THE OSMOTIC EFFECTS OF ELECTRON

MICROSCOPE FIXATIVES

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ABSTRACT

The reflecting cells on the scales of sprat and herring contain ordered arrays of guanine crystals. The spacing of the crystals within these cells determines the wave bands of the light which they reflect, hence volume changes in the reflecting cells can be observed as color changes directly. This property of the scales is used to show that (a) fixation with osmium tetroxide solutions destroys osmotic activity; (b) fixation with aldehyde solutions does not destroy osmotic activity and does not cause volume changes if the aldehydes are made up in salt or sucrose solutions whose osmolarities, discounting the aldehyde, are about 60% of those to which the cells are in equilibrium in life, and (c) after aldehyde fixation the cells are osmotically active but come to a given volume in salt and sucrose solutions of concentrations only 60% of those which give their volume before fixation. Various possible mechanisms underlying the change of osmotic equilibrium caused by aldehyde fixation are discussed.

INTRODUCTION

The reflectivity of the silvery scales of fish (Denton and Nicol, 1965) results from the presence of a cell layer containing a highly ordered system of very thin guanine crystals. A stack of spaced crystals gives the highest reflectivity at a given wavelength λ_0, together with the widest band of high reflectivity, when both the crystals and the cytoplasmic material separating them have optical thicknesses of $\frac{1}{4}\lambda_0$ (Huxley, 1969). Denton and Land (1967) have shown that a close approach to this condition obtains for scales from several animals, including the sprat (*Clupea sprattus* L.). Fig. 1 (A and B) shows diagrammatically a reflecting platelet from a fish scale; such a platelet lies within one cell.

The wave band reflected by a scale from a fish depends on where it comes from on the body. At near normal incidence those scales from the ventral region of the juvenile sprat reflect light maximally in a wave band centered in the red part of the spectrum, and in white light they appear coppery colored by reflection. The colors of sprat scales arise by interference, so that the fraction of the light which is not reflected by a scale is transmitted, and these scales appear peacock blue in color when viewed in transmitted light (see Denton, 1970).

It is evident that the wave bands of the light reflected and transmitted will alter if the optical thickness of any component of the ordered array of cytoplasm-crystal-cytoplasm-crystal within the cell is altered; for example, if the intercrystal spacing alters, or if the crystals themselves change in optical thickness. Unless there exists within the reflecting system interconnections between the crystals which fix the intercrystal spacing, we should expect, therefore, that volume changes of the entire cell will alter this spacing and manifest
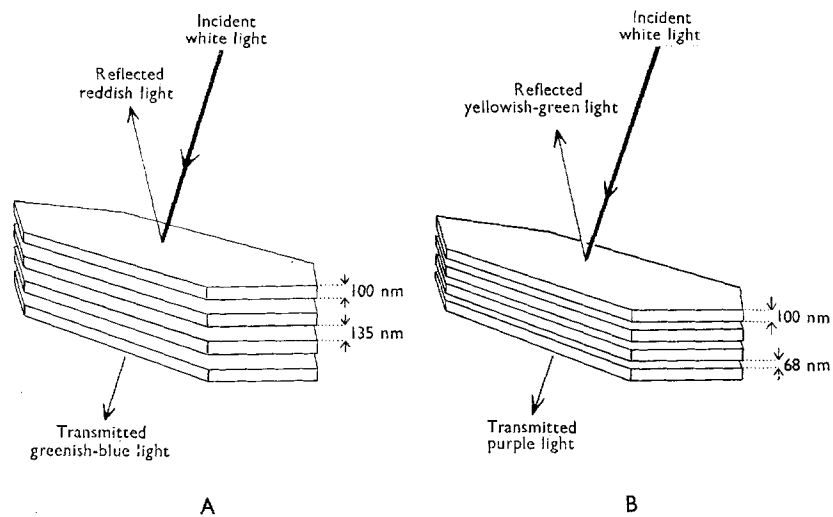
FIGURE 1 (A) Diagram of a platelet showing a stack of crystals of thickness and spacing to give the best reflexion for light of 720 nm. The thickness of the crystals and their spacing is relatively exaggerated. A typical platelet from a scale of a sprat would have a broad surface of about 25 μm × 5 μm, and the whole stack of crystals and spaces would be about 1 μm thick. Platelets of this kind are shown in cross-section in Fig. 2 C. (B) The same platelet as that shown in (A) but the distances between the crystals have been halved and therefore the wave bands of light reflected and transmitted have been very greatly changed. The wave band best reflected is now around 540 nm.

themselves by changes in the colors of light reflected and transmitted. In this paper we show that changes in the osmolarities of external solutions can alter the colors of the reflecting cells, and we use this property to study the osmotic effects of fixatives.

