

DECLARATION

I declare that the experimental work described in this thesis
is my own work and that the thesis has been prepared by myself.

1971

IMMUNOHISTOLOGICAL STUDIES OF CONNECTIVE TISSUES

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M.D. Thesis University of Edinburgh 1971

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IMMUNOHISTOLOGICAL STUDIES OF
CONNECTIVE TISSUE

INTRODUCTION AND SUMMARY

This thesis is concerned with immunohistological studies of adult human connective tissues and with connective tissues in the adult and embryonic rat.

The studies of human connective tissue arose out of the researches of Hill and Cruickshank who had demonstrated, by immunohistological means, that there is some overlap in antigenic specificity between isolated renal glomeruli and other connective tissues (Cruickshank and Hill 1953a and b; Hill and Cruickshank, 1953). The present studies were undertaken to determine whether this overlap is complete or partial.

Globulin fractions of antisera raised in rabbits against human renal glomeruli and against human synovial membrane were labelled with fluorescein isocyanate. The distribution of specific fluorescence produced in tissue sections by the fluorescein-anti-human glomerulus conjugate was compared with that produced in serial sections by the fluorescein-anti-human synovium conjugate. In later work, globulin fractions of anti-human glomerulus and anti-human synovium antisera, each labelled with one or other of two fluorochromes of contrasting colour, fluorescein

isocyanate and lissamine rhodamine B200, were applied simultaneously or in sequence to a single section of tissue.

Membranes at the boundaries between epithelia and connective tissues were found to be composed of two antigenically distinct constituents. One of these, the basement membrane "proper", reacted with antibodies present only in anti-human glomerulus antisera, and manifested in direct staining, cross inhibition and cross absorption procedures, antigenic properties identical with those of the basement membranes of renal glomerular capillaries. The second constituent of epithelial boundary membranes, although reactive with antibodies present in anti-human glomerulus and in anti-human synovium antisera, was related by its antigenic properties more closely to splenic reticulum than to the basement membranes of renal glomerular capillaries.

A third group of connective tissues antigenically related to, but not antigenically identical with basement membranes and reticulum, was found to include fibroblasts and fibrils lying between bundles of collagen in the capsules of joints and in vascular adventitia.

These observations, since they pointed to a loose correspondence between the histological distribution and the antigenic specificities of the various

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connective tissues, stimulated speculation concerning the possibility that antigenic differences between connective tissues might, in part, be attributable to molecular configurations intimately involved in the function of the different tissues.

A basis for these speculations had, in fact, already been provided by the work of Krakower and his colleagues. These workers had shown that canine renal glomerular capillary basement membranes were related to capillaries throughout the body by their content of antigens responsible for the production of nephrotoxic antisera, and to renal tubular basement membranes and collagenous fibrils by their content of at least two groups of non-nephrotoxinogenic antigens (Goodman, Greenspon and Krakower, 1955). Studies of antigens responsible for the production of nephrotoxic antisera, NTS antigens, had shown that there were differences between the concentration of these antigens in different capillary beds (Krakower and Greenspon, 1958) and also between the concentration of NTS antigens in renal glomeruli from neonatal and adult animals (Krakower and Greenspon, 1954). It was found possible to relate the quantitative differences between the concentration of NTS antigens in different capillary beds to the hydrostatic or filtration pressures to which capillaries in different locations are normally exposed.

These observations of Krakower and his colleagues heightened speculation concerning the possibility that the antigenic properties of connective tissues might reflect not only their structural differentiation but their functional differentiation also. It became of interest, therefore, to determine whether adult and embryonic connective tissues exhibit a partial or complete overlap in antigenic specificity.

Studies of connective tissue in adult and embryonic rats are incomplete. They have, in the main, been concerned with the reactions of anti-adult rat connective tissue antisera and those of anti-embryonic rat connective tissue antisera with renal and dermal connective tissues in adult, young, neo-natal and embryonic rats.

Observations arising out of these studies have indicated that:

- (i) embryonic and adult connective tissues exhibit only a partial overlap in their antigenic specificity;
- (ii) the histological maturation of renal glomeruli is accompanied by a change in the antigenic constitution of their capillaries;
- (iii) differences exist between embryos and groups of embryos as regards the antigenic constitution of their dermal and

juxta-epidermal connective tissues.

These findings, it is suggested, may have implications for the control of enzymic activity.

Changes in the antigenicity of connective tissue during development, or the molecular configurations these changes represent, may do more than reflect differentiation. They may also form part of a series of feed-back systems which ensure that the various gene-enzyme systems, ultimately responsible for development and differentiation, are activated in appropriate sequence. It is suggested also that the maintenance of the local integrity of connective tissues in post-natal life depends on a series of self-regulating systems, each concerned with the synthesis of one constituent of connective tissue and that hormones, acting as sensors and activators, monitor and co-ordinate the synthesis of the different constituents of connective tissue.

These propositions neither deny nor conflict with the thesis advanced by Burwell (1963), that immunological mechanisms operate in the control of the growth of, and in the maintenance of the balance between, differentiated or differentiating tissues throughout the body. The propositions outlined here are concerned with the role of the cellular environment in the activation and repression of gene-enzyme systems concerned with the synthesis of

constituents of connective tissue. The present propositions do not ascribe to the cellular environment any role in determining the population of cells responsible for the synthesis of any one constituent of connective tissues, or in determining the balance between the cell populations concerned with the synthesis of the different constituents of connective tissue.

The concept that the constitution of connective tissues represents a response by synthesising cells to their environment, provides a basis for conjecture concerning the processes involved in ageing and rheumatoid disease.

In connection with ageing, the possibility is considered that subtle changes in the structure of the collagen molecule may contribute to the age associated alterations in the rheological properties of collagen. This is not to deny the proposition that the increased structural stability of aged collagen is to be attributed to an increase in the number and strength of cross links between and within collagen macromolecules, but merely to suggest that the nature of the intra- and intermolecular cross links, the rate at which they can form, and the ease with which cross linking agents can react with adjacent collagen macromolecules, are governed by the subtle structure of the collagen molecule. The proposition that ageing is associated

with changes in the subtle structure of the collagen molecule may be open to investigation by means of a study of the patterns of immunological reactivity manifested by neutral salt-soluble, acetic acid-soluble and insoluble collagen obtained from subjects of different ages.

The concept that a mutual interplay between a cell and its environment is involved in the maintenance of the integrity of connective tissues has, it is suggested, relevance to rheumatoid disease. Alterations in the representation of any one constituent of connective tissue, whether attributable to abnormal genetic endowment or somatic mutation in the relevant synthesizing cell, are likely to initiate changes in the environment, and therefore the function of cells synthesizing related constituents of connective tissue. Although this concept takes into account the view that rheumatoid disease involves a restricted number of somatic mutations, responsible perhaps for the formation of a tissue constituent which is abnormally susceptible to damage by trauma or infection, a major difficulty arises. The present concept appears to require that to the rheumatoid factors be ascribed a role in the metabolism, or in the control of the synthesis of one or other of the constituents of adult connective tissue. This view is hardly tenable. It may be, however, that the rheumatoid factors reflect the activity of a gene-enzyme system which

is responsible for the synthesis of a constituent of connective tissue peculiarly susceptible to degradation by trauma or micro-organisms. It is not necessary, however, to deny immune mechanisms as a role in the production of rheumatoid factors. The view that rheumatoid factors reflect intense immunological activity in rheumatoid disease is not necessarily irreconcilable with the proposition that one of the initiating events in rheumatoid disease may be the synthesis of a constituent of connective tissue which is abnormally susceptible to degradation by the action of micro-organisms or trauma.

IMMUNO-HISTOLOGICAL STUDIES OF
HUMAN CONNECTIVE TISSUE

The studies reported here followed the demonstration by Hill and Cruickshank that an antigenic overlap exists between renal glomeruli and other connective tissues (Cruickshank and Hill, 1953 a, b., Hill and Cruickshank, 1953). They were undertaken to determine whether this overlap is complete or partial. The approach adopted involved:-

- (1) The immunisation of rabbits with (a) isolated human renal glomeruli and (b) human synovial tissue.
- (2) The conjugation of globulin fractions of anti-human glomerulus and anti-human synovium antisera with fluorescein isocyanate.
- (3) Direct staining experiments. The mapping of the specific staining produced in quick-frozen unfixed sections of tissue by fluorescein labelled anti-human renal glomerulus and anti-human synovium conjugates.
- (4) Cross inhibition experiments. A study of the staining produced by fluorescein labelled conjugates of the one specificity, (e.g. anti-glomerulus conjugates), in

sections of tissue which had been pre-treated with unlabelled globulins of the other specificity (e.g. anti-synovium).

- (5) Cross absorption and cross absorption inhibition experiments. Studies of the modifications in their anti-tissue reactivity brought about by absorbing labelled and unlabelled anti-glomerulus preparations with synovial tissue, and anti-synovium preparations with renal glomeruli.
- (6) Mixed staining experiments. The use of fluorochromes of contrasting colour made possible a more direct comparison of the specificities of anti-glomerulus and anti-synovium preparations. In mixed staining experiments, anti-glomerulus and anti-synovium preparations, each labelled with a red or a green fluorochrome (lissamine rhodamine B200 or fluorescein isocyanate) were applied to a single section of tissue either simultaneously or in sequence.

DEFINITIONS.Fluorochrome, Conjugate, Conjugation.

The fluorescent antibody technique is an immunological procedure in which globulins separated from an immune serum and labelled with a fluorescent marker (fluorochrome) are used as specific reagents for the detection of the corresponding antigens in sections of tissue.

The term conjugate is used to refer to the solution of fluorochrome labelled globulins used as immunological reagents in immunohistological studies, and the term conjugation to the process of labelling globulins with fluorochromes.

Autofluorescence.

Normal tissues when bombarded by blue-violet light emit a blue, blue-grey or white fluorescence. The term autofluorescence is used to refer to such fluorescence, attributable to the tissues themselves, and to distinguish it from fluorescence attributable to fluorochrome.

Staining.

It is convenient to use histological terminology when referring to immunohistological procedures. Thus the term staining is used to refer to the treatment of sections with conjugates. The term is used also to refer to specific fluorescence seen in treated sections and attributable to labelled antibody molecules specifically fixed to the corresponding antigens in tissue sections.

The test of specific staining routinely used during the studies described here was designed to demonstrate that specific staining owed its origin to a factor present both in the labelled and in the unlabelled fractions of a particular antiserum, but not present in normal sera nor in antisera raised against unrelated antigens. This test demanded that the specific staining produced by a conjugate be blocked by pretreatment of sections with the corresponding unlabelled immune globulins, but not by pretreatment with unlabelled normal globulin or with unlabelled globulin fractions of antisera raised against unrelated antigens.

Non-specific Staining.

The term non-specific staining is used to describe fluorescence attributable to the non-specific fixation of fluorochrome labelled proteins, or free fluorochrome molecules, to tissues.

During the process of conjugation some proteins become excessively labelled with fluorochrome, while some fluorochrome molecules remain unattached to protein. Both the uncombined fluorochrome and the heavily labelled protein exhibit a non-specific affinity for a wide variety of tissues. Thus before it can be used as an immunohistological reagent a conjugate must be freed of material responsible for non-specific staining, and it must be shown to be free of such material on

every occasion that it is used, for with time, a gradual release of label from protein appears to occur.

Specific Staining Attributable to Contaminating Antibodies.

The antisera from which were prepared the immunohistological reagents used in the studies described below were raised against homogenates of tissue. They, therefore, contained contaminating antibodies having affinity for serum proteins and red blood cells of the species (rat or human) from which the tissues had been obtained.

Immunohistological studies have shown that blood group antigens A and B and plasma proteins have a widespread distribution in human tissues. The histological distribution of blood group antigens was mapped by Szulman (1960), and that of plasma proteins by Gitlin (1953).

The distribution of blood group A and B substances in tissues was found to be to some extent dependent on the secretor status of the subjects concerned. However, these antigens were found to have a widespread distribution, irrespective of secretor status, in endothelia of veins, arteries, capillaries (including renal glomerular capillaries), and the sinusoidal cells of the spleen.

Plasma proteins, albumin, gamma globulin, β metal combining globulin and fibrinogen, were

found in connective tissue, interstitial spaces, lymphatics and blood vessels.

It follows from the work of Szulman and Gitlin that every effort must be made to remove contaminating anti-plasma protein and anti-erythrocyte antibodies from anti-tissue conjugates. If this effort is not made the specific staining of connective tissue produced by these conjugates cannot confidently be ascribed to the fixation of labelled anti-connective tissue antibodies by connective tissue antigens. The manipulations involved in absorption procedures, designed to remove contaminating antibodies from anti-tissue antisera, are set out in Appendix 4B.

Basement Membrane.

The term "basement membrane" will be used to refer to the PAS positive structure seen at the boundaries between epithelium or endothelium and connective tissue, in paraffin embedded blocks of tissue.

Boundary Membrane.

Structures having the topographical distribution of basement membranes are seen in sections stained with fluorochrome labelled anti-tissue antisera. In certain situations, however, the structure revealed by the immunohistological technique appears to have a more complex constitution than the Periodic Acid Schiff technique suggests that the corresponding basement membrane has. For this reason, the term "boundary memb-

rane" will be used to refer to membranes seen at boundaries between epithelium or endothelium and connective tissue, in sections stained with anti-tissue conjugates.

Reticular Fibres and Reticulum.

The term "reticulum" will be used to refer to the fine branching argyrophilic fibres seen in formalin fixed paraffin embedded material to form the framework of epithelial organs, lymph glands and the spleen. Fibres having the distribution of reticulum are regularly delineated by anti-tissue conjugates. These will be referred to as "reticular fibres".

ILLUSTRATIONS.

Monochrome prints and colour transparencies are used to illustrate the experimental findings described below.

Monochrome Prints.

These are mounted in Appendix 7. The abbreviation "Fig." is used to refer to these prints.

Colour Transparencies.

These are placed together in Appendix 8, from which they may be removed. The abbreviation "C.T." is used to refer to colour transparencies.

COMPARISON OF ANTI-TISSUE SPECIFICITIES
OF ANTI-GLOMERULUS AND ANTI-SYNOVIUM CONJUGATES

Direct Staining Experiments

In this series of experiments the specific staining produced by fluorescein labelled anti-human glomerulus conjugates was compared with that produced by anti-human synovium conjugates labelled similarly.

The specificity of staining was tested by means of inhibition experiments. The manipulations involved in inhibition experiments are described in detail in Appendix 5B, and summarised in Table 1 below.

In essence, in these manipulations one of a pair of frozen sections, mounted on the one slide, was exposed to normal rabbit globulin (2 to 18 hours), and the other to unlabelled rabbit anti-tissue globulin (2 to 18 hours). Both sections were then exposed to a conjugate prepared from the anti-tissue globulin.

Staining which could be shown to be inhibited by prior exposure of a section to the unlabelled immune globulin, but not prevented by prior exposure to normal serum globulins, or to globulins separated from unrelated antisera, was judged to be specific.

TABLE 1
DIRECT STAINING EXPERIMENTS

SECTION	PRETREATMENT Unlabelled Globulin 2 hrs. at 20°C. and 16 hrs. at 4°C.	STAINING Conjugate $\frac{1}{2}$ to 1 hr. at 20°C.
1	Normal rabbit	F-anti-glomerulus
2	Anti-glomerulus	F-anti-glomerulus
1	Normal rabbit	F-anti-synovium
2	Anti-synovium	F-anti-synovium

1. Studies with Anti-human Renal Glomerulus Conjugates

(A). Staining Attributable to Contaminating Antibodies

Preliminary experiments designed to investigate the extent to which contaminating antibodies contributed to the specific staining produced by anti-glomerulus conjugates, established that most anti-glomerulus antisera contained antibodies reactive with human serum proteins and with human red blood cells (see Appendix 4B). Absorption of conjugates separated from such antisera with (i) human serum, (ii) human fibrin separated from recalcinated plasma, (iii) human plasma, and (iv) human red blood cells, did not appreciably alter the distribution of specific staining produced by these conjugates, except in the case of the

endothelium of blood vessels.

Before their absorption with human serum and human red cells, anti-glomerulus conjugates frequently, but not invariably, produced a dull or sometimes bright green specific fluorescence in the endothelium of blood vessels. This staining was, in the main, attributable to constituents of human serum. Antibodies against red cell antigens were found occasionally to contribute to endothelial staining.

(B). Specific Anti-tissue Reactivity

The observations described below concern specific staining produced by anti-glomerulus conjugates absorbed with human serum and human group AB, or A and B, rh+ red blood cells.

Boundary Membranes

Staining of boundary membranes occurred at all boundaries between epithelium and endothelium and connective tissue.

Epithelial boundary membranes varied in thickness in different organs. The boundary membrane was usually seen as a single brightly fluorescent layer, closely applied to epithelial cells. In some situations, the testis for example, the membrane could be resolved into two components, while in others, the salivary gland and, very occasionally,

in the kidney around renal tubules (Fig. 1), splits each enclosing an unstained space were seen in an otherwise simple membrane.

Both of these variations in the appearance of epithelial boundary membranes were seen in sections of the thyroid gland, although splitting was much more common here than re-duplication. The staining produced around thyroid acini by anti-glomerulus conjugates varied in its pattern from gland to gland. At one extreme contiguous splits in the membrane gave the impression that the acini were surrounded by a basket work of capillaries, Fig. 2 and C.T. 12). At the other extreme the acini were enclosed by a relatively broad membrane showing an occasional split only. Here, in one or two instances, it was possible to resolve the membrane between splits into two components (Fig. 4).

A consistent pattern of staining was produced in sections of kidney by anti-glomerulus conjugates. Renal tubules were enclosed by a well-defined membrane (Fig. 6), more prominent around cortical than medullary tubules. The renal glomerular tuft presented a complex picture. Glomerular capillaries, their lumina outlined by a fine membrane, were seen as brightly fluorescent circles, loops and convolutions (Figs. 6 and 8 and C.T. 1). Here and there amongst the complexity

of the capillary loop pattern could be discerned, round, spindle-shaped, or oval structures which often contained a circular central or eccentric unstained area (Fig. 8 and 10). These structures were at first thought to represent glomerular capillaries cut obliquely; it later appeared, however, that they differed antigenically from glomerular capillaries. While their identity remains obscure, it has been found convenient to refer to them as "glomerular epithelial cells". The production of specific staining in glomerular epithelial cells by anti-glomerulus conjugates had previously been described by Hill and Cruickshank (1953).

Capillaries in most organs were outlined by a single fluorescent line. In certain situations, however, capillaries were seen as rings or tubes with thick walls, showing unstained fenestrations. Capillaries, presenting this appearance, were seen in the synovium (Fig. 17 and C.T. 22), the tela choroidea (C.T. 31) and the dermis (C.T. 30). The so-called arteries of the red pulp of the spleen presented similar appearances (C.T. 19).

Reticular Fibres

Anti-glomerulus conjugates reacted with reticular fibres in a variety of situations. They produced specific staining of the sarcolemma of

voluntary and heart muscle (Fig. 6), and of the neurolemma of peripheral nerves. Adipose tissue was seen as a brightly fluorescent open mesh outlining fat-filled spaces.

In the red pulp of the spleen and the medulla of lymph nodes, anti-glomerulus conjugates delineated fine fibres forming the framework of the organs and outlining sinusoids (Fig. 15).

A reticulate pericellular network was demonstrated in the synovial cell layer (C.T. 22).

A reticulate network was demonstrated also in the walls of vasa vasorum of elastic arteries, in the media of arterioles of the renal glomerulus and in the media of the larger muscular arteries (Fig. 6, 11 and 12).

Fine branching reticular fibres were seen occasionally in the renal interstitium and more frequently in the interstitium of the thyroid gland.

Elastic Tissue and Collagen

Neither elastic tissue nor collagen was stained by anti-glomerulus conjugates.

In general, elastic tissue emitted a white or silver auto-fluorescence (C.T. 30), but internal elastic laminae of arteries were often tinged with blue or purple (C.T. 27).

Collagen was seen as wavy non-branching

fibres, having a blue auto-fluorescence (C.T.24 and 33).

Collagenous Fibrils

In some situations specifically stained fibrils were seen lying closely apposed to bundles of collagen (C.T.24 and 33). These "collagenous fibrils" differed morphologically from reticular fibres in that they did not branch, and immunologically, from collagen in that they showed specific staining.

The tendentious terms "collagenous" and "fibrils" are used here in a descriptive sense only. They carry no special connotations as regards the nature, (developmental or structural relationship to collagen) or the form in vivo of the material which they describe.

Collagenous fibrils were seen amongst bundles of collagen in the capsules of joints and in tendons, but were not normally found in association with dermal collagen. They were also found in the adventitia of arteries (C.T. 26), in the capsule of the kidney and the serous coat of the alimentary tract.

Fibrocytes

Anti-glomerulus conjugates reacted specifically with the cytoplasm and cytoplasmic processes

of fibrocytes, in, for example, the leptomeninges and dermal histiocytoma (C.T. 33 and 32).

Cartilage

Articular cartilage remained unstained in sections exposed to anti-glomerulus conjugates. The nuclei of cartilage cells in these sections invariably showed a stippled green fluorescence. This fluorescence was, however, also seen in sections pretreated with unlabelled anti-glomerulus preparations before their exposure to the corresponding conjugate. It was therefore non-specific.

2. Studies with Anti-human Synovium Conjugates

(A). Staining Attributable to Contaminating Antibodies

Preliminary experiments designed to test the anti-tissue specificity of anti-glomerulus conjugates had shown that the contaminating antibodies which these conjugates contained did not add significantly to the staining produced by them. The situation was quite different in the case of anti-synovium conjugates. Not only did these conjugates stain the endothelium of blood vessels before their absorption with human serum and erythrocytes, but they also produced specific staining in renal glomeruli. Absorption

with serum and red cells abolished the endothelial staining produced by these conjugates and altered the pattern of glomerular staining (see below).

(B). Specific Anti-tissue Reactivity

The observations described here concern the specific staining produced by anti-human synovium conjugates after their absorption with serum and red cells.

Boundary Membranes

Anti-synovium conjugates did not stain boundary membranes of the choroid plexus, nor of renal glomerular capillaries. Specific staining was, nevertheless, seen in renal glomeruli, in sections which had been exposed to anti-synovium conjugates. This staining emanated from round or oval bodies which often contained a circular unstained central or eccentric area, and which were usually distributed along four or five cords arising from a point or points near the circumferences of the sectioned glomerular tuft (Fig. 7 and 9, C.T. 3). These bodies were taken to correspond to those lying between capillary loops in glomeruli, stained with anti-glomerulus conjugates (Fig. 8 and 10).

In situations other than the choroid plexus and the renal glomerular tuft, there was little

to distinguish boundary membranes in sections stained with anti-synovium conjugates from the corresponding structures in sections stained with anti-glomerulus conjugates - with the possible exception of boundary membranes around thyroid acini.

The pattern of peri-acinar fluorescence seen in sections of the thyroid gland, stained by anti-synovium conjugates, was, in the main, similar to that seen in sections stained with anti-glomerulus conjugates (compare Figs. 2 and 3). However, while it was occasionally possible to resolve acinar boundary membranes made visible by anti-glomerulus conjugates into two components, successful resolution into two components of acinar boundary membranes stained with anti-synovium conjugates was never achieved. In two instances the pattern of peri-acinar fluorescence produced by anti-synovium conjugates differed from that produced in serial, or adjacent sections, by anti-glomerulus conjugates. In these instances anti-synovium stained acini were surrounded by a series of small, apparently independent loops, irregularly distributed in groups of three to five, while in sections stained with anti-glomerulus conjugates, the loops took the form of splits in a specifically stained boundary membrane (Fig. 2).

Reticular Fibres

Anti-synovium conjugates produced specific staining of framework reticular fibres in; the red pulp of the spleen (Fig. 15); the medulla of lymph nodes, and the synovial cell layer. They were occasionally found to have reacted also with scanty reticular fibres in the interstitium of the kidney and the thyroid gland.

While reticular tissue forming the framework of organs did react with anti-synovium conjugates, reticular tissue in the walls of muscular arteries and arterioles and in the walls of vasa vasorum of elastic arteries did not (compare Figs. 13 and 12).

Collagenous Fibrils and Fibrocytes

No difference was apparent between the interactions of anti-synovium conjugates, on the one hand, and anti-glomerulus conjugates on the other, with collagenous fibrils or with the cytoplasm and cytoplasmic processes of fibrocytes.

Collagen, Elastic Tissue and Cartilage

Collagen and elastic tissue, which were left unstained by anti-glomerulus conjugates, were left unstained by anti-synovium conjugates also.

Anti-synovium conjugates reacted with articular cartilage in the same way as anti-glomerulus conju-

gates did. That is, they produced non-specific staining in the nuclei of cartilage cells and left the intercellular matrix unstained.

Table 2 summarizes the distribution of specific fluorescence produced by both conjugates with various substrates.

These conjugates appeared to be similar in their reactions with collagen, collagenous fibrils and fibrocytes.

Both conjugates produced specific staining of collagenous fibrils and fibrocytes in kidney and in other organs. But only the anti-lysine conjugate stained cartilage, suggesting that the antigenic determinant of the antigen is located in the intercellular matrix of cartilage.

Differences exist also between the two conjugates. The anti-lysine conjugate stained the nuclei of cartilage cells and the intercellular matrix of cartilage. The anti-lysine conjugate stained the nuclei of cartilage cells and the intercellular matrix of cartilage. The anti-lysine conjugate stained the nuclei of cartilage cells and the intercellular matrix of cartilage.

SUMMARY OF OBSERVATIONS ARISING OUT OF
DIRECT STAINING EXPERIMENTS WITH ANTI-GLOMERULUS
AND ANTI-SYNOVIUM CONJUGATES

Table 2 summarises the distribution of specific fluorescence produced by anti-glomerulus and anti-synovium conjugates.

These conjugates appeared to be similar in their reactions with collagen, collagenous fibrils and fibrocytes.

Both conjugates produced specific staining of reticular fibrils forming the framework of lymphatic organs and in the interstitium of the kidney and other organs. But only the anti-glomerulus conjugates produced specific staining in the media of arterioles and muscular arteries and in the walls of the vasa vasorum of elastic arteries.

Differences existed also between the interactions of the two conjugates with certain boundary membranes. Anti-glomerulus conjugates stained the boundary membranes of the renal glomerular capillary tuft, anti-synovium conjugates did not. In the case of the thyroid gland, morphological differences were occasionally apparent in boundary membranes after interaction with anti-synovium conjugates on the one hand, and anti-glomerulus conjugates on the other. In general, however, where anti-glomerulus conjugates delineated a boundary

membrane anti-synovium conjugates also did so, and there was little by which to distinguish boundary membranes made visible by anti-glomerulus conjugates, from those delineated by anti-synovium conjugates.

TABLE 2
DIRECT STAINING EXPERIMENTS

	PRESENCE (+) OR ABSENCE (-) OF SPECIFIC STAINING	
	F-ANTI-GLOMERULUS	F-ANTI-SYNOVIUM
<u>BOUNDARY MEMBRANES</u>		
A. <u>EPITHELIAL</u>		
RENAL TUBULES		
SEMINIFEROUS TUBULES		
THYROID	+	+
PANCREAS		
SALIVARY GLAND		
-----	-----	-----
CHOROID PLEXUS	+	-
B. <u>CAPILLARIES</u>		
RENAL GLOMERULAR	+	-
-----	-----	-----
EXTRA GLOMERULAR "THICK WALLED"	+	+
<u>RETICULAR FIBRES</u>		
A. <u>FRAMEWORK</u>		
LYMPH NODES AND SPLEEN		
LIVER		
SARCOLEMMMA	+	+
NEUROLEMMMA		
ADIPOSE TISSUE		
SYNOVIAL CELL LAYER		
B. <u>INTERSTITIAL</u>		
KIDNEY		
PANCREAS	+	+
THYROID		
C. <u>VASCULAR</u>		
ARTERIAL MEDIA		
ARTERIOLAR MEDIA	+	-
VASA VASORUM		
<u>COLLAGENOUS FIBRILS</u>		
TENDON AND JOINT		
ARTERIAL ADVENTITIA	+	+
CAPSULES OF ORGANS		
<u>FIBROCYTES</u>		
CYTOPLASM	+	+
CYTOPLASMIC PROCESSES		
<u>COLLAGEN</u>		
CARTILAGE	-	-
ELASTIC TISSUE		

CROSS INHIBITION EXPERIMENTS

Methods

In cross inhibition experiments sections were pretreated with unlabelled globulins of the one specificity (anti-glomerulus or anti-synovium) and then exposed to fluorescein-labelled globulins of the other specificity (F-anti-synovium or F-anti-glomerulus). Thus, there were two groups of cross inhibition experiments:-

(a) anti-synovium ---> F-anti-glomerulus

(b) anti-glomerulus ---> F-anti-synovium

Direct staining experiments and direct inhibition experiments were run in parallel with cross inhibition experiments. Direct staining experiments can be summarised thus:

(a) Normal Rabbit Globulin ---> F-anti-glomerulus

(b) Normal Rabbit Globulin ---> F-anti-synovium

and direct inhibition experiments:-

(a) anti-synovium ---> F-anti-synovium

(b) anti-glomerulus ---> F-anti-glomerulus

The manipulations involved in these experiments are described in more detail in Appendix 5, and summarised in Table 3 below.

TABLE 3
CROSS INHIBITION EXPERIMENTS
Experimental Manipulations

Slide	Section	UNLABELLED GLOBULIN 1½ hrs. at 20°C. and 16 hrs. at 4°C.	CONJUGATE ½ - 1½ hrs. at 20°C.
1	1	anti-synovium	F-anti-glomerulus
	2	NRG	F-anti-glomerulus
2	3	anti-synovium	F-anti-synovium
	4	NRG	F-anti-synovium
3	5	anti-glomerulus	F-anti-synovium
	6	NRG	F-anti-synovium
4	7	anti-glomerulus	F-anti-glomerulus
	8	NRG	F-anti-glomerulus

Observations

It was predicted on the basis of direct staining experiments that:

- (a) no staining would appear in sections pre-treated with unlabelled anti-glomerulus globulins and subsequently exposed to anti-synovium conjugates.
- (b) prior exposure of tissue to anti-synovium globulins would block all staining normally produced by anti-glomerulus conjugates except that produced in renal glomerular capillaries and the media of arteries and arterioles.

In the event these predictions were not completely fulfilled.

Anti-glomerulus ---> F-anti-synovium Experiments

The interaction between F-anti-synovium conjugates and tissue sections was completely prevented by pretreatment of the sections with unlabelled anti-glomerulus globulins.

Anti-synovium ---> F-anti-glomerulus Experiments

After pretreatment of sections with unlabelled anti-synovium globulins the staining produced by F-anti-glomerulus conjugates was found to be:-

- (i) unaltered,
- or (ii) altered but not blocked,
- or (iii) blocked.

In the following summary of observations made during anti-synovium ---F-anti-glomerulus experiments, each of these patterns of F-anti-glomerulus activity is considered separately.

(1) Staining produced by F-anti-glomerulus unaltered.

Structures stained in a normal manner after the anti-synovium ---F-anti-glomerulus procedure included the boundary membranes of renal glomerular capillaries and the choroid plexus, the media of muscular arteries and arterioles, and the vasa vasorum of elastic arteries.

(2) Staining produced by F-anti-glomerulus conjugate altered.

It was predicted on the basis of direct staining experiments that, with a possible exception in the case of the thyroid gland, unlabelled anti-

synovium globulins would completely inhibit interaction between F-anti-glomerulus conjugates and renal tubular and other epithelial boundary membranes. This they did not do. Renal tubular, thyroid acinar and other epithelial boundary membranes continued to show staining in anti-synovium ---> F-anti-glomerulus sections. The anti-synovium globulins were not, however, without effect on the interaction between F-anti-glomerulus conjugates and epithelial boundary membranes. These membranes appeared finer and showed less brilliant fluorescence after anti-synovium ---> F-anti-glomerulus cross inhibition experiments than they did after the Normal Rabbit Globulin ---> F-anti-glomerulus direct staining experiments.

The effect of pretreatment with unlabelled anti-synovium preparations was most marked where the membrane had, in Normal Rabbit Globulin ---> F-anti-glomerulus experiments, been seen to split to enclose unstained spaces (thyroid and salivary glands), or where it had been resolvable into two components (testis and thyroid gland). In the anti-synovium ---> F-anti-glomerulus preparation the membrane was seen as a fine line, which showed neither splitting nor reduplication (Fig. 5).

It will be noted that the boundary membrane of the choroid plexus differed from other epithe-

lial boundary membranes in that its staining by anti-glomerulus conjugates was neither blocked nor altered by pretreatment with unlabelled anti-synovium globulins.

(3) Staining produced by F-anti-glomerulus blocked.

Tissues which remained unstained in sections treated by the anti-synovium ---> F-anti-glomerulus procedure included: glomerular epithelial cells; extra-glomerular capillaries; reticular tissue in the spleen; lymph nodes and synovial cell layer and in the interstitium of organs; collagenous tissues and fibrocytes.

The observations made during cross inhibition experiments are summarised and compared with predictions based upon direct staining experiments in Table 4.

TABLE 4.
CROSS INHIBITION EXPERIMENTS.

	PREDICTIONS (P) AND OBSERVATIONS (O) REGARDING PRESENCE (+) OR ABSENCE (-) OF SPECIFIC STAINING.			
	F-ANTI-GLOMERULUS.		F-ANTI-SYNOVIUM.	
	P	O	P	O
<u>BOUNDARY MEMBRANES.</u>				
A. <u>EPITHELIAL.</u>				
RENAL TUBULES				
THYROID	-	+		
SALIVARY GLAND		ALTERED		
PANCREAS				
SEMINIFEROUS TUBULES				

CHOROID PLEXUS	+	+		
B. <u>VASCULAR.</u>				
RENAL GLOMERULAR CAPS.	+	+	N	N
-----			O	O
EXTRA GLOMERULAR CAPS.	-	-		
"THICK WALLED" CAPS.				
<u>RETICULAR FIBRES.</u>				
A. <u>FRAMEWORK.</u>			S	S
LYMPH NODES AND SPLEEN			T	T
LIVER			A	A
SARCOLEMMA	-	-	I	I
NEUROLEMMA			N	N
ADIPOSE TISSUE				
SYNOVIAL CELL LAYER				
B. <u>INTERSTITIAL.</u>				
KIDNEY	-	-	I	I
PANCREAS			N	N
THYROID				
C. <u>VASCULAR.</u>				
ARTERIAL MEDIA			G	G
ARTERIOLAR MEDIA	+	+		
VASA VASORUM				
<u>COLLAGENOUS FIBRILS.</u>				
TENDON AND JOINT				
ARTERIAL ADVENTITIA	-	-		
CAPSULES OF ORGANS				
FIBROCYTES	-	-		

CROSS ABSORPTION AND CROSS
ABSORPTION INHIBITION EXPERIMENTS

The observations made during direct staining and cross inhibition experiments appeared to carry the following implications:-

- (a) that if human synovial tissue contained synovium-specific antigens, these were not to be detected by means of the antisera used. Anti-glomerulus globulins blocked all anti-tissue reactivity, including anti-synovial tissue reactivity, of fluorescein-anti-synovium conjugates;
- (b) an antigenic distinction existed between synovial tissue and the boundary membranes of renal glomerular capillaries but not between synovial tissue and epithelial boundary membranes.

Cross absorption and cross absorption inhibition experiments were performed to compare more directly the antigenic constitution of synovial and glomerular tissue.

METHODS

A. Reagents

The following reagents were used in cross absorption and cross absorption inhibition experi-

ments:-

- (i) F-anti-glomerulus x synovium
fluorescein labelled anti-glomerulus
conjugates pooled and
absorbed with synovial tissue.
- (ii) F-anti-synovium x glomerulus
fluorescein labelled anti-synovium
conjugates pooled and
absorbed with isolated renal glomeruli.
- (iii) Anti-glomerulus x synovium
a pool of unlabelled globulins repre-
senting the conjugates making up the
F-anti-glomerulus pool and similarly absorbed
with synovial tissue.
- (iv) Anti-synovium x glomerulus
unlabelled anti-synovium globulins
absorbed as in (ii) above.

The synovial tissue used for the absorption of F-anti-glomerulus conjugates and for the absorption of anti-glomerulus globulins, was used in amounts which preliminary experiments had shown to be just sufficient to remove from the F-anti-glomerulus pool its ability to produce detectable staining in the synovial cell layer in sections of synovial tissue. Similarly, renal glomeruli were used for the absorption of the F-anti-synovium reagent in amounts which had been shown to be just sufficient to remove staining reactivity for renal glomeruli from the F-anti-synovium pool.

The methods employed in the preparation of cross absorbed conjugates and unlabelled globulins are described in Appendix 5B.2.

B. Experimental ManipulationsCross Absorption Experiments

In cross absorption experiments, the staining produced by a cross absorbed conjugate was compared with that appearing in sections treated by the corresponding cross inhibition procedure (e.g. anti-glomerulus ---> F-anti-synovium, in the case of the F-anti-synovium x glomerulus preparation).

The manipulations involved in these experiments are outlined in Table 5 and described more fully in Appendix 5B.2.

TABLE 5
CROSS ABSORPTION INHIBITION EXPERIMENTS
Experimental Manipulations

Slide	Section	UNLABELLED GLOBULIN 1½ hrs. at 20°C. and 16 hrs. at 4°C.	CONJUGATE ½ hr. at 20°C.
1	1		∅F-anti-glom. x Synovium
2	2	anti-syn.	F-anti-glom.
	3	NRG	F-anti-glom.
	4	anti-glom.	F-anti-glom.
1	1		∅F-anti-syn. x Glomerulus
2	2	anti-glom.	F-anti-syn.
	3	NRG	F-anti-syn.
	4	anti-syn.	F-anti-syn.

∅ Sections were exposed to the cross absorbed conjugates for 2 hrs. at 20°C. and 16 hrs. at 4°C.

F-anti-glom. x Synovium = anti-glomerulus conjugate absorbed with synovial tissue.

F-anti-syn. x Glomerulus = anti-synovium conjugate absorbed with isolated renal glomeruli.

Cross Absorption Inhibition Experiments These experiments tested the residual inhibitory activity of anti-glomerulus and anti-synovium globulins, after they had been absorbed with synovial tissue and isolated renal glomeruli respectively.

Sections were pretreated with, for example, the unlabelled anti-glomerulus x synovium preparation and subsequently exposed to the anti-glomerulus conjugate. Staining seen in sections treated in this way was compared with that seen in sections submitted to the corresponding cross inhibition (anti-synovium ---> F-anti-glomerulus) and direct staining (normal rabbit globulin ---> F-anti-glomerulus) procedure (Table 6).

TABLE 6
CROSS ABSORPTION INHIBITION EXPERIMENTS
Experimental Manipulations

Slide	Section	UNLABELLED GLOBULIN 1½ hrs. at 20°C. and 16 hrs. at 4°C.	CONJUGATE ½ hr. at 20°C.
1	1	anti-glom. x Synovium	F-anti-glom.
	2	anti-syn.	F-anti-glom.
	3	NRG	F-anti-glom.
2	4	anti-glom. x Synovium	F-anti-syn.
	5	NRG	F-anti-syn.
1	1	anti-syn. x Glomerulus	F-anti-syn.
	2	anti-syn.	F-anti-syn.
	3	NRG	F-anti-syn.
2	4	anti-syn. x Glomerulus	F-anti-glom.
	5	NRG	F-anti-glom.

The experiments carried out on slides No.2 were run only when sections 1, 2 and 3 represented previously unexamined blocks of tissue.

C. Observations

1. Cross Absorption Experiments

The observations made during cross absorption experiments are summarized in Tables 7 and 8 where they are compared with observations arising out of direct staining and cross inhibition experiments.

F-anti-synovium x Glomerulus Experiments

The fluorescein-anti-synovium conjugates lost all staining reactivity on absorption with isolated renal glomeruli (Table 7).

TABLE 7
CROSS ABSORPTION EXPERIMENTS
F-anti-Synovium x Glomerulus

	Presence (+) or Absence (-) of Specific Staining		
	Staining Procedure		
	1 Cross Absorption	2 Direct Staining	3 Cross Inhibition
<u>BOUNDARY MEMBRANES</u>			
Epithelial	-	+	-
Glomerular Capillaries	-	-	-
<u>RETICULAR FIBRES</u>			
Framework	-	+	-
Interstitial	-	+	-
Vascular	-	+	-
<u>COLLAGENOUS FIBRILS</u>	-	+	-
<u>FIBROCYTES</u>	-	+	-
<u>GLOMERULAR "EPITHELIAL" CELLS</u>	-	+	-

The experiments summarised in this table involved exposure of sections to:

Col. 1 F-anti-Synovium x Glomerulus.
Col. 2 NRG ---> F-anti-Synovium.
Col. 3 anti-glomerulus ---> F-anti-Synovium.

