A STUDY OF THE ANTIGENICITY OF BASEMENT MEMBRANE AND RETICULIN

D. G. SCOTT

From the Oxford Regional Rheumatic Diseases Research Centre, Stoke Mandeville Hospital, Aylesbury, Bucks.

Received for publication December 14, 1956

THE immuno-histological technique of Coons (Coons and Kaplan 1950 ; Coons 1956), which uses antibody protein conjugated with fluorescein as a stain for the corresponding antigen, has made possible the accurate localization in tissue sections of antigens native to the tissue (Marshall, 1951 ; Hill and Cruickshank, 1953 ; Mellors, Siegal and Pressman, 1955).

Using this technique, Cruickshank and Hill (1953*a*, *b*) demonstrated that an antiserum against a crude homogenate of glomeruli reacted *in vitro* with reticulin and basement membrane in the kidney and other organs. These observations indicate that there is some overlap of antigenic components between renal glomeruli and other tissues. They do not establish whether this overlap is complete (*i.e.*, that glomeruli and other tissues share a single antigen or an identical set of antigens) or partial. To resolve this question, experiments have been undertaken to compare the reactivity of antibodies evolved by immunizing animals with extra-renal tissues, with the reactivity of an antiserum against a crude homogenate of glomeruli. Synovium was selected for the first experiment on these lines and the purpose of this paper is to report a comparison of the properties of conjugated anti-glomerulus and anti-synovium antibodies, to present evidence for the presence in the glomerulus of two distinct antigens (or groups of antigens), and to describe the distribution of these antigens in the body.

Globulin fractions prepared from the sera of rabbits immunized with isolated human renal glomeruli or with human synovial membrane were conjugated with fluorescein by the method of Coons and Kaplan (1950). Unfixed sections of healthy human organs and tissues were examined under the fluorescence microscope after exposure to one or other of the conjugated antisera. In each case certain tests of specificity (*vide infra*) were carried out. The distribution of the specific fluorescence produced by one conjugate was compared with that produced by the other. Cross-inhibition experiments in which sections were pre-treated with the unconjugated anti-glomerulus globulin before exposure to the conjugated anti-synovium globulin (and vice versa) were performed to compare the antibody content of the two antisera.

MATERIALS AND METHODS

Preparation of homogenate and glomeruli

Glomeruli were isolated by a method based on that of Krakower and Greenspon (1951) from histologically normal human kidneys obtained at autopsy and stored at $-15°$ until required. The isolated glomeruli were suspended in buffered saline (pH 7·2) containing 1/10,000 merthiolate. The suspension, containing naked glomerular tufts, empty parietal capsules, whole glomeruli and clumps of tubule cells, was fragmented in a Griffith tube,

packed by centrifugation at 3000 r.p.m. for 30 min., and finally adjusted to a 10 per cent v/v concentration in buffered saline. Tubule cells formed up to 10 per cent of the suspension before fragmentation. No attempt was made to estimate the proportions of whole glomeruli and glomerular derivatives.

Preparation of homogenate of synovium

Human synovial membrane was obtained at operations for internal derangement of the knee joint and at autopsy from the knee joints of cases of accidental death.

The synovial membrane was freed as far as possible from sub-synovial tissue by blunt dissection and the material remaining, which consisted of synovial cells, capillaries and sub-synovial tissue, was pooled and stored at $-15°$ until required.

The antigen suspension was prepared by grinding pooled synovium in sand. The sand was separated by sedimentation in buffered saline. The ground synovium was packed in the centrifuge, re-suspended in buffered saline, fragmented in a Griffith tube, packed by centrifugation and finally brought to a 20 per cent v/v suspension in buffered saline.

The antigen was injected into rabbits as a 10 per cent v/v suspension in saline and also (following the method of Freund) as a 10 per cent water in oil emulsion, containing killed tubercle bacilli, made up as follows : 5 ml. of 20 per cent suspension of synovium in saline were emulsified with 5 ml. of liquid paraffin in which 25 mg. of killed tubercle bacilli had previously been incorporated. Crill 19 (Croda Ltd., Snaith, Yorks) was used as an emulsifying agent.

