Experiments on the Action of Mordants

I. 'Single-bath' Mordant Dyeing

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Summary

Aluminium sulphate was chosen as the simplest mordant, and purpurine (1,2,4-hydroxyanthraquinone) as the simplest dye suitable for use in experiments with mordants.

It was shown by a series of experiments that a solution containing about 16 atoms of aluminium to each molecule of purpurine has special properties. A section already dyed with an aluminium/purpurine solution till chromatin is dark and cytoplasm lightly tinged, is in equilibrium with such a solution: that is to say, it neither takes up more dye nor loses any part of what it already holds. If the proportion of dye to mordant is increased, the section will take up more of the dye; if the proportion of the mordant to the dye is increased, the section will lose part or all of the dye it held.

The experiments described in the paper, taken in conjunction with existing knowledge of the behaviour of aluminion ions in solution and of their capacity to form chelate compounds with anthraquinonoid dyes, afford a basis for the following theory of single-bath mordant dyeing by aluminium/purpurine.

One purpurine ion chelates with one hydrated aluminium ion to form a complex ion with double positive charge; this is attracted to sites of negative charge in the tissues, such as the phosphoric groups of the nucleic acids. Electrostatic attraction is replaced by covalent bonds, resistant to solution by neutral ethanol. In extraction ('differentiation') by the mordant, the dye partitions itself between the tissue/mordant complex on one hand and the dissolved mordant on the other.

Contents

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Introduction

A GREAT deal of study has been devoted to the action of mordants in the textile industry, and much knowledge of the chemical reactions involved has been obtained. The recent work on this subject, however, has nearly all been done with basic chromium salts as mordants and with azo-compounds as dyes. This combination is never used in microtechnique, where salts of aluminium and iron are the usual mordants for haematein and anthraquinonoid dyes. Beyond this, the textile worker has no easy means of discovering whether his mordant/dye complex acts as a basic or an acid dye, or indeed as neither (compare Gaunt, 1949; Race and others, 1946), while the microtechnician can generally tell by a glance down the microscope. Further, the textile worker is not concerned with the problem of differentiation (differential extraction).

A rather full account of our knowledge of the action of mordants in microtechnique has been published recently (Baker, 1958). It was emphasized that despite the important contribution of Wigglesworth (1952), large gaps in our knowledge remain.

It was thought best to concentrate at first on the simplest mordant (aluminium sulphate) and the simplest dye (purpurine) that could be used, and to try to solve one of the most straightforward problems: namely, the numerical relations between the metal atoms on one hand, and the dye molecules on the other.

If a mordant and a suitable dye are dissolved together at appropriate concentrations, and a piece of fixed tissue (section or whole mount) is placed in the solution, the tissue is dyed. If it is now placed in a solution of the mordant, the dye is seen to be extracted in clouds. The mordant solution is to some extent coloured by the dye: yet it extracts the dye. Thus one solution of mordant and dye adds colour to the tissue, while another such solution removes it. Can a balance be struck, so that a solution neither adds nor subtracts colour? And if so, what are the numerical relations between the atoms of the mordant metal and the dye molecules in such a solution?

In attempting to answer this and related questions, one is concerned with the 'single-bath' method of mordant dyeing, in which mordant and dye are dissolved in a single solution. The 'two-bath' method, involving separate solutions of mordant and dye, will be considered in subsequent papers.

In the early part of this paper it will be convenient to refer to the atoms and molecules of mordants and dyes. A consideration of the ions of these substances will be deferred to the section beginning on p. 267.

Symbols for Expressing the Concentrations of Solutes

A lot of unnecessary words will be avoided in this series of papers by the adoption of a set of conventions for expressing the concentrations of mordants and dyes.

Since we are concerned here with numbers of atoms and molecules,

solutions are made up (where appropriate) in concentrations proportionate to the molecular weight. In the case of the salts used as mordants, however, the word 'molecule' is inappropriate. The 'molecule' of potassium alum, for instance, can be written as $Al_2(SO_4)_3 . K_2SO_4 . 24H_2O$ or as $AlK(SO_4)_2 . 12H_2O$. For this reason it is necessary to select a formula for such substances and to make up solutions by reference to it. A solution containing the formula-weight in g contained in 1 l. of solution is often called an F solution. It is rather troublesome, however, to keep changing from formula-weights to molar weights as one turns from mordants to dyes. No misunderstanding will arise from the use of the symbol M throughout, if it is clear that in the case of salts, M solutions are really F solutions (the selected formula being given in every case where there could be any doubt). In this series of papers the formula with $24H_2O$ is chosen for the alums, because it shows the structure of these substances more clearly.

