J. clin. Path., 1972, **25**, 373-396

Renal hyalin

A study of amyloidosis and diabetic fibrinous vasculosis with new staining methods

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SYNOPSIS This describes the sodium sulphate-Alcian Blue (SAB) method for staining amyloid in paraffin sections. Its value lies in the possibility of subsequent counterstaining and thus of revealing the structural relationships of amyloid.

In the kidney the topical disposition of amyloid closely resembles the disposition of fibrin in the kidney of diabetics; this suggests that upset in vascular permeability plays a part in determining the site of the amyloid deposits. Furthermore, an aging process in amyloid can now be envisaged resembling the aging of extraluminal fibrin. Both materials proceed to a hyalin material that, staining like collagen, merits the name pseudo-collagen. This term we apply to a hyalin, staining like collagen, for which we can postulate a specific precursor.

The light microscopist has generally accepted amyloid as a hyalin substance, an acellular firm gel, situated interstitially, and distinguished from other hyalins by particular staining reactions, notably the metachromatic reaction with methyl violet and an affinity for Congo red. Neither of these methods is ideal for the study of the structural relationships of amyloid.

Our attempts to demonstrate and study amyloid, as seen in postmortem material, were progressing unprofitably when suddenly one divagation revealed possibilities. A rationally evolved although perhaps inaccurate idea led one of us (W.S.) to produce a modified Alcian Blue solution. This enabled us to stain amyloid, in sections from paraffin, with a dye that could be stabilized *in situ* and thus allow a variety of counterstainings. Although, as with most other dye-staining methods, the results were not chemically specific for amyloid, with suitable counterstaining they proved sufficiently selective to allow study of the precise situation of the amyloid deposits. Unfortunately the dye makers, in 1955, changed the constitution of the dye, and our results altered miserably.

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Received for publication 13 January 1972.

Another study meanwhile captured our attention. This, the formulation of new trichromic methods for staining fibrin (Lendrum, Fraser, Slidders, and Henderson, 1962; Lendrum, Fraser, and Slidders, 1964; Slidders, 1968), led to the discovery that these methods could graphically demonstrate and elucidate changes in the diabetic kidney and in many other vascular lesions (Lendrum, 1963). On turning our attention again to amyloid, one of us (W.S.) found a way of using the new, and still current, Alcian Blue. This we could now complement with new and apt counterstains derived from our studies on fibrin staining; and the changes seen in the diabetic kidney proved an illuminating counterpart to what we were now finding in the amyloid kidney.

Our present purpose is to describe the new methods, and the facts made manifest in the amyloid kidney. This study with the light microscope revealed an unexpected similarity with the diabetic kidney not only in the topical disposition of the two deposits but also in the indication that amyloid, like interstitially deposited fibrin, undergoes changes on aging and loses its characteristic staining reactions, becoming just anonymous hyalin. When obviously derived from fibrin we called this material pseudo-collagen (Lendrum, 1961; Lendrum *et al*, 1962, 1964) because with our trichromic methods it stains

as collagen, and similarly, we now believe that amyloid also can become a pseudo-collagen.

At a time when world-wide intent is converging on the experimental production of amyloid, and on its strikingly profitable study by the electron microscope (Mandema, Ruinen, Scholten, and Cohen, 1968), it is worthy of note that at this same symposium Cohen (1968), summing up a day's proceedings said, 'One must return to more classical light-microscopic studies to give the proper perspective'. We are prompted to offer our observations on the grounds that the pathologist, particularly, is likely to maintain his interest in both the wood and the trees.

PART I

The Staining of Amyloid

I i THEORETICAL

The demonstration of amyloid has for the light microscopist always been visually unsatisfactory. Of the generally used methods the metachromatic stain may show good colour contrast but gives only poor definition. Staining with Congo red is often feeble, and, even at their best, neither of these classical methods nor the new fluorochrome method permits subsequent counterstaining of a type to demonstrate the intimate relationship of amyloid with the stromal tissue.

Given well fixed tissue a trichrome of the Mallory-Masson family can show in the kidney the characteristic strong blue of collagen and of basement membrane, contrasting moderately well at high power with the pale grey-blue of the amyloid substance (Figs. 1, 2, and 6, and colour Fig. 14). Some use van Gieson in a similar fashion, the pale buff colour of amyloid contrasting with the fuchsinophil collagen. These rather negative methods of staining offered the nearest approach to a cartographic method to show amyloid's relation to the kidney's preexistent structure. Clearly, however, something better is needed, some way of obtaining a positive staining of amyloid, and of fixing the stain so that counterstaining of the preexistent structures can be added without ousting or degrading the colour fixed in the amyloid. Such a method is the sodium sulphate-Alcian Blue (SAB) method described by Lendrum, Slidders, and Fraser (1969) and reproduced below.

Alcian Blue (Alcian Blue 8GX, Colour Index designation Ingrain Blue 1[1]) is prepared by Messrs ICI from their copper phthalocyanine pigment Monastral Fast Blue B by the introduction of certain novel solubilizing groups; these confer on the molecule the properties of a cationic (basic) dye. On textiles Alcian Blue is fixed and rendered fast by after-treatment with alkali; this, by causing the solubilizing groups to decompose, deposits the copper phthalocyanine pigment within the fibre.

The earlier and somewhat different dye, Alcian Blue 8GS, was the form of Alcian Blue introduced by Steedman (1950) as a stain for mucin. The fact that both mucin and amyloid are revealed by metachromatic dyes first prompted us to test Alcian Blue 8GS as a stain for amyloid.

Our investigations soon revealed that Alcian Blue 8GS in aqueous-acetic solution had little tendency to colour amyloid in sections from paraffin and efforts were directed to methods of activation. Eventually, one of us (W.S.) by adding sodium sulphate to the usual aqueous-acetic solution of the dye found a distinctly adjuvant effect. The production of Alcian Blue 8GS was discontinued around 1955 and thereafter Alcian Blue 8GX became the standard product, although several other Alcian Blues, two Alcian Greens, and an Alcian Yellow were also made available during this time.

When Alcian Blue 8GS was replaced by Alcian Blue 8GX a marked deterioration in our results diverted our interests away from amyloid. On our return, W.S. found that sodium sulphate-induced staining of amyloid could be obtained with the new dye if the solvent contained alcohol; unfortunately this brought about a loss of selectivity regained, however, by increasing the acetic acid content of the stain. The present version of the method was mentioned and illustrated but not detailed in 1963 (Lendrum).

The original, apparently irrational manoeuvre of adding sodium sulphate to Alcian Blue was prompted by an appreciation, albeit imperfect, of the factors involved in the dyeing of textile fibres.

The staining methods commonly used by histologists are regarded as comparable with the dyeing of wool and thus as depending primarily on the formation of salt-like linkages between cationic (basic) or anionic (acid) dyes and, respectively, ionized acidic (eg, carboxyl) or basic (eg, amino) groups in the tissues.

On the other hand, in the dyeing of cotton with the so-called 'direct' dyes (large-molecule anionic dyes used without mordants) interaction of the dye and fibre occurs despite the fact that the only available ionizing groups in cellulose (hydroxyl) are themselves anionic, although weak. Attachment of the dye in this instance is considered by some to depend on hydrogen-bond formation. Bonding of this sort could occur between, for example, the hydrogen atom of the hydroxyl groups in the fibre and the nitrogen atom of an azo group in the dye.

[1]See part I v, Identification of dyestuffs (the colour index).

To initiate this non-ionic bonding the dyer generally has to add sodium sulphate or chloride to the dyebath, the salt being thought to act by annulling the antagonistic ionic forces, thereby permitting the close contact, of dye and fibre, essential for hydrogen bonding.

Even in the dyeing of wool, when using anionic dyes, the dyer generally adds sodium sulphate to the dyebath. Here the function of the salt is to retard the 'strike' of the anionic dye by diminishing the forces of attraction between the dye anions and cationic groups in the fibre, thereby ensuring a more even or 'level' colouring of the fibre. Thus in wool dyeing with its ionic dye-binding the presence of the salt ions is suppressive whereas in cotton dyeing with direct dyes it is adjuvant by annulling the ionic forces that otherwise would tend to keep the dye and fibre apart.

The fact that Congo red is one of the very few direct dyes used by histopathologists suggested a possible similarity in the mechanism of amyloid staining and the direct dyeing of cotton. Alcian Blue resembles the direct dyes in being substantive to cellulose but differs from them in being cationic, and it seemed, therefore, that the failure of Alcian Blue to stain amyloid might be attributable to an opposing ionic force between the dye cations and similarly charged groups in the substrate, a force corresponding to the mutual ionic repulsion that exists between the hydroxyl groups of cellulose and the anions of direct dyes. Pursuing the analogy a step further, we thought it not unreasonable to try to bring about Alcian Blue staining of amyloid by adding a salt to the dye solution.

It is interesting to note that Puchtler, Sweat, and Levine (1962) found the addition of sodium chloride intensified Congo red staining of amyloid, and that they postulated hydrogen bonding as the mechanism of attachment of this direct dye to amyloid as in the dyeing of cotton. It does, however, seem anomalous that the attachment to amyloid of cationic Alcian Blue and of anionic Congo red should both benefit from the intervention of salt ions; after all, an ionic force that opposed the one would presumably favour the near approach of the other. A possible explanation would be that amyloid, unlike cellulose, possesses both cationic and anionic groups and thus is essentially amphoteric. To suppress general background colouring Alcian Blue has to be used at an acid *p*H; this represses ionization of anionic groups in the tissues, ionization of cationic groups being incidentally increased. On the other hand, selective staining of amyloid with Congo red requires a distinctly alkaline *p*H at which ionization of cationic groups in the tissues is repressed and that of anionic groups increased. Thus it may be that in each instance ionizing groups in the amyloid material itself exert a repelling force on the dye ions, in the first instance against cationic Alcian Blue, and in the second against anionic Congo red; and it may be that these mutual ionic repulsions are annulled by the salt in the solution, thereby encouraging non-ionic dye binding to take place. Substrates that are deficient in hydrogen-bonding sites or have a molecular configuration that militates against this form of dye adsorption will of necessity remain unstained.

An alternative explanation for the increased uptake of Alcian Blue and Congo red by amyloid in the presence of salt ions has been advanced by Mowry and Scott (1967) and Scott, Dorling, and Stockwell (1968). This is an extension of the 'critical electrolyte concentration' concept of Scott (1960) and postulates that in amyloid substance numerous carbohydrate polyanionic groups are masked by being coupled with the polycationic groups of the associated basic protein. At a particular level of added salt (the critical electrolyte concentration) these ionic bonds are broken and the carbohydrate polyanions become capable of combining with the cations of Alcian Blue. The polycations of the protein moiety of amyloid are correspondingly made available to interact with the anions of Congo red.

In an earlier paper, Scott and his coworkers (Scott, Quintarelli, and Dellovo, 1964) suggest another mechanism whereby added salt may increase the uptake of Alcian Blue by tissue polyanions. Each molecule of Alcian Blue has at least two and possibly three or four cationic sites available for binding with polyanionic groups in the tissues. When salt is added, its anions may satisfy one or more of these cationic sites in this huge dye molecule, thereby reducing the number of anionic sites in the tissues with which each molecule of dye can combine. Since the dye is in excess, a given amount of tissue polyanion will now be able to bind a greater number of dye molecules and stronger staining will result.

In their *histochemical* investigation of amyloid Mowry and Scott (1967) added magnesium chloride as the functioning electrolyte. In our *histological* studies we have continued with our empirical formula containing sodium sulphate.

Whatever the correct explanation for the increased avidity shown by Alcian Blue for amyloid in the presence of added salt we have no doubt about the practical value of this method of staining amyloid. The fact that the Alcian Blue, thus encouraged to stain the amyloid, can now be fixed in place by the insolubilizing action of alkali allows the subsequent use of various trichromic counterstainings and so the critical study of amyloid in tissue sections from paraffin.

I ii New Staining Method for Amyloid

METHOD 1: SAB WITH VAN GIESON COUNTERSTAINING

Stock solutions
A 1% Alcian Blue 8GX in 95% ethanol
B 1% sodium sulphate hydrate in distilled water

SAB working solution
Stock solution A .. .. 45 ml
Stock solution B .. .. 45 ml
Glacial acetic acid .. .. 10 ml
Mix: stand 30 minutes before use; prepare freshly each day.

Acetic-alcohol rinse
95% ethanol .. .. .. 45 ml
Distilled water .. .. .. 45 ml
Glacial acetic acid .. .. 10 ml
Mix: ready for use; prepare fresh each day.

