Studies of differential staining with acid dyes in the human adenohypophysis

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Summary

With Mallory techniques the differential staining characteristics in the human adenohypophysis are mainly dependent upon the treatment with phosphotungstic acid. Though this does not remove dye which is already bound to the acidophils it prevents the subsequent access there of anilin blue or acid fuschin, but not of orange G. The molybdenum blue reaction, used after treatment of sections with phosphomolybdic acid, shows that hexavalent molybdenum is strongly bound to the basiphils but more weakly to the acidophils; it can be inferred that phosphotungstic acid reacts with the tissues in a similar manner. Deamination, acetylation, and benzoylation produce a marked reduction in dye-binding, though the granules of the basiphils are resistant to the benzoylation. Methylation results in a marked increase in the staining of all elements. The differential effect of the phosphotungstic acid is abolished, so that the dyes are able to gain admission to the acidophils after the polyacid treatment.

The differential staining is most probably dependent upon the differing permeabilities of the tissue proteins to molecules of differing sizes, the acid fuchsin, anilin blue, and phosphotungstic acid competing with each other for basic groups. On the other hand, it is possible that phosphotungstic acid has a different type of reaction with the acidophils, involving chelate linkages to carboxyl and other groups of the tissue proteins.

Introduction

The mechanism of the differential staining of tissue sections with acid dyes, used in association with phosphotungstic or phosphomolybdic acids, is incompletely understood. Baker (1958) considers that it depends upon the differing permeabilities of tissue constituents to dye molecules of differing molecular sizes, and recent work appears to support this view (Bulmer, 1962). On the other hand, claims have been made for particular chemical specificities in some of the staining reactions (Monné and Slautterback, 1951; Hršel, 1957).

Differential staining methods are frequently employed for the demonstration of the various cell types in the adenohypophysis, and the present work describes an attempt to elucidate some of the factors concerned there.

Methods and results

Paraffin sections of formalin-fixed blocks of the human hypophysis, obtained from the post-mortem room, were employed as test material. Since its chemical effects are obscure, and complicate rather than simplify studies
of differential staining mechanisms (Bulmer, 1962), mercurial treatment was omitted, either in fixation or mordanting. The standard staining method used was a variant of the Mallory technique. Sections were first stained in 1% aqueous acid fuchsin for 5 min, then exposed to a 1% solution of phosphotungstic acid for 5 min and finally stained in a mixture of 1% anilin blue in 0.5% acetic acid for 10 min. With this sequence the acidophil cells of the adenohypophysis stain with the acid fuchsin and the cytoplasm of the basiphils with the anilin blue. Most of the granules of the basiphils are blue, but a few stain with the acid fuchsin. Individual cells may contain both red and blue granules.

The precise differential staining obtained is due to the interposition of the phosphotungstic acid treatment. If this is omitted, and the two dyes used in the same sequence, the acidophils are stained mainly by the acid fuchsin but with a considerable admixture of the anilin blue. The basiphils are stained by the anilin blue. In other words, the intervening phosphotungstic acid treatment prevents the admission of anilin blue to the acidophils but does not prevent the replacement of the acid fuchsin by the anilin blue in the basiphils.

The staining characteristics also depend upon the order in which the different solutions are used. If sections are exposed to a sequence of (1) aqueous anilin blue, (2) phosphotungstic acid, and (3) acidified acid fuchsin, the acidophils are stained blue and the basiphils a mixture of the red and the blue. Some basiphil granules are blue and some are red. The staining is rather intense with this reversed Mallory procedure, but with reduced staining times a clear histological picture is obtained. Anilin blue and acid fuchsin are thus rather similar to each other in their behaviour, which is not surprising in view of their similarity in chemical structure. After treatment with phosphotungstic acid neither is able to replace the other in the acidophils, though anilin blue is replaced by acid fuchsin in the basiphils less completely than acid fuchsin is replaced by anilin blue. Since anilin blue has a larger molecular weight than acid fuchsin this is in accord with the view that larger dye molecules bind to tissue proteins more strongly than smaller molecules (Baker, 1958).

