ANTIGENIC COMPONENTS OF RAT CONNECTIVE TISSUE*

II. FLUORESCENT ANTIBODY STUDIES WITH ANTISERA TO CONNECTIVE TISSUE ANTIGENS

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IT was shown in a previous publication (Sulitzeanu, 1965) that a variety of antigenic substances could be extracted from rat organ preparations rich in connective tissue. The procedure consisted in separating the insoluble portion of organ homogenates by low speed centrifugation, washing the sediment fairly extensively and sonicating the residue. When tested by agar diffusion against the corresponding antisera, the sonicates produced a large number of diffusion lines. The antigenic content of lung and kidney sonicates was practically identical. The sonicates of liver and diaphragm shared some antigens with the lung and kidney, but they also contained antigens not found in the latter organs.

It was concluded from these preliminary studies that many of the antigens reacting in agar were probably components of connective tissue. Additional evidence was needed, however, to prove that this was indeed true. An obvious requirement was that the anticonnective tissue sera mentioned above should stain, by the fluorescent antibody technique, the connective tissue structures of the organs involved. We undertook therefore to test in the present work the reactivity of our antisera with the connective tissue by fluorescence microscopy. We also prepared a few additional antisera by injecting to rabbits fractions containing less complex mixtures of antigens. This was done by taking advantage of the preparative disc electrophoretic method (Sulitzeanu and Goldman, 1965), which makes it possible to obtain antibodies to selected antigens from complex mixtures, without first isolating the antigens. These additional antisera were similarly tested for their reactivity with connective tissue by agar diffusion and by fluorescence microscopy.

Anti-organ sera have been extensively used in the past as immunohistochemical reagents with specificity for connective tissue structures. Cruickshank and Hill (1953) were probably the first to use fluorescein labelled anti-organ sera in order to study the distribution of connective tissue antigens. Their antisera to rat glomeruli or lung revealed a widely distributed antigen found in connective tissue fibrils and membranes (basement membranes, reticulum, sarcolemma and neurilemma) of various organs, but not in collagen. Their findings were confirmed and extended by subsequent investigators, using fluorescent antibodies (Mellors, Siegel and Pressman, 1955; Hiramoto, Jurandowski, Bernecky and Pressman,

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1959 ; Taylor, 1961 ; Teodoru, Brancato and Volk, 1962 ; Steblay, 1962 ; Boss, 1963*a* and *b*, 1965*a*) or radiolabelled tissue localizing antibodies (reviewed by Tamanoi, Yagi, Hiramoto and Pressman, 1961). Scott (1957, 1959) was able to distinguish 3 classes of connective tissue antigens on the basis of their reactivity with antisera to glomeruli and synovia. The basement membranes antigens reacted only with anti-glomerulus serum. The reticulin and fibrous tissue antigens reacted with both anti-glomerulus and anti-synovium sera, but the fibrous tissue antigens reacted preferentially with the antiserum to synovium. The presence in placenta of antigens crossreacting with the basement membrane of rat glomerular capillaries, inferred from the ability of antiplacenta sera to induce nephrotoxic nephritis (Seegal and Loeb, 1943) was demonstrated by fluorescence microscopy by Boss and Craig (1963). Antisera to human placenta (Steblay, 1962 ; Boss, 1963*a* and *b*) and to human skeletal muscle (Boss, 1965*a*) were practically identical to antisera to human kidney, when compared by fluorescent antibody staining. The 3 antisera stained similar fibrillar and membranous structures in all organs tested.

Scott (1957) has suggested that the study of connective tissue antigens might contribute towards the understanding of the pathogenesis of connective tissue diseases. However, since it has not been possible to obtain them in a soluble form, no significant progress has been made in the isolation, purification and biochemical study of these antigens. The work detailed in this communication describes the reactivity with connective tissue of antisera to soluble connective tissue antigens. The results indicate that a combination of agar diffusion and fluorescent antibody techniques should enable the separation and the study of antigenic connective tissue components.

MATERIALS AND METHODS

Animals.—Rats 150–250 g. were used.

Saline.—Solutions of antigens and antisera were prepared in borate buffered saline pH 8·0, unless otherwise stated.

Soluble sonic fractions (SSF) of connective tissue (CT).—SSF were prepared from rat liver (LSSF), lungs (LgSSF), kidneys (KSSF) and diaphragm (DSSF), by sonicating sediments of organ homogenates as previously described (Sulitzeanu, 1965). Since the diffusion patterns given by the lung and kidney SSF with their corresponding antisera were nearly identical, SSF prepared from these organs were mixed (Lg-KSSF), thus providing larger amounts of antigen to work with.

Antisera.—The following antisera were studied :

(a) Antisera to whole organ CT antigens of : liver (ALGG- anti-liver serum γ-globulin), kidney (AKGG), lung (ALgGG) and diaphragm (ADGG). The preparation and diffusion patterns of these antisera has been described (Sulitzeanu, 1965). γ-globulin fractions of antisera to whole organ CT, prepared by precipitation with ammonium sulphate at $\frac{1}{3}$ saturation, were used in both diffusion and fluorescent antibody work.

(b) Antisera to fractions of CT obtained by the preparative disc electrophoretic method (Sulitzeanu and Goldman, 1965) : Lg-KSSF and LSSF were fractionated by electrophoresis in large columns of polyacrylamide gels. Gel slices were cut, homogenized, emulsified with complete Freund's adjuvant and injected into rabbits. The animals received 0·5 ml. of the emulsion in the leg muscles and 0·5 ml. under the abdominal skin. After 3–4 injections at 10 day intervals, the rabbits were bled and the sera were tested for antibodies to CT antigens by the ring test. To prevent confusion due to antibodies to rat plasma proteins, which were invariably present in all antisera, the latter were mixed with rat plasma before carrying out the ring test.

Antisera against antigens in Disc 4 and Disc 7–8 were obtained as follows : 3 ml. of Lg-KSSF were fractionated by preparative disc electrophoresis. The antigenic content of

the discs was analyzed by diffusion in agar against AKGG. Discs 7 and 8 appeared to contain the same antigens while Disc 4 contained 2 antigens different from those in Discs 7 and 8. Discs 4 and 7–8 were injected into 2 sets of rabbits to prepare the corresponding antisera, (Anti-Disc 4 and Anti-Discs 7–8, AD 4 and AD 7–8 respectively).

Antiserum against Disc 1 (AD1) was prepared with Disc 1, obtained after fractionating LSSF on a polyacrylamide gel by preparative disc electrophoresis.

(c) Anti " precipitate " serum (A Prec). An antiserum directed solely against soluble components of Lg-KSSF was obtained as follows: Lg-KSSF was ultracentrifuged in a Spinco Model L ultracentrifuge, at 100,000 × *g* for 1 hr. The supernate (2·5 ml.) was reacted with an amount of ALgGG (5 ml.) chosen so as to give maximum precipitation, in the presence of 5 ml. rat serum. The latter reagent was added in large excess, in the hope of preventing formation of immune precipitates between rat serum proteins present as contaminants in Lg-KSSF and corresponding antibodies present in ALgGG. The precipitate, consisting of ultracentrifuged Lg-KSSF and its antibodies, was emulsified with Freund's adjuvant and injected as above.

AD 1, AD 4, AD 7–8 and A Prec were used as whole sera. All antisera, whether fractionated or not, will be referred to as " antisera ". All antisera (even A-Prec) contained antibodies to rat plasma proteins. These antibodies were removed by absorbtion with lyophilized or non lyophilized rat plasma (40–100 mg. rat plasma/ml. antiserum).

Antiserum to rat plasma (A Rat P1) was prepared by injecting into rabbits rat plasma in Freund's adjuvant, according to the usual schedule (see above). It was used as the γ-globulin fraction (A Rat P1 GG).

