ability to elute all of the antibodies from the beef cell antigen is probably related to a difference in the avidity of the heterophile antibody molecules(13).

It was also observed that on occasion, small amounts of IgG were adsorbed to the beef cell antigen, necessitating further purification by sucrose density gradient ultracentrifugation. The purified material had the immunological characteristics of heterophile antibody reported by other workers(1-3). However, the lability of this material has proven to be a problem, as noted by others for certain 19S immunoglobulins(14). The stability of our preparation was found to be greatest in 50% glycerin or 30% sucrose at 4°C.

Summary. Specific heterophile antibody was prepared by adsorption to beef erythrocytes and elution with diethyl ether. Using a formula based on the antigen-antibody relationship permitting maximum elution, up to 10% of the antibody was recoverable. The eluted material, further purified by sucrose density gradient ultracentrifugation, had the characteristics of heterophile antibody. The material was found to be unstable at −20°C or 4°C storage, but was relatively stable in 30% sucrose and 50% glycerin at 4°C.

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Influence of Prior Sensitization with Mycobacteria on Antibody Response To Protein Antigen in Freund's Adjuvants. (31779)

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The wide use of water-in-oil emulsions with or without killed mycobacteria to enhance the immune response reflects the general acceptance of the Freund-type preparations as effective and potent immunologic adjuvants. In general, injection of an antigen in a water-in-oil emulsion (Freund's incomplete adjuvant) results in antibody production greater than that obtained by injection of the aqueous antigen alone and an even greater response is obtained when various mycobacteria are added to the emulsion (Freund's complete adjuvant). However, the antibody enhancing effect of such adjuvants may be altered by the immunization regimen. Weigle and his associates(1) found that the effect of the complete adjuvant on production of precipitating antibody to a constant amount of protein antigen in guinea pigs was no greater than that of the incomplete when the preparations were administered in multiple, large volume, doses. In contrast, the complete adjuvant had an extreme enhancing effect when an equal amount of antigen was given in a single, small volume, injection. Because the mycobacteria themselves function as an antigenic stimulus and sensitize the host to tuberculin, the possibility exists that prior sensitization with mycobacteria may also influence the antibody en-

hancing effect of the complete adjuvant. It seems reasonable to believe that the previously-sensitized host may exhibit an accelerated inflammatory response to the mycobacteria in the complete adjuvant and, consequently, its response to the antigenic stimulus may differ from that of the nonhypersensitive host. The purpose of this report is to describe a comparison of the antibody response in tuberculin-positive and tuberculin-negative guinea pigs to a soluble protein antigen when administered in either the complete or the incomplete Freund adjuvant preparation.

Material and methods. Sensitization and immunization. Twenty, male, albino, Hartley strain guinea pigs (520-530 g) were divided into 4 equal groups. Each animal of Groups II and IV was rendered hypersensitive to tuberculin by intramuscular injection of 5 mg (dry weight) of heat-killed *M. tuberculosis* (H37Rv strain) suspended in 1 ml of incomplete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Each animal in Groups I and III received 1 ml of incomplete adjuvant intramuscularly. Two weeks later, all animals were injected intradermally with 5 μg of PPD (Merck, Sharp and Dohme, West Point, Pa.); the diameter of induration at the skin test site was measured 48 hours later. Skin reactions of approximately 10 mm were recorded for animals of Groups II and IV; all animals of Groups I and III were negative. Two weeks after tuberculin testing, all animals were immunized with bovine γ globulin (BGG, Fraction II, B grade, Pentex, Inc., Kankakee, Ill.). A 15% solution of the BGG antigen in 0.85% sodium chloride, sterilized with ethylene oxide, was diluted with equal volumes of sterile incomplete or complete Freund's adjuvants (Difco) to prepare the immunizing materials. Each animal of Groups I and II received a single injection containing 15 mg of BGG in 0.2 ml of incomplete adjuvant while those of Groups III and IV received the same amount of antigen in an equal volume of Freund's complete adjuvant. The immunizing material was administered intramuscularly in the hind leg opposite that used for the sensitizing injection. All animals were bled by cardiac puncture at 3 and 7 weeks after BGG injection; the sera were stored at —20°C for subsequent study.

