Histochemistry

Proteins and Amino Acids:

Proteins are the third of the components that go to make up cell cytoplasm, the other two being lipids and carbohydrates. Similar problems of identification apply to the proteins as to the other two, mainly because of the intermingling of all three components. Proteins are to be found in all cells and tissues. The actual demonstration of protein material is not difficult, but the identification of any particular protein is.

Proteins may be very simply classified as the following:-
1- Simple proteins:- Albumins, Globulins, Fibrous proteins Histones and others.

2- Conjugated protein:- Lipoproteins, Mucoproteins, Glycoproteins and Nucleoproteins.

The simple proteins are the naturally occurring proteins that upon hydrolysis will yield amino acids only. These can be divided into fibrous proteins (e.g. collagen, keratin and fibrin), and globular proteins (e.g. albumin, globulin). The first group are mainly insoluble in water, but the second are freely soluble. Because of their insolubility, it is much easier to demonstrate the fibrous proteins than the globular ones. The conjugated proteins consist of simple proteins combined with a non-protein material, for example, lipids in lipoproteins and nucleic acids in nucleoproteins.

demonstration of fibrous proteins:

The commonly occurring fibrous proteins such as collagen, reticulin, elastin, fibrin / fibrinoid and keratin are easily demonstrated using routine histopathological staining methods. All, with the

exceptions of reticulin, are easily seen in haematoxylin and eosin stained sections, and are usually strongly eosinophilic.

Collagen: can be demonstrated by the red acid fuchsin in the Van Gieson stain, and by certain components of the various trichrome stains.

Finer fibres of reticulin are demonstrated by silver reduction methods,

Elastic fibres can be stained by resorcin or orcein.

Fibrin / fibrinoid, derived from circulating plasma proteins, is stained dark-blue by Mallory's PTAH method and bright red by the MSB technique developed by Lendrum *et al.* Because fibrin/fibrinoid has a high tryptophan content it can also be demonstrated in tissues by the DMAB-nitrite method of Adams.

Keratin: has a particularly high content of the sulphurcontaining amino acid, cystine, and the keratin which forms hair is particularly richly endowed with this amino acid. Mature keratin will therefore be demonstrable by the D D D or Mercury Orange methods which depend on the presence of disulphide linkages.

Methods for proteins and amino acids

The methods discussed below do not demonstrate the protein molecule as a whole, but are dependent on the presence of certain types of reactive groups or special types of linkages within the protein molecule. These groups or linkages reside within certain of the amino acids making up the protein, and so these methods enable some conclusion to be drawn about the amino acids present in the tissue section. Unfortunately this whole segment of amino-acid histochemistry is confused because of the uncertainty about the specificity of the methods used. Histochemical

reactions are available for some, but not all, of the reactive groups of the protein molecule. The groups and linkages for which histochemical methods exist are enumerated below.

(1) Protein-bound amino groups (e.g. lysine).
(2) Disulphide and sulphydryl linkages .(e.g. in cystine and cysteine).
(3) Guanidyl groups (e.g. in arginine).
(4) Indole groups (e.g. tryptophan and tryptamine).
(5) Phenyl groups (e.g. tyrosine).

Protein-bound amino groups

Amino groups of lysine, ornithine and terminal peptides cannot be demonstrated individually, but the group as a whole may be demonstrated. The simplest and most reliable methods are the 1- Ninhydrin-Schiff method, 2- the DNFB (dinitrofluorobenzene) method modified by both Danielli and Burstone, and 3- the hydroxynaphthaldehyde method.

Disulphide and sulphydryl linkages

These sulphur-containing groups are found in the amino acids cystine, cysteine and methionine. Reduction of the disulphide linkage (-S-S) will give the sulphydryl group (-S-H). The best methods available for the demonstration of the sulphydryl and disulphide groups are the dihydroxydinaphthyldisulphide method, the D D D method and the Mercury Orange method. The demonstration of disulphide groups with the former method depends upon the reduction of these groups to sulphydryl. Alternatively, the performic acid-alcian blue method may be used for disulphide groups, but it is less sensitive than the others.

Guanidyl groups

The only amino acid containing the guanidyl group which is demonstrable histochemically in human tissue is arginine. The histochemical method is based on the well known Sakaguchi reaction, in which a red colour develops when arginine reacts with α-naphthol and hypochlorite in an alkaline solution.. To obtain satisfactory results the tissue must contain a high concentration of arginine; testis is particularly rich in arginine.

Indole groups

The indole-containing amino acids which can be demonstrated histochemically are tryptophan and tryptamine. Of the many methods available the most reliable is the DMAB-nitrite method The best results are obtainedusing freeze dried sections which have been fixed in formalin vapour; sites of high concentration of typtophan show an intense blue colou.