F-anti-glomerulus x Synovium Experiments

Prior absorption of the F-anti-glomerulus preparation with synovial tissue did not affect its reaction with boundary membranes in the renal glomerular capillary tuft or the choroid plexus, nor with reticular tissue in the walls of arteries, arterioles or vasa vasorum (Table 8). Absorption with synovial tissue abolished the reactivity of the F-anti-glomerulus conjugate with interstitial and framework reticular fibres, collagenous fibrils and fibrocytes. The cross absorbed F-anti-glomerulus x synovium conjugate continued to react with epithelial boundary membranes, but the staining produced at the sites of these membranes by the cross absorbed conjugate was, in general, finer and less bright than that produced by the unabsorbed conjugate. The F-anti-glomerulus conjugate thus reacted in the same way with epithelial boundary membranes in cross absorption (F-anti-glomerulus x synovium) as in cross inhibition (anti-synovium ---> F-anti-glomerulus) experiments.

TABLE 8
CROSS ABSORPTION EXPERIMENTS
F-anti-glomerulus x Synovium Experiments

	Presence (+) or Absence (-) of Specific Staining		
	Staining Procedure		
	1 Cross Absorption	2 Direct Staining	3 Cross Inhibition
<u>BOUNDARY MEMBRANES</u>			
Epithelial	+	+	+
Glomerular Capillaries	+	+	+
<u>RETICULAR TISSUE</u>			
Framework	-	+	-
Interstitial	-	+	-
Vascular	+	+	+
<u>COLLAGENOUS TISSUE</u>	-	+	-
<u>FIBROCYTES</u>	-	+	-
<u>GLOMERULAR "EPITHELIAL" CELLS</u>	-	+	-

/ Staining at the site of epithelial boundary membranes was finer after cross absorption and cross inhibition than after direct staining experiments.

Col. 1
Col. 2
Col. 3

F-anti-glomerulus x Synovium.
F-anti-glomerulus.
F-anti-glomerulus.

2. Cross Absorption Inhibition ExperimentsAnti-synovium x Glomerulus ---> F-anti-synovium Experiments

Unlabelled anti-synovium globulins, when absorbed with isolated renal glomeruli, were found to lose all their inhibitory activity for F-anti-synovium conjugates. The distribution of staining seen in sections treated by the anti-synovium x glomerulus cross absorption inhibition procedure differed in no way from that seen in sections submitted to the NRG --- F-anti-synovium direct staining procedure (Table 9).

TABLE 9

CROSS ABSORPTION INHIBITION EXPERIMENTS
Anti-synovium x Glomerulus ---> F-anti-synovium Experiments

	Presence (+) or Absence (-) of Specific Staining		
	Staining Procedure		
	1 Cross Absorption Inhibition	2 Direct Staining	3 Cross Inhibition
<u>BOUNDARY MEMBRANES</u>			
Epithelial	+	+	-
Glomerular Capillaries	-	-	-
<u>RETICULAR FIBRES</u>			
Framework	+	+	-
Interstitial	+	+	-
Vascular	-	-	-
<u>COLLAGENOUS FIBRILS</u>	-	+	-
<u>FIBROCYTES</u>	+	+	-
<u>GLOMERULAR "EPITHELIAL" CELLS</u>	+	+	-

Col. 1 Anti-synovium x Glomerulus --- F-anti-Synovium
 Col. 2 NRG --->F-anti-Synovium
 Col. 3 Anti-glomerulus --- F-anti-Synovium

Anti-Glomerulus x Synovium ---> F-anti-Glomerulus
Experiments

On their absorption with synovial tissue, unlabelled anti-glomerulus globulins:

- (a) lost their ability to block the interaction between F-anti-glomerulus conjugates and reticular tissue, collagenous tissue and fibrocytes (Table 10);
- (b) retained their ability to block the staining normally produced by the F-anti-glomerulus conjugate in the boundary membranes of the choroid plexus and renal glomerular capillaries, and in reticular tissue in the walls of muscular arteries, arterioles and vasa vasorum;
- (c) produced only incomplete inhibition of the staining normally produced by the F-anti-glomerulus conjugate in epithelial boundary membranes. The boundary membranes of renal cortical tubules and thyroid acini, for example, appeared finer and showed less brilliant fluorescence after cross absorption inhibition experiments than they did after direct staining experiments.

TABLE 10
CROSS ABSORPTION INHIBITION EXPERIMENTS
Anti-glomerulus x Synovium ---> F-anti-glomerulus
Experiments

	Presence (+) or Absence (-) of Specific Staining		
	Staining Procedure		
	1 Cross Absorption Inhibition	2 Direct Staining	3 Cross Inhibition
<u>BOUNDARY MEMBRANES</u>			
Epithelial	+	+	+
Glomerular Capillaries	-	+	+
<u>RETICULAR FIBRES</u>			
Framework	+	+	-
Interstitial	+	+	-
Vascular	-	+	+
<u>COLLAGENOUS FIBRILS</u>	+	+	-
<u>FIBROCYTES</u>	+	+	-
GLOMERULAR "EPITHELIAL" CELLS	+	+	-

+ Denotes finer and less bright fluorescence than +
 Col. 1. Anti-glomerulus x Synovium ---> F-anti-glomerulus.
 Col. 2. NRG. --->F-anti-glomerulus.
 Col. 3. Anti-synovium --->F-anti-glomerulus.

When the staining seen after cross absorption inhibition experiments was compared with that seen after the corresponding cross inhibition experiments, (Tables 9 and 10) it became apparent that the distribution of staining produced by the one procedure was in general complementary to that produced by the other. Tissues not stained by the anti-glomerulus x synovium ---F-anti-glomerulus procedure were stained by the anti-synovium ---> F-anti-glomerulus procedure and vice versa. Similarly, the tissues left unstained in F-anti-glomerulus x synovium cross absorption experiments

(Tables 7 and 8) were those with which F-anti-synovium conjugates reacted in direct staining experiments.

The absorption experiments thus tended to support the implications, derived from direct staining and cross inhibition experiments, that two antigenically active groups of connective tissues could be recognized by immuno-histological means.

Tissues constituting the first of these groups reacted with antibodies detectable in unabsorbed and cross absorbed anti-glomerulus preparations. This is not to say that the antigenic determinants characterising these group one tissues are not present in synovial tissue; had the process of cross absorption been carried further anti-glomerulus preparations might have lost all, rather than some, of their anti-tissue reactivities.

Group two constituents were reactive with antibodies present in anti-glomerulus and in anti-synovium antisera. These constituents appeared to find complete antigenic representation in synovial tissue and in renal glomeruli.

Group one tissues included the boundary membranes in the choroid plexus and renal glomerular capillary tuft, and reticular tissue in the media of muscular arteries and arterioles and the walls of vasa vasorum of elastic arteries.

Group two constituents comprised collagenous

fibrils in arterial adventitia, framework and interstitial reticular tissue and fibrocytes. Group two components were found also as intercapillary cytooid structures in the renal glomerular tuft.

The relationship of epithelial boundary membranes to these two groups of antigenically active constituents of connective tissue was not immediately apparent.

Epithelial boundary membranes showed reactions in common with glomerular capillary boundary membranes and reactions in common with reticular tissue (Table 11). It appeared, therefore, that these membranes contained both the reticular and the glomerular capillary boundary membrane groups of antigens. The possibility that they contained antigens peculiar to themselves and synovial tissue, i.e. antigens present in synovium but not present in renal glomeruli, was excluded by the following observations; all anti-epithelial boundary membrane activity was removed from anti-synovium preparations by absorption with isolated renal glomeruli (Table 11, rows 7 and 9); unlabelled anti-glomerulus globulins blocked the staining normally produced in epithelial boundary membranes by anti-synovium conjugates (Table 11, row 8).

The distribution of staining around renal

cortical tubules in cross absorption inhibition experiments was thought to provide evidence that epithelial boundary membranes might consist of two histological components. The boundary membranes of renal cortical tubules seemed to lie less close to the bases of cells in sections treated by the anti-glomerulus x synovium ---> F-anti-glomerulus cross absorption inhibition procedure, than they did in sections treated by the corresponding anti-synovium ---> F-anti-glomerulus cross inhibition procedure.

However, it was not found possible to convince an independent observer that a topographical distinction existed between the renal tubular boundary membranes seen after cross absorption inhibition experiments, and those seen after cross inhibition experiments.

More persuasive evidence that certain epithelial boundary membranes might be made up of two distinct components was afforded by the thyroid gland.

In direct staining (NRG ---> F-anti-glomerulus) experiments, each thyroid acinus was seen to be enclosed by a relatively thick membrane which split to enclose unstained spaces and which could, in some instances, be resolved into two components. A thinner membrane, which also split to enclose unstained spaces, but which

was not resolvable into two components, was seen after anti-glomerulus x synovium ---> F-anti-glomerulus cross absorption inhibition experiments.

Neither splitting nor reduplication was apparent in the fine acinar boundary membrane delineated by anti-glomerulus conjugates in cross absorption (F-anti-glomerulus x synovium), and cross inhibition (anti-synovium ---> F-anti-glomerulus) experiments (Fig. 15).

Thus, while it seemed to have been established that the reticular and the renal glomerular capillary groups of antigens were both present in epithelial boundary membranes, it was not clear from the staining reactions of these membranes, except possibly in the cases of the thyroid gland, whether two antigenically distinct tissues went into their formation, or whether a single membrane carried two distinct groups of antigenic determinants.

TABLE 11
EPITHELIAL AND GLOMERULAR CAPILLARY
BOUNDARY MEMBRANES
Staining Reactions

Staining Procedure		Presence (+) or Absence (-) of Specific Staining	
Unlabelled Globulin	Conjugate	Glomerular	Epithelial
1 NRG	F-anti-Glom.	-	+
2 NRG	F-anti-Glom. x Syn.	+	/
3 Anti-Syn.	F-anti-Glom.	+	/
4 Anti-Glom. x Syn.	F-anti-Glom.	-	/
5 Anti-Glom.	F-anti-Glom.	-	-
6 NRG	F-anti-Syn.	-	/
7 NRG	F-anti-Syn. x Glom.	-	-
8 Anti-Glom.	F-anti-Syn.	-	-
9 Anti-Syn. x Glom.	F-anti-Syn.	-	/
10 Anti-Syn.	F-anti-Syn.	-	-

/ Denotes finer and less bright fluorescence than +

NRG = Normal Rabbit Globulin.

Syn. = Synovium.

Glom. = Glomerulus.

STUDIES WITH FLUOROCHROMES OF
CONTRASTING COLOUR

Further studies of the antigenic constitution of epithelial boundary membranes were made possible by the introduction of lissamine rhodamine B200 (RB200) as a fluorescent protein tracer (Chadwick, McEntegart and Nairn, 1958, a, b).

On bombardment with blue violet light, RB200 emits a red fluorescence which contrasts well with the green fluorescence of fluorescein. This made the direct comparison of the anti-tissue specificities of anti-glomerulus and anti-synovium preparations an attractive possibility. Accordingly, a series of exploratory experiments were performed.

EXPLORATORY EXPERIMENTS

Exploratory experiments were undertaken to test the feasibility of basing immunohistological studies of connective tissue on the simultaneous exposure of tissue sections to mixtures of anti-synovium and anti-glomerulus globulins labelled contrastingly.

The methods used in the labelling of antisera with RB200 and in the separation of labelled globulins from such antisera, are described in Appendix 3.

Before their use as immuno-histological reagents RB200-labelled globulins were submitted to procedures designed to reduce their propensity to stain tissues non-specifically, and to remove from them contaminating antibodies reactive with serum proteins and red blood cells (Appendix 4).

The manipulations involved in the exploratory experiments are described in Appendix 5 and outlined below:

Exploratory experiments included:-

- (1) Direct staining experiments and tests of specificity of staining.
- (2) Mixed staining experiments involving immune and non-immune globulins labelled contrastingly.
- (3) Mixed staining experiments involving fluorescein and rhodamine conjugates prepared from the one antiserum pool.
- (4) Mixed staining experiments involving contrastingly labelled conjugates of dissimilar specificity (i.e. representing anti-glomerulus and anti-synovium antisera).

(1) Direct Staining Experiments and Tests of Specificity of Staining

In direct staining experiments the distribution of specific staining produced in sections

of human kidney by a fluorescein conjugate prepared from one antiserum pool was compared with that produced in serial sections by an RB200 conjugate, prepared from the same antiserum pool. These experiments are summarised in Table 12, page 48, rows 1 - 8.

(2) Mixed Staining Experiments: Immune and Non-immune Globulins

In these experiments an anti-human glomerulus (or synovium) conjugate was allowed to react with a tissue section in the presence of contrastingly labelled globulins, separated from (a) normal rabbit serum (NRG) or (b) rabbit anti-guinea pig globulin antisera (RAPS). The staining produced by these mixtures, F-RAPS + RB200-anti-glomerulus, for example, was compared with that produced by similar mixtures in which only the anti-tissue preparation bore a fluorescent label, RAPS + RB200-anti-glomerulus (Table 12, rows 9 - 16).

These experiments were performed to investigate the possibility that immunologically irrelevant globulin might become entrapped amongst antibody molecules, during the fixation of antibody by antigen in tissue sections.

(3) Mixed Staining Experiments : Conjugates of Like Specificity

These experiments are summarised in Table 12, rows 17 and 18. They involved the exposure of

sections to mixtures consisting of equal parts of contrastingly labelled anti-synovium globulins or to mixtures containing contrastingly labelled anti-glomerulus globulins. Experiments in which an appropriately labelled normal rabbit globulin conjugate was substituted for one of the anti-tissue conjugates in the mixture, were run in parallel with these mixed staining experiments.

(4) Mixed Staining Experiments: Conjugates of Dissimilar Specificity

The mixture with which sections were treated in this group of experiments contained equal parts of anti-synovium and anti-glomerulus conjugates, labelled contrastingly (Table 12, rows 19 and 20).

TABLE 12
THE USE OF CONTRASTINGLY LABELLED CONJUGATES
IN IMMUNO-HISTOLOGICAL STUDIES
Exploratory Experiments

STAINING PROCEDURES			HISTOLOGICAL COMPONENTS			
Pretreatment	or	Conjugate	Glomerulus		Arteries	
			B.M.	Cells	Media	Adv.
Mixture						
1 RAPS		RB-anti-Glom.	R	R	R	R
2 RAPS		F -anti-Glom.	G	G	G	G
3 Anti-Glom.		RB-anti-Glom.	-	-	-	-
4 Anti-Glom.		F -anti-Glom.	-	-	-	-
5 RAPS		RB-anti-Syn.	-	R	-	R
6 RAPS		F -anti-Syn.	-	G	-	G
7 Anti-Syn.		RB-anti-Syn.	-	-	-	-
8 Anti-Syn.		F -anti-Syn.	-	-	-	-
9 RAPS	+	RB-anti-Glom.	R	R	R	R
10 F-RAPS	+	RB-anti-Glom.	R	R	R	R
11 RB-RAPS	+	F -anti-Glom.	G	G	G	G
12 RAPS	+	F -anti-Glom.	G	G	G	G
13 RAPS	+	RB-anti-Syn.	-	R	-	R
14 F-RAPS	+	RB-anti-Syn.	-	R	-	R
15 RB-RAPS	+	F -anti-Syn.	-	G	-	G
16 RAPS	+	F -anti-Syn.	-	G	-	G
17 F-anti-Glom.	+	RB-anti-Glom.	Y	Y	Y	Y
18 F-anti-Syn.	+	RB-anti-Syn.	Y	Y	Y	Y
19 F-anti-Glom.	+	RB-anti-Syn.	G	Y	G	r-O
20 RB-anti-Glom.	+	F -anti-Syn.	R	Y	R	G

RAPS = Rabbit anti-Guinea Pig Globulin
(globulin fraction of antiserum)
Glom. = Glomerulus.
Syn. = Synovium.
B.M. = Boundary Membrane.
Adv. = Adventitia.
RB = Lissamine Rhodamine B200
F = Fluorescein
G = Green
r & R = Red
Y = Yellow
r-O = Orange Dominant.

Observations.

The following observations were made during exploratory experiments:-

- (1) The distribution of specific staining produced in sections of human kidney by an anti-tissue conjugate, prepared from one antiserum pool, was exactly matched by that produced in serial sections by the contrastingly labelled conjugate prepared from the same pool (Table 12, rows 1 - 4

and 5 - 8, and compare C.T. 1 and 2).

(2) The staining produced by an anti-tissue conjugate which had been allowed to react with a section in the presence of contrastingly labelled "normal" rabbit globulin, differed only slightly in colour from that seen in sections exposed to the unlabelled NRG + labelled anti-tissue conjugate mixtures (C.T.4). This observation held true whether the "normal" rabbit globulin bore the red or the green label, and whether it represented serum from unimmunised rabbits or serum from animals immunised against guinea pig serum globulins (Table 12, rows 9 - 12 and 13 - 16).

(3) In mixed staining experiments involving conjugates of like specificity, all stained tissue components were found to emit a yellow fluorescence (Table 12, rows 17 and 18).

The mixtures used in these experiments were prepared immediately before use, by mixing together equal parts (measured as drops) of the fluorescein and RB200 conjugate. Although it is probable that the relative proportions of the conjugate varied slightly from mixture to mixture, the colour of staining produced by such mixtures did not vary noticeably from experiment to experiment. Nevertheless, the composition of the mixtures did affect the colour of staining produced

by them. Green rapidly emerged as the dominant colour in stained tissues when the proportion of the fluorescein conjugate in the mixture was gradually increased. Increments in the representation of the red label in mixtures had a less marked effect, and orange only slowly came to dominate the colour of staining as the proportion of the RB200 conjugate in the mixture was increased.

(4) Three groups of colours appeared in sections exposed to contrastingly labelled mixtures of anti-glomerulus and anti-synovium conjugates. These groups were:-

- (i) The colour of the anti-glomerulus conjugate.
- (ii) An intermediate yellow colour.
- (iii) A third colour, predominantly, but not solely, attributable to the anti-synovium conjugate.

The staining produced in sections of human kidney by contrastingly labelled mixtures of anti-glomerulus and anti-synovium conjugates is illustrated in C.T. 5 and 10.

The colour produced by a particular mixture, F-anti-glomerulus + RB200-anti-synovium, for example, in a given component of renal connective tissue, remained constant over a series of experiments. The colour of the staining in renal

glomerular boundary membranes and the media of arteries, for example, was always that of the anti-glomerulus conjugate. These tissues were stained green by the F-anti-glomerulus + RB200-anti-synovium mixture and red by the RB200-anti-glomerulus + F-anti-synovium mixture. The colour of the staining produced in arterial adventitia was also influenced by the composition of the mixture, but here the influence was attributable to the anti-synovium conjugate. Collagenous fibrils in arterial adventitia were stained green by mixtures containing the F-anti-synovium conjugate and orange by mixtures containing the red RB200-anti-synovium conjugate. The colour of staining produced in glomerular epithelial cells was not influenced by the composition of the staining mixture. These cells were stained yellow by both combinations of contrastingly labelled anti-synovium and anti-glomerulus conjugates.

Conclusions

The following conclusions were drawn from these observations:-

- (1) Conjugation with RB200 effects no immunohistologically significant alteration in the specificity of antibodies vis-a-vis that of the corresponding F-labelled antibodies.
- (2) Any non-specific co-precipitation of (contrastingly) labelled globulins which may



occur when labelled antibodies are allowed to react with their antigens, in the presence of heterologous globulins, is not of sufficient magnitude materially to modify the colour of specific fluorescence, normally produced by the labelled antibodies.

(3) Since increments in the proportion of the F-labelled antibodies had a greater influence on the colour of staining produced by mixtures of F- and RB-labelled antibodies than similar increments in the proportion of RB200 antibodies had, it would be necessary to run complementary sets of mixed staining experiments in parallel. In these experiments, one of a pair of serial sections would be exposed to an F-anti-glomerulus + RB-anti-synovium mixture, and the other to a similar mixture in which the order of the labels had been reversed.

(4) Because: (a) No intermediate colours were seen in mixed staining experiments involving non-immune and immune globulins (e.g. RB-NRG + F-anti-synovium C.T.4),

(b) only the yellow intermediate colour appeared in sections exposed to contrastingly labelled anti-tissue conjugates of like specificity (e.g. F- and RB200-anti-glomerulus),

the range of colours seen in sections of human kidney exposed to contrastingly labelled anti-glomerulus + anti-synovium conjugates presumably carried some connotations for the antigenic relationships existing between the various histological components of renal connective tissue, and possibly for connective tissues in general.

To test this possibility, the scope of mixed staining experiments was broadened to include organs other than the kidney.

MIXED STAINING EXPERIMENTS

RENAL AND EXTRA RENAL TISSUES

Methods

A. Reagents

The mixtures of anti-tissue conjugates used in these experiments were prepared by adding 1.5 ml. of an anti-synovium conjugate to an equal volume of an anti-glomerulus conjugate labelled contrastingly.

Contrastingly labelled mixtures of anti-tissue globulins and normal rabbit globulins were similarly prepared in "bulk".

The conjugates forming these mixtures had previously been absorbed with guinea pig liver powder, human serum and human red blood cells and they had been shown to produce either no non-specific staining (in the case of fluorescein conjugates), or, in the case of rhodamine conjugates, an acceptably unobtrusive dull brown-red non-specific staining of cytoplasm. It was found impossible to completely rid rhodamine conjugates of material reactive non-specifically with fresh tissues.

B. Staining Procedures

In mixed staining experiments individual sections were exposed for 16 to 18 hours to one

of the following mixtures of contrastingly labelled conjugates:

- 1). F-anti-glomerulus + RB-anti-synovium
- 2).RB-anti-glomerulus + F-anti-synovium
- 3). F-anti-glomerulus + RB-NRG
- 4).RB-NRG + F-anti-synovium

In the early stages of the study, and whenever the sections to be examined represented previously unsectioned blocks of tissue, certain supplementary experiments were run in parallel with mixed staining experiments. These experiments are summarised in Table 13. They were:-

- (i) Tests of the specificity of the staining produced by the anti-glomerulus and anti-synovium conjugates used in mixed staining experiments (Table 13, rows 1, 2, 4, 5, 6, 7, 9 and 10).
- (ii) Cross inhibition experiments (Table 13, rows 3 and 8).

TABLE 13
MIXED STAINING EXPERIMENTS
Supplementary Procedures

	Pretreatment 16 to 18 hours	Conjugate 30 to 45 mins.
1	RB-NRG	F-anti-synovium
2	anti-synovium	F- " "
3	" "	F-anti-glomerulus
4	F-NRG	RB-anti-synovium
5	anti-synovium	RB- " "
6	RB-NRG	F-anti-glomerulus
7	anti-glomerulus	F- " "
8	" "	F-anti-synovium
9	F-NRG	RB-anti-glomerulus
10	anti-glomerulus	RB- " "

Observations

The three groups of colours seen in sections of kidney after mixed staining experiments appeared also when sections of other organs were submitted to mixed staining procedures. It was found that the colour of staining produced in any one histological component of tissue by a particular mixture of anti-glomerulus and anti-synovium conjugates remained constant throughout a series of experiments.

F-anti-glomerulus + RB-anti-synovium mixtures, for example, produced: green staining in the media of all muscular arteries and arterioles;

orange-red staining of collagenous tissue in arterial adventitia; yellow staining of reticular tissue in the spleen, liver and lymph nodes (Table 14).

These tissues (arterial media, collagenous tissue and framework reticular tissue) were stained red, green and yellow respectively by the RB-anti-glomerulus + F-anti-synovium mixture (Table 14).

TABLE 14
MIXED STAINING EXPERIMENTS

Immunohistological Classification of Connective Tissues

Histological Component	Staining F-v-G + R-v-S	Procedure R-v-G + F-v-S	I-H Group
BOUNDARY MEMBRANES			
Renal Glomerular Capillaries Pulmonary Alveolar Capillaries Choroid Plexus	Green	Red	ONE
Epithelial inner	Green	Red	TWO
outer	Yellow	Yellow	
Extra-glomerular Capillaries Arteries	Yellow	Yellow	
RETICULAR TISSUE			
Media of Muscular Arteries Arterioles Vasa Vasorum	Green	Red	ONE
Thick Walled Capillaries	Yellow- Orange	Orange	Related to ONE & TWO
Synovial Cell Layer Spleen, Liver, Lymph Glands	Yellow	Yellow	TWO
Interstitial	Orange	Green	THREE
CELLS			
Glomerular "Epithelial" Cells Fibrocytes	Yellow	Yellow	TWO
COLLAGENOUS TISSUE	Orange	Green	THREE

F = Fluorescein label.
-v-G = anti-glomerulus.

R = Rhodamine label.
-v-S = anti-synovium.

The observations made during mixed staining experiments suggested that the individual components of connective tissue could be placed into one of three, presumably antigenically dissimilar, groups on the basis of their staining reactions (Table 14).

The first of these groups contained tissues reactive with antibodies present only in anti-glomerulus preparations. These tissues were stained green by mixtures containing F-anti-glomerulus and red by mixtures containing RB-anti-glomerulus.

The second group comprised connective tissues reactive with antibodies present in anti-glomerulus and in anti-synovium conjugates. Members of this group were stained yellow on exposure to contrastingly labelled mixtures of anti-glomerulus and anti-synovium conjugates.

Tissues placed into the third group were also reactive with anti-glomerulus and with anti-synovium antibodies. Unlike second group components, however, third group components were not stained yellow by contrastingly labelled mixtures of the two anti-tissue preparations. They were stained green or green-yellow by the RB-anti-glomerulus + F-anti-synovium mixture, and orange or orange-red by the F-anti-glomerulus + RB-anti-synovium mixture.

The interpretations put upon findings in mixed staining experiments carried certain implications. These were:-

- (a) A fundamental difference in their capacity to combine with antigens of the third group exists between anti-glomerulus and anti-synovium conjugates.
- (b) There is no such difference between the reactions of these conjugates with antigens of the second group.

The direct exchange and cross exchange experiments described below were performed to test these implications and thereby to assess the validity of the interpretations placed upon the findings in mixed staining experiments.

Conjugate	No. of slides	Anti-glomerulus			
		1	2	3	4
Anti-glomerulus	18	15	15	15	15
Normal Rabbit	18	15	15	15	15
		Anti-synovium			
Anti-synovium	18	15	15	15	15
Normal Rabbit	18	15	15	15	15

1. The results of the direct exchange experiments are shown in Table I and Table II. The results of the cross exchange experiments are shown in Table III and Table IV. The results of the mixed staining experiments are shown in Table V and Table VI.

DIRECT EXCHANGE AND CROSS EXCHANGE
EXPERIMENTS

These experiments were based upon the following observation:

Pretreatment of a section with unlabelled immune globulin will block the staining normally produced by the corresponding conjugate, but this inhibition can be overcome if contact with the conjugate is prolonged, specific staining then appears and becomes progressively brighter (Table 15).

TABLE 15.

REVERSAL OF SPECIFIC INHIBITION

Unlabelled Globulin	Hours	F-anti-glomerulus			
		$\frac{1}{2}$	1	2	18
Anti-glomerulus	18	U-?	+	+++	++++
	2	?-+	++	+++	++++
Normal Rabbit	18	++	+++	+++	++++
	2	++	+++	+++	++++
		RB-anti-glomerulus			
		$\frac{3}{4}$	$1\frac{1}{2}$	3	18
Anti-glomerulus	18	U	+	++	+++
	2	U	+	++	+++
Normal Rabbit	18	+	++	+++	+++
	2	+	++	+++	+++

U and ? = No and doubtful fluorescence.
+ to ++++ represent visual estimates of
brilliance of fluorescence.

Methods

For direct exchange experiments, sections were stained for 18 hours with a conjugate prepared from the one antiserum pool and then exposed ($\frac{1}{2}$ to 18 hours) to a contrastingly labelled conjugate, prepared from the same antiserum pool.

In cross exchange experiments, sections were stained for 18 hours with a red or green conjugate of the one specificity (e.g. anti-glomerulus) and then exposed ($\frac{1}{2}$ to 18 hours) to a contrastingly labelled conjugate of the other specificity (e.g. anti-synovium).

In both groups of experiments sections were first rinsed and then washed for five minutes in buffered saline pH 7.0 to 7.2, before they were exposed to the second conjugate.

Observations

Observations made during direct exchange experiments are summarised in Table 16.

In direct exchange experiments, the colour of all tissues, initially stained green by the F-anti-glomerulus conjugate, was gradually altered to yellow on exposure to the RB-anti-glomerulus preparation. Similarly, in sections stained first with the RB-anti-glomerulus preparation, the colour of all stained tissues was gradually altered from red to a yellowish-green by the F-anti-glomerulus conjugate.

TABLE 16
DIRECT EXCHANGE EXPERIMENTS

1st Conjugate	2nd Conjugate	$\frac{1}{2}$ -3/4	2-3	18
RB-anti-Glom	F-anti-Glom	R	g-Y	y-G
F-anti-Glom	RB-anti-Glom	G	y-G	G
RB-anti-Glom	F-NRG	R	R	R
F-anti-Glom	RB-NRG	G	G	G

G and g = Green
y and Y = Yellow
g - Y = Yellow dominant
y - G = Green dominant
R = Red.

Sections were exposed to the first conjugate for 18 hours and to the second for 3/4, 3 and 18 hours (RB-conjugates) or $\frac{1}{2}$, 2 and 18 hours (F-conjugates).

A gradual alteration in the colour of stained tissues was found to occur in cross exchange experiments also (Table 17).

In cross exchange experiments, however, anti-synovium conjugates effected:

- (a) no colour change in glomerular capillary boundary membranes or arterial media.
- (b) a relatively more rapid and more complete alteration in the colour of collagenous tissue than in the colour of framework reticular tissue.

In sections which had been stained first with anti-synovium conjugates, anti-glomerulus conjugates brought about a less marked colour change in vascular adventitia (i.e. collagenous tissue) than in splenic reticulum and other framework

reticular tissues.

The observations made during cross exchange experiments are summarised in Table 17.

TABLE 17
CROSS EXCHANGE EXPERIMENTS.

Staining Procedure			B.M.			RETICULAR TISSUE		
1st Conjugate 18 hrs.	2nd Conjugate	hrs.	Glomerular	Inner	Outer	Collagenous Tissue	Framework	Arterial Media
F-v-Syn.	R-v-Glom.	3/4 3 18	R "	R "	G Y Y	G G G	G o-Y o-Y	R " "
R-v-Syn.	F-v-Glom.	1/2 2 18	G " "	G " "	O Y y-G	R O O	O o-Y y-G	G " "
R-v-Glom.	F-v-Syn.	1/2 2 18	R " "	R " "	R y-O Y	o-Y G G	R o-Y Y	R " "
F-v-Glom.	R-v-Syn.	3/4 3 18	G " "	G " "	G y-G Y	G y-O O	G y-G Y	G " "
F-v-Syn. R-v-Syn.	R-NRG F-NRG	18 18	U U	U U	G R	G R	G R	U U
R-v-Glom. F-v-Glom.	F-NRG R-NRG	18 18	R G	R G	R G	R G	R G	R G

-v-Syn. = anti-synovium.
R- = Rhodamine label.
-v-Glom. = anti-glomerulus.
F- = Fluorescein label.

r and R = Red.
y and Y = Yellow.
o and O = Orange.
G = Green.
U = Unstained.

y - G)
o - Y) Capitals denote dominant colour.
y - O)

Conclusions

The final colour of stained tissues after direct exchange experiments suggested that both fluorescein labelled and rhodamine labelled antibodies were attached to these tissues; fluorescein labelled antibodies being, it seemed, better represented after F---> RB experiments

than rhodamine labelled antibodies were after RB---> F experiments. These observations indicated that the interchange between fixed and free antibodies did not go to completion. It was thought also that antibodies conjugated with fluorescein became more firmly bound to their antigens than RB labelled antibodies did (Scott, 1959). This latter conclusion, it is now appreciated, cannot validly be drawn. Significant differences may well exist between the degree of labelling achieved during the preparation of F-conjugates on the one hand and RB-conjugates on the other, nor is the emission intensity of the one label necessarily equivalent to that of the other.

Whatever may be the reason for the predelection, probably more apparent than real, exhibited by tissues in direct staining experiments for fluorescein labelled antibodies, the observation that in cross exchange experiments collagenous tissues reacted preferentially with anti-synovium antibodies, whether these were labelled with fluorescein or rhodamine, whereas reticular tissues did not, suggests that antigenic differences exist between reticular tissue and collagenous tissue.

The implications carried by cross exchange experiments for the immuno-histological classi-

than rhodamine labelled antibodies were after RB---> F experiments. These observations indicated that the interchange between fixed and free antibodies did not go to completion. It was thought also that antibodies conjugated with fluorescein became more firmly bound to their antigens than RB labelled antibodies did (Scott, 1959). This latter conclusion, it is now appreciated, cannot validly be drawn. Significant differences may well exist between the degree of labelling achieved during the preparation of F-conjugates on the one hand and RB-conjugates on the other, nor is the emission intensity of the one label necessarily equivalent to that of the other.

Whatever may be the reason for the predelection, probably more apparent than real, exhibited by tissues in direct staining experiments for fluorescein labelled antibodies, the observation that in cross exchange experiments collagenous tissues reacted preferentially with anti-synovium antibodies, whether these were labelled with fluorescein or rhodamine, whereas reticular tissues did not, suggests that antigenic differences exist between reticular tissue and collagenous tissue.

The implications carried by cross exchange experiments for the immuno-histological classi-

fication of connective tissues were very similar to those carried by mixed staining experiments.

In both groups of experiments connective tissues showed three patterns of reactivity (Table 18). These patterns characterised the following three groups of antigenically active tissues:

- Group 1. Tissues reactive with anti-glomerulus preparations only.
- Group 2. Tissues reacting with anti-synovium and anti-glomerulus conjugates and for which anti-synovium antibodies exhibited no greater or lesser affinity in cross exchange or mixed staining experiments, than anti-glomerulus antibodies did.
- Group 3. Tissues reactive with anti-synovium and anti-glomerulus antibodies but for which anti-synovium antibodies appeared to possess a higher degree of affinity than anti-glomerulus antibodies did.

TABLE 18
CROSS EXCHANGE AND MIXED STAINING EXPERIMENTS

I-H Group,	Histological Component	Colour change following Mixed Staining (-) and Cross Exchange (↓) Experiments					
		F-v-G	F-v-G	R-v-S	R-v-G	F-v-S	F-v-S
		+ R-v-S	↓ R-v-S	↓ F-v-G	↓ F-v-S	↓ R-v-G	+ R-v-G
	BOUNDARY MEMBRANES						
1	Renal Glomerular Capillaries Pulmonary Alveolar Capillaries Choroid Plexus	G	G	G	R	R	R
1	Epithelial inner *	G	G	G	R	R	R
2	Epithelial outer *	Y	Y	y-G	Y	Y-o	Y
2	Extra-glomerular Capillaries Arterial	Y	Y	y-G	Y	Y-o	Y
	RETICULAR TISSUE						
2	Synovial Cell Layer Spleen, Liver, Lymph Glands	Y	Y	y-G	Y	Y-o	Y
3	Interstitial	y-O	O-r	y-G	y-G	G-y	G
1	Media of Muscular Arteries Arterioles Vasa Vasorum	G	G	G	R	R	R
1 to 2	Media of Thick Walled Capillaries	Y-o	g-Y	y-G	o-Y	O-r	O
	CELLS						
2	Renal Glomerular "Epithelial" Fibrocytes	Y	Y	Y	Y	Y-o	Y
3	COLLAGENOUS TISSUE	O	O	O	G	G	G

* In general it was possible to distinguish the two components of epithelial boundary membranes only when red and yellow were in juxtaposition.

Histological Distribution of Group 1 Components

The first immuno-histological group of connective tissues included: the boundary membranes of renal glomerular and pulmonary alveolar capillaries, the sub-epithelial boundary membrane of the choroid plexus, the reticular tissue in the media of muscular arteries and arterioles. These structures were stained green in cross exchange and mixed staining experiments, involving F-anti-glomerulus and RB-anti-synovium conjugates. They were stained red in experiments in which the order

of the labels was reversed (C.T. 5, 6, 13, 28 and 7, 10, 14 and 27).

In certain situations, the sub-synovium and the tela choroidea, thick walled capillaries were seen which gave staining reactions tending towards, but not identical with, those of Group 1 components (Table 18). In RB-anti-glomerulus + F-anti-synovium experiments, these capillaries were seen to have thick orange coloured tunica media, fenestrated by unstained (black) circular or spindle shaped spaces (C.T. 29). In F-anti-glomerulus + RB-anti-synovium experiments, the tunica media of these vessels emitted a yellowish fluorescence and the distinction between the media and boundary membrane was not readily apparent (C.T. 31).

The use of contrastingly labelled conjugates made it clear that two antigenically distinct components went to form epithelial boundary membranes in the kidney, thyroid and pancreas. These membranes, in F-anti-glomerulus + RB-anti-synovium, mixed staining and F-anti-glomerulus ---> RB-anti-synovium and RB-anti-synovium ---> F-anti-glomerulus cross exchange experiments, usually appeared to consist of a single line. Now and then, however, a fine green line was seen to be interposed between the bases of renal tubular cells and an outer yellow membrane. Occasionally

an unstained gap was present between the inner and outer components of renal tubular boundary membranes, the tubular cells and the inner membrane having separated as a unit from the outer membrane. Separation of the inner from the outer component of acinar boundary membranes, was seen also in disrupted sections of the thyroid gland (C.T.13).

The dual constitution of epithelial boundary membranes was more readily apparent after mixed staining and cross inhibition experiments in which the anti-glomerulus conjugate bore the RB-label, than after experiments in which this preparation bore the green label (Table 18).

After RB-anti-glomerulus + F-anti-synovium experiments, renal tubular and thyroid acinar membranes usually showed a fine red inner component lying closely applied both to epithelial cells and an outer yellow stained component (C.T.10).

The two components of epithelial boundary membranes were seen to best advantage in sections which had been stained with the F-anti-synovium conjugate for 18 hours, and subsequently exposed to the RB-anti-glomerulus conjugate for 30 minutes (C.T.14). In such immuno-histological preparations, the inner or basal component of epithelial boundary membranes showed a red fluorescence while the outer or reticular component showed green

staining.

Histological Distribution of Group 2 Components

In addition to their distribution as the outer component of epithelial boundary membranes Group 2 constituents of connective tissue, those staining yellow in all mixed staining and cross exchange experiments (Table 18) were found in the synovial cell layer, the sarcolemma of muscle, pericapillary reticulum, and in reticular tissue forming the framework of the spleen, liver and lymph nodes. A yellow line was constantly present between the media and adventitia of muscular arteries. This line was best seen after procedures involving the use of the RB-anti-glomerulus preparation (C.T.27), but was visible also when the anti-glomerulus conjugate had borne the green label (C.T. 8).

Group 2 components were not distributed solely as reticular tissue. The staining reactions of glomerular epithelial cells (C.T.5) characterised them as Group 2 components.

Histological Distribution of Group 3 Components

The third group of connective tissues was stained green or predominantly green when the RB-anti-glomerulus conjugate was used in conjunction with the F-anti-synovium conjugate and orange when the order of the labels was reversed (Table 18).

Third group components were distributed as fine wavy fibrils in the adventitia of arteries and

as fine fibrillar material between bundles of collagen in tendons and the capsules of joints (C.T.33). Collagen which was recognised by its blue auto-fluorescence did not react with either of the anti-tissue preparations.

Members of the third immuno-histological group of connective tissues were occasionally encountered as scanty interstitial fibrils in the kidney, thyroid gland and other epithelial organs and between muscle bundles in voluntary muscle.

The observation that anti-tyrosine preparation did not react with groups of fibrils of substance which had been subjected to similar treatment each to each and which internal fibrils, potentially reactive with tyrosine, were not observed in substance is possible that the anti-tyrosine preparation was not reactive with the fibrils in substance and that the fibrils were not reactive with the anti-tyrosine preparation.

Observations were made during the course of the study that the fibrils in substance were not reactive with the anti-tyrosine preparation and that the fibrils in substance were not reactive with the anti-tyrosine preparation.

IMMUNO-HISTOLOGICAL STUDIES OF HUMAN
CONNECTIVE TISSUES

Comments and Speculations

The immuno-histological studies of connective tissue described here have indicated that the basal component of epithelial boundary membranes, boundary membranes of renal glomerular capillaries and reticular tissue in the media of muscular arteries and arterioles, all contain determinants reactive with antibodies present in anti-glomerulus antisera. It does not follow that these tissues are antigenically identical.

The observation that anti-synovium preparations did not react with group one constituents of connective tissue, does not exclude the possibility that some of these constituents may contain internal determinants, potentially reactive with antibodies in anti-synovium antisera. It remains possible then, that the anti-reticular tissue reactivity of anti-glomerulus antisera is derived not only from glomerular epithelial cells, but also from the glomerular capillaries themselves.

Observations made during cross inhibition and mixed staining experiments indicated that while there was a difference between the reactions of anti-glomerulus and anti-synovium preparations with collagenous tissue, there was no demonstrable difference between the reactions of the two prepa-

rations with reticular tissue. It followed that collagenous tissue and reticular tissue differ antigenically.