Serologic tests. All sera were analyzed for complement-fixing and precipitating antibodies, the latter by the Ouchterlony double diffusion technique and by immunoelectrophoresis. A sterile 2% solution of the BGG antigen in 0.85% sodium chloride served as a stock for all serologic tests; the material was stored at —20°C and diluted with saline as required. For agar diffusion tests, Petri plates containing 15 ml of 1% Ionagar No. 2 (Consolidated Laboratories, Chicago Heights, Ill.) in 0.85% sodium chloride were prepared. Sample wells were cut in the hardened agar using a plastic template. The center well was filled with BGG (2.5 mg/ml) which was diffused against undiluted antisera in the peripheral wells. The plates were incubated at 5°C for 4 days in a humid chamber and examined daily.

For immunoelectrophoretic analyses, samples were separated in a zone electrophoresis apparatus (Buchler Instruments, Inc., Fort Lee, N. J.) and then allowed to diffuse against specific reactants to form precipitin bands at 5°C for 4 days. Electrophoretic separation was conducted in an agar gel supported on 2 × 3 inch glass slides; a discontinuous barbital buffer system (pH 8.6) was used. Each slide was prepared with 4 ml of a buffer-agar solution which contained 0.55 g barbital, 3.50 g sodium barbital, 0.51 g calcium lactate, and 10 g Ionagar No. 2 per liter of distilled water. Exact placement of sample wells and troughs in the agar gel was accomplished with the aid of an agar cutter (Buchler). The buffer solution used in the electrode vessels contained 1.38 g barbital, 8.76 g sodium barbital, and 0.38 g calcium lactate per liter of distilled water. The BGG antigen (2.5 mg/ml) was separated at a current of 10 ma for 105 minutes while undiluted antisera were separated at 12.5 ma for the same time; all separations were performed at room temperature. Precipitin bands were developed by diffusion of undiluted antisera against the separated antigen or by diffusion of BGG (1 mg/ml) against the separated antisera. Rabbit antiserum to guinea

pig γ globulin (Hyland Laboratories, Los Angeles, Calif.) was used to confirm adequate separation of the guinea pig sera. After precipitin bands had developed, slides were washed for 48 hours in 1% sodium chloride and for 3 hours in distilled water, heat-dried, and stained for protein with amido-schwarz 10B(2).

For complement fixation, antisera were heated at 56°C for 30 minutes on the day of testing and quadruplicate, 2-fold, serial dilutions in 0.85% sodium chloride containing 0.01% magnesium sulfate were prepared. Each tube contained 0.2 ml of diluted antiserum and then received 0.5 ml of the saline solution. Two tubes of each dilution served as controls and received an additional 0.2 ml of saline; each of the remaining 2 tubes received 0.2 ml of saline which contained 0.5 mg BGG. After incubation at 56°C for 30 minutes and cooling to room temperature, 1 ml of guinea pig complement (Baltimore Biological Laboratories, Baltimore, Md.) which contained 2 hemolytic units/ml was added to each tube. Following overnight incubation at 5°C and warming to room temperature, each tube received 0.5 ml of 1:3000 anti-sheep hemolysin (BBL) and 0.5 ml of a 2% suspension of washed sheep erythrocytes (BBL). The tubes were examined for hemolysis after incubation for 60 minutes at 37°C; the titer of complement-fixing antibody was defined as the highest dilution of antiserum showing no visible hemolysis. Appropriate controls for the reaction and indicator systems were included in each test series.

Results. An attempt was first made to compare qualitatively the response of tuberculin-positive and tuberculin-negative guinea pigs to BGG administered in either complete or incomplete Freund's adjuvant. For this purpose, precipitins against BGG were analyzed by the agar diffusion method. A typical precipitation pattern is shown in Fig. 1 in which pooled 7-week serum samples were diffused against BGG. It appeared that the groups immunized with BGG in the complete adjuvant (III and IV) responded more intensely than those receiving BGG in incomplete adjuvant (I and II), both on the basis of the number of precipitin bands and the density of the precipitate. However, no obvious difference between tuberculin-negative and tuberculin-positive animals was seen with either immunizing preparation (Group I *vs* II and Group III *vs* IV). The 3-week serum samples exhibited similar patterns but were less intense.

