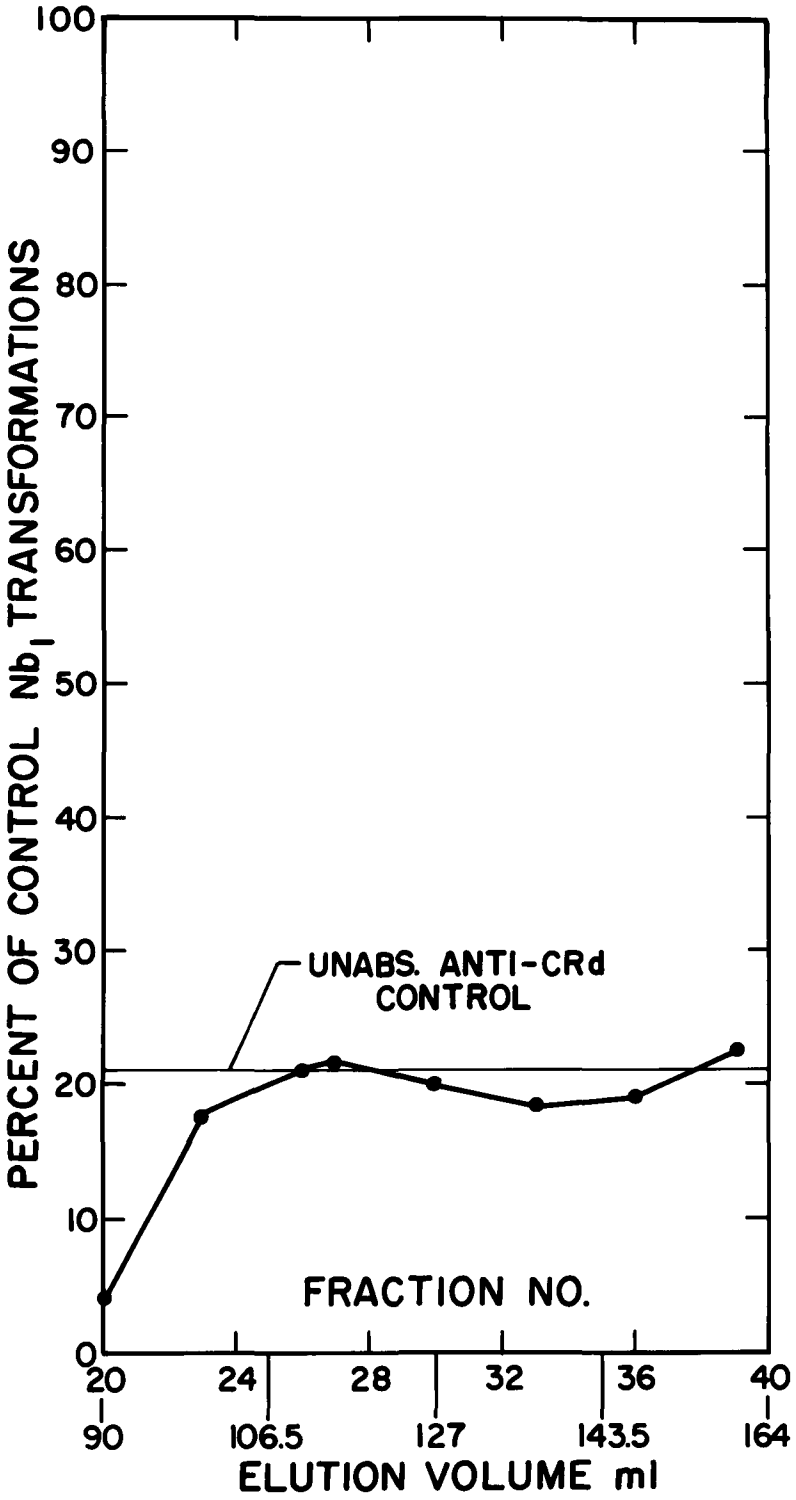
There was no absorption of anti-transformation activity from anti-CRd serum by material from disrupted non-competent cells (Figure 18), but there was moderate absorption of anti-DNA binding activity (Figure 19). The peak activity of anti-DNA binding absorption is indicated by an arrow on the elution profile (Figure 13). The elution volumes for the peak of absorption were about the same for competent and non-competent disrupted cells (about 96-100 ml). The capacity to absorb anti-DNA binding activity from anti-CRd serum was about 50% less for the fractions from non-competent cells as compared to those from competent cells. The fractions from the non-competent cells were concentrated 8-fold, and those from the competent cells were concentrated about 6-fold. The soluble antigens released from competent cells had significantly more anti-DNA binding absorbing capacity than those from non-competent cells.