Antiserum to rabbit γ-globulin (ARGG) was obtained by injecting into guinea-pigs a washed immune precipitate, prepared by reacting guinea-pig serum with rabbit anti-guinea-pig serum. The precipitate was administered first in complete Freund's adjuvant (2 s.c. injections with an interval of 2 weeks). Further injections were given i.d. in saline, twice a week, until a strong Arthus reaction was produced. The guinea-pigs were bled from the heart and the γ-globulin fraction was separated from the pooled antiserum by fractionation with ammonium sulphate. This fraction (ARGG) gave a very strong line in the γ-globulin region, when examined in immunoelectrophoresis against whole rabbit serum. (Fig. 1).

Fluorescent antibody technique.—The γ-globulin fraction of guinea-pig anti-rabbit gamma globulin serum (ARGG) was labeled with fluorescein isothiocyanate (F1 ARGG) (California Corporation for Biochemical Research, Los Angeles) following the method of McKinney, Spillane and Pearce (1964). Non specific staining was removed by absorbing F1 ARGG twice with dried rat liver powder (100 mg./ml. γ-globulin). Fluorescent antibody tests were performed by the " sandwich " technique. γ-globulin fractions of antisera were diluted to 16 mg. γ-globulin/ml. This was the highest concentration at which normal rabbit γ-globulin (NRGG) failed to " stain " the tissue sections. The antisera were absorbed with acetone dried guinea-pig liver powder (100 mg./ml. per absorption) or with wet, washed sediment of guinea-pig liver homogenate (1 ml. sediment/ml. per absorption). The absorptions were repeated 4 times. Specificity of staining was checked by absorbing antisera with SSF in solution or with lyophilized SSF, or both. Control experiments showed that lyophilization did not result in loss of diffusion lines. When absorption of concentrated antiserum proved insufficient to remove all fluorescent staining, the antisera were first diluted with borate saline to ascertain the minimal concentration still giving satisfactory staining. A similar dilution was then prepared with the antigen solution as diluent, to obtain the absorbed antiserum. If necessary, further absorptions were carried out by adding lyophilized antigen. Tests and control antisera were always used at the same concentration. ALgGG was absorbed with lyophilized Lg-KSSF prior to dilution with the antigen solution. AKGG was absorbed with Lg-KSSF which had been first ultra centrifuged in a Spinco Model L ultracentrifuge at 100,000 × *g* for 2 hr, and then lyophilized. Other samples of AKGG were absorbed by diluting AKGG with the supernate of ultracentrifuged Lg-KSSF. Absorption of A-Prec was carried out by diluting it 1 : 16 with ultracentrifuged LgK-SSF, (3·4 mg./ml.), *e.g.* 1 ml. of antiserum was absorbed with 51 mg. antigen. Absorption of AD 1 is described under results.

Rats were perfused through the aorta with borate saline. Organs were frozen quickly in dry ice. Lungs were immersed for a few min. in a 5 per cent gelatin solution before freezing ; the gelatin filling the alveolae facilitated the cutting of sections. Diaphragm was frozen while contained in a block of 5 per cent gelatin. 2–4 μ sections were cut in a cryostat (International Equipment Co, Mass.), at −20°. The sections were dried under a fan for

1 hr. and stored at −20° until used. Antisera were filtered immediately before staining by passing through a millipore filter (Millipore Filter Corp., Bedford, Mass.), 1·2 μ pore size, using a Swinny adapter. Before staining, sections were fixed for 10 min. in cold acetone (−18°), with the exception of liver sections which were fixed in cold alcohol (−18°). Controls included sections stained with: (a) NRGG, (normal rabbit γ-globulin) at the same concentration as the anti-organ γ-globulin, followed by F1 ARGG; (b) F1 ARGG only; (c) antiserum absorbed with corresponding SSF followed by F1 ARGG; (d) antiserum followed by fluorescent normal guinea-pig γ-globulin; (e) antiserum followed by non fluorescent guinea-pig anti-rabbit γ-globulin and then by F1-ARGG (inhibition tests).

A Wild M20 microscope (Wild Heerbrugg Ltd, Heerbrugg, Switzerland) with attachments for fluorescence microscopy was used. Black and white photographs were taken with Kodak high speed Tri X Pan film (Kodak Ltd, London) or Ilford Hp 3 film (Ilford Ltd, Ilford). Exposure times varied from 3½–8 min., according to magnification and strength of fluorescence. To minimize the diffuse, green light of the background and thus improve contrast, a Kodak No. 11 Wratten green gelatine filter (Kodak Ltd, London) was used in the camera eye piece.

Agar diffusion.—Antigens and antisera were analyzed by agar diffusion on microscopic slides following the standard techniques (Sulitzeanu, 1965).

Preparative Disc Electrophoresis.—The instrument and technique of Sulitzeanu and Goldman (1965) were used. Electrophoresis was carried out at 4°.

Protein determination.—Protein content of serum globulins was determined by measuring the optical density at 280 mμ, in N/10 NaOH, with rabbit γ-globulin as standard. Protein concentration of SSF was determined by a micro-biuret technique (Itzhaki and Gill, 1964), with bovine serum albumin as standard. Optical densities were measured in a Unicam SP 500 spectrophorometer.

RESULTS

Antibody content of antisera, as determined by agar diffusion experiments

The antibody content of ALGG, AKGG, ALgGG and ADGG has been described in the previous publication (Sulitzeanu, 1965). The diffusion patterns of the antisera to fractions of CT were examined in this work. These antisera contained in general fewer antibodies than the antigens to whole organ CT.

The Table gives the number of precipitation lines obtained with the antisera to fractions of CT. This varied from 1 line (AD 4) to 5 lines (AD 7–8). The antigens were tentatively identified (Table) by setting up AKGG side by side with the

TABLE.—*Number and Identity of Diffusion Lines Given by Antisera to Fractions of CT Antigens*

Antisera	Number of precipitation lines	Identity of precipitation lines (antigen number)
AD 4	1 weak line	10
A-Prec	2 strong lines	10 and 8 or 9
AD 1	1 weak and 2 strong lines	10, 8 or 9, 1 unidentified
AD 7–8	2 weak and 3 strong lines	12, 10, 8 or 9, 2 unidentified lines

Antigen numbers are identical to the number affixed to CT antigens in the previous publication (Sulitzeanu, 1965).

antisera to be tested, against Lg–KSSF as antigen, or by testing the antisera in pairs. The antigen numbers are identical to the numbers used before (Sulitzeanu, 1965). All antisera contained antibodies to antigen 10. Antibodies to antigen 8 (or 9) were found in 3 of them. Some of the diffusion experiments on which these conclusions were based are seen in Fig. 2.

When AD 1 and A–Prec were reacted in agar against supernates of whole organ homogenates, only weak or faint precipitation lines were obtained, thus confirming that these antisera were directed only to components of organ sediments (presumably CT).

Fluorescent antibody experiments

It was found in preliminary tests that concentrations of control NRGG higher than 16 mg./ml. caused non specific staining of CT structures. Anti-organ sera were therefore examined at concentrations not exceeding 16 mg./ml. Organ sections were tested with and without prior fixation to check for possible adverse effects of the fixative. No such effects were noted.

Staining by ALgGG.—Sections of the following organs were stained with ALgGG: Liver, kidney, lung, diaphragm and heart.

Liver.—Bright staining was observed in the sinusoidal walls and in the vascular system (portal tracts and central veins) (Figs. 3, 5, 7). Strands of connective tissue were seen to radiate from the walls of the central veins into the surrounding tissue (Fig. 3). The reticular network in the walls of the larger veins and arteries was clearly delineated (Fig. 5).