Cross inhibition (e.g. unlabelled anti-synovium ---> F-anti-glomerulus) experiments had excluded the possibility that the staining produced in collagenous tissue by anti-glomerulus conjugates and by anti-synovium conjugates, was to be attributed to the selective staining by each of these conjugates of one of two distinct histological components of collagenous tissue lying side by side. These experiments had not excluded the possibility, however remote, that anti-synovium and anti-glomerulus conjugates reacted with entirely unrelated antigenic determinants, carried on a single histological structure. It will be recalled that in cross inhibition experiments, unlabelled anti-tissue antibodies from the one antiserum, blocked the staining normally produced in collagenous tissues by conjugates prepared from the other antiserum. This cross inhibitory activity is explicable on the basis of a postulate invoking steric hindrance, whereby antibodies of the one specificity, having fixed to their antigens, thereby prevent access of antibodies of different specificity to neighbouring antigens. This postulate demands that in the cross exchange situation anti-glomerulus (or anti-synovium) antibodies, in

order to gain access to their antigens, elute unrelated antibodies from combination with neighbouring determinants, for which the eluting antibodies possess no affinity. It seems more reasonable to suggest that cross exchange experiments predicate a degree of overlap between the anti-collagenous specificities of anti-glomerulus and anti-synovium antisera.

If antibodies in anti-glomerulus and anti-synovium preparations react with the same set of antigenic determinants in collagenous tissue, the difference between the reactions of these two preparations with collagenous tissue in cross exchange and mixed staining experiments must be attributed to a difference in the stability of the union formed by anti-glomerulus antibodies on the one hand and anti-synovium antibodies on the other, with antigens in collagenous tissue. This view might imply that the antibodies which form the weaker link with collagenous antigens (anti-glomerulus antibodies) are cross reacting antibodies. It remains possible, however, that trace amounts of collagenous tissue were present in the glomerular antigen against which anti-glomerulus antisera were raised, and that these elicited antibodies with only poor combining power.

When the various histological components of

connective tissue are tabulated according to their reactions in immuno-histological staining experiments, it becomes evident that there is a loose correspondence between their immuno-histological classification and their histological distribution (Table 19).

TABLE 19
IMMUNOHISTOLOGICAL CLASSIFICATION
OF CONNECTIVE TISSUES

Immuno-histological	Histological
<u>GROUP I</u> Boundary Membranes Renal Glomeruli Choroid Plexus Pulmonary Alveolar Capillaries Epithelial (inner component)	Basal Membranes
Reticular Tissue Media of Muscular Arteries Media of Arterioles Walls of Vasa Vasorum	Vascular Media
<u>GROUP II</u> Media of Thick Walled Capillaries in: Tela Choroidea Spleen Sub-synovium	
Boundary Membranes Epithelial (outer component) Muscular Arteries, Medial- adventitial junction Extra-glomerular Capillaries	Framework Reticular Tissue
Reticular Tissue Synovial Cell Layer Spleen, Liver, Lymph Nodes	Packing Tissue
Cells Glomerular "Epithelial" Fibrocytes	
<u>GROUP III</u> "Collagenous" Tissue Interstitial Reticular Tissue	

It is evident from Table 19 that such correspondence as exists between the immuno-histological classification of connective tissues and their histological distribution is neither complete nor consistent.

Group 1, for example, includes reticular tissue in the media of certain arteries, the basal component of epithelial boundary membranes and the boundary membranes of renal glomerular capillaries but excludes the boundary membranes of extra-glomerular capillaries.

Similarly Group 2 contains framework reticular tissue, but neither interstitial reticular tissue nor collagenous tissue.

The loose correspondence demonstrable between the immuno-histological grouping and the histological distribution of the various components of connective tissue, and especially the antigenic distinction between thick walled and other extra-glomerular capillaries, stimulated speculation concerning the possible relationship between antigenic characteristics and functional differentiation. The observation that fibrocytes differed antigenically from collagenous tissue and collagenous tissue from mature collagen, might provide a basis for similar speculations, if there were any certainty that fibrocytes, collagenous tissue and collagen represented a continuous spectrum in the maturation of collagen. There is, however, no

such certainty.

Speculations concerning the relationship between antigenicity and functional differentiation necessarily give rise to the prediction that antigenic differences exist amongst components of connective tissue currently grouped together.

A possible approach to the testing of these speculations and predictions was suggested by the work of Eroshkina and Kolymikova (1952). These workers reported that the antigenic characteristics of human embryonic connective tissue changed during culture, so that antigens characteristic of the embryo were partially lost and those characteristic of the adult acquired.

It seemed, therefore, that it might be possible to base further analyses of connective tissues on a comparison of the anti-tissue specificities of the corresponding antisera, provided:

- (a) that the antigenic differences which might exist between embryonic and adult tissues were sufficiently gross to be detectable by immunohistological means and
- (b) that the various adult patterns emerged in sequence rather than simultaneously.

The studies of adult and embryonic connective tissue in the adult and embryonic rat, described below, were undertaken to test the first of these provisos.

IMMUNOHISTOLOGICAL STUDIES OF CONNECTIVE
TISSUE IN THE ADULT AND EMBRYONIC RAT

Immunohistological studies of connective tissue in the adult and embryonic rat are incomplete and it is proposed to confine the present report to studies of renal connective tissue in adult, young, neonatal and 3.0 cm. embryonic rats, and of dermal connective tissue in rat embryos measuring from 1.0 cm. to 3.0 cm. in crown to rump length.

It might be well to preface a description of the studies with an explanation of the terms "adult," "young," neonatal" and "embryonic." These terms are used in the following way: "adult" describes rats more than two months old; "young" describes rats from eight to seventeen days old; "neonatal" describes rats from birth to seven days old. The embryonic rats were obtained in the following way: a buck was introduced to each of 2, 3 or 4 colonies of 6 to 12 female rats and allowed to remain with the colony for 3 to 5 days. Females were killed by chloroform anaesthesia on the 7th, 9th, 11th, 12th, 13th, 14th and 15th day after removal of the bucks from the colonies. Embryos harvested from pregnant females were placed into groups on the basis of their general appearance and size. These groups were:

General Appearance	Length in cms.
Amorphic	Less than 0.5 in diameter
Incompletely developed	1.0 \pm 0.3 crown to rump
Rat-like	1.0 to 1.5 crown to rump
Rat-like	1.5 to 2.0 crown to rump
Rat-like	More than 2.5 crown to rump

The term "amorphic" describes small white spheres or discs which measured about 3mm. in diameter and which were firmly attached to the uterine wall.

Incompletely developed embryos showed little evidence of their final form and little differentiation between head and trunk. In the amnion these embryos had the appearance of curved cylinders but on removal from the amnion they flattened out.

Rat-like embryos had undergone external metamorphosis. Although in the smallest of these the development of the face and limbs was incomplete they possessed a definite trunk; a ventrally flexed head, disproportionately large relative to the body; a blunt or rounded rather than pointed face; short limbs with relatively large spatulate paws (C.T. 34).

The larger rat-like embryos clearly represented further stages in development. The face

had become more pointed, the limbs more slender and more definitely jointed than the corresponding structures in the smaller, 1.0 to 1.5 cm., rat-like embryos. There was also a gradual decrease in the size of the head relative to that of the whole body (C.T. 36 to 39). The largest of the rat-like embryos, measuring from 3.0 to 3.5 cm. in crown to rump length, differed little either in size or general appearance from new-born rats (C.T. 40).

EXPERIMENTAL APPROACH

Studies of connective tissues in the rat fell into three parts. These were:

- (1) studies of the interaction between fluorescein-anti-adult rat connective tissue conjugates and embryonic connective tissues;
- (2) studies of the alterations in their specificities brought about by absorbing anti-adult rat connective tissue preparations with embryonic connective tissues. These investigations were concerned mainly with the antigenic constitution of histologically mature and immature renal glomeruli (Scott and Rowell, 1967a), and with the antigenic constitution of dermal connective tissues in rat-like embryos (Scott and Rowell, 1967b).
- (3) a comparison of the anti-tissue specificities of anti-embryonic and anti-adult rat connective

tissue antisera, described in part by Scott and Rowell (1967b).

It is proposed here to attempt to present an overall picture of these studies rather than to describe them in the order in which they were performed.

METHODS

Preparation of Antisera

Antisera were raised in rabbits against adult rat renal glomeruli, adult rat splenic reticulum, adult rat renal connective tissue, connective tissues separated from rat-like embryos and against human renal connective tissues. The methods used in the preparation of these immunising antigens are described in Appendix 6.

The adult rat renal glomerular antigen was essentially similar to the human glomerular antigen used in the studies of human connective tissue. It consisted mainly of intact glomeruli, naked capillary tufts and empty parietal capsules, but contained also clumps of tubular cells which were present in a proportion of 10%.

The splenic reticulum antigen represented the cell-free framework of the spleens of adult rats freed of trabeculae and the larger blood vessels by passage through monel woven wire gauze sieves.

The adult rat renal connective tissue antigen contained equal volumes of isolated renal glomeruli

and cell-free fibrillar tissue prepared from renal tissue from which glomeruli had been isolated.

The human renal connective tissue antigen was similarly prepared from renal glomeruli and cell-free fibrillar tissue obtained from human kidneys.

Anti-embryonic rat connective tissue antisera were raised against cell-free fibrillar tissues obtained from rat embryos measuring less than 1.5 cm. in crown to rump length.

These antigens were injected into rabbits as 10% v/v suspensions in Freund's complete or incomplete adjuvant, and as 10% v/v suspensions in saline, according to the immunization schedules described in Appendix 6 and summarised in Table 20 below.

Table 20	Antigen	Adjuvant	Immunization Schedule	Antibody
1	Embryonic rat	Freund's complete	1, 2, 3, 4, 5	1, 2, 3, 4, 5
2	Embryonic rat	Freund's incomplete	1, 2, 3, 4, 5	1, 2, 3, 4, 5
3	Embryonic rat	Saline	1, 2, 3, 4, 5	1, 2, 3, 4, 5
4	Human renal	Freund's complete	1, 2, 3, 4, 5	1, 2, 3, 4, 5
5	Human renal	Freund's incomplete	1, 2, 3, 4, 5	1, 2, 3, 4, 5
6	Human renal	Saline	1, 2, 3, 4, 5	1, 2, 3, 4, 5

FC - Freund's complete adjuvant
FI - Freund's incomplete adjuvant
S - Saline

TABLE 20

PREPARATION OF ANTI-ADULT AND ANTI-EMBRYONIC
RAT CONNECTIVE TISSUE ANTISERA AND OF ANTI-HUMAN
RENAL CONNECTIVE TISSUE ANTISERA

Summary of Immunisation Schedules

Number of Rabbits	Total Dose of 10% Suspension of Antigen in			Antigen
	FCA ml.	FIA ml.	Saline ml.	
2	2	-	48	Adult rat renal glomerulus
1	2	4.5	-	Adult rat kidney
1	2	1.5	24	
1	-	-	24	
1	2	4.5	-	Embryonic rat C.T.
1	2	1.5	24	
1	2	3.0	12	
1	2	-	12	
2	4	-	-	Adult rat splenic reticulum
2	2	-	12	Human renal C.T.

FCA Freund's complete adjuvant

FIA Freund's incomplete adjuvant

C.T. Connective Tissue

Preparation of Conjugates

Crude globulin fractions of the anti-adult rat renal glomerulus, anti-adult rat renal connective tissue, anti-adult rat splenic reticulum and anti-embryonic rat connective tissue antisera were labelled with fluorescein isothiocyanate by the method of Marshall, Eveland and Smith (1958) - see Appendix 3.

Before their use in immunohistological staining experiments conjugates were freed of non-specifically reacting material by passage down a column of Sephadex G25 and by absorption with pig liver powder. They were then absorbed with rat serum, rat erythrocytes and one quarter of their own volume of packed homogenised human renal connective tissue.

Staining Experiments

Three types of staining experiment were performed, direct staining, cross inhibition and cross absorption. The experimental manipulations involved in staining experiments are summarised in Table 21 below. The principle was adopted of allowing conjugates to react with sections in the presence of an equal volume of unlabelled immune globulins.

In direct staining experiments sections were pre-treated with measured volumes of unlabelled globulins representing anti-human renal connective tissue antisera and then reacted with a mixture containing equal

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In direct staining experiments sections were pre-treated with measured volumes of unlabelled globulins representing anti-human renal connective tissue antisera and then reacted with a mixture containing equal

parts of these unlabelled globulins and one or other of the anti-rat connective tissue conjugates.

Tests of specificity of staining (direct inhibition experiments, Table 21) were run in parallel with direct staining experiments. In these tests anti-tissue conjugates were reacted with sections in the presence of the corresponding unlabelled anti-tissue globulins.

In cross inhibition experiments sections were reacted first with unlabelled globulin fractions of one or other of the anti-adult rat connective tissue antisera. To each of these globulins was then added an equal volume of the fluorescein labelled anti-embryonic connective tissue conjugate. The inhibitory activity of unlabelled anti-embryonic rat connective tissue globulins towards the fluorescein anti-adult rat connective tissue conjugates was similarly tested.

Cross absorption experiments were based upon the use of (a) fluorescein anti-adult rat connective tissue conjugates which had been repeatedly absorbed with small volumes of a crude preparation of embryonic connective tissues, obtained from larval-like and small incompletely developed embryos, and (b) the fluorescein anti-embryonic rat connective tissue conjugate which had been repeatedly absorbed with small volumes of the adult rat renal connective tissue antigen.

TABLE 21
STAINING EXPERIMENTS

Experiment	Pretreatment Unlabelled Globulin 3 hr. at 20°C, 16 hr. at 4°C.	Add	Staining Conjugate 1½ hr. at 20°C.
Direct Staining	NRG	+	F-ARG
	NRG	+	F-ARK
	NRG	+	F-ARS
	NRG	+	F-ARE
Direct Inhibition	ARG	+	F-ARG
	ARK	+	F-ARK
	ARS	+	F-ARS
	ARE	+	F-ARE
Cross Inhibition	ARG	+	F-ARE
	ARK	+	F-ARE
	ARS	+	F-ARE
	ARE	+	F-ARG
	ARE	+	F-ARK
	ARE	+	F-ARS
Cross Absorption	NRG	+	F-ARG x E.c.t.
	NRG	+	F-ARK x E.c.t.
	NRG	+	F-ARS x E.c.t.
	NRG	+	F-ARE x A.c.t.

NRG = anti-human renal connective.
 ARG = anti-adult rat renal glomerulus.
 ARK = anti-adult rat renal connective tissue.
 ARS = anti-adult rat splenic reticulum.
 ARE = anti-embryonic rat connective tissue.
 F = fluorescein conjugate.

x E.c.t. = absorbed with embryonic connective tissue.
 x A.c.t. = absorbed with adult rat renal connective tissue.

TABLE 22
 DIRECT STAINING EXPERIMENTS
 RENAL CONNECTIVE TISSUES IN THE RAT
 GLOMERULI

CONJUGATES	MATURE, TRANSITIONAL AND DEVELOPING Boundary Membranes									PROEMIAL		RATS
	Capillaries			Capsules			Intercapillary			Tubular	Inter-	
	Mat.	Tr.	Dev.	Mat.	Tr.	Dev.	Mat.	Tr.	Dev.	B.M.	tubular	
F-ARG F-ARS F-ARK	+			+			+					Adult
F-ARE	-			+			+					
F-ARG F-ARS F-ARK	+	+	+	+	+	+	+	+	+	+	+	Young and Neonatal
F-ARE	-			+	+	+	+		+	+	+	
F-ARG F-ARS F-ARK	+	+	+	+	+	+	+	+	+	+	+	3. - cm. Embryonic
F-ARE	+	+	+	+	+	+	+	+	+	+	+	

F-ARG = Fluorescein-anti-adult rat renal glomerulus.
 F-ARS = " " " " splenic reticulum.
 F-ARK = " " " " renal connective tissue.
 F-ARE = Fluorescein-anti-embryonic rat connective tissue.
 + = Specific Staining.
 - = No Staining.
 Blank = Structure not present or not recognized.

TABLE 23
DIRECT STAINING EXPERIMENTS
RENAL CONNECTIVE TISSUES IN THE RAT
EXTRA-GLOMERULAR

	Boundary Membranes		Interstitialium	Media of Arteries	
	Tubules	Peri-tubular Capillaries			
F-ARG F-ARS F-ARK	+	+	+	+	Adult
F-ARE	+	+	+	-	
F-ARG F-ARS F-ARK	+	+	+	+	Young and Neonatal
F-ARE	+	+	+	-	
F-ARG F-ARS F-ARK	+	+	+	+	3.0 cm. Embryonic
F-ARE	+	+	+	-	

OBSERVATIONS

DIRECT STAINING EXPERIMENTS

Observations arising out of F-anti-adult and F-anti-embryo direct staining experiments on the kidneys of adult, young, neonatal and embryonic rats are summarised in Tables 22 and 23.

Observations made during immunohistological studies of embryonic rat skin are summarised in Table 28.

Fluorescein anti-adult rat connective tissue conjugates

Renal glomeruli

The fluorescein anti-adult rat renal glomerulus (F-ARG), fluorescein anti-adult rat splenic reticulum (F-ARS) and fluorescein-anti-adult rat renal connective tissue (F-ARK) conjugates all reacted with glomerular capillary and capsular basement membranes and with the cytoplasm of glomerular epithelial cells in the kidneys of adult rats.

Three types of glomeruli were recognised in the kidneys of young, neonatal and embryonic rats in sections stained with the F-ARG, F-ARS or F-ARK conjugates. The different types of glomeruli may be conveniently referred to as:-

- (i) mature

(ii) immature

(a) transitional

(b) developing

(iii) proemial

Mature glomeruli seen in the kidneys of young, neonatal and embryonic rats presented the same general pattern of staining as did glomeruli seen in the kidneys of adult rats. A tuft of specifically stained capillaries lay within a circular, or roughly circular, filtration space, which was outlined by a specifically stained capsular membrane. In the kidneys of the younger rats glomerular capillaries appeared to be of relatively wider bore and less complexly convoluted than those forming the tuft of the adult renal glomerulus.

In immature glomeruli Bowman's capsule was represented by a crescentic tubule which was outlined by a specifically stained membrane. In transitional and developing glomeruli the concavity of the crescentic tubule frequently showed digitate invaginations.

In transitional glomeruli the tubule incompletely enclosed a simple convolution of capillary loops (C.T.41).

Developing glomeruli lacked a capillary tuft. In these glomeruli the position of the tuft was occupied by weakly stained matrix material which

was shot with brightly stained streaks and granules and fenestrated with unstained spaces or brightly stained circles (C.T.41).

In proemial glomeruli the crescentic tubule capped or embraced a second tubule which was seen cut at varying angles to its long axis. The space between the two tubules sometimes showed specific staining distributed in the form of granules (C.T.41). Although apparently made up of two tubules proemial glomeruli were taken, on the basis of appearances seen in conventionally processed and stained sections, to represent the topological interrelationship established between two parts of a single tubule by the invagination of the more distal into the more proximal part, prior to the separate differentiation of these parts into:

- (a) the glomerular capillary tuft and
- (b) Bowman's capsule.

Structures reminiscent of proemial glomeruli were seen in paraffin sections of embryonic tissue stained with haematoxylin and eosin. In these structures the embracing and embraced tubule often appeared to be in direct continuity (C.T.42).

Proemial glomeruli were found in the subcapsular zone of the kidneys of neonatal and young rats.

Mature glomeruli formed the juxta-medullary zone of the renal cortex in the kidneys of 3.0 cm.

embryos and were found intermixed with transitional glomeruli in the mid-cortex of neonatal rats. In 17-day old rats the area of the cortex occupied by mature glomeruli extended almost to the subcapsular zone.

There was some variation in the complexity of the capillary loop pattern presented by mature glomeruli in the kidneys from 12 to 17-day old rats. Capillaries forming the tufts of the more superficial glomeruli tended to be relatively more patent and less complexly convoluted than did those of juxta-medullary glomeruli. Topographical variations in the complexity of the capillary loop pattern shown by mature glomerular capillary tufts were present also, but were less pronounced, in the kidneys of neonatal and 8 to 11-day old rats.

Extra-glomerular connective tissues

The F-ARG, F-ARS and F-ARK conjugates stained the basement membranes of renal tubules and peritubular capillaries in kidneys from rats of all ages. Differences were noticed, however, between the pattern of staining produced by the F-ARG and F-ARK conjugates, on the one hand, and by the F-ARS conjugate on the other in renal tubular basement membranes in the kidneys of adult rats. In F-ARG and F-ARK stained sections renal tubular basement

membranes were usually seen as a single layer of brightly stained material. Occasionally, however, a small unstained spindle-shaped split was seen in the membrane, and it was possible to resolve the membrane on either side of the split into two components (Fig. 19). This splitting and doubling of tubular basement membranes was not seen in F-ARS stained sections of adult rat kidney.

Interstitial tissue was regularly seen as a relatively dense meshwork of fine branching, intertubular fibrils in F-ARS, F-ARK, and F-ARG stained sections of kidneys from 3.0 cm. embryos (C.T. 47). In larger rats the amount of interstitial tissue detected by these conjugates varied from kidney to kidney and also between sections cut at different depths from the one kidney. In some cases none was seen, in other instances intertubular spaces contained a loose meshwork of fine branching fibrils which appeared to fuse with the basement membranes of renal tubules, while in other instances solitary fibrils protruded from tubular basement membranes into the intertubular space (C.T. 46).

Arteries in F-ARG, F-ARS and F-ARK stained sections of kidneys from adult rats were characterized by a reticulate pattern of medial staining (Fig. 18). In younger rats arterial staining was distributed either as a reticulate network or as a

series of concentric laminae.

Embryonic Rat Skin

Dermo-epidermal Junction

The F-anti-adult connective tissue conjugates regularly produced specific staining of the dermo-epidermal boundary membrane in the skin of embryonic rats.

Dermis

The F-anti-adult conjugates did not regularly react with dermal connective tissues in the skin of embryonic rats.

In sections from four of the six 1.0 to 1.5 cm. rat embryos examined dermal staining produced by the F-anti-adult conjugates was limited to the blood vessels. In the remaining two embryos, embryos 2 and 6, Table 28, staining of blood vessels was partially obscured by a lace-work pattern of fluorescence. The pattern of staining produced by the F-anti-adult conjugates in the dermis of 1.0 to 1.5 cm. embryonic rats was taken to represent staining of the cytoplasm and cytoplasmic processes of dermal mesenchymal cells (Fig. 22 and C.T. 48).

Dermal staining in the 1.5 to 2.0 cm. group of embryos tended to have a fibrillar rather than a cellular distribution (C.T. 49). Dermal fibrillar staining was seen in five of the eight

1.5 to 2.0 cm. embryos examined in F-anti-adult rat connective tissue direct staining experiments - embryos 23, 26, 22, 27 and 28, Table 29.

In the 3.0 cm. group of embryos all three of the F-anti-adult conjugates produced staining of reticular fibres in the media of arteries, perimysial reticulum and perineural reticulum. The conjugates reacted also with the dermo-epidermal boundary membrane and with the walls and boundary membranes of sub-epidermal capillaries.

Fluorescein anti-embryonic connective tissue conjugates

Kidneys of adult, young, neonatal and embryonic rats

Mature renal glomeruli

The F-ARE conjugate produced specific staining in the capsular membranes of mature renal glomeruli in the kidneys of adult, young, neonatal and embryonic rats but did not react with capillary boundary membranes of mature renal glomeruli in the kidneys of adult, young or neonatal rats. Specific staining in the capillary tufts of mature glomeruli in these rats was confined to oval or polygonal and occasionally confluent bodies.

The filtration space of juxta-medullary glomeruli in the kidneys of 3.0 cm. embryonic rats contained brightly stained streaks, circles or loops embedded in a brightly stained matrix. It thus appeared possible that the F-ARE conjugate

was reacting with capillaries of mature glomeruli in the embryonic rat kidney, although it did not produce a capillary loop pattern of glomerular staining in the kidneys of adult rats.

Immature, Developing and Proemial Glomeruli

The F-ARE conjugate produced specific staining of the membrane outlining the developing glomerular capsule of immature glomeruli in the kidneys of young, neonatal and embryonic rats.

The F-ARE conjugate appeared to react also with capillaries and with intercapillary material of immature glomeruli. In these glomeruli the site of the developing capillary tuft showed brightly stained streaks, loops or circles embedded in a less brightly stained matrix which was itself fenestrated by unstained spaces - as if perforated by unstained capillaries (C.T. 56).

Extra-glomerular connective tissues

The F-ARE conjugate produced specific fluorescence in the kidneys from rats of all ages. The pattern of peritubular staining produced by the F-ARE conjugate in the kidneys of young, neonatal and embryonic rats did not differ from that produced by the F-anti-adult connective tissue conjugates. The two groups of conjugates, however, appeared to differ in their reactions with renal tubular basement membranes of the adult rat. While these structures were invariably complete in sections stained with the F-anti-adult connective tissue conjugates, peritubular staining in sections of kidney from adult rats stained with the F-ARE conjugate was often found to be incomplete or discontinuous (C.T.54).

The F-ARE conjugate reacted with renal interstitial tissue in the kidneys of adult, young and neonatal rats in the same way as the F-anti-adult connective tissue conjugates did and regularly produced staining of renal interstitial tissue in the kidneys of 3.0 cm. rat embryos.

Peritubular capillaries

Where renal interstitium was absent, or present as a loose meshwork of fine fibrils, as in the kidneys of adult rats, it was possible to detect specific fluorescence of peritubular capillaries

in F-ARE stained sections. Specific staining of peritubular capillaries was not readily detectable in the kidneys of embryonic rats in which the inter-tubular areas contained a relatively dense packing of interstitial fibrils.

The media of arteries.

Arteries which showed a reticulate pattern of medial staining in F-anti-adult direct staining experiments showed no medial staining in F-ARE direct staining experiments (Fig. 21).

Embryonic Rat Skin

Dermo-epidermal junction

The F-ARE conjugate produced specific fluorescence at the dermo-epidermal junction in sections from all three groups of embryos examined.

Dermis

The F-ARE conjugate reacted with the basement membranes of blood vessels and with dermal mesenchymal cells in all of the 1.0 to 1.5 cm. embryos. Thus dermal mesenchymal cells in four of these embryos (1, 3, 4 and 5, Table 28) were found to be reactive with the F-ARE conjugate but not with the F-anti-adult connective tissue conjugates, while embryos 2 and 6 possessed dermal mesenchymal cells reactive with the F-anti-adult conjugates and with the F-anti-embryonic conjugate.

Specific staining of dermal fibrils was seen after F-ARE direct staining experiments in sections from seven of the 1.5 to 2.0 cm. embryos. In this group of eight embryos, then, three (embryos 21, 24 and 25) possessed dermal fibrils reactive only with the F-ARE conjugate; one, embryo 26, fibrils reactive only with the F-anti-adult conjugates, while sections from embryos 23, 22, 27 and 28 showed dermal fibrillar staining after F-anti-adult and F-anti-embryo direct staining experiments.

The F-ARE conjugate did not react with dermal collagen in the skin of 2.5 to 3.0 cm. embryos. In F-ARE stained sections from this group of embryos specific fluorescence was limited to the dermo-epidermal boundary membrane, the walls and boundary membranes of sub-epidermal capillaries and perimysial and perineural reticulum.

TABLE 24

CROSS INHIBITION EXPERIMENTS
RENAL CONNECTIVE TISSUES IN THE RAT
MATURE, TRANSITIONAL AND DEVELOPING GLOMERULI

Histological Components	Rats	ARG + F-ARE	ARS + F-ARE	ARK + F-ARE	ARE + F-ARG	ARE + F-ARS	ARE + F-ARK
Capillary B.M.	Mat. Y & N 3.0 cm.	O O O	O O O	O O O	B b-B b-B	B W W	B b-B W
	Tr. Y & N 3.0 cm.	O O O	O O O	O O O	B b-B	B W	B b-B
	Dev. Y & N 3.0 cm.	O O O	O O O	O O O	b b	O O	b b
Capsular B.M.	Mat. Y & N 3.0 cm.	O O O	O O O	O O O	b-B b-B b-B	W O O	b-B b-B b-B
	Tr. Y & N 3.0 cm.	O O O	O O O	O O O	b-B b-B	W O	b-B b-B
	Dev. Y & N 3.0 cm.	O O O	O O O	O O O	b b	W O	b b
Intercapillary	Mat. Y & N 3.0 cm.	O O O	O O O	O O O	O O O	O O O	O O O
	Tr. Y & N 3.0 cm.	O O O	O O O	O O O	O O O	O O O	O O O
	Dev. Y & N 3.0 cm.	O O O	O O O	O O O	W W	O O	W W

A = Adult rats (2 months old).
Y = Young rats (8 - 17 days old).
N = Newborn rats (1 - 7 days old).
3.0 cm. = Embryonic rats.

B = Bright staining
W = Weak staining
O = No staining

TABLE 25
 CROSS INHIBITION EXPERIMENTS
 EXTRA-GLOMERULAR TISSUES

Kidneys of Adult (A), Young and Neo-Natal (Y) and
 3.0 cm. Embryonic (E) Rats

	B.M. Tubules	Interstitium Peri-Tubular Capillaries	Media of Arteries	
ARE	b	-	B	A
+	b	-	B	Y
F-ARG	b	-	B	E
ARE	b	-	B	A
+	b	-	B	Y
F-ARK	b	-	B	E
ARE	-	-	B	A
+	-	-	B	Y
F-ARS	-	-	B	E
ARG	-	-	-	A
+	-	-	-	Y
F-ARE	-	-	-	E

TABLE 26

CROSS ABSORPTION EXPERIMENTS

MATURE (M), TRANSITIONAL (T) AND DEVELOPING (D)

GLOMERULI

Adult (A), Young and Neo-natal (Y) and 3.0 cm.

Embryonic (E) Rats

	Capillary B.M.			Capsular B.M.			Inter Capillary			RATS
	M	T	D	M	T	D	M	T	D	
F-ARG	B	NS	NS	b	NS	NS	-	NS	NS	A
x	-	b	-	b	b	b	-	-	-	Y
E.c.t.	b	W	-	b	b	b	-	-	-	E
F-ARK	B	NS	NS	b	NS	NS	-	NS	NS	A
x	b	b	-	b	b	b	-	-	-	Y
E.c.t.	b	W	-	b	b	b	-	-	-	E
F-ARS	W	NS	NS	W	NS	NS	-	NS	NS	A
x	W	W	-	W	-	-	-	-	-	Y
E.c.t.	-	-	-	-	-	-	-	-	-	E
F-ARE	-	NS	NS	-	NS	NS	-	NS	NS	A
x	-	-	W	-	-	W	-	-	-	Y
A.c.t.	-	-	W	-	-	W	-	-	-	E

NS = not seen

TABLE 27
CROSS ABSORPTION EXPERIMENTS
RENAL CONNECTIVE TISSUES IN THE RAT
EXTRA-GLOMERULAR TISSUES

Conjugates	B.M. Tubules	Peritubular Capillaries	Interstitial Tissue	Media of Arteries	Rats
F-ARG x E.c.t.	B	O	O	B	Adult
F-ARS x E.c.t.	B	O	O	B	
F-ARK x E.c.t.	B	O	O	B	
F-ARE x A.c.t.	O	O	O	O	
F-ARG x E.c.t.	b	O	O	B	Young and Neonatal
F-ARS x E.c.t.	O	O	O	B	
F-ARK x E.c.t.	b	O	O	B	
F-ARE x A.c.t.	O	O	O	O	
F-ARG x E.c.t.	b-W	O	O	B	3.0 cm. Embryonic
F-ARS x E.c.t.	O	O	O	B	
F-ARK x E.c.t.	b-W	O	O	B	
F-ARE x A.c.t.	O	O	O	O	

CROSS INHIBITION EXPERIMENTS

Cross inhibition experiments tested the ability of fluorescein conjugates of the one specificity (F-anti-adult connective tissue conjugates for example) to react with connective tissues of adult, young, neonatal and embryonic rats in the presence of unlabelled immune globulins of the other specificity (e.g. unlabelled ARE globulins).

The experimental manipulations involved in cross inhibition experiments are described in Appendix 6 and summarized in Table 21, page

ARE + F-Anti-Adult Connective Tissue Experiments

In these experiments sections were pretreated with unlabelled globulin fractions of ARE antisera for a standard length of time and then reacted with a mixture (prepared in situ) containing equal volumes of the ARE globulin and one or other of the F-anti-adult connective tissue conjugates.

Adult rat kidney

The unlabelled globulin fractions of ARE antisera blocked all interaction between F-anti-adult conjugates and intertubular fibrils and peritubular capillaries in the kidneys of adult rats (Table 25).

These structures apart, it can be said that the reaction of the F-ARS conjugate with adult

renal connective tissues was, in general, more completely inhibited by ARE globulins than were the reactions of the F-ARG and F-ARK conjugates (Tables 24 and 25).

Unlabelled ARE globulins completely prevented the F-ARS conjugate from staining tubular boundary membranes, but did not prevent the F-ARG and F-ARK conjugates from reacting with these structures. However, the staining seen in tubular boundary membranes in sections submitted to ARE + F-ARG and ARE + F-ARK cross inhibition procedures was less bright and appeared to be finer than that seen in sections stained by the NRG + F-ARG and NRG + F-ARK direct staining procedures (Table 25).

Similarly, glomerular capillary tufts showed only faint staining in sections reacted with the F-ARS conjugate in the presence of unlabelled ARE globulins, but relatively bright staining in the ARE + F-ARG and ARE + F-ARK experiments (Table 24). This is not to say that the unlabelled ARE globulins brought about no modification of the staining normally produced by the F-ARG and F-ARK conjugates in the mature glomerular capillary tuft. In cross inhibition experiments staining in the glomerular capillary tuft appeared to be confined to the boundary membranes of glomerular capillaries,

whereas both glomerular capillaries and glomerular epithelial cells showed staining in direct staining experiments.

Although unlabelled ARE globulins exerted a greater inhibitory effect on the reaction of F-ARS conjugates than on the reactions of the F-ARG and F-ARK conjugates with renal glomerular capillaries, there was little difference between the intensity of staining produced by the three F-anti-adult conjugates in the media of arteries or glomerular capsular membranes in cross inhibition experiments. In all ARE + F-anti-adult experiments faint staining appeared in the glomerular capsular membranes and relatively bright staining in the media of arteries.

Kidneys of young, neonatal and embryonic rats

There was a difference between the inhibitory effect exerted by the unlabelled ARE globulin on the reaction between F-ARS conjugate on the one hand, and the F-ARG and F-ARK conjugates on the other, with certain connective tissues in the kidneys of young, neonatal and 3.0 cm. embryonic rats.

After the ARE + F-ARS cross inhibition experiments kidneys from young, neonatal and 3.0 cm. embryonic rats showed weak staining of the capillary and capsular membranes of mature and transitional glomeruli and of the boundary membranes of renal tubules and proemial glomeruli. Weak staining appeared also in the capsular membranes of developing

glomeruli (Table 24).

In ARE + F-ARG and ARE + F-ARK experiments glomerular capsular membranes in the kidneys of young, neonatal and 3.0 cm. embryonic rats showed bright rather than weak staining. Relatively bright staining appeared also in the basement membranes of glomerular capillaries of transitional glomeruli and in the formed elements, loops, circles and streaks, seen at the site of the developing capillary tuft in developing glomeruli (Table 24).

Structures remaining unstained in the present group of cross inhibition experiments included renal interstitial tissue and matrix material of developing glomerular tuft in developing glomeruli.

Embryonic Rat Skin

Unlabelled ARE globulins blocked all the staining normally produced by the F-ARS conjugate in the skin of 1.0 to 1.5 cm., 1.5 to 2.0 cm. and 2.5 to 3.0 cm. embryos. The unlabelled ARE globulins also blocked all interaction between the F-ARG and F-ARK conjugates and the skin of 1.0 to 1.5 cm. rat embryos.

In the case of the larger rat embryos, some tissues which had been found to be reactive in direct staining experiments with the F-anti-embryonic and F-anti-adult connective tissue

conjugates continued to react with the F-ARG and F-ARK conjugates in cross inhibition experiments (Table 28). Tissues showing this anomalous reaction were the dermo-epidermal boundary membranes of all 1.5 to 2.0 cm. and 2.5 to 3.0 cm. embryos and dermal fibrils in the 1.5 to 2.0 cm. embryo No. 23.(Table 28).

Anti-adult + F-ARE Experiments

Kidneys of adult, young, neonatal and embryonic rats

Unlabelled globulin fractions of ARG, ARK and ARS antisera brought about complete inhibition of the staining normally produced in the kidneys of adult, young, neonatal and embryonic rats.

Embryonic Rat Skin

It will be seen from Table 28 that in a number of instances where they might have been expected to block interaction between the F-ARE conjugate and embryonic dermal connective tissues, unlabelled anti-adult conjugates did not do so.

Tissues showing anomalous staining reactions in anti-adult + F-ARE cross inhibition experiments included the dermo-epidermal boundary membranes of 1.0 to 1.5 cm. rat embryos, dermal mesenchymal cells in the 1.0 to 1.5 cm. embryos 2 and 6, and dermal fibrils in embryos 22, 27 and 28.

TABLE 28
EMBRYONIC RAT SKIN
DIRECT STAINING, CROSS INHIBITION AND
CROSS ABSORPTION EXPERIMENTS

	1.0 to 1.5 cm.			1.5 to 2.0 cm.					2.5 to 3.0 cm.		
	D.E.J.	Dermis		D.E.J.	Dermis				D.E.J.	Dermis	
F-arG	+	+	-	+	+	+	+	-	+	-	D S
F-arK	+	+	-	+	+	+	+	-	+	-	
F-arS	+	+	-	+	+	+	+	-	+	-	
F-arE	+	+	+	+	-	+	+	+	+	-	
arE + F-arG	-	-	-	(+)	+	(+)	-	-	(+)	-	C
arE + F-arK	-	-	-	(+)	+	(+)	-	-	(+)	-	
arE + F-arS	-	-	-	-	-	-	-	-	-	-	
arG + F-arE	(+)	(+)	+	-	-	-	(+)	+	-	-	I
arK + F-arE	(+)	(+)	+	-	-	-	(+)	+	-	-	
arS + F-arE	(+)	(+)	+	-	-	-	(+)	+	-	-	
F-arG x E.c.t.	-	-	-	(+)	+	(+)	-	-	(+)	-	C A
F-arK x E.c.t.	-	-	-	(+)	+	(+)	-	-	(+)	-	
F-arS x E.c.t.	-	-	-	-	-	-	-	-	-	-	
F-arE x A.c.t.	(+)	(+)	+	-	-	-	(+)	+	-	-	
EMBRYO	A11	2 6	1,3, 4,5	A11	26	23	22 27 28	21 24 25	A11	A11	

(+) Presence and (-) Absence of specific staining.

D.E.J. = Dermo-epidermal boundary membranes.

arG = anti-adult rat renal glomerulus.

arK = anti-adult rat renal connective tissue.

arS = anti-adult rat splenic reticulum.

arE = anti-embryonic rat connective tissue.

F =

() symbols in parentheses draw attention to instances where predictions based upon the results of direct staining experiments proved false.

D.S. = Direct Staining.

C.I. = Cross Inhibition.

C.A. = Cross Absorption.

CROSS ABSORPTION EXPERIMENTS

The reagents used in cross absorption experiments were:

(1) Cross absorbed conjugates

a. F-ARG x embryo, F-ARK x embryo and
F-ARS x embryo

b. F-ARE x adult

(2) Non-specifically absorbed conjugates

F-anti-adult and F-anti-embryo conjugates
absorbed with human renal connective
tissue.

The staining produced in kidneys of adult, young, neonatal and embryonic rats and in the skin of embryonic rats by cross absorbed conjugates was compared with that produced by fractions of the F-anti-adult and F-anti-embryo conjugates which had been non-specifically absorbed.

The cross absorbed F-anti-adult connective tissue x embryo conjugates were prepared by repeatedly absorbing the F-anti-adult conjugates, previously freed of non-specifically reacting material and antibodies to rat serum and erythrocytes, with small volumes of a crude preparation of connective tissues obtained from larval-like and incompletely developed embryos. The process of cross absorption was followed immunohistologically and carried up to but not beyond the point

where the cross absorbed F-anti-adult conjugates no longer produced staining in dermal connective tissues in sections of skin from incompletely developed embryos with which they had been shown to react before cross absorption.

The cross absorbed F-ARE x adult conjugate was similarly prepared by repeatedly absorbing a purified portion of the F-ARE conjugate with the adult rat renal connective tissue antigen until it was found to have lost its reactivity for connective tissues in the kidneys of adult rats.

F-anti-adult x Embryo Experiments

Adult rat kidney

The staining produced in sections of adult rat kidney by the cross absorbed F-ARG x embryo and F-ARK x embryo conjugates differed from that produced by the non-specifically absorbed F-ARG x human and F-ARK x human conjugates in the following respects.

