QUANTITATIVE OBSERVATIONS ON THE
KINETICS AND MECHANISMS OF BINDING
OF ELECTRON STAINS TO THIN SECTIONS
THROUGH HEN ERYTHROCYTES

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SUMMARY

The kinetic studies described in this paper have enabled us to define the staining conditions
which lead to occupation of all available binding sites in sections through biological material.
In a hypothetical section containing stainable regions the concentration, $c$, of bound stain in any
thin layer is a function of its distance, $x$, from the surface in contact with the staining solution
and the staining time $t$. Theoretical considerations indicate that there are 2 extreme types of
kinetics depending on the relative values of 2 diffusion or migration rates: $r_1$, that of the staining
solution into the depth of the section, and $r_s$, that of the solution into the stainable regions.
When $r_1 > > r_s$, called type A kinetics, penetration of stain throughout the depth of the section
is 'instantaneous' and binding to stainable regions is slow. Two families of curves can be
constructed, each member of which has a particular value of $t$: first $c$-vs-$x$ curves and second,
derived from them by integration, $E$-vs-$d$ curves, where $E$ is the electron-scattering density of a
stained region relative to clear resin and $d$ is section thickness. When $r_1 < < r_s$, called type B
kinetics, the staining solution diffuses relatively slowly into the section, and all the binding
sites in each stainable region are occupied 'instantaneously'. Similarly there are 2 families of
curves, $c$-vs-$x$ and $E$-vs-$d$, but of different shapes. When $r_1 \sim r_s$ there is an intermediate type
AB kinetics. At any point on an $E$-vs-$d$ curve the slope is proportional to the concentration of
bound stain. The penetration time, $t_p$, is defined as the time taken for the concentration of the
staining solution to reach the same value at the bottom of a 100-nm section as at the top surface
in contact with the solution.

Models for the cytoplasm and chromatin bodies of erythrocytes are proposed, based on their
physical structure and on interferometric determinations of the fractional volumes occupied by
resin, biological material and free space. Staining mechanisms can be explained in terms of
diffusion or migration through a system of interconnecting spaces comprising the resin, as well
as a second, hydrophilic, pathway connecting the 2 surfaces of the section, arising from long
threads of DNA-histone.

Experimentally, $E$-vs-$d$ curves are derived by treating single grids, each carrying a series of
sections of increasing thickness, for increasing times. $E$ is measured directly in the microscope
with a Faraday cage. Sections have been treated at 20° and at 60°C with aqueous and, or,
ethanolic solutions of (a) magnesium uranyl acetate or uranyl acetate, either alone or followed
by Pb-citrate; (b) PTA; and (c) KMnO₄. From the $E$-vs-$d$ curves upper and lower limits for $t_p$
have been tabulated. Also tabulated are values of $E$ for sections 100 nm in thickness, at equili-
brium when all available sites are filled. $E/100$ nm is approximately proportional to the con-
centration of bound stain per unit volume. The kinetics of stain uptake are discussed in terms of

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the models. The anomalous binding of KMnO₄ and ethanolic PTA previously reported have been explained and the significance of our data for the interpretation of electron micrographs is also discussed.

INTRODUCTION

Our present studies on the binding of electron stains have arisen from our interest in the structure of interphase chromosomes (see Davies & Haynes, 1976). Previously, thin sections through hen reticulocytes and erythrocytes embedded in epoxy resin were treated with either uranyl acetate, which was shown to bind preferentially to DNA (Huxley & Zubay, 1961), followed by lead citrate, or with stains thought to bind preferentially to histones, namely, phosphotungstic acid (Silvermann & Glick, 1969) and potassium permanganate (Davies, 1976). Different patterns of staining were observed and we concluded (Davies, Murray & Walmsley, 1974; Walmsley & Davies, 1975) that condensed interphase chromosomes, or chromatin bodies, are formed from long tubular units (Davies, 1968), diameter about 28-0 nm, by folding and close-packing. We further concluded that the DNA and histones in these units which we call superunit threads, are differentially distributed in space. We did not regard this latter conclusion as rigorously established due to the existence of anomalous binding effects (Davies et al., 1974; Davies, 1976).

We are now attempting to measure the spatial variation in the concentrations of DNA and histones in end-on views of superunits. These data can be expected to be useful in testing models for the higher-order structures in chromatin (Davies & Haynes, 1976; Finch & Klug, 1976; Worcel & Benyajati, 1977; Renz, Nehls & Hozier, 1977). Their intrinsic value lies in the fact that they are obtained on superunits in intact nuclei. The units are, of course, subject to structural changes during preparation for electron microscopy, but these are likely to be less severe than those which occur when nuclei are disrupted and the threads spread out for observation. To obtain the required data it is necessary to measure the stoichiometry of binding of the different electron stains to DNA, histones and v-bodies (Olins & Olins, 1973; Woodcock, 1973) or nucleosomes (Oudet, Gross-Bellard & Chambon, 1975) isolated from chromatin bodies and then to compare these data with the stoichiometry of binding in sections. We show elsewhere (Richardson, Murray & Davies, in preparation) that the numbers of binding sites per unit mass of DNA and histone are similar in section and in solution. The number of available sites is apparently little altered by the resin environment, provided that suitable staining procedures are employed. This paper is essentially concerned with defining these procedures, and involves studies of the kinetics of positive staining. The anomalous binding effects have now been resolved.

There already exist many observations on the binding of electron stains to thin sections through tissues embedded in epoxy resin (references in Hayat, 1975; Horobin & Tomlinson, 1976; Lewis & Knight, 1977). The uptake of stain by any one component depends on many factors which include the fixative, the embedding medium, the nature of the stain and the vehicle in which it is dissolved, and also the concentration,
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temperature and pH of the staining solution. With the introduction of 1-MV electron
microscopes and the possibility of examining thick sections, 500-2000 nm, it became
clear that lack of penetration of stain throughout the depth of the section created
problems which could be overcome by increasing the temperature of the staining
solution and employing ethanol as a vehicle for dissolving the stain (Locke & Krishnan,
1971). Sectioned-section procedures were developed (Peters, Hinds & Vaughn, 1971)
to provide visual estimates of the extent to which electron stains had penetrated into
the section. In this paper we demonstrate that penetration problems can also arise in
thin sections, say about 50 nm thick. Depth of penetration for any given stain is
dependent on the nature and localization of the cellular component, and also on

treatment during preparation for electron microscopy. Furthermore we show that
even when a section is uniformly stained throughout its entire depth, it may not be
fully stained, i.e. only a small fraction of the available binding sites may be occupied.
The electron-scattering densities, $E$, of areas within the cell, relative to surrounding
clear resin, are closely proportional to the concentrations (mass/volume) of bound
stain (Hall, 1955; Cosslett, 1958; Burge & Silvester, 1960; Zeitler & Bahr, 1965).
Measurements of $E$ as a function of both section thickness and staining time clearly
reveal the mechanisms which underlie positive staining, and make it possible to
define the conditions which lead to occupation of all the available binding sites. Our
conclusions and methods should prove generally useful for interpreting electron
micrographs and obtaining quantitative data on the concentrations of molecules
within cells.

