Experiments on the action of mordants
2. Aluminium-haematein

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With one plate (fig. 4)

Summary

Unmordanted haematein in aqueous solution varies in colour from yellowish orange at pH 3 to bluish magenta at pH 9.

In the presence of aluminium sulphate, the colour of solutions of haematein at any particular pH depends on the number of aluminium atoms to each molecule of haematein. At pH 4·6 the colour varies from yellowish orange through dull red to rose as the number of aluminium atoms to each molecule of haematein is increased from ½ to 8. The facts suggest that such solutions contain both uncombined haematein and an aluminium-haematein compound.

From about pH 5 upwards the aluminium-haematein compound is insoluble. Although the dominant colour is blue, a considerable proportion of red light is transmitted.

The blue colour attaches itself to tissue-constituents like a typical cationic (basic dye. It combines principally with the nucleic acids of chromatin; but if these are extracted from the tissues, it attaches itself to the acidic proteins of chromatin, and the appearance given is much the same.

In differentiation by acid, the mordant is set free from the tissue.

Chromatin that has been reddened by acid during or after dyeing becomes blue from about pH 5 upwards. Neutrality or alkalinity is not necessary for blueing.

The dull red aluminium-haematein compound present in acid solutions acts (like the blue) as a cationic dye.

When aluminium and haematein are used (as iron and haematein usually are) by the two-bath method, chromatin is dyed blue but collagen and certain other tissue-constituents *yellowish*. An exhaustive series of experiments shows that the yellowish colour is due to unmordanted haematein.

At room temperature haematein is about 17 times more soluble in ethylene glycol than in ethanol, and 21 times than in water. In 25% aqueous solution ethylene glycol is a suitable solvent for practical use. A solution in this solvent containing aluminium and haematein in the proportion of 8 atoms of the metal to one molecule of haematein may conveniently replace Mayer's *Hämalaun* and similar mixtures.

Contents

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Introduction

THE first paper in this series was concerned with the mordanting of purpurine by aluminium sulphate. Aluminium was preferred to iron and chromium as mordant, because its chemical reactions are less complicated than those of the transition elements. Purpurine was chosen because its chemical composition is simpler than that of any other dye capable of being used with a mordant in practical microtechnique.

Aluminium-haematein has probably been employed more extensively in the study of living organisms than any other dye. It was introduced into microtechnique by Böhmer in 1865. His solution was widely used for many years. It gives excellent results, despite the disparaging remarks of Bolles Lee (1900). Unfortunately, Böhmer used old-fashioned measures of weight and volume, and these have been inaccurately converted to the metric system by the compilers of textbooks of microtechnique. The most familiar aluminium-haematein solutions are those of Delafield (Prudden, 1885), Ehrlich (1886), and Mayer (1891, 1903). These work quickly and are very easy to use, since the blue colour is quite fast to unacidified water and ethanol, yet easily differentiated by dilute acid. Most of the dye-solutions in common use are equally applicable to sections and whole mounts. The blue colour of chromatin in typical preparations lends itself well to the counterstaining of acidiophil tissue-constituents by familiar anionic dyes.

It was the high practical value of aluminium-haematein that led me to undertake the present investigation. An attempt is made in this paper to analyse the various colours shown by haematein, with and without aluminium, and to interpret those seen when tissues are dyed with aluminium-haematein. The questions to which answers are sought are these. What tissue-constituents are coloured by the dye? Why is the colour of the tissue-constituents sometimes blue, sometimes red, and sometimes yellowish? What happens during the process of differentiation by acid?

In the first paper in this series attention was focused entirely on the 'single-bath' method, because the object was to find the effect of varying the proportions of mordant and dye while both were present in the same solution. In practice, aluminium ions and haematein are nearly always dissolved together in a single bath. Nevertheless, most of the experiments described in the present paper were carried out by the 'two-bath' method, because it enables the processes of mordant dyeing to be more exactly analysed in certain respects.

The word 'haematoxylin' is used in this paper in its strict sense to mean the colourless solid that is converted by oxidation to the dye, haematein; but in accordance with the usual custom, a dye-solution is called so-and-so's 'haematoxylin' if the formula prescribes that haematoxylin should be used in

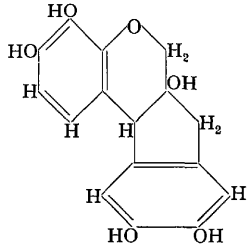
preparing the solution (though some of this substance must necessarily undergo oxidation before the solution can be used).

The word 'chromatin' is used in this paper in the original sense of Flemming (1880), to mean 'that substance in the cell nucleus which takes up the dye in the treatments with dyes known as nuclear colouring'. I do not apply this name to the substance of the nuclei of ripe spermatozoa of the mouse, for this shows strong affinity for anionic dyes on account of its high content of basic protein.

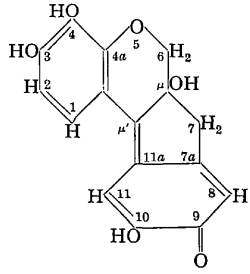
Material and methods

The mordant used throughout this investigation was aluminium sulphate. Solutions were made from the crystalline salt, $Al_2(SO_4)_3 . 16\ H_2O$ (analytical grade). The molecular weight is 630·41. The simple salt was preferred to the alums because the addition of potassium or ammonium ions could not help to elucidate the action of aluminium as a mordant.

The haematoxylin used was obtained from the British Drug Houses, the haematein from G. T. Gurr. The formulae are given here. It is unfortunate that in Harms's valuable *Handbuch* on dyes (Harms, 1957), the formula for haematein is given incorrectly.



Haematoxylin (crystallizes with 3 molecules of water)



Haematein

The numbering of the various positions in the molecule of haematein is copied from that used by Pfeiffer and Döring (1938) for chromindan, the parent substance of haematein and brazilein.

The molecular weight of haematein is 300·3.

In this paper, as in the earlier one in the series (Baker, 1960), the symbol dM is used to mean *decimolar* (one-tenth of molar). Both aluminium sulphate and haematein were generally dissolved at concentrations in the series dM/2, dM/4, dM/8, &c. It must be remarked, however, that perfectly pure haematein was not available, and that the concentrations of the solutions must therefore have been slightly less than the stated figures suggest.

For the purpose of this investigation it was necessary to know the

solubilities of haematein in various solvents. It would not appear that any figures for the solubility of this important substance are available in the literature. The following methods were adopted to measure the solubility of the dye at room temperature. It was not thought necessary to control the temperature exactly, since the dye was not available in a perfectly pure state.

To find the solubility in water, an excess of haematein was placed in more than 1,500 ml of glass-distilled, de-ionized water; this was boiled with reflux condenser. The solution was cooled overnight to room temperature and filtered. 1,500 ml of the filtrate were allowed to evaporate. The solid remnant was dried over phosphorus pentoxide until the weight was constant.

The solubilities of haematein in absolute ethanol and in *iso*-butanol were obtained in the same way. The alcohols were dehydrated with anhydrous sodium sulphate before use. The same method was also used to find the solubility of haematoxylin in absolute ethanol, but for this purpose 250 ml of solvent sufficed, since haematoxylin is very soluble in ethanol.

