An approach to the thermodynamics of histological dyeing, illustrated by experiments with azure A

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Summary

Methods are proposed for the estimation of the rate, activation energy, heat, and affinity of staining of histological sections, and approximate results are given for the staining of mucin, mast-cell granules, chromatin, cytoplasmic ribonucleic acid (RNA), cartilage matrix, and other structures by azure A.

The half-staining time $t_{\frac{1}{2}}$ is the time taken by a substrate under given staining conditions to achieve half the intensity of staining it would reach at equilibrium, and is approximately equal to the time taken to stain in a given, fairly dilute dyebath to the same intensity as at equilibrium in a dyebath of half the given concentration. The activation energy E of staining is given by $E = \ln t_{\frac{1}{2}(1)}/t_{\frac{1}{2}(2)} \times RT_1T_2/(T_2-T_1)$, where $t_{\frac{1}{2}(1)}$ and $t_{\frac{1}{2}(2)}$ are the half-staining times at absolute temperatures T_1 and T_2 respectively, and R is the gas constant. The activation energy of staining reflects the effect of temperature on rate of staining, and may be regarded as an index of substrate permeability. Half-staining times and activation energies of staining with azure A increase in the order mucin, mast-cell granules, chromatin, RNA, and interstitial cartilage matrix. Times of half-destaining and activation energies of destaining also are probably largely determined by substrate permeability. Differential staining dependent on differences in rate of staining may be enhanced by the use of chilled and stirred dyebaths, and by the use of dyes of large particle size.

The heat of dyeing ΔH, sometimes regarded as the sum of the heats of formation of the various dye-substrate bonds, approximately equals $RT_1T_2/(T_2-T_1) \times \ln [D]_1/[D]_2$, where $[D]_1$ and $[D]_2$ are the concentrations of dyebath giving equal intensity of staining of the substrate at equilibrium at temperatures T_1 and T_2. Approximate figures for ΔH in kcal/mole for staining with dilute azure A are: mucin, -8; chromatin and cartilage matrix, -7; cytoplasmic RNA, $-5{\cdot}5$; mast-cell granules, -2 to -4. The higher the value of $-\Delta H$ the more is staining inhibited by a rise in temperature of the dyebath.

The affinity of a dye for a substrate may be regarded as the standard free energy change accompanying the staining process, which under certain conditions is given approximately by $\Delta F^\circ = -RT \ln \theta/(1-\theta)[D]$, where θ is the fraction of available staining sites in the substrate occupied by dye when the substrate is at equilibrium with a dyebath of concentration $[D]$. Differential staining of substrates with a high affinity for the dye is facilitated by the use of dilute dye solutions. Approximate values of ΔF° for staining with azure A at 4° C and pH 4·0, in kcal/mole, are: cartilage matrix, $-3{\cdot}8$ (orthochromasia) and $-5{\cdot}3$ (metachromasia); mast-cell granules, -4 (orthochromasia) and $-4{\cdot}4$ (metachromasia); RNA, $-3{\cdot}1$; mucin, between $-2{\cdot}7$ and $-3{\cdot}4$; chromatin, $-3{\cdot}1$; thyroid colloid, $-2{\cdot}3$; *Xenopus* poison gland secretion, $-2{\cdot}3$. It is suggested that part of the high affinity of sulphate groups for basic

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dyes is due to an increase in entropy during staining, resulting from dispersion of a large hydration shell surrounding the sulphate groups before attachment of the dye.

DESPITE extensive quantitative work on the thermodynamics of textile dyeing (Vickerstaff, 1954), probably only Weiss (1954), who estimated the heat of reaction of elastin with orcein *in vitro*, has attempted to apply similar methods to a histological problem. In the present article a semi-quantitative investigation into the effect of temperature on the staining of sections by the basic dye azure A (M.W. 292) is reported, and certain aspects of the thermodynamics of staining are discussed. Despite the fact, discussed in the text, that some of the results reported in the present article can be accepted only with reservations, it will be shown that the thermodynamical approach to staining promises to throw light on several aspects of the staining process.

General material and methods

All experiments were carried out on formalin-fixed stomach, small intestine, large intestine, kidney, and skin of *Xenopus*, and stomach, small intestine, large intestine, pancreas, and trachea of the rat.

A preliminary investigation with alcoholic solutions of azure A indicated that a rise in the temperature of the dyebath increases the rate of staining, particularly of less permeable tissue components, and decreases the intensity of staining attainable at equilibrium. As these conclusions were in general supported and extended by the results of later experiments, reported in this article, the methods and results of the preliminary investigation will not be further discussed.

Unless otherwise specified, in the present work 6-μ paraffin sections were stained with azure A (National Aniline and Chemical Co., certification No. ANz8, dye-content 85%) in veronal-acetate buffer at pH 4·0 (Hale, 1958), and dehydrated in 95% and absolute alcohol (30 sec in each, with constant agitation), before mounting in DPX. Usually an unstirred dyebath was used, but in certain experiments the dye was agitated by a magnetic stirrer. The duration of staining, and the concentration and temperature of the dye solutions used in the various experiments, are specified below.

The intensity of staining of experimental sections was in most cases estimated semi-quantitatively by comparison with that of a standard series of sections, stained at varying intensities. Because this procedure was the crux of the investigation, it will be described in some detail.

A series of dyebaths was prepared by dissolving 1 g of azure A in 100 ml of veronal buffer (pH 4·0) at room temperature, leaving to stand for 15 min, filtering, and serially diluting with buffer to obtain solutions containing $\frac{1}{2}$%, $\frac{1}{4}$%, $\frac{1}{8}$%, ..., $\frac{1}{16,384}$% (i.e. 2^{-1}%, 2^{-2}%, 2^{-3}%, ..., 2^{-14}%) dye. Sections were stained for 24 h at 4° C in dyebaths containing from 2^{0} to 2^{-10}% of dye, and for 48 h in the weaker solutions. Evidence is presented below regarding the rate of staining in azure A, but we shall assume for the

moment that equilibrium between dye in the dyebaths and dye in the standard sections had been attained.

Now if dye molecules are adsorbed on to definite chemical groups in the tissues, and if (at least at low staining intensities) the uptake of dye at a given site is unaffected by that at neighbouring sites, it can be shown that the amount of dye taken up by a substrate at equilibrium is usually related to the concentration of the dyebath by a Langmuir adsorption isotherm (Vickerstaff, 1954, pp. 99 and 110). It may be noted that van Duijn (1961, 1962) has presented indirect evidence suggesting that the uptake of basic dyes by living sperm heads in fact follows a Langmuir adsorption isotherm fairly closely. On the assumption that the uptake of azure A by histological sections follows a Langmuir isotherm, at staining intensities far short of saturation the uptake of dye will be linearly proportional to the concentration of the dyebath, and a series of preparations stained to equilibrium in a series of dyebaths of regularly increasing concentration will show a similarly increasing intensity of staining. It should be noted, however, that at very high dyebath concentrations the staining of the substrate approaches saturation, and the linear relationship between staining intensity and dyebath concentration is lost.

Having thus obtained a standard series of sections, experimental sections were compared with the standard series with the aid of two identical microscopes and lamps, a comparison ocular and an orange filter. Deep blue and red filters were also used on occasion. The intensity of staining in an experimental section was recorded as being equal to the most closely matching section of the standard series: sometimes finer discrimination was possible, and the experimental intensity was then recorded as being just less than, equal to, or just more than that of a control, or intermediate in intensity between two controls. The procedure gave satisfactorily reproducible results, and largely eliminated difficulties due to metachromasia and irregular distribution of stained material, since the structures compared were morphologically identical and similarly stained. Further, a wide range of staining intensities could be studied, probably far greater than that possible with a microspectrophotometer. In preliminary experiments other semi-quantitative methods of assessing staining intensity were tried: these included the use of a camera lucida and a series of stained pieces of paper (Seki and Kohashi, 1940), and a microscope coupled through a comparison ocular with a comparator containing a solution of azure A in one well, but these procedures proved unsatisfactory in my hands.

On the rate of staining and destaining

The rate of histological staining is interesting from both the theoretical and practical points of view—theoretically, because a study of the kinetics of staining may be informative regarding properties of the substrate and the nature of the staining process, and practically, because in histological technique equilibrium between substrate and dyebath is seldom attained in the

short times usually used, and the rate of staining of different tissue components may to a large extent determine the differential distribution of dye.

