ELECTROPHORETIC PURIFICATION OF A WATER-SOLUBLE GUINEA PIG TRANSPLANTATION ANTIGEN

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The isolation of tissue transplantation antigens has been energetically pursued since Billingham *et al.*[1] found that cell extracts could induce homograft immunity. A major problem in this endeavor has been the solubilization of the antigens which probably reside on cellular membranes. Studies employing tissue homogenization[2] and pressure decompression[3] to disrupt cells have yielded insoluble antigenic preparations of a gross lipide composition. On the other hand, water-soluble materials have been liberated by means of low-frequency ultrasound,[4] by digestion of membranous sediments,[5, 6] and by digestion combined with chelation of divalent cations.[7] The antigen released from murine splenic cells by ultrasound has been analyzed by gel filtration on Sephadex G-200, by equilibrium ultracentrifugation, and by linear sucrose gradients.[4] The active substance had a buoyant density of greater than 1.238, which was compatible with a proteinaceous rather than lipoprotein nature. It behaved on gel filtration and in sucrose gradients as if it had an approximate molecular weight of slightly less than 200,000.

The present report describes the purification of sonically liberated guinea pig transplantation antigen by discontinuous polyacrylamide gel electrophoresis. This method has achieved remarkable resolution of the components of complex mixtures of macromolecules.[8-11] In the present situation the active fraction obtained by gel filtration contained 17 distinct components, and the antigenic activity was confined to a single one of this array of tissue components.

Materials and Methods.—Preparation of the antigen: The spleens of inbred, histocompatible strain 2 guinea pigs[12] were excised, cleansed of fat, and minced in Tris-sucrose buffer (0.05 *M* Tris, 0.008 *M* magnesium chloride, 0.0025 *M* potassium chloride, 0.15 *M* sucrose adjusted to pH 7.45, at 25°C). Single cell suspensions were prepared either by pressing the tissue through a nylon stocking top, or by consecutive passes through nylon screening of 50 and 200 mesh. The erythrocyte contamination of the cell pellet obtained by centrifugation at 950 × *g* was decreased by lysis with 3% acetic acid in Tris-sucrose buffer. After two washes with Tris-sucrose buffer, the residual cells were passed through a double thickness of nylon stocking, and the cell concentration was adjusted to 8–10 × 10^6 cells/ml. The filtered cell suspension was sonicated in a Raytheon model DF 101 150W magnetostrictive oscillator at 10 kc/sec for 3 min at 1.0 amp and a temperature of less than 10°C.

After removal of the cell debris by centrifugation at 2000 rpm for 15 min, the cell membranes were sedimented by ultracentrifugation at 130,000 × *g* for 90 min in a model L2 65 Spinco preparative ultracentrifuge. The supernate was placed in dialysis tubing which had been boiled in 0.1 *M* sodium carbonate and stored in 0.01 *M* ethylenediaminetetraacetate (EDTA), and concentrated against Aquacide I, C grade (Calbiochem). The concentrated supernate was passed at a flow rate of 3.5–6.0 ml/hr over a 2.5 × 100-cm column of Sephadex G-200 (Pharmacia Fine Chemicals), which had been equilibrated with 0.5 *M* glycine, 0.2 *M* Tris, 0.5% mannitol, pH 8.0. Fractions were collected in 2.5-ml aliquots and read for absorbancy at 280 mμ. Fractions were lyophilized following desalting through a Sephadex G-25 column which had been equilibrated with 0.05% mannitol. Antigen was reconstituted with distilled water. Protein concentrations were estimated according to the method of Zamenhof,[13] using ovalbumin as standard.