Absorption of ALgGG with lyophilized Lg-KSSF abolished completely the sinusoidal staining and reduced the staining of the vessel walls to a very low level (Figs. 4, 6). All other controls were completely negative (Fig. 8).

Kidney.—Strong fluorescence was seen in the basement membranes of the glomerular capillary tufts, of the tubules and of the intertubular capillaries (Fig. 9). Additional intensely stained structures were the Bowman's capsule and the blood vessels. In the arteries strong fluorescence was observed in the inner and outer elastic membranes and in the connective tissue fibres of the media. The endothelial cells were outlined by a thin rim of delicate fluorescence.

Lungs.—The connective tissue surrounding the epithelial layer of the bronchi and bronchioles was brightly stained. The epithelial cells were not fluorescent. The alveolar septa and blood vessels showed intense staining, but the adventitia of the arteries was devoid of fluorescence. Good staining was also seen in the sarcolemma sheath surrounding the smooth muscle bundles of the pulmonary artery (Fig. 11–15).

EXPLANATION OF PLATES

FIG. 1.—Immunoelectrophoretic pattern of guinea-pig anti-rabbit γ-globulin (central trough), against rabbit serum as antigen (small holes). A very strong line was produced in the γ-globulin region. Only small amounts of contaminating antibodies seem to be present.

FIG. 2.—Agar diffusion tests. The following abbreviations are used: L—Lung-kidney sonicate (Lg-KSSF); L′—lyophilized Lg-KSSF; H—lung homogenate; H′—kidney homogenate; RS—rat liver sonicate; h—rat liver homogenate; HS—human liver sonicate; 1, 4, 7—antisera to discs 1, 4, 7–8 respectively (AD1, AD 4, AD 7–8); P—anti-precipitate serum (A-Prec); k—anti-kidney serum (AKGG); Anti-precipitate serum (P) gives 2 lines with Lg-KSSF (L) (2a, c, e, f). Anti-precipitate serum gives only weak reactions with lung and kidney homogenates (H, H′) (2f). AD 4 serum (4) gives one weak line with Lg-KSSF (L) (2a, b). AD 7–8 (7) gives 5 lines with Lg-KSSF (L) (4 lines are seen in Fig. 2b). AD 1 (1) gives 2 lines with Lg-KSSF (L) (2e), 3 lines with liver sonicate (RS) (2g), one of which is also found in the liver homogenate (h) (2g). Fig. 2d shows that the antigens in Lg-KSSF are not damaged by lyophilization. The human liver sonicate, tested as a control for rat liver sonicate (HS and RS respectively), did not react with AD 1 (1) (2g).

Fluorescent antibody staining

Staining by ALgGG

FIG. 3.—Liver section. Fluorescent connective tissue strands are radiating from intensely stained walls of central vein and sinusoids. ×635.

FIG. 4.—Control liver section stained with ALgGG absorbed with Lg-KSSF.

FIG. 5.—Portion of large liver portal tract. Fluorescent connective tissue fibres are seen clearly in the walls of the vessels. In the artery the fibres are arranged at right angles to the walls while in the vein the fibres are arranged along the walls, apparently in a helical-like pattern. ×635.

FIG. 6.—Portal area stained with ALgGG absorbed with Lg-KSSF. ×635.
FIG. 7.—Brilliant fluorescence in walls of liver sinusoids. ×635.
FIG. 8.—Liver section showing central vein stained with NRGG. Only grey autofluorescence of the tissue is apparent. ×635.
FIG. 9.—Bright fluorescence seen in several kidney structures: Bowman capsule, basement membranes of the glomerulus and of the tubuli. ×635.
FIG. 10.—Kidney section stained with AlgGG absorbed with Lg-KSSF. Tubular basement membranes are not stained. ×635.
FIG. 11.—Lung section showing bright staining in connective tissue of bronchus, at base of epithelial layer. ×315.
FIG. 12.—Lung section. Unstained central areas are epithelial regions of bronchioles. These are surrounded by fluorescent connective tissue. Alveolar septas are stained. ×635.
FIG. 13.—Lung section containing bronchiole, artery and alveoles. Internal and external elastic laminae of the artery are fluorescent. No staining is seen in the adventitia. ×315.
FIG. 14.—Lung section treated with ALgGG absorbed with Lg-KSSF. ×635.
FIG. 15.—Large artery in section of lung. The walls of the artery as well as the sarcolemma around the muscle fibres are fluorescent. The isolated small bright dots are due to impurities in the Fl-anti-RGG. ×635.
FIG. 16.—Section of diaphragm showing fluorescent staining in walls of vessel, capillary and sarcolemma surrounding muscle fibres. ×635.
FIG. 17.—Fluorescent connective tissue partitions (neurilemma) in nerve of diaphragm. ×635.
FIG. 18.—Treatment of diaphragm section with ALgGG absorbed with Lg-KSSF does not produce any fluorescence. ×635.
FIG. 19.—Section of heart muscle showing stained sarcolemma. ×275.
FIG. 20.—Artery in section of heart muscle. Internal and external elastic membranes are stained. A thin rim of fluorescence can be seen at the periphery of the endothelial cells. ×1120.

Fluorescent antibody staining by AKGG

FIG. 21.—Brilliant fluorescence in liver portal tract. ×275.

Fluorescent antibody staining by ADGG

FIG. 22.—Strongly fluorescent sinusoids and connective tissue fibrils of liver stroma. ×275.
FIG. 23.—Reticulum fibres in section of liver appear to have a double stranded structure. ×1120.
FIG. 24.—Kidney section, showing brilliant staining in intertubular capillaries. Tubular basement membranes are stained weakly. ×1120.
FIG. 25.—Characteristic, tree like fluorescent staining (Mesangium?) in glomerulus. Bowman's capsule is stained weakly. ×635.