Rate of staining of different tissue components in an unstirred dyebath

Method. Sections were stained for 7·5, 15, or 30 min, or 1, 2, 4, 8, 16, or 32 h in 2^{-10} % azure A at 4° C, or in 2^{-7} % dye at 4°, 24°, or 58° C, dehydrated in alcohol, mounted, and compared with the standard series in the usual way.

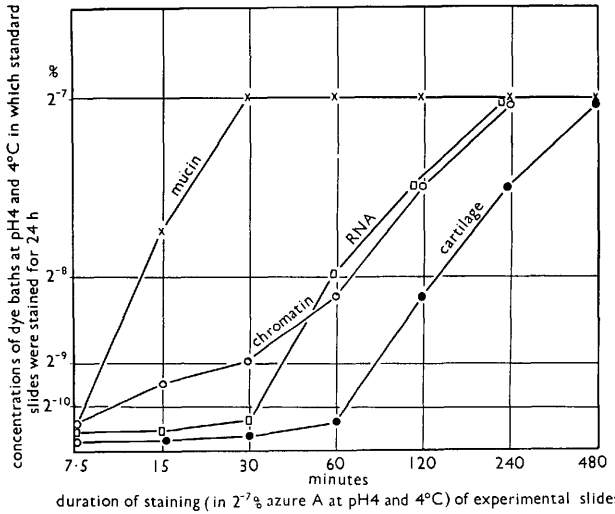

FIG. 1. Rate of staining in 2^{-7} % azure A at 4° C. Note that different substrates stain at different rates.

Results. These are plotted graphically in figs. 1 to 4. Mucin stained too faintly, and mast-cell granules too intensely, for the rate of staining of these substrates to be followed in the 2^{-10} % and 2^{-7} % dye solutions respectively.

Fig. 1 shows that all substrates examined stained as intensely after 8 h in 2^{-7} % azure A at 4° C as after 24 h, and this indicates that 8 h was sufficient for the attainment of equilibrium under these conditions. Two hours were enough for equilibrium to be reached at 24° C (fig. 2), and 60 min enough at 58° C in 2^{-7} % dye (fig. 3). Sixteen hours was sufficient for the attainment of equilibrium of all substrates in 2^{-10} % dye at 4° C (fig. 4).

Pancreatic RNA stained for longer than about 2 to 4 h at 58° C showed slightly *less* staining than after shorter times, presumably due to hydrolysis and removal of the substrate. No other tissue component showed this effect

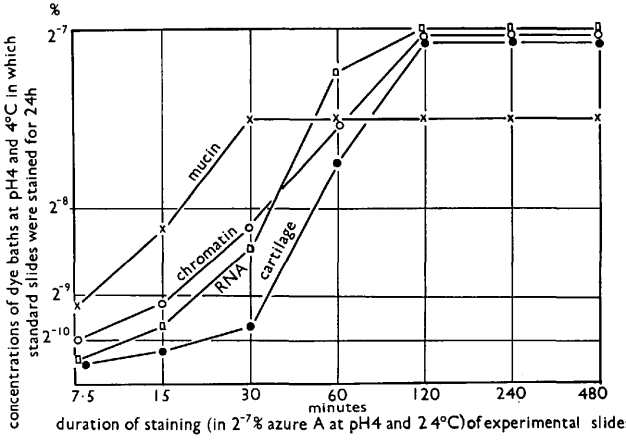

FIG. 2. Rate of staining in 2^{-7} % azure A at 24° C. The rate of staining is higher than at 4° C, but the equilibrium intensity reached is in some cases lower.

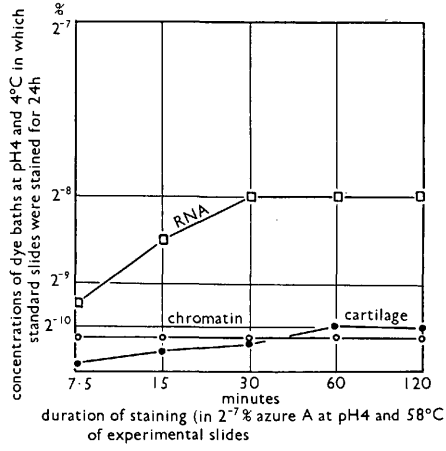

FIG. 3. Rate of staining in 2^{-7} % azure A at 58° C. The rate of staining is higher, and the equilibrium intensity reached lower than at lower temperatures.

in an unstirred dyebath during the staining periods used in the present series, and RNA did not show it at 24° or 4° C.

Figs. 1 to 4 show that staining equilibrium is reached at different times by different tissue components. In the comparison of staining rates, the time taken to reach equilibrium is, however, less suitable than the 'time of half-staining' $t_{\frac{1}{2}}$ defined as the time required for a substrate to achieve, under given conditions, an intensity of staining *one-half* of that which it would reach at equilibrium (Vickerstaff, 1954, p. 147). As was mentioned above, the amount of dye taken up by a substrate at equilibrium with a dyebath is likely to be proportional to the concentration of the latter, provided the staining is far

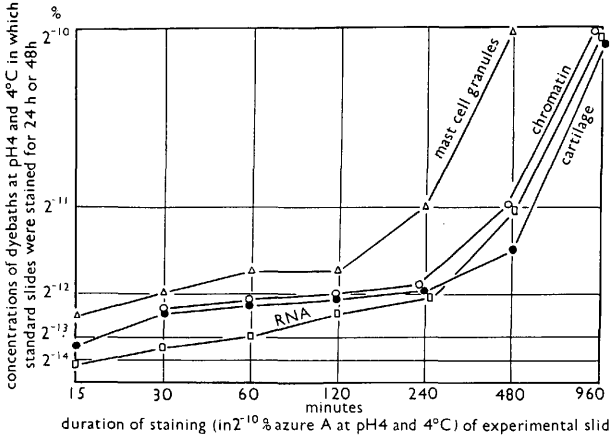

FIG 4. Rate of staining in 2^{-10} % azure A at 4° C. Mast-cell granules stain faster than other substrates examined under these conditions.

short of saturation. This proviso was met in the staining of mucin, chromatin, RNA, and cartilage matrix in 2^{-7} % azure A (24 h at 4° C), since staining in a 1% solution for the same time and at the same temperature gave a far greater intensity of staining. For these substrates, therefore, it may be concluded that the half-staining time in 2^{-7} % azure A under the specified conditions is approximately equal to the time taken to stain as intensely in 2^{-7} % dye, as at equilibrium in a 2^{-8} % solution. Similarly, $t_{\frac{1}{2}}$ in 2^{-10} % azure A is the time taken by a substrate to stain as intensely in 2^{-10} % dye as it would do at equilibrium in 2^{-11} % dye. Mast-cell granules stained almost as intensely in 2^{-7} % as in 1% azure A: for this substrate $t_{\frac{1}{2}}$ was therefore estimated only for 2^{-10} % dye.

The graphical estimation of $t_{\frac{1}{2}}$ is facilitated by the use of a semi-log plot, with an arithmetical scale of staining intensity on the ordinate and a logarithmic

scale of staining time on the abscissa, since the graph so obtained tends to be sigmoid, with a straight line in the centre portion (Vickerstaff, 1954, p. 148). Figs. 1 to 4 are plotted in this way, and the half-staining times read off these figures are summarized in table 1 (see appendix, p. 437). It may be noted in fig. 1 that pancreatic cytoplasmic RNA stained about as intensely after 1 h in 2^{-7} % azure A as in 2^{-8} % dye at equilibrium: comparison with the other experimental points of the RNA curve suggests, however, that this single reading falls somewhat off the main curve (possibly owing to experimental error), and that $t_{\frac{1}{2}}$ for RNA under the given conditions is probably closer to 1·5 h than to 1 h.

We may conclude that the rate of staining with azure A decreases (i.e. the $t_{\frac{1}{2}}$ increases) in the series mucin, chromatin, RNA, and cartilage matrix, and that mast-cell granules probably fall somewhere between mucin and chromatin.

Discussion. In the dyeing of textiles with a dyebath of constant composition, the rate-limiting factor is generally the diffusion of dye in the substrate; the speed of this diffusion is in turn dependent on the size of the dye particle, the 'porosity' or 'compactness' of the substrate, and to a lesser extent on the affinity of the substrate for the dye, a high affinity tending to slow down diffusion (Vickerstaff, 1954, pp. 124 ff.). Under certain conditions the rate of stirring or circulation in the dyebath may also be important, but we shall ignore this factor for the moment since it can scarcely be responsible for differences in the rate of staining of different substrates immersed in a common dyebath.