Where the non-specifically absorbed conjugates produced staining of renal interstitial tissue and peritubular capillaries the cross absorbed conjugate did not (Table 27).

Staining produced in glomerular capsular membranes by the cross absorbed conjugates was less bright than that produced in these membranes

by the non-specifically absorbed conjugates. Similarly, staining seen in the position of tubular boundary membranes in sections exposed to the cross absorbed conjugates was less bright and possibly finer than that seen in sections exposed to the non-specifically absorbed conjugate (Table 26).

Glomerular capillary loops presented a crisper pattern of staining in sections reacted with the cross absorbed F-ARG x embryo and F-ARK x embryo conjugates than they did in sections reacted with the non-specifically absorbed F-ARG x human and F-ARK by human conjugates. This difference between the pattern of staining seen in glomerular capillary tufts in sections stained by the cross absorbed conjugates, on the one hand, and by the non-specifically absorbed conjugates on the other, was attributed to a difference between the reactions of the two groups of conjugates with intercapillary tissue. In cross absorption experiments glomerular staining appeared to be confined to the basement membranes of capillaries, whereas in sections stained by the non-specifically absorbed conjugates glomeruli showed staining of capillary basement membranes and of material lying either between glomerular capillaries or at the outer surface of their basement membranes. Hill and Cruickshank (1953) described a similar pattern of staining in renal glomeruli stained by fluorescein

anti-rat glomerulus and anti-rat kidney conjugates. They attributed the extra-capillary fluorescence to the staining of capillary epithelium.

The F-ARS x embryo conjugate was found to have lost almost all of its reactivity for adult rat renal connective tissue. This conjugate produced only faint fluorescence in glomerular capillary tufts and none in interstitial tissue, peritubular capillaries, the media of arteries or the boundary membranes of tubules in sections from the kidneys of adult rats.

Kidneys of young, neonatal and embryonic rats

F-ARS x Embryo Experiments

The F-ARS x embryo conjugate produced no staining in sections of kidneys of embryonic or neonatal rats and only faint fluorescence of capillaries in mature glomeruli in the kidneys of young rats.

F-ARG x Embryo and F-ARK x Embryo Experiments

Immature renal glomeruli

The majority of transitional glomeruli seen in the kidneys of young, neonatal and embryonic rats showed a tuft of weakly stained capillaries, (Fig. 24), but an occasional transitional glomerulus showed relatively bright fluorescence of its capillary loops.

The F-ARG x embryo and the F-ARK x embryo

conjugates produced specific staining of the membrane outlining the developing glomerular capsule in developing glomeruli, but specific staining in the position of the developing capillary tuft in these glomeruli was either absent or distributed solely as streaks.

Mature renal glomeruli

The staining produced by the F-ARG x embryo and the F-ARK x embryo conjugates in mature renal glomeruli did not, in general, differ from that produced by the non-specifically absorbed conjugates, but in all F-ARG x embryo and F-ARK x embryo stained sections of young, neonatal and 3.0 cm. embryonic rat kidneys an occasional glomerular capillary tuft showed an abnormal pattern of staining. In some instances capillaries forming a glomerular tuft appeared to be incompletely or patchily stained; in other instances the position of the capillary tuft was outlined by a single plicated membrane and occupied by specifically stained material distributed in the form of streaks. Sometimes a combination of these appearances was seen; in these instances a tuft of incompletely or patchily stained capillaries appeared to be enveloped by a folded membrane (C.T. 60).

Extra-glomerular tissues

The F-ARG x embryo and the F-ARK x embryo

conjugates produced no staining of renal interstitial tissue or of peri-tubular capillaries in the kidneys of young, neonatal or embryonic rats. The conjugates did produce staining of tubular boundary membranes and of glomerular capsular membranes in these kidneys but this staining was less bright than that seen in sections stained with the non-specifically absorbed F-ARG x human and F-ARK x human conjugates.

Embryonic Rat Skin

The cross absorbed F-ARS x embryo conjugate was found to have lost all its reactivity for connective tissues in the skin of 1.0 to 1.5 cm., 1.5 to 2.0 cm. and 2.5 to 3.0 cm. rat embryos.

Although the cross absorbed F-ARG x embryo and F-ARK x embryo conjugates produced no staining in the boundary membranes or dermal mesenchymal cells of 1.0 to 1.5 cm. rat embryos, these conjugates were found to have lost only some of their reactivities for connective tissues in the skin of the larger 1.5 to 2.0 cm. and 2.5 to 3.0 cm. rat embryos.

The cross absorbed F-ARG x embryo and F-ARK x embryo conjugates produced specific fluorescence at the dermo-epidermal junction in 1.5 to 2.0 cm. and in 2.5 to 3.0 cm. embryos. They stained also dermal fibrils in two of the 1.5 to 2.0 cm. group of embryos (embryos 23 and 26, Table 28).

F-ARE x Adult Experiments

Kidneys of adult, young, neonatal and embryonic rats

The F-ARE x embryo conjugate which had been exhaustively absorbed with the adult rat renal connective tissue antigen as regards its ability to produce detectable staining in the kidneys of adult rats, produced no staining in the kidneys of young, neonatal or 3.0 cm. embryonic rats.

Embryonic Rat Skin

The cross absorbed F-ARE x adult conjugate produced no staining in sections of skin from 2.5 to 3.0 cm. embryonic rats and was found to have lost also its ability to stain dermo-epidermal boundary membranes in 1.5 to 2.0 cm. embryos.

Although it had lost its ability to react with the 1.5 to 2.0 cm. rat embryonic dermo-epidermal boundary membrane, the cross absorbed F-ARE x embryo conjugate produced staining of dermal fibrils in sections from six of the eight 1.5 to 2.0 cm. embryos. The conjugate reacted also with dermo-epidermal boundary membranes and dermal mesenchymal cells in all of the 1.0 to 1.5cm. embryos examined.

COMMENTS

STUDIES OF CONNECTIVE TISSUE IN THE ADULT
AND EMBRYONIC RAT

Adult Rat Kidney.

The experimental observations summarised in Table 29 indicates that renal connective tissues of the adult rat can be sub-divided into two antigenically dissimilar groups:

Group 1.

Tissues reactive with antibodies detectable only in anti-adult antisera.

Group 2.

Tissues reactive with antibodies detectable in anti-adult and in anti-embryo antisera.

Group 1 tissues, typified by the basement membranes of renal glomerular capillaries, were stained by F-anti-adult conjugates in direct staining, cross inhibition and cross absorption experiments, but were not stained by F-anti-embryo conjugates.

Group 2 tissues, for example renal interstitium, were stained by F-anti-adult and by F-anti-embryo conjugates in direct staining experiments, but by neither of these two groups of conjugates in cross inhibition or cross absorption experiments.

Renal Tubular Basement Membranes.

Renal tubular basement membranes in the kid-

TABLE 29

ADULT RAT KIDNEY

DIRECT STAINING, CROSS INHIBITION AND
CROSS ABSORPTION EXPERIMENTS

	B.M. Glomerular Capillaries GROUP 1	B.M. Tubules GROUP 2	Interstitialium GROUP 3
NRG + F-ARG	+	+	+
NRG + F-ARE	-	+	+
ARE* + F-ARG	+	+	-
ARG + F-ARE	-	-	-
F-ARG* x E.c.t.	+	+	-
F-ARE x E.c.t.	-	-	-

*In general, staining seen after cross inhibition and cross absorption experiments was less bright than that seen after direct staining experiments.

neys of adult rats, although they reacted as group 2 tissues in F-ARG and F-ARE direct staining experiments, behaved as group 1 tissues in the corresponding cross inhibition experiments. Thus, the staining reactions shown by rat renal tubular basement membranes resembled those shown by tubular basement membranes in sections of human kidney.

In studies of human connective tissue it was found that while both F-anti-human glomerulus and F-anti-human synovium conjugates stained tubular basement membranes, only the unlabelled anti-glomerulus reagent brought about complete inhibition of staining in cross inhibition experiments. Unlabelled anti-synovium globulins did not prevent F-anti-glomerulus conjugates from producing peritubular staining in anti-synovium ---> F-anti-glomerulus cross inhibition experiments.

The possibility that two antigenically dissimilar tissues go to form rat renal tubular basement membranes is raised not only by the antigenic properties of these membranes but also by their morphological appearances in F-ARG direct staining experiments. Both group 1 and 2 antigens were detectable in rat renal tubular basement membranes. In F-ARG direct staining experiments a split was occasionally seen in tubular basement membranes and it was sometimes possible to resolve the membrane

on either side of the split into two.

Group 1 Antigens in Embryos - Conflicting Evidence.

The failure to detect staining of renal glomerular capillary basement membranes in F-ARE direct staining experiments suggests that group 1 antigens are not present in embryonic connective tissue. On the other hand, the anti-group 1 reactivity of F-ARS conjugates, and, to a lesser extent, that of F-ARG conjugates was reduced by the cross absorption of the conjugates with embryonic connective tissue (Table 25).

However, the finding that F-ARE conjugates produced no detectable staining of adult group 1 tissues does not necessarily establish that the embryonic connective tissue immunising antigen lacked group 1 antigens. It remains possible that these antigens were present in the tissue in concentrations too low to induce a vigorous immune response, or that the presence of embryonic antigens abrogated the immune response to group 1 antigens.

In cross absorption experiments F-anti-adult conjugates which had been absorbed with embryonic connective tissues to the point where they no longer produced fluorescence in the skin of 0.7 cm. embryonic rats continued to react with adult group 1, but not group 2, tissues. This finding does not necessarily rule out the possibility that

embryonic connective tissues contain group 1 antigens; it may merely indicate that these antigens are present in higher concentration, i.e. more closely grouped together in adult group 1 tissues than on antigenically related tissues in the skin of rat embryos.

This is to suggest that the development of detectable staining in immunohistological staining experiments depends not only upon the concentration of antibodies in labelled antisera but also upon the concentration of the relevant antigenic determinants in tissues. Some support for this view is provided by the gain in sensitivity - assessed as tenfold by Coons (1956) and as four to twelvefold by Pressman, Yagi and Hiramoto (1958) - achieved by the use of the multiple layer or sandwich method in immunohistological studies. The method is essentially a manoeuvre designed to increase the local concentration of antigen in tissues. It was first introduced by Weller and Coons (1954) who treated suspensions of varicella or herpes zoster viruses with specific human antiserum and then traced bound human globulin with fluorescein labelled anti-human globulin. The use of human antiserum as the middle layer of the sandwich increases the chances of fluorescein molecules aggregating in sufficiently close juxtaposition to give rise to detectable fluorescence.

There is no need to postulate quantitative differences between the antigenic constitution of adult renal glomerular capillary basement membranes and adult splenic reticulum to account for the greater degree of anti-group 1 reactivity shown by F-ARG and F-ARE conjugates vis-a-vis F-ARS conjugates after cross absorption. Rabbits furnishing ARS antisera were less vigorously immunised than were those furnishing ARG and ARK antisera. Thus, conjugates likely to contain concentrations of antibody the more nearly approaching the lower limiting concentration required for the detection of group 1 antigens were those from which anti-group 1 reactivity was the more noticeably removed by absorption with embryonic connective tissue.

Kidneys of Young, Neo-natal and Embryonic Rats.

Interest in immunohistological studies of renal connective tissues of young, neo-natal and embryonic rats focussed upon the staining reactions shown by glomerular capillaries. These reactions are summarised in Table 30.

The capillaries of developing glomeruli showed no staining when reacted with F-ARG conjugates in cross absorption experiments, whereas those of mature glomeruli did. It has been argued that the appearance of staining in immunohistological staining experiments depends not only upon the concentration of antibody in antisera but also

upon the concentration of antigen in tissues. On the basis of this argument the presence of staining in mature glomerular capillaries and its absence from developing glomerular capillaries after F-ARG cross absorption experiments may indicate that the group 1 antigenic determinant is present in mature glomerular capillaries in concentration higher than in developing glomerular capillaries, or in the skin of 0.7 cm. rat embryos - used as an indicator in the determination of the end point of the process of cross absorption.

Alternatively the fact that cross absorbed F-ARG conjugates stained the capillaries of mature but not those of developing glomeruli provides a basis for speculation concerning the possibility that differences exist between the extent to which individual members of the group 1 class of antigenic determinants find representation in capillaries of mature glomeruli on the one hand and of developing glomeruli on the other.

This speculation embodies the following proposition:

- (a) The anti-group 1 reactivity of ARG antisera embraces a number of antigenic determinants, related to each other either configurationally, by virtue of their structure, or topologically, in the sense that they represent structural or functional units of one histological component

TABLE 30

RENAL GLOMERULAR CAPILLARIES

YOUNG, NEO-NATAL AND EMBRYONIC RATS

DIRECT STAINING, CROSS INHIBITION AND CROSS
ABSORPTION EXPERIMENTS

RATS	GLOMERULI					
	Mature		Transitional		Developing	
	Yo. NN.	Em.	Yo. NN.	Em.	Yo. NN.	Em.
NRG + F-v-Ad	+	+	+	+	+	+
NRG + F-v-Em	-	+	+	+	+	+
v-Em + F-v-Ad	+	+	+	+	+	+
v-Ad + F-v-Em	-	-	-	-	-	-
F-v-Ad x E.c.t.	+	+	+	+	-	-
F-v-Em x A.c.t.	-	-	-	+	+	+

Yo., NN. Young and Neo-natal rats.

Em. 3.0 cm. Embryonic rats.

+ Incomplete inhibition or absorption.

- Complete inhibition or absorption.

of connective tissues - basement membranes.

- (b) Qualitative as well as quantitative differences exist between adult and embryonic connective tissues as regards their content of group 1 antigens, and therefore
- (c) between anti-adult and anti-embryo antisera in their content of anti-group 1 antibodies.

The proposition that a gradual diversification as well as accumulation of group 1 antigens accompanies increasing embryonic age has certain attractions over the alternative proposition limiting changes in group 1 antigenicity accompanying increasing embryonic age solely to the accumulation of a group 1 antigen. The former proposition, since it carries the implication that the more mature glomerular capillaries contain antigenic determinants not present in the less mature capillaries and not represented by antibodies in anti-embryo antisera, provides a better account than does the latter for the following observations; in ARE + F-ARG cross inhibition experiments the F-ARG conjugate produced brighter staining in the capillaries of transitional glomeruli than in those of developing glomeruli: cross absorbed F-ARG conjugates produced staining of varying intensity in the capillaries of transitional glomeruli but of consistent intensity in the capillaries of mature glomeruli.

Immunohistological studies of renal connective tissues in the rat were undertaken (a) to explore the possibility that embryonic and adult connective tissue may each manifest antigenic properties not shown by the other, (b) to determine whether or not the histological differentiation of renal glomerular capillaries is accompanied by changes in their antigenic make up, (c) to determine whether any relationship exists between the morphological appearance of glomeruli and the antigenic properties of their capillaries. The studies have provided some evidence that differences may exist between adult and embryonic connective tissues as regards their content of the so-called group 1 antigens, and have provided basis for speculation concerning the possibility that a process of accumulation and diversification of group 1 antigens takes place in glomerular capillaries during development. It has, however, not been established that group 1 antigens are absent from embryonic connective tissues (the staining produced by F-ARG conjugates in cross inhibition and cross absorption experiments was in general less bright than that produced in direct staining experiments), nor have any antigens peculiar to embryonic connective tissue been detected in the kidneys of 3.0 cm. rat embryos. The staining seen in immature glomeruli after F-ARE direct staining experiments is to be

attributed to the fixation of anti-group 2 antibodies, since immature glomeruli showed no staining in F-ARE cross absorption experiments.

Further, and possibly more persuasive, evidence that the complex changes - including the obliteration of embryonic antigens - accompany the development of connective tissues is provided by studies of the skin in embryonic rats.

Embryonic Rat Skin.

Epidermal boundary membranes in the skin of embryonic rats exhibited two, and dermal connective tissues four, patterns of reactivity in immunohistological staining experiments (Tables 31 and 32). These patterns can be interpreted to mean that the following groups of antigens occurred in varying concentration in the skin of rat embryos (Table 33).

(a) Adult antigens, reactive with antibodies detectable only in anti-adult conjugates and antisera. Tissues carrying adult antigens showed staining in F-anti adult direct staining, cross absorption and cross inhibition experiments, but no staining in any F-anti embryo experiment.

(b) Common antigens, reactive with antibodies detectable in anti-adult and in anti-embryo antisera and conjugates. Tissues containing common antigens reacted with F-anti adult and F-anti embryo conjugates in direct stain-

ing experiments, but not in cross absorption or cross inhibition experiments.

(c) Embryonic antigens, reactive with antibodies detectable only in anti-embryo reagents. Tissues bearing these antigens were stained in all experiments involving the use of F-anti embryo conjugates, but showed no staining on exposure to F-anti adult conjugates.

Dermo-Epidermal Boundary membranes.

Common antigens were found in association with embryonic antigens in the dermo-epidermal boundary membranes of embryos measuring less than 1.5 cm. in crown to rump length. Evidence for the presence of common antigens is provided by the finding that F-anti adult conjugates produced staining at the dermo-epidermal junction in sections of skin from these embryos only in direct staining experiments. The finding that staining appeared at the dermo-epidermal junction in all experiments involving the use of F-anti embryo conjugates provides evidence for the presence of embryonic antigens in the epidermal boundary membrane of embryos measuring less than 1.5 cm. in crown to rump length.

The possibility that the so-called embryonic antigens are, in fact, tissue specific antigens is excluded by the fact that dermo-epidermal boundary membranes in embryos measuring more than 1.5 cm. in crown to rump length did not show staining in

TABLE 31

EMBRYONIC RAT SKIN

DERMAL-EPIDERMAL BOUNDARY MEMBRANES

DIRECT STAINING, CROSS INHIBITION AND CROSS

ABSORPTION EXPERIMENTS

	1.0 - 1.5cm	1.5 - 2.0cm.	2.5 - 3.0cm.
	EMBRYOS	EMBRYOS	EMBRYOS
	ALL	ALL	ALL
NRG + F-ARK	+	+	+
NRG + F-ARE	+	+	+
ARE* + F-ARK	-	+	+
ARK* + F-ARE	+	-	-
F-ARK x E.c.t.	-	+	+
F-ARE x A.c.t.	+	-	-

*In general staining seen after cross absorption and cross inhibition experiments was less bright than that seen after direct staining experiments

TABLE 32

EMBRYONIC RAT SKIN

DERMAL CONNECTIVE TISSUES

DIRECT STAINING, CROSS INHIBITION AND CROSS
ABSORPTION EXPERIMENTS

	1.0-1.5cm EMBRYOS			1.5-2.0cm EMBRYOS				2.5-3.0cm EMBRYOS
	1 3 4 5	2 6	23	26	22 27 28	21 24 25	ALL	
NRG +	-	+	+	+	+	-	-	
F-ARK								
NRG +	+	+	+	-	+	+	-	
F-ARE								
ARE *								
+	-	-	+	+	-	-	-	
F-ARK								
ARK +	+	+	-	-	+	+	-	
F-ARE								
F-ARK *								
x	-	-	+	+	-	-	-	
E.c.t.								
F-ARE x	+	+	-	-	+	-	-	
A.c.t.								

*In general staining seen after cross absorption and cross inhibition experiments was less bright than that seen after direct staining experiments

TABLE 33

PATTERNS OF REACTIVITY

Characterising

EMBRYONIC, COMMON AND ADULT ANTIGENS

In

DIRECT STAINING, CROSS INHIBITION AND CROSS
ABSORPTION EXPERIMENTS

	ANTIGENS		
	Embryonic	Common	Adult
NRG + F-ARK	-	+	+
NRG + F-ARE	+	+	-
ARE + F-ARK	-	-	+
ARK + F-ARE	+	-	-
F-ARK x E.c.t.	-	-	+
F-ARE x A.c.t.	+	-	-

F-ARE cross absorption or cross inhibition experiments.

The epidermal boundary membranes of the larger embryos, those measuring more than 1.5 cm. in crown to rump length, exhibited the staining reactions of a structure antigenically related to group 1 and group 2 components of adult renal connective tissue, that is containing adult and common antigens. They showed staining in F-anti adult and in F-anti embryo direct staining experiments but reacted only with F-ARG and F-ARK conjugates in cross inhibition and cross absorption experiments. This is to say, that of the three F-anti adult conjugates used, only two, the F-ARG and F-ARK conjugates, produced staining at the dermo-epidermal junction in the skin of embryos measuring more than 1.5 cm. in crown to rump length in cross inhibition and cross absorption experiments.

It is relevant to recall here that the reaction of F-ARS conjugates with the basement membranes of glomerular capillaries in the kidneys of adult rats was modified to a greater extent by the processes of cross inhibition and cross absorption than was that of F-ARG, or of F-ARK, conjugates. This finding was attributed to the relatively gentle course of immunisation to which rabbits furnishing ARS antisera had been exposed. Differences between the strength of ARS antisera,

on the one hand, and ARG and ARK antisera on the other, may also account for the finding that F-ARS conjugates produced no staining of epidermal boundary membranes in the present series of cross absorption and cross inhibition experiments, whereas F-ARG and F-ARK conjugates did. (Table 28).

Dermal Connective Tissues.

In so far as embryonic antigens were not detected in the epidermal boundary membranes of embryos measuring more than 1.5 cm. in crown to rump length and adult antigens were not detected in the epidermal boundary membranes of embryos measuring less than 1.5 cm., some relationship may be said to have been demonstrable between embryonic age and the antigenic make up of epidermal boundary membranes. No such relationship was demonstrable in the case of embryonic dermal connective tissues, except possibly in the special instance of embryos measuring more than 2.5 cm. in crown to rump length, dermal collagen in these embryos reacted neither with anti-adult nor with anti-embryo antisera. (Table 28).

Although embryonic antigens were detected in epidermal boundary membranes only in embryos measuring less than 1.5 cm. in crown to rump length, they were found in the dermis of embryos measuring up to 2.0 cm. They were, in fact, the only group of antigens present in the dermis of three of the

1.5 to 2.0 cm. group of embryos, and were found also on their own in the dermis of four of the smaller embryos. (Table 34).

Adult antigens, which were present in epidermal boundary membranes of all 1.5 to 2.0 cm. embryos, were detected in the dermis only in two of this group of embryos.

Common antigens, regularly found in embryonic epidermal boundary membranes, were not regularly found in embryonic dermal connective tissues. They were found in association with adult antigens in the dermis of one of the 1.5 to 2.0 cm. group of embryos, and in association with embryonic antigens in two of these larger and three of the smaller embryos.

1.5 - 2.0					
2.5 - 3.0					
3.5 - 4.5					
5.5 - 7.0					
8.5 - 11.0					

TABLE 34

EMBRYONIC RAT SKIN

ANTIGENIC CONSTITUTION

Number of instances in which

Embryonic (E), Common (C) and Adult (A) antigens were found in the dermo-epidermal boundary membranes and the dermal connective tissues of rat embryos measuring from 1.0 cm. to 3.0 cm. in crown to rump length

Embryos cms.	ANTIGENS					Site
	E	E + C	C	C + A	A	
1.0 - 1.5	4	2	-	-	-	Derma
1.5 - 2.0	3	3	-	1	1	
2.5 - 3.0	-	-	-	-	-	
1.0 - 1.5	-	6	-	-	-	Boundary Membranes
1.5 - 2.0	-	-	-	8	-	
2.5 - 3.0	-	-	-	6	-	

DISCUSSION

STUDIES OF HUMAN CONNECTIVE TISSUE

Antigenic Inter-relationships Between Connective Tissues and Erythrocytes and Serum Proteins

It was found, early in the studies described here, that rabbit anti-human tissue antisera contained antibodies reactive with human erythrocytes and with human serum proteins. It was assumed that these antibodies owed their origin to the presence of serum and red cells, or red cell stromata, in the tissues used as antigens for the preparation of anti-glomerulus and anti-synovium antisera.

There were some grounds for this assumption. Fluorescein labelled anti-glomerulus and anti-synovium conjugates produced specific staining of vascular intima, but only before they had been freed of anti-erythrocyte and anti-serum protein antibodies; similarly anti-synovium conjugates before, but not after, their absorption with human serum and red cells produced specific staining in the basement membrane of renal glomerular capillaries.

The assumption that the anti-red cell and anti-serum protein reactivity manifested by anti-tissue antisera represented contaminating antibodies, finds some support in the literature. Gitlin et al.

(1953), in their immunohistological studies of the distribution of plasma proteins in the tissues of young human beings, found albumin, gamma globulin and β -metal containing globulin to have a widespread distribution in the blood vessels, lymphatics, connective tissue and interstitial spaces. Fricke and Hadding (1962) found that extracts of human cartilage, intervertebral disc and tendon contained serum α , β and γ globulins, in addition to tissue-specific proteins. Lohman (1963) reported that arterial intima normally contains some plasma proteins (albumin and α globulin) and that the concentration of these proteins in arterial intima increases after death.

Immunohistological studies of blood group antigens have shown that these also are to be found in connective tissue stroma, vascular endothelium and the walls of capillaries (Glynn, Holborow and Johnson, 1957; Kaplan, 1957; Szulman, 1959). It was shown that the A group antigen occurs in connective tissue and capillary endothelium in a water insoluble form (Glynn et al. 1957; Brown and Holborow, 1962). Markowitz reported that the immunization of sheep with canine or rabbit glomerular basement membranes elicited haemagglutinins for the respective species, and that haemagglutinins, apparently independent of the A, B, O system, were to be found in antisera,

raised against a soluble fraction of human renal glomeruli (Markowitz 1960a, b).

The literature, however, makes it clear also that some of the anti-erythrocyte and anti-serum protein reactivity demonstrable in anti-tissue antisera must be ascribed, not to contaminating, but to cross reacting antibodies. There is abundant evidence that a close antigenic inter-relationship exists between certain constituents of plasma and connective tissue. Albumins having electrophoretic and serological properties similar to, but not identical with those of serum albumins, have been isolated from human skin and tendon by Neuberger (1957), and from rabbit tendon by Coleman, Gendrich and Jeffay (1963), and a serum β -globulin-like protein has been found in extracts of collagen (Novak, 1960).

Antisera against rabbit, human and mouse serum proteins has been found to be reactive in immunological tests with homogenates or extracts of connective tissue of the corresponding species. Milazzo (1957) showed that anti-human globulin antisera reacted in capillary flocculation tests with finely ground and thoroughly washed homogenates of human renal glomeruli, and Heller, Yakulis and Zimmerman (1959) detected precipitins for rabbit serum proteins in antisera against saline extracts

of rabbit tendon. Coleman, Gendrich and Jeffay (1963) succeeded in isolating an albumin-like protein from the extracts used by Heller and Yakulis. Tan and Kaplan (1963) raised antisera in rabbits against a chromatographically separated component of mouse serum β globulins. These antisera were found to react in immunohistological tests with a wide variety of mouse connective tissues; renal glomerular and tubular basement membranes; splenic and hepatic reticulum; material applied to collagen fibres; endomysial and perimysial connective tissue of heart and skeletal muscle and connective tissue and basement membrane in the testis.

The observations arising out of studies of human connective tissues described in the present report were interpreted to mean that three antigenically dissimilar groups of human connective tissue can be distinguished by immunohistological means. This interpretation carries no implications as regards the representation of serum protein-like or erythrocyte-like determinants in the different constituents of connective tissue, because it arose out of the use of reagents from which antibodies reactive, or cross reactive, with serum proteins and red blood cells, had been largely, if not completely, removed by specific absorption.

Antigenic Complexity of the Renal Glomerulus

The immunohistological studies of human connective tissue described here were originally undertaken to determine whether the antigenic overlap found by Hill and Cruickshank (1953) to exist between renal glomeruli and other connective tissues is complete or partial.

Evidence has been presented that connective tissues can be divided up into three immunohistologically reactive groups and one immunohistologically unreactive group. The unreactive group comprises the matrix of cartilage, elastic tissue and mature collagen, that is bundles of collagen in the dermis, the capsules of joints and tendons.

The first of the three antigenically active groups of connective tissue included; the inner or basement membrane component of epithelial boundary membranes; the basement membranes of renal glomerular capillaries; reticulum outlining plain muscle in the media of muscular arteries. The second antigenically active group of connective tissues comprised; splenic and hepatic reticulum; sarcolemma and neurilemma; the basement membranes of extra-glomerular capillaries; the outer or reticular component of epithelial boundary membranes. The third group was made up of; interstitial reticulum - fine branching fibrils in the interstitium of organs; collagenous tissue - non-branching

fibrils found in the adventitia of arteries and lying closely apposed to bundles of collagen in tendons and the capsules of joints; fibrocytes. Anti-glomerulus antisera reacted with all members of each of the three immunohistologically reactive groups of connective tissue, while anti-synovium antisera reacted only with the reticulum and collagenous groups. These observations imply that the reactivity of anti-glomerulus antisera with Group 1 components of connective tissue on the one hand, and with Group 2 and Group 3 components on the other derives from at least two groups of antigenic determinants in the human renal glomerulus.

There seems little doubt from the work of Goodman, Greenspon and Krakower (1955); Markowitz (1960b) and Pierce, Beals, Sri Ram and Midgley (1964) that the renal glomerulus incorporates a multiplicity of antigens. The work of Tan and Kaplan (1963) is pertinent in this context. Tan and Kaplan, it will be recalled, demonstrated that antisera to a chromatographically separated fraction of mouse serum β -globulin reacted in immunohistological experiments with a wide variety of connective tissues in the mouse, including the basement membranes of renal glomeruli. These workers point out that this finding does not establish the antigenic homogeneity of connective tissues and they

consider the possibility that individual antigens in the isolated β -globulin fraction are individually related to one or more of the components of connective tissue. In this connection they report that an antiserum of the β -globulin fraction formed three precipitin lines on diffusion in agar against the homologous antigen.

Evidence for the antigenic heterogeneity of renal glomeruli and their basement membranes comes from the work of Goodman, Greenspon and Krakower (1955). These workers carried out a series of serological flocculation and absorption tests with acellular derivatives of canine renal connective tissues, cell free collagenous fibrils obtained from cornea and achilles tendon, epithelial cells from tubules of the renal cortex and cells separated from isolated renal glomeruli. On relating the antigenic specificities of the various tissue fractions and the nephrotoxicity of antisera raised against them, Goodman, Greenspon and Krakower were lead to conclude that renal glomerular capillary basement membranes possess at least three antigens. One of these is responsible for the production of nephrotoxic antisera, while the other two are not nephrotoxinogenic. Of the non-nephrotoxinogenic antigens present in the basement membranes of renal glomerular capillaries, one was

found to be related to basement membranes of parietal capsules and renal tubules, while the other was related to collagenous fibrils of tendon and cornea. The presence of a non-nephrotoxic collagenous antigen in renal glomeruli was confirmed by Markowitz (1960b) who used in vitro assay and bio-assay procedures for the evaluation of the antibody response to glomerular basement membranes, pulmonary basement membranes and tendon fibrils. He found that although dog tendon fibrils were capable of absorbing at least 70% of the anti-glomerulus activity from anti-canine-glomerulus antisera, eluates from these tendon fibrils were not nephrotoxic, whereas eluates from glomerular basement membranes previously exposed to anti-glomerulus antisera were nephrotoxic.

Yagi and Pressman have interpreted their 1957 studies to provide further evidence for the antigenic heterogeneity of the renal glomerular capillary. These authors (Yagi and Pressman, 1957) studied the effects of heat (100°C. x 6 min. at pH 6.0) and trichloroacetic acid (60% x 6 hr. at 25°C. and 16 hr. at 3°C.), on the ability of; (i) a saline insoluble sediment of rat kidney and (ii) a saline soluble tryptic digest of the kidney sediment, to absorb kidney localizing antibodies from 131 I-labelled anti-rat kidney antisera. Before treatment

both the sediment and the digest contained antigens capable of binding kidney localising antibodies on exposure to 1^{131} anti-kidney antisera. In each case the inactivation brought about by heat and that brought about by trichloroacetic acid was only partial. Yagi and Pressman concluded that there are at least two antigens responsible for the localization of anti-kidney antibodies in vivo, and drew the inference that since these antibodies localise in vivo in the renal glomeruli, the glomerulus contains a multiplicity of antigenic components.

The antigenic heterogeneity of renal glomerular capillaries and other components of connective tissue is well illustrated by the work of Pierce and his colleagues. Pierce, Beal, Sri Ram and Midgley (1964), in the course of studies on epithelial basement membranes, examined immunohistologically, the anti-tissue specificities of an antiserum raised against mouse serum β -globulin and one raised against basement material obtained from a murine yolk sac carcinoma. Each of these antisera was found to be reactive with glomerular basement membranes, vascular basement membranes, splenic reticulum and the neoplastic basement membrane material. It was demonstrated, however, that two different groups of antigens were involved in the

interactions between the antisera and tissues. On absorption with β -globulin the anti- β -globulin antiserum lost all its reactivity, but the antiserum to neoplastic basement membrane, similarly absorbed, continued to react with neoplastic, glomerular and vascular basement membranes and with splenic reticulum.

Antigenic Distinction between Basement Membrane and Reticulum

It has been suggested in the present thesis and by Scott (1959; 1960) that basement membranes differ antigenically from reticulum, on the one hand, and collagenous tissues on the other. The studies of Pierce and his colleagues have shown that crude antisera to neoplastic basement membrane contain two families of anti-connective tissue antibodies. One of these has affinity for determinants present in glomerular capillary and epithelial basement membranes, but not present in splenic reticulum. Anti-neoplastic basement membrane antisera, when absorbed with splenic reticulum, lost their reactivity for splenic reticulum and vascular basement membranes, but continued to react with the homologous antigen, neoplastic basement membrane, and with epithelial and glomerular basement membranes (Pierce, Midgley and Sri Ram, 1963).

Studies by Muckerjee, Sri Ram and Pierce (1965) of the chemical composition of neoplastic basement

membrane mucoprotein also tend to set this material apart from reticulum and collagen. The neoplastic basement membrane mucoprotein examined by Muckerjee and his colleagues was obtained from an ascitic form of yolk sac carcinoma and was considered on immunohistological grounds to be almost free of reticulum and collagen. This material was found to contain less glycine, proline and hydroxyproline and more tyrosine and cysteine than reticulum or collagen. It differed also from reticulum and collagen in its carbohydrate, hexosamine and lipid content and in the extent to which it was solubilized by trypsin. Chemical differences between the protein component of basement membrane and collagen have also been demonstrated by Kefalides (1966).

Antigenic Inter-relationship between Framework and Other Reticulums

During the immunohistological studies of connective tissues presented here it was found that anti-glomerulus antisera appeared to react less strongly with fibrils in the adventitia of arteries and in the interstitium of organs (collagenous tissue and interstitial reticulum) than they did with splenic reticulum or with reticular tissue forming the outer component of epithelial boundary membranes. These findings suggest that collagenous tissue and interstitial reticulum differ antigenically from framework reticulum. However, it does not follow from

the fact that the one antiserum reacted with two antigenically dissimilar tissues that these tissues are antigenically related (i.e. carry similar antigenic determinants or hold some determinants in common). The presence in anti-glomerulus antisera of antibodies reactive with collagenous tissue and interstitial reticulum may have reflected the contamination of the glomerular antigen with extraglomerular collagenous tissue or interstitial tissue. These contaminants may have elicited a highly specific but weak antibody response.

The question as to whether Group 2 and 3 components of connective tissue are antigenically related must be left open; the literature provides evidence both for and against the existence of an antigenic inter-relationship between reticulum and collagen.

Antisera raised against acellular tendon fibrils were found by Goodman, Greenspon and Krakower (1955) to give weak flocculation reactions with preparations of renal tubular basement membrane and stroma. Rothbard and Watson, (1961) raised antisera against acetic acid extracts of rat tail tendon. These antisera were thought to be specific for collagen; they did not react with rat serum, and the acetic acid extracts of collagen contained no polysaccharide. On intravenous or intracardiac injection into

rats, globulin fractions of these antisera were demonstrated to localise in the aorta and in nerve sheaths and in the capsules, trabeculae and interstitium of a variety of organs including lung, liver, spleen and jejunum (Rothbard and Watson, 1962). Localization was described also in the basement membranes of renal glomerular capillaries (Rothbard and Watson, 1961). Against these observations of Goodman, Greenspon and Krakower and of Rothbard and Watson, which tend to relate reticulum antigenically to collagen, must be set the findings of Mancini and his colleagues. This latter group of workers (Paz, Davidson, Gomez and Mancini, 1963; Mancini, Paz, Vilar, Davidson and Barquet, 1965) prepared antisera against acetic acid, neutral salt- and citrate-soluble and insoluble constituents of chicken leg tendon. When these antisera were used in immunohistological studies specific fluorescence corresponding to soluble and insoluble fractions of collagen appeared only in collagen fibres and interstitial fibrils. The antisera did not react with reticulum. It is to be noted also that the antisera did not react with cartilage cells, cartilagenous matrix or fibroblasts.

The Problem of the Glomerular Epithelial Cells

In mixed staining experiments the renal glomerular capillary tuft was found to contain two antigenically

dissimilar components - the basement membranes of renal glomerular capillaries, which reacted with antibodies present only in anti-glomerulus preparations, and certain cell-like structures which reacted with antibodies present both in anti-glomerulus and anti-synovium preparations. These latter structures were thought to represent glomerular epithelial cells. There is no direct evidence in favour of this view and there is some difficulty in accepting it. Electron microscopic studies of the glomerulus during development and in chronic aminonucleoside nephrosis suggest that the glomerular capillary basement membrane derives from glomerular epithelial cells (Kurtz, 1958; Kurtz and Feldman, 1962). On this basis, the epithelial cell might be expected to show the same antigenic specificity as the glomerular capillary basement membrane - unless the capillary basement membrane represents a modified secretion of the epithelial cell. This latter view is just tenable. Ashworth, Erdmann and Arnold (1960) studied the rat renal glomerulus by combined histochemical and electronmicroscopic techniques. Their observations indicated the lamina densa of the glomerular basement membrane differs histochemically from the sub-epithelial and sub-endothelial clear zones. They suggest that the clear zones represent secretion products of the contiguous epithelial and endothelial

cells and that the lamina densa represents a physically altered form of material derived from endothelium and epithelium.

Considerations of the nature of the cell-like structures detected immunohistologically in the renal glomerulus, and here called epithelial cells, must take into account also the recently revived, and considerably modified concept of the mesangium (see Suzuki, Churg, Greshman, Mautner and Dachs, 1963; Jones, Mueller and Menefee, 1962). The mesangium is not now considered to be a sheet of connective tissue on which glomerular capillaries are suspended but a complex of cells, extracellular matrix and cement substance lying within the capillary wall. All structures within the glomerulus develop from the one cell mass (Kurtz, 1958). The mesangial (or intercapillary, or third, or stalk) cell is looked upon as a cell which has undergone least differentiation (Suzuki, Churg, Greshman, Mautner and Dachs, 1963). The mesangial cell is found between epithelial and endothelial cells, frequently abutting on the capillary lumen. The cell is associated with an extracellular fibrillar matrix, or sponge fibre lattice (Jones, Mueller and Menefee, 1962), which forms a portion of the wall of the capillary. The matrix appears to be continuous with the basement membrane of the

capillary and to resemble it histochemically, but not electronmicroscopically (Suzuki et al., 1963).

Although the mesangial cell or the mesangial matrix may be responsible for some of the antigenic properties of the glomerular capillary basement membrane, for example, its antigenic relationship to collagen, (Suzuki et al., 1963), it seems hardly possible that either the cell or the matrix would be recognized as discrete structures on fluorescence microscopy.

The nature of the structures here called glomerular epithelial cells therefore remains uncertain. These structures, in their topological relationship to glomerular capillaries and in their topographical distribution throughout the tuft, seemed to correspond to the large epithelial cells seen in PAS-stained sections of kidney, prepared from paraffin embedded material. All attempts to carry out the PAS procedure on immunohistologically treated sections failed. It was not possible, therefore, directly to relate histological and immunohistological appearances.

IMMUNOHISTOLOGICAL STUDIES OF CONNECTIVE TISSUE IN
THE EMBRYONIC RAT

Observations arising out of studies of connective tissue in the rat appeared to carry the following implications:

- (1) The development of renal glomerular capillaries is accompanied by changes in their antigenic properties.
- (2) Three classes of antigens can be recognized in the skin of embryonic rats:
 - (a) Embryonic antigens.
 - (b) Common antigens.
 - (c) Adult antigens.
- (3) In general adult antigens were found only in the skin of embryos measuring more than 1.5 cm. in crown to rump length. Tissues of 1.0 - 1.5 cm. embryos contained embryonic antigens, either alone in the dermis, or in association with common antigens, in the dermis and epidermal boundary membranes. Members of all three groups of antigens were variously represented in the dermis of 1.5 - 2.0 cm. rat embryos, whereas none of the antigens were detected in the dermis of 2.5 - 3.0 cm. embryos.

One general conclusion can be drawn from these observations; that embryonic connective

tissues undergo antigenic change during development.