Preparation of antisera

Suspensions of antigen in saline were injected intra-peritoneally in 1 ml. doses on 4 days of the week for 4 weeks.

The antigen-adjuvant emulsion in an immunizing dose of 2 ml. was injected subcutaneously in four separate sites, one in each limb on a single occasion (Table I).

TABLE I.—*Immunization Schedules*

Rabbit	Antigen in saline 4-week courses	Antigen + adjuvants $4 \times 0{\cdot}5$ ml. injections	Antigen
H/124	2	—	Glomeruli
H/125	2	—	,,
H/131	2	1	Synovium

Conjugation of globulins with fluorescein

The animals were bled from an ear vein 10 days after completion of immunization. Conjugates were prepared, by the method of Coons and Kaplan (1950) from serum globulin separated by half saturation of serum with ammonium sulphate. Before use conjugates were absorbed twice with an equal volume of packed washed human Group O red cells, twice with normal human serum and twice with acetone-dried guinea-pig liver powder (to remove the non-specific staining produced by all untreated conjugates). The absorption with human serum was carried out by adding one part of normal human serum to 100 parts of the conjugate. The serum and conjugate were allowed to react for 12–24 hr. at 4° ; the mixture was then centrifuged and the supernate kept for use.

Preparation of tissue sections

The methods of Coons and Kaplan (1950) and Coons, Leduc and Kaplan (1951) were followed in the preparation of quick-frozen unfixed blocks of tissue and in the cutting of sections (4 μ thick) from them in a cryostat. The sections were mounted on acid-cleaned glass slides without adhesive. After staining in one or other of the ways described below, the sections were mounted in glycerol containing 1 part in 10 of buffered saline, and covered with a glass coverslip.

Fluorescence microscope

The light source and filters used were as described by Hill and Cruickshank (1953), except that the gelatine and sodium nitrite eye-piece filters were replaced by a Lempert filter.

When a photograph of a stained section was to be taken, slides made from OX1 filterswere used in place of ordinary glass slides. Their use increases contrast by replacing glass slides, which produce a blue background fluorescence, with non-fluorescent material. They also absorb some of the blue-violet light transmitted by the Woods glass filter, positioned between the light source and the microscope. Coverslips made of OY12 glass were used in conjunction with the OX1 filters to prevent fluorescence of the microscope lens system excited by ultraviolet light entering the objective (the use of OX1 filters and coverslips of OY12 glass was suggested by Dr. W. R. Alexander).

Staining experiments

The staining of tissue sections by fluorescein-conjugated antibody globulin is in essence an immunological reaction resulting in the fixation of an antibody by its antigen in the tissue. The site of fixation of a fluorescein-conjugated antibody is rendered readily visible by virtue of its yellow-green fluorescence when exposed to ultra-violet light.

Direct staining experiments

In this group of experiments staining produced by the anti-glomerulus conjugate was compared with that produced by the anti-synovium conjugate. Two contiguous sections from the same block of tissue were mounted on one slide. Each section was pre-treated for 2 hr. with unconjugated normal rabbit globulin, rinsed in buffered saline and exposed to one or other of the conjugates for 1½ hr., after excess saline had been carefully removed from the slide. Exposure to the conjugates was followed by a wash for a total time of 10 min. in two changes of buffered saline which was kept gently agitated by a stream of air bubbles.

Tests of specificity

The criterion of specificity of staining was the demonstration that unconjugated antibody globulin would block the fluorescence produced in a tissue section by the corresponding conjugate, whereas unconjugated normal rabbit globulin would not. Of two sections mounted on the same slide, one was pre-treated with normal rabbit globulin (2 hr.), and the other with unconjugated antibody globulin (2 hr.), and both were subsequently exposed to the conjugate under investigation for 1½ hr. Such tests of specificity were routinely applied.