It appears probable from the present results that the permeability of the substrate is the rate-limiting factor in histological as in textile staining; as shown previously (Goldstein, 1962), the density (as judged from the refractive index) appears to increase and the permeability to basic dyes to decrease in the sequence mucin, mast-cell granules, chromatin, cytoplasmic RNA, and interstitial cartilage matrix, which correlates well with the relative rates of staining reported here.

Rate of diffusion is proportional to the concentration gradient involved, so that if diffusion of dye in the substrate is rate-limiting in staining one would expect $t_{\frac{1}{2}}$ to be an inverse function of the concentration of the dyebath. Strict proportionality between rate and dyebath concentration would not, however, be expected, as the diffusion gradient depends not only on the dyebath concentration, but also on the (changing) concentration of dye in the substrate. It appears from table 1 that at 4° C the rate of staining of chromatin is about 6·4 times faster in 2^{-7} % than in 2^{-10} % azure A, while the corresponding figures for RNA and interstitial cartilage matrix are 5·33 and 4·0 respectively. These differences between various substrates may be due to dye particle aggregation in the stronger solution, which would retard staining particularly in relatively impermeable substrates such as RNA and cartilage matrix, but would have less effect on the rate of staining of the more permeable chromatin.

Effect of temperature on rate of staining: the activation energy

Results. Owing to the fact that the staining of most substrates at equilibrium with 2^{-7}% azure A was markedly depressed at 58° C, $t_{\frac{1}{2}}$ at this temperature was estimated only for RNA. Since RNA at equilibrium with 2^{-7}% azure A at 58° C stained about as intensely as in 2^{-8}% azure A at 4° C, $t_{\frac{1}{2}}(2^{-7}\%, 58°)$ was the time required by RNA to stain as intensely in 2^{-7}% dye at 58° C as at equilibrium in 2^{-9}% dye at 4° C.

Figs. 1 to 3 and table 1 show that, as one might expect, the rate of staining increases ($t_{\frac{1}{2}}$ decreases) with rise in temperature.

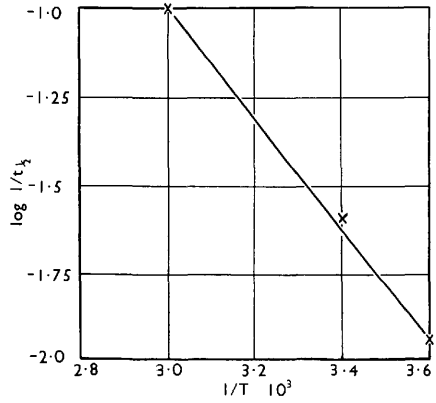

FIG. 5. Effect of temperature on rate of staining of cytoplasmic RNA. log $1/t_{\frac{1}{2}}$ plotted against $1/T$ gives an approximately straight line, as predicted by the Arrhenius equation.

Discussion. According to the Arrhenius equation,

$$\log k = \log A - \frac{E}{2{\cdot}303RT},$$

where k is the specific rate of a reaction, A is a constant, R the gas constant, T the absolute temperature, and E a constant for the reaction called the activation energy (Glasstone, 1950, p. 607). It will be seen from the equation that for any reaction a plot of log k against $1/T$ should be linear. Without concerning ourselves with the significance of k (the specific rate), we may note that for both first and second order reactions k is inversely proportional to $t_{\frac{1}{2}}$ ($t_{\frac{1}{2}} = 0{\cdot}693/k$ and $1/ka$ for first and second order reactions respectively, where a is the initial concentration of the reactants (Glasstone, 1950, pp. 588 and 593)). Thus irrespective of the order of reaction involved in staining, a plot of log $1/t_{\frac{1}{2}}$ against $1/T$ should be linear. Fig. 5 shows that this is probably the case for the staining of RNA in 2^{-7}% azure A at 4°, 24°, and

58° C; the obedience of the data to the Arrhenius equation may be taken as confirmatory evidence of the validity of the methods used.

The activation energy E may be calculated from the relation

$$\log\frac{k_2}{k_1} = \frac{E}{2{\cdot}303R} \times \frac{T_2 - T_1}{T_1 T_2},$$

where k_1 and k_2 are the rate constants at absolute temperatures T_1 and T_2 (Glasstone, 1950, p. 607). As noted above, the rate constant is inversely proportional to $t_{\frac{1}{2}}$, so that

$$E = \log\frac{t_{\frac{1}{2}(1)}}{t_{\frac{1}{2}(2)}} \times \frac{2{\cdot}303RT_1T_2}{T_2 - T_1},$$

where $t_{\frac{1}{2}(1)}$ and $t_{\frac{1}{2}(2)}$ are the half-staining times at temperatures T_1 and T_2. Activation energies calculated from this formula are given in table 1 (appendix, p. 437), where it will be seen that the activation energy of staining tends to rise with decreasing permeability of the substrate. The value of E for the staining of mucin, for example, is considerably lower than that for less permeable substrates.

The activation energy, apart from being a quantitative measure of the effect of temperature on the rate of a reaction, may be regarded as the energy required by the participating molecules to enter a transient activated state necessary for the reaction to occur. An increase in temperature may affect the rate of staining by increasing the rate of diffusion of dye molecules in solution and in the substrate, by inhibiting aggregation of dye molecules, by lessening the affinity of the dye for the substrate, and in other ways. Since part of the effect of temperature on staining rate is probably through dispersion of dye molecule aggregates, and since dye aggregation is a function of dye concentration and ionic strength as well as of temperature, the activation energy of staining would be expected to vary under different conditions. Little fundamental significance therefore attaches to the approximate absolute values of E obtained here under arbitrary sets of conditions, but two points do merit discussion.

First, for a series of similar reactions, E tends to increase as the reaction rate decreases (Glasstone, 1950, p. 615). The fact that the activation energy of staining tends to increase with decreasing substrate permeability is consistent with this generalization, and the activation energy under defined conditions may perhaps be regarded as an index of substrate permeability.

Secondly, the activation energy of dyeing tends to be greater, the larger the dye molecule (Vickerstaff, 1954, p. 421). Vickerstaff states (p. 90) that for the staining of wool by levelling acid dyes E is about 22,000 cal, which is considerably greater than the values obtained in the present study. The difference may be partly due to the fact that azure A has a relatively small molecule; further, the activation energies recorded by Vickerstaff were presumably for staining by relatively concentrated solutions, in which dye aggregation may have been more important than in the present study. More

recently Bird and Stancey (1961) have found the activation energy of staining of wool by a series of basic dyes to vary between 6·2 and 12·5 kcal. These values are similar to those found in the present investigation, which is rather surprising in view of the very different experimental conditions.

We may conclude that where differential histological staining is due to different rates of staining of substrates of varying permeability, chilling the dyebath will enhance the selectivity of the method. This applies particularly to dyes of large effective particle size, whether this is due to the use of a dye of high molecular weight, or to aggregation of dye molecules in solution.

Effect of stirring on rate of staining

According to Vickerstaff (1954, p. 146) the rate of dyeing of wool is greatly affected by the rate of stirring or circulation of the dyebath, particularly at high temperatures. The rate of diffusion within the fibre increases more rapidly with rise in temperature than does the rate of diffusion in the dyebath (i.e. the former has the higher activation energy), so that at high temperatures the rate of diffusion in the dyebath can become the rate-limiting factor in dyeing. The rate of stirring is important also at low dye concentrations, since under these conditions the dye concentration in the immediate neighbourhood of the substrate may be significantly lowered by the staining process.

Although differences in the rate of staining of histological substrates immersed in a common dyebath must depend primarily on the rate of diffusion of dye molecules in the tissues, the rate of diffusion in the bath may presumably affect the absolute rate under certain conditions. In the experiments so far reported, staining took place with the slides standing in a stagnant (unstirred) glass staining jar, and it appeared desirable to study the effect on the rate of staining of stirring the dyebath.

Method. Stirring was accomplished by an electro-magnetic stirrer acting on a small glass-enclosed magnet underneath the slides in a normal staining dish, producing visible movement of the fluid around the slides. The rate of stirring was controlled by a rheostat on the stirrer, and was more or less reproducible.

In a preliminary experiment sections were stained for 1 h in 2^{-7}% or 2^{-10}% azure A at 4° C, with or without stirring.