Some support for this conclusion is provided by histochemical and chemical studies of embryonic connective tissue.

Schoenberg and Moore, for example, in their studies of the cellular origin of mucopolysaccharides of the ground substance found an oligosaccharide, digestible by β -glucuronidase, but not by testicular hyaluronidase or α -amylase, to be abundant in the Whartons jelly of immature human umbilical cords. The amount of this material was found to decrease with increasing cord age. *Pari passu* with this decrease in oligosaccharide occurred an increase in hyaluronic acid and chondroitin sulphate C. It was proposed that the oligosaccharide represented a precursor of hyaluronic acid secreted into the ground substance by fibrocytes, and that the polymer was synthesized extracellularly (Moore and Schoenberg, 1958; Schoenberg and Moore, 1957, 1958).

Frankel and his colleagues, on the basis of their studies of the elastic membranes in the developing human aorta, have suggested that the elastin initially formed consists mainly of an acid mucopolysaccharide. With development, the acid groups of this substance combine with basic amino-acids of proteins to form neutral protein-polysaccharide complexes (Frankel, Bernick, Patek, Edmonson, Peters

and Paul, 1963).

Changes in the nature of mucopolysaccharides in the walls of blood vessels in the embryonic chick have been described by Alekseeva (1964). At the seventh day of incubation, vessel walls contained mucopolysaccharides digestible by testicular hyaluronidase, after the sixteenth day the mucopolysaccharides were found to resist hyaluronidase.

Price (1963) has shown that the composition and dimensions of the tracheal basement membranes in chick embryos changes several times during development. Initially composed of insoluble polysaccharide free of chondroitin sulphate, the basement membrane by the end of the embryonic period has become a complex structure containing collagen fibres, mucoproteins and chondroitin sulphate.

Chemical studies of soluble collagen obtained from the skin of embryonic pigs and cows have shown that the nature of neutral salt soluble collagen changes during development. Early in embryonic life neutral salt soluble collagen is hydroxyproline poor and is not precipitable in fibrillar form. Later this collagen increases its hydroxyproline content and comes to be precipitable as fibrils. The increase in hydroxyproline is associated with a decrease in the proline - hydroxyproline and glycine - hydroxyproline ratios (Myagkaya,

Zaides, Orlovskaya and Tustanovskii, 1963). Later work has confirmed that the low hydroxyproline content of neutral salt soluble collagen from young embryos is to be attributed to structural immaturity of the neutral salt soluble collagen, and not to the contamination of the extract with non-collagenous substances (Tustanovskii, Myagkaya and Volkova, 1964).

Studies of connective tissue in post-natal life have shown that these tissues continue to undergo modification in early life and that aging is associated with further alterations in their mechanical and chemical properties.

Chemical studies of human embryonic, young, adult and aged tissues have been undertaken by a number of workers. Clausen (1962 a,b, 1963) studied the age variations in mucopolysaccharide and hydroxyproline content of aorta, myocardium and skin. The material studied was obtained from foetuses (3 to 38 weeks) and from individuals from 4 months to 87 years of age. The acid mucopolysaccharide content of all three tissues showed a progressive decrease and the hydroxyproline content a progressive increase with advancing age. A similar pattern of change was described by Kaplan and Meyer (1960) for the human aorta, while these workers were unable to relate the total aortic content of mucopolysaccharides or hexosamine to

to age, they found that individual mucopolysaccharides showed variation with age, hyaluronate and chondroitin sulphate C decreasing, and heparitin and chondroitin B increasing with age. Kaplan and Meyer point out that the pattern of aging shown by the aorta is more complex than that shown by costal cartilage and suggest that the aortic pattern represents age changes superimposed on degenerative and reparative changes. The aortic pattern of age change was found by Rugarli, Vallino and Cantalamessa (1962) to differ from that shown by pulmonary arteries. Acid mucopolysaccharides were found to increase with age in the pulmonary arteries and to decrease in the aortic arch.

Lagier and Exer (1960) have described for abdominal skin and abdominal aponeurosis a gradual decrease in the content of mucopolysaccharides and an increase in the content of hydroxyproline during the last four months of ante-natal life and the first ten years of post-natal life. The mucopolysaccharide : collagen ratio was at its highest in the embryo, fell to its lowest at 20 to 30 years and rose again slightly in old age. Achilles tendon showed less marked changes than skin or aponeurosis.

In his review of the ontogeny of non-enzyme proteins Solomon (1965) quotes the studies of Ebert and of Croissille on the development of organ

specific saline soluble proteins in the spleen, heart and brain of 9 to 18 day chick embryos and adult chicks. Ebert found that:

- (a) while the antigenic constitution of the heart did not change during development, that of the brain and spleen did.
- (b) in the case of the brain all animals, i.e. 9, 12, 18 day embryonic and adult chicks contained a brain specific antigen, but 18 day embryos and adult chicks contained two additional groups of antigen not found in younger animals. One of these groups was common to the heart and brain, the other to the spleen and brain.
- (c) the spleen of 9 and 12 day old chick embryos contained two antigens, one spleen specific and one common to spleen and brain. The 18 day chick embryonic spleen and the adult spleen, however, possessed a second spleen specific antigen in addition to the antigens found in the younger animals.

Croiselle used immunoelectrophoretic techniques to study the ontogeny of liver antigens in the chick embryo and found that, of the fourteen antigens detectable in extracts of the adult chick

liver, extracts from livers of -

5 day embryos contained 1

6 $\frac{1}{2}$ day embryos contained 6

8 - 9 day embryos contained 7

7 -18 day embryos contained 9

5 day chicks contained 11

Changes occurring during development are not confined to changes in the chemical composition or antigenic constitution of tissue. Shifts have been found to occur in tissue isoenzyme patterns during development. These shifts have been attributed to changes in (1) the relative proportion of a particular histological component of an organ, (2) genuine changes at the molecular level.

Attempts to relate changes in the pattern of tissue isoenzymes and alterations in the antigenic constitution of tissues to each other run into a number of difficulties:

(a) It is not everywhere clear to what extent alterations in the representation of individual molecular forms of an (iso)enzyme may have on the metabolism of a tissue (Moss, 1964).

(b) In some cases alterations in tissue isoenzymes appear to be related to increasing activity rather than to differentiation - the shift, for example, in creatinine kinase from form I through form II to form III which takes place in

the skeletal muscle of the rat foetus from the eleventh day on (Eppenberger, 1964).

(c) Even where changes in the relative proportions of M and H type LDH isoenzymes appear to denote a shift from (or to) aerobic metabolism, there is no evidence that these changes - from M to H type LDH in the rat heart for example (Fine, Kaplan and Kuftiner, 1963) - coincide with, or precede, the appearance of a tissue specific antigen.

(d) The fact that a shift in the isoenzyme complement can sometimes be correlated with the increasing predominance of a particular histological component of an organ, proximal tubules in the kidney for example (Smith and Kissane, 1963) relates more to the histological development of an organ than to antigenic changes in a particular component of an organ (connective tissue of the skin or renal glomerular capillaries), and is probably irrelevant to the present discussion.

Relationship Between Antigenic Properties and
Function of Tissues

While the changes which take place in their immunological and histochemical properties necessarily reflect the gradual development of the mature structure and constitution of connective tissues, it seems not unreasonable to suggest that these changes relate also to the function of connective tissues.

There is some evidence that a relationship between antigenicity and function may, in certain circumstances, be demonstrable.

It will be recalled that in mixed staining and other experiments involving the use of contrastingly labelled conjugates, it was found that:-

- (a) adult human connective tissue could be divided into three immunologically reactive groups,
- (b) a loose relationship was found to exist between the immunological grouping of components of connective tissue and their histological distribution,
- (c) it was suggested that this relationship pointed to a relationship between the antigenic properties of connective tissues and their function.

A relationship of this kind had already been suggested by Krakower and Greenspon (1958). These workers found that the nephrotoxinogenic propensity

of different tissues was related, not only to their content of capillaries, but more immediately to the hydrostatic pressure to which these capillaries were normally exposed. It was suggested that the antigen(s) responsible for the production of nephrotoxic antisera might be "a specific chemical substance capable of playing an important role in the permeability or filtration qualities of capillaries".

Possibly pertinent to enquiries concerned with a demonstrable relationship between anti-genicity and function are:-

- (1) The demonstration that nephrotoxic antisera are not species specific.
- (2) The loss of immunological reactivity of renal tubular basement membranes associated with the development of renal tubular necroses in mice.
- (3) Studies on the renal distribution of anti-rat kidney antisera injected into new born rats.

Markowitz (1960) has shown that antisera raised in sheep against renal glomerular basement membranes of rabbits elicit nephritis on injection in dogs, and that sheep anti-canine glomerular basement membrane antisera mediate nephritis in rabbits. Nephritis has also been produced in dogs by the injection of rabbit anti-human glomer-

ular basement membrane antisera (Markowitz, 1960 b; Steblay and Lepper, 1961 b). While these observations do not constitute a demonstration of a relationship between anti-genicity and function, they establish that there is a degree of antigenic overlap between tissues subserving the same function, the renal glomerular basement membranes in man and dog, and dog and rabbit.

A temporary change in the immunological properties of renal tubular basement membranes has been found to precede the development of frank tubular necrosis in mice given a single intravenous injection of Streptotysin S. This change consisted of the loss of the ability of tubular membranes to react with fluorescein labelled anti- β -globulin antisera. It occurred soon after the injection of Streptotysin S at a stage when light microscopy showed only hyaline granular change in tubular cells, and decreased PAS staining of tubular basement membranes. Regeneration of damaged tubular segments was accompanied by the return of immunological reactivity of tubular basement membranes with anti- β -globulin antisera (Tan and Kaplan, 1963).

Rabbit anti-rat kidney antiserum was detected, by fluorescein-anti-rabbit serum, in the juxtamedullary and mid-zonal glomeruli, but not in subcortical glomeruli in six week old rats which had been injected at birth with the anti-kidney serum

(Hiramoto, Calcagno and Pressman, 1962). In juxta-medullary glomeruli, fluorescence denoting the presence of anti-rat kidney antiserum had a capillary loop distribution (Hiramoto et al., 1962; Fig. 2a). Mid-zonal glomeruli, however, showed only partial staining. Fluorescence in these glomeruli was distributed as streaks and did not exhibit a capillary loop pattern (Hiramoto et al., 1962; Fig. 2b). Although Hiramoto and his colleagues did not interpret them in this way, two deductions can be made from these observations. From the presence of anti-rat kidney antiserum in mid-zone glomeruli and from the unusual distribution of this antiserum, it can be deduced: firstly, that at the time of injection of the antiserum these mid-zonal glomeruli (presumably then situated near the subcortical zone) were in contact with the circulation; secondly, that at that time their capillaries contained only low concentrations of antigens capable of reacting with anti-glomerular basement membrane antibodies in anti-rat kidney antisera.

SPECULATIONS CONCERNING THE ANTIGENIC
PLASTICITY OF EMBRYONIC CONNECTIVE TISSUE

It will be recalled that the dermal connective tissues of some embryos contained antigens reactive only with anti-embryonic rat connective tissue antisera and that tissues of other rats contained antigens reactive only with anti-adult antisera. An additional group of antigens, reactive with both classes of antisera, was detected in most, but curiously not in all embryos. It was found also that antigens detected in the younger 1.0 to 2.0 cm. embryos were not detected in the derma of embryos measuring more than 2.5 cm. These observations suggest that histogenesis is associated with changes in the concentration or solubility of embryonic adult and common antigens.

The evidence that the development of the dermis may be accompanied by changes in the antigenic constitution of dermal connective tissues stimulates speculations concerning the significance of these changes.

It may be assumed that changes in the antigenic properties of connective tissues during development represent either alterations in the configuration of macromolecules or by the appearance of new molecular species, and that they

therefore reflect the progress of histogenesis.

It can be suggested that these changes do no more than reflect the progress of histogenesis. This is to propose that the balance between synthesis and breakdown of tissues, on which histogenesis depends, is achieved solely by genetic information and that the various gene-enzyme systems involved in the synthesis and breakdown of tissues are called into action in a pre-ordained sequence and at pre-ordained intervals.

An alternative proposition embraces the concept that the manner and sequence in which a synthesising cell expresses its genetic potential is under the control of a variety of factors, and expresses the view that antigenic changes, occurring during histogenesis or the molecular configurations which these changes represent, not only reflect but also play a part in directing histogenesis.

This alternative proposition carries certain implications. These are:-

- 1). Variations in the antigenic properties of connective tissues during development are to be attributed to alterations in synthesising activities of connective tissue cells.

- 2). A sequential activation of certain gene-enzyme systems and a sequential inhibition of others occurs during development.

3). The state of these systems, active or inactive, is regulated, in part, by configurations which derive directly or indirectly from the activities of these cells.

It is not unreasonable to relate the changes which occur in the antigenic properties of dermal connective tissues of the rat during development, to the appearances of new molecular configurations in these tissues.

Two possible explanations for the appearance of these configurations present themselves. They may derive directly from the activities of synthesising cells, or they may owe their origin to the polymerisation in an extracellular situation of molecules synthesised previously. Subsequent maturation of these polymers involving the formation of new inter- and intra-molecular cross links would provide a mechanism whereby continuing antigenic change might take place in connective tissues in the absence of the active synthesis of new molecules.

Physio-chemical processes are thought to be implicated in some of the chemical and histochemical changes found to occur in connective tissues during development. Moore and Schoenberg, for example, attribute the gradual appearances of hyaluronic acid in the human umbilical cord to the polymerisation of an oligosaccharide, sec-

reted by fibroblasts (Moore and Schoenberg, 1958; Schoenberg and Moore, 1957, 1958). Frankel and his colleagues suggest that the protein-polysaccharides complexes, present in human aortic elastin, are formed by the reaction of acid polysaccharides with basic aminoacids of proteins (Frankel et al., 1963).

Less readily reconciled with a proposition which attributes the chemical and histo-chemical changes occurring during development solely to extra-cellular processes, are alterations in the amount of hydroxyproline and mucopolysaccharides found to take place in human bovine and porcine dermal tissues during embryonic life.

Studies of human dermal tissue have shown its mucopolysaccharide content to undergo a gradual decrease and its hydroxyproline content a gradual increase during the last four months of embryonic life (Lagier and Exer, 1960).

Myagkaya and his associates employed chemical, electron microscopic and X-ray diffraction techniques in studies of the development of bovine and porcine dermal collagen. They found that the hydroxyproline content of neutral salt soluble collagen, and the amount of chondroitin sulphate relative to that of hyaluronate produced by mesenchymal cells, increased with in-

creasing embryonic age. Changes in the amount of hydroxyproline present in neutral soluble collagen, with advancing age, were correlated with the number of 640 Angstrom fibrils present. It was shown that early in embryonic life the rate of synthesis of polypeptide precursors of collagen exceeds that of fibril formation, while later in life this situation is reversed. It was found also that the acid mucopolysaccharide content of dermal tissue falls during the formation of collagen. These observations may suggest that alterations in the rate of the synthesis of chondroitin sulphate, relative to the rates of synthesis of hyaluronate, on the one hand, and hydroxyproline on the other, are implicated in fibrillogenesis.

A proposition attributing the chemical changes occurring in connective tissue during development to co-ordinated alterations in the activities of synthesising cells, provides a more satisfactory explanation for the observations of Myagkaya and his associates, and of Lagier and Exer and Clausen, than one which attributes these changes solely to extra-cellular physio-chemical processes. Moreover, physio-chemical processes probably depend for their regulation on the co-ordinated synthesis of a variety of constituents. To ascribe, for example, the formation of collagen fibrils

solely to physio-chemical processes, is to leave out of consideration factors controlling the rate of synthesis of subunits of collagen, their release from the cell and the mechanisms by which a limit is set to the diameter of collagen fibrils. The diameter of collagen fibrils varies from tissue to tissue, but is characteristic for each tissue (Fitton Jackson, 1964). It seems therefore, that fibrillogenesis involves not only the co-ordinated synthesis of α_1 and α_2 polypeptides, but also variations in the amount of, or balance between, agents which inhibit and those which encourage the aggregation of collagen subunits into fibrils.

The proposition that alterations occur in the synthesizing activity of connective cells during development, derives support from the observations of Mancini, Vilar, Stein and Fiorni (1961). These workers used histochemical and radioautographic techniques to study the role of fibroblasts in the production of connective tissue mucopolysaccharides. The material examined consisted of tissue cultures of fibroblasts from subcutaneous tissue and heart explants of 8 day chick embryos, and biopsy material obtained from foetal and adult rats. It was found that the change from embryonic to adult type fibroblasts was accompanied by a gra-

dual reduction in cytoplasmic nucleoproteins, periodic acid-Schiff positive material, succinodihydrogenase, β -glucuronidase and leucylamino-peptidase.

The view that alterations in the activities of synthesizing cells contribute to the chemical and immunological changes which occur in connective tissues during development, embraces the following two propositions. A sequential activation and inactivation of gene-enzyme systems takes place during development. The environmental conditions which a synthesizing cell has helped to create, play a part in determining which facet of its genetic potential the cell will next exhibit.

Some support for these propositions accrues from studies of the giant chromosomes of the midge *Chironomus* and other Diptera (summarised by Clever and Beerman, 1964).

The giant chromosomes found in the salivary glands, intestine and malpighian tubules of Diptera are thought to be equivalent to interphase chromosomes of normal cells. From time to time, these giant chromosomes exhibit localised modifications in structure - chromosome puffs of Balbiani rings. A relationship is thought to exist between the formation of chromosome puffs and the activation of genes. This

view is based on the following phenomena;

- (a) the number and the position of bands on giant chromosomes both correspond with the distribution of genes as mapped out by breeding experiments,
- (b) chromosome puffs are formed by loosening of the filaments forming a single band,
- (c) chromosome puffs are the site of RNA synthesis,
- (d) this RNA is believed to be messenger RNA because:

the administration of actinomycin D brings about the inhibition of its synthesis, and the regression of puffing; RNA's associated with chromosome puffs differ in their base composition from those associated with cytoplasm and nucleoli; puff RNA showing deviations from the 1 : 1 adenine : uracil and guanine : cytosine ratios, suggesting that it is a copy of a single strand of DNA.

Chromosome puffs have come to be looked upon as expressions of gene activity and the sequence of puffing along a chromosome as a reflection of the sequence of activation of different genes. It has been known for a number of years that specific tissues and specific stages in the development of the midge *Chironomus* and other Diptera, are characterised by a definite sequence in the formation of chromosome puffs.

Studies of giant chromosomes therefore provide precedence for the proposition that a sequential activation of genes may occur during the development of connective tissues. These studies also implicate a variety of factors in the regulation of genetic activity. Studies of the sequence of puffing at four sites on a single chromosome of the chironomus larva before and during the molt that begins pupation, taken together with a study of the effects of administration of the hormone ecdosyne, have shown that the hormone controls in a very specific way the puffing of certain genes activated early in molting. Of the two genes activated earliest by ecdosyne, one was found to undergo regression before pupation, this in spite of the fact that the tissue fluids of such larvae contain the activating hormone. Haemolymph from pupating larvae induced puffing at both loci in larvae which had not yet begun to molt. These studies implicate at least two factors in the control of genetic activity in the midge, ecdosyne and another factor which represses puffing at one locus known to be directly responsive to the hormone.

The work of Weiler (1959) provides evidence that the manner in which cells express their genetic potential may be influenced by the nature of their environment. Weiler found that a cellular change, involving the loss of a tissue specific

antigen, took place during the first two cell generations, when hamster kidney cells were grown in tissue culture. This loss of kidney specific antigen appeared to represent a change in the phenotype of the cells. It could not be attributed to a population change caused by random mutation, followed by selection. These observations recall the work of Beadle (1957) who found that the genes for the ciliary antigens C, D and E of *Paramecium aurelius*, were active only at certain temperatures.

The experiments of Kroeger provide evidence that a functional inter-relationship between cytoplasm and nucleus may play a part in regulating genetic activity. Kroeger transferred nuclei from the salivary gland of prepupal larvae of the fruit fly *Drosophila busckii* into preparations containing the cytoplasm of developing eggs in the preblastoderm and blastoderm stages of development. He found that certain puffs in chromosome number 2 disappeared after transfer and a new pattern of puffing developed in the new environment, and that the pattern developing in the preblastodermic environment differed from that developing in the blastodermic environment.

The proposition that molecular configurations appearing in the cytoplasm and extracellular environment of connective tissue cells during development play a role in histogenesis, ascribes to pro-

ducts of one stage of histogenesis, the ability to call into action gene-enzyme systems responsible for the synthesis of constituents required for the next stage. This is to take as axiomatic the ability of each connective tissue cell to synthesise all the subunits going to form extracellular connective tissues (Fitton Jackson, 1964), and to suggest that the various genetic potentials incorporated in a connective tissue cell are sequentially called into activity by a series of stimuli, each stimulus deriving from the operation of previously activated gene-enzyme systems.

The present proposition is not immediately concerned with processes responsible for: the control of the structural organization of the extracellular connective tissues; topographical variations in the constitution of connective tissues; the integration of local tissue anabolic and catabolic processes with total bodily metabolism. These will be considered later.

In their studies of sulphation in connective tissues Fitton Jackson and Randall found that the first acid mucopolysaccharide to appear in osteoblastic tissue cultures was a hyaluronic acid-like material, some five days later a poorly sulphated chondroitin sulphate-like material formed a large proportion of the acid mucopolysaccharides present (Fitton Jackson, 1964). These observations may,

perhaps, point to a sequential activation of gene-enzyme systems, concerned in the synthesis of acid mucopolysaccharides.

The work of Monod and Jacob (1961) and Monod, Changeux and Jacob (1963) provides a basis for conjecture concerning the mechanisms by which serial activation of gene-enzyme systems may be brought about during histogenesis. The activation of gene-enzyme systems responsible for the synthesis of subunits going to form the various constituents of connective tissue may, it is suggested, be dependent on:-

- (i) the sequential appearance of regulating configurations
- (ii) the inhibition by these configurations of repressors binding and inhibiting operator genes concerned in the next stage of histogenesis.

On this basis antigenic changes occurring during development can be attributed either to

- (a) a switch from the synthesis of one species of polysaccharide to another, or to (b) the new synthesis or synthesis of new macromolecular mucopolysaccharide - peptide - protein complexes, or to (c) alterations in the configuration of these protein - polysaccharide complexes consequent upon;
 - (1) the replacement of a primitive linkage peptide by a more "adult" one or (2) a change in the ex-

tracellular ionic environment.

The integrity of connective tissues in post-natal life may depend upon a variety of factors; genetic information; local feed-back systems regulating, and to some extent co-ordinating, the synthetic activities of connective tissue cells, and hormonal stimuli by which local tissue anabolism and catabolism are integrated with the metabolic requirements of the body.

Something is already known about the intracellular feed-back systems implicated in the synthesis of polysaccharides. It has been shown, for example, that the synthesis of Uridininediphospho-acetylglucosamine is a self limiting process, UDP-acetylglucosamine inhibiting the formation of glucosamine-6-phosphate from fructose-6-phosphate (Kornfeld, Kornfeld, Neufeld and O'Brien, 1964). Similarly UDP-xylulose is involved in a feed-back system which prevents the formation of UDP-glucuronic acid, from which the inhibitor UDP-xylulose is derived (Neufeld and Hall, 1965). It seems possible that these two feed-back systems might provide for the alternate synthesis of hexuronic acids and hexosamines. The pathways involved in the synthesis of UDP-glucuronic acid and UDP-acetylglucosamine both stem from glucose-6-phosphate.

Fitton Jackson's (1964) model of a protein

polysaccharide filament is based on the speculation that chondroitin sulphate is linked to a peptide and that two to four polysaccharide peptide units become attached to a molecule of protein.

Speculations concerning the homeostasis of connective tissues must, therefore, invoke a feedback system by which molecular configurations appearing in the environment of a cell control the rate of release of the various components of the mucoprotein-polysaccharide complexes from the cell. The balance between filament formation and the release of filament forming units from the cell, in the final analysis, being achieved by intermittent activation and inhibition of gene-enzyme systems regulating the synthesis of these units.

It has long been known that variations in hormonal state bring about alterations in the chemical and histochemical properties of connective tissue (see Dorfman and Schiller, 1958; Schiller, 1963, for reviews). Evidence is accumulating that a variety of hormones (e.g. growth hormone, ACTH, testosterone, oestrogen, aldosterone, thyroxine, insulin) exert their effects in part, by regulating the genetic apparatus (Talwar and Segal, 1963; Porter, Bogoroth and Edelman, 1964; Carlsen, Trelle and Schjeibe, 1964; Kidson and Kirby, 1964; Wool and Moyer, 1964).

It seems reasonable to suggest that individual

hormones (1a) stimulate or (1b) repress different groups of gene-enzyme systems and (2) selectively but (3) not irreversibly, inhibit the action of analogous hormones. These speculations are put forward to account for:-

(1a) The observation that growth hormone appears to have a selective effect on the metabolism of sulphated mucopolysaccharides, reversing in hypophysectomised rats the reduced synthesis of chondroitin sulphate, but not reducing the abnormally high concentrations of hyaluronic acid found in these rats (Schiller, 1963).

(1b) Priest's (1961) observation that oestradiol reduces the synthesis of sulphated mucopolysaccharides in the rat.

(2) The difference between the response of the comb in the cock and the capon to sex hormones. Androgens produce an increase of hyaluronic acid in the comb of the cock, oestrogens do not. The capon's comb responds to oestrogens and androgens (Ludwig and Boas, 1950; Schiller, Benditt and Dorfman, 1952). In young cockrels oestrogens inhibit the growth of the comb (Boas and Ludwig, 1950).

(3) The oestrogen-stimulated synthesis of yolk proteins, phosphovitin and lipovitellin,

by liver cells of the rooster, provides an example of hormonally induced activation of repressed genes. (Carlsen et al. 1964).

Variations in the responsiveness of different tissues to hormonal stimuli, and differences in the representation of individual mucopolysaccharides in different connective tissues, may perhaps be attributable to the repression of different groups of gene-enzyme systems. The demonstration that the sex skin of the monkey, when transplanted to other sites, continues to respond to oestrogenic stimulation, establishes that this response is a property of the specialised cells of the sex skin. There is evidence, however, that cells, other than those of the sex skin, can be induced to respond to oestrogenic stimulation. Bachman and his colleagues (Bachman et al., 1936) found that when oestrogenic stimulation was continued for a month or more the response to the hormone gradually extended to areas around the transplanted sex skin. This observation suggests (a) that gene-enzyme systems potentially responsive to oestrogenic stimulations are present in the initially unresponsive less specialised cells, (b) that these systems are normally held in an inactive state and (c) that their activity may involve factors released locally by oestrogen stimulated sex skin cells.

SPECULATIONS CONCERNING THE AGEING OF COLLAGEN

The proposition that the integrity of connective tissues is maintained by a series of self-regulating systems may have implications for the processes accompanying the ageing of collagen.

The ageing of collagen is associated with a decrease in the tissue content of soluble collagen and non-collagenous proteins and with an increase in the yield of insoluble collagen obtained from tissues.

The most characteristic feature of the ageing process is the development of increasing structural stability of collagen (Verzar 1963: Fry, Harkness and Harkness, 1964). This change has been attributed to an increase in the number and strength of the intra- and extra-molecular cross links in collagen (Gross, 1961; Verzar, 1963; Bjorksten, 1963; Bornstein and Piez, 1964).

Much evidence suggests that the formation of cross links within and between collagen fibrils increases simply as a function of the time that has elapsed since the formation of their constituent molecules (Jackson and Bentley, 1960; Hruza and Hlavackova, 1963; Brocas and Verzar, 1961).

It has been suggested that cross linking between collagen and other molecules represents the primary phenomenon in ageing and that research should be directed towards the development of means

to break down these linkages (Bjorksten, 1963).

It is proposed to consider here the possibility that subtle changes in the structure of the collagen molecule contribute to the age associated increase in the structural stability of collagen, and that such biological changes in collagen may have the effect of introducing a pattern of cross linkages, which, although apparently excessive in number and strength, may represent the only way in which stability can be conferred on collagen formed later in life.

It is not intended to deny the proposition that cross linking between macromolecules is a continuously occurring process, but merely to suggest that factors, other than chronological, may contribute to age associated alterations in the properties of connective tissues.

There is evidence that the rate of ageing of collagen may be susceptible to modification. The pattern of age change has been found to be influenced by sex (Kao et al., 1960), site (Schaub, 1963 a, b; 1964), caloric intake (McCay, 1952; Chvapil and Holeckova, 1962, and multiple pregnancies (Arvay et al., 1963).

It cannot reasonably be maintained that cross linking between and within collagen macromolecules is under direct hormonal or metabolic control. Collagen, once it has been laid down as fibres, is

known to be metabolically inert. It seems more reasonable to suggest that the nature of the intra- and inter-molecular cross links, the rate at which they can form, and the ease with which cross linking agents can react with adjacent collagen macromolecules, are governed by the subtle structure of the collagen molecule.

Factors influencing the subtle structure of the collagen molecule may well be under hormonal or metabolic control. Constituents of the ground substances are thought to play an important part in fibrillogenesis in vivo (Fitton Jackson, 1964). Histochemical changes, indicative of an increase in neutral mucopolysaccharides and glycoproteins, have been described in the ground substances during the development of renal and hepatic fibrosis (Lupu et al., 1962), and in association with the proliferation of fibroblasts (Taylor and Saunders, 1957).

Although in vitro studies have thrown little light on the part played by proteinpolysaccharides and mycopolysaccharides in fibrillogenesis, it has been reasoned (Fitton Jackson, 1964) that mechanisms are operative in the ground substance which (a) allow tropocollagen molecules to pass through the ground substance, (b) permit aggregation of these molecules into specific fibrils and (c) precisely limit the size of the fibrils formed

to that which is characteristic of the particular tissue.

Apart from their putative role in the control of fibrillogenesis, constituents of the ground substance are thought to become incorporated both in the collagen molecule and in the collagen fibril. Rubin and his colleagues (1963) have provided evidence that non-collagenous peptide chains serve as links in the end to end aggregation of tropocollagen molecules and participate also in the formation of stabilising intra-molecular links. Fitton Jackson (1964, b) has suggested that these peptide chains represent non-collagenous proteins of the ground substances. Small amounts of carbohydrates, in the form of glycoproteins and sialoproteins, appear to be tightly bound to the collagen fibril. These sugars are thought to contribute to the stability of the collagen fibre (Banga and Balo, 1960; Jackson and Bentley, 1960).

Components of the ground substances, then, seem likely to play a vital part in the control of fibrillogenesis and the stabilisation of the collagen molecule.

Several groups of investigators (e.g. Davidson, Small, Perchemlides and Baxley, 1961) have reported that changes occur in the metabolism of polysaccharides with ageing. Age-associated changes have been reported also in the response

of connective tissue to the administration of hormones (Smith and Allison, 1965; Sethi et al., 1961). Chemical and histochemical studies of connective tissues have shown that changes in the histochemical properties and chemical composition of the ground substance accompany ageing in man (Kaplan and Meyer, 1960; Lagier and Exer, 1960; Rugarli et al., 1962; Kanabroki et al., 1963; Clausen, 1962, a and b, 1963; and Van den Hoof, 1964) and in the experimental animal (Smith et al., 1951; Dickerson and John, 1963; Morett and Whitehouse, 1963; Dische and Zelmenis, 1965).

These findings, taken together with the possibility that fibrillogenesis depends upon a precise inter-relationship between the various constituents of the extracellular phase of connective tissues, (Fitton Jackson, 1964) and the evidence that constituents of the ground substances contribute to the structure of the collagen macromolecule, it is suggested, add credence to the proposition that alterations in the subtle structure of the collagen macromolecule may be implicated in the ageing process.

The proposition that the structure of the collagen molecule is subject to gradual change, or a series of sequential changes during maturation, may be open to experimental investigation.

It has been shown by Mancini and his colleagues

(Mancini, Paz, Vilar, Davidson and Barquet, 1965) that it is possible to differentiate immunologically between neutral salt-soluble, acetic acid-soluble and insoluble collagen. It may therefore be possible, by studying the patterns of immunological reactivity manifested by antisera raised against collagen fractions derived from animals of different ages, to decide whether significant changes occur in the antigenic properties of collagen with age, and to determine whether these changes are to be attributed solely to the formation of cross links, or whether they reflect more fundamental alterations in the structure of collagen.

SPECULATIONS CONCERNING THE PATHOGENESIS
OF RHEUMATOID ARTHRITIS

Introduction

The proposals put forward by Jacob and Monod (1961) concerning the regulatory mechanisms involved in the control of gene activity and enzyme synthesis provide a conceptual basis for speculations concerning the possibility that rheumatoid arthritis represents an aberration of connective tissue cell function. Experimental basis for speculations of this nature has possibly been provided by rheological studies in rheumatoid arthritis. These studies have indicated that joint stiffness in rheumatoid arthritis is attributable to changes in the tissues of the joint capsule rather than to frictional changes within the joint itself (Johns and Wright 1961). Rheological studies have shown also that differences,

possibly attributable to rheumatoid arthritis or possibly to cortico-steroid therapy, are demonstrable between the rheological properties of dermal collagen from subjects with rheumatoid arthritis and from those without rheumatoid arthritis (Ridge and Wright 1966).

Basic Assumptions

The present speculations are based on a number of assumptions. These are:

(1) Both heritable genetic factors and random mutational events are involved in the aetiology of rheumatoid arthritis.

The studies of de Blecourt, Polman and de Blecourt-Meindersma (1961) point to the operation of heritable mechanisms in rheumatoid arthritis, while the work of Burch (1963) suggests that the age-specific prevalence of the disease depends upon random mutational events.

(2) Rheumatoid factors have no significance for the initiation of rheumatoid arthritis but their appearance is, nevertheless, related to the cause of the disease.

Epidemiological studies have shown that rheumatoid arthritis and the factor responsible for the sheep cell agglutination test may be aggregated independently (Lawrence and Ball 1958, Lawrence 1966). On the other hand, there is evidence that individuals sero-positive for the S.C.A.T. factor may be more prone to develop rheumatoid arthritis and may fare worse with the disease than sero-negative individuals (Ball and Lawrence 1961, Kellgren 1964).

(3) Every connective tissue cell possesses the genetic information necessary for the synthesis of all constituents of connective tissue.

The various types of differentiated connective tissue cell in the body are all thought to be derived from the one stem cell (Fitton Jackson 1964, Schubert 1964), and Monod and Jacob (1961) argue that differentiation need not involve any alteration of the genome.

(4) The manner in which a connective tissue cell expresses its genetic potential is determined in part by its environment.

This proposition derives from (3) above and from the way in which the polysaccharide composition of connective tissues varies between sites in the body and with age (Fitton Jackson 1964, Schubert 1964, and see Meyer 1964).

(5) The production of linkage peptides is one of the synthetic activities of connective tissue cells. Fitton Jackson (1964) has proposed that a small polypeptide links polysaccharides to globular proteins in the formation of polysaccharide-protein complexes of connective tissues.

(6) The macromolecular configuration of a constituent of connective tissue determines in part its physical properties and is itself influenced by the ionic environment (Katchalsky 1964).

(7) The operon model of Jacob and Monod (1961) is applicable to cells of organisms higher than bacteria.

This point is argued by Monod and Jacob (1961).

Postulates

A. General

The present speculations recognise three states or stages in the development of rheumatoid arthritis. These are:

(a) a state of predisposition to the disease, (b) the pre-proliferative phase of the disease and (c) the proliferative phase of the disease.

Predisposition to rheumatoid arthritis is ascribed to the inheritance of an allelomorphic gene, the RG2 gene, at a particular locus in stem cells of the connective tissue series. The RG2 gene confers on the cell, or on its descendents, the potential but not the ability to synthesise a modified monomeric constituent of connective tissue, the Zr monomer.

The pre-proliferative phase of the disease is initiated by the conversion of some stem cells into modified stem cells and carried forward by changes in the cellular environment which stimulate the differentiation of modified stem cells into modified synthesising cells. Modification of stem cells is brought about by mutational events involving regulator rather than structural genes. Modified synthesising cells possess not only the potential but also the ability to synthesise the Zr monomer. This monomer is incorporated, in the place of the normal Zn monomer, into a polymeric (ZZZr), possibly polysaccharide, constituent of connective tissue. The Zr monomer, it is suggested, gives rise to the appearance of functionally inadequate constituents of connective tissue by altering the macromolecular configuration of protein-polysaccharide complexes containing it.

Speculations concerning the proliferative phase of the disease see this phase as being dependent on the appearance of modified, I_{mr}, metabolites in the environment of unmodified synthesising cells. These metabolites, it is suggested, act as inducers of the as yet inactive RG2 gene present in unmodified synthesising cells. They may be breakdown products of ZZZr containing connective tissue constituents, or they may represent products of enzymes directing steps in the Zr to ZZZr pathway. On the basis of these speculations the pre-proliferative phase of the disease will be followed by the proliferative phase only if modified I_{mr} metabolites are produced in concentrations sufficient to stimulate a significant number of unmodified synthesising cells to express their previously repressed RG2 potential.

Proposals for the genetic mechanisms involved in the initial repression and subsequent induction of the activity of the RG2 gene are set out in Section B below.

Rheumatoid factor, it is suggested, reflects the presence in connective stem cells of a second allelo-morphic gene, the RP gene, which is concerned with the synthesis of a modified linkage peptide. It is further suggested that:

- (a) the modified RP peptide carries determinants relating it to heavy chain sub-units of the IgG molecule,
- (b) under certain circumstances the RP peptide and the normal NP peptide can both be synthesised by the one cell,
- (c) RP peptides can link either normal polysaccharides or modified polysaccharides, those incorporating the Zr monomer, to globular proteins to form protein-polysaccharide complexes,

- (d) normal polysaccharides tend to link with normal NP peptides in the formation of protein-polysaccharide complexes, unless the concentration of RP peptides significantly exceeds that of normal peptides,
- (e) in the absence of activity of the RG2 gene-enzyme system, free RP peptides form antigenically active polymers,
- (f) these polymers elicit antibodies reactive with denatured IgG globulin,
- (g) activation of the RG2 gene-enzyme system leads to the production of modified polysaccharides and to the appearance of protein-polysaccharide complexes containing modified polysaccharides and modified linkage peptides,
- (h) modified ZZZr polysaccharide-RP peptide-protein complexes are less well adapted to withstand physical stress than normal complexes or even modified polysaccharide-normal peptide-protein complexes are.

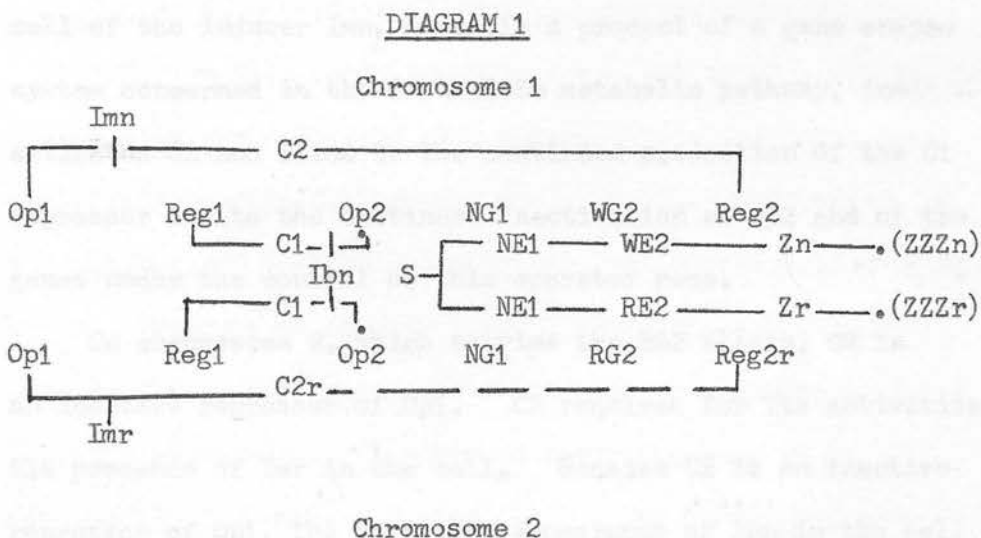
B. Genetic Mechanisms

1. The RG2 Allele.

In the foregoing speculations initiation of the pre-proliferative phase of rheumatoid disease was attributed to de-repression of the RG2 gene by constitutive mutations in stem cells of the connective tissue series. The initiation of the proliferative phase of the disease was attributed to the appearance of inducing molecules (Imr metabolites) in the environment of unmodified synthesising cells - cells in which constitutive mutations have not occurred and in which the RG2 gene is still held in a state of repression.

Speculations concerning the genetic mechanisms involved in the initial repression and subsequent induction of the RG2 gene can be based on one of the schema put forward by Jacob and Monod in their consideration of the operon as a model in differentiation (Monod and Jacob 1961).

It can be suggested, for example, that in the heterozygous individual the RG2 gene and its normal WG2 allele are arranged on their chromosomes in the manner indicated in Diagram 1.