METHODS

Preparation for electron microscopy

The methods used in preparing adult hen erythrocytes for electron microscopy have been
described in detail elsewhere (Small & Davies, 1970; Walmsley & Davies, 1975). Briefly whole
cells were processed by the capillary tube method and fixed in glutaraldehyde (3 %), sucrose
(0.4 M), cacodylate buffer (0.01 M, pH 7-2), MgCl$_2$ (1 mM). Some samples were postfixed in 1 %
OsO$_4$ in the same vehicle. Haemolysed cells were obtained by treatment with Triton X-100
(0-5 % w/v), sucrose (0.4 M), MgCl$_2$ (1 mM), Tris (0.01 M, pH 7-5), n-octanol (4 mM) and gum
arabic (3 %), called Triton-SMTOG. Cells were washed in SMTOG and saline and pellets
were fixed in glutaraldehyde (3 %), NaCl (0.075 M), cacodylate (0.01 M, pH 8-0). Following
dehydration pellets were embedded in Spurr resin (1969). Sections were cut with a diamond
knife on a Reichert OMU3 ultramicrotome. Either, individual sections were picked up on
collodion-coated grids, or, a series of sections of varying thickness were picked up on a single
grid so as to ensure that all members could be treated for an identical time with stain. When
testing whether the resin itself took up stain, sections of equal thickness were obtained from a
block, the face of which was scored vertically with a razor blade.

Sections were stained in (a) 2 % aqueous uranyl acetate (UAc) at either 20 ° or 60 °C, followed
by a 5-s wash in distilled water; (b) 2 % aqueous magnesium uranyl acetate (MgUAc) under
similar conditions to (a); (c) uranyl salt (UAc or MgUAc) followed by lead citrate (Pb-citrate)
at 20 °C (Reynolds, 1963) for 1 to 2 min, referred to as uranyl-Pb. Post-staining in lead for this
range of times did not affect the values of $E/100$ nm; (d) Pb-citrate alone for 0.5 to 2 min;
(e) 2 % UAc in absolute ethanol at 20 °C; (f) 2 % aqueous phosphotungstic acid (PTA) at
20 °C; (g) 2 % PTA in 90 % ethanol, 10 % H$_2$O at 20 °C; (h) 0.9 % potassium permanganate in
0.1 M phosphate buffer, pH 6-5 (Soloff, 1973). Sections were stained by floating on solutions
(a) to (e) and by immersion in solutions (f) to (h). In the description of figures, fixation was in
glutaraldehyde only, unless stated otherwise. A thin carbon layer was deposited on the surface of sections which were examined in a Siemens Elmiskop 1 at 100 kV, with a 50-μm objective aperture, and a 200-μm condenser aperture.

Relative electron scattering density (E), section thickness (d) and resin content

Electron-beam currents were measured with a Faraday cage in the final image plane of the Siemens electron microscope at a total magnification of 40000. Under these conditions the ~1-cm diameter of the cage corresponded to 0.25 μm in the section. The electron-scattering density, E, of a stained area in the specimen relative to clear resin is the logarithm of the ratio of the 2 transmitted beam intensities. When a series of sections from the same block were measured the same region was chosen so as to minimize errors due to possible slight variations in section thickness and, or, packing of molecules within cells. The value of E for a single nucleus was measured 3 times and each point on an E-vs-d curve is the average of about 15 nuclei. Each value of E/100 nm quoted in Table 1, p. 262, is derived from a complete E-vs-d curve. Electron-beam irradiation results in loss of both resin and biological material from the section and E is measured after these losses have occurred. This fact is irrelevant to this paper, but measurements of such losses have been made by low-dose methods, since they are needed for the comparisons of the numbers of binding sites in section and in solution which are reported elsewhere (Richardson et al. in preparation). The values of the electron-scattering density of clear resin relative to support film were obtained for a series of sections of increasing thickness, d, measured by the fold-method (Small, 1968). E was, as expected, linearly related to d and hence values of E could be used to estimate section thickness in the absence of folds. E per 100 nm of resin (E/100 nm) was about 0.18 under our experimental conditions.

It is interesting that Small's (1968) data show that the half-widths of folds in the sections, measured in the electron microscope, are the same as the thicknesses calculated from light-microscope interferometry of sections prior to electron-beam irradiation. Evaporation of resin does not cause the widths of folds to alter and this may be due to the carbon layer deposited on the section. This observation in no way affects our analyses since only ratios of E are involved.

The resin contents of the uniradiated sections about 0.75 μm thick and dry mounted on a no. 14 coverslip were calculated from the optical paths obtained by light-microscope interferometry before and after extraction of resin with sodium ethoxide (Lane & Europa, 1965): the refractive index of Spurr resin is 1.52. A Zeiss microscope with × 100 objective, a 0.5 μm measuring-aperture and a photomultiplier were employed. The optical path was first measured after extraction for 1 h. Measurements after 2 and 3 h extraction showed no further change, suggesting that all the resin was extracted and that sodium ethoxide does not result in continued breakdown and removal of biological material. Data on resin content were obtained on newt erythrocytes and reticulocytes and used in the calculations of binding stoichiometries in section (Richardson et al. in preparation). Chicken erythrocytes are too small to permit accurate measurements on their chromatin bodies, but visual observations suggest that the erythrocytes of different species are basically similar. In one block, later used for electron microscopy, measurements on the cell and on clear resin were used to show that the fractional volumes of the cytoplasm and chromatin body occupied by resin were ~34 and ~14 % respectively. When reasonable assumptions (Davies, 1958, and unpublished data) are made for the refractive indices of dry haemoglobin and nucleohistone, calculations (unpublished) show that an appreciable fraction, about 10 %, of the volume of each chromatin body is occupied by free space, that is, it contains neither resin nor biological material. The cytoplasm in this block contained negligible free space. We commonly find that chromatin bodies are more difficult to section than is cytoplasm. This observation is, quite probably, associated with the relatively lower resin content, and possibly, the presence of the free space. Our present data are insufficient to show how much variation there is in the amount of free space in different samples, but there is no doubt that resin content of both chromatin body and cytoplasm varies in different blocks, and this variation must arise during the preparation.
HYPOTHESES

Models

Models for the cytoplasm and chromatin body described in Fig. 1(i) and (ii) respectively, are based on the known chemical composition and ultrastructure of these cellular regions and on light-microscope interferometry (see Methods). Furthermore, they are constructed so as to be consistent with our experimental data. The cytoplasm of erythrocytes consists almost entirely of haemoglobin and it is likely that the molecules will form aggregates during fixation, although individual aggregates are not usually visible in the electron microscope, due presumably to their small size and low contrast. In Fig. 1(i) the circles (dotted) represent stainable regions and consist of an aggregate of haemoglobin molecules plus some resin. The circular stainable regions are separated by clear resin, represented by unshaded regions. $d$ is the thickness of the section. Model 2 (ii) represents a chromatin body. The 2 cylinders (cross-hatched) are sections through thread-like units and consist of folded DNA and histone molecules plus resin; the cylinders are the stainable regions and are shown as separated by clear resin (unshaded). The wavy channels (w ch), free of both resin and biological material, represent a system of interconnecting channels through which staining solution can pass (see text).
Staining kinetics: types A, B and AB: penetration times