In the main experiment sections were stained for 3·75, 5·33, 7·5, 15, or 30 min, or 1, 2, 4, or 16 h in 2^{-7}% azure A at 4° or 58° C, in a stirred bath, and then compared with the standard series in the usual way.

Results. In the preliminary experiment stirring markedly increased the intensity of staining of all substrates after 1 h, at both dye concentrations.

The half-staining times for RNA found in the main experiment (table 2, see appendix, p. 438) were, unfortunately, not quite comparable with those obtained previously in an unstirred bath, since by mistake 5-μ instead of 6-μ sections were used in this series. It was, nevertheless, clear that stirring the dyebath increases the activation energy of staining, i.e. a rise in temperature increases the rate of staining more in a stirred than in an unstirred bath.

It may be mentioned that stirring markedly accentuated the removal of substrates from the sections by hot buffer. Almost nothing except mast-cell granules stained after 16 h in stirred 2^{-7}% azure A at 58° C, the stainable components of chromatin, RNA, mucin, and cartilage matrix apparently having been removed. Fifteen hours in hot, stirred buffer in the absence of dye inhibited subsequent staining in cold azure A, and this shows that the buffer and not the dye itself was responsible for the removal of substrate in hot azure A solutions. This effect did not, however, vitiate the experiment, since little or no RNA was removed in hot stirred dye during the very short half-staining time.

Discussion. Since the activation energy of diffusion is higher in the substrate than in the dyebath (Vickerstaff, 1954), in a stagnant dyebath, where the rate of staining is influenced by diffusion both in the substrate and in the dyebath, the actual activation energy will be somewhere between the two. In a stirred bath, diffusion in the bath is less important as a rate-controlling factor, and the activation energy of staining will therefore be higher than in a stagnant bath. This theoretical prediction is supported by the results found.

It follows that differential staining dependent on different rates of diffusion of dye in various substrates will be accentuated by stirring the dyebath, since this will reduce or eliminate the non-specific effect on staining rate of dye diffusion in the bath. This applies both to chilled and unchilled dyebaths, so that the enhancing effect of chilling on this type of differential staining (see above) could in theory be further augmented by stirring the dyebath.

Rate and activation energy of destaining

Many histological methods depend for their selectivity on the fact that dye can be removed more rapidly from some stained substances than from others (differentiation). The rate of destaining may be in practice influenced by such factors as the nature, pH, and temperature of the eluting fluid, the amount of agitation, &c. In the present investigation the only eluting fluid used was veronal buffer at pH 4·0 (i.e. the solvent used for the stain solutions).

Method. Slides were stained for 24 h (i.e. to equilibrium) in 2^{-7}% azure A at 4° C, and then destained for varying times in dye-free buffer at 4° or 58° C before mounting in DPX in the usual way. The buffer was stirred continuously with a magnetic stirrer, and renewed whenever it became visibly discoloured. Slides were then compared with the standard series as before.

By analogy with the 'time of half-staining' $t_{\frac{1}{2}}$, used previously, we may define the 'time for half-destaining' $t'_{\frac{1}{2}}$, as the time required under these conditions for a substrate to reach an intensity of staining similar to that seen at equilibrium in a 2^{-8}% dyebath at 4° C. By destaining in buffer at different temperatures it is further possible to calculate the activation energy of destaining E' in the same way as was done for staining.

Results. Approximate values of $t'_{\frac{1}{2}}$ for various tissue components are given in tables 2 and 3, together with the times taken to reach an intensity of staining similar to that seen at equilibrium with a 2^{-10}% dyebath at 4° C.

For the staining of RNA $t'_{\frac{1}{2}(4^\circ C)}$ was found to be about 45 min, and $t'_{\frac{1}{2}(58^\circ C)}$ only about 45 sec, corresponding to an activation energy of destaining (E') of about 13,800 cal (table 2).

Discussion. It will be seen that the rate of destaining correlates well with the permeability of the substrate, permeable substrates such as mucin destaining much more rapidly than less permeable ones. This suggests that the rate of diffusion of dye molecules in the substrate has under these conditions an important influence on the rate of destaining, as it has on staining.

The activation energy E of staining of RNA in a stirred bath was previously found to be about 10,100 cal (table 2). If the activation energies of staining and destaining in a stirred bath were determined solely by diffusion in the substrate, they might be expected to be equal. The difference found may be due to experimental error, but may possibly suggest that factors other than rate of diffusion, such as the affinity of the dye for the substrate, may also influence the rate of staining and/or destaining to some extent. Under some conditions affinity may well be the most important factor controlling destaining, although this does not appear to be the case in the present experiment.

For a reversible reaction the activation energy E of the forward reaction, the activation energy E' of the reverse reaction, and the change in heat content, ΔH are related:

$$E-E' = \Delta H \text{ (Glasstone, 1950, p. 614).}$$

If one knew the activation energies concerned with the actual uptake and loss of dye by sites in the tissues, it would therefore be possible to calculate the ΔH of staining. The experimentally found activation energies of staining and destaining are, however, probably determined primarily by the activation energy of diffusion, and therefore cannot be used to estimate the ΔH of the actual dye uptake. A method for the determination of the ΔH of staining is, however, discussed in the next section.

On the effect of temperature and dye concentration on staining equilibria: the heat of staining

It can be seen from figs. 1 to 3 that a rise in the temperature of the dyebath, in addition to increasing the rate of staining, tends to decrease the intensity of staining finally attained. We shall in this section consider in more detail the effect of temperature on staining equilibria.

Method. Sections were stained for 16 h at 58° C in the same series of dyebaths as was used for the standard series (i.e. 2^0 %, 2^{-1} %, 2^{-2} %, ..., 2^{-14} %), and the two series were compared in the usual way.

As mentioned above, the staining of RNA at 58° C tends to decrease after about 4 h, presumably owing to hydrolysis and removal. To compensate for this, in a supplementary experiment sections were treated in clean buffer at 4° C for 24 h, and then stained for 16 h in 2^{-7} %, 2^{-8} %, 2^{-9} %, or 2^{-10} % azure A at 58° C. These sections were then compared with a control section

which had been for 16 h in clean buffer at 58° C and 24 h in 2^{-10}% dye at 4° C, and which presumably contained a similar final amount of RNA.

Results and discussion. The results of the main experiment are given in fig. 6, in which (in order to include all the results on a single graph) a logarithmic scale of dye concentration is used on both axes.

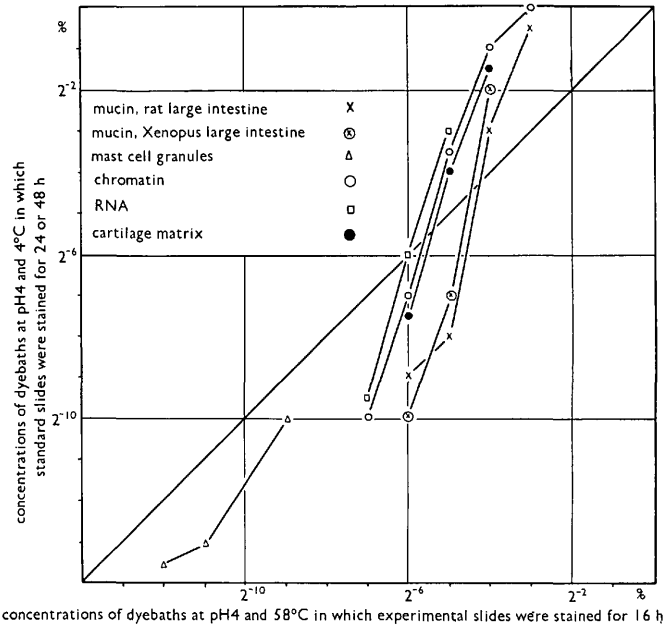

FIG. 6. Effect of temperature on equilibrium staining intensity attainable at various dye concentrations.

If temperature had no effect on dye uptake at equilibrium, staining at a given concentration at 4° C would equal that at the same concentration at 58° C (i.e. all points plotted in fig. 6 would fall on a diagonal passing through the origin). In view of the earlier results expressed in figs. 1 to 3, and bearing in mind the fact that staining is in all known cases exothermic (Vickerstaff, 1954), it was expected that to achieve a given intensity of staining a higher concentration of dye would be needed at 58° C than at 4° C (i.e. points in fig. 6 would be displaced to the right of the diagonal). This was in fact observed at dye concentrations below about 2^{-5}%, but at higher dye concentrations staining appeared to be enhanced by a rise in temperature.