On the chromosome bearing the WG2 gene, chromosome 1, the genes NG1 and WG2 code for enzymes NE1 and WE2. These enzymes direct the synthesis of a normal monomeric constituent of connective tissue, the Zn monomer, from substrate S. The monomer Zn is later incorporated into a polymeric constituent of connective tissue, the (ZZZn) polymer. The operator gene Op2 controlling the activity of the structural genes NG1 and WG2 is under the control of an operator gene - regulator gene system, Op1 - Reg1. The Op1 - Reg1 system is itself under the control of a second regulator gene, Reg2. The products of Reg1 and Reg2, C1 and C2, are active repressors of Op2 and

Op1 respectively.

In this system the transient appearance in the cell of the inducing molecule Ibn, a product of an enzyme active earlier in the sequence, releases Op2 from repression and brings about the activation of the structural genes NG1 and WG2. The regulator gene Reg2 is also activated and synthesizes C2, the active repressor of Op1. Repression of Op1 halts the synthesis of C1 and leads to the continued activity of Op2, NG1, WG2 and Reg2. The transient appearance in the cell of the inducer Imn, possibly a product of a gene enzyme system concerned in the Zn to ZZZn metabolic pathway, inactivates C2 and leads to the continued production of the C1 repressor and to the continued inactivation of Op2 and of the genes under the control of this operator gene.

On chromosome 2, which carries the RG2 allele, C2 is an inactive repressor of Op1. C2 requires for its activation the presence of Imr in the cell. Because C2 is an inactive repressor of Op1, the transient appearance of Ibn in the cell will call no more than a temporary halt to the synthesis of repressor C1. The transient appearance of Ibn will therefore not lead to the continued activation of the operator gene Op2 and the structural genes NG1 and RG2 and the regulator gene Reg2 will remain switched off.

It has been postulated that the ground for the development of rheumatoid arthritis is set by the transformation of potentially rheumatogenic stem cells into modified stem cells which are capable of expressing their RG2 potential should they be called into synthetic activity. The transformation of

potentially rheumatogenic stem cells into modified stem cells might be brought about by constitutive mutations converting the Op1 - Reg1 system on chromosome 2 from one repressible by Imr into one inducible Imr. Alternatively, the postulated constitutive mutations might alter the response of Op2 on chromosome 2 to its repressor C1, converting Op2 from a gene inducible by Ibn to one repressible by Ibn, or into one either inducible or repressible by a second diffusion product Idp present in the cell.

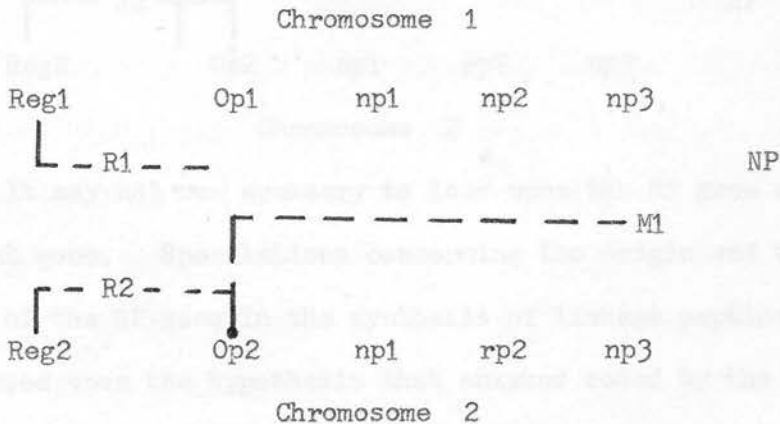
It has been postulated that changes transforming them into modified stem cells occur in some, not in all, of the potentially rheumatogenic stem cells. This postulate implies that the pre-proliferative and early proliferative phases of the disease are characterised by two families of synthesising cells. Members of the first of these families are modified synthesising cells capable of initiating the synthesis of the RG2 derived monomer. The second family is made up of descendents of unmodified stem cells in which the RG2 gene is held in a state of repression. It will be recalled that in unmodified cells the operator gene Op1 is repressible by the Imr metabolite. It is suggested that unmodified synthesising cells are stimulated to express their RG2 determined synthesising potential by the appearance in the cell of the Imr metabolite. This metabolite being derived from the modified (ZZZr) polysaccharide manufactured under the influence of the activated RG2 gene in modified cells.

2. The RP Gene

It has been postulated that the RP and NP peptides may be synthesised simultaneously by the one cell. This postulate does not take into account the epidemiological finding that the incidence of sero-positivity for the sheep cell agglutination test increases with advancing age (Ball and Lawrence 1961).

To account for these epidemiological findings it can be suggested that the NP and RP bearing operons are normally linked in an alternatively locked system favouring the NP operon (Diagram 2) and that constitutive mutations give rise to a system capable of the concerted production of NP and RP peptides or to one in which these peptides are produced in an oscillatory or cyclic sequence (Diagram 3).

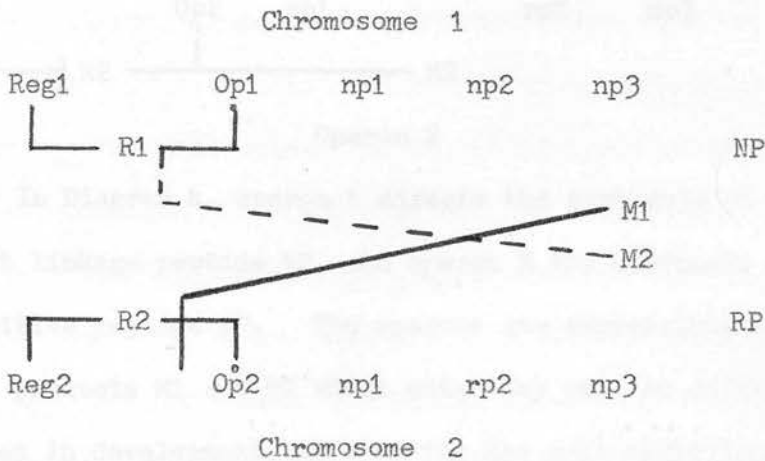
DIAGRAM 2



In the alternatively locked system schematised in Diagram 2 the genes on chromosome 1 code for enzymes concerned in the synthesis of the NP peptide, while the genes on chromosome 2 code for enzymes concerned in the synthesis of the RP peptide. In each case the enzymes also produce molecules M1 and M2 which are capable of activating the repressor

elaborated by the regulator gene present on the parallel chromosome. Neither RP nor M2 are shown in the diagram. It is assumed that M1 normally appears in the cell before M2 and that this precludes the production of M2 and also the synthesis of the RP peptide. Constitutive mutations transforming this system into the oscillatory system schematised in Diagram 3 would bring about the conversion of the Reg2 - Op2 system into one inducible rather than repressible by M1.

DIAGRAM 3

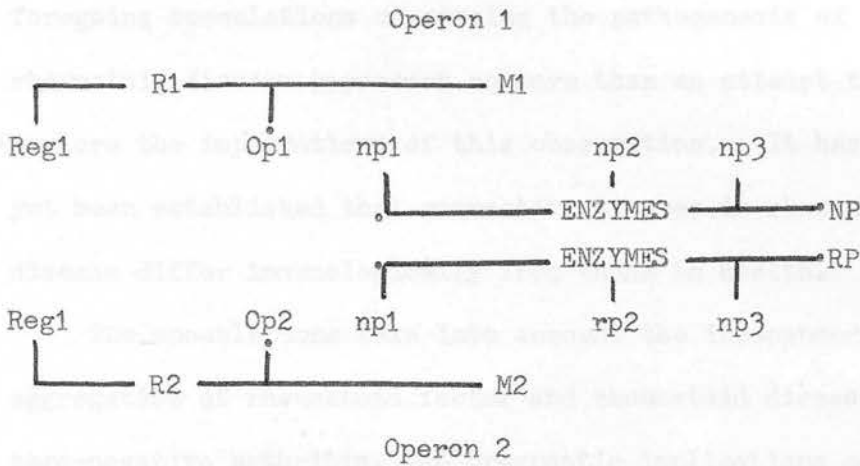


It may not be necessary to look upon the RP gene as a mutant gene. Speculations concerning the origin and the role of the RP gene in the synthesis of linkage peptides can be based upon the hypothesis that enzymes coded by the NP and RP genes are iso-enzymes concerned in the elaboration of alternative forms of linkage peptide. This is to suggest that the nature of linkage peptides synthesised by connective tissue cells changes during development, possibly to match changes putatively occurring in the polysaccharide composition of connective tissues. Evidence that changes in the immunological properties and polysaccharide composition accompany

development has been presented and reviewed above.

The genetic mechanisms demanded by the present hypothesis are set out in Diagram 4. They do not differ significantly from those schematised in Diagram 2.

DIAGRAM 4



In Diagram 4, operon 1 directs the synthesis of the adult linkage peptide NP, and operon 2 the synthesis of the primitive peptide RP. The operons are repressible by diffusion products M1 and M2 which enter the cell at different stages in development, M1 entering the cell early in development and M2 later in development and in adult life. Thus, early in life Op1 is repressed by the activated repressor R1M1, while in later life M2 activates R2 and represses Op2. The disappearance of M1 from the cell at this stage permits the synthesis of the NP linkage peptide. The ability to synthesise both the NP and the RP peptides in adult life might depend upon the re-appearance of M1 in the cell or upon constitutive mutations involving the operator gene Op2. These mutations would alter the response of Op2 to its repressor R2 so that the gene becomes inducible by M2.

Comments

The immunohistological studies of connective tissue in the adult and embryonic rat described in this thesis have provided evidence that changes occur in the antigenic properties of connective tissues during development. The foregoing speculations concerning the pathogenesis of rheumatoid disease represent no more than an attempt to explore the implications of this observation. It has not yet been established that connective tissues in rheumatoid disease differ immunologically from those in health.

The speculations take into account the independent aggregation of rheumatoid factor and rheumatoid disease, sero-negative arthritis, the prognostic implications carried by sero-positivity. They provide also a conceptual basis on which clinical variations in the severity of the disease, whether sero-positive or sero-negative, may be explained.

On the basis of the present speculations the prognostic implications carried by sero-positivity for the S.C.A.T. factor derive from the presence of antigenically active RP peptides. The anti-IgG antibodies elicited by these peptides may serve to initiate an immunologically mediated inflammatory reaction at sites of breakdown of functionally inadequate connective tissues. The antibodies might also stimulate the synthesis of RP peptides by interrupting a feed back control mechanism by which the synthesis of these peptides is regulated and synchronised with that of polysaccharides and proteins. A similar feed back control regulating the synthesis of NP peptides would not be affected in this way. The basic proposition here is that the aggregation of free peptides in the

extra-cellular environment inhibits their synthesis. It is suggested that rheumatoid factors, elicited by RP peptide polymers, might prevent the extra-cellular accumulation of RP peptides, but not of NP peptides.

Variations in the severity of rheumatoid disease and the exacerbations and remissions characterising the disease, it is suggested, reflect (a) the amount of the abnormal (ZZZr) polysaccharide synthesised vis-a-vis that of the normal (ZZZn) polysaccharide, and (b) variations in the activities of the normal NP and abnormal RP genes. It has been proposed that polysaccharide-protein complexes containing ZZZr polysaccharides and RP peptides are more susceptible to traumatic degradation than are complexes containing other permutations of normal and abnormal polysaccharides and linkage peptides.

The present speculations do not provide an explanation for the age incidence of the S.C.A.T. factor or of rheumatoid disease. The age incidence of the factor and the disease may be statistical phenomena, the necessary mutations being more likely to have occurred in older populations. Alternatively, the age incidence of the factor and the disease may be attributable to age dependent changes in connective tissue metabolism, these changes giving rise to the appearance of regulatory metabolites which favour the activation of the RP and WG2 genes.

The sex incidence of rheumatoid arthritis stimulates speculation concerning the possibility that the activity of mutant genes in the connective tissue cell may be influenced by the nature of the interaction between genes on the X

chromosome and autosomal genes. The proposition that factors other than the two alleles themselves may determine the equilibrium between them perhaps finds phenomenological precedence in the Lewis Le^a blood group gene. This gene behaves as a dominant gene in infancy but not in adult life (Boyd 1956).

APPENDIX 1

PREPARATION OF ANTI-HUMAN RENAL GLOMERULUS AND ANTI-HUMAN SYNOVIUM ANTISERA

A. PREPARATION OF HOMOGENATE OF HUMAN RENAL GLOMERULI

Glomeruli were isolated from histologically normal human kidneys by a method based on that of Hill and Cruickshank (1953), itself a modification of the method described by Krakower and Greenspon (1951).

The kidneys furnishing the glomeruli were obtained at autopsy from young adults, not more than 30 years old, the victims of road and rail accidents. At autopsy a block was taken from each kidney for histological examination and the kidneys were bisected and their pelves were removed. Each half, stripped of capsule and perirenal fat, was wrapped in a polythene bag and stored at -15°C .

For the isolation of glomeruli a half kidney was removed from the deep freeze and cut up, while thawing, into slices 3 mm. to 5 mm. thick. The slices were passed first through an ordinary domestic mincer and then through a Mouli-Baby vegetable strainer. The mash so obtained was washed free of haemoglobin in 0.15M saline buffered to pH 7.2 to 7.4 with 0.01M sodium

phosphate and containing 0.01% merthiolate as a preservative.

The washed renal sediment was pressed successively through 50, 80 and 100 mesh monel wire gauze sieves. From 300 to 700 ml. of buffered saline were required to effect the passage of the renal sediment through each sieve. It was convenient, therefore, to concentrate the tissue passing through one sieve by centrifugation before passing it through the next sieve.

The material passing through the 100 mesh sieve was suspended in buffered saline and the heavier particles were allowed to sediment out of suspension for 10 to 15 minutes. The supernatant was then removed by suction and the sediment resuspended in buffered saline. The process of sedimentation and resuspension was repeated until microscopic examination of the sediment showed it to have been largely freed of tubular cells. It was possible, by accepting a considerable loss of glomeruli, to obtain a pure preparation of isolated glomeruli. This loss, however, often threatened to be inconveniently large and a contamination of up to 10% with tubular cells was accepted.

The isolated glomeruli were packed by centrifugation resuspended in a convenient volume of buffered saline and ground in a glass tissue grinder. The

homogenate so obtained was washed by repeated centrifugation from buffered saline until the supernatant no longer contained any protein detectable by 3% sulphonyl salicylic acid.

The washed sediment was resuspended to 20% v/v in buffered saline and stored in 1 ml. volumes at -15°C .

B. PREPARATION OF HOMOGENATE OF HUMAN SYNOVIUM

Human synovial membrane was obtained from the same source as the glomerular antigen and also at operations for internal derangement of the knee joint.

The synovial membrane was freed as far as possible from sub-synovial tissue by dissection and stored at -15°C .

Two methods were used in the preparation of homogenates of synovial tissue.

The first of these involved grinding pooled synovial tissue with sand. The sand was separated from the fragmented tissue by sedimentation in buffered saline and the ground synovium was packed and washed in the centrifuge and homogenized in a tissue grinder.

By the second method pooled synovial tissue was packed by centrifugation in a Seitz filter tube. The packed tissue was removed from the filter tube placed in a suitable container and frozen at -70°C .

in a dry-ice alcohol mixture. Sections 20 μ thick were cut from the frozen block of synovial tissue, collected in a beaker and homogenized in a glass tissue grinder.

The homogenates prepared by these means were washed free of soluble protein and brought to a 20% v/v suspension in buffered saline containing 0.01% merthiolate and stored in 1 ml. volumes at -15°C.

C. PREPARATION OF HUMAN TISSUE ANTIGENS FOR INJECTION

For the preparation of anti-human glomerulus and anti-human synovium antisera the appropriate homogenate of tissue was injected into rabbits either as a 10% v/v suspension in saline or, together with Freund's complete adjuvant, as a water-in-oil emulsion.

The antigen-adjuvant emulsion contained:

Light liquid paraffin	4.25 ml.
Crill K16	0.75 ml.
Killed dried M. tuberculosis	0.50 mg.
20% saline suspension of tissue homogenate.....	5.00 ml.

The emulsion was prepared in the following way. The killed tubercle bacilli were ground into the mixture of liquid paraffin and Crill. The saline suspension of antigen was added in 0.25 ml. amounts

to this mixture and emulsified with it through a 19g. needle by means of a 10ml. syringe.

Emulsification was judged to be complete when a drop of the emulsion placed on ice cold water remained in the form of a tight sphere and showed no signs of spreading out to form a flat disc.

The emulsion was injected into rabbits immediately after its preparation.

D. IMMUNISATION SCHEDULES

Suspensions of antigen in saline were injected intraperitoneally in doses of 1 ml. A course of immunising injections consisted of 4 x 1 ml. injections a week for four weeks.

During their first course of immunisation with antigen-adjuvant emulsions animals received a total of 8 ml. of the emulsion over a period of four weeks. The emulsion was administered once a week in 4 x 0.5 ml. doses which were injected subcutaneously into four separate sites on the back, flank or limbs.

There was some variation in the total dose and in the composition of the emulsion administered to animals undergoing re-immunisation with antigen-adjuvant emulsions. These animals usually received an initial injection of 4 x 0.25 ml. of antigen in complete adjuvant. The amount of emulsion administered in each of the three subsequent weeks and its

composition, whether it contained tubercle bacilli or not, was governed by the appearance of the skin over the sites of previous injections, whether it appeared healthy or inflamed. Thus, the total dose of antigen-adjuvant emulsion injected during a course of reimmunising injections varied from 1.75 to 4.0 ml.

The number and type of immunising courses received by individual animals furnishing anti-glomerulus and anti-synovium antisera are set out in Appendix Table 1.

Animals were bled from the ear vein either on the tenth day or on the sixth, tenth and fourteenth day after a series of injections. Blood was withdrawn in 40 ml. to 60 ml. amounts when animals were bled only on the tenth day, and in 10 ml. to 20 ml. amounts when they were bled on three occasions after an immunising course.

E. ASSESSMENT OF POTENCY OF ANTISERA

The potency of each anti-glomerulus antiserum was assessed by tests of inhibition.

For these tests three sections were cut from a quick-frozen unfixed block of human kidney. The first of these sections was treated with unlabelled normal rabbit globulin, the second with the antiserum under test and the third with an unlabelled

globulin fraction of a potent anti-glomerulus antiserum (the reference globulin). These reagents were allowed to react with the sections for $1\frac{1}{2}$ hours to 3 hours. The sections were then washed in buffered saline and exposed for 20 minutes to a fluorescein-anti-glomerulus conjugate (prepared from the reference globulin).

Under the conditions of the test the section pretreated with normal globulin showed bright staining while no staining appeared in the section which had been pretreated with the reference (anti-glomerulus)globulin.

If the section pretreated with the antiserum under test showed bright staining the antiserum was discarded; if the section was found to be unstained or to show only faint staining the antiserum was judged to be potent.

Crude globulin fractions were separated from potent antisera by half saturation with ammonium sulphate and stored in the deep freeze at -15°C .

In the early stages of the study no attempt was made to assess the potency of anti-synovium antisera. Conjugates were prepared from these antisera and used individually. Later anti-synovium conjugates producing bright staining in $1\frac{1}{2}$ hours were pooled together, a corresponding pool of unlabelled anti-synovium globulins was also formed.

APPENDIX 2

PREPARATION OF ANTI-HUMAN GLOMERULUS ANTISERA

A. IMMUNISATION SCHEDULES

All the anti-human gestulin preparations were

APPENDIX TABLE 1.

IMMUNISING SCHEDULES.

RABBITS FURNISHING ANTI-GLOMERULUS ANTISERA.

Rabbit	Courses of Antigen in		Conjugates Prepared from Bleeds
	Saline	Adjuvant	
124	2½ (1st 2nd 3rd)	1½ (3rd 4th)	1 2 4
125	2 (1st 2nd)	1	1 2 3
128	-	2	2
129	-	2	2

RABBITS FURNISHING ANTI-SYNOVIUM ANTISERA.

101	1 (2nd)	1	1 2
102	2 (3rd 5th)	3	1 2 3 4 5
130	-	1	1
131	2 (2nd 3rd)	1	1 2 3
133	2 (2nd 3rd)	1	1 2 3

PREPARATION OF ANTI-HUMAN GLOBULIN ANTISERAA. IMMUNISATION SCHEDULES

All the anti-human globulin preparations were derived from antisera raised in one rabbit. This animal received 3 courses of immunising injections with human globulin at 6 monthly intervals.

The schedules followed in the three immunising courses were as follows:-

COURSE 1

- | | |
|------------|---|
| Day 0 | 10 ml. blood from ear vein. |
| Days 1,15 | 0.5 ml. human globulin in Freund's complete adjuvant injected into three areas on the back. |
| Days 29,31 | 1.0 ml. of a 1% saline solution of human globulin injected intraperitoneally. |
| Day 37 | 0.5 ml. of 1% saline solution of human globulin intravenously. |
| Day 39 | 40 ml. blood obtained from ear vein. |

COURSE 2

- | | |
|----------|--|
| Days 1,2 | 2.5 ml. alum precipitated globulin injected into each thigh. |
| Day 16 | 5.0 ml. alum precipitated globulin injected into each thigh. |
| Day 27 | 30 ml. blood obtained from ear vein. |

physiological saline containing 0.01% merthiolate and resuspended in 50 ml. physiological saline.

C. PREPARATION OF ANTIGEN-ADJUVANT EMULSION

The human globulin-adjuvant emulsion contained equal volumes of Freund's complete adjuvant (see Appendix 1C) and a 1% saline solution of human globulin separated from group O serum by half saturation with ammonium sulphate.

D. ASSESSMENT OF ANTI-HUMAN GLOBULIN CONJUGATES

Fluorescein conjugates were prepared from globulin fractions of human globulin antisera by the method of Coons and Kaplan (see Appendix 3).

The conjugates were absorbed twice with half their own volume of packed, well-washed, human group AB or A and B red cells, and twice with guinea pig liver powder (100 mg./ml.). Their worth as immunohistological reagents for the detection of human globulin was then assessed in the following way. Sections were cut from quick-frozen blocks of liver, spleen, and kidney obtained from two mice, one killed one hour after an intracardiac injection of 1 ml. of normal human serum and the other uninjected. The sections were fixed in 95% Ethanol at 20°C. for 1 hour and then dried in a steeply sloping position at 37°C. They were then stained with an anti-human globulin of proved potency and with the

conjugate under test according to the following scheme.

Section	Pretreatment	Conjugate
1	NRG 3 hours --->	F-AHG (reference) 20 mins
2	AHG (reference) 3 " --->	F-AHG (") "
3	NRG 3 hours --->	F-AHG (test) 20 mins
4	AHG (test) 3 " --->	F-AHG (") "

NRG = normal rabbit globulin.

AHG = anti-human-globulin.

F = fluorescein labelled.

PREPARATION OF FLUOROCHROME-PROTEIN CONJUGATES

A. CONJUGATION WITH FLUORESCEIN ISOCYANATE

The method of Coons and Kaplan (1950) was followed in the preparation of fluorescein isocyanate from amino fluorescein and in the conjugation of fluorescein isocyanate with globulin separated from serum pools by half saturation with ammonium sulphate.

Amino-fluorescein was converted to the isocyanate by phosgenation. The isocyanate was dissolved in acetone and dioxane and immediately added to a buffered solution of globulin.

Since fluorescein isocyanate is unstable in the presence of water, all apparatus and glass were dried in an oven before use. The acetone to be used in the conjugation was stored over anhydrous calcium sulphate and the dioxane in the presence of metallic sodium.

For the preparation of fluorescein isocyanate a cylinder of phosgene was attached to a securely sealed flow system in a fume cupboard. The system was so arranged that the phosgene was dried by passage through concentrated sulphuric acid before entering a reaction vessel where it was bubbled through 15 ml. of acetone. When this acetone had been saturated with phosgene a solution of amino

fluorescein in 5 ml. of acetone was added to it drop by drop. The effluent from the reaction vessel was passed through solutions of sodium hydroxide before being allowed to disperse.

Phosgenation was allowed to proceed for 15 minutes after the addition of the last drop of amino fluorescein.

The reaction mixture was then evaporated to dryness under reduced pressure at 45°C. The unattractive and unpromising looking residue, the isocyanate, was dissolved in a 2:1 acetone:dioxane mixture and immediately added dropwise to a cold (4°C.) buffered solution of globulin which was kept in constant but gentle agitation by means of a mechanical stirrer. Conjugation was allowed to proceed for 31 hours in a cold room kept at a constant temperature of 4°C.

The conjugate was dialysed against two or three changes of buffered saline for 24 hours to rid it of acetone and dioxane. A variety of methods was used for the further purification of conjugates. These are described in Appendix .

It was found convenient to label a number of globulin solutions at one time. Amino fluorescein (0.05 mg./mg. globulin) was converted to the isocyanate and dissolved in an appropriate volume of the 1:2 dioxane : acetone mixture for distribution

to the various reaction mixtures. The reaction mixtures prepared for two typical series of conjugates are set out below.

Serum	Globulin		Buffer *	Dioxane	Acetone	Saline	FIC	
	ml.	mg.					ml.	ml.
124/4	10.2	250	3.75	2.75	-	5.3	3.1	12
125/3	9.8	250	3.75	2.75	-	5.7	3.1	12
131/2	10.9	250	3.75	2.75	-	4.65	3.1	12
133/2	11.4	250	3.75	2.75	-	4.15	3.1	12
197/2	10.0	250	3.75	2.75	-	5.55	3.1	12

* Carbonate/biCarbonate 0.5M pH 9.0.

65m. of amino fluorescein were converted to fluorescein isocyanate (FIC).

The isocyanate was dissolved in 5.5 ml. of dioxane and 11ml. of acetone and distributed in 3.1 ml. amounts to each reaction mixture.

Serum	Globulin		Buffer *	Dioxane	Acetone	Saline	FIC	
	ml.	mg.					ml.	ml.
124/2	12	228	3.42	2.6	-	2.6	2.6	11.4
125/1	10	200	3.00	2.2	-	2.6	2.25	9.9
101/1	15	180	2.7	2.0	-	-	2.05	9.0
102/1	8	52	0.78	0.5	-	-	0.6	2.6
128/1	10	90	1.35	1.0	-	-	1.0	4.4

* Carbonate/biCarbonate 0.5M pH 9.0.

40 mg. of amino fluorescein were converted to fluorescein isocyanate (FIC).

The isocyanate was dissolved in 3.1 ml. dioxane and 6.2 ml. acetone and distributed to the reaction mixtures in the volumes shown.

B. CONJUGATION WITH FLUORESCEIN ISOTHIOCYANATE

Fluorescein isothiocyanate was purchased from Sylvana Chemicals, New Jersey. The method of Marshall, Eveland and Smith (1958) was followed in the preparation of fluorescein isothiocyanate-labelled-globulin conjugates.

A number of protein solutions were usually conjugated with fluorescein isothiocyanate (FITC) at one time. Each reaction mixture was made up as follows:-

Globulin	10 mg./ml.
1.0M Carbonate/biCarbonate buffer pH 9.0	20 vols. %.
0.15M Saline	to 100 vols.
FITC	0.025 to 0.05 mg./mg. protein.

The buffered solution of globulin was brought to 4°C. before the addition of FITC to it.

When a number of conjugates were being carried out at one time the FITC was weighed out in bulk and taken up in the smallest convenient volume of acetone for distribution to the reaction mixture.

When only one globulin solution was to be conjugated the appropriate amount of FITC was simply sprinkled onto the reaction mixture.

C. CONJUGATION WITH LISSAMINE RHODAMINE B200

The methods used in the labelling of proteins with Lissamine Rhodamine B200 (RB200) were based on

those described by Chadwick, McEntegart and Nairn (1958).

RB200 was converted to the sulphonyl chloride by grinding 0.5g. of the dye with 1.0g. phosphorous pentachloride. When the two components had been thoroughly mixed 5 ml. of acetone were added and the slurry stirred gently for a few minutes and allowed to stand for a further five. It was then filtered and the filtrate added to chilled buffered antiserum. The reaction mixture as finally constituted after the addition of RB200 solution contained:-

Antiserum 1.0 ml.
1.0M carbonate/bicarbonate
buffer pH 9.0 2.0 ml.
RB200 sulphonyl chloride in acetone .. 0.1 ml.

When a globulin fraction of an antiserum rather than the whole antiserum was being labelled with RB200 the reaction mixture was made up as follows:-

Serum globulin 10 to 20 mg./ml.
1.0M carbonate/bicarbonate
buffer pH 9.0.. 20%
0.15 saline to 100%
RB200 sulphonyl chloride 1.6 to 3.2%

Conjugation was allowed to proceed for $\frac{3}{4}$ to $1\frac{1}{2}$ hours. During this time the reaction mixture was kept in constant but gentle agitation and maintained at a temperature of 0°C . to 2°C . and at a pH of 8.6 to 9.0 by the addition of buffer. A

pH meter was used to follow changes in alkalinity.

PURIFICATION OF CONJUGATES

The procedure involved in the purification of conjugates was designed to remove non-specifically reacting material and to remove any remaining antibodies.

1. REMOVAL OF NON-SPECIFICALLY REACTING MATERIAL

(a) Removal of Unreacted Protein and Molecules

Bound and unbound molecules were removed from conjugates by one of the following methods:

- (i) Dialysis against water
- (ii) Passage through a column of water
- (iii) Gel filtration
- (iv) Amalgamation of conjugates with a large amount of water

(b) Dialysis against water

Conjugates were placed in a dialysis bag which was held in a beaker of water. The bag was kept suspended in the water by means of a string. Dialysis in the presence of water was continued until one fluid change of water had been made. The dialysate over a period of 24 hours was changed several times. The dialysate was neutralized with sodium hydroxide and the pH was judged to be near 7.0.

PURIFICATION OF CONJUGATES

The procedures involved in the purification of conjugates were designed (a) to remove non-specifically reacting material and (b) to remove contaminating antibodies.

(a) REMOVAL OF NON-SPECIFICALLY REACTING MATERIAL

(a) 1. Removal of Uncombined Fluorochrome Molecules

Uncombined fluorochrome molecules were removed from conjugates by one of the following methods:-

- (i) Dialysis against saline
- (ii) Passage through ion exchange column
- (iii) Gel filtration
- (iv) Precipitation of globulins followed by
 - (i) or (iii) above in the case of whole anti-sera labelled with RB200.

(i) Dialysis against Saline

Conjugates were dialysed at 4°C. against 0.15M saline in which an ion exchange resin (De Acidite FF) was kept suspended by means of a magnetic stirrer. Dialysis in the presence of resin for 48 hours, with one fluid change, or against changes of buffered saline over a period of four days, in the absence of resin, was usually sufficient to completely free the conjugate of uncombined fluorochrome. The conjugate was judged to be free of uncombined fluorochrome if

the saline against which it was being dialysed showed no green fluorescence when placed in a beam of blue violet light and viewed from above.

(ii) Passage through Ion Exchange Resins

The methods used in the purification of conjugates were based upon those described by Coons (1958). Conjugates were dialysed overnight against buffered saline and then passed through a column of one of the following anion exchange resins:-

De-Acidite FF 20 - 50 mesh 4% cross linked.

Dowex 2-x4 chloride form 20 - 50 cross linked.

The resins were prepared for use by washing them with dilute sodium hydroxide, distilled water, dilute hydrochloric acid, distilled water and finally with buffered saline.

Conjugates greater than 5 ml. in volume were passed down 40 cm. x 1 cm. columns of either resin at a rate of 1 ml. in 40 minutes. For smaller volumes of conjugate it was found convenient either to prepare a column of the Dowex resin in a Pasteur pipette or to add the Dowex resin to the conjugate and to retrieve the conjugate by centrifugation through a seitz or membrane filter. By this latter method the conjugate was recovered with very little alteration in volume.

After passage through ion exchange resins conjugates were concentrated by dialysis against Polyethylene Glycol (15,000 to 20,000 m.w.).

(iii) Gel Filtration

Sephadex G25 of medium particle size was used and columns were prepared according to the manufacturer's instructions. Conjugates were passed down the column at a rate of 1 ml. in 20 minutes. For volumes of conjugate up to 5 ml. a 30 cm. x 1 cm. column was used and for 5 ml. to 20 ml. volumes 30 cm. x 3 cm. columns were prepared.

(iv) Fractionation of Labelled Antisera

Globulins were separated from whole RB200-labelled antisera by half saturation with ammonium sulphate.

A labelled antiserum was diluted with its own volume of distilled water. To the diluted antiserum was then added an equal volume of a half saturated solution of ammonium sulphate. Globulins were allowed to flocculate out of suspension overnight at 4°C. The precipitated globulins were packed loosely by centrifugation, washed twice in a half saturated solution of ammonium sulphate and dissolved in buffered saline. The globulin solution was freed of ammonium sulphate, either by dialysis or by passage down a column of Sephadex G25, and concentrated to about two-thirds of the original serum volume, either by dialysis against Polyethylene Glycol M.W. 20,000 or by means of dry Sephadex G25.

(a) 2. Removal of Heavily Labelled Protein

It is assumed here that the non-specific staining which persists after the removal of uncombined fluorochrome from conjugates is caused by heavily labelled proteins.

Heavily labelled proteins were removed from conjugates by absorption with acetone extracted guinea pig liver powder, used in the proportion of 100 mg. of powder per 1 ml. of conjugate. The powder was weighed out in the dry state and transferred to a centrifuge tube where it was dampened with a few drops of merthiolate buffered saline. The conjugate was then mixed in with powder and the mixture kept in constant but gentle agitation for 1 to 2 hours.

(b) REMOVAL OF CONTAMINATING ANTIBODIES

Most of the anti-tissue antisera prepared contained anti-serum protein and anti-erythrocyte activity. Anti-human tissue conjugates were therefore absorbed twice with half their own volume of washed and packed human group A and B or AB, Rh+ red cells. Anti-rat tissue conjugates were similarly absorbed with rat red cells.

Conjugates were left in contact with the cells for one hour at 37°C. The mixture of cells and conjugate was agitated at intervals during this time.

After their absorption conjugates were tested for residual agglutinating activity by slide agglutination tests using equal volumes of undiluted

conjugate and an 0.5% suspension of red cells.

In the early stages of the study anti-serum protein antibodies were removed from conjugates by absorption with undiluted human plasma. Later, when it was found that the staining produced by a conjugate absorbed with plasma differed neither in its brilliance nor in its distribution from that produced by a fraction of the same conjugate which had been absorbed with serum, Anti-serum protein antibodies were removed from conjugates by the addition of undiluted human serum in the proportion of 2 drops/ml.

After the addition of human serum conjugates were incubated at 37°C. for 30 minutes and then at 4°C. for 48 hours. They were then centrifuged. The absorption was repeated if necessary until centrifugation yielded no precipitate.

TESTS FOR COMPLETENESS OF ABSORPTION

Absorption was judged to be complete when the tests outlined below indicated that a conjugate produced specific staining and only specific staining on its interaction with a section, and that residual anti-serum protein or anti-erythrocyte activity contributed nothing to this staining.

(a) Tests of Specificity of Staining

The term "specific staining" is used to describe fluorescence which can be shown to have been produced

by the interaction of fluorochrome labelled antibodies and the corresponding antigens in tissue sections. Specific staining can be blocked by pretreatment of a section with unlabelled immune globulins representing the conjugate under test, but not by unlabelled immune globulins representing unrelated antisera nor by normal rabbit globulins. The manipulations involved in tests of specificity of staining (direct inhibition experiments) are described in Appendix 5.

(b) Tests for Residual Anti-serum Protein Activity

These tests were run in parallel with tests of specificity of staining. In these tests the staining produced by a conjugate in a section pretreated with normal rabbit globulin was compared with that produced by the same conjugate in a section pretreated with an unlabelled globulin fraction of a rabbit anti-human serum (or anti-rat serum) anti-serum.

(c) Estimation of Residual Anti-RBC Activity

These tests simply involved the comparison of the distribution of staining produced by a fully absorbed conjugate with that produced by a fraction of the same conjugate from which contaminating anti-erythrocyte antibodies had not been removed. This latter fraction had, however, been freed of uncombined fluorochrome, heavily labelled protein and anti-human serum antibodies. This test allowed an assessment to

be made of the extent to which labelled anti-erythrocyte antibodies might contribute to the staining produced by anti-tissue conjugates. It was based on the assumption that conjugates which had been absorbed with red blood cells in the manner described in section (b), page , above contained no labelled antibodies reactive with red cell antigens. Absorption with erythrocytes was found to modify the distribution of specific staining produced by anti-synovium and anti-glomerulus conjugates (see Experimental Observations. Comparison of the specificity of anti-glomerulus and anti-synovium conjugates. Staining attributable to contaminating antibodies).

STAINING EXPERIMENTS

A. GENERAL PROCEDURES

Unlabelled globulins and conjugates were centrifuged before their use in staining experiments and slides were placed in the stream of air from a fan to bring them rapidly to room temperature and minimise condensation. Where slides had been stored in sealed boxes in the presence of a dessicant, for experiments involving soluble antigens, the boxes were not opened until they had reached room temperature.

Sections were exposed to immunohistological reagents in moist chambers made out of Jelonet tins or plastic boxes painted black. A black background facilitated the detection of sections on slides. A paper hand towel affixed to the lid of the moist chamber ensured that condensed water vapour did not precipitate onto the slides.

Two sections, each to be exposed to different immunohistological reagents, were usually mounted on one slide, and it was important, therefore, to guard against the spreading and intermixing of the reagents during incubation. Two or three different experiments could be carried out on one slide provided; sections were mounted in a staggered pattern

if necessary; at least 1.5 cm. apart; the surface of the slide was never handled by the naked finger; the glass between and around the sections was dry, and the slides had been allowed to reach room temperature before application of reagents to the sections.

B. EXPERIMENTS INVOLVING THE USE OF SIMILARLY LABELLED CONJUGATES

B.1. Direct Staining, Direct Inhibition and Cross Inhibition Experiments

These experiments involved the pretreatment of sections with unlabelled globulins and their subsequent exposure to fluorochrome labelled conjugates.

They were performed in two ways. In the first of these sections were left in contact with a drop of unlabelled globulin for 1 to 3 hours at room temperature and for a further 16 to 18 hours at 4°C. Sections were then rinsed free of globulin and washed in two five-minute changes of buffered saline at 20°C. The slide between and around sections was then wiped dry, (this step also had the effect of removing excess buffered saline from the sections). Each section was then covered with a drop of conjugate and interaction between sections and conjugates was allowed to proceed for 30 to 60 minutes at room temperature. The sections were then rinsed free of conjugate, washed for at least

15 minutes in three changes of buffered saline and mounted in buffered glycerol (glycerol 7 parts, 0.01M phosphate buffered saline 3 parts, Merthiolate 0.01%).

In the second method sections were exposed first to unlabelled globulin and then to a mixture consisting of equal parts of unlabelled globulin and labelled globulin (conjugate). For the first of these steps columns of unlabelled globulin 2mm. to 5mm. long were drawn into lymph tubes of standard bore (0.9mm.). Sections were reacted with unlabelled globulins for $1\frac{1}{2}$ to 3 hours at 20°C. and for 16 to 18 hours at 4°C. The moist chambers containing the slides were then removed from the refrigerator and allowed to reach room temperature. A volume of conjugate equal to the volume of globulin originally applied to the section was added to the globulin covering the section and gently mixed in with it. The mixture of labelled and unlabelled globulin was left in contact with the section at room temperature for $\frac{1}{2}$ to 3 hours. Sections were then rinsed free of the staining mixture and washed for at least 15 minutes in three changes of buffered saline. It was sometimes necessary to wash RB200 stained sections with a dilute solution of Bovine Serum Albumin in buffered saline overnight at 4°C. in order to reduce the intensity of background

staining. The experimental manipulations involved in direct staining, direct inhibition and cross inhibition experiments are summarised below.

Experiment	Pretreatment (Unlabelled Globulin) $\frac{1}{2}$ - 3 hrs. 20°C. 16 -18 hrs. 4°C.	Conjugate $\frac{1}{2}$ - 3 hrs. 20°C.
Direct Staining	NRG NRG	F-anti- glomerulus F-anti- synovium
Direct Inhibition	Anti-glomerulus Anti-synovium	F-anti- glomerulus F-anti- synovium
Cross Inhibition	Anti-synovium Anti-glomerulus	F-anti- glomerulus F-anti- synovium

NRG = Normal Rabbit Globulin.

When available, a globulin fraction of a rabbit antiserum raised against an unrelated antigen (e.g. guinea pig liver, human globulin) was used in the place of normal rabbit globulin.

B.2. Cross Absorption and Cross Absorption Inhibition Experiments

1. Reagents

- (i) F-anti-glomerulus x synovium, 2 ml.
- (ii) anti-glomerulus x synovium, 2 ml.

Fluorescein labelled and unlabelled anti-glomerulus globulins each absorbed twice

with 300 mg. of synovial tissue.

(iii) F-anti-glomerulus.