Phenyl groups

The only amino acid which contains the hydroxyphenyl group and which can be demonstrated histochemically is tyrosine. Because tyrosine is such a very frequent component of almost all tissue proteins, the methods for tyrosine can be regarded as demonstrating protein in general. The histochemical methods available are the Millon reaction modified by Baker (1956), and the diazonium coupling method of Glenner and Lillie (1959)

Staining Methods

Ninhydrin-SchifF for amino groups Ninhydrin at neutral pH will react with α-amino groups. Yasuma and Ichikawa (1953) published the first histochemical technique that involved the use of ninhydrin. The α-amino group reacts with ninhydrin and yields an aldehyde. This is then demonstrated by Schiffs reagent. The precise nature of the reaction is not known although the results appear to be specific, Fixation is best carried out in neutral formol saline.

DNFB method for amino, sulphydryl and hydroxy-phenyl groups

Dinitrofluorobenzene (DNFB) has frequently been used as a reagent for protein end groups. It reacts with free amino groups in proteins, the amino groups of lysine and hydroxylysine, the hydroxyphenyl groups of tyrosine and the sulphydryl groups of cysteine. The end product of the DNFB-amino group reaction is coloured yellow, whereas the product of the DNFB-tyrosine reaction is colourless. This colourless product can be made visible by diazotization and subsequent coupling with a phenol or aromatic amine.

The most suitable coupling agent is Ή - acid ' (8-amino-l-naphthol-3: 6-disulphonic acid) which gives an insoluble reddish-purple deposit as a final reaction product. Burstone considered that the DNFB-H-acid combination could be satisfactorily used to demonstrate primary amino groups (e.g. lysine) and hydroxy groups (e.g. tyrosine). More specific separation of staining due to amino, sulphydryl and aromatic hydroxyl groups can be obtained by using specific blocking agents before performing the DNFB reaction.

Hydroxynaphthaldehyde methods for amino groups

Weiss, Tsou and Seligman (1954) suggested the use of 3-hydroxy- 2-naphthaldehyde for the demonstration of protein-bound amino groups at p H 8-5. The primary reaction product is post-coupled with a diazonium salt at p H 7-4 to form a blue colour. Formalin fixation may partially suppress the reaction, although a positive result may still be obtained after routine formalin fixation provided that there are a large number of reactive amino groups.

The dihydroxyldinaphthyldisulphide method for S-S and S-H groups

This method, known as the D D D reaction, was published by Barrnett and Seligman (1953-1954). The method with modifications can be used to demonstrate: (1) sulphydryl groups and disulphide linkages; (2) sulphydryl groups only; and (3) disulphide linkages. The methods are basically the same. For the first method, thioglycollic acid splits the disulphide linkages and forms sulphydryl groups. These, together with any pre-existing sulphydryl groups, are then demonstrated. Both types of sulphydryl groups react with the D D D reagent and the primary reaction product, which is colourless, is then coupled with the diazonium salt fast blue B. The colour of the final reaction product is blue. The method is considered by many authors to be very specific, but unfortunately, the intensity of the positive blue colour is not verystrong. If the reaction is required for the demonstration of preexisting sulphydryl groups only, the thioglycollic acid stage is omitted, and the disulphide linkages are not then demonstrated since the bond is not broken. To demonstrate disulphide linkagesonly, it is necessary to use N-ethyl maleimide to block the existingsulphydryl groups. The disulphide linkages are then reduced tosulphydryl groups and demonstrated by the method. Barrnett

and Seligman (1954) stated that a weak reducing agent must be used, otherwise the N-ethyl maleimide blocking of the sulphydryl may be reversed. They recommended 10 per cent potassium cyanide for this purpose.

The Mercury Orange method for S-H Groups

This was published by Bennett and Watts (1958). The reagent used is now called Mercury Orange. It was originally called Red Sulphydryl Reagent (R.S.R.) but this name has now passed out of use. The method is said to be specific for sulphydryl groups. The sulphydryl groups react with Mercury Orange to form a mercaptide, which is coloured and visible with the light microscope. The colour however, is not intense.

Performic Acid-Alcian Blue method for S-S groups

This technique was introduced by Adams and Sloper (1955), (1956), for the demonstration of cysteine in paraffin sections. The technique can also be applied to cryostat and freeze dried material, the latter giving the best results of the three. T h e authors used Pearse's performic acid (freshly made daily), to oxidize the cysteine to cysteine sulphuric acid. This was subsequently stained using the basic dye, Alcian Blue. It is important that the pH of the Alcian Blue solution should not be less than 0-2 otherwise the localization is unsatisfactory.