This unexpected result suggested that with higher dye concentrations staining equilibrium at 4° C might not have been reached in 24 h, and in an *ad hoc* experiment sections were stained for up to 8 days in 1%, 2^{-7}%, and 2^{-10}% azure A at 4° C. It was found that sections stained in the two weaker dyebaths showed no increase in staining intensity after 24 h, confirming the earlier results regarding the time necessary for the attainment of equilibrium. Sections stained for 8 days in the 1% solution, however, appeared slightly darker than those stained for only 24 h, showing that equilibrium had not been completely reached after the shorter time; sections stained for 8 days in 1% dye at 4° C nevertheless still stained less intensely than sections stained for 16 h in 1% dye at 58° C.

It is most improbable that staining is exothermic at some dye concentrations and endothermic at others: the most probable explanation of the above results is that at high dye concentrations at 4° C the dye molecules are aggregated to such an extent that the rate of diffusion and hence of staining is markedly decreased. Further, owing to aggregation the *effective* dye concentration is lower at 4° C than at 58° C at high dye concentrations, resulting in a diminished intensity of staining at equilibrium at the lower temperature, despite the exothermic nature of the staining reaction. Lenher and Smith (1935) have described a similar phenomenon in the staining of cotton with certain azo dyes.

It may be noted that Rosenbaum and Deane (1959) observed that an increase in the temperature of the dyebath decreases histological staining by dilute solutions of certain basic dyes (including azure A), but increases staining by more concentrated solutions. No explanation was, however, offered by Rosenbaum and Deane for this anomalous result.

It might be expected that the staining at equilibrium of relatively permeable substrates such as mucin would be less inhibited by dye aggregation at high concentrations and low temperatures than that of denser (less permeable) substances. It may be noted that the dye concentration at which staining at 4° and 58° C is equal (i.e. where the graph crosses the diagonal) is higher for mucin than for the other substrates. At this point the inhibitory effect of high temperature on staining, due to inhibition of an exothermic reaction, equals the enhancing effect due to inhibition of dye aggregation. Since at high dye concentrations aggregation is greater, but the exothermic nature of staining is presumably unaffected, this is consistent with the inhibitory effect of dye aggregation on staining being less marked in the case of mucin than in that of other tissue components. It is not, however, conclusive proof of this, since a similar result would be expected if the staining of mucin was more markedly exothermic than that of the other substrates (as indeed it may be—see below).

In the further discussion of the effect of temperature on staining equilibria, the results principally discussed will be those obtained by a comparison of experimental sections with slides stained in 2^{-10}% azure A at 4° C. This is because (*a*) dye aggregation is less at low dye concentrations, and may more

safely be ignored; (*b*) evidence has been presented that the staining time used (24 h) was sufficient for equilibrium to be attained in 2^{-10}% dye at 4° C, but this may not be true of much stronger or weaker solutions; (*c*) at still lower dye concentrations most substrates did not stain, or stained too faintly for accurate comparisons to be made. In the case of mast-cell granules, however, staining was very intense, and the results obtained with 2^{-10}% dye were confirmed with still more dilute dyebaths.

A figure expressing the effect of temperature on staining equilibria may be obtained by dividing a concentration of dye at a low temperature into that concentration of dye at a higher temperature which gives the same intensity of staining. It will be seen from fig. 6 that at 4° and 58° C, and a dye concentration in the colder bath of 2^{-10}% azure A, the ratio is about 8 to 16 for mucin, 8 for nuclear chromatin and interstitial cartilage matrix, and 2 for mast-cell granules. At lower dye concentrations the ratio for mast-cell granules was slightly higher, but still less than that found for other substrates. The ratio for RNA appears from fig. 6 to be about 8, but in the supplementary experiment (in which there was compensation for hydrolysis of RNA in the hot dyebath) it was found to be between 4 and 8. Results of the supplementary experiment confirmed those found in the main experiment for substrates other than RNA.

If an equal intensity of staining of a substrate is achieved at equilibrium in two dyebaths of dye concentrations $[D]_1$ and $[D]_2$ and absolute temperatures T_1 and T_2, the heat of dyeing can be calculated from the relation

$$\Delta H = \frac{RT_1T_2}{T_2 - T_1} \ln \frac{[D]_1}{[D]_2},$$

where R is the gas constant (Vickerstaff, 1954, p. 118. N.B.: the equation is misprinted in Vickerstaff, the ratio of dye concentrations being inverted). It may be noted in passing that the dye concentrations $[D]_1$ and $[D]_2$ in the above equation should really be replaced by the respective activities, but it is customary to use the concentrations of dye solutions, as the activities are not in general known. In the present experiment the *ratio* of concentrations is being considered, and this is not likely to vary greatly from the ratio of activities under most conditions, since the activity coefficients in the numerator and denominator would tend to cancel out. This is, however, probably not always true: the anomalous effect of temperature on staining with concentrated solutions of azure A, attributed above to aggregation of dye molecules in cold, concentrated solutions, may perhaps be regarded as an extreme deviation of such solutions from ideal behaviour in the physico-chemical sense.

Substituting the temperatures 4° and 58° C in the above equation,

$$\Delta H = \frac{1{\cdot}98 \times 277 \times 331}{331 - 277} \ln \frac{[D]_1}{[D]_2} \text{ cal/mole}$$

$$= -3362 \ln \frac{[D]_2}{[D]_1} \text{ cal/mole.}$$

For values of $[D]_2/[D]_1$ of 2, 4, 8, and 16,

$$\Delta H = -2{,}330,\ -4{,}660,\ -6{,}990, \text{ and } -9{,}320 \text{ cal/mole}$$

respectively.

From the approximate values of $[D]_1$ and $[D]_2$ found experimentally, we may conclude that the ΔH of staining with dilute azure A in buffer at pH 4·0 is probably about −8,000 cal/mole for mucin, −7,000 for chromatin and cartilage matrix, −5,500 for RNA, and −2,000 to −4,000 for mast-cell granules.

The slightly different values of ΔH found for chromatin, cartilage matrix, RNA, and mucin should be regarded with caution. It is conceivable that even in 2^{-10}% azure A there might still be some aggregation of dye particles at 4° C, and, as discussed above, inhibition of dye aggregation at high temperatures would enhance the staining of relatively impermeable substances but have less effect on the staining of the more permeable mucin. It is, however, improbable that dye aggregation is sufficient to account for the differences in ΔH found, as increasing $-\Delta H$ does not correlate perfectly with the known order of increasing permeability. This applies particularly to mast-cell granules, which are probably relatively permeable to basic dyes, but appear to have a particularly low $-\Delta H$.

We may therefore conclude that there probably are real differences between the heats of dyeing of various tissue components, even though the estimated values may not be quite accurate.

In considering the significance of the ΔH of staining, one must be careful not to confuse the *rate* of staining and the final *equilibrium* intensity attainable. The rate of a chemical reaction is a function of the activation energy (strictly speaking the rate is also affected by the entropy of activation, but this may often be ignored). For an endothermic reaction, such as the breaking of the dye-substrate bond, the activation energy is always greater than the (positive) ΔH. In general, therefore, the activation of dye elution is likely to increase *pari passu* with the $-\Delta H$ of formation of the dye-substrate bond, so that the higher the $-\Delta H$ of staining, the more slowly will stain be removed from the substrate (other factors, such as substrate permeability, being constant). Note that if a stained substance is placed in a bath of solvent kept essentially dye-free by constant renewal or large volume, *all* dye will eventually be eluted from the substance whatever the ΔH of the dye-substrate bond—only the *rate* of dye removal is affected by the nature of the bond under these conditions, not the final equilibrium. On the other hand, the more heat given off during the course of a chemical reaction (i.e. the larger the $-\Delta H$), the more is such a reaction inhibited at equilibrium by a rise in temperature. In general one may say that the larger the $-\Delta H$ of a dye-substrate bond, the more stable is such a bond likely to be under all conditions, but the more will the equilibrium intensity of staining be inhibited by a rise in the temperature of the dyebath.

In view of the fact that mast-cell granules stain extremely intensely with

azure A, and are under most conditions highly resistant to differentiation, one would have expected the dye-substrate bond to be strong and to have a high $-\Delta H$. The low value actually found was therefore surprising, and raised the question of the nature of 'affinity', which is considered in the next section.