Analytical disc electrophoresis: Analytical acrylamide gel electrophoresis was performed with-

out and with 8 *M* urea in the system described by Reisfeld and Small.[9] A lower gel of the appropriate concentration of monomer and 0.2% cross-linking reagent (methylenebisacrylamide) was polymerized in the presence of an oxidant (ammonium persulfate) and a catalyst (N',N,N'-tetraethylene diamine) in Tris-HCl buffer at pH 9.4. The upper gel was prepared with $2^1/_2$% monomer and 0.2% cross-linking reagent in Tris-phosphoric acid buffer pH 6.74 in a volume of at least twice that of the sample. The entire gel measured 5.0–5.5 cm. The buffer troughs contained Tris-glycine, pH 8.91, in the upper tray, and Tris-HCl, pH 8.07, in the lower tray. Electrophoresis was performed at 25°C with a constant current of $2^1/_2$ ma per tube. Gel electrophoresis at pH 4.3 was performed according to the method previously described,[10] and recently modified[11] by the use of 0.12 *M* potassium hydroxide and 0.75 *M* acetic acid, pH 4.3, in the lower tray, and of 0.12 *M* potassium hydroxide and 0.0125 *M* acetic acid, pH 6.7, in the upper gel.

When the bromphenol blue tracking dye had reached the end of the lower gel, electrophoresis was considered completed, and the gels were either stained or alternatively frozen for sectioning. Staining was performed with Coomassie Brilliant Blue:[14] Gels were fixed for 1 hr in $12^1/_2$% trichloroacetic acid (TCA), stained for several hours in 0.05% Coomassie Brilliant Blue in $12^1/_2$% TCA, and then stored in 10% TCA.

Gels to be submitted to biologic assay or to re-electrophoresis were placed at −20°C for 15 min, and then sliced horizontally in a "guillotine"-type gel cutter designed by Chrambach.[15] Each slice was placed in an individual tube containing 0.25–0.40 ml of isotonic saline (0.85% sodium chloride buffered with 0.01 *M* phosphate, pH 7.4), and eluted by shaking for 24–72 hr at 4°C. This process was found to liberate only a portion of the material since re-electrophoresis of the slice following elution still revealed the presence of protein substances. Components were identified according to their R_f values, considering the band of bromphenol blue to be equivalent to R_f 1.0 and the beginning of the lower gel to R_f 0.0.

Ion-exchange adsorbents: Whatman microgranular carboxymethylcellulose (CM 32) and Whatman microfibrous diethylaminoethylcellulose (DE 22) (Reeve Angel Company) ion-exchange adsorbents were cycled with sodium hydroxide and hydrochloric acid as recommended by the manufacturer, and then packed into 20 × 1.5-cm columns in a 1:1 (v/v) slurry with the starting buffer. Carboxymethylcellulose (CMC) was equilibrated with 0.05 *M* triethylamine, 0.05 *M* acetic acid, pH 6.0, and diethylaminoethylcellulose (DEAE), with 0.05 *M* glycine, 0.02 *M* Tris, pH 8.0. Stepwise elution was performed on CMC with (1) 0.5 *M* triethylamine, 0.5 *M* acetic acid, pH 6.0; (2) 0.5 *M* triethylamine, 0.5 *M* acetic acid, 0.5 *M* sodium chloride, pH 6.0; (3) 0.5 *M* glycine, 0.2 *M* Tris, pH 8.0; and finally, 0.5 *N* sodium hydroxide. Elution from DEAE employed (1) 0.5 *M* glycine, 0.2 *M* Tris, pH 8.0; (2) 0.5 *M* glycine, 0.2 *M* Tris, 0.5 *M* sodium chloride, pH 8.0; (3) 0.5 *M* glycine, 0.2 *M* Tris, pH 10.0; and (4) 0.5 *N* sodium hydroxide. Buffers were pumped onto columns at a rate of 30 ml/hr. Fractions were collected in 2.5-ml aliquots and read for absorbancy at 280 mμ. Following elution, fractions were desalted and lyophilized.

Assay of transplantation antigenic activity: Brent *et al.*[16] have recently demonstrated that immunity to allogeneic tissue can be expressed as cutaneous hypersensitivity phenomena. In previous work[17] it was reported that guinea pigs immunized with allogeneic skin grafts developed specific, direct, delayed skin reactions to 10 μg of active Sephadex fraction I. According to the method described therein, 300–400 gm strain 13 guinea pigs (reared by the Animal Production Unit, National Institutes of Health) are immunized with allogeneic strain 2 skin grafts, and then skin is tested with antigenic preparations from the fifth to twelfth postoperative day. At 3, 6, 12, 24, 36, 48 hr, the reactions are scored for the extent and depth of erythema, the area of induration, the degree of skin thickening, and the presence of discoloration or necrosis.