Procedure
1 Sections to water via iodine and hypo; wash 10 minutes.
2 Transfer to acetic-alcohol, one to two minutes.
3 Stain in SAB solution, two hours.
4 Transfer to acetic-alcohol, one to two minutes.
5 Wash in water.
6 Alkalinize in 80% ethanol saturated with borax, 30 minutes.
7 Wash in water.
8 Stain nuclei in ferrous haematein, five minutes.
9 Wash in water.
10 Take each slide individually and differentiate by dipping in 80% ethanol saturated with picric acid, 20 to 30 seconds; rinse briefly in water to remove the alcohol and stain two to three minutes in picro-fuchsin. Washing in water at this stage weakens van Gieson staining almost instantly. Therefore, flush off the picro-fuchsin, *rapidly*, with 95% and so to absolute ethanol and thence to xylene and mount in BPS.

Result
Amyloid—green; nuclei—black; stroma—red; muscle, cytoplasm and erythrocytes—yellow (colour Figs. 1 and 2).

In addition to amyloid, mast cell granules and some colloids stain strongly with SAB.

Intestinal epithelial mucin is generally less strongly coloured than when stained for the same length of time in 1% Alcian Blue in aqueous 3% acetic acid, but the connective tissue mucin of large vessels reacts more strongly.

Also stained is a curious crystalline material present in one of our amyloid cases. This seems to begin as granules in the cytoplasm of the epithelium of the convoluted tubules, becoming fine spicules and then after the desquamation of the cells coming to lie free in the lumen. The 'crystals' lengthen, but broaden less, and the lath-like structures may come to distend collecting tubules, by which 'time' they are less basophilic and many purely acidophilic.

Aging fibrin at the stage when chromophobic with MSB (Lendrum *et al*, 1962) may show some affinity for SAB, as discussed in part IV.

Cell nuclei and connective tissue fibres also take the stain to some extent but this is effectively masked by the counterstaining.

In some cases all the material we regard as amyloid shows an equally intense green; in others the green is again uniform but of a paler and duller character. A significant finding, however, is the presence in most cases of a range of response in the material we feel justified in accepting as amyloid. This ranges from a pure green, through a grey-green, a bluish and then a brownish shade to a pure fuchsin colour (colour Fig. 4). For reasons to be discussed in part III below we believe this changing response relates to change in amyloid as it ages.

NOTES

1 Fixation

Unneutralized 10% formol-saline may prove an adequate fixative, but primary fixation in Lillie's (1953) neutral (phosphate) buffered 10% formalin followed by secondary fixation in aqueous 5% mercuric chloride, our standard procedure for post-mortem material, is particularly recommended for the study of amyloid in paraffin sections. For biopsy material, when urgent, we compromise with formalin and mercuric chloride in a single solution (formol-sublimate, Lendrum, 1941, 1951, 1966). Whenever possible, however, surgical material of special interest receives the same treatment as postmortem tissue.

Fresh tissue is sliced and immersed in a large volume of buffered formol for 24 to 72 hours; the thickness of the slices should not greatly exceed 5 mm, to allow complete penetration by the fixative within 24 hours. Thereafter slices are trimmed to suitable dimensions and the pieces transferred to aqueous-sublimate for 48 hours.

This schedule is based on the following considerations: first, that buffered formalin is the fixative most suitable for large slices of postmortem tissue, and the least lytic on erythrocytes in such material; secondly, that formalin alone being an inadequate fixative before the rigours of paraffin embedding (Mallory, 1938), such tissue greatly

benefits from the stabilizing effect of subsequent mercuric chloride. As is discussed in part IV below, however, prolonged immersion in aqueous mercuric chloride, beneficial in the case of fibrin (Lendrum *et al*, 1962), progressively diminishes the characteristic staining reactions of amyloid and is therefore to be avoided.

2 Processing and sectioning

From the secondary fixative, tissue blocks after a cursory rinse in water are transferred to the first of the dehydrating alcohols. Mercuric chloride, being more soluble in alcohol than in water, prolonged washing is not indicated.

When possible, a slow dehydration is used in which water and ethanol are gradually replaced by n-butanol (Lendrum, 1951); this is followed by methyl benzoate and double-embedding in nitrocellulose and wax according to Peterfi's method (Drury and Wallington, 1967).

Sections should be cut at not less than 6 microns if vivid colour contrast is wanted between stroma and amyloid.

3 Staining and mounting

We prefer to purchase Alcian Blue 8GX (Ingrain Blue 1) directly from Messrs ICI.

In our early studies acid-resistant nuclear staining was obtained with the oxazine-haemalum sequence (Lendrum and McFarlane, 1940; Lendrum *et al*, 1962), but as the oxazine dyes, celestin blue (Mordant Blue 14) and Solochrome Prune AS (Mordant Violet 54), have been deleted from the lists of the commercial manufacturers, we now use ferrous haematein (Slidders, 1969), a reasonably stable, single-solution iron haematoxylin, viz: dissolve 1 g haematoxylin in 100 ml 95% ethanol. Dissolve 10 g aluminium chloride hydrate and 10 g ferrous sulphate hydrate in 100 ml of distilled water. Combine these two solutions and add 2 ml concentrated hydrochloric acid and 2 ml, or slightly less, stock aqueous 9% sodium iodate. Mix and allow to stand 48 hours; the solution is now ready and with moderate use should remain active for about two months, and longer if kept at 4°C.

The van Gieson variant described is our standard procedure; the picro-alcohol, in addition to differentiating the nuclear staining, enhances the subsequent staining with picro-fuchsin and converts the Alcian Blue-coloured amyloid to a bright green; the picro-fuchsin used is that advocated by Lendrum (1951), viz, 15 parts of aqueous 1% acid fuchsin to 100 parts of aqueous half-saturated picric acid.

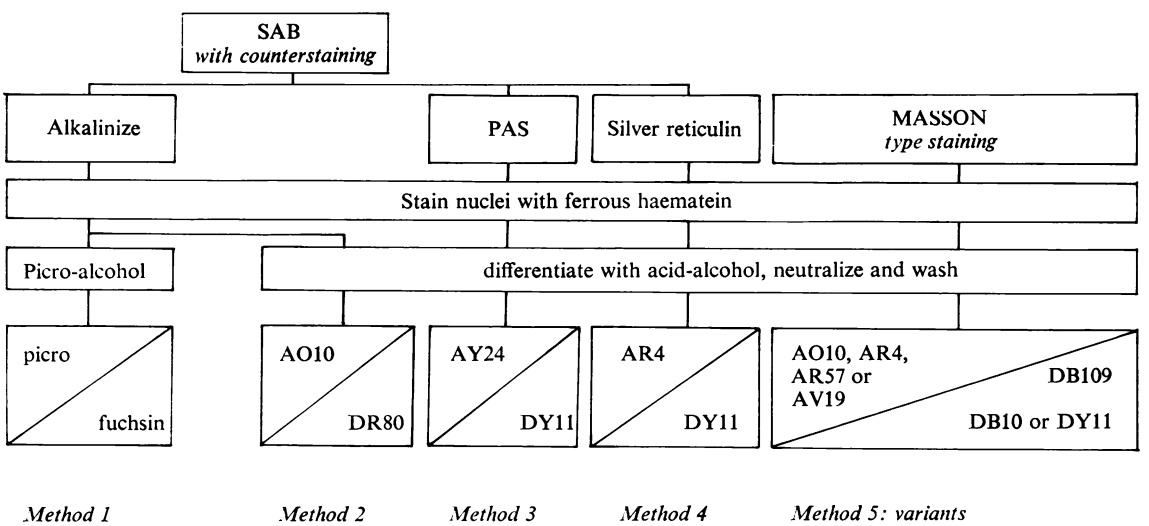
The polystyrene mountant BPS (Kirkpatrick and Lendrum, 1941) that replaced the original polystyrene mountant DPX (Kirkpatrick and Lendrum, 1939) was subsequently modified (Lendrum *et al*, 1962), viz: dissolve 12 g Styron[1] in a mixture of 40 ml xylene and 4 ml dibutyl phthalate.

I iii SAB with special counterstaining

The publication of new trichromic staining methods, variants of Mallory and Masson, has rarely been

[1]Dow Chemical Company's Styron 27/66-7. This is obtainable in Britain from Messrs Caplan & Co (Plastics), Third Avenue, Bletchley, Bucks, as Stock 6660, GP, P/ST crystals.

TABLE OF METHODS



accompanied by any acknowledged rationale. However, by following the ideas of Ehrlich (see Part III), one of us (W.S.) evolved and, in the OFG method for the pituitary (Slidders, 1961a), obtained confirmation of the concept that a small-molecule anionic wool dye dissolved in alcoholic phosphotungstic acid is selectively and firmly fixed in tissue elements with a tight texture, and can then exclude from these sites anionic dyes of larger molecular size.

When later, stimulated by the work of Emig (1941), we discovered the value of direct cotton dyes (anignic dyes of particularly large molecular size) as selective dyes for collagen in our MSB method (Lendrum *et al*, 1962), we saw the possibility of improving the orange and blue method used by the late Professor J. Shaw Dunn in his studies on the kidney (Baird and Dunn, 1933). We transferred the orange G from aqueous phosphomolybdic acid to *alcoholic* phosphotungstic acid, and replaced the aniline blue (Acid Blue 22) by Durazol Brilliant Blue B (Direct Blue 109), calling the new method JSDB109 (Lendrum *et al*, 1964). This worked excellently (Fig. 6), and we realized that if we could find suitable small anionic dyes soluble in alcoholic phosphotungstic acid and associated them with large direct cotton dyes in aqueous-acetic solution we would have a range of almost automatic trichrome methods. The dyes found (listed in part I iv) have provided a number of variants, some of these having special value as counterstains after SAB, as now to be described.

Method 2: SAB with Counterstain AO10. DR80

Proceed to step 7 of method 1.

8 Stain nuclei with ferrous haematein, five minutes, and wash in water; differentiate with 0·5% hydrochloric acid in 70% ethanol and wash in water; neutralize with aqueous 2% potassium acetate for several minutes and again wash in water.

9 Rinse in 95% ethanol and stain in 0·5% Acid Orange 10, and 2% phosphotungstic acid in 95% ethanol, three minutes.

10 Rinse in water.

11 Stain in 0·5% Direct Red 80 in aqueous 1% acetic acid, half to two hours.

12 Take each slide individually and rinse in water; mop and then dehydrate by running on absolute ethanol; clear in xylene and mount in BPS.

NOTE

Acid Orange 10 is the dye known to histologists as orange G. An excellent commercial Acid Orange 10 is available from Messrs Williams (Hounslow) Ltd[1], under the name Acid Orange GG. Direct Red 80 is commercially available from Messrs ICI as Durazol Brilliant Red BS, from Messrs Ciba as Chlorantine Fast Red 5 BRL, and from Messrs Bayer as Sirius Red F 3B.

Staining is best carried out in a Coplin jar. Staining solutions are kept in screw-cap bottles, and returned thither after use. In hard-water areas it is likely that the acid-alcohol used to differentiate the nuclear stain will be adequately neutralized by washing under the tap. In these circumstances it is probably advisable to rinse off the subsequent stains with de-ionized water. These remarks apply equally to the other variants described below.

COMMENT

The results with this AO10.DR80 counterstain are similar to those obtained with method 1 but the small-molecule orange-yellow dye is more sharply confined to erythrocytes and cytoplasmic granules than is the picric acid of van Gieson and thus the amyloid has a bluer colour. The large direct red dye has a stronger affinity for reticulin than does acid fuchsin, and like other direct dyes, has a notable affinity also for pseudocollagen (colour Figs. 5, 6, 7). The long staining time allows this large dye to gain a foothold in fibrin and this may sometimes be seen as a dark brown-red area amidst the amyloid (see page 381).

Method 3: SAB with Counterstain PAS Followed by AY24.DY11

Proceed to step 5 of method 1; there is no need here to alkalinize the SAB staining as the periodic acid seems to stabilize it. Stain with PAS.

After the final wash of the PAS procedure, stain the nuclei with ferrous haematein, differentiate with acid-alcohol, and neutralize with aqueous potassium acetate as in method 2.

Proceed to counterstain AY24.DY11; this is the same as method 2 except that the Acid Orange 10 is replaced by Acid Yellow 24, and the Direct Red 80 is replaced by Direct Yellow 11.

NOTE

We prepare Schiff reagent according to the method of Itikawa and Ogura (1954) as given by Drury and Wallington (1967) and by Pearse (1968). The best form of basic fuchsin for this and most other histological purposes is pararosaniline (Basic Red 9). An excellent commercial Basic Red 9 is available from Messrs. Colne Vale Ltd[2] as Para Magenta.