In addition to reflecting the strength of the dye-substrate bond, the heat of dyeing '. . . may be regarded as the sum of the heats of formation of the various bonds existing between the dye and the fibre, and it can thus give an insight into the mechanism of the combination . . .' (Vickerstaff, 1954, p. 119; but see the critical comments of Derbyshire and Peters, 1955). Typical values of $-\Delta H$ are: covalent bonds, 40 to 100 kcal/mole; hydrogen bonds, 2 to 8 kcal/mole; van der Waals bonds, about 0·5 kcal/mole. Ionic bonds vary considerably in strength depending on the conditions, but are usually about 5 kcal/mole (Albert, 1951).

The values of $-\Delta H$ found in the present experiment for the staining of mast-cell granules and possibly RNA are reasonable for a dye-substrate bond consisting of a single electrostatic link, but the values found for cartilage matrix, chromatin, and especially mucin are perhaps a little large for this. They could, however, be accounted for by supposing that a primary salt linkage (which may be presumed to play a part in the attachment of a basic dye to an anionic substrate), is stabilized in these sites by van der Waals forces or perhaps more probably a hydrogen bond.

On the affinity of dye for substrate

A dye is commonly said by histologists to have a high affinity for a tissue component if it stains it intensely under given conditions. Intensity of staining is, however, often determined chiefly by the density and permeability of the substrate rather than by its affinity for the dye (Baker, 1958, p. 228); even if there is no obstacle to dye penetration, and if adequate time is allowed for equilibrium between dyebath and substrate to be reached, the amount of dye taken up is a function both of the affinity of each reactive site in the substrate for dye, and of the number of such sites present.

Following Vickerstaff (1954), we shall use the term 'affinity' to refer strictly to the tendency of dye to move from a defined standard state in the dyebath to a defined standard state in the substrate. This tendency may be expressed in terms of the equilibrium constant K of the dye-tissue interaction, or in terms of the standard free energy change, ΔF°, accompanying the reaction. Where R is the gas constant, and T is the absolute temperature,

$$\Delta F^\circ = -RT \ln K.$$

It is possible in theory to estimate the equilibrium constant K from the relation

$$K = k/k',$$

where k and k' are the rate constants for the forward and backward reactions at equilibrium. These rate constants cannot, however, be measured in practice, since rates of staining which can be determined experimentally

reflect mainly the rate of diffusion of the dye rather than the actual process of dye uptake by the tissue.

Affinity is studied in the present investigation in terms of the standard free energy change, which is the change in free energy per mole of dye occurring when a small amount of dye is transferred from a defined standard state in solution in the dyebath to a defined standard state in the substrate.

$$\Delta F^\circ = -RT \ln \frac{a_{\text{substrate}}}{a_{\text{dyebath}}},$$

where $a_{\text{substrate}}$ and a_{dyebath} are the activities of dye in the substrate and a given dyebath at equilibrium (Vickerstaff, 1954, p. 102). If the dye formed ideal solutions in both phases, the activities in the above equation could be replaced by the concentrations. This is in fact customary for the dyebath, since little or no information is available regarding the activity of dyes in solution (Vickerstaff, 1954, p. 103): the practice will be followed here, although at high concentrations the deviation of dye solutions from the ideal is probably considerable (see above). Following this convention, the standard state of dye in the dyebath may be taken to be 1 g mole or 1 g equivalent per litre. Unfortunately, histological dyes (with the possible exception of colouring agents for lipids) almost certainly do not form true solutions in tissue components, and therefore even if it were possible to measure the absolute dye concentration in a tissue component at equilibrium with a known dyebath, the activity of the dye in the substrate, and hence the affinity, would still be unknown.

Measurement of the activity of dye in a substrate is, however, possible if one makes certain assumptions, namely, (*a*) that the substrate contains a number of reactive sites capable of attracting dye molecules (a reasonable assumption, supported by experimental evidence); (*b*) that when a site is occupied by a dye molecule it is no longer available for the attraction of further dye (a somewhat less safe assumption, in view of the fact that dye molecules are able to aggregate under some conditions—see especially Pal and Schubert, (1961)); and (*c*) that the sites are so far apart that the adsorption of a dye molecule on one site does not interfere with the adsorption of dye on any neighbouring site (this assumption is probably not strictly valid for tissue components containing a high concentration of staining sites).

Making the above assumptions, the activity of adsorbed dye is given by $\theta/(1-\theta)$, where θ is the fraction of available sites occupied by dye (Vickerstaff, 1954, p. 107). The standard state of dye on the substrate, where the activity has a numerical value of 1, is thus the state of half-saturation, since $0{\cdot}5/(1-0{\cdot}5) = 1$. We may now write

$$\Delta F^\circ = -RT \ln \frac{\theta}{(1-\theta)(\text{dyebath concentration})},$$

where θ is the fraction of available sites in the substrate occupied by dye molecules, at equilibrium with a given dyebath. It should be noted that the

absolute amount of dye taken up is not relevant—two substrates may have the same affinity for a dye, measured in calories per mole of dye taken up, although one substrate may have more available sites per cubic micron, and therefore stain more intensely than the other. This point is illustrated in fig. 7.

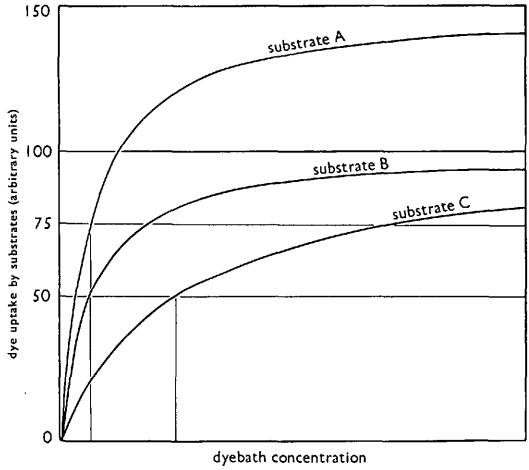

FIG. 7. Theoretical uptake of dye from dyebaths of varying concentration, as predicted by the Langmuir adsorption isotherm. Substrate *A* has the same affinity for the dye as has substrate *B*, but has 50% more staining sites. Substrate *C* has the same number of stainable sites as has substrate *B*, and in a dyebath of infinite activity would stain with the same intensity, but has a lower affinity for dye. Note that the saturation intensity is a measure of the number of available sites, and that the dyebath concentration at which the substrate takes up half the saturation value is a measure of the affinity.

The problem of measuring the affinity of a dye for a tissue component then resolves itself into finding the concentration of dye in a dyebath at equilibrium with the substrate, when the latter has taken up some known fraction of the amount of dye it would take up at saturation.

Experimental procedure and calculation. Tissues of the rat and of *Xenopus*, fixed in formalin solution, were embedded in a single paraffin block, and cut at 3 μ and 6 μ.

A 3-μ section, designated the 'control section', was stained for 8 days (presumably long enough for equilibrium to be reached) in saturated azure A in veronal-acetate buffer at pH 4·0 and 4° C, dipped into tap-water to rinse off loose dye solution, blotted, air-dried for 1 h, and mounted in DPX. To ensure that every site that is capable of taking up dye has in fact done so, it is theoretically necessary to stain with a dyebath of infinite activity, since

according to the Langmuir adsorption isotherm a substrate approaches saturation hyperbolically with increase in dyebath activity. Staining to equilibrium in a dyebath saturated with a soluble dye such as azure A is, however, probably sufficient for the staining of *almost* all possible sites in the substrate, and we shall assume that the control section was for practical purposes saturated with dye.

Sections 6 μ thick, designated the 'experimental series', were stained for 8 days at 4° C and pH 4·0 in a series of dyebaths ranging in concentration from 2^{-1}%, 2^{-2}%, ..., 2^{-11}% azure A, air-dried, and mounted in DPX. In previous experiments slides were dehydrated in alcohol, but this was not possible in this series since alcohol might have removed different amounts of dye from the 3-μ and the 6-μ sections, which would therefore not have been comparable. A low temperature was used to minimize removal of tissue components during the long staining period.

The control section and the experimental series were then compared with a comparator eyepiece in the usual way. An orange filter was used in most cases, but a deep red and a deep blue filter were used for the study of metachromatic substrates.