In the present study of mordants it was never found necessary (and it was often impossible) to dissolve any substance at more than one-tenth of the M concentration (in the sense just defined). All concentrations are therefore related to the M/10 or decimolar concentration. For brevity this will be called the dM concentration. As a general rule, mordants and dyes were used in this investigation at various concentrations in the series dM/2, dM/4, dM/8, and so on: that is to say, in the series $dM/2^1$, $dM/2^2$, $dM/2^3$. It is often convenient to express the degree of dilution in the series by mentioning the index of the denominator only. Thus 'dil. 13' means $dM/2^{13}$ (= dM/8182).

The composition of a solution containing both mordant and dye may conveniently be expressed in terms of what I shall call the *mordant quotient*: that is to say, the number of atoms of mordant metal in the solution, divided by the number of dye molecules. This figure is easily obtained from the concentrations of the two constituents expressed in terms of dM. For instance, if the mordant is at dM/4 and the dye at dM/16, the mordant quotient is $2 \times \frac{16}{4} = 8$. (It is necessary to multiply the fraction $\frac{16}{4}$ by 2, because the formula for aluminium sulphate contains two atoms of aluminium.)

Materials and Methods: General Remarks

Aluminium sulphate. Aluminium was chosen as the mordant metal for the preliminary study reported in the present paper, because its chemical reactions are simpler than those of such transition-elements as chromium and iron. The simple sulphate seemed preferable to the alum (double sulphate with potassium). Solutions were made from the crystalline salt,

$$Al_2(SO_4)_3 . 16H_2O.$$

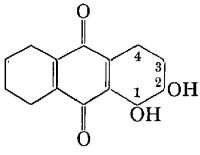
Crystals of analytical grade were used, but even these are not quite pure, and the actual concentrations of the solutions used must have been very slightly less than the figures suggest. The formula-weight is 630·4. In several of the experiments the salt was dissolved at dM/4. Aluminium sulphate is soluble in

water at this concentration; but purpurine is much more soluble in alcoholic solutions, and 60% ethanol was therefore used as solvent. Aluminium sulphate is just soluble in 60% ethanol at dM/4 in a warm room (about 25° C).

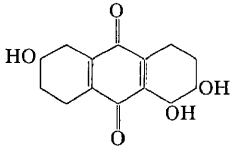
The pH of a solution of aluminium sulphate at dM/4 in 60% ethanol is 3·08.

Purpurine (molecular weight 256·2). This dye was chosen as the main subject of the investigation recorded in the present paper partly because it is simpler in chemical structure than carminic acid and haematein, and its mode of action is therefore easier to interpret; partly because it does not act as a typical basic dye in the absence of a mordant (p. 262), so that one can always be sure whether the mordant has acted or not; and partly because its solutions with aluminium sulphate, unlike those of carminic acid and especially of haematein, have scarcely any tendency to deposit an insoluble lake and thereby change in concentration.

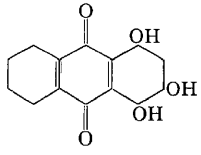
The original intention was to work with the simplest dye that is capable of being mordanted by aluminium sulphate, namely, alizarine. This, however, is not sufficiently soluble in the dye-solvents commonly used in microtechnique, and the same is true of the related anthraquinonoid dye, alizarine



alizarine



alizarine brown



purpurine

brown. Purpurine, another close relative, is, however, soluble in 60% ethanol, especially in the presence of aluminium sulphate. The commercial specimen of the dye was presumably not quite pure, and the actual concentrations must have been slightly less than the stated figures indicate.

For certain purposes it was necessary to study purpurine in acid solutions in the absence of any mordant. Two such solutions were used.

Acetic purpurine (pH 3·23)

Acetic acid (glacial)	15 ml
Ethanol, 60%	up to 500 ml
Purpurine	to saturation

Sulphuric purpurine (pH 2·43)

Sulphuric acid, approx. 0·2 N	25 ml
Ethanol, 60%	up to 500 ml
Purpurine	to saturation

These solutions are yellowish orange.

The following solution was used for diluting or extracting purpurine in certain experiments.

Acid ethanol (pH 2·50)

Sulphuric acid, approx. 0·2 N	25 ml
Ethanol, 60%	up to 500 ml

It will be noted that acid ethanol has the same composition as the solvent in sulphuric purpurine.