MATERIAL AND METHODS

Most of our observations were made upon young sprats at Plymouth but some observations were made upon young herring (Clupec harengus L) during a visit to the biological station of the Fisheries' Research Board of Canada at St. Andrews, New Brunswick. The fish were anesthetized with MS222, and scales from the ventral region anterior to the anus were carefully removed with forceps. The scales from the sprats were placed initially in marine teleost Ringer's solution (MTR) (Pantin, 1946); those from the herrings in MTR of slightly different composition (Hudson, 1968). They were examined with a binocular dissecting microscope fitted with vertical illumination, and the colors of the scale were noted in different solutions. For some purposes, scales were mounted on a tilting table (described in Denton and Nicol, 1965). Usually, the wave band of maximum reflectivity was estimated from the color of the scale when viewed with transmitted

white light but some quantitative measurements were made with an apparatus designed for measurement of the absorption of light by fresh retinas, described by Denton and Walker (1958) as their Method III. The scale to be studied was placed in the appropriate solution in a small chamber made by fixing a thin ring of nylon to a microscope slide. The chamber was covered with a cover glass and light was passed normally through it. Since the reflected and transmitted lights added together make up the incident light falling onto the scale (Fig. 1 A), the spectral reflectivity of a scale could be determined by finding, for a number of wavelengths, the difference between the intensities of incident and transmitted light, and the ratio of this difference of intensity to that of the incident light.

Since interference colors change with change of angle of observation, it is very important in all experiments, such as those described here, to control the angles at which scales are viewed or measurements made. It was shown, by using the tilting table, that very large osmotic changes, such as those involved in the measurements of Figs. 3 and 4, did not cause changes in the angles of the platelets with respect to the scales under which they lay. If, however, scales were placed under very great osmotic stress, e.g. by putting them into distilled
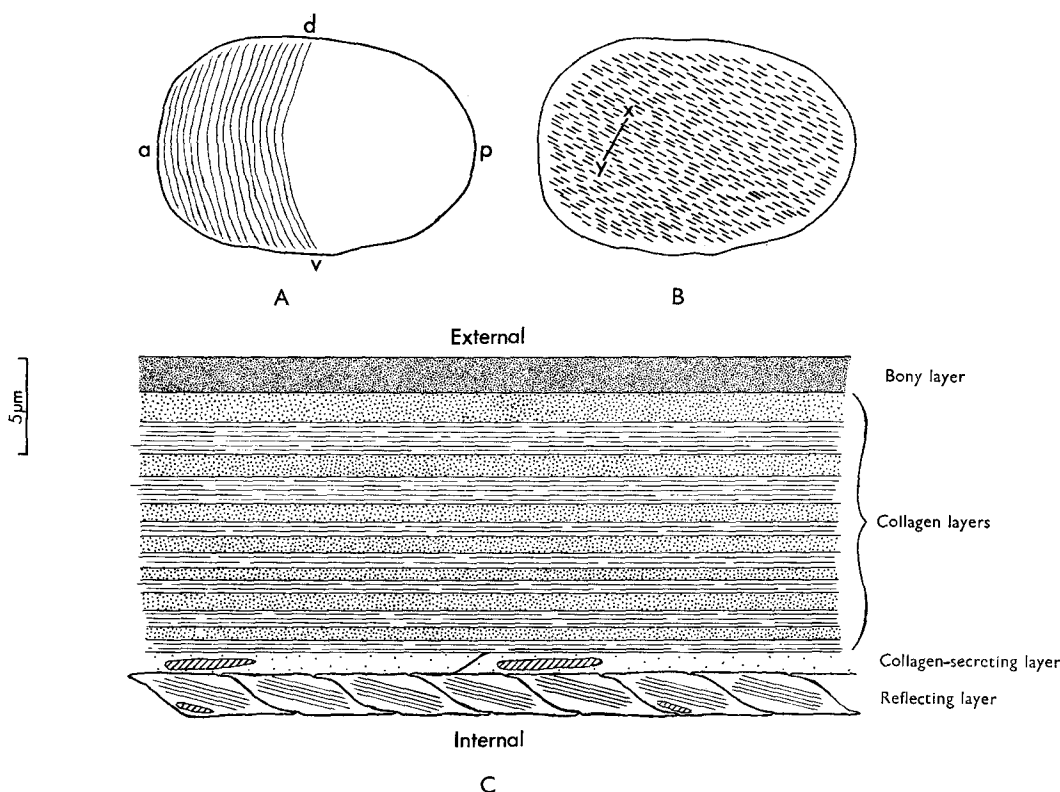
FIGURE 2 Diagrams of a ventral scale from a juvenile sprat. A and B show the flat surface of the scale from the internal aspect. *a*, anterior; *d*, dorsal; *p*, posterior; *v*, ventral. (A) Shows a scale from which the silvery layer has been rubbed away. The anterior part of the scale lies within the scale pocket; this part has small hooked ridges, the circuli, on the external surface of the scale. This scale is about 1 mm long. (B) Like A, but showing the reflecting platelets as dashes parallel to their long axes. The platelets cover almost the whole surface of the scale; they are about 25×5 μm in dimensions and about 1 million are needed to cover each square centimeter of scale surface. The line x–y, perpendicular to the long axes of the platelets, shows the position and direction in which the section C is cut. The circuli are not shown. (C) Cross-section of scale in the region indicated by x–y in (B). The external surface of the scale is upwards. The most external layer of the scale is the bony layer. This layer is laid down only at the edges of the scale and its thickness does not then increase as the scale grows. Collagen is laid down immediately internal to the bony layer as a series of layers between which the fibers differ greatly in the direction in which they run. The collagen layers continually increase in number and are most numerous in the oldest parts of the scale. It is possible that the dimensions and number of the collagen layers in the scales of free-living fish of the same age may differ from those shown, owing to differences in diet between free-living fish and those studied. The collagen is secreted by a single layer of cells which lie against its internal (here lower) surface. Finally, on the inner surface of the scale lie the reflecting cells. Each cell contains a reflecting platelet; here these are cut perpendicular to their long axes. Their broad, flat surfaces are inclined to the plane of the scale.