A different technique had to be adopted to find the solubilities of haematein in the non-volatile solvents, ethylene glycol (ethane-diol), and glycerol. A saturated solution of haematein in each of these solvents was prepared by heating with excess of solute, cooling to room temperature, and centrifuging to remove the undissolved remnant. The solutions were diluted (partly with the original solvents, but mainly with absolute ethanol) until solutions were obtained at 1/1,000, 1/2,000, 1/4,000, 1/8,000, and 1/16,000 of the concentrations of the saturated solutions. These concentrations will be called the 'saturation-fractions'. A saturated solution of haematein in absolute ethanol was also diluted in the same way.

It was found by experiment that the transmission of light by haematein dissolved in ethanol is least at a wavelength of about 465 mμ. Each of the diluted solutions was therefore examined in a spectrophotometer with light at this wavelength. Curves were drawn relating the logarithms of the saturation-fractions to the transmissions expressed as percentages. From the curves representing each solvent (ethanol, ethylene glycol, and glycerol) the logarithm of the concentration-fraction giving a transmission of 50% was read off. The antilogarithms of the figures obtained were noted. These antilogarithms showed that a solution of haematein in ethanol would have to be diluted to 0·00363 of the concentration of a saturated solution in order to give a light-transmission of 50%; the corresponding figures for ethylene glycol and glycerol were 0·0002265 and 0·000248 respectively. It follows that a saturated solution in ethylene glycol is 16·0 times as concentrated, and one in glycerol 14·6 times as concentrated, as one in ethanol. Since the concentration of haematein in a saturated solution in ethanol was already known, the concentration of a saturated solution in each of the other two solvents could be calculated.

The three curves were nearly parallel, and the figures for solubility would scarcely have differed if some transmission other than 50% had been selected for the comparison with ethanol. A possible source of error in the experiment was caused by the high viscosity of ethylene glycol and especially of glycerol,

and by the very dark-brown colour of the saturated solutions in these solvents. It was difficult to be certain that all the undissolved remnant was separated by centrifuging.

The solubilities of haematein in the various solvents are listed on p. 513. The solubility of haematoxylin in ethanol is given on p. 514.

In those experiments in which it was necessary to buffer solutions at various pHs, Michaelis's acetate / veronal / hydrochloric acid solution was used (Michaelis, 1931). The sodium chloride included by Michaelis in this buffer was, however, reduced in amount or omitted since it tends to precipitate dyes and does not affect pH.

For certain purposes it was required to buffer alcoholic solutions at pH 4·6. The following solution was used.

Buffered 60% ethanol

sodium acetate crystals	5 g
acetic acid, glacial	27 ml
ethanol, 60% aq.	up to 1 l.

The tissues used in the investigation were the testis, epididymis, and small intestine of the mouse. These were chosen because they give characteristic reactions with unmordanted cationic and anionic dyes, and thus enable one to determine whether mordanted dyes act as though cationic or anionic; but the choice was somewhat arbitrary, since many other tissues would have served the purpose equally well.

The tissues were fixed in Clarke's fluid, a mixture of glacial acetic acid with 3 times its volume of absolute ethanol (Clarke, 1851; Carnoy, 1886). This fixative was chosen because it contains nothing that could act as a mordant. The tissues were embedded in paraffin and sectioned at 7 μ.

A *standard method* of dyeing was devised, to give 'good' results by the criteria of routine histology (clear blue colouring of chromatin, very slight colouring of cytoplasm). This method involved the use of the following solutions.

Aluminium sulphate, dM/16

$Al_2(SO_4)_3 . 16H_2O$	3·940 g
distilled water	up to 1 l.

Half-oxidized haematoxylin (Baker and Jordan, 1953)

haematoxylin	5 g
sodium iodate	0·5 g
distilled water	1 l.

Heat till the water just boils. Cool.

Weak sulphuric acid

concentrated sulphuric acid	5 ml
distilled water	up to 1 l.

Weak ammonia

ammonia solution sp. g. 0·89	5 ml
distilled water	up to 1 l.

The technique was as follows. Rinsing was done in each case with distilled water from a squash-bottle.

I. Bring section to water.
II. Aluminium sulphate, dM/16, 1 h.
III. Rinse.
IV. Half-oxidized haematoxylin, 20 min.
V. Rinse.
VI. Weak sulphuric acid, 40 sec.
VII. Rinse.
VIII. Weak ammonia, 5 sec.
IX. Rinse.
X. 70% ethanol, ½ min.
XI. 90% ethanol, ½ min.
XII. 1st absolute ethanol, 1 min.
XIII. 2nd absolute ethanol, 1 min.
XIV. 1st xylene.
XV. 2nd xylene.
XVI. Mount in benzene-balsam.

Benzene-balsam was chosen as the mounting medium because dyes have less tendency to fade in it than in xylene-balsam.

The sections were examined by electric light passed through an Ilford 'daylight' filter (no. 810), or by daylight. A 4-mm objective of N.A. 0·70 was generally used. The iris diaphragm of the condenser was set to give an illuminating cone of about ¾ the N.A. of the objective.

It was found that certain acidophil constituents, especially collagen, tended to be dyed yellowish by this technique. To accentuate this tendency as much as possible, the following *method for collagen* was devised:

I to VII, as in the standard method.
VIII. Sodium bicarbonate, 0·1% aq., 1½ min.
IX. Without rinsing, blot twice.
X. Absolute *iso*-butanol, 3 lots, 1 min in each.
XI. Mount in benzene-balsam.

It will be noticed that for this particular purpose 'blueing' is done with a feebly alkaline solution, and ethanol is avoided in the after-treatment. (It is not necessary to pass through xylene, since *iso*-butanol is miscible with benzene-balsam.)

To remove DNA from sections, it is usual to treat with 5% trichloracetic acid at 90° C for 15 min. This was found to be damaging to the tissues. Experiment showed that it is greatly preferable to use a much lower tempera-

ture and to allow the trichloracetic acid to act for a much longer time. If a section of material fixed in Clarke's fluid is left for 30 h in a 5% aqueous solution of trichloracetic acid maintained at 50° C, the tissue suffers no damage, but no definite indication of a positive Feulgen reaction can subsequently be obtained in any part of the tissue. This period of extraction therefore suffices for most purposes. If the period is increased to 40 h, not even a suspicion of a positive Feulgen reaction can be obtained.

Although trichloracetic acid removes part of the RNA from the tissues, it was found that subsequent treatment with ribonuclease was necessary when it was desired to remove the nucleic acids completely. Sections that had been treated with trichloracetic acid were washed in water and then left for 3 h in Bradbury's ribonuclease solution (Bradbury, 1956). They were subsequently dyed with aluminium-haematein by the usual techniques.

To deaminate tissues, sections were brought down to water and left for 4 h or more in the following solution, freshly made, at room temperature.

Van Slyke's reagent (Monné and Slautterback, 1950)

sodium nitrite, sat. sol. aq.	2 vol.
acetic acid, glacial	1 vol.
distilled water	5 vol.

The sections were then washed in running water for 10 min., rinsed with distilled water, and dyed. It was found that increase in the period of deamination beyond 4 h had little effect.

To determine whether haematein, with or without mordant, acts like a typical cationic or anionic dye, it was necessary to experiment with various unmordanted dyes. For this purpose sections prepared in the usual way were dyed in the solutions listed below, for the periods stated. All the solutions were aqueous except where the contrary is stated. The intention was to choose concentrations and periods that would give clear results without overstaining.