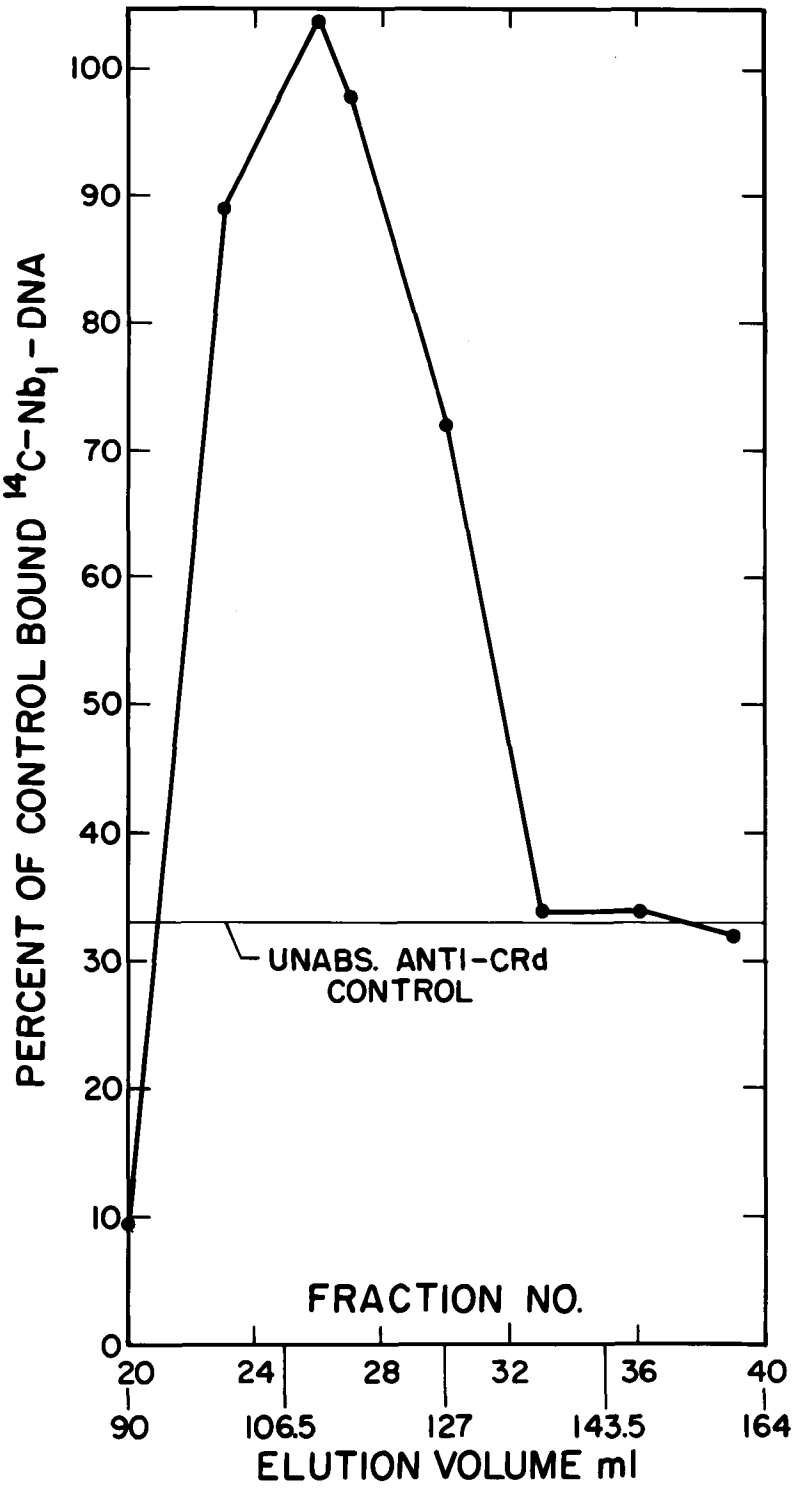
The Sephadex G-100 fractions of glass bead-disrupted competent Rd cells were concentrated 8-fold and assayed for antibody absorbing activity. There was no absorption of anti-transformation activity from anti-CRd serum (Figure 20), but there was strong absorption of anti-DNA binding activity (Figure 21). The peak of anti-DNA binding absorption is indicated by an arrow on the elution profile shown in Figure 14. The approximate molecular weight was calculated to be about 50,000 (see the Appendix), which corresponds to that calculated for the G-200 peak absorption.