Cross inhibition experiments

These were performed in the same way as the tests of specificity except that sections were pre-treated with one antibody globulin (unconjugated) and then exposed to a conjugated fraction of the other antibody globulin. Direct staining with each of the conjugates and tests of specificity were run in parallel with cross inhibition experiments.

RESULTS

Direct staining experiments

The observations made in this group of experiments are set out in Table II. No attempt has been made to record variations in intensity of fluorescence produced in sections stained by different conjugates, or between individual elements in the same preparation. The factors governing the intensity of fluorescence produced are not readily controlled so that a reliable estimate of the relative strengths of two antibody solutions or the relative concentrations of two antigenic components in the tissues cannot be made from a visual comparison of the intensity of fluorescence produced.

Only the anti-glomerulus conjugate produced staining of the renal glomerular capillaries (Fig. 2), the basement membranes of the thyroid acini (Fig. 8), and the media of arteries (Fig. 5). On the other hand both conjugates stained the basement membrane of renal tubules (Fig. 2), sarcolemma (Fig. 4), and the periacinar reticulin in the thyroid gland (Fig. 8 and 9). Both stained fine fibrils lying in the interstitium of the thyroid gland and among the collagen fibres of the splenic

TABLE II.—*Results of Direct Staining and Cross Inhibition Experiments*

Organ	Histological component	Direct staining experiments: Anti-glomerulus conjugate	Direct staining experiments: Anti-synovium conjugate	Cross inhibition experiments: Anti-glomerulus globulin and anti-synovium conjugate	Cross inhibition experiments: Anti-synovium globulin and anti-glomerulus conjugate
Kidney	Glomerular capillaries	+	−	−	+
	Glomerular epithelial cells	+	+	−	−
	Parietal capsule of glomerulus	+	+	−	−
	Tubular basement membrane	+	+	−	−
	Tubular cells	−	−	−	−
	Peritubular capillaries	+	+	−	−
	Media of arteries	+	−	−	+
Thyroid	Acinar basement membrane	+	−	−	+
	Peri-acinar reticulin	+	+	−	−
	Interstitium	+	+	−	−
	Capillaries	+	+	−	−
	Media of arteries	+	−	−	+
Spleen	Reticulin	+	+	−	−
	Fibrils in trabeculae	+	+	−	−
	Media of arteries	+	−	−	+
	Sarcolemma	+	+	−	−

trabeculae. Both stained sub-synovial capillaries and both produced specific pericellular fluorescence in the synovial cell layer (Fig. 6 and 7). Neither conjugate stained collagen.

The anti-synovium conjugate did not stain any structures which were not also stained by the anti-glomerulus conjugate.

The most striking difference between the two conjugates was seen in their reaction with the renal glomerular tufts (Fig. 1). The anti-glomerulus conjugate, which stained the basement membrane of glomerular capillaries, produced a characteristic picture made up of a series of fluorescent loops. The anti-synovium conjugate did not produce a loop pattern in glomeruli but it stained numerous discrete structures, round, oval, polygonal, or crescentic in shape, in the glomerular tuft. Under high power these structures frequently contained a round unstained centre. The appearances suggested that it was the cytoplasm of cells which had reacted with the conjugated anti-synovium preparation. Occasionally two or three such cells lay side by side forming the boundary of an unstained space. Similar cells could be seen in preparations stained with the anti-glomerulus conjugate. In this case they lay closely applied to the basement membrane of glomerular capillaries. No firm conclusion about the relationship of these cells to basement membranes could be formed. It follows that the identity of these cells has not been firmly established but their situation suggests that they may be glomerular epithelial cells. Very occasionally similar cells were seen lying in Bowman's capsule.