Cationic dyes		
basic fuchsine	0·5%	1 min
methyl green	1%	20 min
thionine	0·5%	30 min
toluidine blue	0·5%	15 min
methylene blue	1%	15 min
safranine	0·5%	15 min
Anionic dyes		
acid fuchsine	0·5%	15 min
light green	0·25%	15 min
methyl blue	0·5%	10 min
eosin	0·1%	2 min
azocarmine	0·1% in acetic acid, 5% aq., 1 min	
orange G	sat. in abs. ethanol, 2 min.	

The dyed sections were generally rinsed with distilled water, dehydrated, passed through xylene, and mounted in benzene-balsam. Those dyed with orange G were simply washed in absolute ethanol, passed through xylene, and mounted.

The pH readings recorded in this paper were made with the Model 23A meter of Electronic Instruments Ltd. The second place of decimals is not quite reliable. The readings of optical density and transmission were made with the SP 350 spectrophotometer of Unicam Instruments Ltd.

Preliminary experiments with unmordanted dyes

To be in a position to interpret the reactions of aluminium-haematein with various tissue-constituents, it was first of all necessary to know which of these were basiphil and which acidophil; or, in other words, which were more readily coloured by cationic and which by anionic dyes. For this reason preliminary experiments were made with the dyes already mentioned on p. 499. The test-object was the testis of the mouse.

The cationic dyes were found to resemble one another closely, apart from the fact that some of them would give a stronger colour than others if used at the same concentration for the same length of time. There was more variation among the anionic dyes. In particular, methyl blue differed markedly from the others, especially in its strong tendency to colour chromatin. It was, however, found possible to formulate strict rules by which the cationic dyes could be distinguished from the typical anionic ones. These rules are as follows:

1. The chromatin of the primary spermatocytes, and of those spermatids in which the nucleus is beginning to assume the form characteristic of the spermatozoa, is strongly coloured by cationic dyes and shows up very clearly against the unstained or scarcely stained cytoplasm.
2. The middle-pieces and tails of the spermatozoa are coloured by anionic dyes.
3. The idiozome (centrosphere) of the primary spermatocytes is often slightly coloured by anionic but not by cationic dyes. The failure of the idiozome to colour must not, however, be regarded as indicating that a dye is cationic.
4. The cytoplasm of the interstitial cells is more strongly coloured by anionic dyes than that of the spermatogenetic cells.
5. The collagen of the tunica propria of the seminiferous tubules (and of the tunica albuginea) is coloured by anionic dyes.

The responses of the albuginea are not as clear-cut as one might wish, because Clarke's fluid leaves the superficial part of a piece of tissue in a rather different state from the internal parts, so far as colouring by dyes is concerned. If an organ had been chosen in which a thick collagenous layer underlay other tissue-constituents, it would almost certainly colour strongly with anionic dyes (but scarcely at all with cationic). Red blood-corpuscles are not easily

penetrated by dyes after fixation with Clarke's fluid, and therefore one cannot rely on their acidophilia to distinguish anionic from cationic dyes.

The stainable granules of von Ebner (1888), which are such a striking inclusion in the late spermatids and residual cytoplasm of the mouse, are more strongly coloured by most cationic than by most anionic dyes, but this character does not permit a sharp distinction to be made.

To find whether unmordanted haematein acts as a cationic or an anionic dye, the usual material was dyed for 10 min with unmordanted half-oxidized haematoxylin. The section was rinsed, dehydrated, passed through xylene, and mounted in benzene-balsam. The colour given was a pale yellow. All the 5 criteria by which cationic can be distinguished from anionic dyes showed clearly that unmordanted haematein is a very weak anionic dye. When the period of colouring was extended to 24 h, the tissue was much more strongly dyed, but the results once again showed that haematein is an anionic dye. This might, indeed, be inferred from the structural formula (p. 495).

The colours of solutions containing haematein

Saturated solutions of unmordanted haematein in Michaelis buffers, in layers about 4 cm thick, have these colours:

pH		
3	yellowish orange	
4	yellow	
5	yellowish orange	sharp change in colour
6	orange-red	
7	red	
8	very dark red	

A saturated solution at pH 9 was too dark for a clear view of the colour. Haematein was therefore dissolved at dM/1024 in 60% ethanol and the solution was mixed with an equal volume of Michaelis buffer. The colour at pH 9 was at first bluish magenta. Alkaline solutions of haematein are, however, not stable: the colour changes gradually to reddish orange.

The effect of pH on the colour of unmordanted haematein was investigated more exactly by making solutions in the way described in the preceding paragraph, but at various pHs, and examining them with a spectrophotometer. The results are shown in fig. 1. The curve for pH 8·75 is somewhat unreliable on account of the instability of the solution, but the main features are revealed. In aqueous solution at pH 4·7 haematein shows a maximum absorption at about 450 mμ, but the curve is here a broadly rounded hump. There is a slight shoulder at about 540 mμ. Increase in pH raises the shoulder and flattens out the hump. It is the flattening of the main hump that gives the bluish tinge to alkaline solutions.

The following experiment was carried out to find the effect of aluminium sulphate on the colour of dissolved haematein.

It was necessary to hold the solution at a particular pH, so that the change

of colour due to variation of hydrogen ion concentration might be eliminated. It was inevitable that an acid pH should be chosen, since the mordant/dye complex is precipitated in alkaline solutions, and this would prevent spectrophotometric study. It was decided to work at pH 4·60, because aluminium

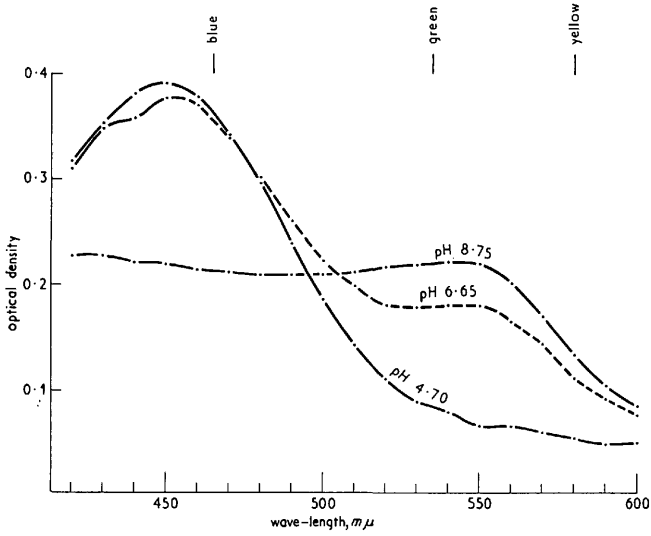


FIG. 1. Graph showing the absorption of light of various wavelengths by layers 1 cm thick of solutions of unmordanted haematein at various pHs. For full particulars see text.

sulphate solutions are easily buffered at this pH without danger of precipitation. To achieve this, the dye and the aluminium sulphate were dissolved separately in buffered 60% ethanol (p. 497) and the solutions were then mixed. The dye was in each case present (after mixing) at dM/1024, while the aluminium sulphate was at such concentrations that the number of atoms of aluminium to each molecule of haematein was $\frac{1}{2}$, 1, 2, 4, or 8. A control solution was also made which resembled the others in every respect except that it contained no aluminium sulphate.