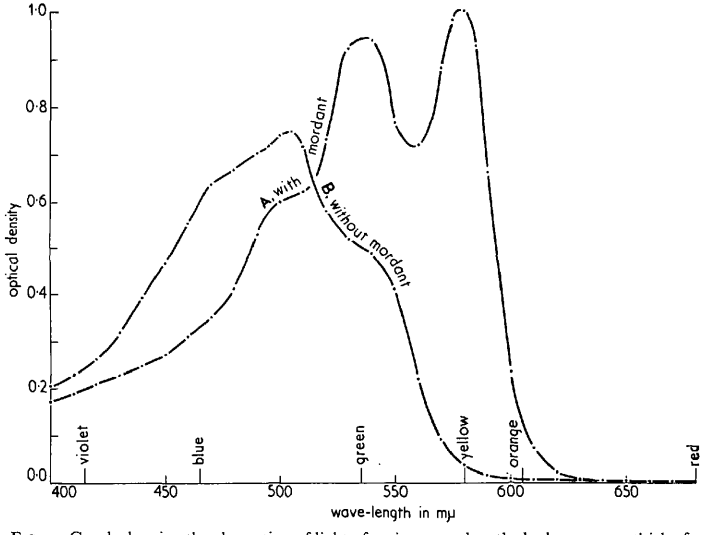


FIG. 1. Graph showing the absorption of light of various wavelengths by layers 1 cm thick of two solutions containing purpurine at the same concentration. One solution (graph *A*) was acidified by the addition of sulphuric acid; the other (graph *B*) was acidified to about the same pH by the addition of a mordant, aluminium sulphate. For full particulars see text.

Purpurine resembles carminic acid, another anthraquinonoid dye, in forming basic dye-lakes with suitable mordants. In the experiments described in the present paper it was dissolved in 60% ethanol at various concentrations from dM/16 downwards (dil. 4, 5, 6, &c.), with aluminium sulphate at various concentrations from dM/4 downwards (dil. 2, 3, 4, &c.).

These solutions are not yellowish orange, but red. The effect of aluminium on the colour of purpurine solutions is shown in fig. 1, the data for which were obtained as follows.

A measured volume of sulphuric purpurine was placed in each of two bottles, *A* and *B*. To the solution in *A* was added an equal volume of acid ethanol; to that in *B* an equal volume of aluminium sulphate at dil. 2 in 60%

ethanol. The solutions in *A* and *B* were now both strongly acid (pH 2·40 and 2·68 respectively). Both contained purpurine at exactly the same concentration; both contained sulphate ions; *B* contained aluminium ions while *A* did not. The graph shows that in the presence of aluminium ions (themselves colourless), the peak of the absorption-curve is raised and the wavelength of maximum absorption shifted from about 505 to about 580 mμ.

A solution of purpurine at dil. 4 with aluminium sulphate at dil. 2 in 60% ethanol is near the upper limit of solubility of both dye and mordant. It is convenient to call this strong solution *purpural*.

Purpural (pH 2·78)

Purpurine	1·601 g
Aluminium sulphate, $16H_2O$	15·760 g
Ethanol, 60%	up to 1 litre

Dissolve by refluxing.

Purpural may be stored indefinitely at 25° C (or equally well at 37° C), but in a cold room there is a tendency towards the slow deposition of hydrated purpurine in the form of yellowish-orange needles. Apart from its use in the present investigation, purpural is a convenient dye for chromatin in routine histology. For such purposes it suffices to remove any precipitate by filtration, but in quantitative work of the kind described in this paper it is essential to work with solutions in which no precipitate has appeared.

Aluminium sulphate at high concentrations greatly increases the solubility of purpurine in 60% ethanol. Thus, as we have just seen, the dye can be dissolved at dil. 4 if the mordant is at dil. 2 (though the solution is on the border-line of saturation); but if the mordant is dilute, at dil. 7, the dye is near the border-line of saturation at dil. 9. In other words, the strong solution of the mordant holds 32 times as much of the dye as the weak solution.

It would appear that purpurine tends conversely to hold aluminium sulphate in solution. Thus, if a solution similar to purpural is made up, containing only one-half of the usual weight of purpurine, there is no precipitate of dye when the solution is left in a cold room; but instead there is a slow formation of crystals of aluminium sulphate. Such crystals do not form in purpural.

The tissue chosen for study. The tissue chosen as the material on which the dye would act was the testis of the laboratory mouse, *Mus musculus*, fixed in Clarke's fluid (1851); that is to say, acetic acid mixed with 3 times its volume of absolute ethanol. (The fluid is usually ascribed to Carnoy (1886).) The testis is convenient for work of this kind, because it possesses constituents ranging from strong basiphilia (chromosomes, and nuclei of spermatozoa) to strong acidophilia (red blood-corpuscles, and collagen of tunica propria and albuginea). Clarke's fluid contains no constituent that could act as a mordant or interfere with the uptake of a dye-lake. Paraffin sections were cut at 7 μ.

In most of the experiments, dyeing continued for a week. This very long

period was chosen to ensure that equilibrium should be reached between dye solution and tissue-constituents.

Mordanted purpurine is insoluble in neutral solutions. Washing and dehydration were therefore carried out at leisure. The dyed sections were washed for 15 min in repeated changes of 60% ethanol, dehydrated, and mounted in benzene-balsam. This mounting medium was chosen because it does not extract basic dye-lakes, as xylene-balsam sometimes does. Certain unmordanted sections were dehydrated quickly, to prevent extraction of the dye (p. 262).

All observations were made with a 4-mm objective, illuminated by a $\frac{3}{4}$-cone (or thereabouts) from the condenser. When dyeing was very slight, it was necessary to use phase-contrast to find the section, but all observations of colour were made by direct microscopy, not by phase contrast. Daylight was used, or light from an electric bulb was transformed into a close approximation to daylight by passage through an Ilford 810 filter.