The precipitin response to the BGG antigen also was analyzed by the immunoelectrophoretic technique to ascertain that the more intense response of the groups receiving complete adjuvant was confined to the principal components of the BGG antigen and not in response to other bovine serum components that may have been present in the antigen as contaminants. A typical immunoelectrophoretic pattern is shown in Fig. 2 in which the BGG antigen was separated electrophoretically and diffused against pooled 7-week antisera. Analysis of the sera by this method indicated that there was no qualitative difference in response to the antigen although the obviously more intense response in the animals receiving BGG in the complete adjuvant remained (Groups III and IV). There was no indication of a response in these groups to bovine serum components other than the γ globulins. It should be noted that animals receiving BGG in incomplete adjuvant (Groups I and II) formed antibodies against the same components of BGG as did the groups receiving the complete adjuvant preparation, but to a lesser degree.

The results of the precipitin analyses suggested that the groups receiving the complete adjuvant preparation produced a quantitatively greater response than those receiving the incomplete adjuvant preparation. This impression was confirmed using the complement-fixing titer of the antisera as a relative measure of the antibody response (Table I). The characteristic antibody enhancing effect of the mycobacteria was clearly shown by the significantly higher ($P<0.01$) titers at 3 and 7 weeks in the tuberculin-negative animals receiving BGG in complete adjuvant (Group III) when compared to their controls which received the same dose of BGG in incomplete adjuvant (Group I). A similar comparison of the tuberculin-positive animals