water, these angles were changed; the platelets are then usually completely and permanently disorganized.

The osmolarities of the solutions in which the scales were placed were found by measuring their freezing points with a Fiske or Advance osmometer.

OBSERVATIONS

In the young sprats, the size of the scales increased as the fish grew, but over the period during which they were studied the wave band of the light transmitted by the ventral scales changed little, if
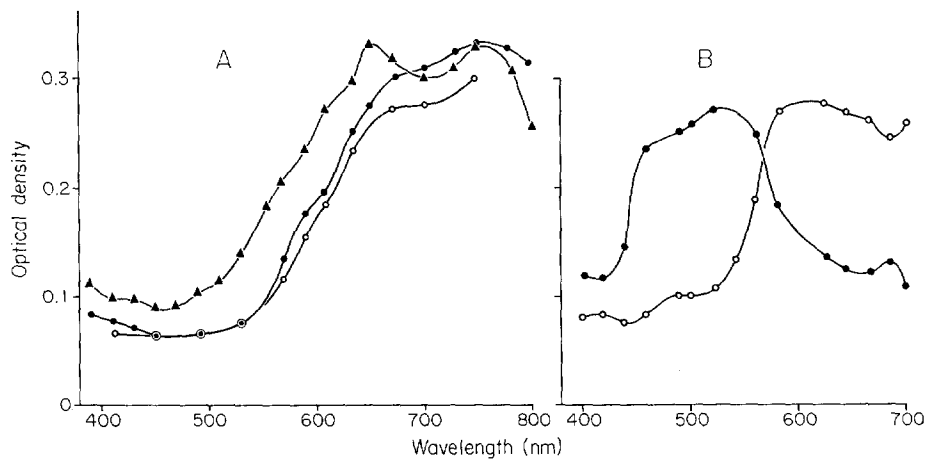
FIGURE 3 The loss of light due to reflexion as it passes through a scale is plotted against wavelength. The ordinate is optical density; the higher its value, the higher is the reflexion of light by the scale. These measurements were made with the light falling at near normal incidence on the scales. A, ventral scales from three different sprats in marine teleost Ringer's; B, a ventral scale from a sprat; O, in marine teleost Ringer's; ●, in a solution in which the salts of marine teleost Ringer's were doubled in concentration. Colored photographs of such scales are given by Denton (1970).