Cross inhibition experiments

Cross-inhibition experiments were performed to confirm the differing antibody content of the two types of antisera. The results are summarized in Table II. It was found that unconjugated anti-glomerulus globulin would inhibit all the staining produced by anti-synovium conjugate, whereas unconjugated anti-synovium globulin would prevent only some of the staining produced by anti-glomerulus conjugates. The anti-synovium globulin failed to prevent the reaction of anti-glomerulus conjugate with the basement membranes of glomerular capillaries (Fig. 3) and with the media of muscular arteries and arterioles. It did prevent the reaction of the anti-glomerulus conjugate with extra-glomerular capillaries, renal tubular basement membrane and the sarcolemma of muscle. A section of thyroid gland pre-treated with the anti-synovium globulin (unconjugated) and subsequently exposed to the anti-glomerulus conjugate, showed fluorescence lying only in the position of a basement membrane (Fig. 10: compare with Fig. 8 and 9).

EXPLANATION OF PLATES

All the photographs are fluorescence photomicrographs of unfixed tissue sections treated with fluorescent globulin solutions. The lighter lines and areas in the photographs represent the yellow-green fluorescence of fluorescein and indicate sites of fixation of conjugated antibody. Most tissue components exhibit a dull blue-grey autofluorescence under the fluorescence microscope. Such autofluorescence is not readily confused in the photographs with the fluorescence of fluorescein. Elastic tissue exhibits a brilliant white autofluorescence which has the same appearance in the photographs as green specific fluorescence.

FIG. 1.—Upper. Human renal glomerulus treated with anti-glomerulus globulin. Specific fluorescence in basement membranes and epithelial cells. (× 350.)

Lower. Human renal glomerulus treated with anti-synovium conjugate. Staining confined to the epithelial cells. The cell seen in the lower right-hand corner lies on Bowman's capsule. (× 350.)

FIG. 2.—Human kidney treated with anti-glomerulus conjugate (top), and with anti-synovium conjugate (bottom). Specific fluorescence occurs in tubular basement membrane in both cases. (× 120.)

FIG. 3.—Human kidney. The section was pre-treated with unconjugated anti-synovium globulin and stained with anti-glomerulus conjugate. Specific fluorescence is present only in the glomerular tuft. (× 100.)

FIG. 4.—Human muscle treated with anti-synovium conjugate. Specific fluorescence is present in the adventitia of the vessel and in the sarcolemma. The white spots in the media of the artery and in the muscle represent granules of pigment showing orange autofluorescence. (× 350.)

FIG. 5.—Splenic artery treated with anti-glomerulus conjugate (G) and with anti-synovium conjugate (S). The arrow lies at the junction of the composite photomicrograph and points to the internal elastic lamina, which has a brilliant white autofluorescence. Only the antiglomerulus conjugate has reacted with the media of the vessels. Specific fluorescence occurs in the adventitia and splenic reticulin in both cases. (× 150.)

