

Cell Shrinkage caused by Fixatives and Paraffin-wax Embedding in Ordinary Cytological Preparations

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SUMMARY

A series of measurements was made of the diameters of the cells and nuclei of the primary spermatocytes of the snail *Helix aspersa* in preparations made by embedding in paraffin wax after fixation in a number of different cytological fixatives. These were compared with similar measurements made on the living spermatocytes mounted in suitable saline solutions, and the shrinkage that the cells underwent as a result of fixation and embedding in each case was calculated from the medians of the size-distributions recorded.

The results showed that the amount of shrinkage varies to some extent according to the fixative employed—picric acid gave the greatest linear cell-shrinkage (about 40 per cent.) and Sanfelice's fluid by far the least (about 13 per cent.); but with this one exception the differences in final cell-size were not very great, and represent a linear shrinkage to about 67 per cent. and a volume shrinkage to about 30 per cent. of the original dimensions of both cell and nucleus.

INTRODUCTION

THE object of this work was a strictly practical one, namely, to measure to what extent the cells seen in ordinary fixed and stained microscopic preparations have shrunk from their size when alive, and how this varied according to the fixative used.

The results obtained may bear to some extent on current theory about fixation, but they will not do so directly, since, although in some cases the fixative is probably responsible for most of the shrinkage in a preparation, any embedding process is an additional modifying factor in reducing the final cell-size still farther, and paraffin-wax embedding after alcohol and xylene or toluene—by far the commonest method, and the one used here—is certainly one of the most drastic processes in this respect. As it happens, measurements of changes in cell-size under the effect of fixatives alone have been made at the same time by Crawford and Barer, and their results, when fully published, should cover this aspect very adequately.

In fact it is rather surprising to find how little work has been done hitherto on size-changes in actual cells. It is true that very similar studies have been made by Berg (1908) and Tarkhan (1931) on pieces of tissue, and by Patten and Philpott (1922) on whole embryos, measured before and after fixing; but not very much can be inferred from these results, because many of the extracellular substances, which form a considerable proportion of these larger [Quarterly Journal of Microscopical Science, Vol. 94, part 2, pp. 125-139, June 1953.]

bodies, may react with fixatives in an entirely different manner from the protoplasm of the cells. Sometimes they are practically inert. In addition, some organs may have large intercellular spaces which usually tend to collapse after paraffin embedding, and cavities are, of course, always present in whole organisms.

In fact, the only really comparable work appears to have been done by Kaiserling and Germer, who in 1893 measured egg cells from cow ovaries, but this was partly invalidated by their unfortunate use of a hypotonic salt solution in the course of their technique—nor was their fixed material subsequently embedded. Thus it was with an eye to filling a gap in elementary data that this present work was attempted.

MATERIAL

It was decided to use the primary spermatocytes of the snail *Helix aspersa*, the gonad of which was invariably found to contain primary spermatocytes at all stages of development in spite of the fact that the work was done in the middle of the winter. The ovotestis was always very small at this time and it was seldom possible to make more than one or two preparations from a single organ; this, however, did serve to establish that there were no very great differences in cell-size between different individuals. In addition the cells themselves have the following advantages:

(1) They are easily recognizable in all preparations—the only other round cells present being Sertoli cells, which are heavily granular and usually larger, and the quite distinctive secondary spermatocytes and young spermatids, which are relatively uncommon in winter.

(2) They have no very specialized organelles and the ratio of nucleus to cytoplasm is fairly normal. They resemble the 'typical animal cell' (in so far as such a concept may be valuable), and, while not very much larger than the average, they are at the same time large enough for dimensions to be measured accurately at least with no larger error than 7 per cent. of the whole, with the technique employed.

(3) The cells are spherical or nearly spherical and in the majority of cases show no great distortion after fixing and embedding, which means not only that only one diametrical measurement is necessary, but that estimations of volume and surface area based on nuclear- and cell-diameters also have validity within the limits of accuracy of the work.

METHOD

During dissection the animal was not placed in any saline solution. The ovotestis was removed rapidly and a portion of it placed for at least 6 hours in the fixative and then passed through alcohol and toluene and embedded in paraffin wax (1 part of melting-point 54° C. to 3 parts 52°). 8- μ sections were cut and the preparations were subsequently stained in iron haematoxylin and mounted in Canada balsam, the whole process being standardized throughout. (The 'post-chroming' and iodine treatments often employed after the use of reagents containing chromium and mercuric chloride respectively were

omitted, since it did not seem likely that they, or any other treatment subsequent to embedding, would affect cell-size.) Cell-diameters were then measured with an eyepiece micrometer and a 2-mm. oil-immersion objective. The diameters of fifty cells and their nuclei were thus measured in each preparation—often all in a single section—and their size-distribution compiled in histogram form with eyepiece divisions forming the units of the abscissa. The microscope was actually set up so that one eyepiece-division equalled 1.25μ ; but in estimating shrinkage in percentages, absolute units are, of course, unnecessary.

These results were compared with the size of living cells similarly observed in teased preparations.

The work was then repeated, generally with fresh reagents and snails from a different locality. As the results obtained did not differ markedly from the previous ones, the two were combined and presented as the sum of 100 cell-measurements. Just how far this step was in fact justified will be discussed below.