at all, and in MTR the scales appeared uniformly peacock blue over their whole surfaces. The structure of these scales is seen diagrammatically in Fig. 2; details of the structure will be given elsewhere (E. J. Denton and Jane Whish, in preparation). The surface in contact with seawater in the living fish is a thin epithelial layer. This layer covers about one-third of the under surface of the scale; the remainder of this surface lies loosely within the dermis. Around the outer border of the scale are special cells concerned with the growth of a thin bony plate, which is of almost constant thickness over the whole surface of the scale. Internal to the bony plate, over the whole area of the scale, apart from a narrow band around its periphery, there are layers of collagen which increase in number towards the center of the scale, i.e., its older parts. These layers of collagen are secreted by a single layer of cells which lie on the inner side of the scale. The reflecting cells lie immediately internal to these collagen-secreting cells.

Living Cells

When a scale was detached from the fish, a varying amount of the epithelial layer came with it, so that sometimes about a third of the internal surface of a scale was still covered by this epithelium.

Immediate color changes were observed when sprat scales were detached from a fish under seawater. These changes of color could be readily reversed by placing the scales in MTR. It was evident, therefore, that the reflecting cells of at least the posterior two-thirds of the scale were not exposed in life to seawater, but were in osmotic equilibrium with the body fluids. Where portions of the reflecting layer of the sprat scale were still covered by the epithelium in the isolated scale, they were initially less affected by changes in salt or sucrose concentration in the external medium than the remainder of the reflecting layer; and it appears that, in life, the reflecting layer in the sprat is, at least partially, protected from seawater by the epithelial layer.

In MTR the scales exhibited spectral reflectivities and spectral transmissions very close to those seen in scales in their normal array on the fish. When the scales were transferred to diluted MTR or to 2MTR (made up by doubling the quantities of salts used to prepare MTR), the wave band of the light reflected or transmitted altered, and these changes in spectral reflectivity could be reversed by returning the scale to MTR (Fig. 3). These changes were rapid, the main effect occurring within 2 sec. Color photographs showing such changes are given in a review by Denton (1970).

TABLE I

Color Changes Observed in Light Transmitted by Scales When the Composition of the
Surrounding Medium Was Changed

Initial medium	Final medium	Color change
MTR	2MTR*	Blue to purple
2MTR	MTR	Purple to blue
MTR	½MTR	Blue to pale green
MTR	KCl‡ isosmotic with MTR	No change
MTR	NaCl‡ isosmotic with MTR	No change
MTR	K₂SO₄‡ isosmotic with MTR	No change
MTR	Sucrose‡ isosmotic with MTR	No change
MTR	KCl + MTR to make solution isosmotic with 2MTR	Blue to purple
MTR	Distilled water	Blue to silver

* All salts except calcium chloride were doubled in concentration.

‡ These solutions also contained a few millimoles of calcium chloride.

The changes in the wave bands of light transmitted and reflected are those expected if the cells containing the reflecting platelets behaved like simple osmometers, i.e., swelling in solutions hypotonic to MTR and shrinking in solutions hypertonic to MTR. Unpublished observations by Dr. Denton and Dr. M. F. Land have shown that the reflecting crystals of fish scales are not changed in thickness or refractive index when subjected to osmotic changes or to fixatives such as those used in the experiments described in this paper.

Table I shows the results obtained when scales are transferred from MTR into solutions of different composition.

All changes were reversible, except for the last. These observations show that transfer of the scale from MTR to isosmotic solutions containing Na^+, Cl^-, K^+, SO_4^-, and sucrose results in little change of volume of the reflecting cells. When scales were transferred to solutions containing the same substances but more concentrated than MTR, the wave band of light *transmitted* moved towards the red end of the spectrum; in less concentrated solutions it moved towards the blue end of the spectrum. Changes in the external medium of about 50 milliosmols gave changes in color which could be detected by eye. The similarity of the effects of Na^+ and K^+ in different concentrations in the external solution suggests that the reflecting cells are relatively impermeable to both Na^+ and K^+, although the results could be explained if the reflecting cells were impermeable only to the anions Cl^- and SO_4^-. The addition of urea to

solutions bathing the scales caused only transient changes in color as would be expected if the cell membranes of the reflecting cells were permeable to urea, yet less permeable to urea than to water.

Since the colors of the light reflected and transmitted by scales are sensitive to osmotic changes, the scales offer an opportunity of examining easily and directly the osmotic effects of fixatives. We have examined the effects of three fixatives commonly used in electron microscopy.