In thicknesses of about 4 cm these solutions showed the following colours:

No. of atoms of aluminium to each molecule of haematein	*Colour*
0	yellow
$\frac{1}{2}$	yellowish orange
1	orange
2	reddish orange
4	dull orange-red
8	rose (dull red with a tinge of blue)

The spectrophotometric readings are shown graphically in fig. 2. It will be noticed that the addition of an atom or more of aluminium to each molecule of haematein causes a considerable darkening of the solutions (increased absorption of light) at all wavelengths from violet to yellow. The

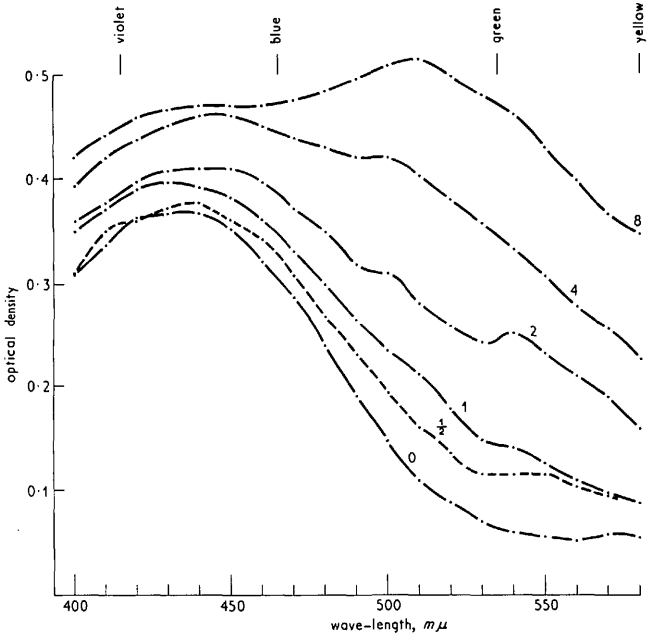


FIG. 2. Graph showing the absorption of light of various wavelengths by layers 1 cm thick of solutions of haematein in the presence of varying amounts of aluminium sulphate. The figures 0, ½, 1, 2, 4, and 8 represent the numbers of atoms of aluminium to each molecule of haematein. The pH of each solution was 4·60. For full particulars see text.

curves show shoulders at about 500 and about 540 mμ. These effects are best seen in the curve representing the addition of two atoms of aluminium to each molecule of haematein. When more aluminium is added, the shoulders at about 500 and 540 mμ merge together to give a hump showing maximum absorption at 510 mμ. Meanwhile the hump at about 440 mμ, characteristic of haematein in buffered 60% ethanol in the absence of the metal, rises progressively with each addition of aluminium. The transmission of bluish-green light (wavelength 510 mμ) falls from 70% in the absence of the metal to 22% in the presence of 8 atoms of aluminium to each molecule of haematein:

and although less blue and violet light is transmitted in the presence of aluminium, yet the proportion of such light is increased, and this accounts for the eventual production of a rose tint.

It is unlikely that one molecule of haematein could combine with more than two atoms of aluminium; yet the changes that started with the addition of aluminium in the proportion of half an atom to each molecule continue progressively as the number of atoms is increased. The facts suggest that such solutions as these contain both uncombined haematein and also the (soluble) mordant/dye complex. This hypothesis is strongly supported by the following experiment.

Haematein and aluminium sulphate were dissolved separately in 60% ethanol buffered with acetic/acetate at approximately pH 5·60 instead of 4·60. When the solutions were mixed in equal volumes, the resulting solution contained 4 atoms of aluminium to each molecule of haematein. The solution darkened as usual, but a precipitate formed over night. The precipitate, when filtered off, was seen to be blue-black with a slight purple tinge; the filtrate, on the contrary, was orange-yellow. The filtrate was examined spectrophotometrically. The density-curve closely resembled the usual curve for unmordanted haematein, with an absorption-maximum at about 450 mμ and no shoulder characteristic of the mordant/dye complex. It is clear that even when there is an excess of aluminium, only a part of the haematein forms a complex with the metal; at pH 5·6 this complex is precipitated.

The colours of tissues dyed with aluminium-haematein

The blue colour

Examination of the colour. It is one of the most familiar facts of microtechnique that aluminium-haematein, as usually applied, gives a bluish colour to chromatin and certain other tissue-constituents. Since the bluish substance is insoluble, it cannot be examined spectrophotometrically in the same way as the soluble compounds of the metal and dye. The following procedure was adopted to obtain a graph that would represent the colour.

A gel was prepared by dissolving powdered gelatine in warm water in the proportion of 25 g to 100 ml, and cooling the solution. The gel was hardened in 10% formalin and sectioned at 40 μ on a freezing microtome. The sections were dyed for 15 min in 'haematal 8' (p. 515), a single-bath aluminium-haematein solution. They were then rinsed with distilled water, blued for 5 min with 0·1% aq. sodium bicarbonate solution, rinsed again, dehydrated, and mounted on a slide in a row, touching one another, in Canada balsam. A black mask was placed round the sections, so that no light could pass through the spectrophotometer without traversing the sections. Undyed gelatine sections were treated in exactly the same way, to serve as a control and to prevent the slight yellowish colour of the gelatine itself from influencing the result. The mask round the undyed sections gave the same area of transmission as that which surrounded the dyed sections.

The dyed gelatine appeared to the eye blue with a purple tinge. The spectrophotometric readings of optical density are represented graphically in fig. 3.

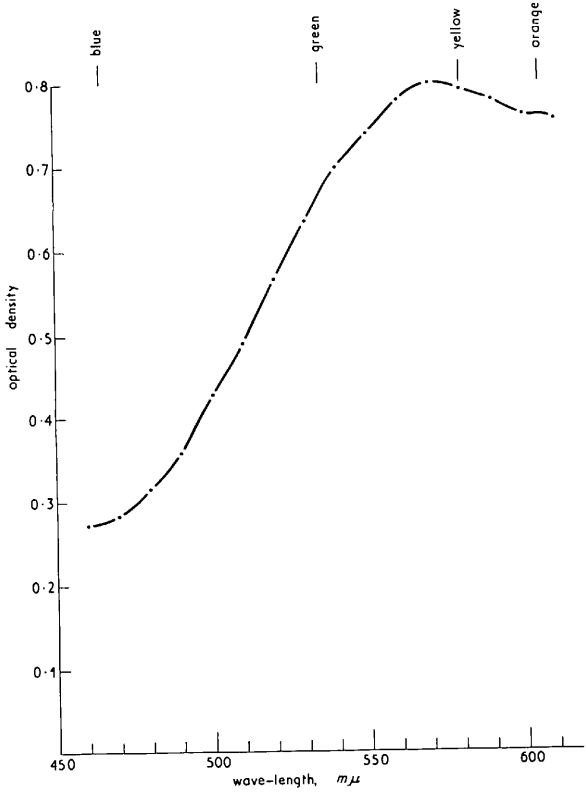


FIG. 3. Graph showing the optical density of gelatine sections dyed with aluminium-haematein. For full particulars see text.

It will be noticed that the maximum absorption is not in the violet-blue at about 440 mμ (haematein without mordant), nor in the blue-green at 510 mμ (haematein with excess of mordant at pH 4·60), but in the yellow at 570 mμ. Although the dominant colour is blue, yet a considerable proportion of red light is transmitted. The sections transmit 53% of blue light (460 mμ), 38% of red (660 mμ). It is a familiar fact that although chromatin appears blue in

preparations dyed with Ehrlich's haematoxylin and similar solutions if daylight is used for illumination, they may appear purple (even reddish purple) when unfiltered electric light is used.

The colours shown by haematein, with and without aluminium, are considered further on p. 516.