Used alone, without phosphotungstic acid, anilin blue or acid fuchsin stains the acidophils quite strongly and the basiphils intensely. When the staining is preceded by treatment with phosphotungstic acid, however, the basiphils are still quite strongly stained but the acidophils are only faintly coloured. If instead of being employed as a pretreatment the phosphotungstic acid is mixed with the anilin blue, the staining of the acidophils is abolished while the basiphils are a pale blue colour. If orange G is included in this mixture, as in the final stage of another variant of the Mallory technique, the orange G binds strongly to the acidophils while the basiphils have a pale blue tinge which is heavily overlain by the orange G. The anilin blue stains the basiphils so faintly that this mixture is unsatisfactory for differential staining in the pituitary.
While it is not possible to demonstrate directly the binding of phosphotungstic acid to tissue sections, this can be inferred indirectly from the binding of the comparable phosphomolybdic acid. After treatment of sections with phosphomolybdic acid the bound hexavalent molybdenum can be demonstrated by the molybdenum blue reaction (Puchtler and Isler, 1958). In the adenohypophysis a strong molybdenum blue reaction is obtained under these conditions in the basiphils, with a weaker reaction in the acidophils. Because of its colour the molybdenum blue reaction cannot be profitably used after anilin blue staining; but if after exposure to acid fuchsin sections are treated with phosphomolybdic acid and subjected to the molybdenum blue reaction, the acidophils are red and the basiphils blue.

If sections which have been deaminated with nitrous acid for 24 to 28 h (Pearse, 1960) are submitted to the acid fuchsin / phosphotungstic acid / anilin blue sequence the staining of all elements is greatly reduced. The acidophils are only very faintly coloured with the acid fuchsin, while the blue stain of the basiphils and their granules is very weak. A similar reduction occurs after deamination with the phosphotungstic acid / anilin blue sequence; the acidophils are unstained and the basiphils coloured very weakly. On the other hand, anilin blue alone, without previous phosphotungstic acid treatment, gives moderate staining of acidophils and stronger staining of basiphils in deaminated sections. Similarly, sections continue to bind orange G quite strongly after deamination. When a deaminated section is exposed to phosphomolybdic acid and then subjected to the molybdenum blue reaction there is slight staining of the acidophils and stronger staining of the basiphils, though in both situations the reaction is much weaker than in undeaminated control sections.

Acetylation, by the method of Lillie (1954), has effects similar to those of nitrosation, with marked reductions in all the staining reactions of both basiphils and acidophils. Here, however, the staining with anilin blue alone is markedly impaired.

Benzoylation (Pearse, 1960) has less uniform effects on the staining characters, since the granules of the basiphils are partly resistant to its action. Staining of the acidophils is abolished with all the dyes which were used, with the exception of orange G. The staining of the basiphil cytoplasm is abolished, but some of the granules are still stained. With the acid fuchsin / phosphotungstic acid / anilin blue sequence some of the residual granules are blue and some are red. If the benzoylation procedure is preceded by heating the section to 60° C for 10 min, more basiphil granules become resistant. Now, however, all are stained blue with the acid fuchsin / phosphotungstic acid / anilin blue sequence. These results are essentially comparable with those described by Pearse (1952) on the effects of benzoylation on the coupled tetrazonium reaction of the basiphil granules.

Exposure of sections to a hot methylation procedure (Lillie, 1954) greatly increases the staining intensity with anilin blue. The effect on subsequent treatment with the acid fuchsin / phosphotungstic acid / anilin blue sequence
is to produce marked blue staining of the acidophils, while the basiphils are intensely blue. After methylation, phosphotungstic acid, used in the phosphotungstic acid / anilin blue sequence, no longer prevents subsequent staining of the acidophils with anilin blue. A further interesting feature is that methylation is the only pretreatment which induces appreciable staining of the neutrophils.