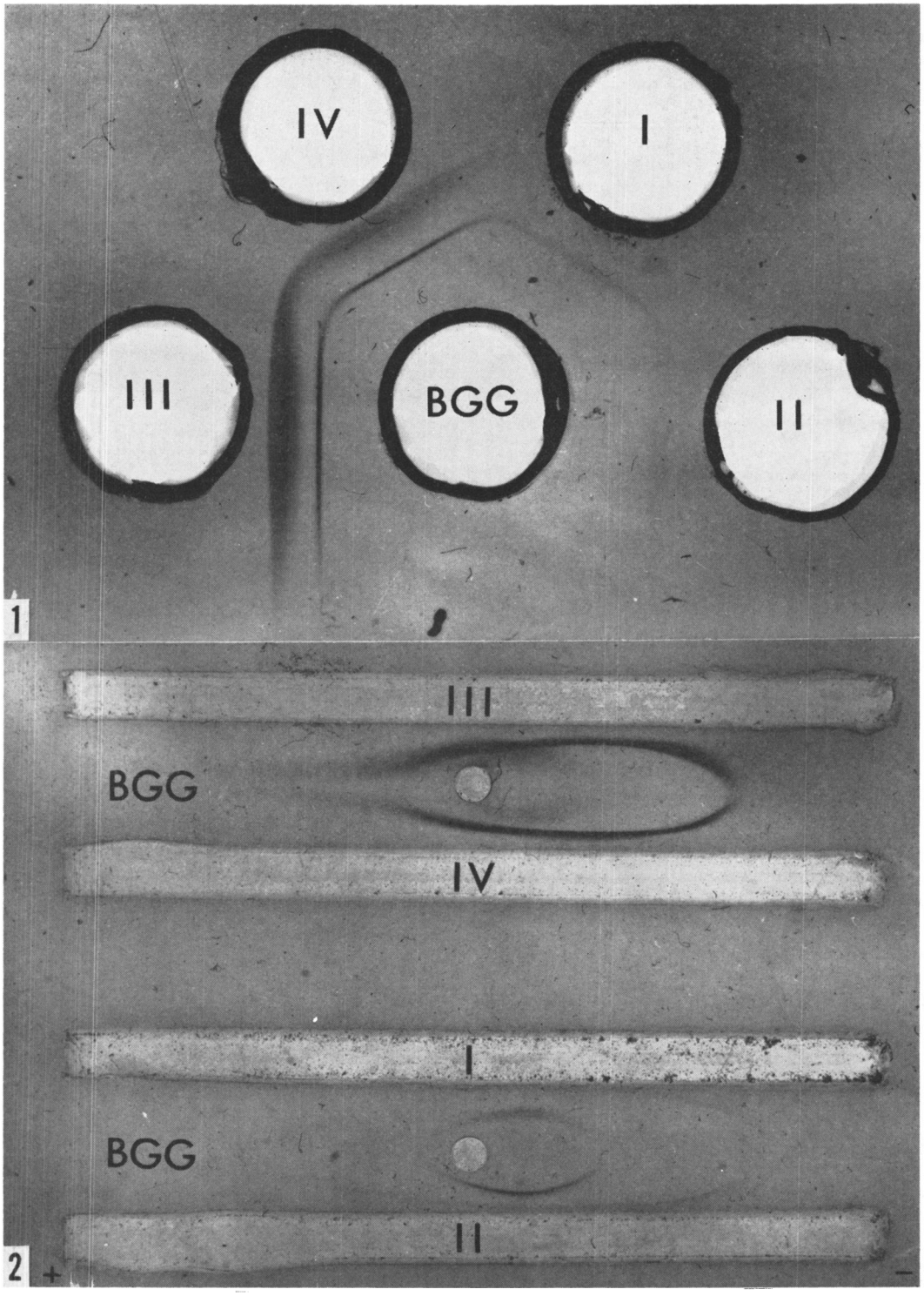

FIG. 1. Influence of Freund's adjuvants on precipitin response to bovine gamma globulin in tuberculin-negative and tuberculin-positive guinea pigs. (BGG in center well; pooled 7 week sera representative of experimental groups in peripheral wells.)

FIG. 2. Influence of Freund's adjuvants on precipitin response to bovine gamma globulin in tuberculin-negative and tuberculin-positive guinea pigs. (BGG in wells; pooled 7 week sera representative of experimental groups in troughs.)

TABLE I. Influence of Prior Sensitization with Mycobacteria on Guinea Pig Complement-Fixing Antibody Response to Bovine Gamma Globulin in Complete and Incomplete Freund's Adjuvant.

Guinea pig group	Anti-BGG titer* at 3 weeks	7 weeks
I (PPD—, BGG in inc. adjuvant)	0.0	0.5 ± 0.6
II (PPD+, BGG in inc. adjuvant)	1.6 ± 1.5	2.2 ± 1.6
III (PPD—, BGG in comp. adjuvant)	3.6 ± 1.7	5.5 ± 1.0
IV (PPD+, BGG in comp. adjuvant)	1.8 ± 2.4	5.0 ± 0.7

* Values represent mean ± standard deviation of the reciprocal of the titer (expressed as $\log_2$) for sera collected 3 and 7 weeks after BGG injection.

showed a significantly higher titer at 7 weeks, but not at 3 weeks, in those receiving the complete adjuvant. There was no significant difference when the influence of prior sensitization with mycobacteria was examined in the groups receiving either the complete adjuvant (Group III *vs* IV) or the incomplete adjuvant (Group I *vs* II). However, although comparison of Groups I and II indicated a difference of only questionable significance ($P = 0.05$), the consistently higher titers in Group II appeared suggestive that the mycobacteria used for the sensitizing injection may have served as an adjuvant when the BGG was administered 4 weeks later. The possibility seemed to be supported by close inspection of the precipitation patterns shown in Fig. 1 and 2; the density of the precipitates obtained with the Group II sera appeared greater than that of the Group I sera in both cases.

Immunoelectrophoretic analyses of the antisera also were performed to compare the anti-BGG immunoglobulin response of the various groups. This approach seemed pertinent because it has been reported(3,4) that two distinct types of precipitating antibody (γ_1 and γ_2) are formed 3 weeks after injecting guinea pigs with a soluble protein antigen in the complete adjuvant preparation. Only the γ_1 type is formed when the antigen is administered in incomplete adjuvant. The precipitation patterns obtained after electrophoretic separation and diffusion of representative 3-week antisera against BGG are shown in Fig. 3. The results of such analyses performed on all sera indicated that the Group I animals formed only the γ_1 type of anti-BGG precipitin while the majority of the animals in Groups II, III, and IV responded with both γ_1 and γ_2 anti-BGG immunoglobulins.