What is coloured blue by aluminium-haematein? It will be remembered that in the standard method, described on p. 498, sections are mordanted with aluminium sulphate, dyed with haematein, differentiated with sulphuric acid, blued with ammonia, and mounted in benzene-balsam.

When this method is applied to the standard material (p. 497), the distribution of the blue colour in the finished preparation shows that by all the five criteria listed on p. 500, aluminium-haematein is a typical cationic dye. Chromatin and von Ebner's granules are dark blue, while the cytoplasm of various cells, the contractile substance of smooth muscle, and the tails of spermatozoa are in varying shades of pale blue or pale grey. The idiozome of the primary spermatocytes is just visible, but not distinctly blue (very pale grey). Since chromatin is dark and cytoplasm pale, the preparation is 'good' by the criteria of routine histology (fig. 4, A). Collagen and the nuclei of spermatozoa are not blue or grey, but *orange-brown*. This unexpected result is discussed in detail below under the heading of *The yellow colour* (p. 511).

The nucleic acids were removed from sections by treatment first with trichloracetic acid and then with ribonuclease (p. 499). The extracted sections were then dyed by the standard method. Hardly any blue was seen in the final preparations, apart from a tinge in the cytoplasm of the epithelial cells of the epididymis. In most cells the cytoplasm was scarcely tinged with grey; collagen was unstained or very pale grey, von Ebner's granules and the tails of the spermatozoa very pale grey; the chromatin was just touched with yellowish grey. It is evident from this experiment that the blue colour seen in an ordinary section of the testis or epididymis of the mouse, dyed by the standard method, is due almost entirely to the reaction of the nucleic acids with aluminium-haematein.

If sections are extracted with trichloracetic acid, but not afterwards with ribonuclease, the effect of the dye is somewhat different. The chromatin is now a pale blue instead of very pale yellowish grey, and von Ebner's granules are blue-grey, much darker than when ribonuclease was used. This suggests that the treatment with trichloracetic acid does not fully remove the RNA, and that the colouring of ordinary (unextracted) sections by the standard method is due in part to the reaction of RNA with aluminium-haematein. It

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FIG. 4 (plate). Sections of the testis of the mouse, fixed in Clarke's fluid.

A, a blued aluminium-haematein preparation (standard method).

B, an unblued (red) preparation. The section was treated in exactly the same way as A, except that blueing was omitted. Note resemblance to A.

C, a preparation from which the nucleic acids were extracted before dyeing with aluminium-haematein. Note the strong coloration of chromatin. See p. 507.

For full particulars see text.

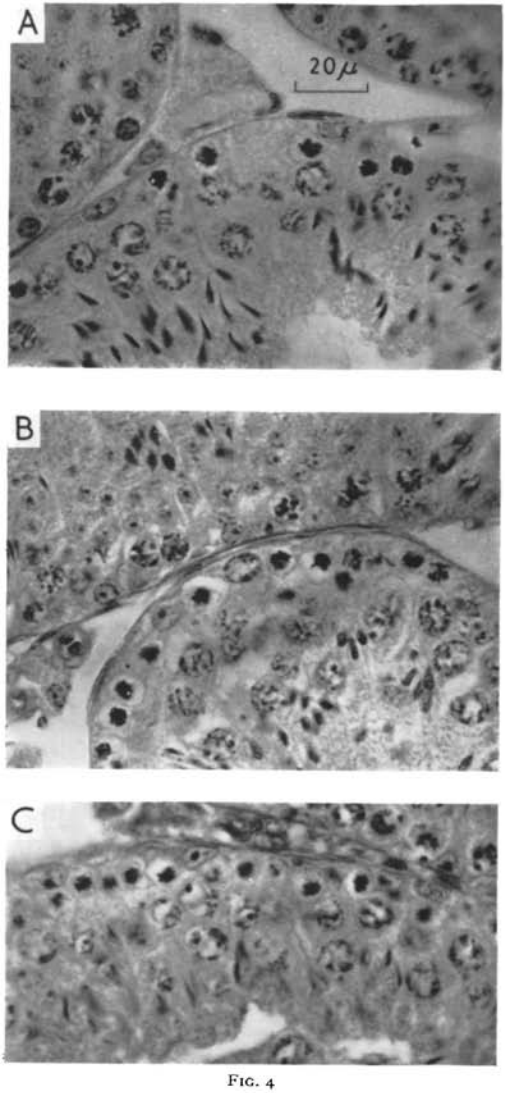

FIG. 4

J. R. BAKER

is probable, however, that DNA plays the predominant role, except in the dyeing of von Ebner's granules. Sud (1961) has shown that these contain RNA but no DNA (in the rat).

Experiments on differentiation by acid. Differentiation of a tissue/mordant/dye complex by the mordant itself was considered in some detail in the first paper in this series (Baker, 1960).

To reveal the effect of differentiation by acid, sections of testis, epididymis, and intestine that had been treated by the standard method were compared with others that had been treated in exactly the same way, except that differentiation in sulphuric acid had been omitted. The two sets of sections were markedly different. When differentiation was omitted, everything was blue, except collagen and the nuclei of spermatozoa, both of which are markedly acidophil. Cytoplasm was pale blue (but much darker than when differentiation was carried out); the tails of spermatozoa medium blue; chromatin, von Ebner's granules, and the contractile substance of smooth muscle, dark blue. Thus the preparation was not 'good' by the standards of routine histology. 'Good' preparations always result if the standard method is carried out in full, with differentiation by sulphuric acid.

When the sulphuric acid bath was omitted in the treatment of sections that had been extracted with trichloracetic acid and ribonuclease, the appearance (fig. 4, c) was remarkably similar to that seen in a 'good' preparation (though the chromatin was a slightly brownish blue in places, and there were other minor differences). One would not have guessed that this result had been obtained in the absence of nucleic acids. Although the dark blue of chromatin in a differentiated preparation is almost entirely due to its nucleic acid content, yet the proteins of chromatin are capable of giving a closely similar appearance, after the nucleic acids have been removed. The Stedmans (1947) noted that chromosomin is capable of being strongly dyed by Mayer's *Hämalaun*.

Although part of the mordant/dye compound is taken up by the protein of chromatin, and this part is susceptible to acid differentiation, yet the latter process does not make very much difference to the colour of chromatin, since so much of the complex is firmly held in it by the nucleic acids. In those tissue-constituents in which the colour is taken up principally by protein, differentiation had a marked effect. This applies to cytoplasm, the tails of spermatozoa, and the contractile substance of smooth muscle, all of which are nearly or quite free from the blue colour after the standard differentiation. It must be remarked, however, that in the tissues chosen for this investigation, the cytoplasm of the various cells does not contain much RNA. Somewhat different results would have been obtained if the pancreas had been chosen for study.

An experiment was performed to find out where the sulphuric acid attacks the tissue/mordant/dye complex. Does it set free the dye from the mordant, or the mordant from the tissue? One section of testis was treated by the standard method: another by the same method with one modification, namely, that the treatment with sulphuric acid was carried out before instead of after dyeing (but after mordanting and then rinsing, as usual). The two sections

were carefully compared. They were found to be indistinguishable. This shows clearly that the acid attacks the tissue/mordant link.