Results.—Fractionation of strain 2 antigen on Sephadex G-200: Chromatography of the 130,000 × *g* supernate on Sephadex G-200 revealed five fractions in the inner volume (Fig. 1). Only fraction 1, which moved at the front of the inner volume, elicited specific, direct, delayed cutaneous reactions and participated in transfer reactions.[17] This fraction lost its antigenic activity after storage at 4°C for 48 hours, but retained its potency following lyophilization.

Electrophoretic analysis of the active fraction: Sephadex inner volume fraction 1 was subjected to electrophoretic analysis on discontinuous polyacrylamide gels.

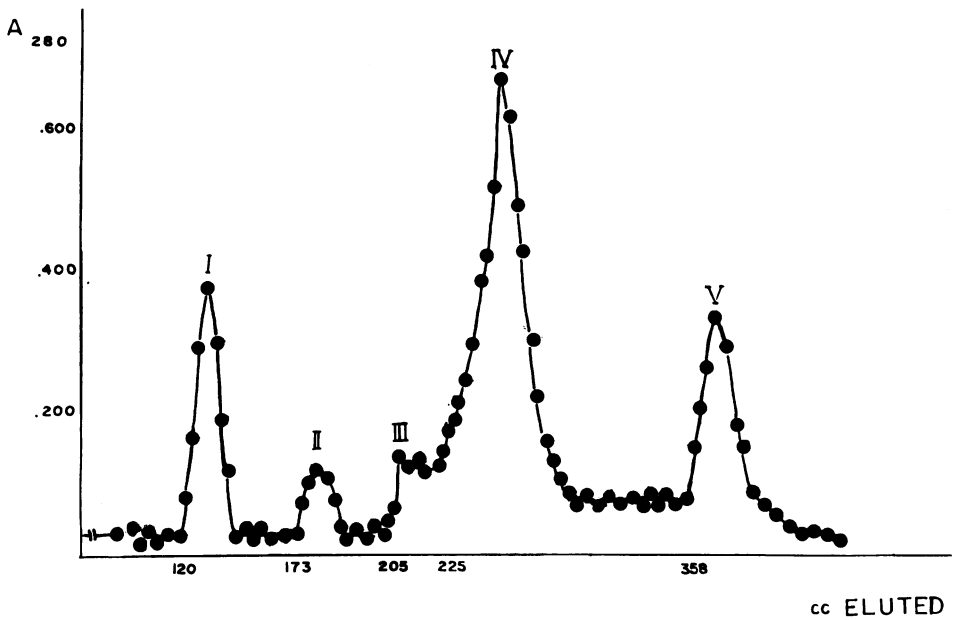
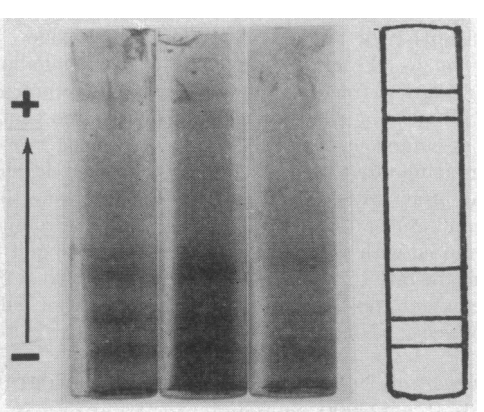
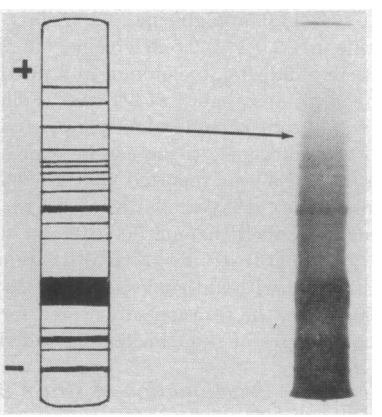


FIG. 1.—The inner volume elution pattern of strain 2 antigen from gel filtration on Sephadex G-200. Only fraction I elicited delayed-type hypersensitivity reactions.

When 250 μg of protein were applied to a $7^1/_2$ per cent gel, five components were resolved at pH 9.4 (Fig. 2), and eight components at pH 4.3. When 1000 μg were applied, 17 components were identified at pH 9.4 (Fig. 3).