Acid Yellow 24 is the dye known to histologists as Martius yellow. We use a commercial brand,

[1]Messrs Williams (Hounslow) Ltd, Hounslow, Middlesex, England.

[2]Messrs Colne Vale Dye and Chemical Co. Ltd., Box A33, Huddersfield, England.

Manchester Yellow (Na salt) from Messrs W. S. Simpson Ltd[3]. Direct Yellow 11, which histologists know as sun yellow, is commercially available from Messrs Williams (Hounslow) Ltd as Direct Yellow R.

COMMENT

The particular value of this sequence is that in general it gives a pale blue on fresh amyloid; as the amyloid ages it becomes more PAS-positive and the colour darkens through deep blue (colour Fig. 8) to plum-purple (Kornerup and Wanscher, 1963). Then as the amyloid's affinity for SAB diminishes, the colour changes gradually to magenta (colour Fig. 9); more slowly then the amyloid loses its ability to react with PAS and so comes to stain the pure yellow colour of the collagen dye (colour Fig. 10). In different sites (eg, tubular membranes, vessel walls, glomerular tufts, and capsular linings) the rate, direction, and completeness of these colour changes may be somewhat different.

Method 4: SAB with Counterstain Silver Reticulin Followed by AR4.DY11

Proceed to step 5 of method 1; there is no need here to alkalinize the SAB staining as it is adequately stabilized by the phosphotungstic acid of the reticulin method (Slidders, Fraser, and Lendrum, 1958). We have slightly modified this as follows:

After formalin reduction, Jar IV of the method (*loc cit*), tone by flooding with gold chloride for five minutes and wash in water; treat with hypo, Jar V, for up to 10 minutes and wash again.

Stain the nuclei with ferrous haematein, differentiate with acid-alcohol, and neutralize with aqueous potassium acetate as in method 2.

Proceed to counterstain AR4.DY11; this is the same as method 2 except that the Acid Orange 10 is replaced by Acid Red 4 and the Direct Red 80 is replaced by Direct Yellow 11 as in method 3.

NOTE

SAB staining is bleached, even after alkalinization, by treatment with acid-permanganate; therefore, reticulin methods necessitating this step cannot be used after SAB. Our method has the further advantage of completeness of reticulin pattern and minimal interference with subsequent counterstaining.

Acid Red 4 is the dye known to histologists as azoesin. An excellent commercial Acid Red 4 is available from Messrs L. B. Holliday Ltd[4] as Azo Eosine. For Direct Yellow 11 see under method 3.

COMMENT

With thin sections the reticulin picture can be excellent (colour Fig. 11), although the intensity of the SAB staining is diminished. This combination reveals the relation of amyloid to the argyraspid (silver-coated) membranes (colour Fig. 12).

I iv Masson-type Staining

As noted already (Part I i), amyloid material in the kidney is moderately recognizable in a well stained trichrome. The definition is distinctly better if the small first dye is sharply confined to cytoplasmic structures and the large second dye to the stromal ones, as in the variants described above as counterstains to SAB. These antipodal trichromes, thanks to the lack of overlap of the two colours and to the ease of performance, are also excellent Massons in their own right, and we now offer some others with comments on particular applications.

Method 5 with Variants

1 Take sections to water.
2 Stain the nuclei with ferrous haematein, differentiate with acid-alcohol, and neutralize with aqueous potassium acetate as in method 2 (see part I iii).
3 Rinse with 95% ethanol and stain in 0·5% small-molecule anionic dye, and 2% phosphotungstic acid in 95% ethanol, three minutes.
4 Rinse in water.
5 Stain in 0·5% large-molecule anionic dye in aqueous 1% acetic acid, half to two hours.
6 Rinse in water; dehydrate, clear, and mount as in method 2.

SMALL-MOLECULE ANIONIC DYES

Acid Yellow 24 (Martius yellow); see method 3.
Acid Orange 10 (orange G); see method 2.
Acid Red 4 (azo-oesin); see method 4.
Acid Red 57; see notes below.
Acid Violet 19 (acid fuchsin); see notes below.

LARGE-MOLECULE ANIONIC DYES

Direct Yellow 11 (sun yellow); see method 3.
Direct Red 80; see method 2.
Direct Blue 10; see notes below.
Direct Blue 109; see notes below.

NOTES

Acid Red 57 is commercially available from Messrs ICI as Propalan Red 3GX, and from Messrs Sandoz as Azo Rubinole 3 GP.

[3]Messrs W. S. Simpson and Co, Linden Way, Old Southgate, London N14, England.
[4]Messrs L. B. Holliday and Co Ltd, Box B22, Huddersfield, England.

Acid Violet 19 has a rather variable quality and in this method must be freely soluble in alcohol. An excellent product is commercially available from Messrs Colne Vale as Acid Magenta PR, this being a sulphonated derivative of pararosaniline.

Direct Blue 10 is supplied by Messrs Bayer as Benzo New Blue GS, and Direct Blue 109 by Messrs ICI as Durazol Brilliant Blue B.

Selected Masson Variants

VARIANT 1
AO10.DB109, which is the same as the JSDB109 method (Lendrum *et al*, 1964), the precursor of all the permutations described in this section, by itself provides a fine picture of renal reticulin. Some material does better with AO10.DB10, thanks to the darker shade of the blue and its red contaminant of smaller molecular size. These combinations can also be used as counterstains after PAS (see Figs. 8, 9, 10, and 13 of Lendrum, 1969), thus providing a notable improvement on Lillie's allochrome method (1951).

VARIANT 2
AO10.DR80, like the preceding, is an interesting occasional counterstain to PAS, providing with cyan-filtration excellent detail in black and white photography of glomerular reticulin. After SAB, as in method 2 above, and after silver impregnation, it has the advantage of emphasizing pseudo-collagen (see Fig. 16 of Lendrum, 1969).

VARIANT 3
AY24.DY11 is a delicate stain with an occasional place as counterstain after PAS because of its minimal tendency to alter the magenta colour, as, for example, in method 3 above (part I iii).

VARIANT 4
AR4.DY11 is a good counterstain after Alcian Blue staining for mucin, and after Weigert's elastica method, ideally Lawson's modification (1936). See also method 4 above (part I iii).

VARIANT 5
AR4.DB109 produces a brilliant scarlet in the erythrocytes contrasting well with the clear blue of collagen and reticulin; there is also spread of the scarlet to granules, fibrin, and variably to muscle and to myelin.

VARIANT 6
AR57.DB109 is a good standard Masson, as this red gives a little more cytoplasmic staining than AR4.

VARIANT 7
AV19.DY11 is the first choice when smooth muscle is to be demonstrated, but is generally unnecessarily strong on normal striated muscle. It is useful in the search for striated fibrils in neoplasms and for staining myoepithelium.

With fixation other than described above (part I ii), some variation of the stated staining times may be necessary, but improved results are more likely to be obtained by subjecting the sections to procedures designed to overcome deficiencies in fixation. The simplest of these is a 24-to-48-hour treatment with picro-alcohol (80% ethanol saturated with picric acid) which, in addition to removing any formalin pigment present, has a beneficial effect on most trichrome stains and on van Gieson's stain. Sometimes the efficacy of the treatment is improved by the addition to the picro-alcohol of 4% mercuric chloride. Treatment with picro-alcohol or picro-mercuric-alcohol should be carried out before sections come in contact with water, and after picro-mercuric-alcohol sections must be passed through iodine and hypo. The degreasing procedure (Lendrum *et al*, 1962) may also bring about an improvement in staining; this is a simple immersion of the section immediately after its dewaxing, for 24 to 72 hours in pure trichloroethylene. Tissue fixed in a chrome-containing fixative is usually improved by a modified Overton's dechroming technique: sections are immersed for half an hour in sulphurous acid prepared by bubbling sulphur dioxide from a syphon into 20% ethanol (Mann, 1902).

I v Identification of Dyestuffs (the Colour Index)

The accurate identification of dyes is of vital importance in histological staining. Much of the common confusion arises from the arbitrary naming of dyes; different dyestuff manufacturers supply chemically identical dyes under quite different names, and sometimes quite dissimilar dyes are given names that to the layman seem to imply a similarity that does not in fact exist. For example, Acid Orange GG (Williams), Fast Light Orange G (Holliday), Naphthalene Fast Orange 2G (ICI), and Xylene Fast Orange G (Sandoz) are only a few of the synonyms for the dye better known to histologists as orange G, but neither Phloxine G (American Aniline Products) nor Acronol Phloxine FF (ICI) are in any way similar to the standard histological dye phloxin B. The difficulties thus created can be obviated if all writers would refer to dyes by both name and Colour Index (CI) number, as has been done in the current Biological Stains (Conn, 1969).

The Colour Index (2nd edition, 1956) was compiled jointly by the Society of Dyers and Colourists

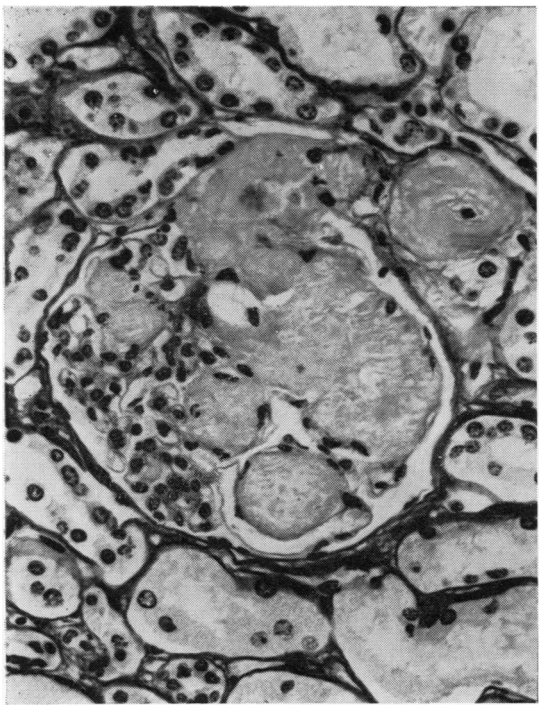


Fig. 1.

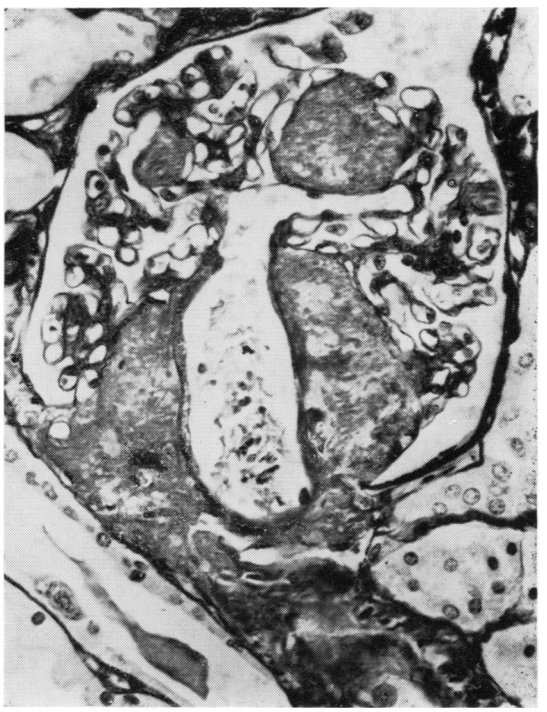


Fig. 2.

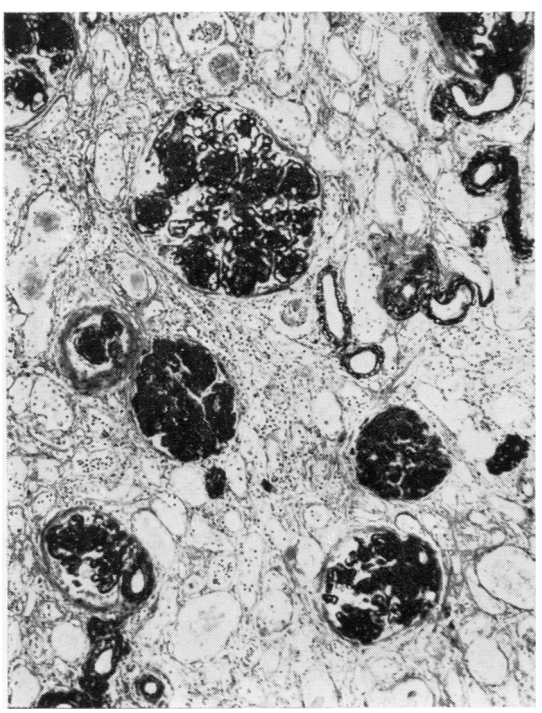


Fig. 3.