Discussion. The results of the present study demonstrated that prior sensitization with mycobacteria had no qualitative or quantitative effect on the antibody response to a protein antigen when administered to guinea pigs in Freund's complete adjuvant. The tuberculin-positive animal (Group IV) appeared to respond to the same antigenic determinants of the BGG antigen (Fig. 1 and 2) to the same degree (Table I) and synthesized the same type precipitating antibodies (Fig. 3) as the tuberculin-negative animal (Group III). In contrast to the complete adjuvant, sensitization with mycobacteria appeared to enhance the response to the BGG antigen when administered in the incomplete adjuvant. Although animals receiving the incomplete preparation (Groups I and II) responded to the same determinants of the antigen, there was an indication that the tuberculin hypersensitive animal (Group II) produced more antibody (Fig. 1 and 2, Table I) than the unsensitized animal (Group I).

The formation of γ_1 and γ_2 anti-BGG precipitins (Fig. 3) and the higher anti-BGG complement-fixing titer (Table I) in the Group II hypersensitive animals suggest that the mycobacteria remaining from the sensitizing injection may have provided these animals with the equivalent of the complete adjuvant when the BGG antigen in incomplete adjuvant was injected. The implication that the mycobacteria of the sensitizing injection functioned in an adjuvant role in this case seems justified when the general characteristics of the immune response to an antigen in complete adjuvant are considered. White(4) listed the formation of both γ_1 and γ_2 types of precipitating antibody and the induction of delayed-type hypersensitivity as essential features. The former was observed in the

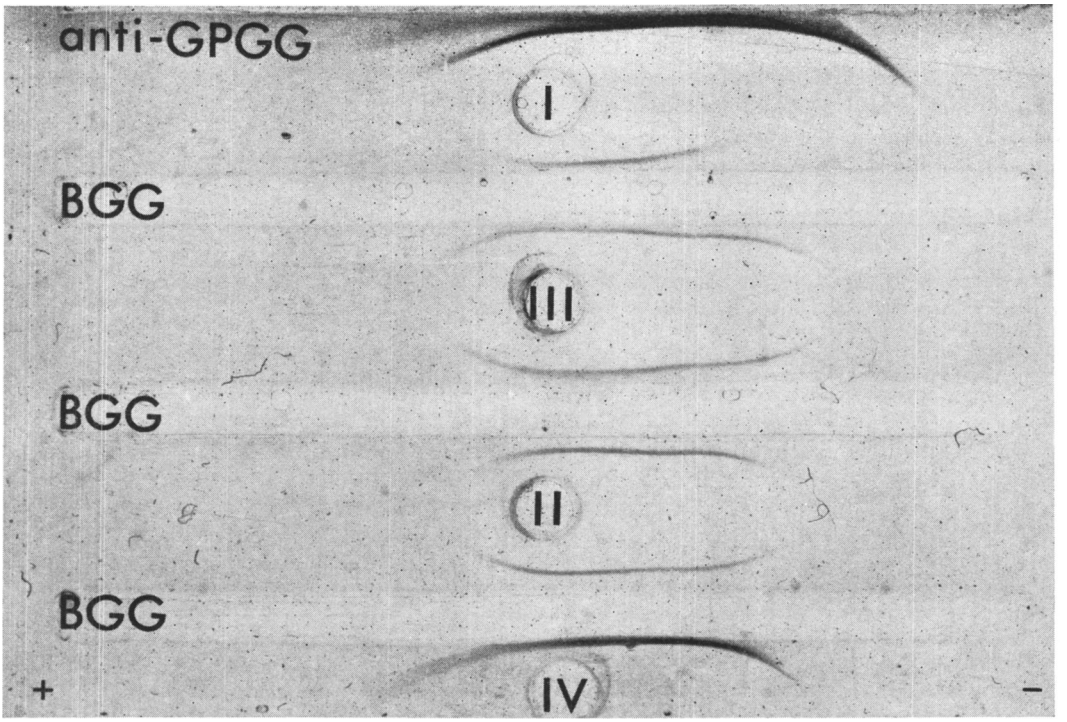

FIG. 3. Influence of Freund's adjuvants on immunoglobulin response to bovine gamma globulin in tuberculin-negative and tuberculin-positive guinea pigs (Anti-guinea pig γ globulin in top trough, BGG in other troughs; 3 week sera representative of experimental groups in wells.)