Fluorescent antibody staining by AD 1

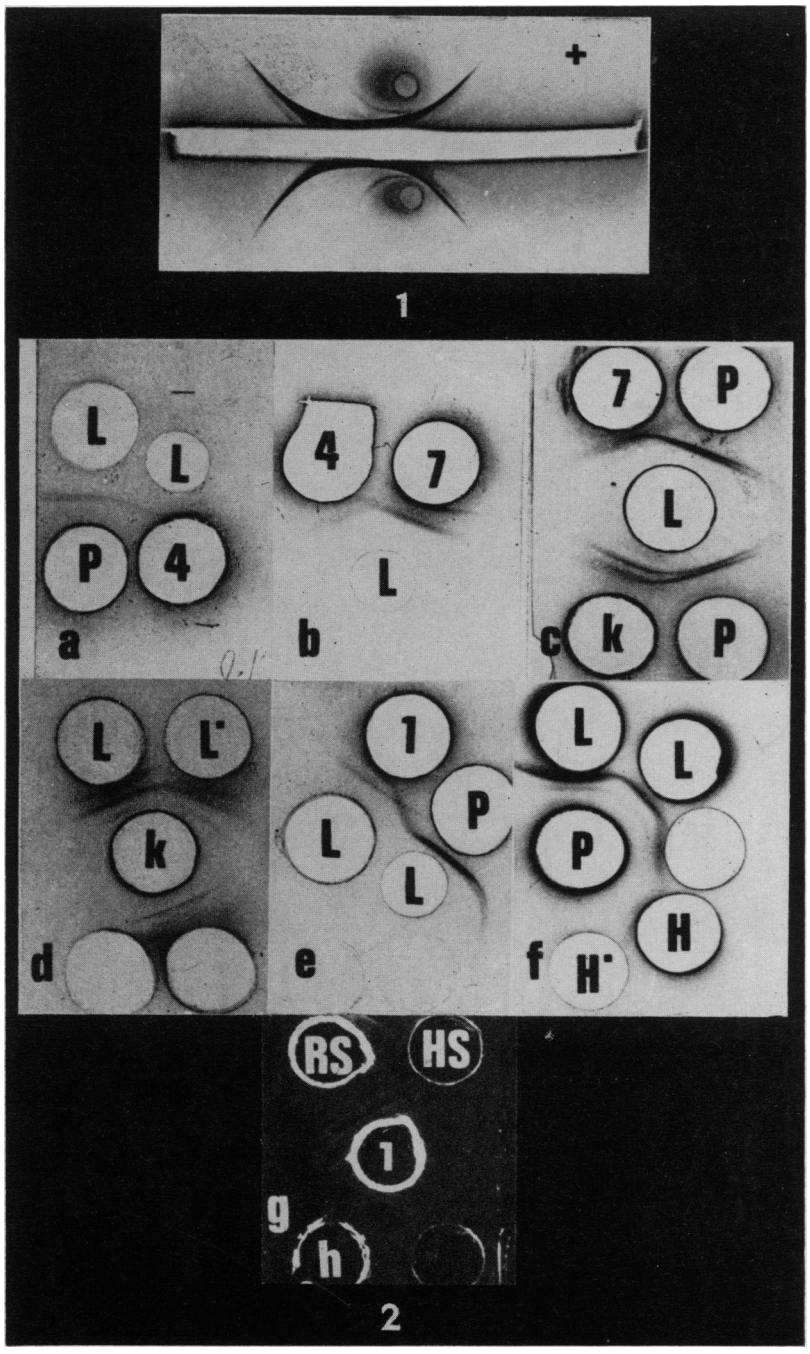
FIG. 26.—Brilliant fluorescence in glomerular capsule and basement membrane of glomerular capillaries. ×500.
FIG. 27.—Transverse section through tubules and capillaries. Capillary walls are intensely stained. ×500.
FIG. 28.—Longitudinal section through kidney capillaries. ×500.
FIGS. 29–33.—Different types of reticular network in walls of kidney blood vessels. The fibres are arranged in concentric, radial or reticular patterns. Fluorescent internal elastic lamina can be seen in Figs. 29 and 31. ×500.
FIG. 34.—Longitudinal section through strands of heart muscle cells. Cross striations appear fluorescent. ×500.
FIG. 35.—Cross-section through heart muscle. Intense fluorescence is seen in sarcolemma sheaths and capillary walls. ×500.

Fluorescent antibody staining by A-Prec

FIG. 36.—Brightly stained glomerulus. Staining in Bowman's capsule is very weak. ×750.
FIG. 37.—Cross-section through diaphragm, showing fluorescent sarcolemma and neurilemma. ×250.

Fluorescent antibody staining by Anti-rat plasma serum

FIG. 38.—Section of heart muscle. There is a great deal of fluorescence in particular in lumen and walls of small and large vessels. Connective tissue sheaths enclosing large bundles of muscle cells are also stained. Structures are blurred and indistinct, contrasting with the clean and clear cut staining given by anti CT sera.



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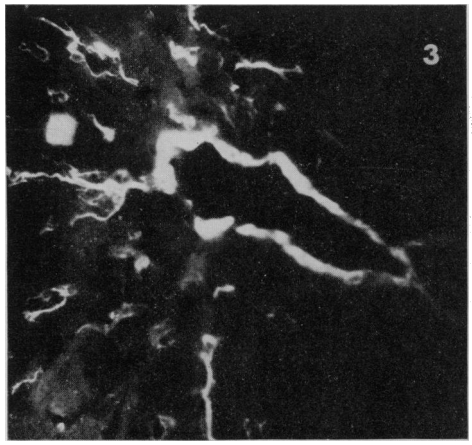