The 3 types of staining kinetics can most easily be explained in terms of a Model, like Model 1 for the cytoplasm (Fig. 1(i)). In practice the 3 types of kinetics were not found in the cytoplasm with the stains used. Consider a section constructed out of stainable regions like Model 1, the top surface of which is in contact with the staining solution. We assume that the solution enters the section and traverses the clear resin at a rate \( r_1 \) which will determine the concentration of staining solution surrounding the stainable regions. The build up of bound stain in the stainable regions depends on this concentration and a second rate, \( r_2 \), that at which the solution penetrates, or traverses, the stainable regions; \( r_1 \) and \( r_2 \) need not be precisely defined. Three types of staining kinetics arise depending on the relative values of \( r_1 \) and \( r_2 \). When \( r_1 \gg r_2 \) and \( r_1 < < r_2 \) the kinetics are referred to as types A and B respectively. Type AB lies between these 2 extremes.

Consider a very thin layer of thickness \( \delta x \) situated at a distance \( x \) from the top surface of the section, that in contact with the staining solution. The average concentration of stain, \( c \), bound in this layer will be a function of 2 variables, the staining time, \( t \), and the distance, \( x \). If \( r_1 \gg r_2 \) (type A) then the staining solution will, we can suppose, penetrate the entire thickness of the clear resin in a time which is short compared with the minimum staining time. Hence all stainable regions, independent of their depth, will be surrounded by an equal concentration of stain so that the value of \( c \) reached in a time \( t \) will be independent of \( x \). That is, the section will be uniformly stained throughout its depth. The value of \( c \) increases with time reaching a maximum value \( c_m \) when all the available binding sites are occupied. The hypothetical family of curves relating \( c \) and \( x \) for a series of values of \( t \) is shown in Fig. 2(i). Let \( \delta E \) be the electron-scattering density of the thin layer of thickness \( \delta x \). Then by integration, that is summation,

\[
\int_0^d \delta x = d.
\]

Also,

\[
\delta E = k c \delta x,
\]

where \( k \) is a constant which depends on the electron-optical operating conditions and is approximately independent of the chemical composition of the stain (Hall, 1955). If \( E \) is the electron-scattering density of a section of thickness \( d \) stained for a time \( t \) then

\[
E = \int_{x=0}^{x=d} \delta E = k \int_0^d c \delta x.
\]

A second family of curves (Fig. 2(ii)) showing the relationship between \( E \) and the section thickness, \( d \), can be derived from equations (3) and (1) and Fig. 2(i). Since the value of \( c \) is independent of \( x \), then

\[
E = kc \int_0^d \delta x = kcd.
\]

\( E \) is linearly related to section thickness. The slope at any point is proportional to
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concentration of bound stain at that point and is everywhere constant. The slope \( E/d \) increases with the value of \( c \).

When \( r_1 < r_2 \) (type B kinetics) we suppose that the staining solution slowly diffuses into the resin and rapidly fills all the stainable regions so that the concentration of stain bound to them reaches its maximum value \( c_m \) immediately the staining solution reaches them (Fig. 3(i)). Thus, after a time \( t_1 \), say, the staining solution will

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**Figs. 2-4.** Diagrams showing the 3 hypothetical types of staining kinetics, type A (Fig. 2i, ii), type B (Fig. 3i, ii) and type AB (Fig. 4i, ii). They are explained (see text) in terms of a structure like that shown in Model 1 (Fig. 1). In Figs. 2i, 3i, 4i, \( c \) is the average concentration of bound stain at a distance \( x \) from the surface of a section the top surface of which is in contact with the staining solution. \( c \) reaches a maximum value \( c_m \). In Figs. 2ii, 3ii, 4ii, \( E \) is the electron-scattering density relative to clear resin; \( t_1, t_2, \) etc. are increasing staining times and \( d \) is section thickness. \( x_1 \) and \( d_x \) are particular values.
penetrate a depth $x_1$ into the section, and a layer of thickness $x_1$ will be maximally stained to a concentration $c_m$; for $x > x_1$ the concentration of bound stain is zero. From equations (3) and (1) and the family in Fig. 3(i) the family relating $E$ and $d$ can be deduced (Fig. 3(ii)). If, for example, the staining time is $t_1$ all sections of thickness up to $d_1$ will be maximally and uniformly stained. Sections of thickness greater than $d_1$ will be stained to a depth of $d_1$ only.

Two families in which $r_1$ and $r_2$ have similar values (type AB kinetics) are shown in Fig. 4(i), (ii). The slope at any point on a member of the family in Fig. 4(ii) is, for reasons already given, proportional to the concentration of bound stain at the selected distance from the top surface.

When comparing the behaviour of the different kinds of staining solutions it is convenient to have an approximate measure of the time taken for the solution to penetrate the entire depth of the section. The penetration time, $t_p$, can be defined as the time taken for the concentration of the staining solution at the bottom of a 100-nm section to reach the same value as at the top. The concentration of the staining solution at any depth depends upon the rate of diffusion of stain into the section and time. Conceivably it may reach its maximum value when the concentration of bound stain is still increasing. That is, if at time $t$ the concentration of bound stain has not reached the value found at the top of the section, this does not mean that $t_p > t$. Nevertheless, the following simple considerations indicate that the slopes of the $E$-vs-$d$ curves derived experimentally can be used to set limits to the values of $t_p$. It $t_1$ is the shortest staining time which gives rise to a linear plot then, obviously, $t_p < t_1$. If $t_2$ is the maximum staining time for which the $E$-vs-$d$ curve has zero slope at 100 nm i.e. the concentrations of bound stain and therefore, necessarily, of staining solution are zero, then $t_p > t_2$. When sections are stained both sides the times deduced from the $E$-vs-$d$ curves are doubled, so as to make them comparable to penetration times deduced from sections which have been stained from one side only. Experimental data are given in Table 2, p. 265.

$E$-vs-$t$ curves and comparison with $E$-vs-$d$ curves

In a previous study on permanganate staining (Davies, 1976) $E$ for chromatin bodies in haemolysed erythrocytes was plotted against staining time, $t$, for a series of sections cut to nominally the same thickness, the actual thickness being calculated from measurements of the electron-scattering density of the resin. The value of $E$ initially increased rapidly and reached a constant value at a staining time for which all the available binding sites were occupied. Such $E$-vs-$t$ curves can be useful (see Fig. 15, p. 273) but they have disadvantages. For example what may appear to be a plateau in the $E$-vs-$t$ curve could be a slowly rising curve which is missed due to inherent experimental errors. If the kinetics approximate to those of type B then the time required to stain fully a section will be a function of the chosen thickness, and data obtained on one series of thin sections will not apply to thicker ones. This latter difficulty could be overcome by plotting a family of $E$-vs-$t$ curves. Also, without knowledge of the type of kinetics it is not clear whether the values of $E$ in the steeply-rising part of the curve should be normalized to take into account variations in section
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thickness and this would hinder analysis of an $E$-vs-$t$ family. The major advantages of $E$-vs-$d$ curves are as follows. First they can be obtained to a higher accuracy, since a series of sections of different thickness can be stained and washed under identical conditions, all being on the same grid. They immediately give complete insight into the type of kinetics involved, and hence it is simpler to deduce from them the staining time required for occupation of all the available binding sites. Most important, it is only in $E$-vs-$d$ curves that the origin is a fixed and essentially useful point in determining the value of the slope required for assessing this staining time.