The same conclusion resulted from a different experiment. A section of the testis was treated by the standard method, slightly modified; dyeing lasted for only 10 min instead of 20, and the period of differentiation was increased from 40 to 60 sec. Naturally the resulting preparation was paler than usual, but in other respects it was normal. A second section was treated in precisely the same way throughout, except that when it had been rinsed after blueing, it was put into half-oxidized haematoxylin solution (never previously used, and therefore free from mordant), and left in this for 10 min. It was rinsed and blued again at once, without a second differentiation, and then carried through to balsam. So far as the blue colour was concerned, the two preparations were *indistinguishable*, though one had been dyed twice. If the sulphuric acid had split part of the dye from the mordant, but had left the mordant untouched in the tissue, the second section would necessarily have been more strongly dyed than the first.

Experiments on blueing. The standard method used in the present investigation leaves tissues red when they are rinsed after dyeing. Strongly acid solutions of aluminium-haematein, such as Ehrlich's 'haematoxylin' (pH 2·5), also dye tissues red. Certain other aluminium-haematein solutions make tissues blue or violet, but the colour becomes red when acid is used for differentiation. It is almost universal practice to change the colour to blue, usually by use of an alkaline solution. The red colour will be considered below (p. 509).

It may be remembered that in the standard method, weak ammonia solution (pH 11·1) is used for blueing. This is a suitable fluid for the purpose if the period of treatment is kept very short (5 sec), but magenta clouds come out of the sections if treatment is prolonged (see p. 509). Squire (1892) used a solution of 10 grains of sodium bicarbonate in a pint of water. A 0·1% solution of this salt (slightly weaker than Squire's) is a convenient blueing agent, used in the present investigation in the 'method for collagen' (p. 498). It works much more slowly than the weak ammonia solution, but does not extract the dye; the pH is 8·05. A 1% aqueous solution of potassium acetate is still gentler (pH 7·45); it was occasionally used in the present investigation. Squire (1892) found that even distilled water may be used to blue Ehrlich's haematoxylin.

An experiment was performed to find the effect of pH on the blueing of aluminium-haematein. Sections of testis and epididymis were dyed by the standard method, differentiated with sulphuric acid as usual, rinsed, and then placed in Michaelis buffers at pH 9·0, 8·0, 7·0, 6·0, 5·0, 4·0, and 3·0. They were left in the buffers for 10 min. Coverslips were applied and the sections were examined while still immersed in the buffers. Chromatin and von Ebner's granules were dark blue from pH 9 to pH 6, blue (but not dark) at pH 5, dull red with a tinge of blue at pH 4, and red at pH 3. The cytoplasm of most of the various cells in the two organs was only definitely blue at

pH 9 and 8; the colour was, of course, pale. Collagen was pale blue at pH 9, 8, and (in most places) 7. A magenta cloud came out of the section into the buffer at pH 9.

The extraction of colour by strongly alkaline solutions was studied by the following experiments, carried out with sections of the testis or the mouse. The preparations were examined in benzene-balsam, after the treatments described below.

A section was treated by the standard method, but the period in weak ammonia was extended from 5 sec to 1 h. Von Ebner's granules retained their dark blue colour, but chromatin was pale blue. The albuginea and the nuclei of spermatozoa did not show their usual yellowish colour, but were very pale warm grey and blue or bluish brown respectively. It is evident that the alkaline solution extracted the unmordanted haematein. The magenta clouds that came out of the sections presumably consisted mostly of unmordanted haematein showing the characteristic colour at high pH.

Another section was treated in the same way, except that the period in weak ammonia was extended to 4 days. The colour was now almost completely bleached away. The nuclei of spermatozoa were grey, but everything else was extremely pale warm grey or grey. A third section was treated similarly, but after the ammonia had been rinsed away, the section was dyed again in half-oxidized haematoxylin for 20 min; it was then blued for 5 sec in weak ammonia. Von Ebner's granules and chromatin were now grey (not blue), much darker than in the previous case; the nuclei of spermatozoa were dark grey.

These experiments seem to show that long treatment with a solution at high pH results in partial but not complete loss of the mordant from the sections.

The red colour

When a section is dyed with aluminium-haematein and then differentiated with an acid solution, the predominant colour is at first dull red. The same colour is produced without differentiation if a rather strongly acid aluminium-haematein solution, such as Ehrlich's haematoxylin or Mayer's acid haemalum (1903), is used for dyeing. What is the cause of the colour, which in the ordinary practice of microtechnique is transformed by blueing?

The question can be answered most shortly if acid differentiation alone is considered: the conclusions reached can easily be applied to the case of strongly acid single-bath solutions, used without subsequent differentiation.

When tissues are red after differentiation by acid, two possibilities present themselves. Either (1) the dye itself has been set free from the mordant, and is showing the red colour of haematein at a particular pH; or (2) the dye is still linked to aluminium and is showing the red colour of the mordant/dye complex in acid solution.

It has already been shown (pp. 507, 508) that acid differentiation certainly separates the mordant from the tissue; but it is not unthinkable that the

haematein might also be separated from the aluminium, and in its free state it might then attach itself to the tissues as an anionic dye. If so, blueing would involve the re-establishment of the mordant/dye compound, which, being cationic, would rearrange itself in the tissues.

To resolve this problem, two sections of testis were brought down to water, mordanted as usual, dyed with half-oxidized haematoxylin, and differentiated with weak sulphuric acid. One was blued (with potassium acetate); the other only briefly rinsed with distilled water. Both were dehydrated and mounted. What was blue in one preparation was red in the other. The dyeing, in blue or red, was in both sections cationic, by all the 5 criteria listed on p. 500. Chromatin and von Ebner's granules were strongly coloured red in the unblued preparation. Black-and-white photomicrographs of the two sections were indistinguishable (fig. 4, A, B). (There was a certain amount of yellowish colouring in the unblued preparation, in sites not occupied by the red dye. This subject will be considered in detail on p. 511.)

To find whether the red colour is soluble in ethanol, a section of the testis was treated in the way described in the preceding paragraph, without blueing; it was dehydrated, left for 24 h in absolute ethanol, and then mounted. The red colour persisted unchanged: no part of it had been extracted. (An unblued section left for 24 h in distilled water becomes blue.)

A section treated in exactly the same way, except that mordanting was omitted, showed no trace of red.

It follows from these experiments that the red colour is the cationic mordanted dye, aluminium-haematein, in its reddish range.

Why, then, does anyone bother to blue the red dye? It colours the same objects as the blue, and is equally insoluble in ethanol. The colour itself is probably the reason. The one is a dull or dirty-looking red, the other bright blue. It is easy to choose an anionic dye that will provide a striking contrast with the blue, but this is difficult with the red.

The fact that aluminium-haematein, when red, has an unsatisfactory colour is easily shown by comparing it spectrophotometrically with a brilliant red dye, such as safranine O. For this purpose haematein was dissolved at dM/256 in buffered 60% ethanol, and the solution was mixed with an equal volume of aluminium sulphate dissolved at dM/128 in the same solvent. The resulting fluid (pH 4·65) was a dull, indistinct red ('wine-colour'). Safranine O was dissolved at dM/512 in 60% ethanol (on the assumption that the specimen of the dye consisted of equal weights of the two closely related components of the usual mixture); the fluid was bright red. It will be noticed that haematein and safranine were both present at dM/512 in these solutions—the former presumably combined (or most of it combined) with aluminium. The transmissions of the two solutions are shown in fig. 5. The steep rise of the safranine curve through yellow to orange, with a transmission of nearly 90% all the way from orange to red, must necessarily represent a brilliant dye; the gradual slope of the aluminium-haematein to a maximum of less than 50% in the red must, on the contrary, provide a graphical picture of dullness.

