# Soluble Antigens from Rat Liver Connective Tissue.* (28705)

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The insolubility of the connective tissue has limited its analysis with respect to the individual protein components. There have been reports of the antigenicity of some saline extractable materials other than collagen (for example Heller *et al*(1)). Reports such as those of Cruickshank and Hill(2), Scott(3) and Hiramoto *et al*(4) on the antigenic components of connective tissue were not concerned with the isolation and characterization of the antigens but dealt with the staining properties of antibodies prepared against different types of connective tissue.

The present work describes the preparation and preliminary fractionation of a soluble material obtained by sonicating an extensively washed low speed sediment of liver homogenate. This material contained antigenic components capable of reacting in Ouchterlony tests with an antiserum prepared against sonicated rat liver sediment. We were able to show that at least some of these antigens are derived from rat liver connective tissue. Two of them were separated in a state pure enough to give a single line by the Ouchterlony test.

*Materials and methods. Preparation of the soluble sonic fraction.* Sprague-Dawley male rats were perfused through the aorta with borate saline. The livers were homogenized in a Servall Omni-Mixer in ice cold borate saline, pH 8 at maximum speed (14,000 rpm), for 1 minute. The insoluble material was separated by centrifugation. It was then washed by suspending in 5-10 volumes of ice cold saline and centrifuging repeatedly (21 times), until the supernate was fairly clear. Only material sedimenting at 2000 *g* for 20 minutes was retained. The sediment finally obtained appeared microscopically to consist largely of fiber-like material (presumably connective tissue) with no intact cells (Fig. 1). Only very rarely could a neucleus be observed. The sediment was sonicated for 30 minutes in a 10 KC Raytheon Magnetostriction Oscillator, followed by 5 minutes in a Biosonik Ultrasonic Vibrator. The sonicate was centrifuged for 2 hours at 20,000 rpm (35,000 *g*). The sediment was again sonicated and ultrasonicated and the whole procedure repeated a third time. The supernates were all pooled. They are referred to subsequently as the *soluble sonic fraction.* Protein determinations were carried out by dissolving the materials in N/10 NaOH and measuring the optical density at 280 mμ, with rabbit γ-globulin as standard. From 424 g wet liver tissue (27 livers), 15 g insoluble sediment determined as protein were obtained. The sonication of the insoluble material yielded 3.6 g of the soluble sonic fraction also determined as protein.

The antiserum used has been described previously(5). It had been made in rabbits against the sonicated insoluble sediment from rat liver.

*Fractionation of the soluble sonic fraction by agar electrophoresis*(6). The solution was treated with 1.5 volumes of 3.5 M phosphate buffer pH 6.8. The precipitate which formed was filtered off, dissolved in a minimum amount of borate saline, and dialyzed against veronal buffer μ: 0.025, pH 8.2. Electrophoresis was carried out in an apparatus described by Smithies(7) for starch gel electrophoresis (30.7 cm × 12.2 cm). An agar layer 6 mm thick was made with 300 ml of 0.5% Ionagar‡ in the same veronal buffer. After the agar had set, a rectangular channel 0.5 cm wide and 11.2 cm long was cut in the center. 2.4 ml of the sample solution was

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‡ Ionagar No. 2, (Consolidated Laboratories Inc., Chicago Heights, Ill.).

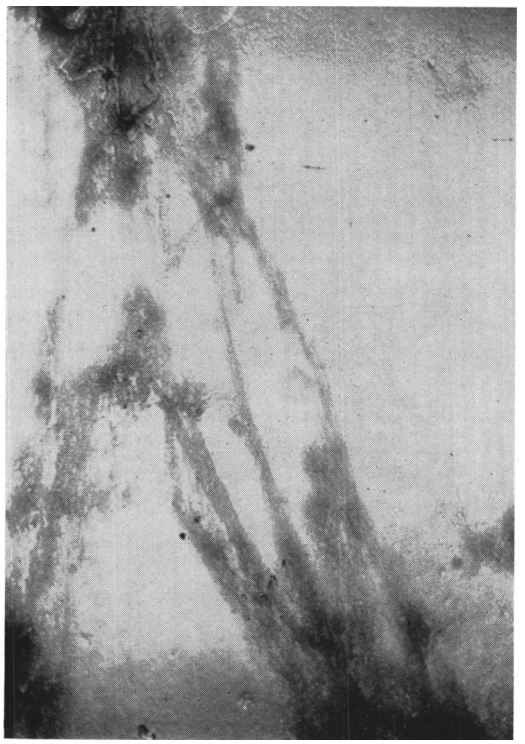

FIG. 1. Washed rat liver insoluble sediment, stained with toluidine blue 1:1000. The fibrous structure of the material is easily seen. (× 300)