RESULTS

MgUAc, UAc, Pb-citrate: chromatin bodies

The family of $E$-vs-$d$ curves (Fig. 5) for the chromatin bodies in erythrocytes from adult hen treated with UAc-Pb at 20°C (see Methods) follow type A kinetics. The data can be explained in terms of Model 2 (Fig. 1(ii)). The linear relationship between $E$ and $d$ which holds for the shortest staining time of 1 min and upwards, shows that the solutions of both UAc and Pb must penetrate and attain the same concentration throughout the entire depth of the section in a time short compared with 1 min. Hence $t_p < 1$ min (Table 2, p. 265). We suppose that the solution has free access to and traverses the wavy channels shown in Model 2. The staining solutions then diffuse out of these channels and bind relatively slowly to the DNA-histone complex.

$E$-vs-$t$ curves can be simply constructed from the data in Fig. 5. Fig. 6 suggests that
all the available binding sites are occupied, that is equilibrium is reached, in about 10 min. Equilibrium values for \( E/100 \) nm, which are proportional to the concentration of bound stain, are given in Table 1. \( E\)-vs-\( d \) curves (not shown) for haemolysed nuclei are also linear. When UAc is compared with MgUAc about 30% more is bound (Table 1) to chromatin bodies in both intact and haemolysed cells and also to cytoplasm. Post-staining the chromatin bodies in haemolysed nuclei with Pb-citrate for 1 min after either MgUAc or UAc leads to increases in \( E/100 \) nm by factors of 3.14 and 3.16 respectively (Table 1). There is negligible binding of Pb-citrate to the chromatin bodies when treated for 1 min with Pb-citrate alone (Table 1).

![Fig. 6. An \( E\)-vs-\( t \) curve for a 100-nm section through a chromatin body calculated from data in Fig. 5.](image)

Table 1. Values of \( E/100 \) nm for sections through adult hen erythrocytes treated with various staining solutions

<table>
<thead>
<tr>
<th>Region</th>
<th>MgUAc</th>
<th>MgUAc-Pb</th>
<th>UAc</th>
<th>UAc-Pb</th>
<th>Pb</th>
<th>PTA</th>
<th>Unstained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatin body in intact nucleus</td>
<td>0.16</td>
<td>—</td>
<td>0.22</td>
<td>—</td>
<td>0.015</td>
<td>0.39</td>
<td>~0.005</td>
</tr>
<tr>
<td>Chromatin body in haemolysed nucleus</td>
<td>0.15</td>
<td>0.46</td>
<td>0.19</td>
<td>0.59</td>
<td>0.015</td>
<td>0.36</td>
<td>~0.005</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>0.12</td>
<td>—</td>
<td>0.15</td>
<td>—</td>
<td>0.04</td>
<td>0.38</td>
<td>~0.005</td>
</tr>
</tbody>
</table>

Values are for various staining solutions (see also Methods) at 20 °C for times which result in occupation of all the available binding sites, uncorrected for unstained value: \( E/100 \) nm is approximately proportional to the concentration (m/v) of bound stain.

Table 1 shows that for uranyl, and indeed for all stains, the value of \( E/100 \) nm for chromatin bodies in intact nuclei decreases after haemolysis. This decrease could be due to a less-close packing of the superunit threads accompanied by an increase in the volume of the chromatin body and/or to a loss of haemoglobin from the chromatin body. This latter possibility arises from the conclusion based on microspectro-
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photometry of suitably large amphibian erythrocytes (Small & Davies, 1970) that about 10% of the volume of chromatin bodies in intact cells is occupied by haemoglobin at a concentration equal to that of the cytoplasm. Nuclear sap and cytoplasm consist almost entirely of haemoglobin at the same high concentration and their stain uptake (see Table 1 and Figs. 12, 13) approaches that of chromatin bodies. If we assume that the decreases upon haemolysis are due only to loss of haemoglobin then it can be shown from data in Table 1 that the fractional volume of the chromatin body occupied by haemoglobin at the same concentration as cytoplasm is, on average for all stains, about 12%. The fact that this is very similar to the above value of 10% and that chromatin bodies in hen erythrocytes are likely to be similarly constructed to those in amphibia implies that the decrease in $E/100$ nm can be ascribed purely to loss of haemoglobin without any volume change. Loss of haemoglobin from spaces between and perhaps within units and replacement by clear resin could account for the increase in their visibility after haemolysis. Other data on $E/100$ nm (Davies, 1976, and unpublished data) indicate that, additionally, separation of the units can sometimes occur during haemolysis, by amounts which depend upon the vehicle used for haemolysis and fixation. Comparison of figs. 12, 17 in Davies et al. (1974) with figs. 6, 8 in Walmsley & Davies (1975) demonstrate the increased visibility after haemolysis.

Changes in the visibility of the structural units in chromatin can also occur in intact cells. When reticulocytes in a preparation of blood from 4-day chick mature into erythrocytes the visibility of the units diminishes. This is associated with a decrease in the volume of the nucleus and an increase in $E/100$ nm for uranyl-Pb, both consistent with a closer packing of the structural units (Davies et al. 1974)

The above considerations are relevant to the study of the structure and organization of superunit threads in chromatin bodies, as well as in understanding the data presented in this paper.

MgUAc, UAc, Pb-citrate: cytoplasm

The family of $E$-$d$ curves (Fig. 7) for the cytoplasm of hen erythrocytes stained in aqueous MgUAc at 60 °C, followed by Pb-citrate, exhibit type AB kinetics. Binding of stain to the haemoglobin aggregates depicted in Model 1 (Fig. 1(i)) is limited by penetration both into the stainable regions and the clear resin surrounding them and both processes are very much slower than is diffusion into chromatin (Table 2). Consider, for example, a staining time of 30 min. The curve is approximately linear up to about 30 nm. That is, sections of thickness up to this value are evenly stained throughout their depth. In a 120-nm-thick section the concentration of stain decreases between distances of 30 and 60 nm from the top surface and is zero between about 60 and 120 nm. In 30-nm sections the staining intensity in the cytoplasm continues to increase with time, from 10 to 120 min. It is interesting that in silver-gold sections through reticulocytes there is an increase in the observed number of polysomes per unit area as the staining time increases from 10 to 120 min. Evidently after 10 min only the polysomes near the surface are stained, and they appear with high contrast. With increasing time the contrast in the polysomes decreases due to the staining of an increasing thickness of surrounding haemoglobin.
Comparison of $E$-vs-$d$ curves for MgUAc and UAc (not shown), both followed by Pb-citrate, shows that UAc penetrates the cytoplasm more rapidly (Table 2). Measurements (Richardson et al. in preparation) of the binding of uranyl to aqueous solutions of haemoglobin were made at 20°C. Hence, for purposes of comparison, binding of MgUAc and UAc were also studied at 20°C in sections. $E$-vs-$d$ curves for sections up to about 50 nm in thickness, stained for 4 and 24 h were linear with a small difference in slope. We concluded that 24 h is sufficiently long for all the available binding sites to be occupied. Equilibrium values of $E/100$ nm are shown in Table 1. When sections of cytoplasm stained in uranyl at 20°C for 24 h were treated with Pb-citrate for 1 min linear $E$-vs-$d$ curves were obtained, showing that Pb-citrate penetrates sections up to 50 nm thick in less than 1 min.