Fixed Cells

OSMIUM TETROXIDE: Solutions of osmium tetroxide, with or without indifferent salts, and buffered in various ways, have long been employed in electron microscopy. We examined the effects upon scales of transferring them from MTR to 1% osmium tetroxide buffered to pH 7.2 with Veronal buffer (Palade, 1952). Immediately a scale was placed in this fixative, the blue color was replaced by an irregular greyish silver color; this change was irreversible. After fixation for 2 min or less, the silver-grey color of the scale was unaltered when the fixing solution was replaced by MTR, alcohol, or various concentrations of sucrose. Once fixed in osmium tetroxide the scale no longer showed color changes, and the crystal array in the reflecting cell was disorientated.

GLUTARALDEHYDE: The effects of glutaraldehyde solutions were very different from those of osmium tetroxide. Scales were placed in solutions containing glutaraldehyde taken from three

TABLE II

Color Changes Observed in Light Transmitted by Scales on Fixation

Glutaraldehyde-fixing solution	Osmolarity	Color change
	milliosmols	
5% in distilled water	650	Blue to pale green
5% + 1% NaCl*	1000	Blue to purple
5% + 3% sucrose*	750	Blue to green
5% + 7.5% sucrose*	914	Blue to very slightly darker blue
5% in MTR	865	Blue to purple
5% in ½MTR	687	Blue to slightly greener
5% in ⅔MTR	790	Little change

* These solutions also contained a few millimoles of calcium chloride.

different stock solutions: (a) glutaraldehyde 25% (Koch-Light); (b) glutaraldehyde 25% stabilized for electron microscopy (Taab); (c) glutaraldehyde (a) shaken with metallic zinc to reduce glutaric acid. When freshly prepared, this solution has a pH around 7.0.

No differences which could be attributed to the glutaraldehyde were observed when fixing solutions were made up with these different stock glutaraldehyde solutions, and most of our experiments were carried out with the third solution. 5% glutaraldehyde solutions were made up and buffered to pH 7.2–7.4 with sodium cacodylate; the osmolarity of these solutions was increased in some cases by the addition of salts or sucrose.

Table II shows the color changes produced when herring scales were transferred from MTR to fixing solutions of different composition but all containing 5% glutaraldehyde. These changes, like the similar ones observed before fixation, were rapid and complete within a few seconds.

These observations show that glutaraldehyde solutions of even greater osmolarity than MTR give rise to color (volume) changes which are like those found when scales are placed in *hypotonic* solutions of salts or sucrose. After addition of salts or sucrose to make the osmolarity of the fixing solution around 850–900 milliosmols for sprat scales at Plymouth, and around 800 milliosmols for herring scales at St. Andrews, little color change was observed; the cells of the reflecting layer behaved as if they were in solutions isosmotic with MTR. Since it has been found by direct measurement that fixation does not alter the thickness of the guanine crystals, it seems reasonable to ascribe the color changes observed upon fixation with

glutaraldehyde to changes in crystal spacing resulting from changes in volume of the reflecting cells.

It appears from Table II that the original cell volume was best maintained by fixing in solutions about isosmotic with 60% MTR when the glutaraldehyde which they contained was neglected. Electron micrographs of material which has undergone dehydration and embedding can only give indirect evidence of the osmotic effects of fixation, but "good" fixation is obtained when scales are fixed in glutaraldehyde made up in ½MTR. These results suggest that, in fixation with glutaraldehyde, cell volume is dependent only on the concentration of salts and sucrose in the fixing solution, and not on the glutaraldehyde concentration; and indeed, in electron microscopy, glutaraldehyde has been employed at concentrations ranging from 0.5–7.5%.

When scales were transferred from the fixative solution to salt or sucrose solutions, the fixed reflecting cells could still show marked color changes.

Fig. 4 shows the effect of glutaraldehyde fixation. The wave band best reflected in a Ringer's solution of 450 milliosmols is changed greatly by fixation. To give approximately the same spectral reflectivity after fixation, the salt concentration of the Ringer solution has had to be reduced from 450 to 260 milliosmols. Table III gives the results of some direct observations on the color changes caused by fixation.