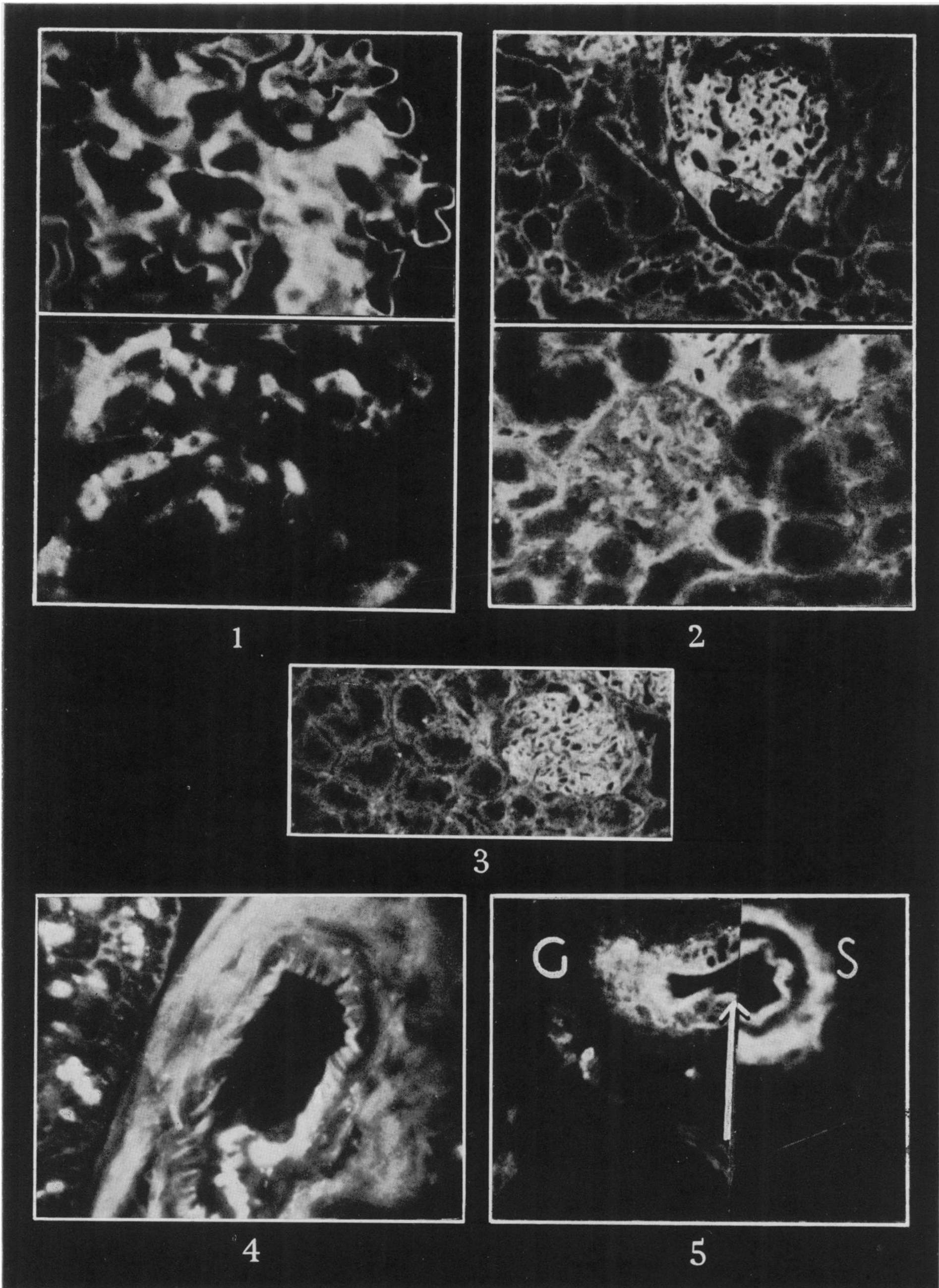
FIG. 6.—Synovium treated with anti-glomerulus conjugate. Specific fluorescence is present around the synovial cells and in the sub-synovial capillaries. (× 250.)

FIG. 7.—Synovium treated with anti-synovium conjugate. The specific fluorescence has the same distribution as in Fig. 6. (× 250.)

FIG. 8.—Thyroid treated by anti-glomerulus conjugate. The green fluorescence of the basement membrane is represented by a thick white line. The periacinar reticulin lying beneath the basement membrane is also specifically stained. (× 200.)

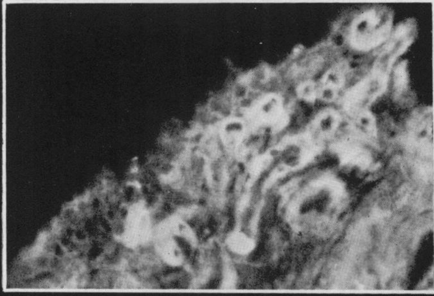
FIG. 9.—Thyroid treated with anti-synovium conjugate. The periacinar reticulin appears as a single line which splits to enclose round unstained spaces. (× 250.)

FIG. 10.—Thyroid pre-treated with unconjugated anti-synovium and stained with conjugated anti-glomerulus. Only the basement membrane is stained. (× 200.)