Fig. 1 *Stain AR57.DB109, method 5 var 6 × 315. Filters minus: blue 1, green 2. Amyloid (pale blue) is seen as grey compared with the black (dark blue in section) of the capsular and tubular membranes. It distends arteriolar wall and spreads into the glomerulus forming characteristic acellular discs at glomerular periphery with persistence of covering capillaries. R.I. Dundee N4061.*

Fig. 2 *Stain AO10.DB109, method 5 var 1 × 280. Filters minus: blue 1, green 2. Amyloid grossly involves an arteriole, almost certainly afferent, with enlargement of lumen. This feature of many plasmatic vasculoses in the kidney was regarded in the distant past as evidence of hypertension! R.I. Dundee N4061.*

Fig. 3 *Stain SAB-van Gieson, method 1 × 70. Filters minus: blue 1, green 2. Green-stained amyloid (black in photograph) is confined to arteries, arterioles, and glomeruli, the last being heavily involved. There is hardly any sign of tubular involvement. W.I. Glasgow A6485.*

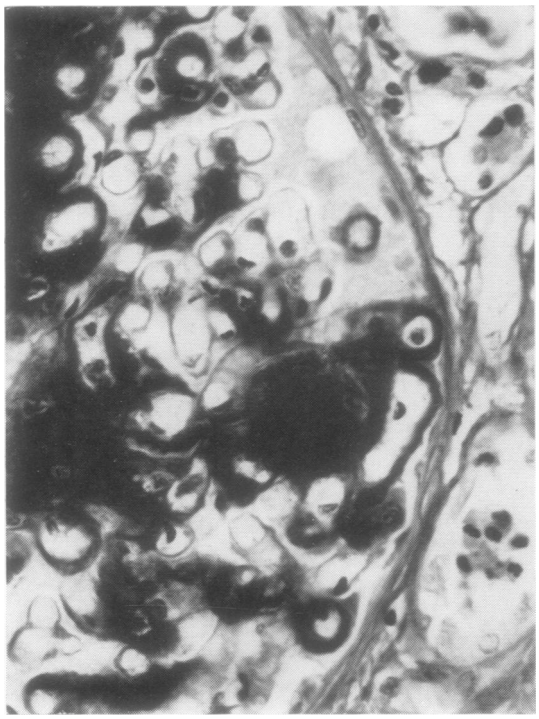


Fig. 4.

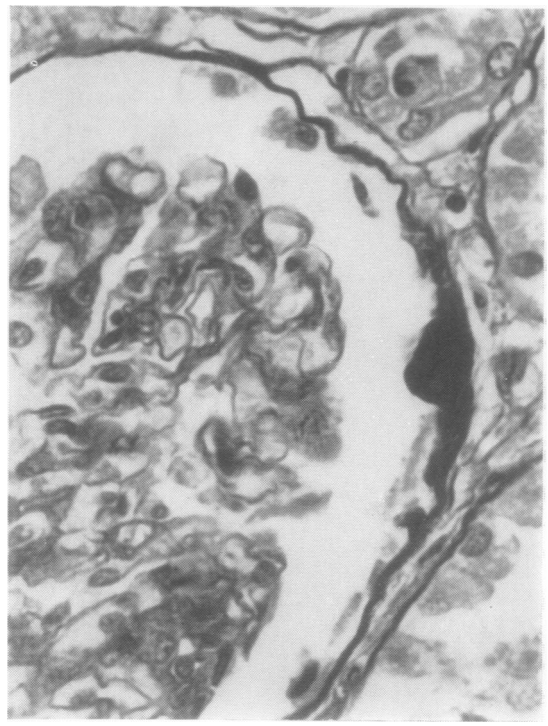


Fig. 5.

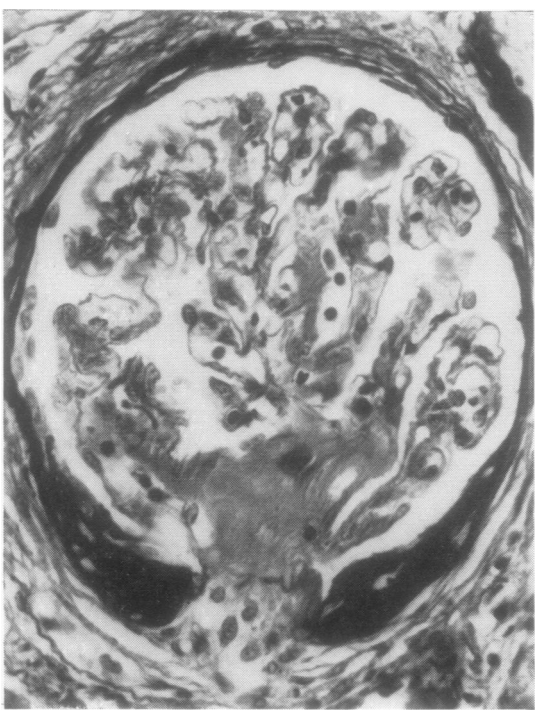


Fig. 6.

Fig. 4 *Same section as Fig. 3* × *392. Filters minus: blue 1, green 1. The deposition of amyloid (black) in the glomerular capillary walls varies greatly in degree; the intense involvement of some peripherals recalls the intramural deposition of material, staining like fibrin, in systemic lupus erythematosus.*

Fig. 5 *Stain SAB-van Gieson, method 1* × *784. Filters minus: blue 1, green 2. This papulose deposit of amyloid under the epithelium of Bowman's capsule is rarely so obvious as the comparable fibrinous deposit in the diabetic (Fig. 13, Lendrum* et al, *1962). R.I. Dundee N6170.*

Fig. 6 *Stain AO10.DB109, method 5 var 1* × *350. Filters minus: green 1, blue 2. The pericarp formation of pseudo-collagen, stained dark blue (black in photograph) forms thick nodes at the glomerular root, as also happens with fibrin-derived pseudo-collagen. Amyloid at the base of the stalk is relatively chromophobic (pale blue in section). R.I. Dundee N5842.*

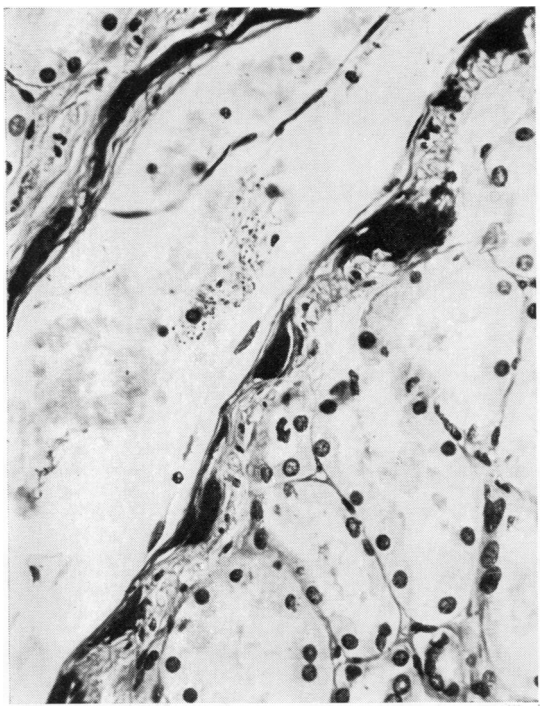


Fig. 7.

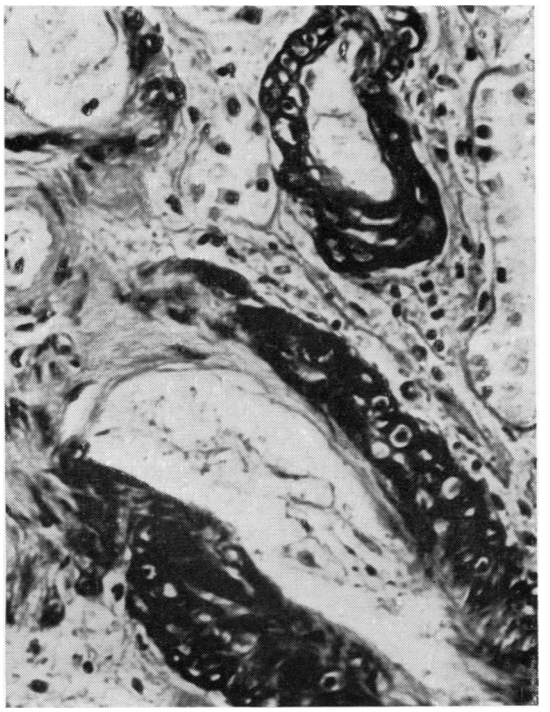


Fig. 8.

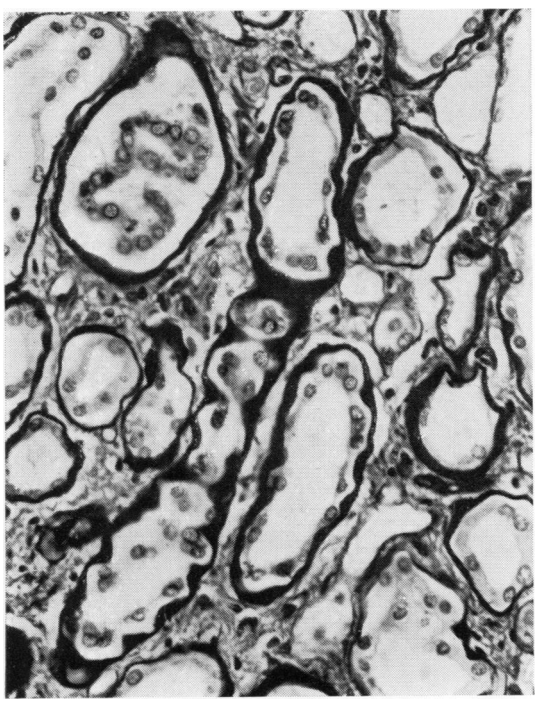


Fig. 9.

Fig. 7 *Stain SAB-van Gieson, method 1 × 280. Filters minus: blue 1, green 2. The subendothelial deposits (black) of amyloid push outwards against the media of this small artery, as the fibrinous ones do in diabetes (Figs. 9 to 12, Lendrum* et al, *1962). R.I. Dundee N4061.*

Fig. 8 *Stain SAB-AO10.DR80, method 2 × 294. Filters minus: green 1, blue 2. The amyloid (black) has infiltrated into the media, surrounding the myocytes with but slight evidence of ill effect. R.I. Dundee N6763.*

Fig. 9 *Stain SAB-van Gieson, method 1 × 336. Filters minus: blue 1, green 2. This stains amyloid thickening of renal tubules a uniform green (black in photograph), without distinguishing between the original membrane and the new deposit (compare colour Fig. 12). The cog-like internal spurs seem to encircle the tubule, as does the pseudo-collagen in colour Fig. 7, and also the fibrin-derived pseudo collagen in Figs. 9, 10, and 17 (Lendrum, 1969). Maryfield H. Dundee E318.61*

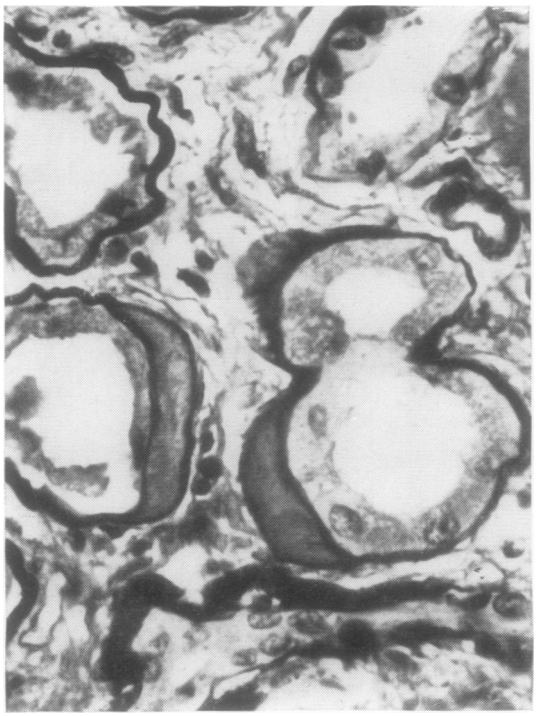


Fig. 10.