In the work described in the present paper, the most superficial layer of seminiferous tubules was disregarded, because its reactions to dyes were somewhat different from those of all the seminiferous tubules in the rest of the piece of tissue.

Symbols for Recording the Intensity of Dyeing

The standard of comparison is a section that has been dyed for 30 min in purpural, washed, dehydrated, and mounted in benzene-balsam. This has the appearance of a 'good' routine microscopical preparation, coloured by a basic dye. The chromatin is strongly dyed (red), the cytoplasm feebly.

The symbols used are as follows:

++++	More strongly coloured than the standard of comparison. The cytoplasm in particular is obviously darker.
+++	Indistinguishable or scarcely distinguishable from the standard.
++	Paler than the standard, but colour can still be seen in the cytoplasm.
+	No colour is visible in the cytoplasm, but a tinge remains in the chromatin. (The chromosomes at synizesis retain the last trace, when everything else, including even the nuclei of the spermatozoa, has lost it.)
O	No colour can be seen in the section, which can scarcely be found without the aid of phase-contrast.

These symbols are applicable to most of the experiments recorded in this paper.

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 (fig. 1) were made with the SP 500 spectrophotometer of Unicam Instruments Ltd.

Description of the Experiments

It was necessary to do a lot of preparatory work in order to design critical experiments that would give clear answers to particular questions. To save space, the results of this work will not be recorded here. It will suffice to say that no results were obtained that conflicted with those given by the experiments in their final form, as described below.

The experiments are numbered for ease of reference.

Experiment in which the dye was used without mordant

Experiment 1. If a substance were capable of acting as a basic dye in the absence of a mordant, it would be unsuitable for use in a study of the kind described in this paper, because there would be doubt whether the mordant had acted as such in any particular experiment. In simple ethanolic solutions, purpurine is a very weak acid dye. Certain acid dyes, however, become basic when acidified (Seki, 1933 *a*, *b*, *c*). Since solutions of aluminium sulphate are acid, it might be thought that this salt could convert purpurine into a basic dye by acidity alone. It was therefore necessary to find out whether acidity would convert purpurine into a basic dye.

A section of the standard material was brought down to 60% ethanol and dyed for a week in acetic purpurine (pH 3·23). It was then passed directly into absolute ethanol, left in changes of this for only 1½ min, and mounted in benzene-balsam. The action was that of an extremely feeble acid dye. No special affinity for chromatin was shown. The cytoplasm of the spermatogenetic cells was a very faint brownish yellow, that of the interstitial cells slightly darker; the collagen of the tunica propria of the tubules somewhat darker again.

If purpurine is made up at dil. 9 in a solution of aluminium sulphate at dil. 3 in 60% ethanol, the pH of the fluid is 3·28; that is to say, the acidity is almost exactly the same as that of acetic purpurine. This solution, however, acts on the tissue in an entirely different way. It is a typical, bright red, basic dye, with special affinity for chromatin, but none for the collagen of the tunica propria.

This experiment shows that when purpurine acts as a basic dye in the presence of aluminium sulphate, it is mordanted to the tissue. This is, indeed, what one would expect, for the chemical formula for purpurine does not suggest any capacity to act as a basic dye in the absence of a mordant.

Experiment on the extraction of the dye-lake by the mordant and by acid

Experiment 2. It is a familiar fact that solutions of mordants are able to extract mordanted dyes from tissues. It is important to know whether aluminium sulphate extracts mordanted purpurine by simple acidity, or by some other property of the dissolved salt.

Sections of the standard material on two slides were brought down to 60%

ethanol and dyed for 30 min in purpural. They were then washed for 15 min in changes of 60% ethanol. One slide was placed in aluminium sulphate solution at dil. 2 in 60% ethanol (pH 3·08), the other in sulphuric ethanol (pH 2·50). After 1 h both slides were washed as usual in 60% ethanol, dehydrated, and mounted in benzene-balsam.

The aluminium sulphate solution removed much more of the dye from the tissue than the sulphuric ethanol did, though it was less strongly acid. It is clear that aluminium sulphate does not extract mordanted purpurine simply by its acidity.

Experiments in which the mordant was used at constant concentration, the dye at various concentrations

Experiment 3. The main object of the experiments recorded in this paper was to determine whether particular mixtures of mordant and dye had the effect of increasing or decreasing the amount of dye held by the tissue. It was therefore necessary to use material that had already been coloured to a moderate degree. Sections of the chosen material were brought down to 60% ethanol, left in purpural for 30 min, and then washed for 15 min in several changes of 60% ethanol. One slide, the 'standard of comparison', was dehydrated and mounted. The grade of colouring was +++ (p. 261).

The other slides were placed in solutions all of which contained aluminium sulphate at dil. 2, while the concentration of the purpurine varied. In the strongest solution the dye was at dil. 4, so that the fluid was identical with purpural. In the other solutions the concentration of the dye was successively halved (dil. 5, 6, 7, &c.).