There was strong inhibition of DNA binding and transformation


PERCENT OF CONTROL Nb1 TRANSFORMATIONS
100
90
80
70
60
50
40
30
20
10
0
UNABS. ANTI-CRd
CONTROL
FRACTION NO.
14
18
22
26
30
34
38
49.5
65
80.5
96
111.5
ELUTION VOLUME ml


PERCENT OF CONTROL BOUND ^{14}C-Nb_1-DNA
100
90
80
70
60
50
40
30
20
10
0
UNABS. ANTI-CRd CONTROL
FRACTION NO.
14
18
22
26
30
34
38
49.5
65
80.5
96
111.5
ELUTION VOLUME ml


100
90
80
70
60
50
40
30
20
10
0
PERCENT OF CONTROL Nb1 TRANSFORMATIONS
UNABS. ANTI-CRd
CONTROL
FRACTION NO.
20
24
28
32
36
40
90
106.5
127
143.5
164
ELUTION VOLUME ml


PERCENT OF CONTROL BOUND ^{14}C-Nb_1-DNA
100
90
80
70
60
50
40
30
20
10
0
UNABS. ANTI-CRd CONTROL
FRACTION NO.
20
24
28
32
36
40
90
106.5
127
143.5
164
ELUTION VOLUME ml

by the first few fractions from the columns (Figures 16-21). This was attributed to DNA from the disrupted cells. When the remaining fractions from each column were assayed on competent cells without antiserum, they had no effect on transformation or DNA binding.

These results are interpreted to mean that material related to DNA binding is soluble and released (all or in part) from the cell by disruption. The material involved in subsequent steps of DNA uptake after binding remains securely attached to the cell wall material after disruption of the cell.

DISCUSSION

Simple diffusion is ruled out as a possible DNA uptake mechanism by a calculation which shows that, when the concentration of DNA on the outside of the cell is 10 molecules/ml, the concentration on the inside of the cell is 1×10^{13} molecules/ml (Barnhart, 1962). Active transport must be an integral part of getting large molecules of DNA (1.5×10^{7} molecular weight) across the cell wall and membrane of *H. influenzae* cells. Evidence for active transport comes from the fact that inhibitors of the cell's energy production stop the uptake of transforming DNA (Fox and Hotchkiss, 1957; Barnhart and Herriott, 1963; Young and Spizizen, 1963). However, active transport requires more than just energy. It also requires some structural moiety, usually protein in nature (Stein, 1967; Pardee, 1968), to mediate the transport.

The involvement of mediating-proteins in DNA uptake is evidenced by the requirement for protein synthesis during the development of competence. Inhibitors of protein synthesis such as chloramphenicol (Stuy, 1962), erythromycin, novobiocin, and streptomycin (Ranhand and Lichstein, 1969) inhibit the development of competence at concentrations where the viability is unaffected.

Genetic evidence points to the probable existence of DNA uptake sites on competent *H. influenzae* cells. Mutants of transformation have been found for various steps in the uptake process. Some mutants, e.g., mutant number 74 (Table 1), bind significant amounts of DNA, but they are unable to integrate or express the bound DNA. On the other

hand, mutant number 67 (Table 1) neither binds transforming DNA nor takes it up for integration and expression. There have been several classes of mutants described by Caster *et al.* (1969) that show deficiencies at different steps of transformation. These genetic mutations indicate that there are probably a number of components involved in the entire mechanism of transformation.