The components of pH 9.4 gels were assayed for their ability to elicit direct cu-





(*Left*) FIG. 2.—Patterns obtained following disc electrophoresis of three different samples containing 250 μg of strain 2 Sephadex inner volume fraction I on $7^1/_2$% polyacrylamide gels at pH 9.4. Note five major components of this fraction. (Arrow indicates direction of electrophoresis.)

(*Right*) FIG. 3.—Pattern obtained following disc electrophoresis of 1,000 μg of strain 2 Sephadex inner volume fraction I on $7^1/_2$% polyacrylamide gels at pH 9.4. Note 17 components resolved at this concentration of starting material. The arrow indicates component 15, which elicited cutaneous hypersensitivity reactions.

taneous reactions in strain 13 animals sensitized with strain 2 skin grafts. Only one component was found to elicit specific, direct, delayed-type reactions (component 15: R_f = 0.73–0.74/pH 9.4). The presence of this component on gel electrophoresis was correlated with the transplantation antigenic activity of fractions obtained by chromatography on Sephadex and on ion-exchange adsorbents. Aliquots of fractions collected following gel filtration were analyzed on $7^1/_2$ per cent acrylamide gels at pH 9.4, and were individually tested for cutaneous reactivity. The profile of the Sephadex inner volume fraction 1 was found to be asymmetric with respect to both antigenic activity and the protein components present in each aliquot. There was a correlation between transplantation activity and component 15: all antigenically active aliquots obtained by gel filtration contained this electrophoretic component. Furthermore, all aliquots which contained this component elicited direct cutaneous hypersensitivity reactions (Fig. 4). Similar results were obtained for fractions isolated by ion-exchange chromatography. Four components of Sephadex fraction 1 were resolved on CMC (Fig. 5). Only one component (step 3) was found to elicit specific, direct, delayed reactions, and only this fraction contained component 15 on disc electrophoresis. Chromatography on diethylaminoethyl cellulose (DEAE-cellulose) separated five components of Sephadex fraction 1 (Fig. 6); one component (step 1) was active and only it contained component 15.

Tissue distribution of component 15: Antigen was liberated from guinea pig lung, liver, and kidney by the same method as was employed to liberate splenic material. The relative activities of the 105,000 × *g* supernatant antigens liberated by low-frequency sonic oscillation from murine tissues have been reported by

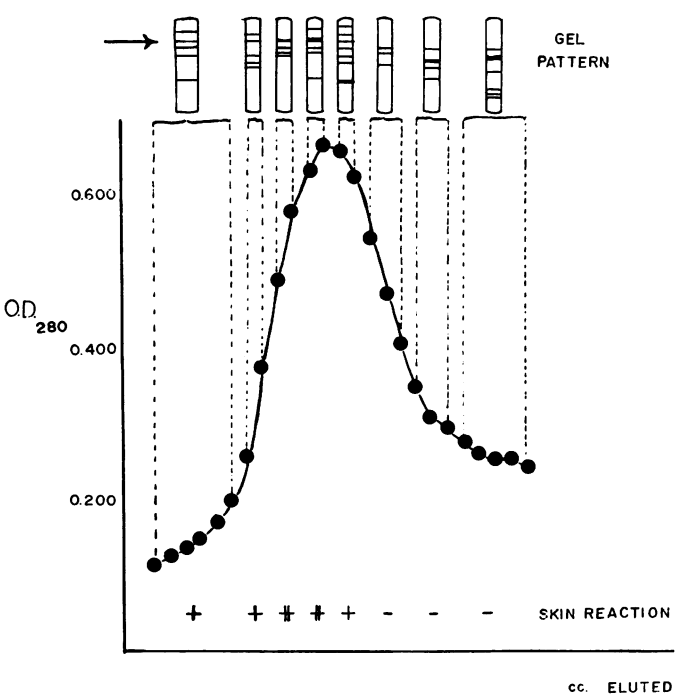


FIG. 4.—Correlation between the gel components and the cutaneous reactivity of aliquots of fraction I by gel filtration. The $7^1/_2$% gel patterns at pH 9.4, the elution pattern of fraction I, and the cutaneous reactivity of each aliquot are illustrated. Note that only aliquots containing component 15 elicited cutaneous reactions and that each aliquot which was active contained this component on analytical electrophoresis.