Other treatments which were employed had no significant effects on the staining reactions. Mild alkaline hydrolysis (5% sodium borate solution), in contrast to its effect on the staining reactions of smooth muscle (Bulmer, 1962), has no effect on the staining of the acidophils. Strong alkaline treatment (10% sodium hydroxide solution for 24 h) markedly reduces the binding of acid fuchsin to the acidophils in the acid fuchsin / phosphotungstic acid / anilin blue sequence, but does not increase the admission of anilin blue. Acid hydrolysis, ribonuclease digestion, performic acid, and periodic acid are without appreciable effects.

**Discussion**

It is apparent that the differential staining is dependent upon the phosphotungstic acid treatment, and particularly upon the effect of phosphotungstic acid on subsequent staining of the acidophils. The effect of deamination demonstrates that when the dyes are used in the sequence of acid fuchsin / phosphotungstic acid / anilin blue the binding is principally to primary and secondary amino-groups of the tissue proteins, and this may also be correlated with the effects of acetylation and benzoylation and with the greatly increased binding of anilin blue after methylation. The persistence of marked staining when anilin blue is used alone after deamination, but not after acetylation and benzoylation, suggests that in the absence of phosphotungstic acid this dye may bind to other protein groups. It has been suggested that anilin blue may stain collagen by a hydrogen bonding mechanism which involves hydroxyl groups (Bulmer, 1962)—as Gustavson (1957) has suggested in the binding of benzopurpurine 4B to collagen. It is likely that some acid dyes may stain protein fibres by hydrogen bonding to peptide linkages (Vickerstaff, 1950), and this may account for the persistence of staining with orange G after deamination. There is no confirmatory evidence that hydrogen bonding is involved in the staining of hypophyseal cells with anilin blue. Agents which rupture hydrogen bonds, such as strong urea and lithium bromide solutions, do not produce any rapid elution of anilin blue from the hypophysis. There is also no evidence to confirm the opinion of Puchtler and Isler (1958) that anilin blue, when used after phosphotungstic acid, acts as a basic dye, binding to the free acidic groups of the polycacid. After deamination, the molybdenum blue reaction shows that a small amount of phosphomolybdic acid binds to the acidophils. If anilin blue is used after the polycacid treatment, however, none of the dye is admitted there.
To a large extent the findings are compatible with the views on differential staining expressed by Baker (1958). Thus, phosphotungstic acid may be considered to act as a 'colourless acid dye', competing with acid fuchsin and anilin blue for accessible amino-groups of the tissue proteins. In the basiphils this competition appears to be fairly straightforward: if anilin blue and phosphotungstic acid are used together, little anilin blue is bound to the basiphils; while if the anilin blue is used after phosphotungstic acid treatment, the dye is able to replace some of the bound phosphotungstic acid. In the acidophils the picture is rather different. Here phosphotungstic acid, used as the second stage in the staining sequence, does not remove the acid fuchsin or anilin blue which is already bound, but it does prevent any subsequent access of these dyes. It does not prevent, however, even the coincident admission of orange G.

It is likely that the tissue proteins of the acidophils are less permeable than those of the basiphils, and that this low permeability is still further reduced by the entry of only a small number of the large polyionic molecules of phosphotungstic acid. The relative impermeability will prevent the entry of a sufficient amount of phosphotungstic acid to displace a dye which is already bound, but the small amount which is admitted will further reduce the permeability. Subsequently, only small dye molecules, such as those of orange G, will be admitted. The more permeable protein of the basiphils may be supposed to permit more ready movement of dye molecules and phosphotungstic acid molecules, both inwards and outwards. On the basis of this explanation it is likely that the increased binding to the acidophils after methylation is due not only to the liberation of amino-groups which were previously held by tissue acidic groups, but also to an increased permeability of the tissue proteins resulting from the rupture of internal bonds by the methylating procedure. In contrast to their effect on smooth muscle (Bulmer, 1962), alkaline hydrolysis and performic acid treatment do not affect the permeability of the acidophils.