Extrapolation of the initial linear portion of the curves for 120 min in Fig. 7 suggests that the equilibrium value for uranyl-Pb at 60°C is about 0.42. Pb-citrate alone has an $E/100$ nm value of 0.04 (Table 1), that is about 10% of the uranyl-Pb value. The comparable figure for chromatin is only 2%.

**Binding sites for Pb-citrate**

Very little Pb-citrate is bound to chromatin when used alone. When treatment is for about 1 min $E/100$ nm is 0.01 corrected for the unstained value (Table 1). Similar values are obtained when staining is extended to 15 min. When chromatin bodies in haemolysed nuclei are treated first with UAc, $E/100$ nm is 0.19 (Table 1): further treatment with Pb-citrate produces a large increase to 0.59, i.e. by a factor of about 3.0.
Kinetics of positive staining

The increase due to Pb-citrate, 0.40, is very large compared with 0.01 and hence we can conclude that, in chromatin, Pb-citrate combines almost entirely with bound uranyl. Indeed it may do so exclusively if the sites occupied by Pb-citrate when used alone are occupied by uranyl when it is used first. Calculations based on measurements of $E/100$ nm in thin sections through newt erythrocytes (Richardson et al. in preparation) treated first with uranyl acetate, then with Pb-citrate, showed an increase in $E/100$ nm of both chromatin and cytoplasm by similar factors, which is to be expected if Pb-citrate merely forms a complex with the uranyl, irrespective of the chemical composition of the substance to which the uranyl is bound. The factors are the same, within error limits, if we assume that uranyl acetate combines with those sites which are occupied by Pb-citrate when it is used alone.

| Table 2. Penetration times, $t_p$ in min, for various staining solutions |
|-----------------------------|----------------|-------------------|----------------|----------------|----------------|---|
| Region                      | MgUAc aq.     | UAc aq.          | UAc eth.       | Pb after uranyl| PTA aq.        | PTA eth.       | KMnO₄ |
| Chromatin body in intact nucleus | < 1           | < 1              | —              | < 1            | 8              | 8              | 480   | < 32  |
| Chromatin body in haemolysed nucleus | < 1           | < 1              | —              | < 1            | 8              | 8              | 480   | < 32  |
| Cytoplasm                   | > 120*        | > 120*           | > 120*         | > 120*         | > 120*         | > 120*         | < 32  |

Various staining solutions were applied (see also Methods) all at 20°C, unless at 60°C (*). $t_p$ is defined as the time taken for the concentration of the staining solution at the bottom of a 100-nm-thick section to reach the same value that it has at the top surface in contact with the stain. The value (*) of $t_p$ for Pb-citrate staining of cytoplasm was calculated from data on sections of thickness up to only 50 nm. Limits for $t_p$ are deduced from experimentally derived $E$-vs-$d$ families (see text). There was zero binding of aqueous PTA after 120 min and $t_p$ is arbitrarily marked infinite (∞). Clearly this Table does not present a systematic investigation into penetration times; the limits given are determined by the somewhat arbitrary values chosen for $t_p$ in the $E$-vs-$d$ curves.

Analysis of the structure of chromatin bodies (Richardson et al. in preparation) requires determination of the distribution of uranyl-binding sites within them. These measurements are best performed on cells stained with Pb, since the higher values of $E/100$ nm lead to greater accuracy of measurement. Hence it was important to show that Pb-citrate merely amplifies the scale of the pattern of uranyl stain.

PTA: chromatin bodies

Chromatin bodies in reticulocytes and erythrocytes bind negligible amounts of PTA when thin sections are treated by immersion in aqueous solutions at a pH of 1.5 to 2.0 for times up to 120 min. The family of $E$-vs-$d$ curves (Fig. 8) for the chromatin bodies in intact nuclei stained by immersion in ethanolic PTA follow type AB kinetics. Penetration into sections thicker than about 40 nm is very slow. Even after 240 min,
the staining solution has not reached the centre of 100 nm sections. Fig. 8 shows that to ensure all binding sites are fully occupied it is necessary to use sections less than 50 nm in thickness and staining times in excess of 4 h. Equilibrium values of $E/100 \text{ nm}$ obtained from further $E$-$vs$-$d$ curves (not shown) are given in Table 1.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig8}
\caption{A family of $E$-$vs$-$d$ curves for the chromatin bodies in nuclei of intact erythrocytes from adult hen. Staining was in 2 \% ethanolic PTA for 8 min (○), 60 min (□), 120 min (+), and 240 min (▲).}
\end{figure}

$E$-$vs$-$d$ curves for chromatin bodies in haemolysed nuclei show that penetration is more rapid (compare Figs. 8, 9 and data in Table 2). Sections 100 nm in thickness are fully stained in about 60 min. That is, $t_p$ is about 120 min, allowing for the fact that staining in ethanolic PTA is by immersion.

**PTA: cytoplasm**

Two members of an $E$-$vs$-$d$ family for the cytoplasm of hen erythrocytes are shown in Fig. 10. Sections of about 50 nm thickness treated by immersion in ethanolic PTA are fully stained in about 8 min, a much shorter time than the 240 min required to stain chromatin bodies in intact nuclei.

**PTA: anomalous binding to chromatin bodies**

In a previous study (Davies et al. 1974) of the binding of uranyl-Pb and ethanolic PTA to intact erythrocytes the latter stain was found to behave in an anomalous way. The anomalies were of 2 kinds which will later be shown to be related: bulk effects occurring throughout most of the chromatin body, which we discuss first, and edge effects. We have already noted that maturation of reticulocytes in the 4-day chick is accompanied by an expected increase in $E/100 \text{ nm}$ for chromatin bodies stained in uranyl-Pb, due to a closer packing of the units; but there is an unexpected decrease in the value of $E/100 \text{ nm}$ for ethanolic PTA. In the intact cells from 17-day embryos...
Fig. 9. A family of $E$-vs-$d$ curves for the chromatin bodies in haemolysed nuclei of erythrocytes from adult hen. Staining was in 2% ethanolic PTA for 10 min (○), 20 min (□), 60 min (+), and 120 min (▲). The dotted curve is for the chromatin body in an intact nucleus stained for 30 min.

Fig. 10. A family of $E$-vs-$d$ curves for the cytoplasm of adult hen erythrocytes. Staining was in 2% ethanolic PTA for 8 min (○), and 60 min (□).
which exhibit the edge effects with PTA described below, similar anomalies in bulk staining were also encountered.