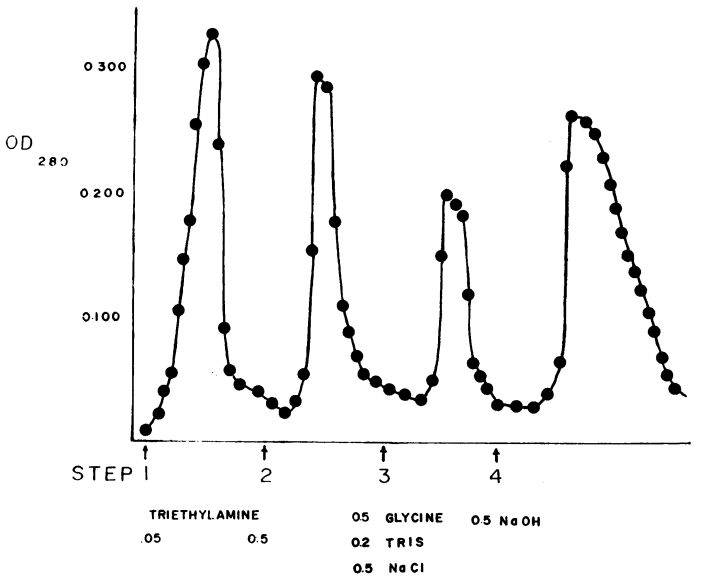


FIG. 5.—Elution pattern of Sephadex inner volume fraction I from CMC adsorbent. Of the four fractions resolved, only one fraction (step 3) elicited specific cutaneous hypersensitivity reactions.

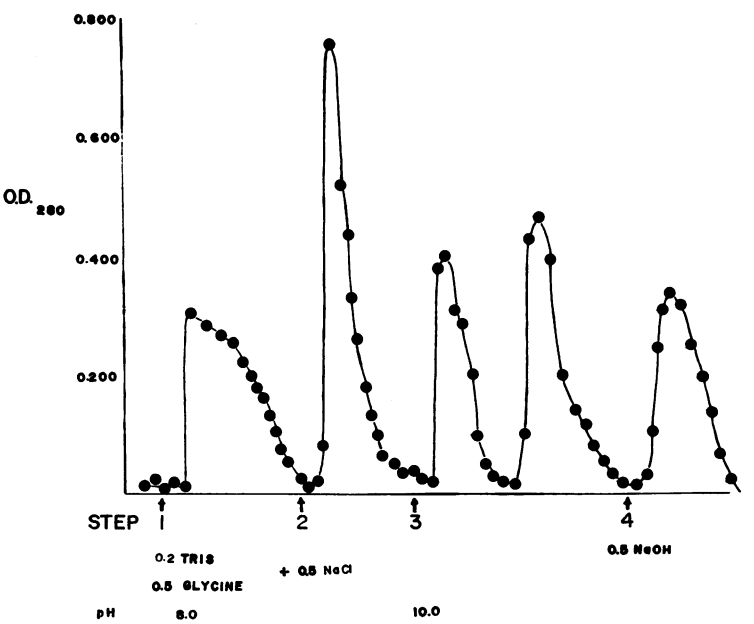


FIG. 6.—Elution pattern of Sephadex inner volume fraction I from DEAE adsorbent. Of the five fractions resolved, only one fraction (step 1) elicited specific cutaneous hypersensitivity reactions.

Zajtchuk *et al.*[18] Lung and spleen were most potent, kidney was moderately active, and liver had minimal activity. Sephadex fraction I was prepared from each organ and electrophoresed in $7^1/_2$ per cent gels at pH 9.4. The gels were sectioned and the eluate of each slice was individually assayed for cutaneous reactivity. The specific, direct reactivity was in each case localized in the zone of component 15. In order to prove that this very component (R_f = 0.73–0.74) was responsible for the transplantation activity of each tissue, the gel cuts from which active materials had been partially eluted were re-electrophoresed in another $7^1/_2$

per cent gel at pH 9.4. The "active cuts" of spleen and lung contained only component 15 upon re-electrophoresis, and their eluates were most potent in eliciting cutaneous reactions. Eluates of kidney were active and contained in addition to component 15 a faint, slightly less mobile component (R_f = 0.65). Liver, which had only slight activity, contained a very faint component 15 (R_f = 0.73) and two additional components (R_f = 0.65 and 0.57) (Fig. 7). On the basis of their potency and of the absence of other components as judged by electrophoresis on $7^1/_2$ per cent gels at pH 9.4, spleen and lung were chosen as the most suitable organs for the routine preparation of transplantation antigen. Re-electrophoresis of a mixture of the active component 15 prepared from each organ revealed a single component with R_f 0.73 (Fig. 8).