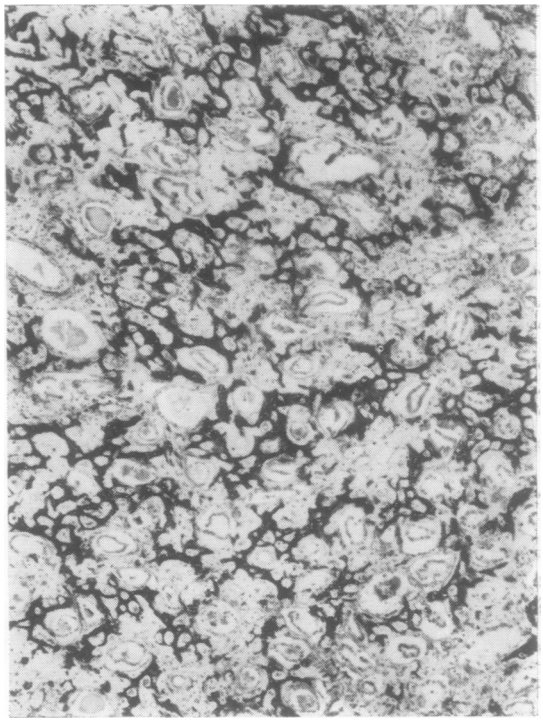


Fig. 11.

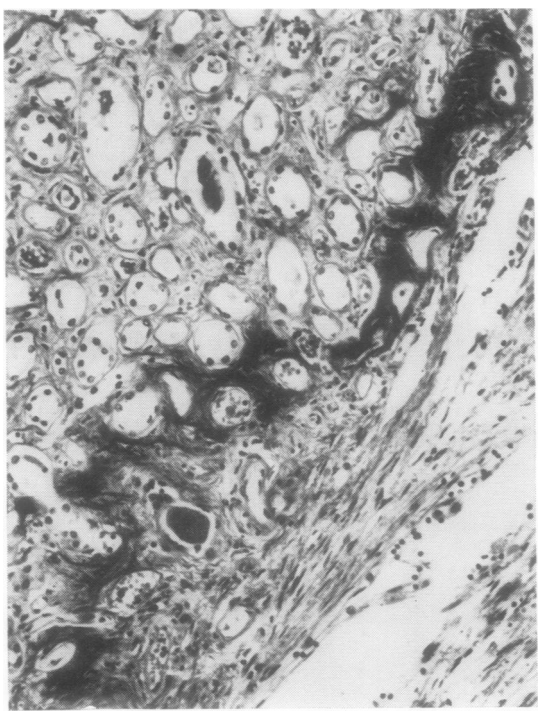


Fig. 12.

Fig. 10. *Stain SAB-PAS.AY24.DY11, method 3 × 525 Filters minus: red 1, blue 3. Two of the tubules show bluish deposit of amyloid (grey) with dark purple (black) line on both sides of the deposits. 'Duplication of basement membranes' is a feature of the severely affected diabetic kidney (Lendrum, 1963). R.I. Dundee N5842.*

Fig. 11 *Stain SAB-AO10.DR80, method 2 × 60. Filters minus: green 1, blue 2. In the juxtapapillary medulla the amyloid deposit (black) is intertubular and assumes this wild array, maintaining it to the stage of pseudo-collagen (colour Fig. 4). This is probably a diagnostic picture. R.I. Dundee N5370.*

Fig. 12 *Stain SAB-AO10.DR16, variant of method 2 × 168. Filter minus red 1. Edge of medulla (see page 389) with amyloid-engendered pseudo-collagen (black) strongly stained by large-molecule red dye. The topical disposition is identical with that of pseudo-collagen of fibrinous origin in diabetics and some primary hypertensives; this seems to be closely related to venous sinuses. Maryfield H. Dundee E348.62.*

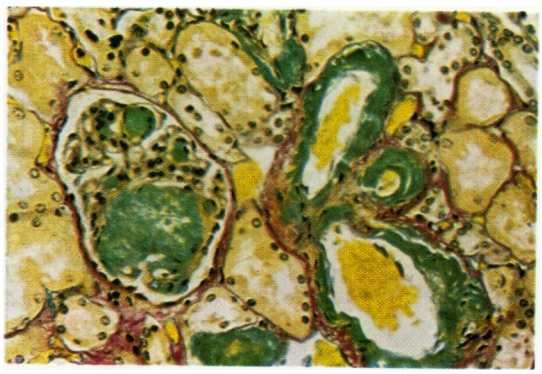


Fig. 1.

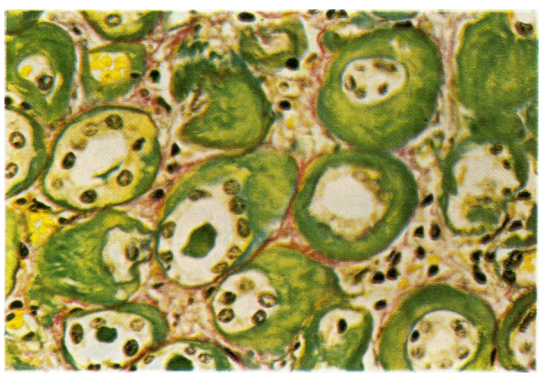


Fig. 2.

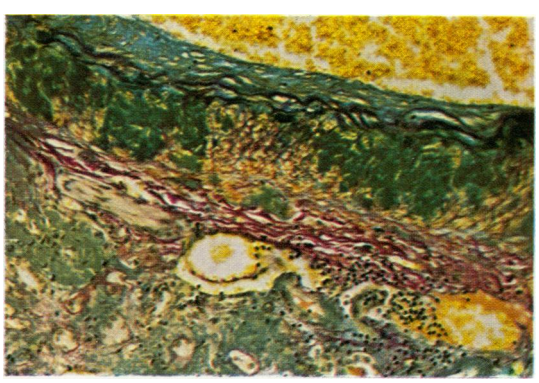


Fig. 3.

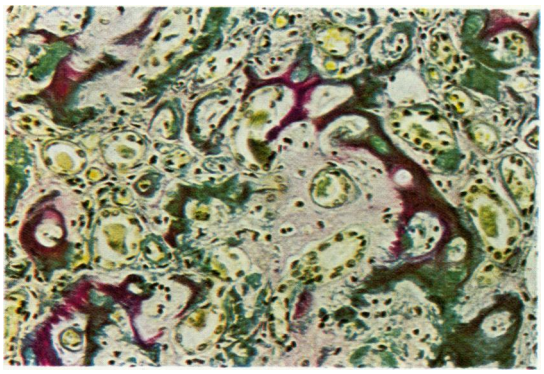


Fig. 4.

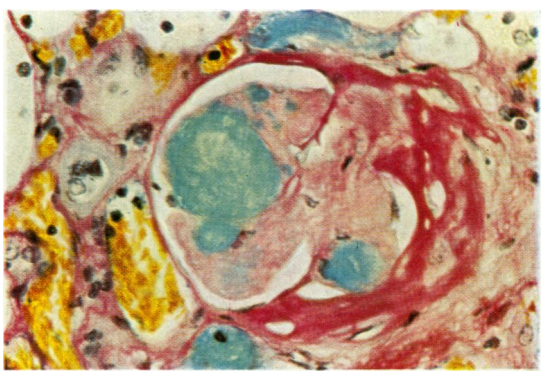


Fig. 5.

Fig. 1 *SAB-van Gieson, method 1 × 90. Amyloid in arterial wall of stronger green than in glomerular deposits; latter disc-like as are fibrinous deposits in diabetic. R.I. Dundee N4061.*

Fig. 2 *Stain as Fig. 1 × 180. Amyloid deposit in tubules, between base of epithelium and basement membrane. Erythrocytes yellow. R.I. Dundee N5842.*

Fig. 3 *Stain as Fig. 1 × 56. Kidney with massive amyloidosis; deposit grey-green except in large arteries where brighter colour may mean recent deposition. Dr. Eggink's case, Groningen 4912 of 1966.*

Fig. 4 *Stain as Fig. 1 × 90. Intertubular amyloid (Hexentanz) showing aging, by diminishing affinity for SAB and replacement of green by red of fuchsin. Maryfield H. Dundee E318.61* (cf *Fig. 11*).

Fig. 5 *SAB-AO10.DR80, method 2 × 180. Amyloid bluer than with method 1, fading to dull pink in remnants of atrophic glomerulus. Pseudo-collagenous pericarp, closing down on tuft, shows usual strong affinity for large-molecule anionic dye. R.I. Dundee N4061.*

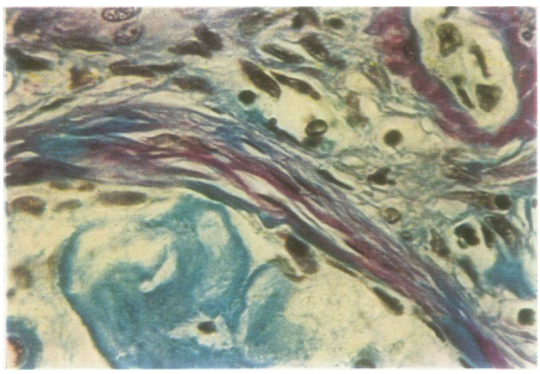


Fig. 6 *Stain as Fig. 5 · 288. Subepithelial deposit in Bowman's capsule with blue amyloid and dull red of partial change to pseudo-collagen. Blue mass below is part of tuft showing more recent amyloid. R.I. Dundee N4081.*

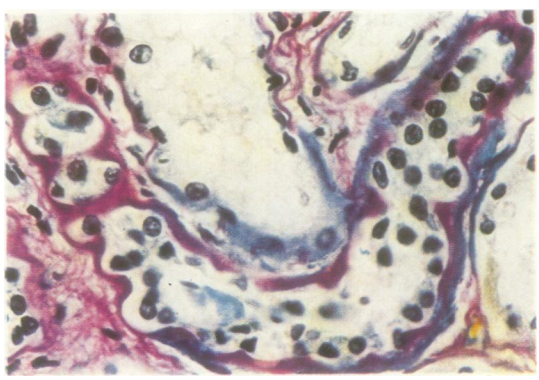


Fig. 7 *Stain as Fig. 5 · 225. Right limb of tubule shows blue layer with red internal spurs; left limb now all red (pseudo-collagen). Compare Figs. 5* et seq *in Lendrum (1969). Maryfield H. Dundee E318.61.*

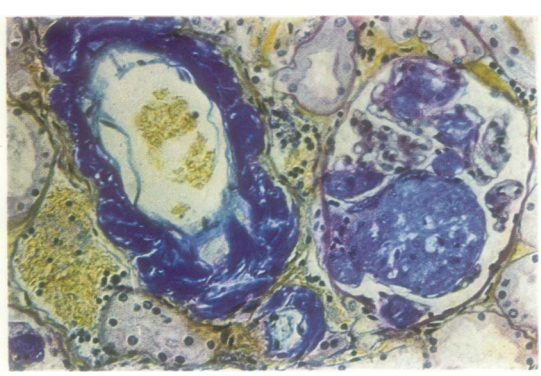


Fig. 8.

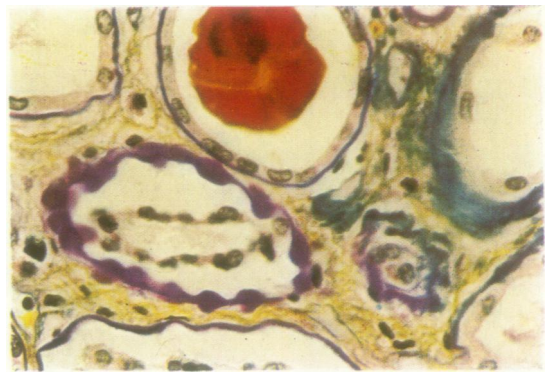


Fig. 9.

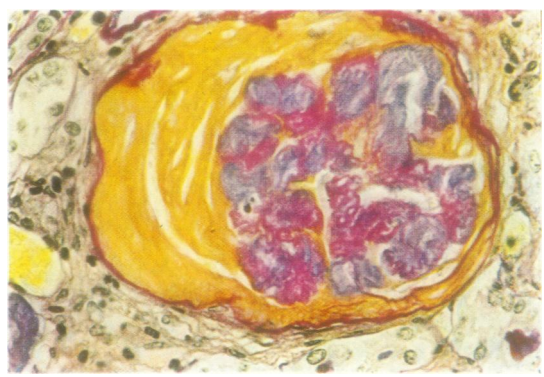


Fig. 10.