Sakaguchi method for arginine

It is difficult to obtain consistent results using this technique. The intensity of the final colour is sometimes weak and is unstable, so sections must be examined immediately. Various modifications

have appeared since the original paper. In his paper, Sakaguchi described the reaction of α-naphthol with arginine in the presence of strong alkali, to give a red colour. Baker's (1947) modification of this method is probably the best. Other naphthols have been tried and in some cases a more stable colour reaction can be obtained; among these may be mentioned 8-hydroxyquinoline (Warren and McManus, 1951) and 2:4-dichloro-a-naphthol. It is important that the sections used for this method are not too thin; satisfactory results are obtained using sections of about 15 microns. They show the weak colour reaction better and are more able to withstand the technique which is very destructive to sections.

The DMAB-nitrite method for tryptophan

Histochemical techniques for the demonstration of tryptophan have been given by Glenner (1957), Adams (1957) and Glenner and Lillie (1957). The methods described by these authors employ jfr-dimethylaminobenzaldehyde (DMAB). The difference between the Glenner and Adams techniques is in the composition of the oxidizing solutions. The Glenner and Lillie method is a post-coupling technique. The method of Adams is recommended because it is easy to carry out and the results are usually reproducible. In the Adams method, the tryptophan reacts with dimethylaminobenzaldehyde to produce a compound known as ß-carboline. Oxidation of this product by nitrite solution produces a blue pigment

The Millon reaction for tyrosine

This reaction, known to biochemists for many years, has been modified for histochemical purposes. The two best and most popular methods are those of Bensley and Gersh (1933) and of Baker (1956). The method, which is specific for tyrosine, has one disadvantage

in that it gives a weak colour reaction. The recommended method, and the one considered here, is a modification of Baker's (1956). It was shown by Meyer (1864) that if mercuric chloride is applied to tyrosine in the presence of potassium nitrite in an acid solution, a red colour is produced. The various modifications of the method have centred around the choice of the mercuric salt to be used. Baker (1956) found that Folin's reagent gave him the strongest reaction (Folin and Ciocalteu, 1927; and Folin and Marenzi, 1929). In this reagent, mercuric sulphate is used with sulphuric acid. Baker (1956) also experimented with fixation, and found that formaldehyde-fixed tissue gave stronger coloration than tissues treated with other routine fixatives. To produce a satisfactory colour reaction it is necessary to heat the mercuric sulphate solution. Generally speaking, the higher the temperature, the more intense the colour. Baker heated celloidin sections to above 90°G. Other types of sections are heated to 70°C, when a reddish pink colour may be obtained at the sites of tyrosine.

Diazotization-coupling method for tyrosine

This method, developed by Glenner and Lillie in 1959, was based on earlier work by Lillie (1957) who claimed that prolonged nitrosation of tyrosine led to the formation of diazonium nitrates. These diazonium nitrates could subsequently be coupled with amines in alkaline solution to give coloured products. The histochemical method uses cS-acid' (8 amino l-naphthol-5-sulphonic acid) as the coupling amine and the incubations are carried out in the dark.

Protein Staining Methods

Method	*Application*	*References*
Millon Reaction	**Tyrosine (Phenyl group)**	**Millon, 1849** **Bensley and Gersh, 1933** **Baker, 1956**
Sakaguchi	**Arginine (Guanidyl group)**	**akaguchi, 1925** **Baker, 1947**
Mercury Orange	**Sulphydryl groups**	**Bennett and Watts, 1958**
DDD Reaction	**Sulphydryl groups** **Disulphide linkages**	**Barrnett and Seligman, 1952/1953; 1954**
Diazotization with H-Acid	**Tyrosine (Phenyl group)**	**Glenner and Lillie, 1959**
DMAB Method	**Trytophan (Indole groups)**	**Adams, 1957** **Glenner, 1957** **Glenner and Lillie, 1957**
Performic Acid-Alcian Blue	**Disulphide groups**	**Adams and Sloper, 1955; 1956**
DFNB Method	**Sulphydryl groups** **Amino groups** **Tyrosine**	**Sänger, 1945** **Sanger, 1945** **Danielli, (1950)** **Burstone 1955)**
Ninhydrin-SchifT	**Amino groups**	**Yasuma and Itchikawa, 1952**
Hydroxynaphthaldehyde	**Amino groups**	**Weiss, Tsou and Seligman (1954)**

This list is not complete, many other methods exist, but it indicates the more common techniques.

FIXATIVES:

For most purposes formalin fixation is satisfactory for protein histochemistry, despite the fact that formalin modifies many of the reactive groups in the protein molecules. This does not interfere with most of the protein methods, since adequate washing in water after the formalin fixation will leave enough of the active groups sufficiently unchanged to react normally with the histochemical reagent. However, a non-formalin fixative is preferred for the hydroxynaphthaldehyde method for amino groups. Barnett and Roth (1958) tested a series of fixatives in certain protein histochemical methods and found that only osmium tetroxide completely inhibited any of the reactions. In general, freeze dried sections give the best staining intensity and localization in protein histochemistry, although the warm formalin vapour used for

fixation tends to interfere with the reactive groups more than routine formalin solutions.