(iv) anti-glomerulus.

The conjugate and immune globulin pools from which the cross absorbed reagents were prepared each contained 22 mg. protein/ml..

Before cross absorption the conjugate was freed of non-specifically reacting material and contaminating anti-erythrocyte and anti-serum protein antibodies. Its protein concentration was then matched with that of the unlabelled globulin solution by over-concentration and subsequent dilution. Concentration of the conjugate was carried out by suspending in the conjugate a bag made out of Visking cellulose tubing 1/4" in diameter containing powdered polythethylene glycol M.W. 20,000 damped with buffered saline.

(v) F-anti-synovium x glomerulus, 2 ml.

(vi) anti-synovium x glomerulus, 2 ml.

Fluorescein labelled and unlabelled anti-synovium globulins each absorbed twice with 220 mg. of homogenized renal glomeruli.

(vii) F-anti-synovium.

(viii) anti-synovium.

Labelled and unlabelled globulin fractions of anti-synovium antisera, each containing 21 mg. protein/ml.. The F-anti-synovium conjugate was prepared for cross absorption in the same way as the F-anti-glomerulus conjugate.

2. Preparation of Cross Absorbed Reagents

For the preparation of the reagents used in these experiments saline suspensions of homogenates of synovial tissue and isolated renal glomeruli were freed of fluid by centrifugation through Whatman No.50 paper, supported in a seitz filter tube on a seitz filter pad.

Three hundred milligrammes of synovial tissue and 220 mg. of the glomerular homogenate were added to and suspended in 2 ml. of the appropriate conjugate or unlabelled globulin in test tubes.

The tissues and immunohistological reagents were incubated, with agitation at 10 minute intervals, for 1 hour at 37°C. They were then separated by filtration in a centrifuge.

The process of absorption was repeated.

After the final centrifugation Merthiolate to 0.01% was added to each reagent, even though they had all contained this preservative in this concentration before absorption.

3. Staining Procedures

The second of the staining methods described in Section B.1. above was used for cross absorption inhibition experiments. By this method sections were exposed to a mixture of labelled and unlabelled globulins after having been pretreated with the unlabelled globulin alone. This method, however, was not applied in the case of the cross absorption procedure. F-anti-glomerulus x synovium and F-anti-synovium x glomerulus conjugates were applied directly to previously untreated sections.

4. Estimation of Amount of Antigen Required to Effect Cross Absorption

Homogenates of isolated renal glomeruli and synovial tissue in suspension were prepared according to the methods described in Appendix 1.

Separation of tissue from saline was achieved by centrifugation through cellulose acetate membrane filters or through Whatman No.50 filter paper, supported in a seitz filter tube by a seitz filter pad.

The centrifuged tissue was weighed out in 25, 75, 125 and 150 mg. amounts and transferred to centrifuge tubes. The F-anti-glomerulus conjugate was added in 0.5 ml. volumes to tubes containing synovial tissue and the F-anti-synovium conjugate in the same volumes to tubes containing glomerular tissue. Tissues were suspended in the conjugates

and incubated with them in a water bath for one hour. The contents of each tube was gently re-mixed six times during incubation. After incubation tissues and conjugates were separated by centrifugation.

Direct staining experiments in which sections of synovial tissue were exposed to the absorbed and unabsorbed fractions of the F-anti-glomerulus conjugate and sections of kidney to the F-anti-synovium preparations, provided a guide to the amount of tissue required to effect cross absorption.

The experiments were repeated. On this occasion volumes of conjugate increasing from 0.27 ml. to 0.40 ml. were added to a fixed amount of tissue.

These experiments indicated that not less than 300 mg. of synovial tissue were required to remove staining reactivity for the synovial cell layer from 1 ml. of the F-anti-glomerulus conjugate, and that about 220 mg. of the renal glomerular homogenate would be required to remove staining reactivity for glomerular epithelial cells from the F-anti-synovium conjugate (see table overleaf).

ABSORPTION OF F-ANTI-GLOMERULUS WITH
SYNOVIAL TISSUE

Synovial Tissue mg.	F-anti-glomerulus ml.	Staining Produced in Synovial Cell Layer
25	0.5	Bright
75	0.5	Bright
125	0.5	Present
150	0.5	Absent
100	0.27	Absent
100	0.30	Absent
100	0.33 *	Absent
100	0.35	Absent
100	0.37	?Present
100	0.40	Present
-	Unabsorbed F-anti-glomerulus	Bright

ABSORPTION OF F-ANTI-SYNOVIUM WITH
RENAL GLOMERULAR HOMOGENATE

Glomerular Tissue mg.	F-anti-synovium ml.	Staining of Glomerular "Cells"
25	0.5	Bright
75	0.5	Bright
125	0.5	Absent
150	0.5	Absent
75	0.27	Absent
75	0.30	Absent
75	0.33	Absent
75	0.35 *	Absent
75	0.37	Absent
75	0.40	?Present
-	Unabsorbed F-anti-synovium	Bright

* Synovial tissue was used in the proportion of 300 mg./ml. to absorb the anti-glomerulus reagent, and Glomerular tissue in the proportion of 220 mg/ml. to absorb the anti-synovium reagent.

Methods used for the cross absorption of the F-anti-glomerulus and the F-anti-synovium conjugates were followed also in the cross absorption of the unlabelled globulin fractions of anti-glomerulus and anti-synovium antisera.

C. EXPERIMENTS INVOLVING THE USE OF CONTRASTINGLY LABELLED CONJUGATES

The manipulations involved in mixed staining and related experiments were based on those described in Section B above, and the same precautions were taken to avoid intermixing of the conjugates, or mixtures of conjugates, covering the two sections mounted on each slide.

Sections were exposed to equal volumes of anti-glomerulus and anti-synovium conjugates labelled contrastingly. In mixed staining experiments the conjugates were applied to sections simultaneously and in cross inhibition and cross exchange experiments they were applied in sequence.

Volumes were measured in terms of the length of the column of the reagent in capillary tubes of standard (0.9 mm.) diameter.

C.1. Mixed Staining Experiments

For mixed staining experiments slides, either freshly prepared in the cryostat or stored at 4°C. and bearing two or three sections, were dried in front of a fan until they had reached room temperature. The staining mixtures to be used were prepared either immediately before application to sections on clean microscope slides or shortly before use in precipitin tubes. Sections were incubated in moist chambers at room temperature for two to three hours and usually for a further 16

to 18 hours at 4°C. They were then rinsed free of the staining mixtures, washed for at least 15 minutes in three changes of buffered saline and finally mounted in glycerol saline. The proforma of a typical mixed staining experiment is set out below.

MIXED STAINING EXPERIMENTS

Tissue	Section	Staining Mixture 2 hrs. - 20°C. + 16 hrs. - 4°C.
Spleen	1	F-anti-glomerulus + RB-anti-synovium
	2	F-NRG + RB-anti-synovium
	3	F-anti-glomerulus + RB-NRG
	4	RB-anti-glomerulus + F-anti-synovium
	5	RB-NRG + F-anti-synovium
	6	RB-anti-glomerulus + F-NRG

C.2. Cross Inhibition and Cross Exchange Experiments

In these experiments sections were pretreated with anti-tissue conjugates of the one specificity (F-anti-glomerulus, for example) and then exposed to contrastingly labelled anti-tissue conjugates of the other specificity (e.g. RB-anti-synovium). A saline wash was interposed between the two treatments and the glass between and around sections was wiped dry before application of the second reagent to the sections.

In cross inhibition experiments sections were incubated with the first conjugate for 18 hours (2 hours at room temperature and 16 hours at 4°C.) and the second conjugate for 30 to 45 minutes at room temperature.

Cross exchange experiments also involved pre-treatment for 2 hours at room temperature and 16 to 18 hours at 4°C. Exposure to the second conjugate, however, varied in duration from $\frac{1}{2}$ to 18 hours (up to 3 hours at room temperature followed in some cases by a further period of 16 hours at 4°C.).

C.3. Direct Exchange Experiments

Direct exchange experiments were performed in the same way as cross exchange experiments, but the two contrastingly labelled conjugates to which individual sections were exposed had both been prepared from the one antiserum.

APPENDIX 6

METHODS USED DURING IMMUNOHISTOLOGICAL STUDIES OF CONNECTIVE TISSUE IN THE ADULT AND EMBRYONIC RAT

A. PREPARATION OF TISSUE ANTIGENS

1. Adult Rat Renal Glomerulus

For the preparation of the adult rat renal glomerular antigen, glomeruli were isolated from the kidneys of adult rats in the way already outlined for the isolation of human renal glomeruli (Appendix).

2. Adult Rat Renal Connective Tissue

Homogenates of connective tissue derived from the kidneys of adult rats and enriched with isolated rat renal glomeruli were used as an antigen in preference to whole rat kidney because;

(a) antisera against homogenates of whole rat kidney invariably react strongly with the cytoplasm and brush borders of renal cortical tubules (C.T.), see Hill and Cruickshank (1953);

(b) the presence in anti-whole kidney antisera of antibodies to constituents of renal tubular cells might, it was thought, add unnecessary complications to studies of embryonic tissues if such antisera were used for these studies;

(c) antisera raised against isolated renal glomeruli and the majority of antisera raised against renal connective tissues, isolated renal glomeruli enriched with cell-free renal connective tissues, do not react in immunohistological staining experiments with renal tubular epithelium.

Homogenates of adult rat renal connective tissue were used as antigens in addition to isolated renal glomeruli because studies of human connective tissues had suggested that certain connective tissue antigens, present for example in vascular adventitia, might be poorly represented in isolated renal glomeruli.

Material from which the adult rat renal connective tissue antigen was prepared was obtained in the course of isolation of renal glomeruli. During isolation of rat renal glomeruli material retained by the monel woven wire gauze sieves through which the renal mesh was successively passed was harvested into chilled (4°C.) phosphate buffered saline (0.05M phosphate pH 8.6) containing 0.01% Merthiolate. Salvage of material held back on a sieve was achieved by inverting the sieve and washing it free of retained renal tissue with jets of buffered saline from a plastic wash bottle.

Renal tissue reclaimed from the sieves was

packed in a refrigerated centrifuge, resuspended in 60 ml. volumes of buffered saline and homogenized in an M.S.E. homogeniser.

Homogenization yielded (a) relatively large fragments and fibres of connective tissue, (b) fine connective tissue fibrils and (c) lumps of renal tissue which had resisted homogenization.

The larger connective tissue fibres were found suspended in the froth generated during homogenization. These were immediately harvested by suction and stored at 4°C. Intact renal tissue was allowed to settle out of suspension. The supernatant fluid, containing intact cells, singly or in groups, connective tissue fibrils and cellular debris, was transferred to centrifuge tubes and packed at 4000 r.p.m. (R.C.F. 2384).

The intact renal tissue remaining in the homogenization flask was submitted to further cycles of homogenization and sedimentation until the renal tissue had been more or less completely broken up. Each cycle of homogenization yielded connective tissue fibres and blood vessels floating in froth at the surface of the saline, and smaller fibrils, intact cells and cellular debris suspended in the saline. These were harvested separately and stored as:

- (a) Fraction 1. Centrifuged (4000 r.p.m.) deposit from froth.

(b) Fraction 2. Centrifuged (4000 r.p.m.)
deposit from saline.

These fractions were re-suspended separately in pH 8.6 buffered saline and fragmented in glass tissue grinders. The two homogenates so obtained were each washed repeatedly by centrifugation at 3200 r.p.m. (R.C.F.1548) for 1 to 3 minutes. Re-suspension between periods of centrifugation was carried out by means of glass tissue grinders and centrifugation was repeated until clear supernatant fluids were obtained.

The two deposits representing washed fractions 1 and 2 were re-suspended in buffered saline. Each suspension was then centrifuged at R.C.F.'s. increasing from 6 through 24, 96, 216, 384 and 600 to 1355. The seven secondary deposits obtained at the serial centrifugations were re-suspended in buffered saline and a sample of each suspension was examined microscopically. Suspensions heavily contaminated with cells or cellular debris were discarded. Fractions 1 and 2 of renal connective tissue were then re-constituted as purified fractions. These were packed at 18,000 r.p.m. (R.C.F.25,000) and re-suspended in 0.01M phosphate buffered saline pH 7.2 to give 40% v/v suspensions.

The renal connective tissue antigen as finally constituted contained one volume of the 40% suspension of fraction 1, one volume of the 40%

suspension of fraction 2 and two volumes of a 40% suspension of packed homogenised renal glomeruli.

3. Adult Rat Splenic Reticulum Antigen

The adult rat splenic reticulum antigen was prepared from the spleens of rats furnishing the renal glomerular and renal connective tissue antigens.

Spleens were sectioned longitudinally into slices 3 mm. thick. These slices were freed of cells by vigorous agitation in chilled (4°C.) phosphate buffered saline (0.05M phosphate pH 8.6). It was sometimes necessary to supplement agitation by cutting into islands of retained cells.

The white spongy cell-free pieces of splenic tissue so obtained were freed of trabecular tissue and the larger blood vessels by passing them, with gentle pressure, successively through 50, 80, 100 and 120 mesh sieves. Material passing the 120 mesh sieve was fragmented in a glass tissue grinder and freed of cellular debris by repeated centrifugation and re-fragmentation. It was then re-suspended from centrifugation at 18,000 r.p.m. to give a 20% v/v suspension in pH 7.2 phosphate buffered saline containing 0.01% Merthiolate and stored at -15°C.

4. Embryonic Connective Tissue

(a) Immunising Antigen. Anti-embryonic connective tissue antisera were raised against cell

free fibrillar tissues obtained from embryos measuring up to 1.5 cm. in crown to rump length.

Embryos previously removed from the uterus, freed of placental membranes, quick frozen in liquid nitrogen when available or in an alcohol/dry-ice freezing mixture and stored at -15°C . were removed from store. They were then decapitated. Sections 10 to 20μ thick, cut from these embryos on a freezing microtome, were vigorously agitated in chilled pH 8.6 buffered saline. Agitation yielded:

- (i) fibrils suspended in the froth generated during agitation;
- (ii) fibrillar and membranous material retained by 140 mesh sieves;
- (iii) fibrils, cells and cellular debris passing 140 mesh sieves.

The third of these fractions was set to one side at 4°C . for 20 to 30 minutes. Meanwhile fractions 1 and 2 were harvested and pooled.

During their period of passive sedimentation the constituents of fraction 3 had become redistributed as sediment and supernatant. The sediment contained cells and groups of cells, some with attached basement membranes. The supernatant contained fine connective tissue fibrils and cellular debris. The supernatant was decanted into a separate container and the fine connective tissue fibrils

it contained were allowed to settle out of suspension at 4°C. over a period of $\frac{1}{2}$ to 1 hour. These fibrils were then harvested and added to the 1st and 2nd fractions of embryonic connective tissue. The pooled tissues were then packed by centrifugation, re-suspended in a convenient volume of buffered saline and fragmented in a glass tissue grinder. The homogenate was freed of cellular debris by repeated centrifugation and refragmentation and finally re-suspended after centrifugation at 18,000 r.p.m. to make a 20% v/v suspension in buffered saline (0.01M phosphate pH 7.2).

(b) Absorbing Antigen. Embryonic connective tissues used for the cross absorption of F-anti-adult rat connective tissue antisera was obtained from larval-like embryos measuring up to 0.7 cms. in overall length and from small incompletely developed rat-like embryos measuring up to 0.7 or 1.0 cm. in crown to rump length.

The embryos which had previously been quick frozen and stored at -15°C. were allowed to thaw out completely. They were then homogenised briefly (30 seconds) in chilled pH 8.6 buffered saline. The tissue suspension so obtained was washed three times in buffered saline. The washed sediment contained not only sheets and fibrils of connective tissue but also clumps of intact cells, some with adherent basement membrane. No attempt was made to purify

the homogenate further. It was simply fragmented in a glass tissue grinder, packed at 18,000 r.p.m. and suspended in 4 volumes of pH 7.2 buffered saline.

PREPARATION OF ANTISERA

Antigens were injected into rabbits as a 10% v/v suspension in saline or as a water in oil emulsion in Freund's complete or incomplete adjuvant.

The antigen-complete adjuvant emulsion contained:

Antigen in saline, 20% v/v suspension ...	5 ml.
Light liquid paraffin	4.25 ml.
Crill 19 (Croda Ltd., Snaith, Yorks.) ...	1.5 ml.
Killed dried M. tuberculosis	2.5 mg.

Animals received 1 to 4 four-week courses of immunising injections. A course of immunising injections was initiated by the subcutaneous injection of 4 x 0.5 ml. of antigen in complete adjuvant into four separate sites in the back or limbs of animals on a single occasion. During the next three weeks animals received either 3 x 0.5 ml. of antigen in incomplete adjuvant or 12 intraperitoneal injections of antigen in saline, administered in 1 ml. amounts on 4 days of the week.

The immunising schedules adopted in the preparation of antisera used in studies of connective tissue

in adult and embryonic rats are set out in the table below.

PREPARATION OF ANTI-ADULT AND ANTI-EMBRYONIC
RAT CONNECTIVE TISSUE ANTISERA AND OF ANTI-HUMAN
RENAL CONNECTIVE TISSUE ANTISERA

Immunisation Schedules

Rabbit	Immunising Injections Number of 4-week courses each course comprising antigen in complete adjuvant 4 x 0.5 ml. followed by antigen in A or B Incomplete Adjuvant Saline 2 x 0.25 ml./wk. 4 x 1 ml./wk. x 3 wks. x 3 wks.		Antigen
S28 S27	-	4 4	ARG
L3 L4 S29	3 1 -	- 2 2	ARK
L5 L6 L9 L10	3 1 2 -	- 2 1 1	ARE
S26 S25		1 1	ARK
S17 S18	Two 2 ml. injections of antigen in complete adjuvant, one 4 months before and the other 2 weeks before venepuncture.		ARS

ARG = adult rat renal glomerulus.
 ARK = " " " connective tissue.
 ARE = embryonic rat connective tissue.
 ARK = adult human renal connective tissue.
 ARS = adult rat splenic reticulin.

CONJUGATION OF ANTISERA AND PURIFICATION OF CONJUGATES

Animals were bled from an ear vein on the 7th, 10th and 12th day after the completion of a course of immunising injections. Crude globulin fractions of the antisera so obtained were conjugated with fluorescein isothiocyanate by the method described in Appendix 3. Conjugates of like specificity were later pooled and pools were similarly prepared from unlabelled anti-adult and anti-embryo globulins.

Before their use in staining experiments conjugates were freed of unbound fluorochrome by passage down a column of Sephadex G25. They were then absorbed with guinea pig liver powder (100 mg./ml. conjugate) and twice with half their own volume of packed washed rat erythrocytes. To each conjugate was then added normal rat serum in the proportion of 0.125 ml./ml. conjugate. Finally, conjugates were concentrated by means of dry Sephadex G25. Concentration was carried out by adding 1g. of dry Sephadex G25 to 4 ml. of the conjugate. The slurry so formed was allowed to stand for 15 to 30 minutes. The conjugate and Sephadex were then separated by centrifugal filtration through membrane filters.

PREPARATION OF CROSS ABSORBED CONJUGATES

The purpose of cross absorption experiments was to determine whether quantitative differences

were detectable between the antigenic constitution of embryonic and adult connective tissues. F-anti-adult conjugates were repeatedly absorbed with small amounts of the embryonic cross absorbing antigen. The process of cross absorption was followed immunohistologically and carried up to, but not beyond the point where the cross absorbed conjugate was found to have lost its ability to produce detectable staining in dermal connective tissues of small rat embryos, measuring about 0.7 cm. or 1.0 cm. in crown to rump length.

In earlier experiments weighed amounts (15 to 30 mg./ml.) of the cross absorbing antigen were suspended in 3 ml. of a conjugate. In later experiments it was found more convenient to add 3 ml. of a conjugate to the high speed sediment obtained from 3 ml. of a 5% v/v suspension of the embryonic cross absorbing antigen.

Before their use in staining experiments the volume of the cross absorbed conjugates was adjusted to 2.75 ml. by preliminary over-concentration with dry Sephadex G25 and subsequent dilution with 0.01M phosphate buffered saline pH 7.2.

STAINING EXPERIMENTS

Four types of staining experiments were performed in immunohistological studies of connective tissue in the adult and embryonic rat. These were direct

staining, direct inhibition, cross inhibition and cross absorption experiments.

The manipulations involved in these experiments did not differ significantly from those described in Appendix 5B.

In direct staining experiments sections were pretreated with unlabelled globulin fractions of anti-human renal connective tissue antisera. They were then incubated with a mixture containing equal parts of the unlabelled anti-human renal connective tissue globulin and one or other of the anti-rat connective tissue conjugates.

Direct inhibition experiments were run in parallel with direct staining experiments. These experiments were performed in the same way as direct staining experiments but involved the use of unlabelled globulin fractions of anti-rat connective tissue antisera in the place of the anti-human renal connective tissue globulins. Thus sections were finally reacted with an F-anti-rat tissue conjugate in the presence of an equal volume of the corresponding unlabelled anti-rat tissue globulin.

Cross inhibition experiments. In these experiments the F-anti-embryo conjugate was reacted with sections in the presence of unlabelled anti-adult globulins. The inhibitory activity of unlabelled anti-embryo globulins towards F-anti-adult connective

tissue conjugates was similarly tested, by pre-treating sections with unlabelled anti-embryo preparations and then reacting them with a mixture containing equal parts of the anti-embryo preparation and an F-anti-adult connective tissue conjugate.

Cross Absorption Experiments. In these the staining produced in sections by cross absorbed F-anti-adult rat connective tissue conjugates was compared with that seen in sections stained by F-anti-adult rat connective tissue conjugates which had been absorbed with one-quarter of their own volume of the human renal connective tissue antigen.

MICROTOMY

A. PREPARATION OF QUICK FROZEN UNFIXED BLOCKS OF TISSUE

Human tissues obtained at autopsy or biopsy were cut into blocks not larger than 1 cm. x 5 mm. x 5 mm. The blocks were immediately placed into test tubes and the tubes, sealed with rubber bungs and plastic adhesive tape, were plunged into a dri-ice-acetone slush, not deeper than half the length of the tubes. Tubes and slush were then transferred to the deep freeze where the tubes were removed from the slush and drained free of acetone over a period of hours. Blocks were eventually transferred either in the deep freeze or cryostat to pre-cooled bijou bottles or to small polythene containers with tightly fitting lids.

Rat tissues were treated in much the same way as human tissues, except that liquid nitrogen was the freezing agent used in most instances. Rat embryos up to 1 cm. in crown to rump length were frozen as a single block, and embryos larger than this were sectioned sagittally or transversely into two or more blocks for freezing.

B. PREPARATION OF TISSUE SECTIONS

1. Cryostat

Sections about 6 μ thick were cut from quick

frozen unfixed blocks of tissue in a refrigerated microtome cabinet at a temperature of -15°C . to -20°C . The cabinet, constructed by the Pressed Steel Company, Oxford, was essentially similar to that described by Coons and his colleagues (Coons and Kaplan, 1950; Coons, Leduc and Kaplan, 1951).

A Cambridge plane cutting microtome and a wedge knife with the "window" attachment described by Coons, Leduc and Kaplan (1951) were used for the preparation of sections.

Sections were lifted off the knife blade with a paint brush, transferred in the cabinet to acid cleaned slides which had been placed in the cabinet some time before the start of the cutting session. If necessary, sections were flattened onto the slide by holding the tip of a naked finger immediately below the section. The mounted sections were withdrawn from the cabinet without being allowed to re-freeze, and dried at room temperature in front of a fan for one hour. It was customary to mount two, or occasionally more sections on one slide. In these cases all sections were positioned on the slide before any one had been thawed and all were thawed simultaneously by placing the slide on the ball of the thumb.

It was usually found convenient to start a staining experiment soon after the sections had been dried and to allow the experiment to proceed at 4°C .

overnight. Sections not used immediately were stored at 4°C. for periods up to 10 days.

2. Freezing Microtome

Some of the rat tissues examined were frozen in a solid carbon dioxide acetone mixture and cut in the refrigerated microtome cabinet. Most of them, however, were frozen in liquid nitrogen and cut on a modified Reichert OmP freezing microtome, incorporating a thermo-electrically cooled stage and the conventional carbon dioxide knife cooling device. This is a relatively inexpensive piece of equipment, with which it is possible, with patience, to obtain several acceptable sections from one block of tissue. Since there is no way of controlling the temperature of the knife, it is not possible to cut sections of consistent thickness nor to prepare serial sections.

Frozen sections cut with the modified freezing microtome were mounted onto slides at room temperature in the following way. Sections were either cut onto the cold stage or picked off the knife with tissue forceps, or stored while not in use in solid carbon dioxide. Sections cut onto the cold stage were picked up off the stage by means of a microscope slide and immediately placed in front of a fan. Sections prepared and mounted in this way, unlike those prepared and mounted in the refrigerated microtome cabinet, very readily become detached from

their slides during staining procedures. The use of gelatinised slides has not offered a way round this difficulty.

APPENDIX 7

MONOCHROME PRINTS

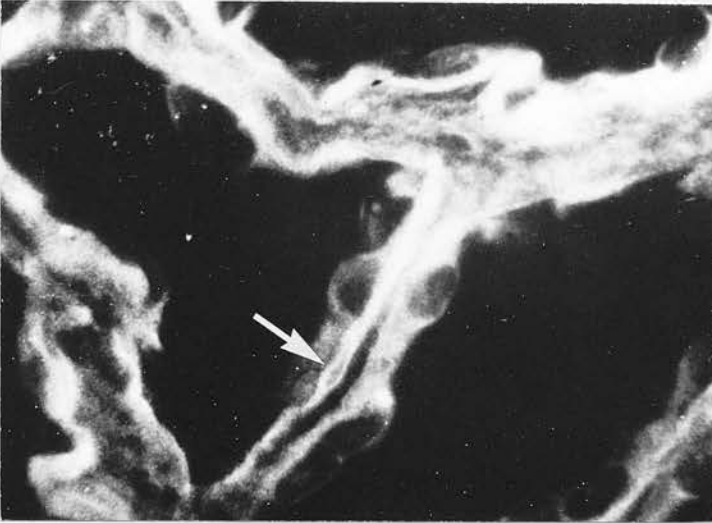


Fig. 1. Human kidney treated with a fluorescein anti-Glomerulus conjugate. The boundary membranes of tubules show specific staining. A small unstained spindle-shaped split can be seen in the membrane around one of the tubules. The faint blueish autofluorescence of the cytoplasm of tubular epithelial cells is exaggerated in this monochrome copy of a colour transparency. Enlarged x 2.5 from x 400.

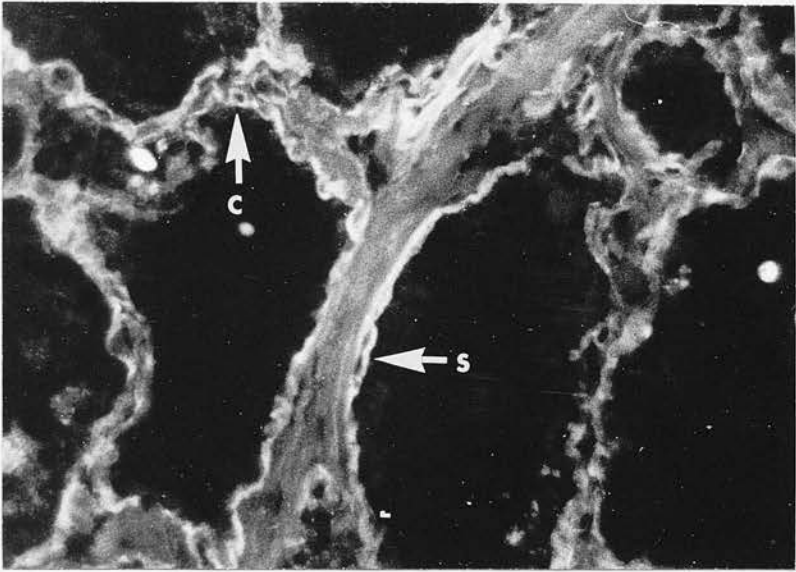


Fig. 2. Human thyroid treated with a fluorescein anti-Glomerulus conjugate. The acinar boundary membrane is seen as a white line which splits to enclose unstained spindle-shaped (S) or circular (C) spaces.

Enlarged x 3.5 from x 80.

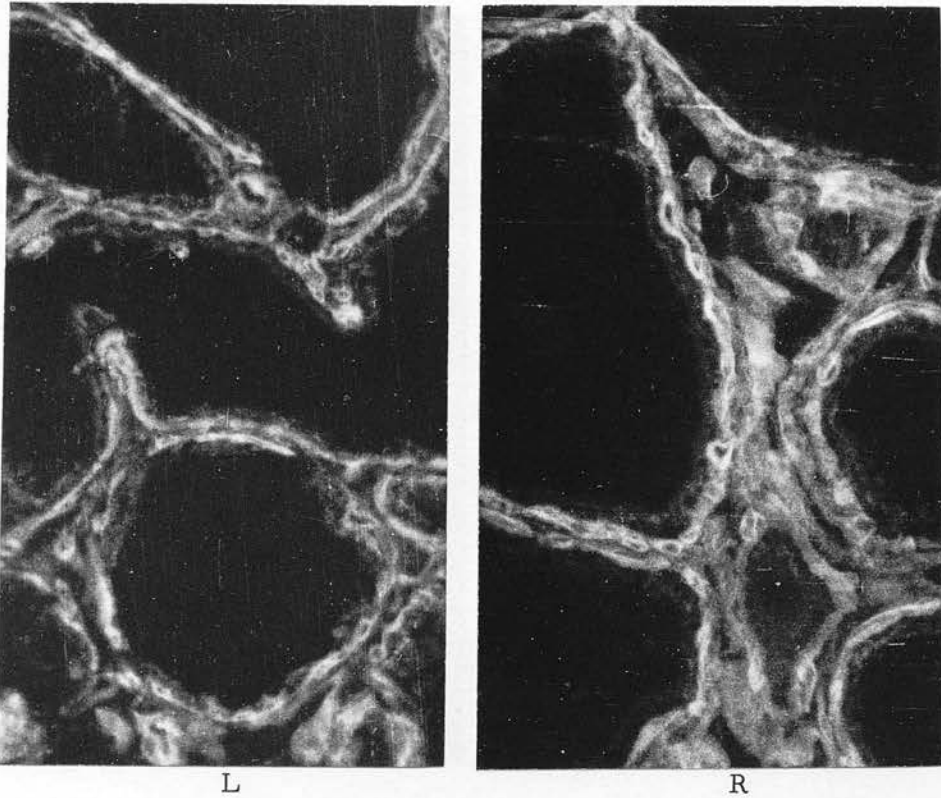


Fig. 3. Human thyroid glands stained with a fluorescein anti-Synovium conjugate. In some anti-Synovium stained sections of the thyroid gland an occasional acinus was seen to be surrounded by capillaries but not completely invested by a continuous membrane (L). More usually, however, the pattern of peri-acinar staining produced by anti-Synovium conjugates could not readily be distinguished from that produced by anti-Glomerulus conjugates, compare (R) with Fig. 2 (C) and with C.T. 12. Enlarged x 3.5 from x 50 and x 100.

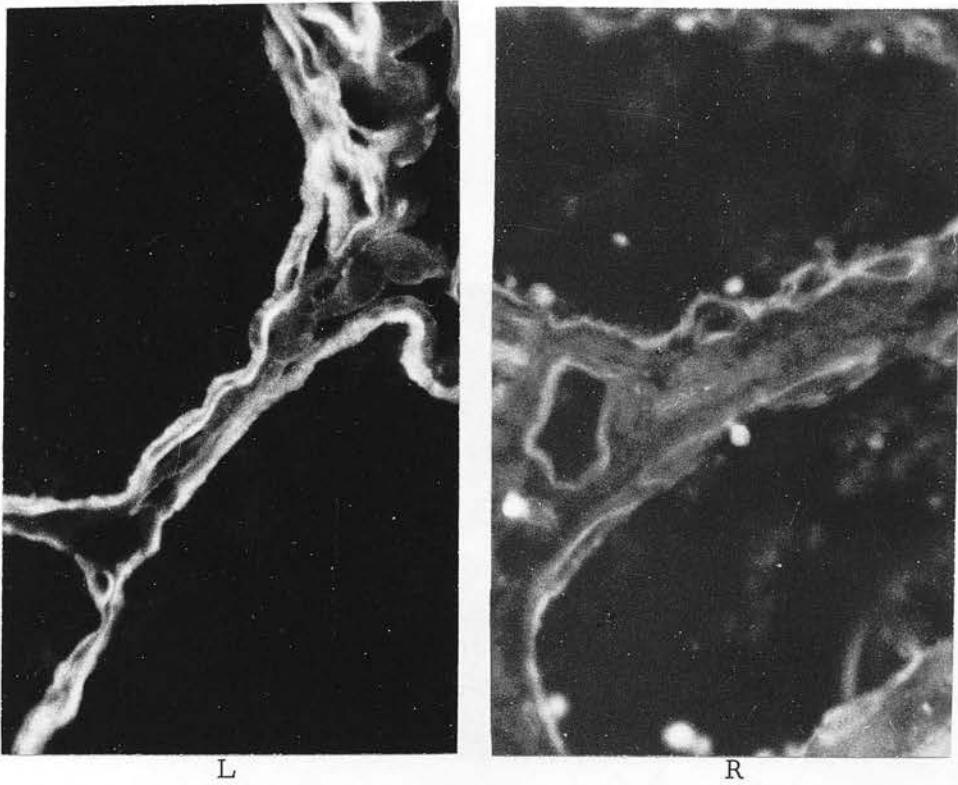


Fig. 4. Human thyroid glands treated with a fluorescein anti-Glomerulus conjugate. (L) and (R) illustrate the different patterns of peri-acinar staining produced by anti-Glomerulus conjugates in the thyroid gland. In (L) the acinar boundary membrane is seen as a thick structure which can be resolved into two components. The appearances in (R) suggest that the acinus is bounded by a single membrane and surrounded by capillaries. Enlarged x 3.5 from x 180 and x 320.

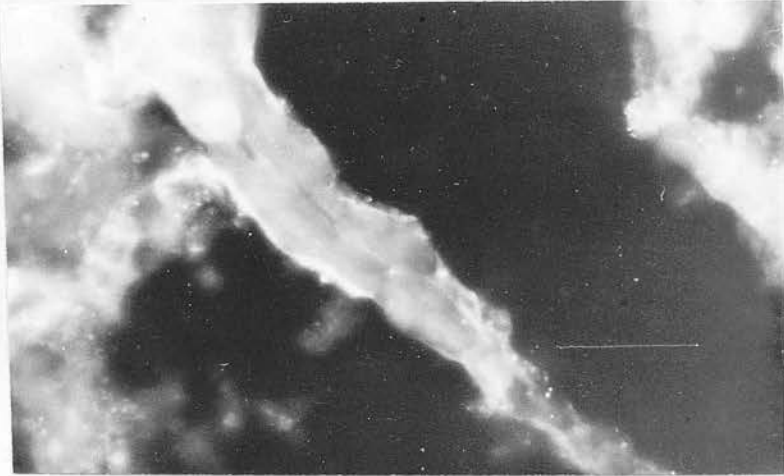


Fig. 5. Anti-Synovium ---> fluorescein anti-Glomerulus cross inhibition experiment human thyroid gland. The section was pre-treated with unlabelled globulins from an anti-Synovium anti-serum for 18 hours and then reacted with a fluorescein anti-Glomerulus conjugate for 1 hour. Peri-acinar staining appears as a single fine line, capillaries are unstained. Enlarged x 2.5 from x 400.

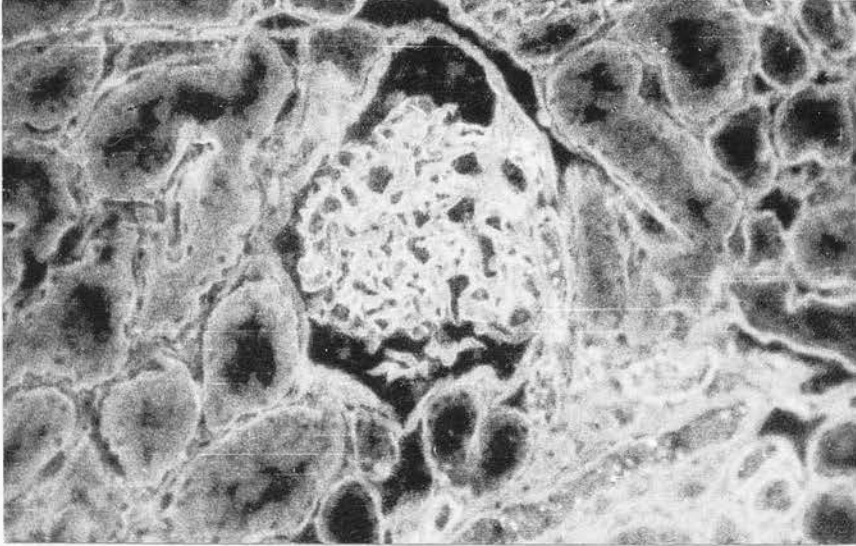


Fig. 6. Human kidney stained with a fluorescein anti-Glomerulus conjugate. Specific staining outlines glomerular capillaries and is seen also in the media of an arteriole and in the boundary membranes of tubules.

Enlarged x 3.5 from x 80.

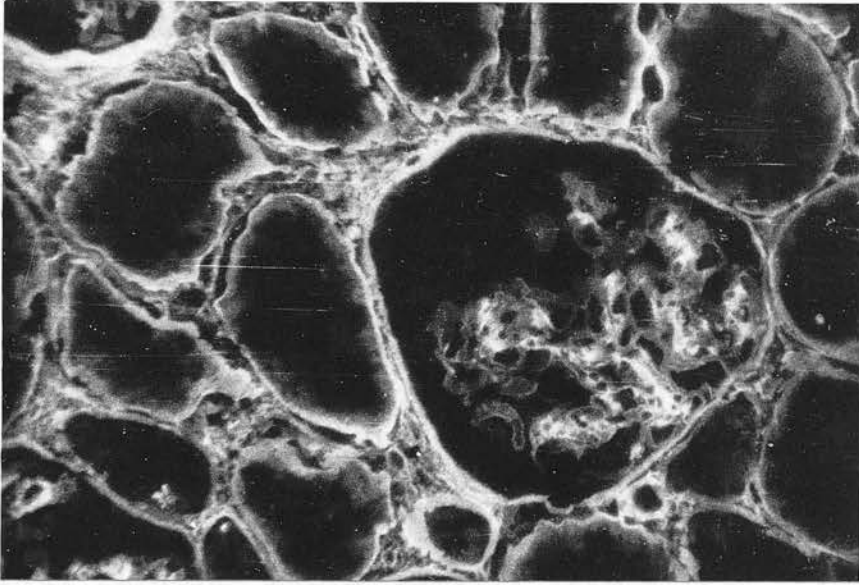


Fig. 7. Human kidney stained with a fluorescein anti-Synovium conjugate. Specific staining is seen in the boundary membranes of tubules. Staining in the glomerular capillary tuft is confined to cell-like bodies, the glomerular capillaries show a dull autofluorescence. Enlarged x 3.5 from x 120.

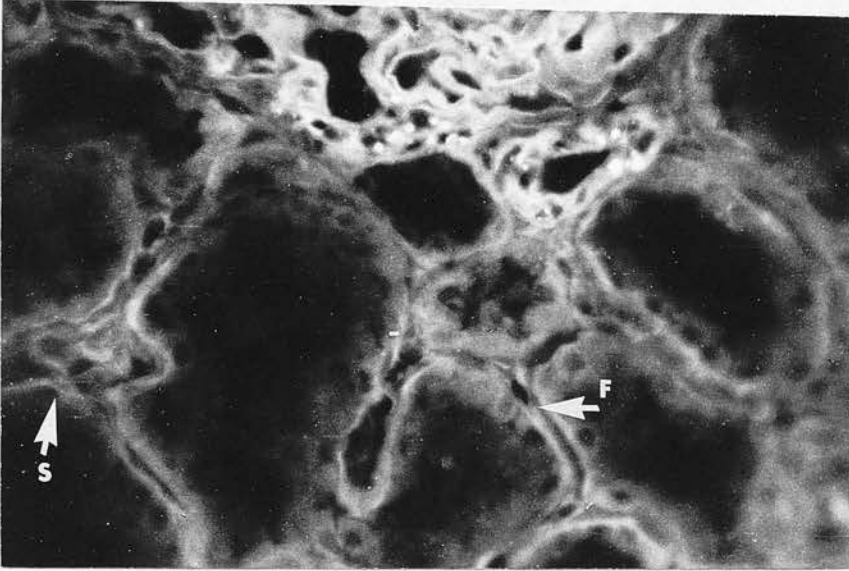


Fig. 8. Human kidney stained with a fluorescein anti-Glomerulus conjugate. Specific staining is seen around and between tubules, in the media of an artery and in glomerular capillaries. The tubular boundary membrane at S appears to split to enclose a narrow unstained slit. Staining between tubules involves capillaries and fine fibrils (F) which fuse with the boundary membranes of tubules.