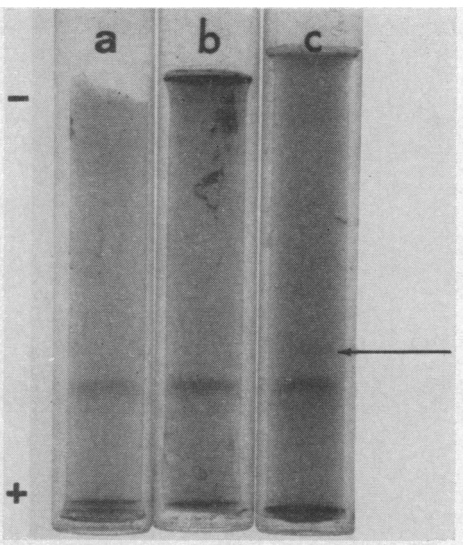


FIG. 7.—Patterns obtained following analysis of (*a*) spleen, (*b*) lung, and (*c*) kidney on $7^1/_2$% polyacrylamide gels at pH 9.4. Note that all three organs contained component 15 and that in addition, kidney (*c*) contained a slightly less mobile component.

Immunologic specificity of component 15: The above data, collected employing strain 2 antigens, were duplicated with strain 13 materials. The active component was again localized to component 15 (R_f = 0.73 at pH 9.4). In order to ascertain whether strain 2 and strain 13 antigens were indeed electrophoretically identical, splenic Sephadex fractions I were prepared from both strains and were electrophoresed individually and mixed (1:1) on $7^1/_2$ per cent gels at pH 9.4. The three sets of gels strain 2, strain 13, strain 2–13 mixture were each divided into 40 slices, and the eluate of each slice was individually tested both in a strain 2 animal which had been immunized with a strain 13 graft, and in a strain 13 animal which had been immunized with a strain 2 graft. In all three instances specific, direct, delayed cutaneous reactions were elicited only by those slices which upon re-electrophoresis contained only component 15 (Fig. 9).

Electrophoretic homogeneity of component 15: Re-electrophoresis of the active component was performed in gels of varying porosity in the presence of 8 *M* urea. Re-electrophoresis in $7^1/_2$ per cent gels either without or with 8 *M* urea revealed only a single component, whereas other, less mobile components of Sephadex fraction 1 dissociated into multiple components upon re-electrophoresis in urea. Since increased gel concentrations result in decreased porosity, thereby facilitating resolution through a sieving effect, component 15 was re-electrophoresed in 15, 20, and 30 per cent gels in the presence of 8 *M* urea (Fig. 10). Under these conditions the component remained in a thin, distinct, individual band. It was considerably retarded by the 15 per cent gel (R_f = 0.25), by the 20 per cent gel (R_f = 0.20), and by the 30 per cent gel (R_f = 0.13). By these criteria the active component appeared to be a single electrophoretic species.

Discussion.—The chemical characterization of the antigenic determinants me-

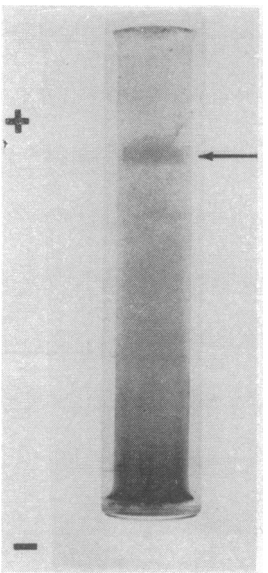


FIG. 8.—Pattern obtained following re-electrophoresis of the active zone of 7½% gels prepared from a mixture of spleen and lung antigens. Note the presence of only a single component, $R_f = 0.73$.