There is, however, a further possibility, that the phosphotungstic acid has a specific chemical effect on the acidophils other than a simple ionic bonding to the protein amino-groups. The comparable effect of phosphomolybdic acid on the staining of the acidophils, together with the absence of a strong molybdenum blue reaction there, suggests that this specific effect may be associated with the binding of molybdenum or tungsten in other than a hexavalent form. This is particularly significant in that tryptophane is known to be capable of reducing phosphotungstic acid. The possibility of an association between plasma staining in trichrome techniques and the presence of tryptophane has been discussed elsewhere (Bulmer, 1962) in relation to the work of Hršel (1957), and the only relevant finding of the present investigation is the negative evidence that performic acid, which destroys tryptophane, has no significant effect on the staining of the acidophils. On the other hand, it is possible that the reaction of phosphotungstic acid on the acidophils is dependent upon the presence of carboxyl groups,
which would explain the abolition of the differential staining by the methylation procedure. While this suggestion was discounted in the staining of smooth muscle, where alkaline hydrolysis has a similar effect to methylation, the possibility cannot so easily be excluded in the acidophils of the adenohypophysis.

It may be that phosphotungstic and phosphomolybdic acids have two types of reaction with tissue proteins. First, as in collagen and in the basiphils of the adenohypophysis, they may form ionic linkages with amino-groups, competing with acid dyes. Their free acidic groups will be available for binding basic dyes (Puchtler and Isler, 1958). Secondly, it may be suggested that in other sites, such as the acidophils of the adenohypophysis, the complex acids may have an additional type of reaction with the tissues, probably binding to them in a reduced form. This reaction would involve carboxyl groups of the tissue proteins, as well as other groups—amino- or hydroxyl—which are the normal binding sites of anilin blue and acid fuchsin. This second type of binding may be of non-ionic form, involving chelate bonds, and this would explain the inability of phosphotungstic acid to remove acid fuchsin or anilin blue which is already bound to the acidophils and the inability of either dye to replace phosphotungstic acid which is already bound. Phosphotungstic acid and anilin blue might be expected to compete with and replace each other only where both form similar types of bonds with the tissue proteins. After methylation, when the carboxyl groups are blocked, phosphotungstic acid will no longer bind in the non-ionic form, but only by ionic linkages. Very much more phosphotungstic acid will now be bound, because of the liberation of amino-groups by the methylation. This suggestion is, of course, only tentative, but one point which might be mentioned in its support is that after deamination, though the molybdenum blue reaction demonstrates that very little hexavalent molybdenum is bound to the acidophils, the treatment with polyacid is still effective in preventing the subsequent admission of anilin blue, though this dye binds quite strongly in the absence of polyacid. In addition, it has been pointed out previously (Bulmer, 1962) that the binding of phosphotungstic acid to nuclei appears to involve acidic groups of the nuclear proteins, in that the normal nuclear basiphilia is abolished. There is a problem here which requires further chemical study.

It is not possible to suggest any relationship between the differential staining of the cell types of the adenohypophysis and their established histochemical characteristics, since the evidence suggests that the staining depends mainly upon physical factors. A point which may be noted, however, is the presence of a few red-stained granules in the basiphils after the acid fuchsin / phosphotungstic acid / anilin blue sequence. In other words, with this technique a few of the basiphil granules have the same staining characters as the acidophils. With Mallory methods Pearse (1952) found that the β-granules stained dark blue, while the γ-granules were pale blue or, occasionally, red. After benzoylation, the coupled tetrazonium reaction
of the γ-granules was abolished, while that of the β-granules persisted. In the present study the application of the acid fuchsin / phosphotungstic acid / anilin blue sequence after benzoylation still resulted in some red-stained granules, and only when the benzoylation was preceded by heating were all the residual granules stained blue. Moreover, if the staining sequence was reversed, when anilin blue / phosphotungstic acid / acid fuchsin was used on benzoylated sections, both red and blue granules occurred, whether or not the benzoylation was preceded by heating. This suggests that the β- and γ-granules (or the R- and S-granules of Adams and Pearse, 1959) may be less firmly distinguished by their histological staining characteristics than Pearse implied.

References