These observations show that after fixation with glutaraldehyde the cells of the reflecting layer are still osmotically active, but their osmotic properties have changed in a significant way. Solutions of salts and sucrose with an osmotic pressure of
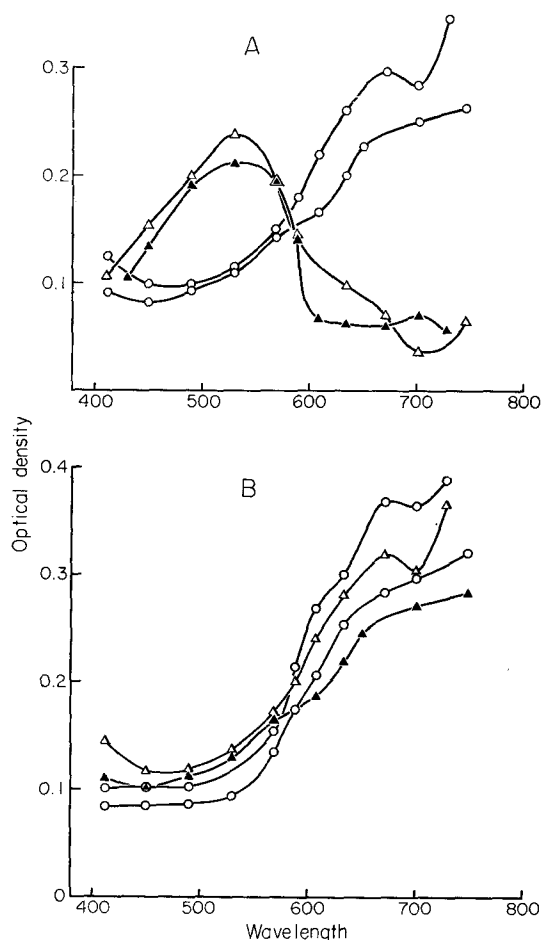
FIGURE 4 Similar to Fig. 3 but measurements made on scales after they had been fixed in a solution of 5% glutaraldehyde containing also the salts of MTR in a concentration equivalent to 260 milliosmols. (A) This shows that after fixation the spectral reflectivity of a sprat scale is still changed by changes in the concentration of the medium in which it is placed. △, ▲ In MTR (450 milliosmolar) the spectral band best reflected is in approximately the same position as that given in 2MTR before fixation (see the left-hand curve of Fig. 3 B). ○, In a solution in which the salts of MTR have been reduced in concentration to give an osmolarity of 260 milliosmols. We see that the spectral band best reflected is in approximately the same position as in MTR before fixation (see the right-hand curve of Fig. 3 B). (B) This shows that the spectral band best reflected by a sprat scale in MTR (450 milliosmols) before fixation is given after fixation in a solution of only 260 milliosmolar concentration. ○, before fixation in MTR; △, after fixation: in 260 milliosmolar sucrose; ▲, after fixation: in 260 milliosmolar Ringer's solution.

around 60% of the original MTR gave the scales the color which they had in MTR before fixation, while the color given in MTR after fixation was not far from that given in 2MTR before fixation. That is, the reflecting cells behaved as if osmotically active indiffusible constituents within the living cells had been reduced to about 60% of their former value after the cells were fixed in glutaraldehyde. We may note particularly that no color change is given on going from a solution containing glutaraldehyde to one which contains no glutaraldehyde, provided that the concentration of such substances as salts and sucrose remains unaltered.

After fixation, most workers wash out the fixative in sucrose, by using either 0.2 M or 0.3 M sucrose solutions, and then postfix in osmium tetroxide solution, before dehydration and embedding.

After postosmication, the scales no longer exhibited color changes (the effects observed were similar to those found when living scales are placed in osmium tetroxide), and it is unlikely that there are significant osmotic effects as the tissue passes through the alcohol series during embedment. However, if scales were transferred from glutaraldehyde, or from 0.2 M sucrose solution, into various concentrations of ethyl alcohol, color changes were observed in the lower alcohols. When scales were transferred from glutaraldehyde to 70% alcohol made up with approximately ½MTR little change in color from that seen in MTR before fixation was observed. After 5 min in the alcohol-½MTR solution, it appeared that the distance between the crystals had been fixed, for little color change was observed when the scales were then placed in distilled water, or in different concentrations of salts. Such a scale appears blue to transmitted light even when it is transferred from alcohol to distilled water and thence to 2MTR.