warmed to 37°C and mixed with 0.8 ml of 0.8% melted Ionagar cooled at 45°. The mixture was poured in the channel and left to set. Electrophoresis was carried out horizontally in the cold room for 4 hours at constant current, 41mA. The starting voltage was 125 V, dropping to 90-100 V by the end of the experiment. A fan was used to improve cooling. After electrophoresis, the agar was cut into 20 strips 1 cm wide, placed in tubes and left overnight in the cold. Each agar strip was then broken up in a glass homogenizer, extracted for 2 hours in the cold on a mechanical shaker with 0.45% saline and centrifuged at 16,000 rpm for one hour. The supernates were concentrated by pervaporation, dialyzed against borate saline and tested in agar diffusion for presence of precipitation lines with the globulin fraction of anti-rat liver sediment serum(5).

*Fractionation of the soluble sonic fraction with $CO_2$ saturated distilled water.* A portion of the soluble sonic fraction was dialyzed against 3 changes of $CO_2$ saturated distilled water in the cold for 2 days. The precipitate formed was separated by centrifugation, suspended in borate saline and centrifuged. Only part of the precipitate redissolved. The insoluble portion could be redispersed by sonic vibration, however, and it then remained in solution even after ultracentrifugation.

*Fluorescent-antibody technique.* The globulin fraction of horse anti-rabbit globulin serum was conjugated with fluorescein isothiocyanate (FITC) using fluorescein isothiocyanate-on-celite containing 10% FITC (California Corp. for Biochemical Research, Los Angeles(8)). The labeled globulin was absorbed 3 times (30 minutes each at room temperature) with rat liver sediment which had been prepared by centrifuging the saline homogenate of liver at 17,000 *g* for 10 minutes and washing with saline until the supernatant appeared clear. Frozen tissues from perfused animals were cut in sections 3 to 4 $\mu$ thick, incubated for 30 minutes with the rabbit antibody preparations and rinsed for 15 minutes. The sections were treated for one hour with the fluorescein-labeled globulin fraction of horse anti-rabbit globulin serum. In a simultaneous control experiment, the tissue was treated with the specifically purified rabbit antibody which had been absorbed with the corresponding purified antigen to show that no staining is obtained with neutralized antibody. Other controls included incubation of the tissue with the following prior to staining with the fluorescent horse globulin: (a) normal rabbit $\gamma$-globulin absorbed with the insoluble part of guinea pig liver, and (b) specifically purified rabbit antibody followed by unlabeled horse anti-rabbit globulin also absorbed with guinea pig liver.

*Results.* The soluble sonic fraction gave 4 well defined lines in agar diffusion tests (Fig. 2) (numbered 1-4 starting from the serum reservoir) and 2-4 additional very thin lines. The precipitation lines were not due to rat plasma antigens. Absorption of the antiserum with rat plasma removed all precipitating activity with rat plasma, but left the antibodies which formed the lines with the soluble fraction. None of the diffusion lines was

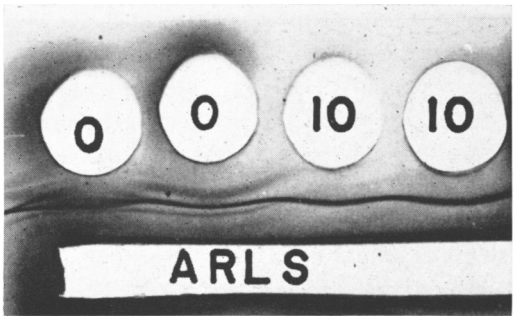


FIG. 2. Diffusion lines given by soluble sonic fraction (0) and eluate of electrophoretic strip 10 (10) with globulin fraction of anti-rat liver sediment (ARLS).

due to collagen§. Rat collagen (tested at several concentrations up to 1 mg/ml) did not precipitate with the antiserum in tubes or agar. It is very unlikely that the lines were due to antigens found primarily in the easily soluble portion of the liver cells. The soluble sonic fraction gave 2 lines at a concentration of 0.7 mg protein/ml, while the supernate of the homogenate gave no such lines even at a protein concentration 10 times higher. These data, together with the extensive washing carried out before extraction of the soluble sonic fraction by vibration strongly suggest that most, if not all, of the antigens were derived from the originally insoluble tissue.