Fig. 8 *SAB-PAS.AY24.DY11, method 3 · 90. Strong blue in vessel wall compounded of SAB and PAS staining. In glomerulus SAB is less strong, the mixture fading to pink. R.I. Dundee N4061.*

Fig. 9 *Stain as Fig. 8 · 225. Blue subepithelial layers are seen, changing to purple in small tubule; late stage of cogs seen as purple string of beads, the purple being stage between blue of SAB and magenta of PAS. Light red of cast compounded of PAS and yellow counterstain. Maryfield H. Dundee E318.61.*

Fig. 10 *Stain as Fig. 8 · 180. Section, six years old when photographed, shows replacement of SAB in glomerular remnants, now mainly PAS positive, and replacement of PAS by large-molecule yellow dye, in the acellular pericarp of pseudo-collagen. Outer membrane retains PAS reactivity, as also in diabetic glomeruli. Compare Figs. 11 and 12 in Lendrum (1969). R.I. Dundee N5842.*

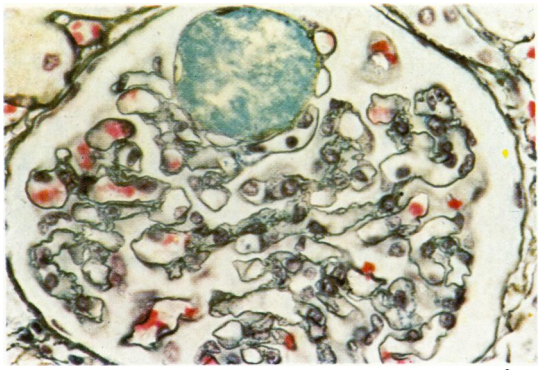


Fig. 11.

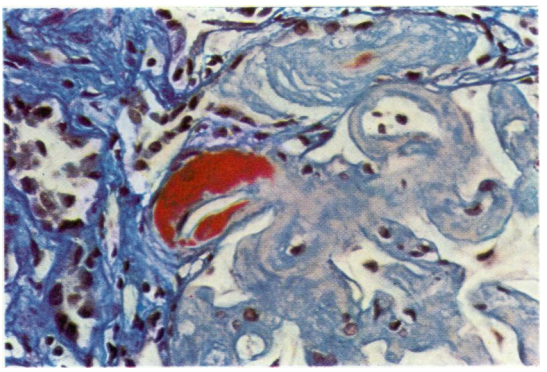


Fig. 14.

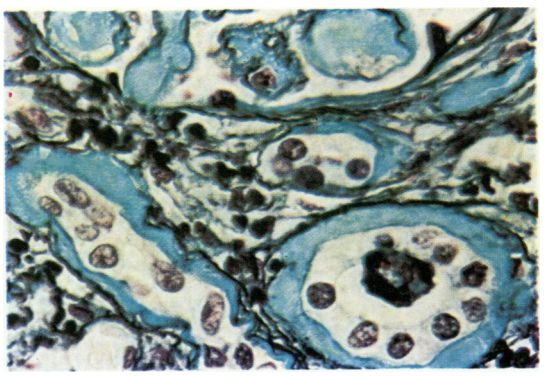


Fig. 12.

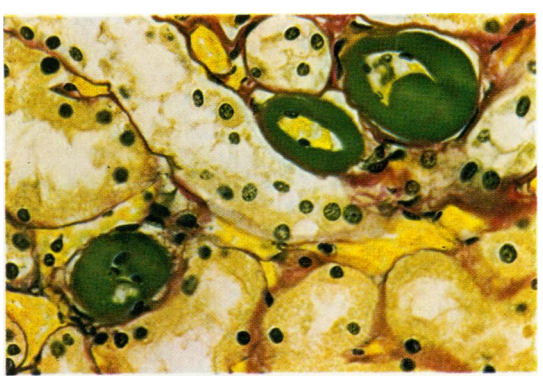


Fig. 13.

Fig. 11 *SAB-Retic.AR4.DY11, method 4× 225. Early involvement of glomerulus, peripheral disc of amyloid with coronet of uninvolved capillaries. Red erythrocytes. R.I. Dundee N4061.*

Fig. 12 *Stain as Fig. 11 × 288. Section, 10 years old when photographed, shows in cortical tubules relation of blue amyloid layer to the black argyraspid (silver-coated) reticulin. R.I. Dundee N4061.*

Fig. 13 *SAB-van Gieson, method 1 × 180. Diabetic arteriolar walls infiltrated with fibrin, now old enough to react with SAB. Only one other diabetic case seen with as vividly confusing a reaction. W.I. Glasgow B5302.*

Fig. 14 *MSB stain × 225. Familial Mediterranean fever with amyloid, typically pale blue compared with deep blue of stroma to left. Fibrin in glomerular arteriole strongly red; fading pink in various parts of glomerulus and in other arteriole. Dr A. G. Stansfeld's case (Dormer and Hale, 1962).*

and the American Association of Textile Chemists and Colorists, and is, we believe, the only internationally recognized and reputable index. Part I groups dyes mainly on the basis of their method of use, eg, acid, direct, mordant. Within each category the dyes are arranged in order of spectral hue and each dye is given a part I—CI number, eg, Congo red is Direct Red 28. All the commercial names under which the dye is sold are listed together with the code names of the manufacturers. In part II of the Colour Index dyes are grouped according to their chemical constitution, eg, azo, triarylmethane, xanthene. Where known, the structural formulae are shown and each dye is given a five-figure part II—CI number, eg, Direct Red 28 appears as C.I. 22120. Part III of the Colour Index contains an alphabetical list of commercial names together with part I and part II—CI numbers, and conversion tables equating the CI numbers in the second edition of the Colour Index with those in the first edition.

Dyes are accurately designated either by their part I or part II—CI numbers. We prefer to use Part I numbers as being more easily remembered and, in addition, giving an indication of the character of the dyes and the histological use to which they may be put[1].

PART II

The Topical Disposition of Amyloid Deposits in the Kidney and its Similarity with that of Fibrin in Diabetic Kidneys

The kidney provides an ideal field wherein to compare the situation of these two different deposits. The SAB method with subsequent trichromic counterstaining demonstrates with graphic exactitude the site of amyloid material and its relation to the normal structures. Previously our methods for fibrin staining (Lendrum *et al*, 1962, 1964) had revealed with similar clarity the site and structural relations of fibrinous deposits within the tissues. Thus demonstrated, both these depositions are seen to be bedded between normal structures, recalling the description of amyloid by Barnard, Smith, and Woodhouse (1938), 'As far as the interstitial tissue is concerned the appearance is that which would be found if the organ had been infiltrated by a coagulable fluid which had coagulated.'

Extraluminal deposition of fibrin has now been studied in the kidneys from 89 necropsies on cases of diabetes mellitus, material supplying abundant examples of the phenomenon (Lendrum, 1963, 1969). This provided information unexpectedly useful when we saw, thanks to the SAB method, the precise situation of amyloid deposits in the amyloidosis kidney. For comparison with the diabetic kidneys, we studied kidneys from 60 cases of amyloidosis related to chronic infection, rheumatoid arthritis, or vague disease combinations; in addition, thanks to Professor Wolman and to Dr A. G. Stansfeld, we obtained sections from cases of familial Mediterranean fever.

In the amyloid kidney the early changes are obvious only in the interlobular arteries, the related arterioles, and some of the glomeruli. In many of the least involved glomeruli the amyloid occurs as spherules at the periphery of the glomeruli with a coronet of patent capillaries (colour Figs. 1 and 11), exactly comparable with the fibrinous spherules seen in the early stages of the diabetic kidney (Lendrum, 1963). The similarity in H and E sections of early amyloid glomeruli to the *late* nodular lesion of diabetic glomerulosclerosis observed by Kimmelstiel and Wilson (1936) had been noted by Smith, Bolton, and Turnbull (1955) who clearly imply that they regard both types of nodule as resulting from extravasation of something 'present in the blood'. Although Heptinstall (1966) regarded this as merely a 'superficial resemblance', Martin, Brown, and Daugherty (1966) think this amyloid glomerulus could easily be confused with the nodular diabetic, and Dustin (1969) accepts the appearances as very comparable. With the new staining methods, glomeruli showing *early* stages of either of these diseases are unlikely to cause difficulties for the microscopist. Certainly, however, these topical and morphological similarities raise the possibility, as discussed below, of a similarity in the mechanisms of origin of the spherules.

Deposition of amyloid also occurs under the epithelium lining Bowman's capsule (Lendrum, 1963); this rarely bulges so freely into the space as fibrin may (Fig. 5), but the deposit seems to build up in layers as we believe happens with fibrin. This is discussed further in part III below. In the interlobular arteries and related arterioles the amyloid may be seen as subendothelial deposits with a somewhat triangular shape, the long side toward the lumen and the peak pushing down into the media (Fig. 7), thus exactly copying the intramural fibrinous

[1]Since this paper was submitted, Messrs ICI have revised the names of many of their products. Of the dyes mentioned in the text, those renamed are given below:

C.I. Number	*Old Name*	*New Name*
Acid Orange 10	Naphthalene Fast Orange 2GS	Lissamine Orange 2G
Acid Red 57	Propalan Red 3GX	Lissamine Red 3GX
Direct Red 80	Durazol Brilliant Red BS	Durazol Red B
Direct Blue 109	Durazol Brilliant Blue BS	Durazol Blue B
Ingrain Blue 1	Alcian Blue 8GX 300	Alcian Blue 8G

deposits of diabetes as well as those of benign hypertension (Lendrum *et al*, 1962); more commonly, however, it seems to insinuate quietly throughout the wall surrounding the myocytes like a magma (Fig. 8). In the proximal interlobular and larger arteries there may be amyloid in greater amount than is usual with diabetic fibrin, but as with fibrin the main site of deposition is in the wall of a branch just as it leaves the media of the parent vessel, and as in all the fibrinous vasculoses the intruding amyloid spreads laterally in the juxta-adventitial part of the parental media.

In the tubules the amyloid, like fibrin, can form a layer between the epithelium and the basement membrane (Carone and Epstein, 1960; Lendrum *et al*, 1962; Lendrum, 1963), a relationship particularly well shown if the reticulin is impregnated with silver after SAB staining (method 4, colour Fig. 12). Again as with fibrin, this layer of amyloid may project between the bases of the cells (Fig. 9), producing a cog-like layer (Lendrum, 1963). Here it is of interest that those studying amyloidosis in guinea pigs and hamsters have shown by electron microscopy a comparable intercellular projection of the subepithelial deposit (Caesar, 1963a; Swaen and van Haelst, 1966; Shirahama and Cohen, 1968). A further site of comparable deposition is along the edge of the columns of Bertin where the parenchymal medullary tissue meets the pelvic fat (Fig. 12). Here the extravasation seems to be from veins (Lendrum, 1963, 1964), and thus raises the question of an upset of renal haemodynamics. The ultimate accumulation of pseudo-collagen appears to lie round venous sinuses and looks identical in amyloidotic and diabetic cases; a similar deposition of pseudo-collagen occurs in some cases of hypertensive nephrosclerosis.

In sum, the disposition of amyloid deposits in the human kidney spectacularly resembles the topical deposition in the diabetic kidney of fibrin.

The origin of the fibrin deposited in the diabetic kidney presumably lies in the advent of a soluble form, and since fibrin's known precursor is the fibrinogen in the plasma we can reasonably regard the result as a manifestation of what Schürmann and MacMahon (1933) called 'dysoria'. This concept of dysoria (upset of permeability) was crystallized for these authors mainly from their studies on the intramural and extravascular deposition of fibrin in cases of severe hypertension and of hyperergy. The latter state is dysoric probably in virtue of a local leakiness which may be extreme even when the blood pressure is not raised. Rapid elevation of blood pressure in experimental animals (Giese, 1967; Hüttner, Jellinek, and Kerényi, 1968; Byrom, 1969; Still and Dennison, 1969) produces morphological results satisfactorily compatible with the dysoric concept. At the other extreme the lesser but persistently maintained pressure of benign hypertension in the human may also bring about dysoria, of diffuse character and mild degree, perhaps the origin of eventual vascular hyalinosis. Speculation apart, we have shown in the diabetic kidneys (Lendrum, 1969) that the amount of extraluminal fibrin and the distance from vessels of its deposition (eg, into the lining of glomerular capsules, and into tubular walls between the epithelium and the basement membrane) are both much greater than would be expected from the degree of hypertension present. It is on these grounds we presume in the diabetic vessels an abnormal excessive permeability. There seems little doubt that the types of plasmatic vasculosis revealed by the deposition of fibrin, namely the fibrinous vasculoses (Lendrum, 1955), be they hypertensive, hyperergic, hypoxic, or metabolic, can reasonably be regarded as manifestations of dysoria caused by excessive *vis a tergo*, or abnormal porosity, or varied combinations thereof.