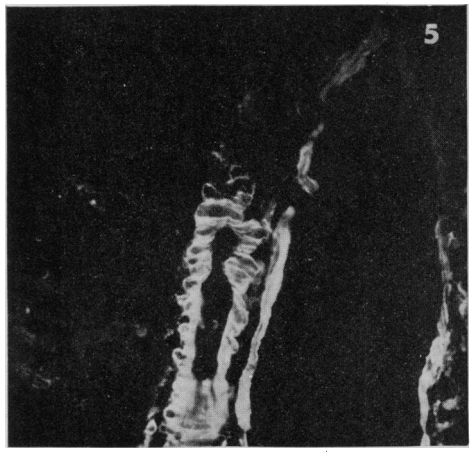

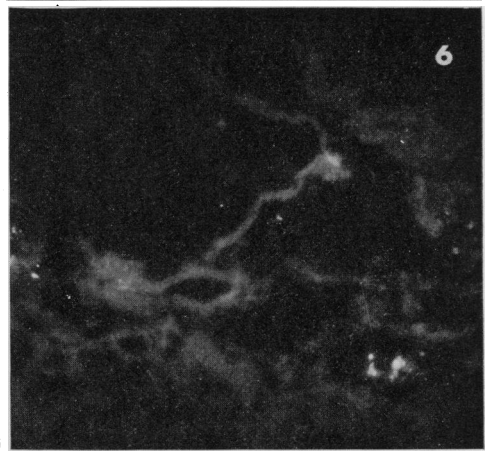

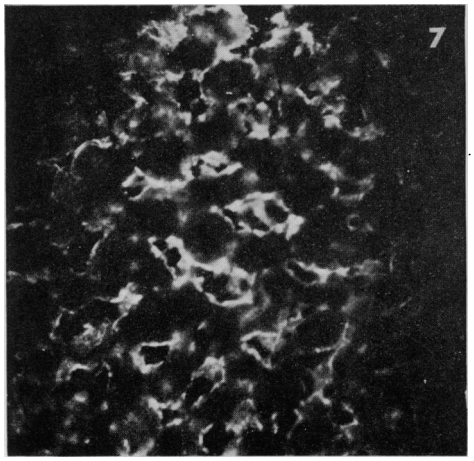

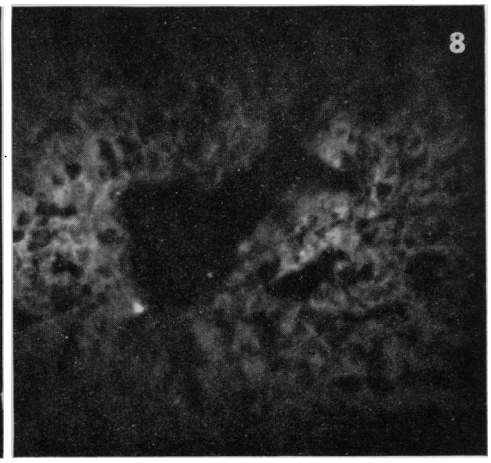

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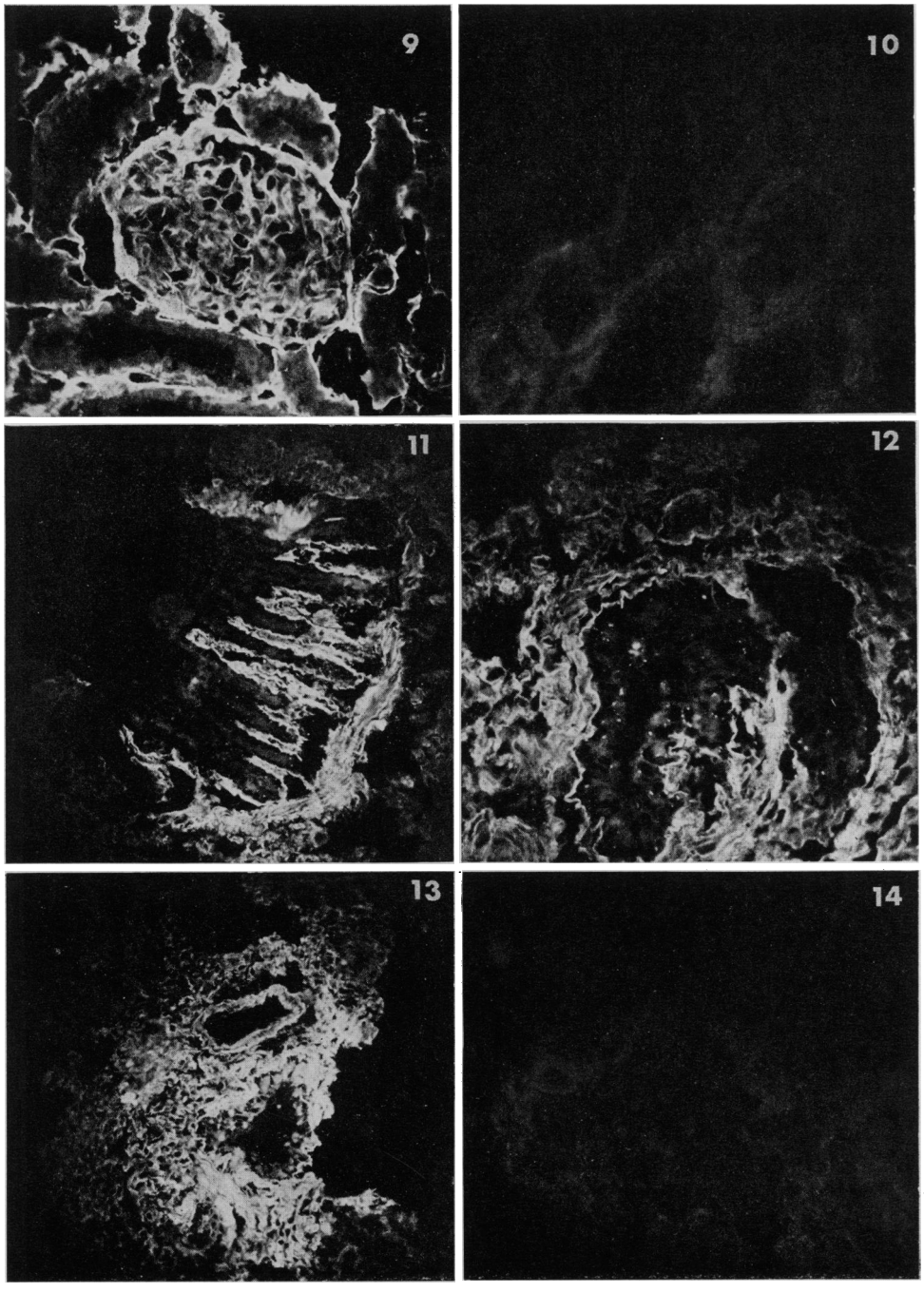
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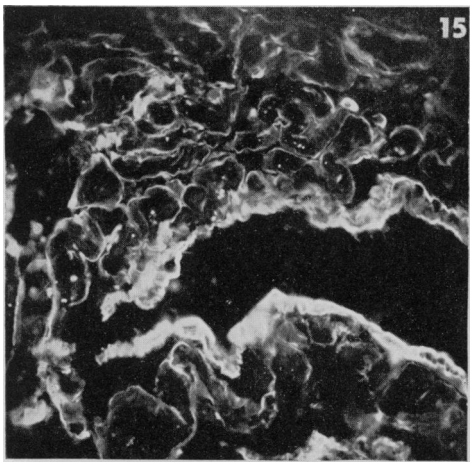

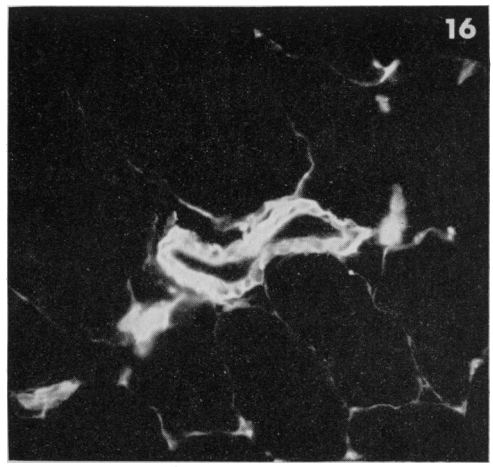

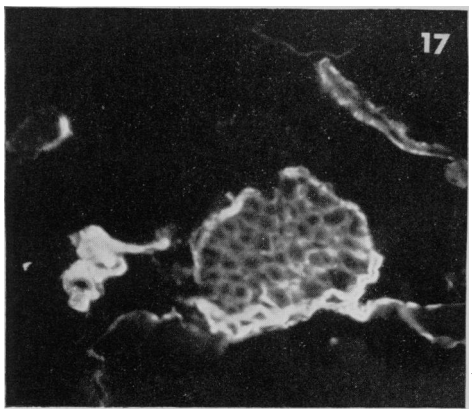

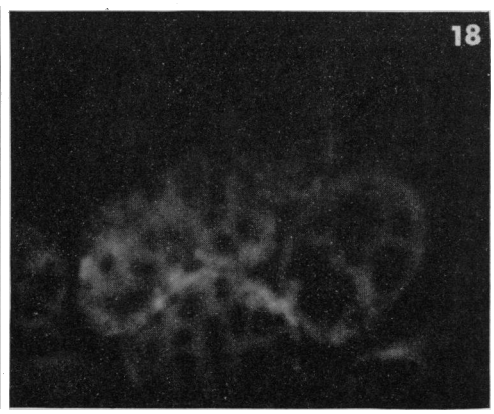

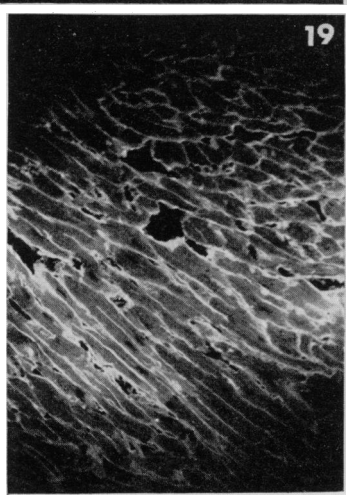

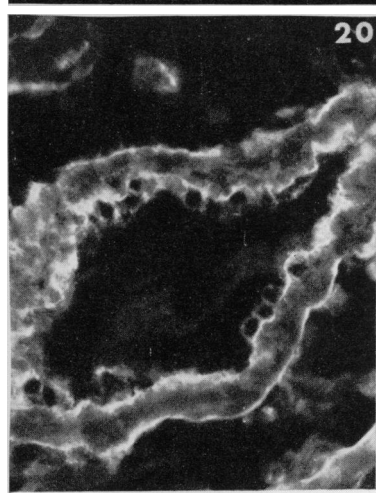

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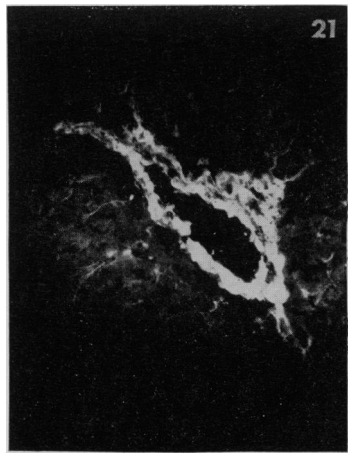