In another study (fig. 3 in Davies, 1976) of sections through a block of intact adult hen erythrocytes, containing a small number of chance-haemolysed cells, haemolysis was accompanied by a small (10%) decrease in the value of $E/100 \, \text{nm}$ for uranyl-Pb, accompanied by an increase in visibility of the units (previously attributed to a separation of the units) but by a very large increase ($\sim 73\%$) in PTA-binding.

Previously (Davies et al. 1974) we proposed that a closer packing of the molecules in chromatin bodies leads to a decrease in concentration of sites available to PTA for steric reasons. This hypothesis was based on the fact that PTA has a much higher molecular weight than uranyl, about 3000 compared with 270, and hence a larger size. From the studies in this paper (see also Davies & Richardson, 1976) we are able to demonstrate, without doubt, that this hypothesis is incorrect. It also seems likely that a number of EM cytochemical methods will need to be re-evaluated (see Discussion).

We now conclude that the anomalous bulk effect is due to a relatively lower rate of penetration into chromatin bodies in which the concentration of the molecules is higher. The number of available binding sites per unit volume for PTA is not reduced, but they merely take longer to fill, and the staining time of 30 min previously employed was too short. This is demonstrated in Fig. 9 which shows that in gold sections ($\sim 80 \, \text{nm}$), stained for 30 min, $E/100 \, \text{nm}$ for chromatin bodies in haemolysed cells is more than double that in intact cells, due to very limited penetration in the latter. In erythrocytes, under equilibrium conditions (Table 1) the expected changes in PTA binding are the same, within error limits, as those found for uranyl-Pb. However, packing of nucleo-protein may be so close in some cell types, for example the mature sperm heads of the dog whelk ($Nucella lapillus$ L.), that penetration of ethanolic PTA is essentially negligible even after prolonged staining, although there is intense staining with uranyl-Pb (this laboratory, unpublished observations).

Anomalous edge-effects were encountered in PTA-stained sections through a particular block of 17-day-embryo erythrocytes (figs. 3–8 in Davies et al. 1974). In one region of the section there was a narrow (10–20 nm) dense band of stain immediately adjacent to the inner membrane, extending around the entire periphery of the nucleus and referred to as an $\alpha$-pattern. In an adjacent region a relatively dense band surrounded each individual chromatin body, the so called $\beta$-pattern: see Fig. 12 and the haemolysed nucleus, fig. 9 in Walmsley & Davies (1975). In another region adjacent to the $\beta$-pattern the entire chromatin body was evenly stained, the so called $\gamma$-pattern: see Fig. 13. The values of $E/100 \, \text{nm}$ for uranyl-Pb stained chromatin bodies decreased in going from $\alpha$ to $\beta$ to $\gamma$ whereas the values for PTA increased, i.e. bulk-effects were also present. The edge-effects were explained, incorrectly, by postulating that the molecules at the periphery of a chromatin body underwent different amounts of compaction compared with those in its interior.

The text to Fig. 11 describes how edge- and bulk-effects are related and shows how they arise due to incomplete penetration of PTA into chromatin bodies. Under the idealized conditions used to construct Fig. 11, that is with equal rates of penetration into the sides and top and bottom surfaces of the chromatin body, the width ($w_1$) of
Fig. 11. Schematic diagram showing the origin of anomalous bulk- and edge-effects which arises when sections through chromatin bodies are stained by immersion in ethanolic PTA. Type B staining kinetics are assumed, but similar effects can arise with Type AB. In (i) $E$ is plotted against section thickness, $d$, for cytoplasm (dotted line), and chromatin body (full line) stained for a time which is too short for all the binding sites in chromatin to be occupied for all values of $d$. In (ii) are 4 side-on views of sections of thickness corresponding to positions A-D in (i). The plane of the section is normal to the 2 membranes comprising the nuclear envelope (ne). $w_0$, $w_1$ and $w_2$ are thicknesses of stained regions; $p_1$, $p_2$ and $p_3$ are geometrical paths through a chromatin body, at the edge in contact with the envelope, in the interior and at an interior edge respectively. The cytoplasm and interchromatin regions contain haemoglobin at similar concentrations (Davies, 1961) and are shown as circles containing dots. The $E$-vs-$d$ curve for chromatin has an inflexion at 40 nm. Hence in sections of thickness $d$, where $d \leq 40$ nm, all the available binding sites are fully occupied. In sections where $d \geq 40$ nm top and bottom surface layers are stained to a depth $w_0$, where $2w_0 = 40$ nm, i.e. $w_0 = 20$ nm. The edges of the chromatin body will be stained to a depth $w_0$ and if we assume that stain penetrates embedded haemoglobin in a time which is short compared with chromatin, then $w_1 = w_0$.

An electron micrograph depicts the amounts, mass/unit area ($m/A$) of stain in the different geometrical paths parallel to the electron beam. Detail becomes visible when the value of $m/A$ sufficiently exceeds the surround. The $\alpha$, $\beta$, $\gamma$-staining patterns referred to in the text arose due to differences in the concentration of molecules in different nuclei in one section stained for a constant time. They can also arise when sections of the same thickness through chromatin bodies with the same concentration are stained for increasing times. They can also arise, as in this schematic diagram when sections of increasing thickness through a chromatin body of constant concentration are stained for a fixed time. For sections where $d \leq 40$ nm the chromatin body is uniformly stained, the $\gamma$-pattern (see Fig. 13). In sections where $d > 40$ nm then $p_1 > p_2$ and $p_3$, and $p_2 > p_3$. As $d$ becomes greater than 40 nm first $p_1$ will become sufficiently large for a narrow band of stain to appear around the periphery of the nucleus, the $\alpha$-pattern. As $d$ increases, $p_2$ sufficiently exceeds $p_3$ for a band of stain to appear around the perimeter of each individual chromatin body, the $\beta$-pattern (see Fig. 12).
the densely staining zone around the periphery of the nucleus seen in electron micrographs is equal to half the section thickness at the point of inflexion in the $E$-$vs$-$d$ curve (Fig. 11(i)). Each individual experimentally obtained $E$-$vs$-$d$ curve for chromatin bodies in intact nuclei stained with ethanolic PTA closely resembles the type B kinetics, but the relatively abrupt change depicted in Figs. 3(ii) and 11(i) from finite to zero slope is replaced by a more gradual change. The actual rate of change in slope cannot be well defined due to the paucity of experimental points. The rate of change in slope is less in the chromatin bodies of haemolysed nuclei: compare Figs. 8 and 9. The more rapid the change in slope the better defined will be the denser-staining zone in the electron micrographs. A comparison of electron micrographs of intact nuclei with $E$-$vs$-$d$ curves shows that the widths of the zones (Fig. 12, double-headed arrows) adjacent to the nuclear envelope, in fact, are equal to about half the section thickness at the region of inflexion in the $E$-$vs$-$d$ curves. This is a clear demonstration that our hypothesis which relates edge and bulk effects is correct. The above equality implies that the penetration time ($t_p$) for the regions containing cytoplasmic and nuclear haemoglobin must have an appreciably smaller value than for chromatin, and this is observed: compare the data in Figs. 8 and 10 and see also Table 1.