The uniformly coloured sections were left in these solutions for a week. They were then washed and mounted.

The results are shown in table 1. It will be noticed that when the dye was at dil. 5, no colour was added to or removed from the tissue during the week. The dyed tissue was in equilibrium with the mordant/dye solution. At higher or lower concentrations of purpurine the tissue gained or lost colour.

The mordant quotient of the solution in equilibrium with the standard of comparison will be called the *critical mordant quotient* (or *critical quotient*). Since the aluminium sulphate in this solution was at dM/4 (dil. 2) and the purpurine at dM/32 (dil. 5), the critical mordant quotient was $2 \times \frac{32}{4} = 16$. That is to say, in the solution that was in equilibrium with the standard, there were 16 atoms of aluminium to each molecule of purpurine. It will be understood that the critical mordant quotient was not determined exactly, because experiments were only made at concentrations of dye and mordant in the series dM/2, dM/4, dM/8, &c., not at intermediate concentrations, and because the amount of dye in the tissue was not measured accurately.

Experiment 4. The result of experiment 3 leads one to ask what would happen if *unstained* sections were placed in the same purpurine solutions as before. An experiment was therefore performed in exactly the same way as experiment 3, except that there was no preliminary dyeing of the sections

with purpural. The sections remained for a week in the dye solutions. They were mounted and compared with the usual standard, namely a section dyed for 30 min in purpural.

The result of the experiment is shown in table 1.

TABLE 1

Results of experiments 3 and 4

Concentration of purpurine	*Experiment 3*	*Experiment 4*
dil. 4	++++	++++
dil. 5	+++	+++
dil. 6	++	++
dil. 7	++	++
dil. 8	++	++
dil. 9	+	+
dil. 10	+	+
dil. 11	+	+
dil. 12	+	○
dil. 13	○	○

For explanation of the symbols +, ++, &c., see p. 261.

It is clear that when the mordant quotient was at the critical figure, dyeing took place *up to* but not beyond the intensity of the standard of comparison (grade +++). Overdyeing could not occur in a solution of this critical composition.

Even when the dye was very dilute (dil. 11 = dM/2048 = 0·00125%), it was capable of imparting to chromatin a tinge that was just detectable. At this concentration there were in the solution 1024 atoms of aluminium to each molecule of purpurine.

Experiments in which the mordant was used at various concentrations, the dye at constant concentration

Experiment 5. It was necessary to use a low concentration of the dye, for otherwise it would not be possible to dissolve it at low concentrations of the mordant (even though the solvent was in all cases 60% ethanol). Dil. 9 was chosen. Aluminium sulphate was used at dils. 2, 3, 4, 5, 6, and 7. It could not be used at dil. 8 or lower concentrations, because the dye would not dissolve even at dil. 9 at lower concentrations of the mordant than dil. 7.

The sections were first dyed for 30 min in purpural and washed in the usual way. One section, the standard of comparison, was dehydrated and mounted; the rest were put in the solutions described in the preceding paragraph and left for a week. They were then washed, dehydrated, mounted, and compared with the standard.

The results are shown in table 2. The more dilute the mordant (provided that there was enough to dissolve the dye), the more deeply coloured the section.

Equilibrium with the standard was not exactly obtained. A solution with aluminium sulphate somewhere between dil. 5 and dil. 6 would give exact equilibrium. If the figure had been dil. 6, the critical mordanting quotient would have been 16, as before (experiment 3).

TABLE 2

The results of experiments 5 and 6

Concentration of aluminium sulphate	*Experiment 5*	*Experiment 6*
dil. 2	+	+ or ++
dil. 3	++	++
dil. 4	++	++
dil. 5	+++*	++
dil. 6	++++	+++
dil. 7	++++†	++++†

* Slightly paler than the standard of comparison.
† Cytoplasm almost as dark as the chromatin.

Experiment 6. This was performed in the same way as experiment 5, except that there was no preliminary dyeing with purpural. It will be seen from table 2 that the results were remarkably similar. In other words, the preliminary dyeing did not make much difference to the final result.

When the mordant quotient was at the critical figure of 16 (mordant at dil. 6, dye at dil. 9), the section took up dye until it exactly resembled the standard of comparison (+++).

The symbols shown on the bottom line of table 2 record the most strongly coloured sections seen in the whole of the present investigation. This is all the more remarkable, since the dye was extremely dilute (dil. 9). The fact is that in *all* the other experiments the mordant was at too high a concentration to allow maximum colour. It must be understood, however, that colouring is very slow when the dye is at dil. 9. The solution would not be suitable for practical use.