Further evidence that the antigens in the soluble sonic fractions are derived from the insoluble portion of the liver homogenate is provided by the following experiment. The sonicated insoluble portion is still able even after the heat extraction to absorb specifically antibodies which react with the soluble sonic fraction. This was shown by labelling the antibody with $I^{131}$ and absorbing it onto the insoluble residue. After washing, radioactivity was eluted from the residue by heating at 60° for 20 minutes. Significant amounts of radioactivity (of the order of 20% of the total) were precipitated from eluate, after mixing with a carrier amount of the globulin fraction of the antiserum and either the soluble sonic fraction or subfractions derived from it. Only negligible amounts of radioactivity were brought down by adding the eluate to the globulin fraction of antinormal rat plasma and precipitating it with rat plasma.

The antigens were not affected by freezing but they were altered by heating. Some were more sensitive than others. Antigen 3 was appreciably altered by heating for 10 minutes at 50°. All antigens were destroyed when placed for 10 minutes in boiling water.

*Electrophoretic properties of the antigens in the soluble sonic fraction.* The fractional precipitation of the soluble sonic fraction with increasing concentrations (10%–60%) of 3.5 M phosphate buffer pH 6.8(9) showed that various fractions could be obtained, differing in the relative amounts of the different antigens. However, no complete separation of any of the fractions could be achieved by this method. Further immunoelectrophoretic experiments indicated that electrophoresis might be useful to effect at least a partial separation. The soluble sonic fraction was therefore subjected to electrophoresis in agar and the results were evaluated by diffusion in agar on microscope slides. Of the 20 strips into which the agar was cut, 14 yielded antigenic material on elution (strips 2–15 numbered from the anode end). A schematic distribution of the antigens in the various strips is shown in Fig. 3. Strips 2–8 contained only antigen 3, but in very small amounts. Strips 9 and 10 also contained only antigen 3 but in much larger amounts. Fig. 2 compares the eluate of strip 10 with the original soluble sonic fraction in diffusion test and serves to identify the line due to antigen 3. Antigen 4 was separated from antigen 3 in a relatively

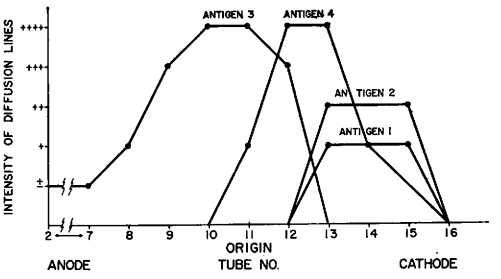


FIG. 3. Schematic representation of the migration of antigens in the electrophoretic field.

§ A sample of rat skin collagen extracted with 1M-NaCl was obtained through the courtesy of Dr. K. Piez, Nat. Inst. for Dental Research, Bethesda.

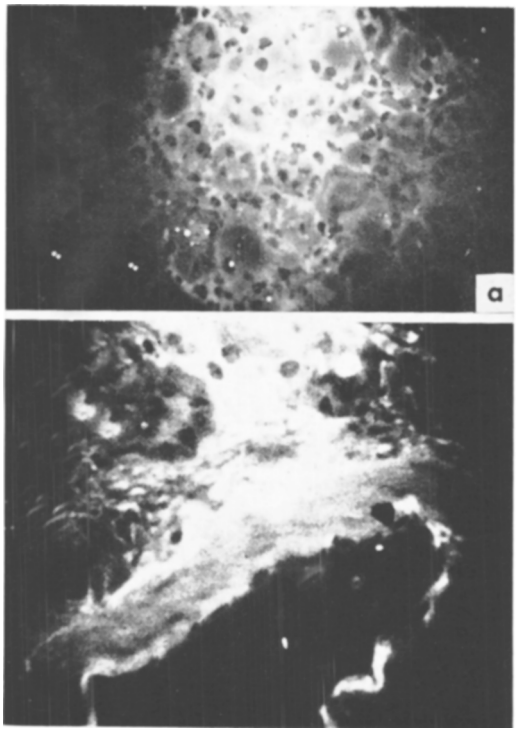


FIG. 4. Fluorescent staining of rat kidney with specifically purified antibody to antigen 3. a: Connective tissue surrounding the tubuli fluoresces brightly. b: Strong fluorescence is seen in adventitia of artery. Endothelial cells show also some staining. (× 240)

pure state from the mixture in tube 12, by dialysis against distilled water saturated with $CO_2$. In this process both precipitated. Antigen 4 was recovered essentially free of antigen 3, as it can be redissolved in borate saline without apparent loss of precipitability with the corresponding antibody. Antigen 3 was irreversibly precipitated. When the whole soluble antigen was similarly dialyzed, antigen 4 was recovered together with antigen 2 from the precipitate.