The genetic evidence, along with the finding that DNA acted to inhibit the action of NEM on competent cells (Appendix B), gave strong support to the hypothesis that some sort of surface material is responsible for the uptake of transforming DNA. Further support for this idea came from results showing that competent cells are unique in stimulating antisera which inhibit DNA binding and transformation. Because the antisera had no effect on transforming DNA, the effects must have been exerted on the cell, specifically on the cell surface, since antibodies do not penetrate bacterial cells (Pardee and Watanabe, 1968). It was concluded that the inhibiting component in the rabbit antiserum was antibody because it was in the gamma globulin fraction, was insensitive to trypsin (Table 4), was digested into active non-agglutinating forms by papain (Table 7), was heat-sensitive only at high temperatures (Table 8), and gave linear dose-response curves (Figure 1). Furthermore, the inhibitory antiserum was produced only in response to competent cell injections (Table 2). These experimental results showed that surface uptake sites for transforming DNA do exist on competent cells.

It was not understood why inhibition of transformation should be 4 times stronger than inhibition of irreversible DNA binding

(Table 2) or why the slopes on the dose-response curves (Figure 1) should be different for inhibition of DNA binding and transformation. It was also puzzling why the inhibition of transformation as a function of time (Figure 3) should have a two-phase character. These results did suggest that there may be a group of antibodies against DNA binding and another group against some uptake step subsequent to binding. In which case, the event of transformation would depend on both processes (binding and uptake) being functional; consequently, transformation would be more susceptible to antiserum inhibition than would irreversible DNA binding.

If the system only needed irreversible binding for DNA uptake, then one would expect absorption of anti-DNA binding activity to be reflected to about the same degree in absorption of anti-transformation activity. Such is not the case. Absorption of anti-CRd serum with competent cells resulted in more absorption of anti-transformation activity than anti-DNA binding activity (Figures 4 and 5).

The idea of multiple steps being involved in DNA uptake is also supported by Erickson, Young, and Braun's (1969) experiment using anti-DNA serum to block transformation. They found that irreversibly-bound transforming DNA is still susceptible to anti-DNA serum inactivation. This is interpreted to mean that irreversibly-bound DNA remains on the cell surface, susceptible to anti-DNA antibodies, before it is taken into the cell and integrated. The conclusion made from these experimental results is that irreversible binding is only a part of the active uptake mechanism for transforming DNA.

When anti-CRd serum was absorbed with non-competent Rd cells

(Figures 4 and 5), it was expected that little or no absorption of antibody against DNA binding or transformation would occur, since non-competent cells do not stimulate the production of either of these two groups of antibodies (Table 2). When the experiment was done, very little absorption of anti-DNA binding antibody occurred (Figure 4), but nearly 50% of the anti-transformation activity was absorbed (Figure 5). These results indicated one of two things: (1) either non-competent cells have part of the DNA uptake mechanism at all times, or else (2) the L-valine block only prevented part of the DNA uptake mechanism from developing. To answer this question one must know what L-valine does to the cell and what is necessary for a cell to be completely uninducible to competence.

L-Valine blocks metabolism of L-isoleucine in *E. coli* (Manten and Rowley, 1953; Cohen, 1958), *Salmonella typhimurium* (Glanville and Demerec, 1960; Wagner and Bergquist, 1960), and *Neurospora crassa* (Bonner, 1946). The major effect of L-valine is interference with the metabolism of L-isoleucine (Leavitt and Umbarger, 1962), but a secondary action may be the production of faulty proteins (Cohen, 1958). The elucidation of L-valine inhibition in *H. influenzae* has not been worked out. However, it is known that the addition of L-isoleucine to M-II medium reverses the L-valine effect on the normal development of competence (Spencer and Herriott, 1965).

Haemophilus influenzae cells that are grown in the presence of inosine and lactate are potentially capable of becoming competent, while cells grown in the absence of these two nutrients cannot develop competence (Ranhand and Herriott, 1966; Ranhand, 1969 and 1970). According to Ranhand's (1969) model for the role of inosine and lactate, inactive

sites are formed during the growth phase for uptake of DNA, and then the sites are modified to an active state by subsequent incubation in M-II medium. If this model is true, then cells blocked in their development of competence by L-valine during incubation in M-II medium have part of the uptake mechanism in an inactive form.