Experiments in which both mordant and dye were used at various concentrations, but in constant proportion to one another

Experiment 7. It was found in experiment 3 that a solution containing the mordant at dil. 2 and the dye at dil. 5 was in equilibrium with a section that had been dyed for 30 min in purpural. If the concentration of both mordant and dye were halved, quartered, &c., the number of atoms of aluminium to each molecule of purpurine would remain the same. Would there still be equilibrium?

Sections of the usual material were dyed for 30 min in purpural, washed, and left for a week in solutions containing aluminium sulphate and purpurine dissolved in 60% ethanol at the concentrations shown in table 3. It will be observed that in every case the mordant was in the same proportion to the

dye, but a wide range of dilutions was tried. It was not possible to make satisfactory experiments with the mordant at lower concentrations than dil. 10, because the dye gradually precipitated and the fluid became colourless.

The sections were washed, dehydrated, and mounted in benzene-balsam.

The results recorded in table 3 show that if the mordant quotient is maintained at the same figure, dilution has no effect on the final result. Thus the critical mordanting quotient is independent of concentration. Equilibrium with the standard of comparison is maintained, provided that there are 16 atoms of aluminium to every molecule of purpurine. (Experiment 5 was not in exact conformity with this finding, but the departure was small. See table 2.)

TABLE 3

Results of experiments 7 and 8

Concentration of aluminium sulphate	*Concentration of purpurine*	*Experiment 7*	*Experiment 8*
dil. 2	dil. 5	+++	+++
dil. 3	dil. 6	+++	+++
dil. 4	dil. 7	+++	+++
dil. 5	dil. 8	+++	+++
dil. 6	dil. 9	+++	+++
dil. 7	dil. 10	+++	++
dil. 8	dil. 11	+++	++
dil. 9	dil. 12	+++	++
dil. 10	dil. 13	+++	++

Experiment 8. This was carried out in the same way as experiment 7, except that the initial dyeing in purpural was omitted. The results are shown in table 3. It will be noticed that the omission of the initial dyeing made no difference to the result when rather strong solutions were used, but the sections were more weakly coloured by the dilute solutions. The instability of the dye in very weak solutions of the mordant may have affected the result.

REMARKS ON THE RESULTS OF THE EXPERIMENTS

The most significant of the 8 experiments recorded in this paper were nos. 3, 5, and 7. The others help in the interpretation of these three.

It follows from the results of experiments 3, 5, and 7 (especially 3 and 7) that a solution containing about 16 atoms of aluminium to each molecule of purpurine has special properties. A section already dyed with an aluminium/purpurine solution till chromatin is dark and cytoplasm lightly tinged (grade +++), is in equilibrium with such a solution: that is to say, it neither takes up more dye nor loses any part of what it already holds. If the proportion of dye to mordant is increased, the section will take up more of the dye; if the proportion of the mordant to the dye is increased, the section will lose part or all of the dye it held. An unstained section will take up dye from a solution containing 16 atoms of aluminium to each molecule of purpurine,

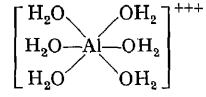
until the standard degree of colouring (+++) is reached; no further colouring beyond this will occur.

It follows that a solution containing 16 atoms of aluminium to each molecule of purpurine is very easy to use in practice, since it cannot overstain; but it acts slowly. A solution that contains more than 16 atoms of aluminium to each molecule of purpurine is unsuitable for practical use, since it cannot colour sections up to grade +++.

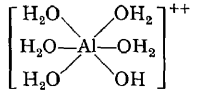
If the proportion of aluminium is reduced below the critical mordant quotient, the solution can overstain, and microscopical control is therefore necessary in practical use. If the mordant quotient is very low, there will be a strong tendency to overstain. A solution containing 8 atoms of aluminium to each molecule of purpurine is convenient. The solutes should be at high concentration in solutions intended for practical work, partly to avoid their rapid depletion by repeated use, partly to achieve fairly quick colouring of the tissues. These conditions are fulfilled by purpural (p. 260).

A Theory of Single-bath Mordant Dyeing

The experiments described in this paper, taken in conjunction with existing knowledge of the behaviour of aluminium ions in solution and of their capacity to form chelate compounds with anthraquinonoid dyes (especially alizarine), afford a reasonable basis for a general theory of single-bath mordant



The hydrated aluminium ion



The hydrated aluminium ion after loss of a proton

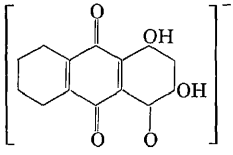
dyeing by aluminium/purpurine. A sketch of such a theory will be attempted here, in the belief that it will clarify thought on the subject and help in the design of future experiments. Carminic acid and haematein probably react with aluminium in much the same way as purpurine does, but both these dyes (especially haematein) have a tendency to deposit insoluble lakes when mixed with mordants.

When aluminium sulphate is dissolved in water, each atom of the metal loses the 3 electrons in its outer (*M*) shell. The ion is now in a position to accept 12 electrons from suitable donor atoms. The 6 orbitals concerned are sp^3d^2 orbitals; they are hybridized, so that all are equivalent. A dative covalency of 6 is established by the attachment of 6 water molecules through their oxygen atoms.