A given tissue component in a 6-μ section, stained with the same apparent intensity as a similar structure stained to saturation in a 3-μ section, may be presumed to have taken up half the maximal amount of dye, provided Beer's law is obeyed. Concentrated solutions of basic dyes are known to deviate from Beer's law, but since the concentration in a 3-μ section is only twice that in a 6-μ section stained with the same apparent intensity, this source of error is probably negligible. Under these conditions the dye activity in the substrate in the 6-μ section is numerically equal to 1. The dye used was 85% pure, with a M.W. of 291·8, so that a nominal 1% solution was 0·02913 M. If half-saturation of a tissue component occurs at equilibrium with a dyebath of y%, the affinity at 4° C is then given by

$$\begin{aligned}\Delta F^\circ &= -RT\ln\frac{1}{0{\cdot}02913y}\\ &= -1{\cdot}987\times 277\times 2{\cdot}3026\log\frac{1}{0{\cdot}02913y}\ \text{cal/mole}\\ &= -1267\log\frac{1}{0{\cdot}02913y}\ \text{cal/mole.}\end{aligned}$$

The standard states are implicitly defined here as being half-saturation in the substrate and 1 g equivalent in the dyebath. Each successive step in the experimental series (e.g. between slides stained in the 2^{-1} and 2^{-2}% dyebaths, or between slides stained in the 2^{-7} and 2^{-8}% solutions) corresponds to a difference in ΔF° of about 380 cal/mole; an error in matching the control slide with the experimental series, of one slide either way, would therefore give an error in estimation of ΔF° of ± 380 cal/mole.

Several important further sources of experimental error remain to be mentioned. (1) It is difficult to cut paraffin sections accurately at a given thickness.

The use of a single control section makes results obtained with different substrates somewhat more comparable, but does not influence possible absolute errors. (2) If substrates in the control section were not in fact stained to saturation, the values of $-\Delta F^{\circ}$ found would be too high. Since, with a given dyebath, a substrate of high affinity stains closer to its saturation value than a substrate of low affinity (see fig. 7), this applies particularly to the latter. (3) The method assumes that a 6-μ section contains twice as much substrate as a 3-μ section. This is not always true of heterogeneous objects such as cell nuclei or mast cells, and even if great care is exercised in the selection of objects which apparently fill the full thickness of the section, results obtained on such objects must remain suspect. (4) The method can measure only the *mean* affinity of sites in a given tissue component, and (except in the special case of metachromatic substrates) cannot detect the presence or measure the affinity of sites of different types occurring in a single tissue component.

Results. These are summarized in table 5, and given more fully below.

Cartilage matrix stained red-purple in 6-μ sections stained in dilute (e.g. 2^{-11} %) azure A, and progressively purple, blue-purple, and an almost pure, deep blue at higher dye concentrations. In the control section cartilage matrix appeared blue. Owing to the difference in colour between the 3-μ and the 6-μ sections, the matching obtained depended on the filter used. With a deep blue filter cartilage in the control section appeared to be approximately of the same intensity as in the 6-μ section stained in 2^{-9} % dye, while with a deep red filter matching was with the section stained in 2^{-5} % dye, corresponding to affinities of about $-5{,}400$ and $-3{,}900$ cal/mole respectively.

Mast-cell granules were extremely difficult to match, for several reasons. For the method to be valid, it was essential to choose cells in the 6-μ section which completely filled the thickness of the section, and it was not always easy to be sure that this was the case. Further, a considerable variation in the intensity of staining of granules in individual cells could be seen in a single section. Finally, mast-cell granules stained rather intensely even in dilute dye solutions, making matching difficult.

Mast-cell granules stained purple in weak dye solutions, and blue in stronger ones, although the difference in colour was not as marked as in the case of cartilage matrix. With deep blue and red filters, the control section matched approximately with experimental slides stained in 2^{-6} to 2^{-7} % and 2^{-5} to 2^{-6} % dye respectively, corresponding to affinities of about $-4{,}400$ and $-4{,}000$ cal/mole. This difference, although slight, was definite.

Ribonucleic acid in rat pancreas in the control section matched approximately with the experimental slide stained in 2^{-9} % dye, but the latter was markedly more cracked and shrunken by the air-drying, and therefore appeared more darkly stained than it should have. Cytoplasmic RNA in rat gastric glands matched at about 2^{-2} to 2^{-3} %, while that of *Xenopus* stomach matched at about 2^{-2} %, giving a value of ΔF° of about $-3{,}100$ cal/mole.

Chromatin is not an ideal object for the present method, as nuclei are not

uniform structures. Chromatin of rat smooth-muscle nuclei in the control section matched approximately with that in the experimental section stained in 2^{-3}% dye, giving a value of ΔF° similar to that found for RNA.

The matching values obtained with mucin varied somewhat, from close to 2^{-4}% in the case of rat and *Xenopus* intestinal goblet cell mucin, to about 2^{-3}% for *Xenopus* renal mucin and 2^{-2}% for *Xenopus* dermal mucin. The affinities calculated from these values varied accordingly from about −3,500 down to about −2,700 cal/mole.

Rat thyroid colloid stained only faintly even in concentrated dye solutions; the control section was of about the same intensity as the experimental section stained in 2^{-1}% azure A. *Xenopus* dermal poison gland secretion stained even more faintly, and in the control section stained more intensely than in the experimental section stained in 2^{-1}% dye. The affinity of these substrates is only about −2,300 cal/mole, or less. If the control section was not fully saturated, even these values were probably too high (see discussion above).

Discussion. The difference found between the affinity of orthochromatic and metachromatic staining of cartilage matrix was probably less than the true difference, as it was not possible to measure the affinity of the two types of staining completely independently with the filters available. This difficulty was even more marked with the densely stained mast-cell granules, and it is possible that the values of $-\Delta F^{\circ}$ found for the orthochromatic and metachromatic staining of this substrate should have resembled those found for cartilage more closely.

It appears, then, that two distinct types of staining occur in both cartilage matrix and mast-cell granules: metachromatic staining with a high affinity, seen well even in dilute dye solutions, and orthochromatic staining of lower affinity, requiring a more concentrated dyebath for its demonstration. This explains the effect of excess dye in 'masking' metachromasia (Sylvén, 1954; Kramer and Windrum, 1955).

It may be mentioned that Sylvén (1954) was able to estimate the approximate bonding energy between dye molecules in solution from the metachromatic spectral shift, by the use of Planck's formula, and found values of about 6·4 and 10·6 kcal/mole for the metachromasia of azure A on chondroitin sulphate and heparin respectively. These values are higher than those found here for metachromatic staining, but Sylvén's figures may relate to the bond between adjacent dye molecules, rather than to the bond between dye molecule and substrate, as in the present experiment.

It is of interest to compare the present results with those of Stacey and Wildy (1960), who estimated colorimetrically the dye eluted from tissue cultures which had been stained with methylene blue under various conditions. Although they did not discuss their results from the point of view of thermodynamics, it is possible to calculate the affinity of staining of nucleic acid from their data (about 90% of the staining of their material was due to deoxyribonucleic and ribonucleic acids). Thus one of their graphs shows

that at pH 7 and presumably at room temperature, an amount of dye was taken up from a dyebath containing 1 mg methylene blue per ml equal to about 22·5/26 times that taken up at saturation. Knowing the molecular weight (about 320) and purity (85·5%) of their dye, one can calculate that the $\Delta F°$ of staining under these conditions equals −4,510 cal/mole. In another diagram, Stacey and Wildy indicate that from a 0·125% dyebath about 0·4 times as much dye is taken up at pH 4 as at pH 7. Assuming that the saturation staining intensity at pH 4 would be the same as that at pH 7 (this point was not reported on), it can be calculated that the $\Delta F°$ of staining of nucleic acid by methylene blue at pH 4 and room temperature is about −3,065 cal/mole. In the present study, a value of about −3,100 cal/mole was found for the $\Delta F°$ of staining of RNA and chromatin by azure A at pH 4 and 4° C. From the values of ΔH of staining of RNA and chromatin (−5,500 and −7,000 cal/mole respectively) also found in the present investigation, it can be calculated that the $\Delta F°$ of staining of RNA and chromatin by azure A at pH 4 and 20° C would be about −2,960 and −2,870 cal/mole respectively, which in view of the very different methods applied may be considered to be in reasonable agreement with the value calculated from the results of Stacey and Wildy.