The effect of L-valine, the necessity of inosine and lactate for competence, and the absorption of anti-transformation antibody by non-competent cells indicate that the sites are at least partly present on the non-competent cells used for this research. The addition of L-valine during the incubation period in M-II medium probably blocked L-isoleucine metabolism which is, in some way, necessary for modification of the DNA-uptake sites into active forms. The inactive uptake sites acted like complex haptens by absorbing antibody against part of the active uptake mechanism but were not themselves able to stimulate antibody production that would inhibit DNA binding or transformation.

The antibody experiments support the hypothesis of multi-step uptake sites on or near the surface of competent *H. influenzae* cells. With antisera against DNA-uptake sites, it became possible to fractionate the cells and to assay the cellular fractions for absorption of anti-DNA binding and anti-transformation antibodies. This gave indirect but substantial evidence that more than one component is involved in uptake of transforming DNA.

When cells were treated with EDTA, small fragments of the cell wall were released. These fragments absorbed anti-transformation antibodies very well (100% in some experiments) but only absorbed a small fraction of the anti-DNA binding antibodies (Figures 7 and 8). This

indicated that antigens involved in steps subsequent to DNA binding were tightly bound to the cell wall but were separate from antigens against anti-DNA binding antibodies. These results were supported by the finding that cell walls from glass bead-disrupted cells behaved in the same way [e.g., they absorbed anti-transformation antibodies and not anti-DNA binding antibodies (Figures 9 and 10)].

The antigens against anti-DNA binding antibodies were found in the 50,000 molecular weight group of soluble material released into the supporting buffer from glass bead disruption of cells (Figures 17, 19, and 21). These results supported earlier conclusions about there being more than one component to the active DNA-uptake system. It was concluded from these experimental results that there were at least two separate functional structures involved in DNA uptake. One was involved in binding DNA to the cell irreversibly, and the other was involved in some uptake step subsequent to binding.

The uptake of transforming DNA occurs in at least three separate steps. First is the non-energy requiring reversible binding of DNA (Barnhart and Herriott, 1963) which is an ionic attraction between the DNA molecules and competent cells. The second is an energy requiring binding step during which the DNA becomes deoxyribonuclease-insensitive. This step seems to be associated with material described in Figures 17, 19, and 21 which is released by cellular disruption in a soluble 50,000 molecular weight form. The third is an uptake step that is subsequent to irreversible binding and is responsible for moving the DNA molecule into the competent cell. This step seems to be associated with cell wall material that is not released by cellular

disruption (Figures 8 and 9) but is attached to wall fragments released from cells treated with EDTA (Figure 8).

Various investigators (Barnhart, 1962b; Bingham, 1970) have tried unsuccessfully to get competent cell walls to bind transforming DNA. The lack of success in these attempts may have been due to release and removal of material involved in binding DNA from the cell wall preparation. One may now be able to perform this experiment by adding the soluble material that absorbs anti-DNA binding antibodies to the cell wall preparation before trying to get DNA to bind to the cell walls.

Once these antigens that absorb with anti-transformation and anti-DNA binding antibodies have been isolated free of other cellular antigens, it will be possible to make antiserum against them individually to see if they stimulate antibody production specific for their individual step in DNA uptake. Once this is accomplished, it should be possible to conjugate the antibodies they stimulate with ferritin or fluorescein, to let them attach to their specific site on bacterial cells, and to observe the attachment sites under the electron or ultraviolet light microscope. This would give a picture of where the uptake sites occur on the cell surface and may elucidate some of the differences between the competent and non-competent state of transformable *H. influenzae*.

If and when it is conclusively shown that the antigens stimulating anti-DNA binding and anti-transformation antibodies are part or all of the actual DNA-uptake site, other systems such as *B. subtilis* and *D. pneumoniae* can be investigated and compared for similarities and

differences in the mechanism for DNA uptake. It would be interesting to know if these sites in various bacterial systems are similar in functional organization. Possible experiments and tests that can be done are many and will involve many years of work to elucidate satisfac-factorily DNA uptake in bacterial transformation. This research has revealed some heretofore unknown features of transformation and succeeded in partially isolating material that is believed to be involved in the mechanism of DNA uptake.