$KMnO_4$: chromatin bodies and cytoplasm

In an earlier study (Davies, 1976) the use of $KMnO_4$ as a possible stain for protein was explored. It seemed likely that steric hindrance effects would be absent since the molecular weight of permanganate is similar to that of uranyl. The $E$-$vs$-$d$ curves for the cytoplasm and chromatin bodies in both haemolysed and intact hen erythrocytes, fixed in glutaraldehyde and subject to a short wash (10 s) have a particular shape: their slope (Fig. 14 and unpublished data), for the smaller values of $d$, increases with $d$, in contrast to the curves for ethanolic PTA. After a prolonged wash (15 min) all the $E$-$vs$-$d$ curves become linear (see, e.g., Fig. 14). The increasing slope of the short-wash curve could, conceivably, be explained by the permanganate staining very thin surface layers of epoxy resin. The chromatin bodies, in particular, do not contain much resin and hence, $E$ being measured with respect to clear resin, the values of $E$ would be lowered proportionally more for thinner sections. Further, the section thickness, $d$, would be overestimated, particularly for thinner sections since part of the measured
value of $E$ would be due to surface layers of stain. This explanation cannot be correct since potassium permanganate was found not to react with epoxy resin. Another possible explanation for the non-linearity is that as a result of permanganate oxidation biological material and associated manganese atoms are rendered soluble and removed first from the surface layers. However, sections stained for 32 min had similar $E$-vs-$d$ curves to these stained for 16 min, so this explanation involving the loss of biological material can also be eliminated. Hake (1965) has concluded that peptide linkage is not affected by KMnO$_4$. The linearization which occurs with prolonged washing in phosphate buffer suggests the following explanation. We must suppose that the manganese part of the reaction complex has 2 components, one firmly bound and the other loosely bound, the latter being retained in KMnO$_4$ solution but removed by washing. The long- and short-wash curves are identical up to about 40 nm and we assume that the short wash is sufficiently long to remove the loosely bound component from surface layers only. Further washing then removes loosely bound stain from the entire section, leaving it uniformly stained. Calculations (not given) show that KMnO$_4$ itself within the section at a concentration of 1% is not sufficient to account for the effect.

**Fig. 14.** Two $E$-vs-$d$ curves for the chromatin bodies in haemolysed nuclei of erythrocytes from adult hen stained in KMnO$_4$ for 16 min (○), and 32 min (□), both followed by a short wash of about 10 s, and also in KMnO$_4$ for 16 min followed by a long wash of 15 min (+).
Kinetics of positive staining

KMnO₄: anomalous binding to chromatin bodies

Previously one of us (Davies, 1976) demonstrated that chromatin bodies stained in KMnO₄ exhibited anomalous bulk-effects, anomalous edge-effects being absent. In sections from the block of intact hen erythrocytes which contained a small fraction of chance-haemolysed cells, the value of \( E/100 \) nm for chromatin bodies was approximately twice as large in haemolysed nuclei as in intact cells (fig. 3 in Davies, 1976). These anomalous bulk effects were attributed to histone-histone interactions in the superunits which are more concentrated in intact nuclei, leading to a reduction in the number of available binding sites. The \( E \)-vs-\( t \) curves (Fig. 15) for this block, fixed in glutaraldehyde-OsO₄ and used for constructing fig. 2 in Davies (1976) show this hypothesis to be incorrect. The values of \( E \) for cytoplasm in intact cells and chromatin bodies in haemolysed cells both reach a plateau in about 15–20 min. However, \( E \) for intact nuclei reaches a maximum and then decreases, suggesting that KMnO₄ may be removing as well as adding to binding sites. These complete \( E \)-vs-\( t \) curves explain the anomalous low value for chromatin bodies in intact cells previously reported (Davies, 1976). Cells fixed in glutaraldehyde-OsO₄ show another peculiarity. The \( E \)-vs-\( d \) curves for the chromatin bodies in haemolysed nuclei are non-linear but do not become linear after prolonged washing. No data are available to show whether similar effects occur in intact cells. When cells are fixed in glutaraldehyde alone the \( E \)-vs-\( t \) curves for chromatin bodies in both intact and haemolysed cells both reach a plateau.
We have concluded that KMnO$_4$ may be useful as a quantitative stain provided that OsO$_4$ is avoided and that washing conditions are chosen which lead to linear $E$-vs-$d$ curves. The nature of the binding sites for potassium permanganate and the effects of prolonged washing on uranyl and PTA binding are considered elsewhere (Richardson et al. in preparation).

**DISCUSSION**

Our results confirm and extend previous conclusions (Locke & Krishnan, 1971) that an increase in temperature of the staining solution and alcoholic solvents promotes penetration of stains into sections through cells embedded in epoxy resin. The uptake of stain can be considered in terms of the pathways for diffusion of stain, especially those which connect the 2 surfaces of the section. In Model 1 (Fig. 1(i)) there is a continuous pathway of epoxy resin. In Model 2 (Fig. 1(ii)) there are 2 continuous pathways, the epoxy resin and the DNA-histone complex. Staining solutions can be envisaged as diffusing or migrating through the epoxy resin via a system of interconnecting spaces called the resin-pathway. Since the resin is hydrophobic it might be thought that aqueous solutions will not rapidly migrate along it, but this does not necessarily appear to be so. The other pathway in Model 2 consists of hydrophilic DNA-histone threads, chains of nucleosomes. These form continuous molecular links between the 2 surfaces and are, we suppose, surrounded by spaces permitting migration of staining solution. These interconnecting spaces may correspond to the experimentally observed space free of both resin and biological material (see Methods), about 10% in one particular block of intact newt erythrocytes. Further studies are needed to evaluate the presence and significance of this interferometrically determined free space: for example, whether it occurs in the chromatin bodies of haemolysed nuclei and how its magnitude may affect the value of $t_p$. We suppose that the circular stainable regions depicted in Model 1 do contain resin, and clearly there must be free space around individual haemoglobin molecules to which the staining solution has access and which is too small to be assayed by interferometry. The monograph on the physical chemistry of dye adsorption in textiles by Rattee & Breuer (1974) contains a useful discussion of models and the mechanisms of dye diffusion or migration.

Aqueous uranyl at 20°C penetrates the chromatin bodies in a very much shorter time ($t_p < 1$ min) than it does the cytoplasm ($t_p > 20$ min): see Table 2. No doubt aqueous uranyl traverses the hydrophilic pathway in chromatin which interconnects the surfaces. The resin-pathway in the cytoplasm is not readily traversed by aqueous uranyl at 20°C but the value of $t_p$ can be reduced either by increasing the temperature to 60°C, or by using ethanol as the solvent (Table 2). The effect of temperature and ethanol is presumably to increase the movement and average separation of the resin polymer chains, thereby facilitating migration.