The yellow colour

It has already been mentioned (p. 506) that in sections dyed with aluminium-haematein, certain tissue-constituents sometimes show a colour that contrasts with the blue and red that have already been considered. I

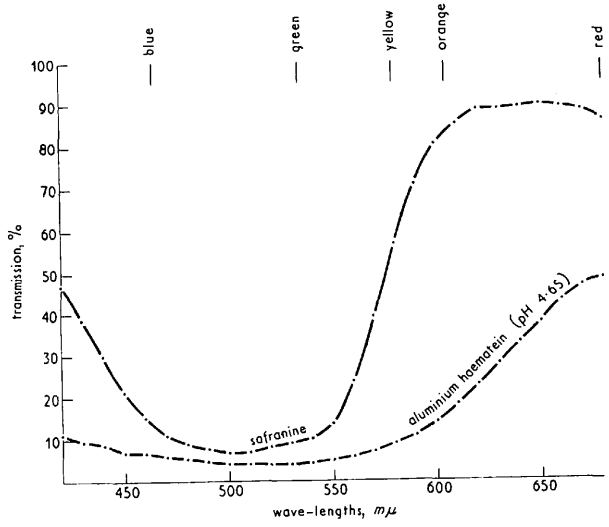

FIG. 5. Graph showing the transmission of light of various wavelengths by layers 1 cm thick of safranine (dM/512) and aluminium-haematein (the haematein at dM 512). For full particulars see text.

have studied this subject in some detail, because an apparently analogous case has been investigated by Wigglesworth (1952), who used iron-haematein. It may be mentioned at the outset that my conclusions are quite different from his. It is possible, though perhaps unlikely, that different causes operate in the two cases.

The colour here referred to varies from pale greyish yellow through dull orange to orange-brown. It will be called the yellow colour for short, since there is always a yellowish element in it, but the colour is never a clear, bright yellow.

Collagen is the tissue-constituent that shows the 'yellow' colour most noticeably. The 'method for collagen' (p. 498) was worked out empirically by changing the standard method in those particular ways that were found to contribute to the yellow coloration of collagen. It was shown (1) that blueing should be done in a weakly alkaline medium, not in the usual ammonia

solution; (2) that the section should not be rinsed in water after blueing; and (3) that *iso*-butanol should be substituted for ethanol as a dehydrating agent.

When these changes are made, rather striking preparations result. Chromatin is blue, von Ebner's granules greyish blue. The following tissue-constituents all show some variety of the yellow colour: nuclei of spermatozoa, cytoplasm (of germ cells, interstitial cells of testis, and epithelial cells of epididymis and small intestine), contractile substance of smooth muscle, tails of spermatozoa, red blood-corpuscles, free border of intestinal epithelium. Collagen (in the tunica albuginea and tunica propria of the testis, and in the connective tissue of the epididymis and of the intestinal submucosa) is orange: so is the elastin of arterioles. With the exception of elastin, these tissue-constituents are all to some degree acidophil. The facts suggest that the yellow colour is due to the anionic dye, unmordanted haematein.

The following experiment was performed to discover whether or not the yellow is a mordanted colour, stable in neutral fluids. Sections of testis and epididymis were carried through the method for collagen as far as stage VIII; the blued sections were then transferred to various solvents. In those cases in which the solvent to be tried was immiscible with water, the sections were first dehydrated by stages IX and X of the method for collagen. They were then left for 24 h in the solvents, and examined under the microscope while still immersed in them. The identity of the slides was hidden until the intensity of the yellow coloration had been judged and recorded. The results are shown in the centre column of table 1.

TABLE 1

This shows the effect of solvents on colouring by the 'method for collagen'.

	Intensity of yellow colour	
Solvents	*mordanted with aluminium sulphate, dM/16*	*sulphuric acid, 3/8 decinormal, substituted for mordant*
benzene	+++	++++
turpentine	++++	++
chloroform	+++	++++
iso-butanol	++++	+++
iso-propanol	++	++
ethanol	++	++
distilled water	+	++
glycerol	±	+
ethylene glycol	±	o

The plus signs indicate the intensity of the yellow colour in acidophil tissue-constituents. Small differences are indicated by plus signs in small type.

The fact that some of these neutral solvents removed the yellow colour suggested strongly that unmordanted haematein was responsible for it. The experiment was therefore repeated, with one difference only: sulphuric acid

was substituted for the mordant. The acid was used at 3/8 decinormal, so that the concentration of sulphate ions remained unchanged: the only difference from the previous experiment was that hydrogen ions replaced aluminium ions. There was now no blue or blue-grey colouring of basiphil constituents. The degree of colouring of the acidophil constituents by unmordanted haematein is represented by symbols in the right-hand column of table 1. Once again the identity of the slides was hidden until the intensity of the colour had been judged.

The resemblance between the two columns of symbols is striking: indeed, apart from turpentine and distilled water the differences are almost negligible. It is scarcely possible to doubt that in both experiments unmordanted haematein was the cause of the yellow colour.

It must be remarked that the tunica albuginea is sometimes of a slightly redder orange when the mordant is used than when it is omitted. This is probably due to a small amount of mordanted haematein adding its effect to a much larger amount of the unmordanted dye. (Only the red component of the mordanted dye shows through the orange of the unmordanted.)

The question arises, whether those fluids in which haematein is soluble are the ones that remove the yellow colour. The solubility of haematein in 8 of the solvents, at room temperature, is shown here (see p. 496):

	Solubility %, *w/v*
benzene	insoluble
turpentine	,,
chloroform	almost insoluble
distilled water	0·079
iso-butanol	0·083
ethanol	0·105
glycerol	1·53
ethylene glycol	1·68

It is evident that there is a general correlation between the solubility of haematein in a solvent, and the capacity of that solvent to remove what is here called the 'yellow' colour. It must be noticed, however, that distilled water removes the colour more readily than *iso*-butanol does, though haematein is slightly more soluble in *iso*-butanol than in distilled water. Solubility is clearly the main cause, but some other undisclosed factor must also be involved.

To obtain information about the substances in tissues that react with unmordanted haematein to give the yellow colour, sections were deaminated by van Slyke's method (p. 499) and then treated by the method for collagen. Many of the tissue-constituents that were yellow with the ordinary method for collagen were now pale blue or pale bluish-grey, or only showed a trace

of the yellow colour here and there. This suggests strongly that the yellow colour is given by haematein, reacting as an unmordanted anionic dye with amino-groups in the tissues. Collagen and elastin, however, are still orange after deamination. It is uncertain which amino-acids in these substances are dyed by haematein. Some yellow also remains in the free border of the intestinal epithelium and in the contractile substance of smooth muscle.

Some practical applications

The standard method and the method for collagen were designed to throw light on the process of dyeing: they were not intended for routine use in microtechnique. The standard method has some practical value, however, since collagen is almost the only tissue-constituent that is dyed a yellowish colour, and it shows up well against the blue of the mordanted dye. Preparations are particularly striking in this respect if differentiation in sulphuric acid is omitted. When the method for collagen is used, even the thinnest layers containing this substance (such as the tunica propria of the seminiferous tubules) are strongly dyed and show up clearly; but other acidophil tissue-constituents are also yellow.