CONCLUSIONS AND SUMMARY

The objectives of this research were to demonstrate the existence on competent *Haemophilus influenzae* cells of specific antigenic sites that stimulate the production of antibody inhibitory to the irreversible binding of DNA and transformation. If such sites were demonstrated, they would be fractionated by centrifugation and column chromatography to identify their cellular location.

Antisera were prepared against competent and non-competent *H. influenzae* cells. The gamma globulin fraction of rabbit serum prepared against competent cells inhibited the ability of other competent cells to undergo transformation by as much as 20-fold and inhibited irreversible binding of transforming DNA by 5-fold. The transforming ability of DNA was not altered by exposure to the inhibitory antiserum. When competent cells were treated with antiserum prepared against non-competent cells, there was no inhibition of transformation or irreversible DNA binding.

The inhibitory antiserum was sensitive to heat (92°C for 10 minutes) and to high (420 μg/ml) concentrations of papain. The antiserum was converted to a monovalent non-agglutinating form by smaller (16 μg/ml) concentrations of papain, but it retained its capacity to inhibit transformation and irreversible DNA binding.

The capacity of the antiserum to inhibit irreversible DNA binding and transformation was absorbed by both competent and non-competent whole cells. When the antiserum was absorbed with cell walls,

the capacity of the antiserum to inhibit transformation was reduced to a much greater extent than its capacity to inhibit irreversible DNA binding. It was later found that small amounts of the material responsible for absorbing the anti-transformation activity could be extracted from the cell walls with non-ionic detergents such as Triton X-100 and Nonidet P-40.

The soluble material released from glass bead-disrupted *H. influenzae* cells was chromatographed by Sephadex gel filtration. The fractions of chromatographed material that eluted around the 50,000 molecular weight range exhibited a strong ability to absorb the anti-DNA binding capacity of the inhibitory antiserum.

It was concluded that all or part of the material responsible for absorbing the capacity of antiserum to inhibit irreversible DNA binding is released from the cell in a soluble form upon mechanical cellular disruption. The material actually responsible for absorbing the anti-DNA binding capacity of the antiserum has a molecular weight of around 50,000 according to estimations made from the gel filtration results. The material involved in absorbing anti-transformation activity is tightly bound to the cell wall and is not solubilized when the walls are separated from other cellular components by mechanical disruption or EDTA treatment.

It is finally concluded that antigenic determinants related to cellular uptake of transforming DNA exist on competent *H. influenzae* cells and that these determinants are only partly present or in a masked form on the non-competent cells. Because of the specificity of antibody-antigen reactions, it is presumed that the material detected

by the antibody absorption assay is directly involved in DNA-uptake sites on the surface of *H. influenzae* cells. The results of this study indicate that the DNA uptake mechanism involves at least two steps subsequent to the initial ionic interaction: one responsible for irreversible binding of the transforming DNA to the cell surface and the other involved in uptake of DNA into the cell for integration into the bacterial genome.

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APPENDIX

Section A — Glossary

A. Anti-CBC200 serum: antiserum prepared against competent *Haemophilus influenzae* BC200 cells.

B. Anti-CRd serum: antiserum prepared against competent *Haemophilus influenzae* Rd cells.

C. Anti-NCBC200 serum: antiserum prepared against non-competent *Haemophilus influenzae* BC200 cells.

D. Anti-NCRd serum: antiserum prepared against non-competent *Haemophilus influenzae* Rd cells.

E. Bacterial transformation: a mechanism of genetic exchange whereby a population of bacteria can obtain genetic information as a result of cellular uptake and integration of extracellular deoxyribonucleic acid.

F. Competent cells: cells that have the ability to bind extracellular DNA irreversibly and subsequently to integrate and express the DNA.

G. HP1Cl phage: a temperate phage that infects *Haemophilus influenzae* Rd and BC200 cells.

H. Irreversibly bound DNA: DNA bound to competent cells that is not removed by DNase or washing with high salt solutions.

I. Low transforming mutants: mutants of *Haemophilus influenzae* Rd or BC200 that have less than a 0.05% frequency of Nb_1 transformation.

J. Reversibly bound DNA: DNA bound to competent cells that can be removed by DNase or washing with high salt solution.

K. "Sevag:" a procedure for deproteinizing nucleic acid preparations with chloroform and octanol as described by Sevag (1938).