FORMALDEHYDE: Formaldehyde (prepared from paraformaldehyde) is used in electron microscopy both in conjunction with glutaraldehyde, and occasionally, on its own. The results obtained when herring scales are fixed in formaldehyde solutions were essentially similar to those when glutaraldehyde was used. Only slight change in color was seen when scales were transferred from MTR to 5% formalin made up in ⅔MTR; 5% formalin in distilled water (which has a higher osmolarity than 5% glutaraldehyde) causes

Color Changes Observed in Light Transmitted by Glutaraldehyde-Fixed Sprat Scales upon
Transfer to Different Solutions

Initial solution				Final solution	Osmolarity of final solution	Color
					milliosmols	
5% glutaraldehyde	+ 7.5%	sucrose*		MTR	458	Blue to purple
5% "	" 7.5%	"		7% sucrose	250	Little change
5% "	" 7.5%	"		½MTR	225	Little change
5% "	" 7.5%	"		14% sucrose	450	Blue to purple
5% "	" 7.5%	"		KCl*		
				Isosmotic with ½MTR	250	Little change

* These solutions also contained a few millimoles of calcium chloride.

changes similar to those found when the living tissue is placed in hypotonic solutions; and 5% formol in MTR causes changes similar to those of the living tissue in hypertonic solutions.

After formalin fixation the tissue was osmotically active, but, again, the osmolarities of salt and sucrose solutions required to give particular colors were between $\frac{1}{2}$ and $\frac{2}{3}$ of those needed before fixation.

DISCUSSION

We have shown by direct observation of a single layer of teleost cells that: (a) fixatives containing osmium apparently destroy osmotic activity even after prior aldehyde fixation; (b) fixatives containing glutaraldehyde or formaldehyde do not destroy osmotic activity and do not cause volume changes if the aldehyde is made up in solutions of salts or sucrose whose osmolarity is about 60% of that to which the cells are in equilibrium in life; (c) after aldehyde fixation, even when the aldehydes are taken away, the cells of the reflecting layer behave osmotically as if they contained about 60% of the concentration of indiffusible molecules which they possessed before fixation.

Some of these conclusions have been reached by previous workers, on less direct grounds, using nonteleost material.

Osmium Fixatives

Several workers have found that osmium tetroxide solutions made up in distilled water produce results indistinguishable from those obtained when salts are added to the fixative (Malhotra, 1962; Baker, 1965). Wood and Luft (1965) observed

that the resting potential of frog sartorius fibers fell rapidly upon exposure to osmium tetroxide, and concluded that cell membranes probably quickly lose their relative impermeability to Na^+ when exposed to osmium, "Isotonicity in connection with osmium fixatives being largely irrelevant."

Lenard and Singer (1968) studied the changes brought about in the proteins of the red blood cell membrane after various fixatives (using the reduction in mean molar ellipticity as a criterion of the conversion of helical portions of protein molecules to a more random configuration), and found that the residual helical content in the membrane was greatly diminished after fixation with osmium tetroxide.

Significantly, little change took place after glutaraldehyde fixation, but, after postosmication of glutaraldehyde-fixed membranes, results similar to those following initial osmium tetroxide fixation were observed.

These observations accord with the view that osmium tetroxide so alters cell membranes that tissues are no longer osmotically active after fixation; possibly (as Wood and Luft suggest) the equivalent pore size of the fixed cell membrane is large enough that ions and small molecules can pass without restriction. Elbers (1966) has found that the cell membrane of the *Limnaea* egg very quickly becomes freely permeable to ions on fixation with osmium tetroxide.

Glutaraldehyde and Formaldehyde Fixatives

Previous workers have observed that glutaraldehyde solutions very hypertonic to tissue fluids may

cause tissues to swell, and have found that the best results are obtained when the osmolarity of the buffer solution used in the fixative approaches that of the tissue, the total osmolarity of the fixative including glutaraldehyde being much higher (Torack, 1965; Schultz and Karlsson, 1965). Thus Schultz and Karlsson found that rat brain perfused with glutaraldehyde gave results similar to those with hypotonic fixatives when the total osmolarity of the fixative was the same as that of cerebrospinal fluid, whereas good results were obtained when the buffer osmolarity was that of the cerebrospinal fluid and the total osmolarity of the fixative including glutaraldehyde much higher. It has long been known that formaldehyde fixatives have a very much higher osmotic pressure than the tissues to be fixed (e.g., 4% formalin in distilled water has five times the osmotic pressure of mammalian blood [Baker, 1933]), and that it is necessary to add salts to achieve good fixation for light microscopy. Baker (1965) has found that better fixation for electron microscopy is achieved with formalin solutions when sucrose is added to increase the osmotic pressure. In the case of the reflecting cells of the scale, the osmotic pressure of the aldehyde in the fixative may be neglected and least volume changes are observed when the solution used to dilute the glutaraldehyde has an osmotic pressure around half of that of the living tissue. Most recently, Maunsbach (1966) has carried out an exhaustive study of the effects of varying osmolarity and fixative concentration on mammalian kidney tissue, assessing the results obtained on electron micrographs of processed tissue. He observed that changes of 55–60 milliosmols in the fixative osmolarity were detectable by their effects on fine structure, but did not appreciate that the tissues were still osmotically sensitive after fixation, although Wood and Luft (1965) had earlier observed that "the tissue, even after perfusion with glutaraldehyde is still quite labile and susceptible to change."