On the other hand, in amyloidosis we have no known reason to inculpate either hypertension or abnormally high penetrability. At cellular level, Cohen's studies with the electron microscope (1965, p. 171 *et seq.*) suggest a bizarre formative activity in the endothelial cells of the glomerulus, and Kennedy's work with isotopes (1962) rather implies that the activity of the endothelial cells is a joining together of some substance arriving in the plasma with a second substance produced in the endothelial cells, the subsequent extrusion of this new compound into the extravascular tissues being the birth of amyloid material. This may well be so, and it is of interest that Glenner, Terry, Harada, Isersky, and Page (1971) have recently described amyloidosis as 'a disease caused by the deposition in tissues of an amino-terminal fragment of the variable region of the light (or possibly heavy) chain of monoclonal immunoglobulins by a mechanism yet to be defined'. For the histologist, however, the fact remains that in the kidney the site and shape of the amyloid deposits resemble with remarkable exactitude those of the fibrinous deposits of the diabetic. A further geographical'[1] fact may be added here; the disposition of fibrinous deposits in the Malpighian corpuscles of the spleen in cases of polyarteritis nodosa (Lendrum *et al*, 1962, Fig. 19) is virtually identical with the disposition of early amyloid material in both the experimental and human spleen (Jaffé, 1926; Dick and Leiter, 1941; Turnbull, 1945; Dahlin,

[1] "Medicine without morbid anatomy is like history without geography." Donald G. McKay, Professor of Pathology, San Francisco, in seminar at Dundee, 1964.

1949; Druet and Janigan, 1966; Scholten, van den Broek, Ruinen, Mandema, and Keuning, 1968).

The history of the happenings before the event can hardly be deduced from our sections. We may reasonably wonder, however, how the amyloid material or its soluble precursor comes to be carried away from the vessels, presumably in some still fluid and pushable form, to such paravascular recesses as the subepithelial layer of Bowman's capsule and the subepithelial spaces in the renal tubules. That fibrinogen can reach these sites in plasma, extravasated because of a dysoric state, seems a reasonable presumption (Lendrum, 1969); we therefore feel that in relation to the deposition of amyloid we have similarly to presume some degree of dysoria, but without a hypertensive component. Thus we have to postulate either a focal hyperpermeability of the vessel wall as is seen in hyperergic diseases, or the presence in the plasma of a qualitatively abnormal solute able to pass through the wall in Aschoff's 'invading stream of plasma' (Lendrum, 1967). Perhaps the fragmented globulin of Glenner *et al*, mentioned above, is just such an abnormal solute.

To return for a moment to fibrin, we know (see part III) that in the diabetic there is an excessive escape of fibrinogen and that the deposited fibrin alters to a hyalin material with an affinity for collagen dyes (pseudo-collagen); from this it is not unreasonable to suggest, as already mentioned, that the hyalinized vessels of old age are the end result of slow minimal intramural deposition of fibrin over the years. In like manner gross deposition of amyloid might be the result of an escape and deposition of a proteinous compound present in excess in the plasma because of an intense immunological upset, whereas a slow minimal deposition over the years could be the explanation of the various amyloids of later life—in the pancreatic islets, the heart, and the senile plaques in the brain.

The possibility that the dysoria in the amyloid kidney is, in part at least, due to a focal abnormal hyperpermeability gains some support from the finding in many of our amyloid cases of a positive fibrin reaction. This was perhaps more obvious in the two cases of familial Mediterranean fever, a disease in which amyloidosis commonly occurs and in which the plasma fibrinogen may then reach very high levels (Frensdorff, Sohar, and Heller, 1961). Histological localization of fibrinogen within amyloid deposits by fluorescent antibody has been demonstrated by Horowitz, Stuyvesant, Wigmore, and Tatter (1965) who suggest that the deposition of fibrin and the consequent tissue alterations permit or predispose to 'the deposition of yet other proteins and lead to the morphologic entity of amyloidosis'. This hypothesis gains no support from the fact that in 89 diabetic kidneys we found only one example of amyloidosis and this was one with very little fibrinous vasculosis.

The presence in the amyloid kidney of young or mature fibrin is revealed, although not very strikingly, in sections stained by SAB-van Gieson, as a yellow-buff area; at these stages in its history it has no affinity for any of the amyloid stains (see part IV below). With the MSB and MSDB109 fibrin stains, the young and mature fibrin stands out prominently red in the amyloid cases, the fibrin occurring as well defined deposits, comparable in shape with the diabetic ones, either within the glomerular masses or in an afferent arteriole containing little or no obvious amyloid (colour Fig. 14). Where the fibrin is aging and losing its affinity for the red dye in these methods, it is less easy to determine the dividing line between the fibrin and the amyloid. In an occasional amyloid case the peripheral glomerular capillaries show the heavy deposition of fibrin and fat that in the diabetic we regarded as a terminal event (Lendrum, 1963). Our impression certainly is that in well fixed tissue amyloid itself never at any time in its life story gives a positive reaction for young or mature fibrin. Thus the presence among the glomerular amyloid of a substance reacting as young or mature fibrin seems to us proof of a dysoric state.

At any rate some degree of dysoria in both diabetes and amyloidosis is the only explanation we can offer as a common factor to explain the topical similarity in the kidney of the deposits of fibrin (diabetic) and amyloid. Leaving their early history in doubt we can now turn with a little more certainty to the subsequent history of both.

PART III

The Similarity of the Subsequent History of Deposits of Amyloid and of Fibrin

The functional relation of the fine structure of acidophil tissues to the molecular size of the anionic dyes that stain these tissues preferentially (Slidders, 1968) was first suggested by Ehrlich (Baker, 1958) and clearly stated by Pappenheim (1901). Here is his significant statement in translation: 'Every dye goes into that material which has for it the most adequate pore size.'

The preferential colouring of tissues, however, seems to depend on more than merely pore size. For example, anionic dyes of smaller molecular size seem to 'invade' the substrate more smartly, gaining access and planting their colours ahead of their clumsy larger brothers. If the intermicellar spaces (pores) be small enough a small dye may fill and indeed become so jammed within the pores that it

can exclude a larger dye. If, however, the pore size be somewhat larger, the larger dye, given a prolonged staining time, may be able to work its way in and oust the smaller dye. With bigger pores still, the small dye apparently feels no inducement to stay; whereas the big dye, finding ingress relatively easy, colours this type of tissue without any degradation of its shade by a smaller rival. At this level of comparative magnitude of dye and pore, the histologist can by trial ensure that such a dye receives only enough staining time to fill the largest pore.

Being concerned with normal tissues, Pappenheim had apparently not considered the structure of abnormal materials such as fibrin, amyloid, or other hyalins. These of course were well known in his day; what was scarcely realized then was the possibility that these materials might undergo alteration with time. This latter idea was brought to our notice by the varied staining reactions revealed by our trichromic methods in the fibrinous deposits present in the kidneys of elderly diabetic patients (Lendrum, 1961; Lendrum *et al*, 1962), and this led us to conclude that fibrin deposited interstitially in tissues away from vascular lumina undergoes with time a change in its pore size.

Fibrin in sections stained with the MSB method is satisfactorily obvious when stained by the red dye, which in relation to the other two dyes in the method is of intermediate molecular size. A short-lived material, not often seen, which we believe to be newly formed young fibrin is stained by the smaller (yellow) dye used to colour the erythrocytes. The red-staining fibrin, what may be called mature fibrin, has a longer life as judged by its greater frequency in our material. There follows a shorter phase in which the fibrin becomes almost chromophobic; thereafter the fibrin gradually takes an increasing amount of the large blue dye used to colour collagen, and thus becomes the hyalin we term pseudo-collagen. Probably the old methods for demonstrating fibrin were positive for some part of the mature stage as defined here, but they were fickle and insufficiently automatic to allow valid comparison. We cannot deduce from our material whether the change with age in pore size moves at a uniform rate, nor can we exclude the possibility of temporary periods of reversed direction. The change, however, runs its course through to pseudo-collagen despite total absence of leucocytic invasion or even surrounding aggregation; it certainly seems to begin at the periphery of the fibrinous deposit, and eventually the whole mass becomes of large pore-size character in that it takes a large-molecule dye. This final phase of pseudo-collagenous hyalin may in some sites be permanent.

Before leaving the story of fibrin we feel obliged to restate why we do not use the term 'fibrinoid'. The word lacks precise meaning, for it has been used by some to describe a hyalin that stains like fibrin but on other grounds is thought to be something else, and by other writers to describe a hyalin that may have some relation to fibrin but fails to react with fibrin stains. A further reason for abjuring the term fibrinoid is that we believe the hyaline material thus described is in fact fibrin which, as described above, stains as fibrin when fresh, and thereafter loses its affinity for the fibrin stains and comes to stain like collagen (pseudo-collagen). As mentioned earlier (Lendrum, 1963), the term fibrinoid may have a place to describe hyalins that stain like fibrin but are shown by immunological methods to be globulins. That cryoglobulins and immunoglobulins, producing their own forms of plasmatic vasculosis, may behave in this way is suggested by some of our observations, but that is another story.

The results with our new staining methods for amyloid now suggest that amyloid's staining reactions, like those of fibrin, change with the passage of time, and that eventually both amyloid and fibrin come to stain with the large-molecule anionic dyes used in trichromic methods to stain collagen. Using the SAB-van Gieson sequence (method 1) we find that sections from kidneys with only slight to moderate, and therefore probably early, deposition of amyloid tend to show a brilliant green colouring in the amyloid, whereas material from cases with widespread and gross amyloidosis generally shows a dulling of the green to a flat grey shade, apart from an occasional artery with a subintimal zone of the brilliant green that we take to be the mark of recently deposited amyloid (colour Figs. 1 and 3). Objective assessment of age is difficult when studying a deposit that undergoes continuous change with age, while at the same time further accumulation may be happening continuously or intermittently and in small or large amount. Fortunately the following particular changes in the kidney allow reasonably accurate comparative assessment of the age of the amyloid deposits.

In certain tubules amyloid is deposited between the epithelium and the basement membrane, as mentioned in part II, and here can be seen transitions from fresh green layers through dull green to a brownish shade and thence to the frank fuchsin shade that van Gieson gives on normal collagen. By an intriguing and useful conformity these subepithelial layers of amyloid sometimes show the same curious internal spur or cog-like arrangement as do the fibrinous layers in the tubules of the diabetic kidney (Lendrum, 1963; Lendrum *et al*, 1969); during aging these cogged layers with their unusual shape provide easily recognizable structures

that can be seen to alter their shape more slowly than their affinity for different dyes (colour Figs. 7 and 9). In the *diabetic* kidney, stained with MSB or MSDB, the red fibrinous cogs change gradually through a chromophobic phase to blue cogs still with the same shape; in other words, the fibrin undergoes some change in its fine structure with a consequent change in the size of dye molecule it will accept, but without obvious change in the size or shape of the deposit, what we have elsewhere called mutation without metamorphosis. Later, when the tubular epithelium desquamates, the cogged layer shows a change in its shape; the cogs now round off to produce a string of beads, staining strongly throughout with the large-molecule blue dye (Fig. 7 of Lendrum, 1969). In the *amyloidosis* kidney, stained with SAB-van Gieson, the green cogs of amyloid alter gradually through brown toward magenta and eventually, as with the fibrin cogs, are transformed into a string of beads now stained by the acid fuchsin (compare colour Fig. 9). We feel therefore reasonably confident in regarding these changes as a timous sequence depending on and revealing the aging of the amyloid deposit.

Another site where age changes in amyloid seem to occur is in the subepithelial deposits of the glomerular capsule. Similar changes have already been described in the diabetic kidney (Lendrum, 1963) where the transition is obvious. In the amyloidosis kidney the deposition under the epithelial lining of the capsule is seen to fade, and to be infiltrated by more fresh amyloid (colour Fig. 6); this gradual accumulation eventually forms a thick acellular pericarp of pseudo-collagen closing down on the atrophying tuft (colour Figs. 5 and 10). Such affected glomeruli occur in clusters, mainly in zones of ischaemic atrophy, and presumably in both diabetes and amyloidosis vascular upset plays at least some part in producing this change, possibly by hypoxic intensification of the dysoria. The 'thickening of Bowman's capsule', described as hyalinized connective tissue by Auerbach and Stemmerman (1944), is recognized by them to contain small amounts of amyloid (compare colour Fig. 6), but they regard the thickening as response to an irritative action of amyloid on the glomerulus. They do not stress the mainly zonal arrangement of these altered glomeruli, nor do they mention the possibility that amyloid itself may be changing into the hyalin material that they regard as hyalinized connective tissue but which for us is a pseudo-collagen formed of age-altered amyloid.