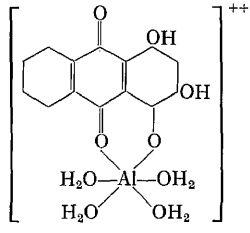
One (or more) of the water molecules bound to an aluminium ion loses a proton, and this accounts for the acidity of solutions of aluminium salts (Brönsted & Volqvartz, 1928). The loss of the proton reduces the charge on the ion from +++ to ++.

Other potential donors, such as alizarine or purpurine, may now replace

water and thus become bound to aluminium. If the hydroxyl group in the 1-position of purpurine becomes ionized, the dye has the structure shown here. Two oxygens (the one in the 1-position, and the carbonyl oxygen nearby) are capable of replacing the oxygens of two water molecules bound to aluminium. A (soluble) chelate compound may thus be formed.



Ionized purpurine



The mordant/dye complex, according to the hypothesis here presented

Since the aluminium ion has two positive charges and the purpurine ion one negative, it might be thought that the chelate compound would bear only a single positive charge; but in fact the formation of the chelate compound is likely to be accompanied by the taking back of a proton from a hydronium ion in the solvent water, and as a result the compound ion must be supposed to possess two positive charges (Venanzi, 1959).

The reaction of aluminium sulphate with alizarine was investigated by Liechti and Suida (1885). The product was stated to be a purplish black solid, *soluble* in water and in alcohol. These authors found that in the absence of other substances, aluminium sulphate does not react with alizarine to form an insoluble lake.

It might be supposed that 3 alizarines or purpurines could replace all 6 water molecules bound to aluminium, with precipitation of an insoluble lake, and indeed this story is told in many chemical textbooks. It is true that an insoluble lake (Turkey red) is formed if calcium ions are present in addition to aluminium and alizarine; but in the compound formed by this reaction, only 2 alizarines are bound to each aluminium. Of the 2 remaining co-valencies of this metal, one still retains a water molecule, and the other is linked through oxygen with calcium (Fierz-David & Rutishauser, 1940).

A comparison of the formulae for alizarine and purpurine (p. 258) might suggest that these two dyes would react in different proportions with mordant metals. The formula for purpurine gives the impression that the hydroxyl group in the 4-position, as well as that in the 1-position, is so situated in relation to a carbonyl group as to provide means of chelation with suitable metals. There is evidence, however, that the hydroxyl group in the 4-position is unreactive, and that purpurine chelates with metals in the same proportions as alizarine (Morgan & Smith, 1922). Similarly, the hydroxyl group in the

1-position does not react with mordants unless there is another hydroxyl beside it in the 2-position (Liebermann & Kostanecki, 1887).

It was shown in experiment 4 (p. 263) that a solution containing 1024 atoms of aluminium to each molecule of purpurine is capable of imparting colour (though only a trace of it) to chromatin. In this case it seems certain that those few aluminium atoms that bound any purpurine at all must have bound only one molecule of it: for one aluminium can hardly be supposed to have held two purpurines, when 2047 other aluminiums held none at all. Since dyeing can take place in these and similar circumstances, the simplest hypothesis is that there is one purpurine or none to each aluminium, whether the aluminium be free in solution or attached to tissue-constituents. It is to be remembered that in all practical dyeing solutions the mordant is present in great excess.

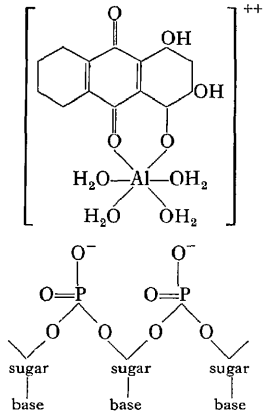
Nevertheless, it is quite possible that when the mordant quotient is low, two dye-molecules may chelate with some of the aluminiums. If so, the resultant complex ions would carry only a single positive charge. Liechti and Suida (1885) incinerated their soluble aluminium/alizarine lake, and compared the original weight with that of the resultant aluminium oxide. Their own figures indicate that 2·69 alizarines were burnt up for each molecule of Al_2O_3 left behind. They themselves gave the figure as 2·9, and they concluded that in fact the molecule of the lake contained 3 alizarines and 2 atoms of aluminium. It is probable, however, that it consisted mostly of one alizarine chelated with one aluminium, but partly of another compound formed by the chelation of two alizarines with one aluminium.

It is thinkable that a mordant and dye, though present in the same solution, might remain separate until the mordant had attached itself to the tissue. This idea, however, is contradicted not only by the findings of Liechti and Suida, but also by independent evidence. Aluminium ions enormously increase the 'solubility' of purpurine (p. 260), and aluminium ions in solution strongly attract purpurine that has already been mordanted to the tissues (experiment 2). When the mordant is dissolved with the dye, the peak of the absorption-curve of the latter undergoes a marked heightening and shift (fig. 1, p. 259), as often happens when a chelate compound is formed.