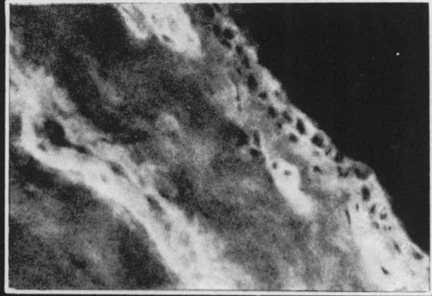


1
2
3
4
5

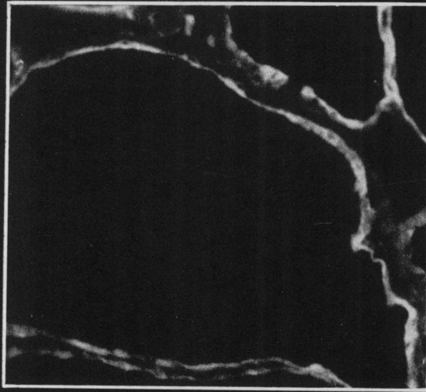
Scott.



6

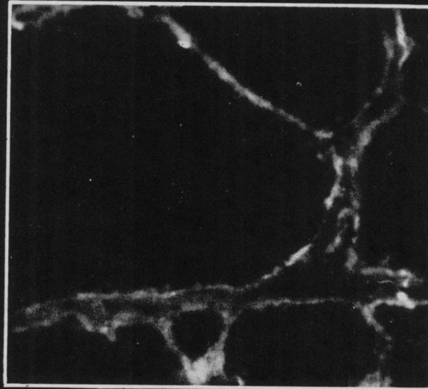


7



8

9



10

Scott.

DISCUSSION

The reactivity of the antiserum to isolated human glomeruli was the same as that described by Cruickshank and Hill (1953*a*) in that it was fixed *in vitro* by reticulin and basement membrane in the kidney and other organs. Mellors *et al.* (1955), using an *in vitro* technique, found a more limited uptake of anti-kidney antibody. They injected rabbit antisera to rat kidney into rats during life and traced the distribution of the injected antibody after death by means of conjugated chicken anti-rabbit globulin. These observations, based as they were on experiments *in vivo*, cannot be directly compared with those of Hill and Cruickshank. The differences may reflect variations in accessibility of the antigen to the antibody, which in one case is brought to the intact kidney by the blood stream, and in the other is applied to tissue sections.

The present observations suggest that the renal glomerulus contains at least two antigens. One of these, present in the basement membrane of the glomerular capillaries, has been shown to be shared by basement membrane in other organs. A study of the distribution of this antigen throughout the body has not yet been completed but preliminary observations indicate that it is present in the choroid plexus, the intra-hepatic bile ducts, the thyroid gland, and in the media of muscular arteries and arterioles, but not in the media of elastic arteries. The other glomerular antigen is more widely distributed throughout the body, and is found in much the same sites as argyrophilic reticulin.

It should be borne in mind that the preparations used for immunization in these experiments were crude tissue homogenates. Although the resulting antisera enabled certain tissue elements to be distinguished from each other, it is not justifiable to assume that each group so distinguished contained a single identical antigen. Such an assumption could only be made if the antisera were prepared by immunization with a single chemically pure antigen. For simplicity of description the term antigen is used here in circumstances where " antigen or group of antigens " would be more accurate.

Physical separation of tissue components differing in antigenic specificity has been accomplished by Goodman, Greenspon and Krakower (1955). They used a flocculation technique to study the antigenic inter-relationships of the various anatomical components of the dog's kidney isolated by sonic vibration and they identified two groups of antigens in the glomerulus. One of these was capable of stimulating the production of a heterologous nephrotoxic anti-serum. The other group of antigens, which did not evoke a nephrotoxic antiserum, was apparently present also in the glomerular parietal capsules and in a fraction consisting of the basement membranes of tubules (and stroma from the cortex) as well as in collagenous fibrils derived from tendon and cornea. The present observations are largely in agreement with those of Goodman *et al.* in that all these tissues specifically fixed an antibody against synovium which reacted also with certain elements (not including the capillary basement membrane) in the glomerulus.