L. Transfection: "infection of cells by the isolated nucleic acid from a virus resulting in the production of a complete virus" (Spizizen, Reilly, and Evans, 1966).

M. Transforming ^{14}C-Nb_1-DNA: deoxyribonucleic acid which bears ^{14}C-labeled thymidine bases and the genetic marker for resistance to 2.5 µg/ml of novobiocin.

N. Transforming Nb_1-DNA: deoxyribonucleic acid which bears the genetic marker for resistance to 2.5 µg/ml of novobiocin (Nb_1).

Section B

Cellex E (ECTEOLA) anion exchange resin was prepared to chromatograph material solubilized from competent *Haemophilus influenzae* cell walls. Five g of resin were added to 75 ml of 0.5 N hydrochloric acid (HCl) and allowed to stand at room temperature for 30 minutes. The 0.5 N HCl solution was separated from the resin by filtration on a Buchner funnel fitted with a Whatman No. 1 filter disc. The resin was washed on the filter with 150 ml of distilled water and resuspended in 75 ml of 0.5 N sodium hydroxide (NaOH). After standing at room temperature for 30 minutes, the 0.5 N NaOH solution was separated from the resin by filtration, and the resin was washed with 150 ml of distilled water and resuspended in 75 ml of 1% Triton X-100. The pH of the resin was adjusted to 7.4 with 0.1 N HCl. After the bulk of the resin had settled out of suspension, the fluid was decanted and fresh 1% Triton X-100 solution (pH 7.4) added to the resin. This process was repeated 8 times over a 30-hour period to remove fine particles from the resin.

The Cellex E resin was finally suspended as a thick slurry in 1-2 volumes of 1% Triton X-100, chilled to 2°C (all further manipulations were done at 2°C), and poured into a column. The resin column was 20.0 cm by 1.0 cm. One liter of 1% Triton X-100 (pH 7.4) was passed over the column to allow the flow rate to equilibrate. After the resin column was packed and equilibrated, 2.0 ml of Triton X-100 extract of competent *Haemophilus influenzae* cell walls were added to the column. The resin was eluted with a 500-ml linear NaCl gradient from 0 to 1 M

in 1% Triton X-100. The flow rate was 16 ml/hour, and fractions were collected at 15-minute intervals. Each fraction was assayed for antigens against anti-CRd serum on Ouchterlony plates using a procedure similar to the one described in Figure 15. Three separate antigens were detected that eluted at 0.05, 0.07, and 0.13 M NaCl, respectively. These results indicated that the chromatographic technique using Cellex E was successful in separating some of the extracted antigens. However, the fractionation diluted the antigens against anti-transformation antibody too much to be assayed by the antibody absorption technique. More efficient methods of extracting the antigens will have to be developed before Cellex E chromatographic fractions can be successfully assayed.

Section C

Fox and Kennedy's (1965) procedure for demonstrating M protein in the β-galactoside transport system was modified and used to indicate DNA-uptake sites on competent *Haemophilus influenzae* cells. Cells were made competent in Ca-BHI medium. Two 0.5-ml aliquots of competent cells were taken from the culture. One aliquot was added to 0.9 ml of Ca-BHI containing 1 g of DNA, and the other aliquot was added to 0.9 ml of Ca-BHI not containing DNA. The cultures were incubated for 2 minutes at 35°C and chilled to 10°C to retard the rate of DNA uptake and integration (Barnhart and Herriott, 1963). One-tenth ml of N-ethylmaleimide [NEM (5×10^{-2} M)] was added to both tubes and incubated for 5 minutes at 10°C to permit binding with exposed sulfhydryl groups on the cell surfaces. One-tenth ml of β-mercaptoethanol (5×10^{-1} M) was added to each culture for 2.5 minutes to stop the action of the NEM.

The cells were washed twice at 4°C by centrifugation, resuspended in 0.9 ml Ca-BHI, and incubated at 35°C for 5 minutes. One-tenth ml of 1 mg/ml DNase in 0.1 M $MgSO_4$ was added to each culture and incubated 5 minutes to effect destruction of reversibly bound DNA and to allow the cells to free the irreversible binding sites by moving bound DNA toward completion of the uptake process.