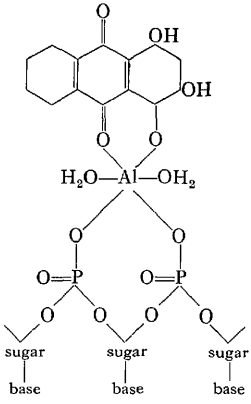
In the presence of tissue, the positively charged aluminium/purpurine complex will be drawn towards sites of negative charge. It will accumulate in greatest abundance where these sites are most numerous. Phosphoric groups are close to one another along the whole length of the nucleic acid molecule, and the complex will therefore associate itself abundantly with chromatin. It will be attracted to protein in much smaller quantity, for the carboxyl side-groups of dicarboxylic acids will not be very plentiful even in rather strongly acidic proteins. For this reason the ground cytoplasm will be feebly dyed when the chromatin is already dark red, unless there happens to be much cytoplasmic RNA in the cell.

The results of dyeing with aluminium/purpurine show clearly that electrostatic forces determine the uptake of the mordant/dye complex, for it

attaches itself to the same sites as basic dyes do. It is probable, however, that covalent linkages eventually replace the ionic bonds; for the dye is firmly held and cannot be extracted by mixtures of ethanol and water, which, as is well known, remove basic dyes from the tissues if enough time be allowed.



The mordant-dye complex attracted to a site of negative charges (a nucleic acid) in the tissue



The tissue/mordant/dye complex

Structural formulae showing hypothetically how purpurine may be linked to nucleic acid through aluminium

The hypothetical structural formula shows aluminium joined to two phosphoric groups in the same polynucleotide chain. This is not necessarily so. The two phosphoric acids might be in different chains, perhaps one in each of the two parallel chains of the DNA helix.

It will be noticed that the opposite charges of the aluminium/purpurine complex and of the two phosphoric groups will satisfy one another, and the insolubility of the product may be attributed to this. The fact that two water molecules are still attached need not confer solubility. It will be remembered that each aluminium has one water molecule attached to it even in the insoluble lake, Turkey red (p. 268).

It is not impossible that aluminium/purpurine may attach itself to some tissue-constituents by chelation. Thymine and certain other bases of the nucleic acids provide possibilities for this type of attachment. Dicarboxylic amino-acids in a polypeptide chain might also form chelate bonds with aluminium, through the $—O^-$ of the free carboxyl group and the nearest —NH—of the polypeptide backbone. Further speculation along these lines would not be profitable in the existing state of knowledge.

When a mordant/dye solution has a high mordant quotient (that is, when it contains a high ratio of aluminium to purpurine), it is probable that many

aluminium ions to which no purpurine is bound are deposited on suitable sites in the tissues.

Experiments 2, 3, 5, and 7 throw light on the problem of *differentiation*. The amount of dye in a section that has been coloured by aluminium/purpurine to grade +++ (p. 261) can be reduced by exposing it *either* to an acid (for instance, dilute sulphuric acid), *or* to the mordant, *or* to any solution of mordant and dye in which there are more than 16 atoms of aluminium to each molecule of purpurine.

Acids have two possible points of attack, indicated by the oblique strokes in the expression *tissue/mordant/dye complex*. The second of the strokes indicates the easier link to break. It must be supposed that protons from the hydronium ions would tend to attach themselves to the purpurine so as to reconstitute the hydroxyl group that originally ionized and thus gave attachment to aluminium. One half of the chelate bond would thus be broken and the other thereby weakened; the purpurine molecule, unionized, would separate from the metal. In some cases (as, for instance, when acid differentiates aluminium/haematein), a striking colour change (bright blue to dull red) announces the setting free of the dye.

Sufficient acidity would also endanger the bond indicated by the first oblique stroke. Protons would reconstitute the hydroxyl groups of the phosphoric acid, thus setting free aluminium ions.

Since aluminium sulphate, on dissolving, produces protons (p. 267), it presumably acts to some extent in the same way as sulphuric or any other acid, though it would not set free any aluminium. It has been shown in experiment 2, however, that the mordant is a much stronger differentiator than an acid at the same pH, and it is therefore clear that the mordant must also act in another way when it extracts the dye. The simplest hypothesis is that the purpurine forming part of the tissue/mordant/dye complex is attracted by the hydrated aluminium ions that are still in solution, and that when these ions outnumber the purpurine molecules in the solution by more than 16 to 1, their attraction for the attached purpurine suffices to free it. The purpurine therefore partitions itself between the insoluble tissue/mordant/dye complex and the dissolved mordant/dye complex.

The metal ions must be present in great excess if they are to extract the colour from a dyed section. It thus appears that the attraction of aluminium for the dye varies according to whether the metal is free in solution as ions, or bound to tissue-constituents. Bound aluminium attracts purpurine much more strongly than its ions do.

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