More recently Baxter and Goodman (1956) have shown that an antigen capable of evoking a heterologous nephrotoxic serum is present in the renal medulla, lung and placenta of the rat in about the same concentration as in the renal glomerulus, while other organs (intestine, heart, liver stroma) contain this antigen in smaller amounts.

The present observations indicate that an organ rich in one antigenically active component of connective tissue is not necessarily rich in the other. It is not possible to relate the concentration of the nephrotoxic antigen, as defined by Baxter and Goodman, in individual organs of the rat, with the distribution in homologous human organs of either of the glomerular antigens demonstrated by the present application of Coons' technique. If the present observations could be taken to show that the glomerulus contains only two antigenically active components, this comparison might be possible. However, they only indicate that there are at least two such components. The antigenic relationship between capillaries and reticulin suggested by the present observations has not been finally established. Both capillaries and reticulin reacted with an antibody to a homogenate of synovium, but the latter contained capillaries which may have evoked an antibody distinct from that which reacted with reticulin.

The results of the present investigation and those of Goodman and his co-workers indicate that connective tissue is an antigenically heterogeneous substance. A study of the antigenicity of the various components of connective tissue and of the distribution throughout the body of the antigenically related components may help towards a better understanding of the pathogenesis of the diffuse mesenchymal diseases. The technique of Coons, which enables antigenically active components of connective tissue to be accurately localized, should prove a useful supplement to chemical and enzymic studies of connective tissue.

SUMMARY

The immuno-histological method of Coons was used to study the distribution of the antigenically active components of human synovial membrane and of human glomeruli.

Reticulin and basement membrane appear to be antigenically distinct. While the renal glomerulus was shown to possess both antigens, only the reticulin antigen was demonstrated in the synovial membrane and only the basement membrane antigen in the media of arterioles.

The relationship between these findings and those of other workers is discussed. It is suggested that the immuno-histological technique of Coons should prove to be a useful supplement to chemical and enzymic studies on connective tissue, and that a study of the distribution throughout the body of the several antigenically active components of connective tissue might help towards an understanding of the sites of the lesions in the diffuse mesenchymal diseases.

I am most grateful to Dr. A. G. S. Hill for patient tuition in the use of Coons' technique and to Dr. Hill and Dr. C. L. Greenbury for their helpful comments during the preparation of the script for publication.

The work was supported by a grant from the Nuffield Foundation.

REFERENCES

BAXTER, J. H. AND GOODMAN, H. C.—(1956) *J. exp. Med.*, **104**, 487.
COONS, A. H.—(1956) *Intern. Rev. Cytol.*, **5**, 1.
Idem AND KAPLAN, M. H.—(1950) *J. exp. Med.*, **91**, 1.

Idem, LEDUC, E. H. AND KAPLAN, M. H.—(1951) *Ibid.*, **93**, 173.
CRUICKSHANK, B. AND HILL, A. G. S.—(1953*a*) 'Nature and Structure of Collagen'. London (Butterworth's Scientific Publ.)—(1953*b*) *J. Path. Bact.*, **66**, 283.
GOODMAN, M., GREENSPON, S. A. AND KRAKOWER, C. A.—(1955) *J. Immunol.*, **75**, 96.
HILL, A. G. S. AND CRUICKSHANK, B.—(1953) *Brit. J. exp. Path.*, **34**, 27.
KRAKOWER, C. A. AND GREENSPON, S. A.—(1951) *Arch. Path.* (*Lab. Med.*), **51**, 629.
MARSHALL, J. M.—(1951) *J. exp. Med.*, **94**, 21.
MELLORS, R. C., SIEGAL, M. AND PRESSMAN, D.—(1955) *Lab. Invest.*, **4**, 69.