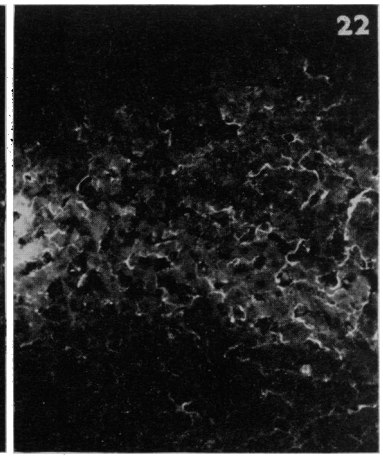

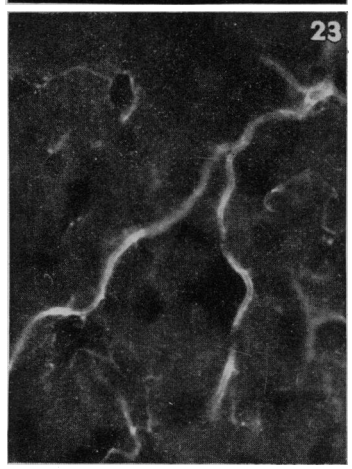

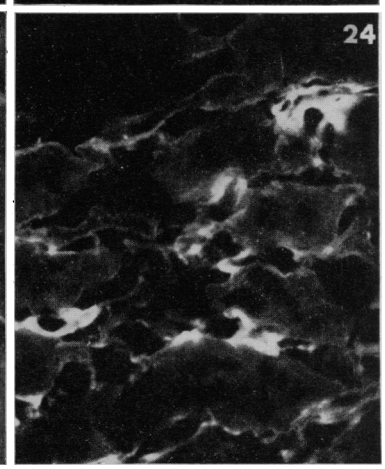

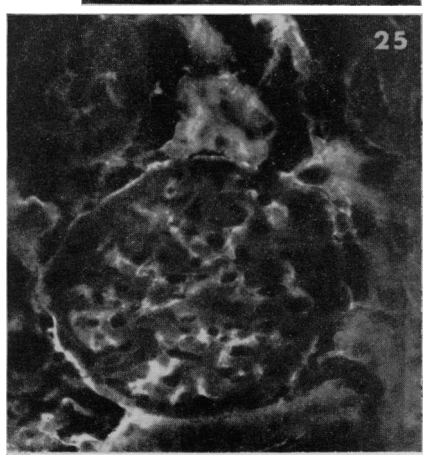

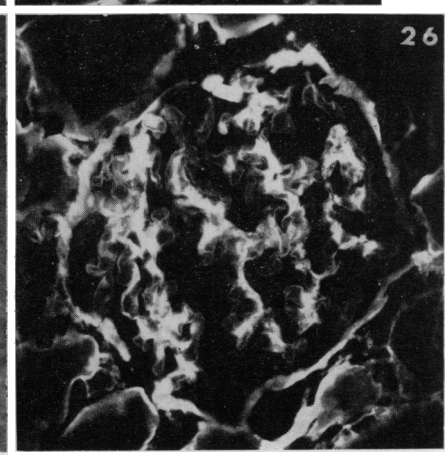

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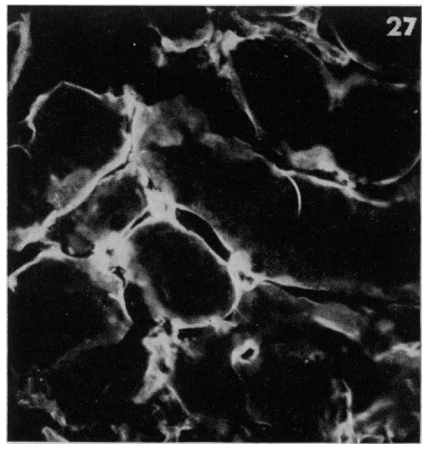
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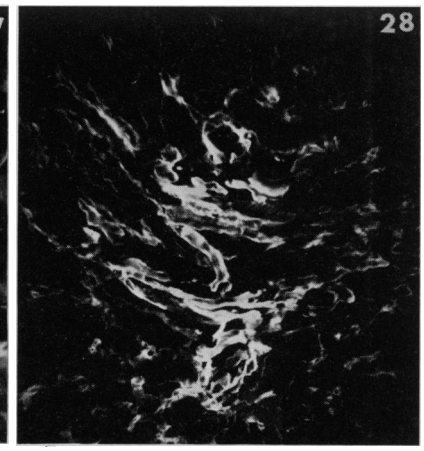
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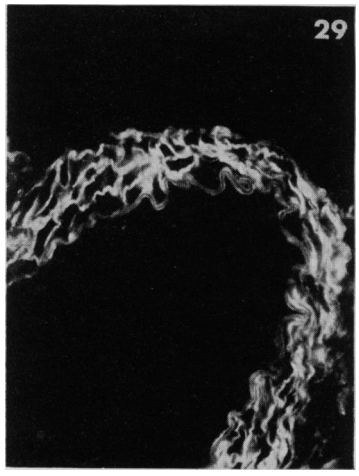
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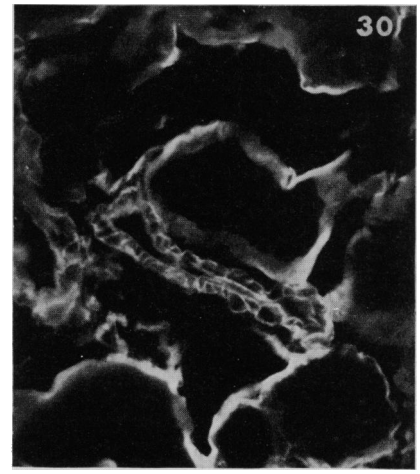
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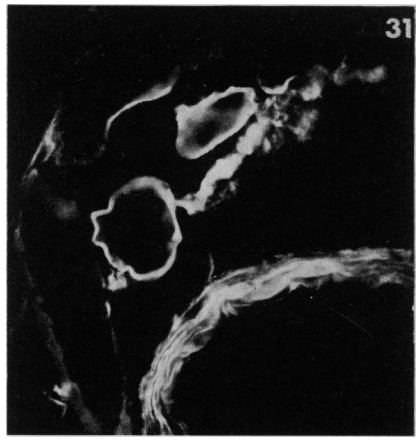
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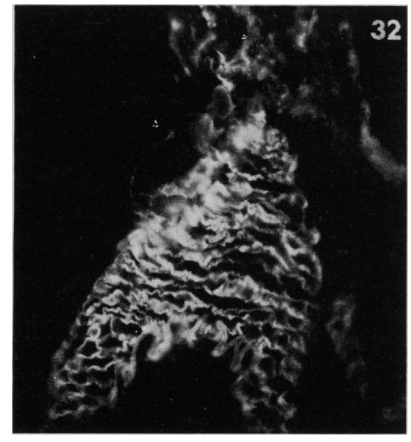
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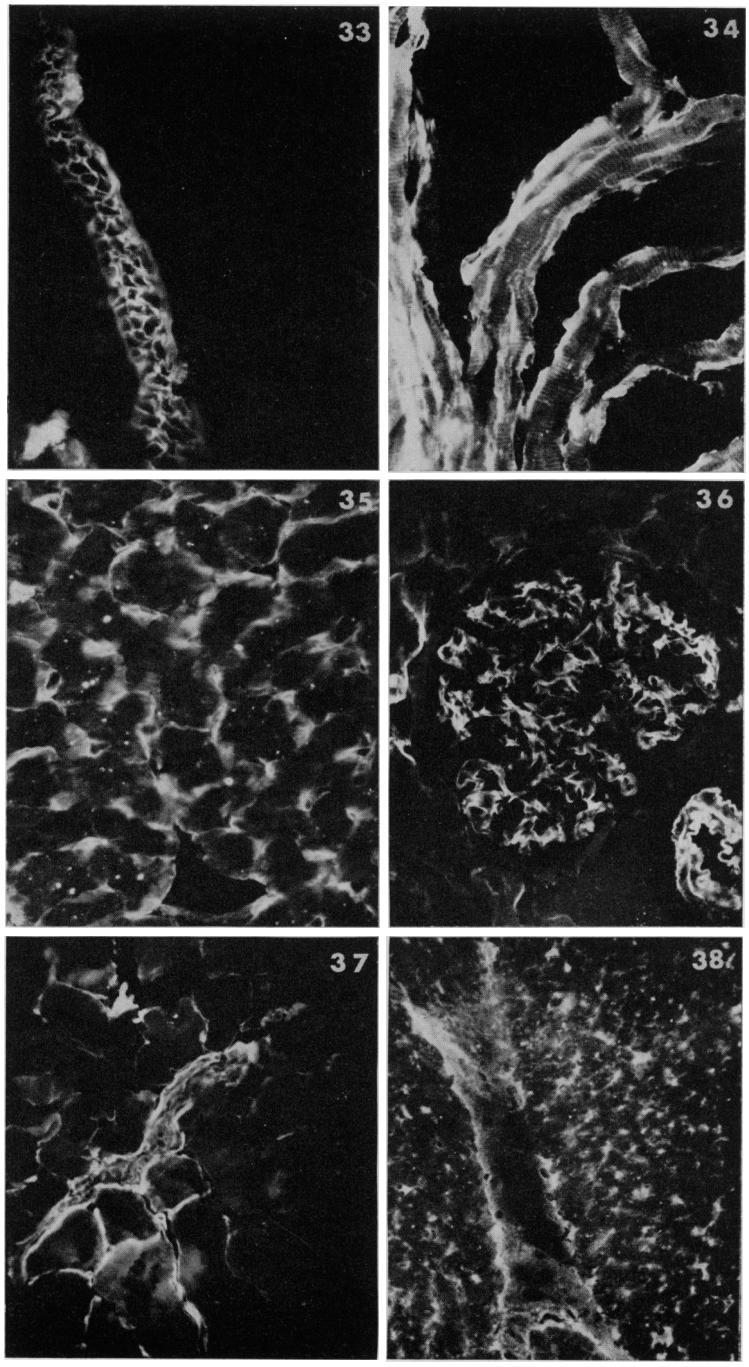