The differences in affinity found in the present study probably reflect real differences in the nature of the dye-binding sites. The highest values, those for the metachromatic staining of cartilage matrix and mast-cell granules, may be due to sulphate groups, while the fact that *Xenopus* and rat intestinal mucin showed a higher affinity than (say) mucin of *Xenopus* skin may indicate the presence in the former of a certain number of sulphate groups, serving to increase the mean affinity of the dye-binding sites. Sulphate groups have in fact been demonstrated in some intestinal mucins by autoradiographic methods (Curran and Kennedy, 1955; Jennings and Florey, 1956). Moderate values of $-\Delta F°$ found for the staining of some mucins, nuclear chromatin, and cytoplasmic RNA, and the orthochromatic staining of mast-cell granules and cartilage matrix may be due to binding by carboxyl and/or phosphate radicles. The very low values found for thyroid colloid and *Xenopus* poison glands (not normally considered to be basiphil) are consistent with a weak, non-specific binding by hydrogen bonds and/or van der Waals forces.

The fact that the affinity of sulphate groups for azure A appears to be significantly higher than that of phosphate or carboxyl groups requires comment. It can hardly be due to the fact that sulphuric acid is a stronger acid than the other two: at the pH used (4·0) probably all three acids were dissociated, and a strong acid would only be expected to be more basiphil than a weak one at a low pH, where only the stronger acid carries a negative charge. Two possible explanations will be discussed.

With substrates of high charge density, such as mast-cell granules, a number of neighbouring charged sites may contribute to the anchoring of a single dye ion. According to this interpretation the higher affinity of sulphate groups in sections is more apparent than real, and closely packed carboxyl or phosphate

groups would show the same phenomenon. The fact that staining of high and low affinity appears to occur simultaneously in mast-cell granules could be explained by supposing that at low dye concentrations a number of neighbouring charged sites contribute to the affinity, but that the importance of this factor diminishes as the number of 'masked', occupied sites increases.

An alternative, and possibly more likely, explanation of the high affinity of sulphate groups involves the concept of entropy. It has been shown (tables 4 and 5) that while the staining of mast-cell granules has a fairly high $-\Delta F^\circ$, it has a rather low $-\Delta H$. Now

$$\Delta F^\circ = \Delta H - T\Delta S,$$

where ΔS is the entropy change accompanying the reaction. A high negative value of ΔF° (i.e. a high affinity) together with a relatively low negative value of ΔH therefore implies an increase in entropy. It was expected *a priori* that staining, especially metachromatic staining, would be accompanied by a *decrease* in entropy, due to the dye molecules being randomly dispersed before staining, and probably highly oriented after staining. The observed results can, however, be accounted for by supposing that sulphate groups are initially surrounded by a large shell of water molecules, which is broken up and dispersed by the attachment of dye to the substrate. The resulting increase in the disorder of the system is reflected in an increase in entropy, contributing to the affinity. Similar explanations have been advanced to account for increases in entropy observed during the staining of albumin *in vitro* (Klotz and Urquhart, 1949), and during the formation of antigen-antibody complexes (Boyd, 1962).

The sulphate group is known to be surrounded by a particularly large hydration shell, due probably to the radius of the group being small and the attraction of gegen-ions being a function of distance (Höber, 1945); the contribution of an entropy increase to the affinity of staining of less hydrated radicles, such as carboxyl and phosphate groups, may well be less marked, accounting for their lower affinity for azure A.

It may be mentioned at this point that with many substrates, including RNA, staining with azure A gives a somewhat more purple colour at high than at low temperatures. This may indicate that metachromatic (red or purple) staining may have a lower $-\Delta H$ than orthochromatic staining in these sites as in those previously discussed. It is, however, not yet possible to say whether an increase in entropy invariably accompanies metachromatic staining.

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Appendix

TABLE I

Effect of temperature and nature of substrate on rate of staining

	Approximate half-staining times (in min) under various conditions				*Ratios of half-staining times, and activation energies E calculated therefrom*			
	(*a*)	(*b*)	(*c*)	(*d*)	*a*/*b*	*b*/*c*	*c*/*d*	*b*/*d*
Dye concentration:	2^{-10}%	2^{-7}%	2^{-7}%	2^{-7}%				
Temperature:	4° C	4° C	24° C	58° C				
Mucin	—	13	11	—	—	1·18 *1354*	—	—
Mast-cell granules	240	—	—	—	—	—	—	—
Nuclear chromatin	480	75	35	—	6·4	2·14 *6221*	—	—
Cytoplasmic RNA	480+	90*	40	10	5·33	2·25 *6631*	4·0 *8153*	9·0 *7415*
Cartilage matrix (interstitial)	600	150	50	—	4·0	3·0 *8984*	—	—

Notes

1. All staining was in unstirred azure A in veronal buffer, pH 4·0.
2. Activation energies were calculated from the formula

$$E = \log t_{\frac{1}{2}(1)}/t_{\frac{1}{2}(2)} \times 2{\cdot}303RT_{(1)}T_{(2)}/(T_2 - T_1).$$

3. Activation energies, although given to four figures, are probably accurate only to about the nearest 1,000 calories.
4. Mast-cell granules did not stain uniformly, even in a single section.

* See discussion on p. 419.

TABLE 2

Effect of temperature on rate of staining and destaining of cytoplasmic RNA in a stirred bath

	Approximate times of half-staining and half-destaining, at		*Activation energy (in calories) calculated from preceding columns*
	4° C	58° C	
Staining	90 min	4·5 min	10,110
Destaining	45 min	0·75 min	13,820

Notes

1. Staining was in 2^{-7}% azure A, pH 4·0, agitated with a magnetic stirrer.
2. Destaining was in a stirred bath of clean buffer at pH 4·0, of slides previously stained to equilibrium in 2^{-7}% azure A at pH 4·0 and 4° C.
3. The time of half-staining or half-destaining is the time taken under the given conditions to reach an intensity of staining similar to that seen at equilibrium with 2^{-8}% azure A at 4° C. The activation energy is calculated in the same way as in table 1.

TABLE 3

Rate of destaining of various substrates

	Approximate time (in min) taken to reach an intensity of staining similar to that seen at equilibrium with a dyebath at 4° C of the stated concentration	
	2^{-8}%	2^{-10}%
Mucin	about 15	—
Mast-cell granules	—	about 30
Nuclear chromatin	15–30	about 60
Cytoplasmic RNA	30–60	120–240
Cartilage matrix (interstitial)	60–120	120–240

Note. Slides were stained for 24 h in 2^{-7}% azure A at pH 4·0 and 4° C, and then destained for varying times in clean, stirred buffer at pH 4·0 and 4° C. The times given in the first column may be regarded as half-destaining times.

TABLE 4

Effect of temperature on staining equilibria

	Concentration of dyebath giving at 58° C an intensity of staining similar to that seen in 2^{-10}% dye at 4° C	*Approximate heat of staining ΔH (in cal/mole)*
Mucin (rat large gut)	2^{-7} to 2^{-6}%	−8,000
Nuclear chromatin, Cartilage matrix	about 2^{-7}%	−7,000
Cytoplasmic RNA	2^{-8} to 2^{-7}%	−5,500
Mast-cell granules	about 2^{-9}%	−2,000 to −4,000

Note. The heat of staining was calculated from the formula

$$\Delta H = RT_1T_2/(T_2-T_1)\times \ln \frac{[D]_1}{[D]_2}.$$

TABLE 5

Affinity of various substrates for azure A

	Concentration of dyebath which stains a 6-μ section at equilibrium as intensely as a 3-μ section stained to saturation	*Affinity, $-\Delta F^\circ$ (in cal/mole)*
Cartilage matrix	2^{-5}% (red filter)	3,850
	2^{-9}% (blue filter)	5,380
Mast-cell granules	2^{-6} to 2^{-5}% (red filter)	about 4,000
	2^{-7} to 2^{-6}% (blue filter)	about 4,400
RNA, rat pancreas	2^{-9}%	5,380
RNA, other sites	about 2^{-3}%	3,090
Mucin (rat and *Xenopus* gut)	2^{-4}%	3,470
Mucin (*Xenopus* kidney)	2^{-3}%	3,090
Mucin (*Xenopus* skin)	2^{-2}%	2,710
Chromatin, rat smooth muscle	2^{-3}%	3,090
Smooth muscle cytoplasm (rat)	2^{-2}%	2,710
Thyroid colloid	2^{-1}%	2,330
Poison glands (*Xenopus*)	$> 2^{-1}$%	$< 2{,}330$

Notes

1. All staining was in azure A at pH 4·0 and 4° C.
2. Rat pancreatic RNA was markedly clumped and shrunken by air-drying in the 6-μ section, giving a falsely high affinity.
3. The affinity was calculated from the formula

$$\Delta F^\circ = -RT \ln \frac{1}{\text{dyebath concentration}},$$

and is probably subject to an error of at least ±200 cal/mole.