The cultures were centrifuged, resuspended in 1.0 ml of saline containing 0.5 μc of ^{14}C-labeled NEM. The cells were incubated with the ^{14}C-labeled NEM at 10°C for 5 minutes, washed 3 times by centrifugation, and resuspended in 1.0 ml of saline. A 0.1-ml sample

of each culture was dried on a filter disc pretreated with 5% TCA. The discs were washed in cold 5% TCA 3 times to remove any unreacted ^{14}C-labeled NEM and dried. The ^{14}C-labeled NEM that had reacted with cellular sulfhydryl groups was measured by counting the radioactivity from the ^{14}C-label in a Packard Model 3320 Tri-Carb scintillation spectrometer.

The cells that were treated with DNA prior to exposure to unlabeled NEM took up 11% more ^{14}C-labeled NEM than cells that were not treated with DNA. This is interpreted to mean that the DNA acted to prevent the NEM from attacking some of the sulfhydryl groups on the cell. Once the cell had taken up the DNA, the sulfhydryl groups that were protected by the DNA became exposed so that ^{14}C-labeled NEM could react with them.

Section D

The buffer containing soluble cellular material from glass bead-disrupted *Haemophilus influenzae* cells was chromatographed on Sephadex G-200 and G-100 gel columns. The molecular weight was estimated for the point on the elution profile showing the peak absorption of anti-DNA binding antibody. The estimation of molecular weight was made by calculating the partial coefficient between the liquid phase and the gel phase (K_{av}) as outlined on page 49 of the Sephadex handbook (Beckman Hansson, 1966) and comparing the K_{av} values to their corresponding molecular weights (Beckman Hansson, 1966, p. 13). The molecular weight of absorbing material from competent cells chromatographed on Sephadex G-200 (Fig. 17) was estimated from the following data:

1. Bed volume (V_t) = 143 ml
2. Void volume (V_o) = 45 ml
3. Elution volume (V_e) = 98 ml
4. $K_{av} = \frac{V_e - V_o}{V_t - V_o} = 0.54$
5. On Sephadex G-200, a K_{av} value of 0.54 corresponds to a molecular weight of approximately 50,000

The molecular weight of absorbing material from competent cells chromatographed on Sephadex G-100 (Fig. 12) was estimated from the following data:

1. V_t = 235 ml
2. V_o = 71 ml

3. V_e = 114 ml
4. $K_{av} = \frac{V_e - V_o}{V_t - V_o} = 0.26$
5. On Sephadex G-100, a K_{av} value of 0.26 corresponds to a molecular weight of around 50,000

The molecular weight of absorbing material from non-competent cells chromatographed on Sephadex G-200 (Fig. 19) was estimated from the following data:

1. V_t = 143 ml
2. V_o = 45 ml
3. V_e = 98 ml
4. $K_{av} = \frac{V_e - V_o}{V_t - V_o} = 0.52$
5. On Sephadex G-200, a K_{av} value of 0.52 also corresponds to a molecular weight of around 50,000

BACTERIAL RECEPTOR SITES FOR UPTAKE OF TRANSFORMING DNA

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ABSTRACT

Antisera prepared against competent and non-competent *Haemophilus influenzae* cells were compared as to their effect on DNA binding and transformation. The gamma globulin fraction of rabbit antiserum prepared against competent cells inhibited irreversible binding of DNA and transformation to 5 and 20% of the control values, respectively, while antisera prepared against non-competent cells had no such effect. The kinetics, dose-response, and sensitivity to heat and enzymatic digestion of the antibody were studied. Material of about 50,000 molecular weight from the aqueous portion of glass bead-disrupted cells absorbed the anti-DNA binding capacity of the inhibitory antiserum. Material bound to cell wall fragments absorbed the ability of the antiserum to inhibit transformation. It is concluded that antigenic determinants related to cellular uptake of transforming DNA exist on competent *H. influenzae* cells but are only partially present or in a masked form on non-competent cells. These antigenic determinants are presumed to be part of the DNA uptake mechanism due to the specificity of antibody-antigen reaction.

COMMITTEE APPROVAL: _____

VITA

Douglas Pierre Bingham