In the past, haematoxylin has been used much more frequently than haematein in the preparation of practical dye solutions. The low solubility of haematein in the usual solvents—water and ethanol—is probably the cause. Haematoxylin dissolves in absolute ethanol at 10·5% w/v; haematein at 0·105% w/v. It has therefore been found convenient to make up stock solutions of haematoxylin and to rely, in many cases, on gradual transformation to haematein through the action of atmospheric oxygen. Such solutions, however, soon become cloudy, and precipitates often form. The insoluble matter is commonly regarded as a product of 'over-oxidation', but in fact the precipitate may consist of haematein itself. It would seem more logical to choose a solvent in which haematein dissolves readily.

Glycerol would be suitable for general use if it were not so viscous, and indeed it is sometimes used (with water and ethanol) in solutions of *haematoxylin* (for instance, by Regaud (1910)). Probably it was found empirically that no precipitate was formed in the presence of glycerol when the haematoxylin gradually oxidized to haematein. For stock solutions, however, glycol is more convenient, on account of its much lower viscosity. One does not need to dissolve haematein at anything approaching saturation in this solvent.

When aluminium is to be the mordant for haematein, it is generally best to use the single-bath method. Experiments were performed to discover what proportions of haematein and aluminium sulphate should be mixed. It was found best to use a mordant quotient of 8 (Baker, 1960): that is to say, a solution in which there are 8 atoms of aluminium to each molecule of haematein. Such a solution may be distinguished by the name 'haematal 8'. It is prepared as follows:

Aluminium sulphate, dM/4 aq.

$Al_2(SO_4)_3.16H_2O$	15·760 g
distilled water	up to 1 l.

Haematein, dM/16 in ethylene glycol, 50% aq.

haematein	1·876 g
ethylene glycol, 50% aq. v/v	up to 1 l.

Haematal 8

aluminium sulphate, dM/4 aq.	1 vol.
haematein, dM/16 in ethylene glycol, 50% aq.	1 vol.

In practical microtechnique it suffices to weigh the solids to the nearest decigram.

The stock solutions are ready for use as soon as they have been made. A new supply may be prepared instantly whenever it is required.

A mordant quotient of 8 gives a solution that is particularly easy to use either progressively or regressively, for sections or whole mounts. There is a general similarity in action to Mayer's *Hämalaun* (1891). Sections of material fixed in Clarke's fluid may be dyed progressively for about 2 to 5 min. (Sections of Zenker material take longer.) Afterwards it is only necessary to rinse the section with a stream of distilled water from a squash bottle: an alkaline solution need not be used. If regressive dyeing is preferred, the period in haematal 8 should be increased to ½ h. Weak sulphuric acid (p. 497) is a convenient differentiator in regressive dyeing, since there is no loss of strength by evaporation of the acid, but hydrochloric may be substituted. The section should only be left in weak sulphuric acid for a few seconds. Alternatively one may differentiate at greater leisure in aluminium sulphate, dM/8 or dM/16 aq. After differentiation (whether in acid or in the mordant) it is best to blue in weak ammonia (p. 498) for 5 sec or in sodium bicarbonate solution, 0·1 aq., for 1½ min.

Like all the other aluminium-haematein dyes, haematal 8 gives a blue (or slightly purplish-blue) colour to chromatin and other basiphil tissue-constituents. It is best to use a good 'daylight' filter, such as Ilford 810. Since aluminium is present inconsiderable excess, the yellow colour of unmordanted haematein is not seen.

A solution similar to haematal 8 but with double the mordant quotient is conveniently prepared thus:

Haematal 16

aluminium sulphate, dM/4 aq.	2 vols.
ethylene glycol, 50% aq. v/v	1 vol.
haematein, dM/16 in ethylene glycol, 50% aq.	1 vol.

The high mordant quotient makes this solution slower in action. Sections of material fixed in Clarke's fluid may be dyed progressively for about 10 min. Since there is scarcely any tendency to overstain, haematal 16 may be specially recommended to beginners in microtechnique.

Biebrich scarlet (0·2% aq.) is a particularly suitable counterstain for all aluminium-haematein dyes, including haematals 8 and 16. Eosin may of course be used.

Whole mounts should be overstained with haematal 8 or haematal 16 and differentiated with aluminium sulphate at dM/8.

Hypothetical remarks on the colours shown by haematein

An attempt is made here to formulate briefly a hypothesis, consistent with all the facts disclosed in the present investigation, to account for the varying colours of haematein. This hypothesis is put forward with some diffidence, in the hope that it will suggest further experiments by which a nearer approach to the truth may be achieved.

In the absence of aluminium, there are at least three —OH groups in haematein, at positions 3, 4, and 10 (see p. 495), which may be supposed to influence colour by changes in the dissociation of the hydrogens. It is not impossible that the —OH group at position μ may also ionize. It is presumably the possession of several ionizable —OH groups that makes the colour changes of haematein so complicated. The colours shown by unmordanted haematein from pH 3 to pH 9 are mentioned on p. 501. The undissociated or scarcely dissociated dye in very strongly acid solution is red.

The structure of the aluminium ion and its attachment to the purpurine molecule were discussed at some length in the first paper of this series (1960). It seems almost certain that one aluminium ion can chelate with haematein in exactly the same way, since an ionizable hydrogen and a potentially donor oxygen atom are situated in the right relation to one another at positions 10 and 9. According to Bruhn (1951), another aluminium ion can attach itself at positions 3 and 4, by substitution for the hydrogens of the —OH groups.

When the linkages between haematein and aluminium are complete, the possibility of colour change by dissociation has been greatly reduced or eliminated. The resultant colour is the familiar blue (or slightly purplish blue) given by Ehrlich's haematoxylin and similar solutions, such as haematal 8. The blue compound, as we have seen (p. 508), can exist from about pH 5·0 upwards.

In this section of the paper we have so far been concerned with two substances: (1) free haematein, varying in colour with pH, and (2) fully linked blue haematein, each molecule attached to two aluminium ions. It is evident, however, that at least one other compound of haematein with aluminium must exist; for at lower pHs than 5·0 there is a dull red compound, capable of attachment (like the blue one) to acidic groups in the tissues. The difference in colour suggests the presence of one or more ionizing —OH groups. If so, it must be supposed that at least one of the two aluminium ions is only

attached to the haematein molecule by one linkage instead of two. The existence of this dull red compound would necessarily be favoured by acidity, for hydrogen ions would compete with aluminium ions for the sites at 3, 4, and 10. With rising pH the competition by hydrogen ions would become less, and the blue compound would replace the red. This is presumably the change involved in the process of blueing (which can occur whether the aluminium ions are attached to tissue-constituents or not).

Aluminium can certainly attach itself to haematein without any need for each of two ions of the metal to make *two* links with the dye. This is proved by the fact that aluminium can be used as a mordant for brasilein, which lacks one of the —OH groups possessed by haematein but is in all other respects identical. Unfortunately, we do not know for certain which —OH group is missing in brasilein. According to Pfeiffer and Döring (1938) it is the one at position 4. If so, one aluminium ion could chelate at positions 9 and 10, and another might attach itself loosely at position 3.

It is not possible that the dull red haematein compound differs from the blue in having only one aluminium ion attached to the dye; for if this were so, blueing would be impossible in the absence of a further supply of the dye.

Mrs. B. M. Luke gave much practical help during this investigation, especially in the task of finding the solubility of haematein in various solvents. Miss Elizabeth Collins also gave skilful assistance in the work on practical applications. Miss J. S. Birkbeck gave secretarial help. I thank all three.

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