The application of other counterstains confirms and enlarges our idea of the aging process. For example, method 2 (SAB-AO10.DR80), thanks to its precision, gives clear distinctions on thin sections and so allows study of the many tiny deposits of amyloid, while its strong affinity for pseudo-collagen makes the transitions more convincing (colour Fig. 5).

Combination of SAB staining with periodic Schiff counterstaining (method 3) reveals further interesting points, particularly because these stains show their own individual affinity for the amyloid at different times in the latter's history, and also because both methods have a peculiar fastness permitting subsequent counterstaining. This combination shows that early amyloid takes the SAB but the PAS scarcely at all: in the next stage the PAS staining is gradually superadded going through deep blue (colour Fig. 8), to a 'plum purple' (Kornerup and Wanscher, 1963) which then, lightening and warming as the SAB diminishes, changes to a pure positive PAS colouring: finally this also fades to negative and the aged amyloid now accepts a large-molecule anionic dye, for example Direct Yellow 11 (colour Figs. 9 and 10); use of this transparent yellow in place of the more usual blue or green collagen stain reveals the lack of nuclei in the pseudo-collagen of the glomerular pericarps. Our earlier studies on fibrin (Lendrum *et al*, 1964; Lendrum, 1969) showed that fibrin reacts positively with PAS at only one stage, that of early postmaturity, in its process of aging. The many arguments in the past about the PAS reaction of amyloid can now also be quietly forgotten.

Both fibrin and amyloid, interstitially deposited and given time and freedom from interruption, lose the staining reaction of their prime. The processes may well be different, and certainly the age changes of amyloid, in relation to the molecular size of the dye bound at different stages, are not fittingly comparable with those of fibrin as shown by a range of anionic (so-called acid) dyes from small to large molecular size. Not only is Alcian Blue a cationic (so-called basic) dye, but its molecular size is in fact huge. Admittedly the similarity of the sites of deposition and of the shapes of the deposits suggests (see part II) a similarity in the method of advent of the soluble precursors of both fibrin and amyloid. But this need bear no relation to the predominant pore-size characteristic of each deposit when fresh or mature. Nevertheless their end is similar in that both attain a pore size suited to take the large anionic dyes that in trichromic methods stain collagen. The resultant pseudo-collagen, as acellular and by the light microscope as structureless a gel as the early stages of both deposits, may seem to the unwary nothing more or less than 'hyalin'. As we wrote in 1969 (Lendrum *et al*), 'The question remains: Are there still other hyalins not yet defined?'

PART IV

Possible Confusion of Postmature Fibrin with Amyloid

During the aging of extravascular deposit of fibrin it may come to give staining reactions that suggest amyloid, and so can mislead. Thus with the MSDB 109 method (Lendrum *et al*, 1964) amyloid is relatively chromophobic compared with collagen (colour Fig. 14 and Figs. 1, 2, and 6), and this is mimicked by the chromophobic stage of postmature fibrin. These hyaline materials share other staining reactions, thus postmature fibrin (MSDB chromophobic) can give a positive reaction not only with SAB (see part I ii, and colour Fig. 13), but also with thioflavine T (fluorescent method for amyloid of Vassar and Culling, 1959), may be moderately metachromatic with methyl violet (Lendrum, 1963), and in the methods of Bennhold and of Highman sometimes binds Congo red. When fibrin is older still and has reached the stage of pseudo-collagen (blue with MSDB), it may not only retain the Congo red in Bennhold's or Highman's method, but may also then, like collagen itself, exhibit a green birefringence when viewed between crossed polarizers. The staining reaction that seems to us most nearly to achieve specificity for amyloid is a green birefringence with the alkaline Congo red of Puchtler *et al* (1962), which, thanks to the character of the solution, does not with our fixation at least stain collagen or pseudo-collagen. On the other hand Klatskin (1969), maintaining that substances other than amyloid may show green birefringence with Puchtler's method, regards as proof of amyloid's identity its ability to give not only green birefringence with Puchtler's Congo red but also, on an adjoining section, fluorescence with thioflavin T, whereas Cooper (1969) believes that for positive identification there should be Congo red staining and a positive DMAB-nitrite reaction. Examination of toluidine blue-stained preparations between crossed polarizers is claimed by Wolman (1971) to give less equivocal results than other methods for amyloid but the sensitivity of the method in comparison with Congo red-birefringence is low. It may be added that this identification parade generally pays no attention to the age of the suspect material.

In practice, postmature fibrin rarely gives as strong a reaction with any of the amyloid stains as does fresh and mature amyloid, but since amyloid itself undergoes changes producing diminished staining reactions (see parts III and V), it seems likely, as was suggested in 1963 (Lendrum), that aging fibrin has not infrequently been erroneously described as amyloid. The microscopist's difficulty in distinguishing interstitially deposited fibrin from amyloid has not been adequately appreciated, the observations of Kelly and Craik (1952) on 'laryngeal nodes and so-called amyloid tumour of the cords' standing almost alone in this context. We certainly have found several singer's nodes and other benign nodules of the larynx to be eloquent examples of fibrinous vasculosis. In the possibly comparable territory of the islets of Langerhans it may well be that some of the hyalin described as amyloid is in fact fibrin. Here of course there is no question of a fibrinous vasculosis caused by torsion of a polyp, but the correlation of this so-called amyloid deposit with diabetes is said to be high (Arey, 1943; Ehrlich and Ratner, 1961; Warren, Le Compte, and Legg, 1966; Wright, Calkins, Breen, Stotte, and Schultz, 1969), and we now know a most striking feature of diabetes is fibrinous vasculosis.

Another interesting similarity between postmature fibrin and amyloid is revealed in the reaction to fixatives. It had earlier been found that the sharpest and most consistent distinction between *mature* fibrin and other material, as revealed by our published methods (Slidders, 1961b; Lendrum *et al*, 1962, 1964), depends on relatively long secondary fixation in mercuric chloride. On the other hand, amyloid stained by SAB or any other amyloid method shows the most brilliant colouring if the duration of fixation in mercuric chloride is kept down to 48 hours. Postmature fibrin, as defined above, behaves exactly like amyloid in this respect; thus a reduced time in mercuric chloride results in its showing a greater affinity for SAB, for thioflavine T, and for Congo red in the methods of Bennhold and of Highman. Before leaving the matter of fixation we would emphasize that the reaction of the amyloid methods on amyloid substance is most intense and specific on fresh-frozen material, as King noted in 1948, and progressively less so with paraffin embedding after formalin, formalin plus short mercury, or plus long. The value of the SAB method is that it can be used on tissues that are well enough fixed to provide good detail in sections from paraffin; the processing schedule we use for study of amyloid is detailed above in part I ii. For diagnosis, given suspicion at the right time, the cryostat offers the best as well as the quickest answer.

It may be mentioned here that knowledge and nous have to be used along with special stains, and that there exist morphological changes of diagnostic help. Thus, for example, in some cases the deposition of amyloid in the interstitium of the medulla is like a witches' dance, and this Hexentanz (Fig. 11 and colour Fig. 4) is obvious at low magnification. We have failed to find anything so eldritch in the intertubular deposits of the diabetic kidneys. Another feature pointing, probably unambiguously, to

amyloidosis is the presence of well defined SAB-positive hyaline deposits within the nerves in the kidney; methods 1 and 2 demonstrate these clearly.

The fact that in paraffin sections postmature fibrin and amyloid tend to react rather similarly to dyes may be no more than chance coincidence, but this at least merits attention as a possible cause of diagnostic confusion.

PART V

The Aging Process in Amyloid

The possibility that amyloid undergoes changes with time, what we have called aging, has occasionally been suspected in the past. Possibly the first to do so was Fahr (1925), who describes 'regressive metamorphosis' as a sequential diminution and eventual disappearance of the metachromatic reaction, the resultant material being just hyalin. Other writers had earlier suggested that a hyalin might become amyloid, but Fahr found absolutely no evidence of a precursor-hyalin and since then no one else has. Not everyone, however, has accepted his regressive metamorphosis; for example, Auerbach and Stemmerman (1944) write: 'Like Koch (1927) we have never found glomeruli which underwent the transition from amyloid degeneration to hyalinization described by Fahr (1934).' In more accord with Fahr is Cameron's statement (1952): 'Regressive changes also influence the reaction, both colour tests and the iodine test being weaker in old deposits.' This change in staining reaction had already been noted by Noble and Major (1929), who state that amyloid deposited in the kidney 'may undergo some change which alters its staining characteristics', and 20 years later Dahlin observed amyloid in glomeruli, 'that appeared to be changing into hyalin'. Finally, on the basis of Bennhold's Congo red, Johannson and Pfeiffer (1954) accept amyloid as constantly undergoing metabolic change and that eventually it 'may develop into true hyalinization, especially in the kidneys'.

Change in staining reaction in relation to age, but without reference to hyalin, is mentioned by Waldenström (1928) who observed reduction in basophilia as amyloid underwent resorption. Avoiding the question of resorption, Turnbull (1945) describes in experimental mice ring-like deposits round Malpighian bodies with the older amyloid on the inner side holding the violet, on differentiation, less well, than the younger outer surface and the younger scattered deposits in the pulp. Also in experimental mice, Christensen and Rask-Nielsen (1962) saw the heaviest PAS reaction in early amyloid, fading later and showing faint reaction in the largest deposits; they add that high PAS positivity occurred rarely in amyloid in glomeruli. The following year Caesar (1936b) stated that older amyloid failed to show double refringence, and in 1965 Nakagawa using changes in birefringence colour and EM observations recognized in a vague way 'three conceptional components' he accepts as sequential; the last he calls postamyloid and regards this as synonymous with para-amyloid. Also in 1965, Gardner notes that EM fibrils become more obvious in the more advanced and longer-standing cases, and more recently Heptinstall (1966) says that metachromasia tends to be diminished in glomerular tufts as they shrink.

The comparative rarity of reports recognizing amyloid as an altering substance would have shocked Virchow with his expressed 'desire in the future to elucidate not merely anatomical state but also processes in the living body' (quoted by Rather, 1966). This is worth recall because new and valuable techniques are being applied to this strange substance amyloid, and deductions made apparently on the basis that amyloid is an unaltering or static substance. Thus, for example, Pearse says in 1963: 'The classical reactions, metachromasia with crystal violet and positive birefringence after staining with Congo red, are the only ones used to identify amyloid. Proteins which give neither reaction must be called something else.' But he does not say they might merit the name, old amyloid. In the field of electron microscopy, Wright *et al* (1969) point out that the neurofibrillary tangles of Alzheimer's disease show Congophilic birefringence and fluorescence with thioflavine S, but not the EM fibrils characteristic of amyloid. To this they add, 'This fact illustrates the hazards of assuming that positive reaction with these various staining methods of necessity identifies the lesions as amyloid deposits.' Like others, they do not mention the possibility that the EM characteristics of amyloid are not exactly synchronous with the staining characteristics during the course of the life history of an amyloid deposit. Likewise the 'three conceptional components' of Nakagawa, mentioned above, might be more accurately regarded as zones within a continuous process of aging, their characteristic being that within each imperceptibly demarcated zone there is a predominance of one or other indicative phenomena that by its eminence creates the zone. Our histological staining methods have by themselves shown this for amyloid, as they already have for fibrin, and we suggest that the concept of process be kept in mind by those who study hyalin, be it the recognizable form we call fibrin, or the other recognizable one we call amyloid, or just anonymous hyalin. Within this last group we are seeing the terminal stages of

fibrin, or of amyloid, or even perhaps of something else.

Conclusions

Amyloid in paraffin sections can be permanently stained with Alcian Blue; with suitable counterstaining its relation to other structures is clearly revealed.

The disposition of amyloid deposits in the amyloidosis kidney closely resembles that of the fibrinous deposits in the diabetic kidney thus suggesting a dysoric mechanism for both.

Amyloid undergoes changes on aging, the end product being a hyaline material that no longer reacts with any of the special stains for amyloid. It is already known that extra vascular fibrin ages to become a similar anonymous hyalin. These recognized end products, now staining as if they were collagen, we designate as pseudo-collagens.

Fibrin in its postmature phase reacts with many of the amyloid stains, not as vividly as mature amyloid but strongly enough to be a cause of confusion.

We are glad to thank Mr R. S. S. Fawkes for his skilled assistance with the illustrations, many colleagues here and elsewhere, too numerous to name, for giving us material to study, the various manufacturers who have helped us with dyes and information, especially Mrs Neal, Academic Relations Department of Messrs ICI Ltd, for consistent aid.

The publication of the coloured plates was made possible by generous financial support from the Carnegie Trust for the Universities of Scotland, and the publishers. We are very conscious of our debt to them and the blockmakers.

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