Our observations on aldehyde-fixed tissue have shown that cells after fixation come to a given volume in a solution which is markedly hypotonic to that which gives this volume before fixation. If each reflecting cell is enclosed by a semipermeable membrane and if the cell volume is determined by its *content* of indiffusible molecules in solution and by the concentration of indiffusible molecules in the solution in which the cell is placed, the effect of aldehyde fixation could be explained: (*a*) If the

cell's *content* of indiffusible solutes were reduced by about 40% either by their escaping from the cell or being bound or condensed into larger units. As we shall see below, amino acids might be expected to condense into larger units and the effect of the removal, for example, of indiffusible anions might be accompanied by a further loss of diffusible cations. (*b*) If about 40% of the water which the cell contained were normally bound and this water were released from binding, or (*c*) by a combination of possibilities (*a*) and (*b*).

We do not know which, if any, of these explanations is true. Several authors (Flitney, 1966; Hopwood, 1967) have examined the effects of glutaraldehyde upon tissue slices or model protein systems (slices of egg albumen), and have observed shrinkage and loss of protein from the system to the fixative solution. Flitney found, for example, that after 2 hr fixation 11% of the original protein in the albumen slices had leached out. The chemistry of the reaction of glutaraldehyde with proteins has been studied most recently by Habeeb and Hiramoto (1968) who demonstrated cross-linkages of various types between proteins as a result of aldehyde fixation. Direct measurements of the amount of cross-linkage when formaldehyde is added to protein solutions were given by Fraenkel-Conrat and Mecham (1949) using a cryoscopic method to determine apparent molecular weight; we have made similar observations when glutaraldehyde is added to amino acid solutions. These processes are relatively slow, so that neither leaching out of proteins within the cell nor cross-linkage to give larger units could explain the rapid adjustment to a new osmotic equilibrium which is observed on fixing the reflecting cells with aldehyde. It could be supposed that the action of glutaraldehyde upon the cell membrane is such that the pore sizes within the membranes are slightly increased, so that the smallest ion species could escape, but our experiments have shown that after fixation the cell membrane seems to be still impermeable to Na^+ and K^+, and Elbers observed in the *Limnaea* egg that ions are lost from the cell only very slowly after glutaraldehyde fixation.

There probably are differences in the way in which different cell types behave when fixed with aldehydes, perhaps in part due to differences in permeability to various ions in the living state; for example, the effects of dilute glutaraldehyde solutions on muscle cells led Fozzard and Dominguez (1969) to conclude tentatively that glutaraldehyde

increased Na^+ permeability; and, as we have seen, the reflecting cells seem to differ from nerve and muscle cells in their K^+ permeability in the living state. However, it does seem that a wide range of cells show osmotic activity after glutaraldehyde fixation, and that in some of these, at least, changes in osmotic equilibria are brought about by fixation. We have examined crab nerve fibers, and although it is less easy to determine volume changes with such material, it seems that, as in the reflecting cells of the teleost scale, aldehyde fixation leads to a new osmotic equilibrium in which the original volume of the living cells is maintained after fixation by solutions *hypotonic* to those in which the living cells retain their initial volume. From the data given by Maunsbach (his Table I), it appears that the same is also true for mammalian kidney material, for least shrinkage artifacts were observed when the fixing solutions were made up in Tyrode's solutions *hypotonic* to normal Tyrode's solution for living material.

Although the work reported here has been upon a single cell type, peculiarly suited for the study of osmotic effects, the effects observed after aldehyde fixation in the reflecting cells of teleosts apparently are to be found also in other cell types in other animals. In terms of electron microscope fixation practice, this means that it would be worth examining the effects of fixation with aldehyde to which buffer or sucrose solutions (made up to about 60% of the osmolarity of the Ringer solution for the animal) had been added; and further, some advantage might be gained by adding sucrose or indifferent salts to the lower alcohols of the dehydration series.

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