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Diaphragm.—Brilliant fluorescence was seen in the connective tissue membranes around the muscles and in the blood vessels, notably in the capillaries. The connective tissue sheaths in the nerve fibres were well stained (Figs. 16–18).

Heart.—All connective tissue elements were stained brightly : sarcolemma, blood vessels and capillaries (Fig. 19–20).

Staining by AKGG.—AKGG gave in general the same pattern of staining as the ALgGG. No photographs are therefore given (except for one—Fig. 21 as an example). Absorption of antibodies from AKGG with Lg-KSSF was much more difficult than absorption from ALgGG. Satisfactory differences between absorbed and non-absorbed serum were finally obtained by diluting the AKGG with either saline (for the test sample) or Lg-KSSF (for the absorbed, control sample) and by using diluted Fl anti-RGG. AKGG was used at 4 mg./ml. to stain the lungs, at 2 mg./ml. to stain the liver and at 1 mg./ml. to stain the kidneys, diaphragm and heart. Under these conditions the test samples gave good staining while the absorbed controls were completely negative.

Staining by ADGG.—This antiserum was tested on liver and kidney sections. In the liver, intense fluorescence was seen in the walls of the sinusoids and in the reticular fibres of the liver stroma (Fig. 22). These fibres showed often an unmistakeable double-banded structure (Fig. 23). The portal areas and the central veins were stained in the same fashion as seen with the other antisera.

ADGG stained the kidney in a somewhat different fashion than the other antisera. The basement membranes of the Bowman's capsule and of the tubules stained very weakly, while the glomerular basement membrane was almost free of fluorescence. On the other hand, a fluorescent, tree-like structure was often observed in the glomerulus (Fig. 25). The intertubular capillaries were brilliantly stained (Fig. 24). DSSF absorbed only partially the staining given by ADGG. Since the amount of DSSF available for absorption was limited, no attempt could be made to absorb the antiserum exhaustively. The other controls were negative.

Staining by ALGG.—ALGG was tested on liver sections. The usual connective tissue structures were stained, but contrast was poor because of some green background staining (possibly due to antibodies against constituents of the hepatic cells).

Antisera to fractions of CT (AD 1, 4, 7–8 and A-Prec) were tested on kidney sections and they gave a staining pattern similar in general to that of the antisera to whole organ CT. Two of the antisera, AD 1 and A-Prec were selected for further study, to check whether they would show any difference in the intensity and pattern of staining, as they had been prepared with antigens from different organs (liver and lung-kidney respectively).

Staining by AD 1.—AD 1 was examined on sections of kidney, liver, lung, heart and diaphragm.

Kidney.—Exceedingly strong staining was seen in the glomerular capsule, basement membrane of the glomeruli (Fig. 26), tubules (Fig. 27) and capillaries (Fig. 27, 28), in the internal elastic lamina, the membrane separating the media from the adventitia and the reticular network of blood vessels (Figs. 29–33). The variability in the structure of this network in the various vessels was remarkably well evidenced by the fluorescent staining.

Liver.—The usual CT structures were well stained. Double stranded reticular fibres, similar to those obtained with ADGG were seen.

Heart, Diaphragm and Lung.—Staining pattern was similar to that obtained with the antisera to whole organs, but the fluorescence was more intense. The adventitia of the blood vessels was not stained.

AD 1 was absorbed with either Lg-KSSF or with ultracentrifuged LSSF. Very large quantities of antigen (measured as total protein) were needed to abolish completely the fluorescent staining (up to 160 mg. protein/ml. of antiserum). Both Lg-KSSF and LSSF were effective in completely absorbing the antibodies, confirming the presence in both organs of the antigens involved. The controls were negative.

Staining by A-Prec.—The staining obtained with this antiserum was similar to that obtained with AD 1, with 2 exceptions :

(a) A-Prec stained only weakly the reticulum fibrils in the liver, while staining as intensely as AD 1 the portal tracts and the central veins.

(b) A-Prec stained very poorly the Bowman's capsule of the glomeruli (Fig. 36).

Staining by A Rat Pl GG.—It has been shown by Gitlin, Landing and Whipple (1953) that antibodies to rat serum would stain CT structures, among others. It was considered therefore advisable to compare the staining of anti-CT sera with that of A Rat Pl GG. The fluorescent patterns obtained with the latter reagent was altogether different from that obtained with anti-CT sera. The fluorescence was widespread and diffuse, the various CT membranes being poorly delineated. Fig. 38 shows the fluorescence seen in sections of heart muscle stained with A Rat Pl GG. The difference from anti-CT sera is noticeable in particular in sarcolemma, which was poorly stained, hazy and irregular. Cross-striations of the heart muscle were well stained. The fluorescence was completely abolished by absorption with rat plasma.

DISCUSSION

The results presented above confirm that all antisera studied in this work were directed against components of connective tissue and exclusively against these components. They all stained by the fluorescent antibody method, identical structures in all organs tested but they did not stain any other structures. The fluorescence seen in the cross striations of the heart muscle was very probably due to an antibody to a rat plasma component, since similar fluorescence was produced by A Rat Pl GG and this fluorescence could be abolished by exhaustive absorption with rat plasma. It can be concluded, therefore, that many, possibly most of the diffusion lines produced by these antisera were due to antigens of connective tissue.