Aqueous PTA cannot penetrate sections through either chromatin or cytoplasm (Table 2) and we assume that this is due to the relatively large size of the PTA molecule, associated with its high molecular weight. Aqueous PTA does not readily penetrate chromatin bodies even in the absence of embedding media. When frozen-
thawed purple sections (~100 nm) through haemolysed hen erythrocytes, prepared by cryoultramicrotomy, are stained for 15 min in aqueous PTA they still exhibit edge effects (this laboratory, unpublished observations). This indicates, for reasons already given, that there is incomplete penetration. The use of ethanol as a solvent for PTA does facilitate penetration into chromatin bodies embedded in resin but the values of \( t_p \) are still high, for example > 480 min for chromatin bodies in intact nuclei (Table 2). The suggestion that ethanol facilities transport via the resin-pathway receives some support from the fact that the value of \( t_p \) decreases as the fractional volume occupied by the resin in the cellular compartment increases. Precise data on the resin content of chicken erythrocytes are not available because they are too small to measure by interferometry. In the block of newt erythrocytes for which data are available the resin content of the cytoplasm was greater than that of the chromatin bodies, 34 compared with 14%, and data in Table 2 show that cytoplasm is relatively more easily penetrated than chromatin bodies in intact nuclei. The value of \( t_p \) for cytoplasm lies between 16 and 120 min and for chromatin bodies in intact nuclei is > 480 min. The resin content of chromatin bodies is higher in haemolysed erythrocytes than it is in intact cells, since the superunits can be more easily seen, and data in Table 2 show that the value of \( t_p \) is lower. However, the value of \( t_p \) is not simply inversely proportional to resin content: the presence of chromatin does impede migration of PTA. When staining is with aqueous uranyl the resin content has no affect on penetration. Thus for chromatin bodies and cytoplasm the resin contents differ only by a factor of about 2, whereas the values of \( t_p \) differ by a factor of > 240 (data on aqueous UAc at 20 °C, Table 2). These data support our suggestion that uranyl follows the hydrophilic pathway.

It is interesting that when cytoplasm is stained with KMnO₄, or Pb-citrate after aqueous uranyl, penetration is quite rapid (Table 2). Presumably these molecules in aqueous solution are able to penetrate the resin pathway very much more rapidly than uranyl. Quite likely, simple diffusion laws applicable to uncharged spheres moving through liquids where the rate varies inversely with the cube root of the volume do not apply to movement of stains through macromolecules embedded in resin. Thus the molecular weights of MnO₄⁻, Pb⁺, UO₂⁺, and PTA are 114, 207, 270 and about 3000 respectively, whereas the values of \( t_p \) for cytoplasm treated with aqueous solutions are < 32 min, < 1 min, > 240 min, and \( \infty \) respectively. There may be a critical average pore size in the resin which is just too small to permit rapid passage of uranyl. On the other hand, Horobin & Tomlinson (1976) suggested that Pb may be present in a hydrophobic form which facilitates transport through the hydrophobic resin. Penetration rate of PTA may be reduced by binding of PTA to tissue components thereby reducing the size of pathways for diffusion. If there was a build-up in the surface regions which completely denied access to the interior this would become apparent by constructing an E-vs-d family.

Our conclusion regarding the importance of a hydrophilic pathway connecting the surfaces of the section was reached earlier by Shalla, Carroll & Detzoeten (1964) who studied the apparent length of randomly oriented rod-shaped tobacco mosaic virus as a function of section thickness: staining was in aqueous solutions of strontium permanganate. They concluded that only virus particles with cut ends, i.e. those
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intersecting both surfaces of the section, took up stain. They found, however, that when the sections were sandwiched between thin layers of epoxy resin, staining of virus was prevented, and this appears at variance with our conclusion that KMnO₄ rapidly traverses epoxy resin.

In a study of spermatogenesis in the house cricket (Acheta domestica L.), Kierszenbaum & Tres (1978) have shown that ethanolic PTA can be used as a probe to evaluate the closeness of packing of the protein in the nuclei of cells in the developmental series. During the condensation of the nucleus, associated with a changeover from histones to more arginine-rich proteins, binding sites for PTA become inaccessible and Kierszenbaum & Tres (1978) point out that either PTA fails to penetrate the nucleus, or that PTA gains access and the reactive sites are masked due to cross-linking. Clearly a family of $E$-vs-$d$ curves would enable a choice to be made between these possibilities. The edge effects shown by the mature nuclei in their micrographs suggest that PTA fails to penetrate fully. Our data indicate that if PTA is to be used as a probe in this way then staining times chosen must be sufficiently short to deny penetration into the most condensed states.

It seems likely that, as a result of the experiments reported here conclusions, other than our own, about positive staining of sections will need re-evaluation. For example Pease (1966) concluded that aqueous PTA at a low pH acted as a specific stain for polysaccharides. Glick & Scott (1970) considered this improbable on chemical grounds since PTA does not precipitate many polysaccharides from solution, but readily precipitates most proteins (see also Pease, 1970; Scott & Glick, 1971; Scott, 1971). Pease (1966) found that in tissues embedded in hydroxypropyl methacrylate, treatment with aqueous 5% PTA for 30 min resulted in a high uptake in the PAS-positive (polysaccharide) regions of various cell types but, in rat thoracic aorta, there was negligible uptake in 'elastica', thought to consist mainly of protein. However, there was a dense band of stain around the rim, termed the pre-elastica. When sections were stained at a higher temperature, 37°C, all the 'elastica' stained intensely, as did the previously unstained chromatin bodies of the cell nuclei. Pease (1966) suggested that 'elastica' material was constructed in such a way that the newly reactive groups only became available at 37°C. The 2 staining patterns of 'elastica' closely resemble the $\beta$- and $\gamma$-patterns in chromatin bodies described in this paper. We suggest that the basis of the differential staining reported by Pease (1966) lies merely in differences of penetration rate and that chemical specificity is lacking. Bernhard (1969) has evolved a technique for distinguishing between DNA and RNA. The possibility that the method depends on the cellular location of the structures containing nucleic acid rather than on chemical specificity is being investigated.

In their study of the influences of the embedding medium on positive staining, Horobin & Tomlinson (1976) used a sectioned-section procedure and reported that, in thin sections, only a few structures were deeply penetrated by aqueous and ethanolic solutions of uranyl and PTA at 20°C. Using stains selective for the embedding medium they noted that only structures poorly infiltrated by the non-polar epoxy resin were readily penetrated by polar stains. They concluded that differential infiltration of resin, leading to differential penetration by polar stains, largely controls the
patterns of staining of ultrathin sections by PTA and uranyl. Our data show that penetration of ethanolic PTA is somewhat dependent upon resin content but that penetration of aqueous uranyl is quite independent. Furthermore our data are in disagreement with their conclusion that ethanolic solutions of PTA and uranyl cannot penetrate into the embedding medium and hence cannot stain structures, such as ribosomes, which do not interconnect the 2 surfaces of the section. Our main conclusion which follows from analyses of families of $E$-$vs$-$d$ curves is that, under suitable conditions of staining, the distribution of stain accurately reflects the distribution of available binding sites irrespective of resin content. The relationship between the number of binding sites available per molecule in resin and in solutions or gels of molecular components derived from cells will be described elsewhere (Richardson et al. in preparation).

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