*The separation of antibody against antigen 3.* Since antigen 3 was available in a fairly satisfactory degree of purity, it was used to separate specifically purified antibody from the globulin fraction of the antiserum. The $CO_2$-distilled water method described by Tozer *et al*(10) was employed. A small amount of purified antibody was recovered (400 $\mu$g), which gave in agar diffusion against the soluble sonic fraction a strong line corresponding to antigen 3 and a very weak line corresponding to antigen 4.

*Fluorescent antibody studies.* Rat liver and kidney sections were treated with the unfractionated antiserum as well as with the above antibody purified with antigen 3. With both purified and unpurified antibodies, the staining in the liver was limited to the connective tissue areas. The hepatic cells were not stained. Both antibody preparations reacted also with the connective tissue of the kidney. Fig. 4 shows the reaction of the purified preparation with this organ. Good staining was seen around the tubules and in the adventitia of the large blood vessels. Weak staining was also seen in the glomeruli.

*Summary.* The work described here has revealed the presence of a large number of antigenic components in a soluble fraction obtained from an originally insoluble residue of rat liver. The major source of these antigens appears to be the liver connective tissue. The material used for their preparation was an extensively washed insoluble residue of rat liver, morphologically appearing as bundles of fibers, with no cells present. The antigens were absent from the rat plasma. One of them (No. 3) was not found in the supernate of the liver homogenate, the others were found there in much smaller concentrations. Both the original antiserum prepared against liver sediment and a purified antibody fraction derived from it reacted only with the connective tissue elements of the liver and kidney. The presence of some of these antigens in both liver and kidney indicates that they are probably widespread constituents of the connective tissue and are not specific for the liver. The use of sonic vibration for solubilizing connective tissue components while maintaining their antigenic reactivity may open the way to an analysis of the composition of connective tissue unattainable heretofore.

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## Passive Interferon Protection in Mouse Influenza. (28706)

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Since its discovery(1), the majority of reports on interferon have been concerned with the basic nature of this protein, the optimal conditions for its production and assay, and its mechanism of action(2). There have been comparatively few efforts to influence infection of the whole animal through administration of interferon before or after exposure to virus(3). The following communication describes experiments demonstrating that intranasal administration of interferon will enhance resistance of mice to infection with influenza virus.

*Methods.* Interferon was prepared in eggs and mice using the GL strain of Type B influenza as infective virus. Allantoic fluids and mouse lung suspensions were clarified by low-speed centrifugation. Virus in these fluids was then absorbed with chick red blood cells following which the pH was adjusted to 2.0 to destroy any remaining virus. The fluids were next sterilized by filtration and stored at —70°C. The interferon from allantoic fluid was freeze-dried and concentrated by reconstituting in one-fifth original volume of distilled water, but mouse lung materials were not tested as concentrates. Interferon was assayed by titrating varying amounts of interferon against serial 10-fold dilutions of egg-adapted vaccinia virus in a tube culture system. Triplicate tubes were exposed to 0.3 ml of interferon for 18 hours at 37°C; the virus dilution was then added in 1 ml of Eagle's basal medium in Earle's saline (EBME). Interferon potency was established as the maximal dilution which would reduce the $TCID_{50}$ of vaccinia one log on the 6th day of observation. The standardized interferons were then used to treat mice experimentally infected with the B/GL strain of influenza virus.

*Results. Tissue culture assays.* In our first experiments interferon was assayed against vaccinia by a standard plaque reduction method(4). However, since titers were found to be 4- to 8-fold less than with the tube culture method, the latter was adopted for assays. That vaccinia virus infection of chick embryo tissue culture (CETC) provided an adequately sensitive system can be seen from dose-response data in Fig. 1. Although this interferon was most active in reducing the infective titer of the homologous B/GL virus, it was also operative against vaccinia and Sendai(5) and dilution endpoints were similar with the 3 viruses. The potency of that lot of egg interferon was calculated to be 380 units/ml since 0.3 ml was used to treat the cultures. It is of interest that within certain dilution ranges the relationship appears to be linear(6), and a similar dose response has been observed when this interferon was measured for its capacity to reduce the $LD_{50}$ of mouse-adapted B/GL strain in mice. Interferon from mouse lung preparations was always at lower titer in the CETC-vaccinia assays and varied in the range of 6–24 units/ml. However, as shown below, their capacity to protect mice against influenzal infections was equal to that of the most active interferons from egg fluids.

*Protective effect of interferons when ad-*