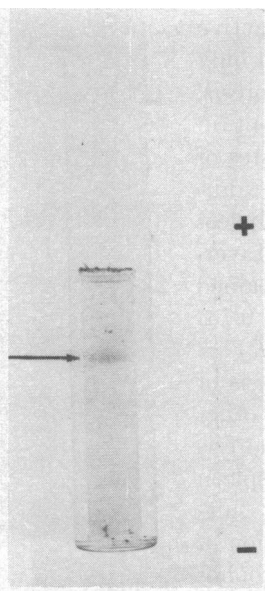


FIG. 9.—Pattern obtained following re-electrophoresis of the active zone of 7½% gels prepared from a mixture of strain 2 and strain 13 antigens. Note the presecne of only a single component, $R_f = 0.73$.

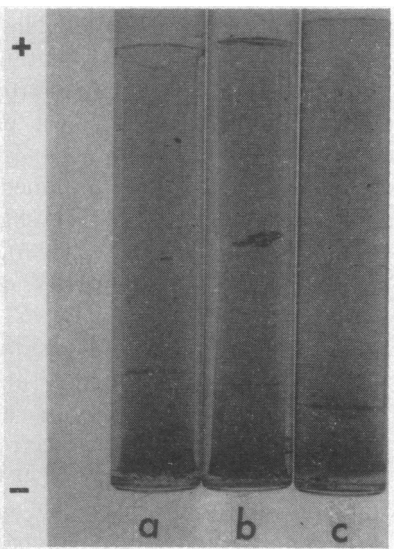


FIG. 10.—Patterns obtained following re-electrophoresis of the active zone of 7½% gels prepared from spleen and lung antigens in (*a*) 15%, (*b*) 20%, and (*c*) 30% polyacrylamide gel in the presence of 8 *M* urea. Note that only a single, thin band could be resolved in each case.

diating tissue transplantation immunity has heretofore been impeded by the lack of a sensitive cell-mediated assay system and by the gross heterogeneity of the antigenic extracts. Recently, it was shown that 10 μg of water-soluble proteinaceous transplantation antigen (Sephadex inner volume fraction I) elicited specific, direct, delayed hypersensitivity reactions. Employing this sensitive, rapidly developing assay, it was possible to apply polyacrylamide gel electrophoretic analysis to the purification of the antigenic determinant contained in soluble tissue extracts. This analysis revealed marked heterogeneity of Sephadex fraction I: a total of 17 components was resolved at pH 9.4. Transplantation antigenic activity was limited to a single component (component 15: $R_f = 0.73$–0.74). Not only was there a correlation between antigenically active fractions obtained by gel filtration and by ion-exchange adsorbents and the presence of component 15 on acrylamide gels prepared from these fractions, but in addition, specific cutaneous hypersensitivity reactions were only elicited by the administration of component 15.

This component was shown to be present in lung, spleen, kidney, and liver, and in each instance it alone elicited direct cutaneous reactions. The antigenic determinants of strain 2 and strain 13 materials were inseparable on polyacrylamide gels at pH 9.4, directly demonstrating that these specificities are superimposed on an electrophoretically similar protein, as had been previously inferred from indirect evidence based upon antibody absorption experiments.[7, 8] The lack of hetero-

geneity of this component upon re-electrophoresis in gels of varying pore size and containing 8 *M* urea suggests that a relatively homogeneous molecular species has now been identified with cell-mediated transplantation antigenic activity.

Summary.—Water-soluble guinea pig transplantation antigen which had been liberated from cells by low-frequency ultrasound and fractionated by gel filtration contained 17 components on discontinuous electrophoresis at pH 9.4. Only a single component (component 15: R_f = 0.73–0.74) elicited direct, delayed-type hypersensitivity reactions in allogeneic animals sensitized by the application of a donor strain skin graft. The reactivity of preparations of strain 2, strain 13, and a mixture of both strains, as well as the reactivity of preparations of splenic, lung, kidney, and liver antigen was limited to this component. Re-electrophoresis of this component in gels of varying porosity and in the presence of 8 *M* urea revealed only a single band, suggesting that a homogeneous molecular species had now been identified with transplantation antigenic activity.

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