Enlarged x 3.5 from x

9



10

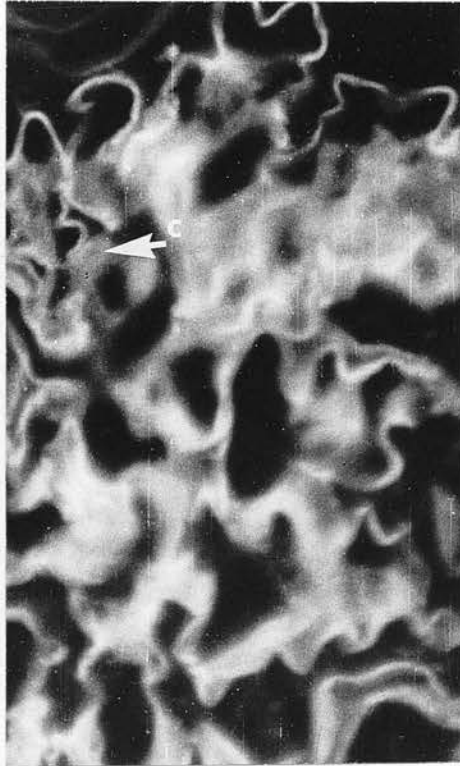


Fig. 9. Human renal glomerulus stained with a fluorescein anti-Synovium conjugate. Specific staining of cell-like bodies in the capillary tuft. Enlarged x 3.5 from x 320.

Fig. 10. Human renal glomerulus stained with a fluorescein anti-Glomerulus conjugate. The basement membranes of capillaries are seen as convoluted lines, loops and filmy tubes. Structures thought to be epithelial cells (C) are seen as masses, sometimes grouped around unstained spaces. Enlarged x 3.5 from x 400.

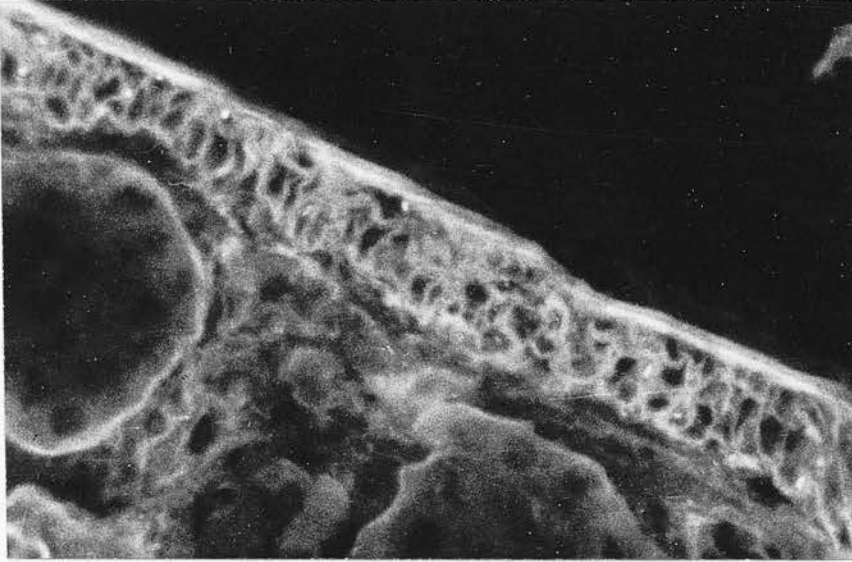


Fig. 11. Artery in human kidney stained with a fluorescein anti-Glomerulus conjugate. The conjugate has produced a reticulate pattern of specific staining in the media of the vessel. The internal elastic lamina is rendered visible by virtue of its autofluorescence, the intima is unstained. Enlarged x 3.5 from x 300.

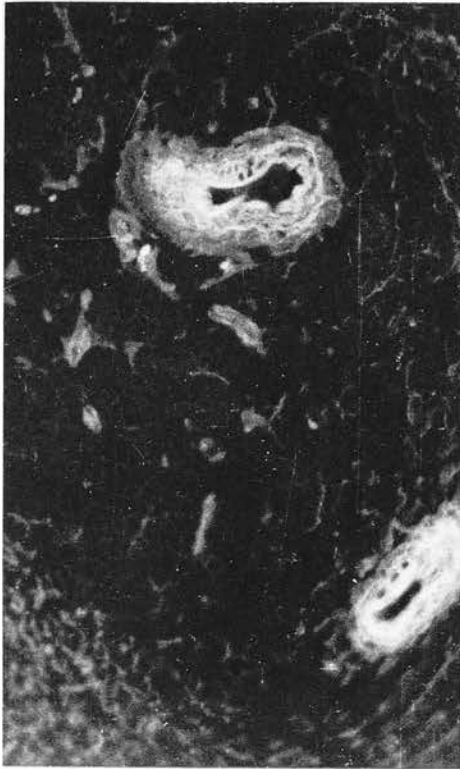
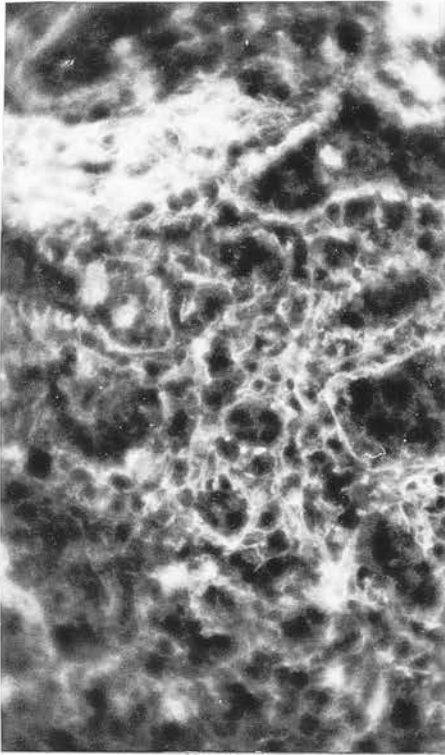


Fig. 12. Arteries in white pulp of human spleen stained with a fluorescein anti-Glomerulus conjugate. The media of the vessels show specific staining. Enlarged x 3.5 from x 40.

Fig. 13. Arteries in white pulp of human spleen stained with a fluorescein anti-Synovium conjugate. The media of the vessels are unstained. Enlarged x 3.5 from x 40.



L



R

Fig. 14. Human spleen stained with a fluorescein anti-Glomerulus conjugate. The reticulum of the red pulp shows specific staining. Staining is seen also in the wall of a branch of a trabecular artery. Enlarged x 3.5 from x 120.

Fig. 15. Human spleen stained with a fluorescein anti-Synovium conjugate. The reticulum of the red pulp shows specific staining and the trabeculum autofluorescence. Enlarged x 3.5 from x 120.

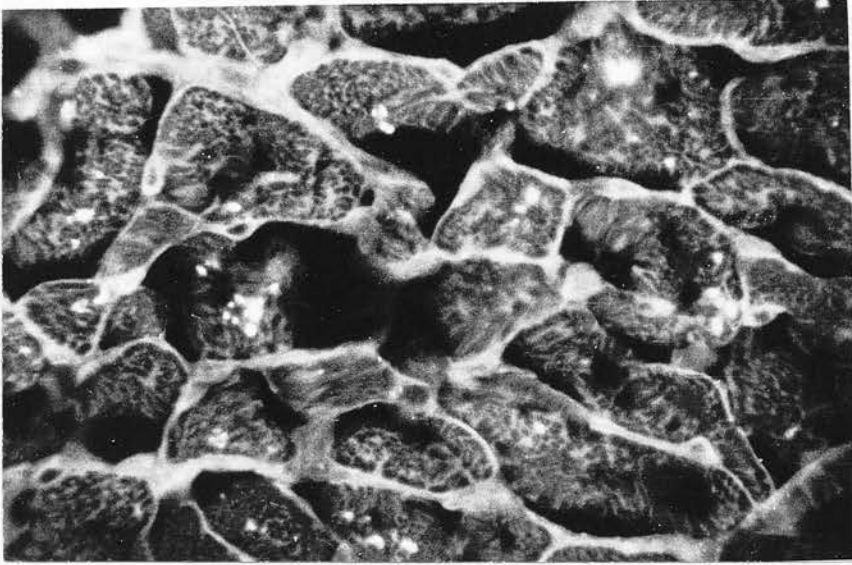


Fig. 16. Human heart stained with a fluorescein anti-Glomerulus conjugate. Specific staining is seen in the sarcolemma and in the walls of capillaries. The muscle fibres show autofluorescence, distributed as grey stripes and red pigment granules in the original section.

Enlarged x 3.5 from x

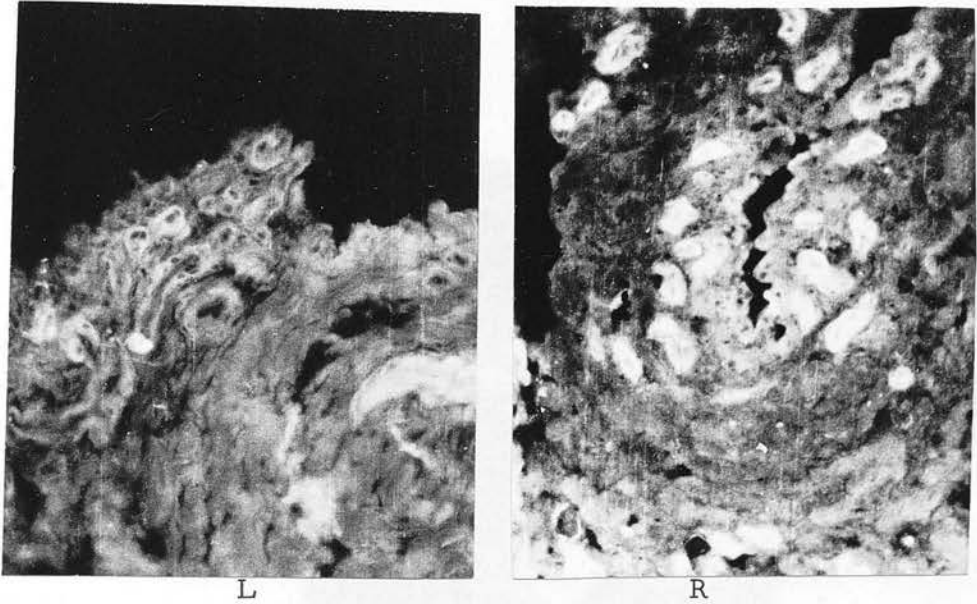


Fig. 17. Human synovium stained with a fluorescein anti-Glomerulus conjugate (L) and a fluorescein anti-Synovium conjugate (R). Specific staining has the same distribution in each case; it is present around synovial cells and in the walls of capillaries. Enlarged x 3.5 from x 120.

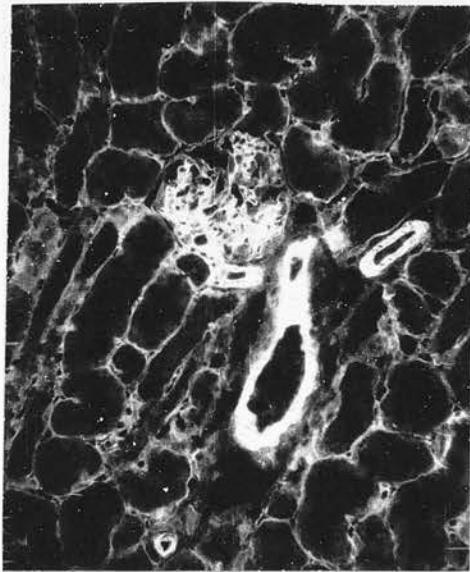


Fig. 18. F-ARG direct staining experiment.
Adult rat kidney. Specific staining
is seen in glomerular and peri-tubular
capillaries, in the boundary membranes
of tubules and in the media of arteries.
x 120.

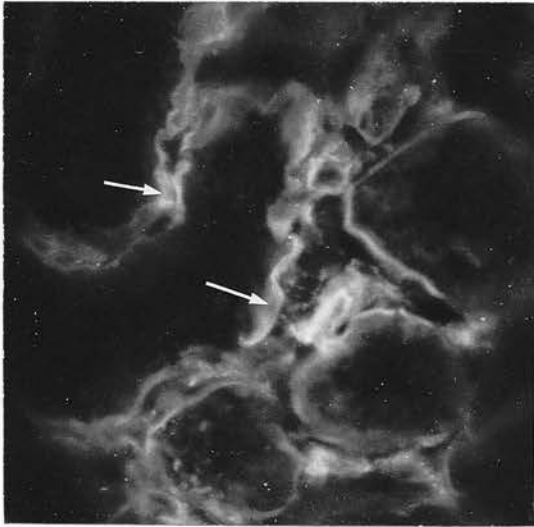


Fig. 19. F-ARG direct staining experiment.
Adult rat kidney. Arrows point to
the small splits or unstained spaces
sometimes seen in renal tubular
boundary membranes in F-ARG stained
sections of adult rat kidney.
Enlarged from x 300.

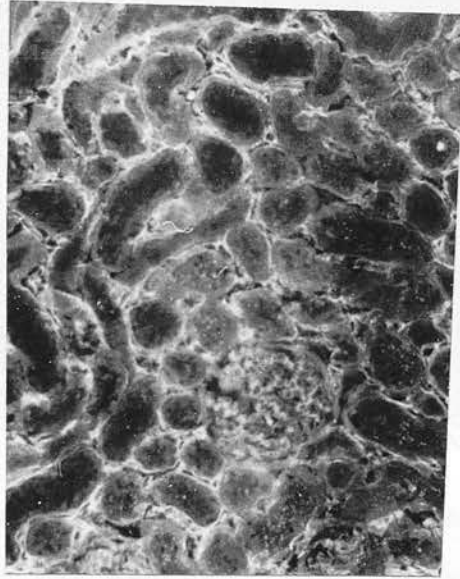


Fig. 20. F-ARE direct staining experiment.
Adult rat kidney. Specific staining
appears between and around tubules and
in the glomerular capillary tuft.
Staining in the glomerular capillary
tuft presents a less complex pattern
than that produced by F-ARG conjugates
(see Fig. 18). Enlarged from x 90.

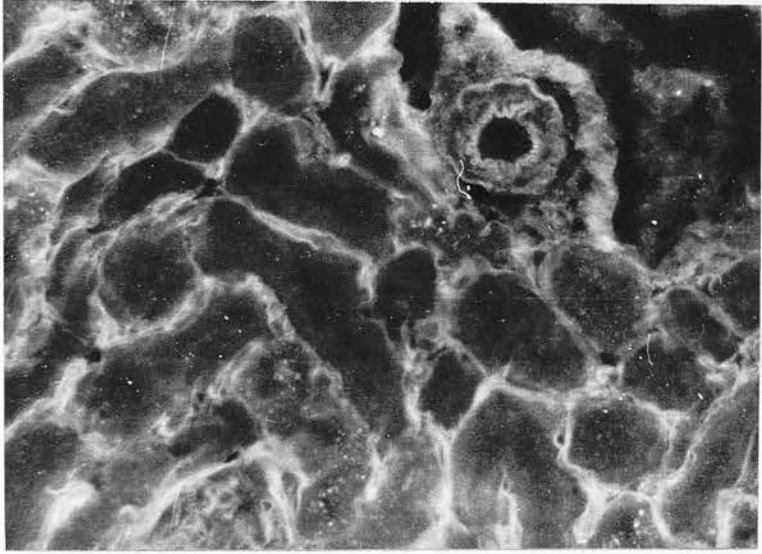


Fig. 21. F-ARE direct staining experiment.
Adult rat kidney. Specific staining
appears between and around tubules,
in perivascular connective tissue and
at the junction of the media and
adventitia of the artery. The media
of the artery is unstained, its
internal elastic lamina shows auto-
fluorescence. Enlarged from x 160.

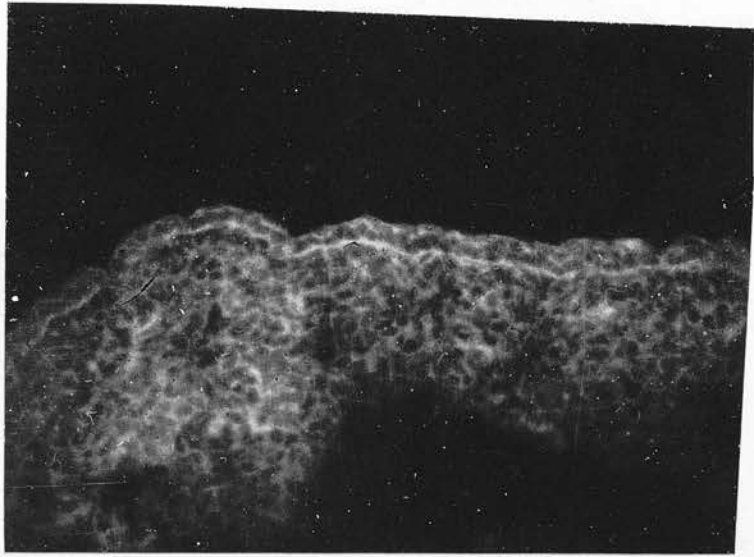


Fig. 22. F-ARE direct staining experiment. Skin of 1.4 cm. rat embryo. Specific staining appears at the dermo-epidermal junction and in the dermis. The peripheral layer of the dermis shows non-specific staining. Staining in the dermis has a lace-work pattern of distribution and is thought to emanate from the cytoplasm of dermal mesenchymal cells. Enlarged from x 160.

Below specific fluorescence of dermal cells in an F-ARE stained section of 1.4 cm. rat embryo skin. x 400.



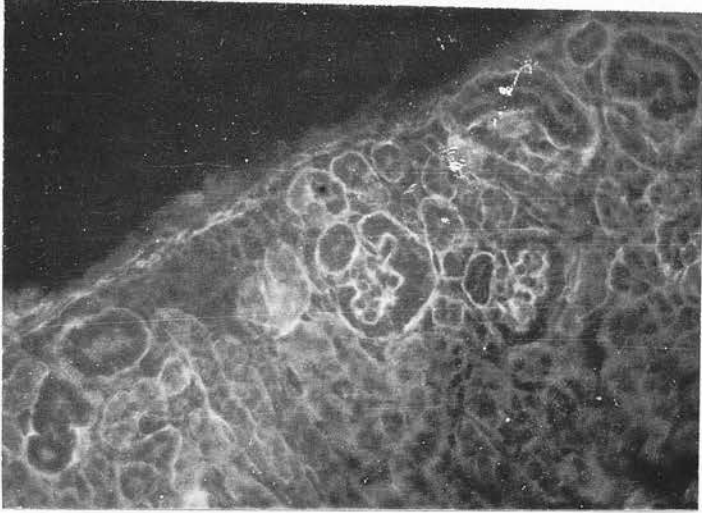


Fig. 23. F-ARG direct staining experiment. Kidney of 3.0 cm. rat embryo. Specific staining appears in the boundary membranes of tubules and in developing glomeruli. These glomeruli take the form of crescentic tubules outlined by a specifically stained membrane, the developing capillary tuft in the concavity of the tubule shows specific staining distributed in the form of circles and streaks. Enlarged from x 160.

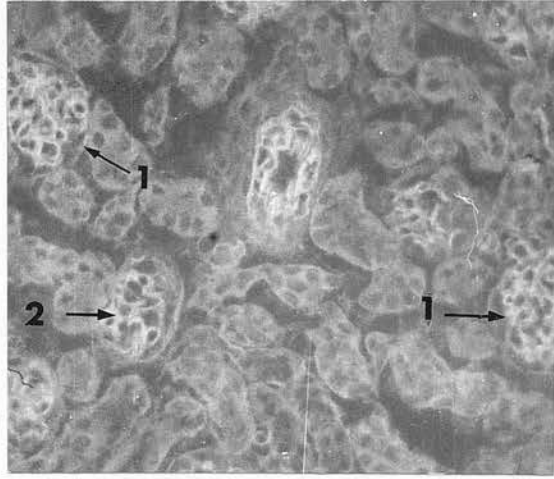


Fig. 24. F-ARG x embryo cross inhibition experiment. Kidney of young rat. The F-ARG conjugate after absorption with embryonic connective tissue continues to react with capillaries of mature (1) and transitional (2) glomeruli, but does not react with the boundary membranes of renal tubules. Enlarged from x 120.

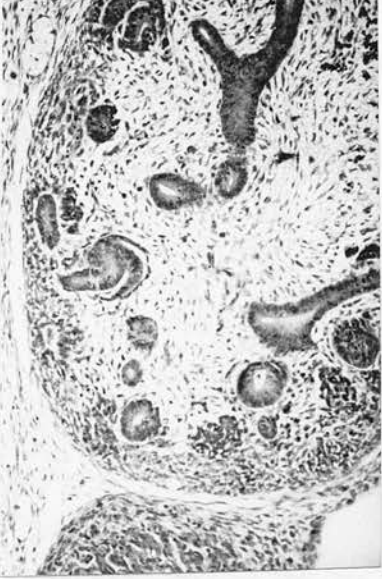
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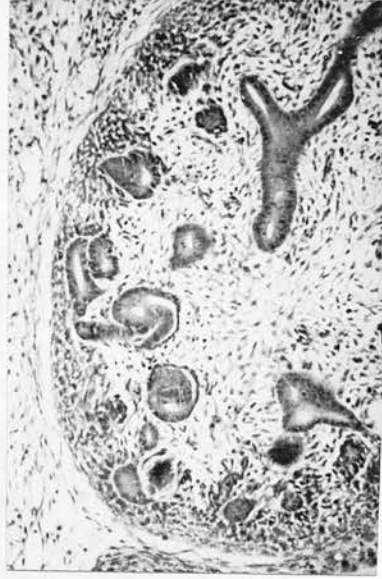
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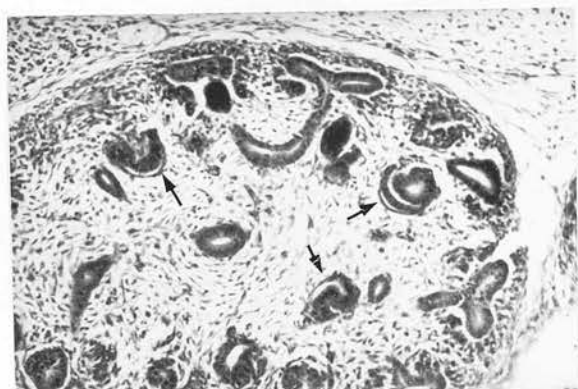


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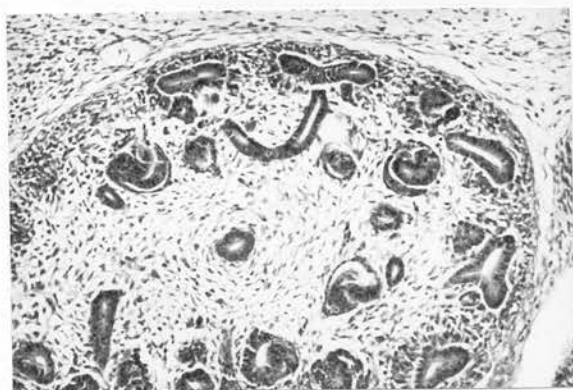


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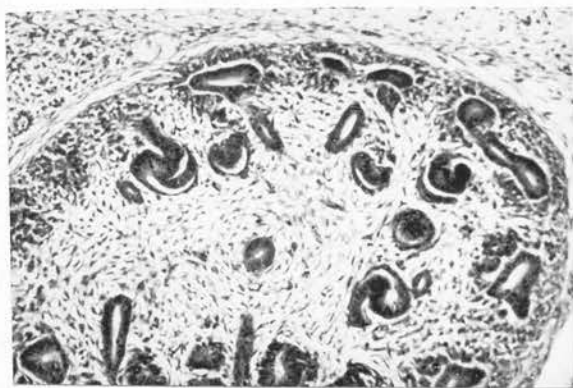




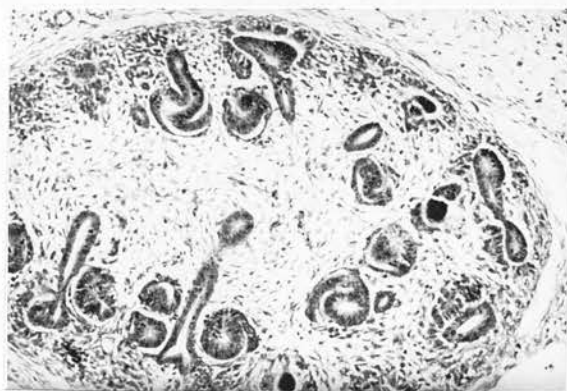
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COLOUR TRANSPARENCIES

APPENDIX 8

Experiments

Expt 1 and Expt 2

Clotting reactions of rabbit anti-human globulin serum with various antigens. The first reaction (I) is for rabbit anti-human globulin conjugate.

APPENDIX 8

COLOUR TRANSPARENCIES

Expt 3 and Expt 4

Clotting reactions of rabbit anti-human globulin serum with various antigens.

Experiments

Expt 1 and Expt 2

The first and second series of experiments in which sections were prepared in mixtures of rabbit anti-human globulin and anti-human globulin conjugate, respectively.

Expt 3 and Expt 4

The third and fourth series of experiments in which sections were prepared in mixtures of rabbit anti-human globulin and anti-human globulin conjugate. In these experiments sections were reacted with the first conjugate for 10 hours and then with the second for up to 2 hours.

COLOUR TRANSPARENCIES

Abbreviations

Conjugates.

RvG and FvG

Globulin fractions of rabbit anti-human glomerulus antisera bearing the fluorescein (F) or the rhodamine (R) label - anti-glomerulus conjugates.

FvG and FvS

Fluorescein and rhodamine anti-human synovium conjugates.

Experiments.

FvG + RvS

The symbol + denotes mixed staining experiments in which sections were exposed to mixtures of anti-human glomerulus and anti-human synovium conjugates labelled contrastingly.

FvG ---> RvS

The symbol ---> denotes cross exchange experiments. In these experiments sections were reacted with the first conjugate for 18 hours and then with the second for up to 3 hours.

COLOUR TRANSPARENCIES.

C.T. 1. FvG Direct Staining Experiment.

Human kidney treated with a fluorescein anti-glomerulus conjugate. Green specific staining appears in glomerular capillary basement membranes and in glomerular epithelial cells. Blue autofluorescence is seen in the cytoplasm of tubular epithelial cells, the nuclei of these cells show neither staining nor autofluorescence. x 160.

C.T. 2. RvG Direct Staining Experiment.

Human kidney treated with a rhodamine anti-glomerulus conjugate. Specific staining (red colour) has the same general distribution as in C.T.1. An artery lies to the left of the glomerulus, its internal elastic lamina shows a silvery autofluorescence, its media is specifically stained. x 160.

C.T. 3. FvS Direct Staining Experiment.

Human kidney treated with a fluorescein anti-synovium conjugate. The basement membranes of glomerular capillaries are unstained. Specific staining in the glomerular tuft is confined to oval or polygonal bodies, many of these bodies contain small circular unstained areas. x 270.

C.T. 4. R-NRG + FvS Mixed Staining Experiment.

Human kidney treated with a mixture containing equal parts of a rhodamine normal rabbit globulin conjugate and a fluorescein anti-synovium

conjugate. Non-specifically reacting material had not been completely removed from the R-NRG conjugate. The presence of the rhodamine label in the mixture has not significantly altered the colour of the green fluorescence normally produced by anti-synovium conjugates in renal connective tissues. x 60.

C.T. 5. RvS + FvG Mixed Staining Experiment.

Glomerular capillary basement membranes have taken the colour of the anti-glomerulus conjugate. Glomerular epithelial cells show an intermediate yellow colour, suggesting that they have fixed anti-glomerulus and anti-synovium antibodies in roughly equal amounts, while peri-glomerular connective tissues, which show an orange colour, appear to have reacted predominantly with anti-synovium antibodies. x 160.

C.T. 6. RvS --->FvG Cross Exchange Experiment.

The implications carried by cross exchange experiments were similar to those of mixed staining experiments. In the experiment illustrated here a section of human kidney was exposed to an RvS conjugate for 18 hours and then to an FvG conjugate for 3 hours. Glomerular epithelial cells (yellow) appear to have given up a larger proportion of RvS antibodies in favour of FvG antibodies than have peri-glomerular connective tissues (orange). x 200.

C.T. 7, C.T. 8 and C.T. 9. RvG ---> FvS Cross Exchange Experiments

Observations arising out of RvG ---> FvS cross exchange experiments were complementary to those made during FvS ---> RvG experiments (C.T. 6). In the experiments illustrated here sections of human kidney were exposed to RvG conjugates for 18 hours and then to FvS conjugates for $3/4$ (C.T.7), $1\frac{1}{2}$ (C.T.8) or 3 hours (C.T.9). Peri-vascular connective tissues have undergone a more rapid and complete change in colour, from red through yellow to green than glomerular epithelial cells have.

C.T. 10. RvG + FvS Mixed Staining Experiment.

Human kidney exposed to a mixture of rhodamine labelled anti-glomerulus and fluorescein labelled anti-synovium conjugates. The boundary membranes of renal tubules show a red inner and a yellow outer component. x 240.

C.T. 11. RvG ---> FvS Cross Exchange Experiment.

Human kidney section treated first with an RvG conjugate for 18 hours and then with an FvS conjugate for $1\frac{1}{2}$ hours. The dual constitution of renal tubular boundary membranes was less evident after RvG ---> FvS cross exchange experiments than after RvG + FvS mixed staining experiments. x 400.

C.T. 12. FvG Direct Staining Experiment.

Human thyroid gland stained with an FvG conjugate after pre-treatment with unlabelled normal rabbit globulin. Acinar boundary membranes

in FvG stained sections of the thyroid gland were usually seen as relatively broad structures containing round or oval unstained spaces, but sometimes, as in this photomicrograph, thyroid acini were surrounded by a fine membrane which split to enclose spindle-shaped spaces. x/60.

C.T. 13. FvG + RvS Mixed Staining Experiment.

Human thyroid - a disrupted section stained with an RvG + FvS mixture. The yellow periacinar reticulum has pulled away from the green basement membrane. x 270.

C.T. 14. FvS -->RvG Cross Exchange Experiment.

Human thyroid gland. The section was treated with the FvS conjugate for 18 hours and then exposed to the RvG conjugate for 3/4 hour. The acinar boundary membrane contains a red inner and a yellow-green outer component. x 320.

C.T. 15. FvS + RvG Mixed Staining Experiment.

The dual constitution of the acinar boundary membrane in the thyroid gland was less readily apparent after FvS + RvG mixed staining experiments than after the corresponding FvS --> RvG cross exchange experiments. x 240.

C.T. 16. FvG Direct Staining Experiment.

White pulp of human spleen. The media of the artery shows specifically stained lamellae arranged concentrically around the autofluorescent internal lamina. Reticulum of the white pulp also shows specific staining. x 160.

C.T. 17. RvS Direct Staining Experiment.

Human spleen. The anti-synovium conjugate has produced specific fluorescence in the reticulum of the red and of the white pulp, but has not reacted with the media of the artery. x 80.

C.T. 18. RvS --> FvG Cross Exchange Experiment.

Human spleen. The section was exposed to the RvS conjugate for 18 hours and to the FvG conjugate for 1¹/₄ hours. Prior exposure of the section to the anti-synovium conjugate has to some extent prevented anti-group 2 antibodies in the FvG conjugate from fixing to splenic reticulum, but has not prevented anti-group 1 antibodies in this conjugate from reacting with the media of arteries. x 120.

C.T. 19. FvS ---> RvG Cross Inhibition Experiment.

Human spleen pre-treated with an FvS conjugate for 18 hours and then exposed to an RvG conjugate for $\frac{1}{2}$ hour. The red colour of the penicilli suggests that these vessels react only with the anti-glomerulus conjugate. The green colour of the splenic reticulum, however, points only to specificities held in common by the two conjugates - prolonged exposure of anti-synovium stained sections of spleen to contrastingly labelled anti-glomerulus conjugates results in a gradual appearance of intermediate colours in splenic reticulum (C.T.18). x 240.

C.T. 20. FvS -->RvG Cross Exchange Experiment.

Human synovium reacted with an FvS conjugate for 18 hours and with an RvG conjugate for $1\frac{1}{2}$ hours. Green-yellow staining appears in the synovial cell layer, where it has a peri-cellular distribution. The walls of capillaries have an orange colour. x 120 .

C.T. 21. RvS Direct Staining Experiment.

Human synovium. Bright specific staining is seen in the synovial cell layer and in the walls of capillaries. Less bright staining can be made out in the blue autofluorescence of the sub-synovium, this represents specific staining of material lying between bundles of collagen. x 240 .

C.T. 22. FvG Direct Staining Experiment.

Human synovium. Specific staining produced by anti-glomerulus conjugates in synovial tissue had the same distribution as that produced by anti-synovium conjugates. x 160 .

C.T. 23. RvG Direct Staining Experiment.

Margin of human articular cartilage. The interstitial substance of the cartilage (lower left) has a bright blue autofluorescent colour, while the chondrocytes have been stained red. The red colour of the chondrocytes represents non-specific staining. Specific staining is seen between collagen fibres of the perichondrium (upper right). x 160 .

C.T. 24. FvG Direct Staining Experiment.

Human joint capsule. The photomicrograph shows collagen, blue bundles, and elastic tissue, white fibrils overlying the collagen bundle in the centre of the field. Fine faint green specifically stained material outlines some of the collagen bundles. x 160 .

C.T. 25. RvS Direct Staining Experiment.

Arteries in human joint capsule. The anti-synovium conjugate has reacted only with perivascular connective tissue, the tunica media of the vessels are unstained and the internal elastic lamina show autofluorescence. x 140 .

C.T. 26. RvG Direct Staining Experiment.

The anti-glomerulus conjugate has reacted with the media as well as with the adventitia of the vessels. x 160 .

C.T. 27. RvG + FvS Mixed Staining Experiment.

Artery in human joint capsule. The vessel shows autofluorescence of its internal elastic lamina and yellow adventitial staining. Medial staining, underexposed in this photomicrograph, had a red colour. x 160 .

C.T. 28. FvG + RvS Mixed Staining Experiment.

Artery in human joint capsule. The FvG + RvS mixture, like the RvG + FvS mixture in C.T.27, has produced yellow staining in perivascular connective tissue. Medial anti-glomerulus staining is

more convincingly demonstrated in this transparency than in C.T. 27. x 130.

C.T. 29. RvG + FvS Mixed Staining Experiment.

Artery in human tela choroidea. The yellow-stained membrane usually seen bounding the outer aspect of the media of arteries exposed to contrastingly labelled conjugates is well seen here, where perivascular connective tissue shows only autofluorescence. The red, RvG stained, media is limited internally by the purplish autofluorescence of the internal elastic lamina. x 160.

C.T. 30. FvG Direct Staining Experiment.

Human skin, showing, in the epidermis, non-specific staining of the stratum lucidum, autofluorescence of the stratum corneum and stratum Malpighii. The dermo-epidermal boundary membrane and dermal capillaries show specific staining. Elastic fibres are rendered prominent by virtue of their silver-blue autofluorescence. x 60.

C.T. 31. FvG + RvS Mixed Staining Experiment.

Human tela choroidea. A thick walled capillary with specifically stained (orange) media and pericapillary reticulum (yellow). Capillaries of this general structure were also seen in the skin (C.T.30) and synovium (C.T.21). x 160.

C.T. 32. RvG + FvS Mixed Staining Experiment.

Fibrocytes in human leptomeninges. Fibrocytes, and especially their cytoplasmic processes,

while apparently reacting predominantly with the anti-synovium conjugate in mixed staining experiments (the yellow colour seen in the body of some of the cells in this duplicate transparency exaggerates the appearances seen in the original section), were found to react with anti-glomerulus conjugates in direct staining experiments (C.T.33).
x 270.

C.T. 33. FvG Direct Staining Experiment.

Histiocytoma in human dermis. The section shows autofluorescence of collagen and specific staining in the cytoplasm and cytoplasmic processes of fibrocytes. x 200.

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IMMUNOHISTOLOGICAL STUDIES OF CONNECTIVE TISSUE IN THE ADULT
AND EMBRYONIC RAT

COLOUR TRANSPARENCIES

34. and 35. Rat-like embryos of the 1.0cm to 1.5cm group.
36. and 37. Rat-like embryos of the 1.5cm to 2.0 cm group.
38. Rat-like embryo of the 2.5cm to 3.0 cm group.
39. New born rat.
40. Young rat.
41. F-ARG direct staining experiment. Kidney of a 3.0cm rat embryo. The field includes a proemial glomerulus (left upper quadrant), a transitional glomerulus (right upper quadrant) and a developing glomerulus (centre). In each case the glomerular capsule takes the form of a crescentic tubule outlined by a specifically stained membrane. The concavity of the capsule is occupied in the proemial glomerulus by a second tubule, and in the developing glomerulus by matrix material. In the transitional glomerulus the capsule incompletely embraces a simple convolution of widely patent capillary loops. x 72.
42. 1.4cm rat embryo kidney, paraffin section, H. and E. Structures resembling proemial glomeruli were seen in paraffin sections of embryonic rat kidney and it was frequently possible to trace a continuity between the two tubules forming these structures. (See C.T.43. to 45.)
46. F-ARG direct staining experiment. Adult rat kidney. Staining in the intertubular space is limited to capillaries and a few fine fibrils which appear to fuse with renal tubular boundary membranes. x 120.
47. F-ARG direct staining experiment. Kidney of a 3.0cm rat embryo. Specifically stained interstitial tissue is abundantly present in the intertubular space. x 240.
48. F-ARG direct staining experiment. Skin of an embryo of the 1.0cm to 1.5cm group. Specific staining is seen at the dermo-epidermal junction and in the dermis, where it has a lace-work pattern of distribution. x 160.

49. Fibrillar pattern of dermal staining produced by the F-ARG conjugate in the skin of an embryo of the 1.5cm to 2.0cm group. Specific staining is seen also at the dermo-epidermal junction. Direct staining experiment. x 160.
50. F-ARG direct staining experiment. Skin of an embryo of the 1.5cm to 2.0 cm group. In this embryo specific staining was seen at the dermo-epidermal junction and in dermal blood vessels, the dermal connective tissues showed no staining. x 300.
51. F-ARG direct staining experiment. Skin of new born rat. Staining appears at the dermo-epidermal junction, in the walls of blood vessels and in perimysial reticulum, but not in dermal collagen. x 160.
52. F-ARE direct staining experiment. Adult rat kidney. Specific staining is seen around and between tubules and in a glomerulus - the dome shaped structure in the lower half of the field. Staining in the glomerulus does not present the capillary loop pattern characterising F-ARG stained glomeruli. x 320.
53. F-ARG direct staining experiment. Adult rat kidney. Specific staining appears at the boundary membranes of tubules and in the glomerular capillary tuft, where it has a capillary loop pattern. (See also C.T.59.) x 54.
54. F-ARE direct staining experiment. Adult rat kidney. Investment of tubules by boundary membrane appears to be incomplete. x 400.
55. F-ARG direct staining experiment. Adult rat kidney. Tubules are completely enveloped by boundary membrane. x 400.
56. F-ARE direct staining experiment. Kidney of new born rat. The field includes a developing glomerulus. Specific staining in the capsular space of this glomerulus is distributed as loops, circles and streaks embedded in a less brightly stained matrix, which is itself fenestrated by unstained spaces. x 120.
57. ARE + F-ARG cross inhibition experiment. Adult rat kidney. Specific staining is seen in glomerular capillaries. Although the boundary membranes of tubules also show staining, the presence of unlabelled ARE globulins was thought to modify the interaction between F-ARG conjugates

and peri-tubular tissues. Tubular boundary membranes appeared finer in sections treated by the ARE + F-ARG cross inhibition procedure than in those treated by the F-ARG direct staining procedure e.g. C.T.59. x 240.

58. F-ARG x embryo cross absorption experiment. The absorption of F-ARG conjugates with embryonic connective tissues removed the reactivity of these conjugates for interstitial tissue and peri-tubular capillaries. The conjugates continued to react strongly with glomerular capillaries and the media of arteries and less strongly with the boundary membranes of tubules. These latter structures are, however, not distinguishable in the photomicrograph. x 120.
59. F-ARG direct staining experiment. Adult rat kidney. Specific staining is seen in glomerular tufts and in tubular boundary membranes. Compare with C.T.57 and C.T.58. x 300.
60. F-ARG x embryo cross absorption experiment. Transitional glomerulus. The absorption of an F-ARG conjugate with embryonic connective tissue effected a varying modification in the reactivity of the conjugate for capillary tufts of transitional glomeruli. The majority of transitional glomeruli in F-ARG x embryo stained sections showed a tuft of weakly stained capillaries, but an occasional glomerulus showed the appearances seen here. In the glomerulus illustrated staining is confined to a membrane which appears to outline the site of the capillary tuft, but capillary basement membranes are unstained. x 240.