The antisera against fractions of CT contained, as expected, a smaller number of diffusion lines than the antisera against whole organ CT. They had at least one antibody in common but differed in the content of their other antibodies. However, the fluorescent staining pattern of these antisera was almost identical. Moreover, there were no differences between the " simple " antisera and the complex, anti-whole organ CT sera, as regards staining properties. This discrepancy between variability in antibody content, as revealed by agar diffusion and constancy in fluorescent staining, could be due to one or more of the following reasons:

(1) All antisera might contain antibodies to the same antigens, but the concentrations of some of the antibodies might be too low for detection in agar. This seems unlikely, however, especially as regards the antisera to antigens separated by electrophoresis in polyacrylamide gels. Agar diffusion tests had confirmed that

separation of antigens had taken place during electrophoresis and therefore gel discs such as 1 and 7 could not have contained identical antigens.

(2) The antibodies reacting in agar might be totally unrelated to the antibodies involved in the fluorescent staining. The antigens in CT might form multilayered structures, with the internal layers unavailable for reaction with the fluorescent antibodies. The internal antigens would be freed by sonication and would thus be able to react in agar.

(3) One of the antibodies at least (*e.g.* antibody to antigen 10) might be present in all antisera. If this antigen were present in all CT structures, all antisera would give identical staining.

(4) The CT structures might have a complex composition and contain many antigens. Different antibodies, reacting with different antigens present at the same sites, would give identical fluorescence. This appears to us a most likely explanation, being in agreement with results obtained by previous investigators. Scott (1959, 1960) recognized the existence of 3 classes of connective tissue antigens on the basis of fluorescent antibody work. Krakower and Greenspon (1963, 1964) reached a similar conclusion on the basis of skin reactions to i.d. injection of anti-tissue sera. According to Krakower and Greenspon, all basement membranes contain both collagenous and non-collagenous antigens, while basement membranes of capillaries and venules contain also nephrotoxic antigens. Yagi and Pressman (1958) showed that several rat kidney antigens were responsible for the localization of radiolabelled, anti-rat kidney antibodies. Tan and Kaplan (1963) demonstrated the presence of an antigen related to mouse serum β-globulin in a variety of connective tissue elements. Pierce, Midgley and Sri Ram (1963) and Mukerjee, Sri Ram and Pierce (1965) described an insoluble protein antigen of epithelial origin in epithelial basement membranes. Finally, at least 2 basement membrane antigens were postulated by several investigators, using various techniques and various types of antisera (Goodman, Greenspon and Krakower, 1955 ; Steblay, 1965 ; Boss, 1965*a*).

The chemical structure of the antigens in CT cannot be determined until the individual antigens are first isolated in a reasonably pure form. To what extent collagenous materials are involved cannot be ascertained at present. The antigenicity of collagen, although weak, has been well documented. Following the initial demonstration of complement fixing antibodies to collagen (Watson, Rothbard and Vanamee, 1954), Rothbard and Watson showed that such antibodies, when injected into the living animal, localized at all sites were collagen and reticulin are present (Rothbard and Watson, 1961, 1962, 1965). They concluded that the antibodies were directed to an antigen present in both collagen and reticulin. The antigenicity of collagen has been confirmed by Steffen, Timpl and Wolff (1964), Steffen (1965), Paz, Davidson, Gomez and Mancini (1963) and Mancini, Paz, Vilar, Davidson and Barquet (1965). However, the latter group of workers failed to stain reticular fibres and basement membranes with antiserum to purified collagen fractions, although their antiserum stained collagen fibres and bundles. Cruickshank and Hill (1953), Scott (1957) and Boss (1963*a*) were unable to stain collagen fibres with anti-basement membrane and reticulin sera.

The extensive crossreactivity of CT elements in various organs has been noted by every investigator in the field. Our own diffusion studies and the absorption experiments of Boss (1965*a*,*b*) have suggested that differences in antigenic content might exist between CT of different organs. However, only further work with

purified antigens and antibodies will make it possible to clarify the extent of these differences.

Relatively little work has been done so far on the isolation of CT antigens, mainly because of the difficulty of obtaining them in solution. The reticulin prepared by Milazzo (1957) and the purified basement membrane material isolated from the mouse yolk sac carcinoma by Mukerjee *et al.* (1965) were insoluble. Attempts to solubilize the CT by using trichloroacetic acid or 8M urea (Robert and Robert, 1965) resulted in the denaturation of the proteins, but several CT antigens have been recently extracted in a soluble form (reviewed by Sulitzeanu, 1965). In the present work, particular attention was paid to the question of solubility, since the ability to obtain the CT antigens in true solution is an essential prerequisite to their further isolation and purification. The results seem to justify the conclusion that some of the antigens have indeed been obtained in solution :

(a) The antigens migrated unhindered in the polyacrylamide gel, in which even medium sized molecules such as fibrinogen are considerably retarded (Ornstein, 1964).

(b) Lg-KSSF retained the ability to induce antibodies to CT after removing the heavy material by centrifuging for 2 hr. at 100,000 *g* (A-Prec serum).

(c) The fluorescence of several of the antisera (AKGG, A-Prec, AD 1) was completely abolished by absorption with supernates of ultracentrifuged antigen. It is true that the quantities of antigenic material required for complete absorption were very large. However, this is believed to have been due to the very small concentration of the antigens. Judging by the results of the agar diffusion tests, the concentration of many of the antigens could have hardly exceeded 1 per cent of the total protein content of SSF.

We found in experiments not detailed here that AD 7–8 caused nephrotoxic serum nephritis when injected into rats. The soluble antigenic mixture used in preparing this antiserum must have contained, therefore, soluble nephrotoxic antigen(s), confirming a report by Cole, Cromartie and Watson (1951) that this antigen can be obtained in solution. The material obtained by Cole and co-workers could absorb out the nephrotoxic activity of the antiserum. It was not assayed, however, for ability to induce formation of nephrotoxic antibodies, as it was done here.

With pure antigens and antibodies as analytical reagents it should be possible to get some information on the chemical structure not only of the normal, but also of the pathological CT. The nature of the tissue alterations in collagen diseases still awaits clarification. Likewise, little is known of the composition of the CT in diseases such as liver cirrhosis. While experimentally induced cirrhosis, whether induced by CCl_4 or by ethionine might be similar at the microscopical level, differences might be found at the molecular level. Alterations in the constitution of CT in pathological conditions have already been described (Hamerman and Sandson, 1963 ; Sandson and Hamerman, 1964 ; Boss, 1965*b*). It is hoped that the methods developed in the present work will constitute a useful contribution to the developing discipline of molecular pathology.

SUMMARY

Eight antisera to rat connective tissue were tested by the fluorescent antibody technique for their reactivity with connective tissue of various organs. Four of

these antisera were obtained by injecting into rabbits whole organ connective tissue, derived from liver, kidney, lung or diaphragm. Three of the antisera were prepared against connective tissue fractions separated by preparative disc electrophoresis and the remaining antiserum was prepared against a " light fraction " (supernate of ultracentrifuged connective tissue extract). Agar diffusion analyses showed that the antisera to connective tissue fractions contained fewer antibodies than the antisera to whole organ preparations. The antisera stained, exclusively, the connective tissue in all organs tested (one or several of the following organs : liver, kidney, lung, diaphragm, heart), suggesting that most, if not all of the antigens with which they reacted in agar were connective tissue components. In spite of the great variability in antibody content, which ranged from 1–14 diffusion lines in agar, the antisera gave practically identical staining patterns, with some minor exceptions. It was concluded that the connective tissue contains a complex mixture of antigens, the similarity in staining being due to different antibodies reacting with different antigens located at the same sites.

Several lines of evidence indicate that many of the antigens were obtained in true solution : an ultracentrifuged extract retained the ability to produce antibodies which stained the connective tissue by fluorescence microscopy ; the fluorescent staining of several of the antisera was abolished by absorption with ultracentrifuged extracts ; many of the antigens migrated easily in polyacrylamide gel, which greatly retards even medium-sized molecules (fibrinogen).

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