Group II animals, as well as in animals which received BGG in the complete adjuvant (Groups III and IV). The latter characteristic has been demonstrated previously in an apparently analogous experimental model by Dienes and Schoenheit(5) and confirmed by Hanks(6). They found that delayed-type hypersensitivity to soluble protein antigens could be induced in guinea pigs by injecting the material into or near a lesion containing tubercle bacilli; injection of the antigen into a distant site or into inflammatory lesions caused by other irritants elicited the development only of immediate-type hypersensitivity. These findings and those of the present study provide reasonable evidence for the conclusion that the mycobacteria introduced previously in the host functioned in an adjuvant capacity.

The results of the present study thus indicate that, although prior sensitization with mycobacteria may have no apparent qualitative or quantitative influence on the response of guinea pigs immunized with a protein antigen in Freund's complete adjuvant, prior sensitization may provide the host with the capacity to respond to an antigen administered in Freund's incomplete adjuvant in much the same manner as if the antigen were administered in the complete adjuvant preparation. Furthermore, it is likely that the interval between the sensitizing and the immunizing injections is critical in this case because the persistence of a sufficient number of tubercle bacilli in the host at the time of the immunizing njection appears to be responsible for this effect.

Summary. Sensitization of guinea pigs to tuberculin by administration of heat-killed human-type, tubercle bacilli 4 weeks before immunization with bovine γ globulin (BGG) emulsified in Freund's complete adjuvant had no apparent qualitative or quantitative effect on the subsequent antibody response to BGG. However, there was an indication that administration of BGG in Freund's incomplete adjuvant to similarly sensitized animals resulted in a response resembling that of

animals which received BGG in the complete adjuvant. Evidence was presented to indicate that the mycobacteria used to establish hypersensitivity to tuberculin may also have functioned in an adjuvant role in this case.

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A Study of Teratogenic Effects of Particulate Compounds in the Amniotic Cavity of Chick Embryos.* (31780)

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In previous studies a significant percentage of gross defects occurred after thalidomide was injected into the amniotic cavity of 3-to 5-day-old chick embryos. The defects included eye defects, encephalocele, beak defects, retarded feathering and retarded ventral closure. However, similar defects were produced by other chemically unrelated compounds, the only obvious characteristic possessed by all being a high degree of insolubility in ordinary diluents and in the amniotc fluid(1). Two compounds of the series tested were colloidal and these produced, in addition, severe distortions of the body axis and limbs. To investigate further the nature of the teratogenic action of the particles several other compounds were studied. Carbon black, easily identified in tissue sections, was used to determine the physical relationship of the particles to the defects produced. Included also was a colloidal silica of known particle size. Several preparations of polystyrene latex balls of graded size were tested in an attempt to determine the relationship of particle size to production of defects. The latex balls agglutinated in the amniotic fluid and thus were not suitable for the purpose, although they did produce the characteristic defects. Due to similarities in the effects of all the compounds, the most obvious supposition is that the developmental anomalies are related to the physical rather than to the chemical nature of the materials. There is, however, no evidence that the compounds may not also produce changes related to their chemical activities. For instance, silica particles, which are etiologically associated with silicosis are known to have specific toxic effects for macrophages which engulf them(2) whereas other particles, including those of alumina and carbon, do not have these toxic effects. In chick embryos the colloidal silica produced the same defects as colloidal alumina with certain slight dissimilarities.

Methods and materials. Materials used. Reagent grade, sugar carbon from Fischer Scientific Co. had maximum impurities of 1% sulfates (calcium, magnesium, sodium, etc.) and 1% ash (alkali metal). For injection it was ground in a tissue grinder and suspended in distilled water or saline buffered at pH 7.2. A suspension of colloidal silica (Ludox®) from Du Pont Co. contained 15% SiO_2, 1% Na_2O (titratable alkali), 0.001% NaCl and 0.003% Na_2SO_4. The silica particles were dense, nonporous spheres approximately 7 mμ in diameter. They remained dis-

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