PROBLEMS OF THE TECHNIQUE

Various difficulties, however, cropped up as the work proceeded which might have cast doubt on its final validity, and these will have now to be discussed in fuller detail.

Of these, perhaps the most important lay in the fact that in one respect the fixed and living material were not exactly comparable, namely, in the relative numbers of the smaller cells. The ovotestis is a partitioned structure with the larger, more fully developed spermatocytes floating freely in the cavities, while the smaller cells usually lie close to and often adhere to the walls; and, naturally, in teased preparations these tend to get left behind in the debris and fewer are included. This, coupled with the fact that the smaller cells are in any case rather numerous, made it very desirable to devise some criterion, other than size alone, by which the juveniles could be rejected and the study confined to the larger and more mature groups. Fortunately this was possible owing to the fact that during growth the size of the nucleus does not increase proportionately to that of the whole cell: the larger cells have relatively much more cytoplasm, while in the smaller size-groups the diameter of the nucleus approaches very closely the diameter of the whole cell, and often the difference between the two is less than one eyepiece division (1.25μ). Thus, by measuring only those cells with a measurably different diameter of cell and nucleus, one's object is largely achieved. I am grateful to Dr. D. I. Finney of the Bureau for the Design and Analysis of Scientific Experiments (Oxford) for his advice as to the justifiability of this step. It is, however, open to the objection that the assumption is made that the nucleus and cytoplasm shrink equally as a result of fixation and embedding, which is not quite true. (In practice, as will be seen, the nucleus usually shrinks slightly less than the cytoplasm.)

I am indebted to Dr. C. F. A. Pantin for pointing out a second important

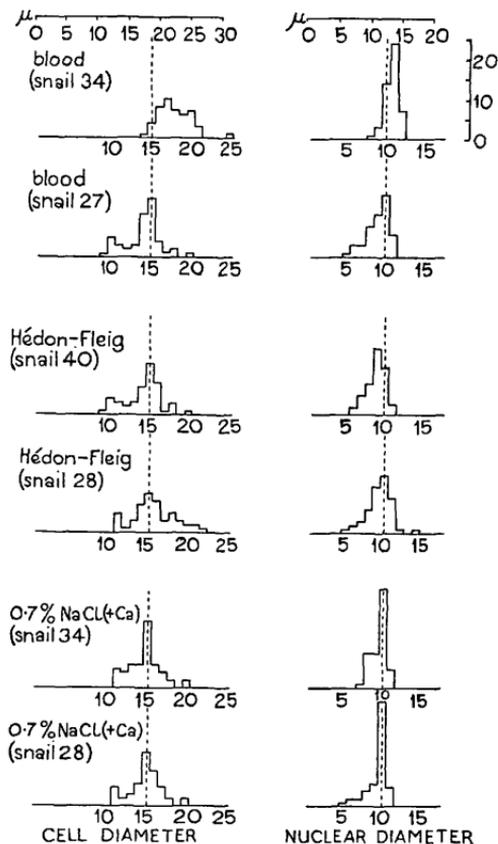


FIG. 1. Frequency polygons showing the size-distribution of living *Helix* primary spermatocytes in snail's blood and various saline media. Fifty cell measurements and fifty nuclear measurements from each preparation.

In this and in all the other figures, the vertical scale (at top right) shows the number of measurements; it is applicable to all the polygons in the figure. The numbers under each polygon constitute a scale of eyepiece-divisions (1 division = 1.25μ); corresponding scales in μ are drawn at the top of the figure.

difference affecting the comparison of size measurements in the fixed and living preparations, and this is that, owing to the aqueous (saline) mounting medium of the latter being of lower refractive index than Canada balsam, the objective has a shorter focal length, and living cells measured in the manner described are bound to appear slightly larger than in balsam; and an experiment had to be performed to investigate the importance of this effect. The

engraved scale of a stage-micrometer was measured with an eyepiece micrometer, first in Canada balsam and then under exactly the same conditions with the balsam replaced by a layer of saline of approximately the same thickness as those in which the living cells were normally measured. The difference was in fact so small that it was difficult to measure accurately, but it was found that the increase of apparent size of the scale in saline was, at the very most, $\frac{3}{4}$ per cent., and this factor can therefore safely be ignored when considering cell-size measurements which are accurate only to the nearest 7 per cent. approximately.

With the living cells it was necessary to find a standard mounting medium. The obvious choice would seem to be snail's blood, and this was in fact used initially, but it was difficult to obtain in quantity except by the crude method of decapitating the animal, with the consequence that it was more or less contaminated with mucus and digestive enzymes, and measurements obtained in it were rather variable. It was therefore decided to use saline media. Hédon-Fleig's (gastropod) saline gave consistent results, but as they were in every way identical with those given in a simple 0.7 per cent. NaCl solution (with the addition of 0.02 per cent. of CaCl_2), the latter was chosen in favour of any more elaborate mixture (fig. 1). (In addition, it was found that this simple saline solution was equally efficient as a medium for culturing snail amoebocytes from pieces of mantle tissue—the technique described by Gatenby and Hill (1934) and used by Crawford and Barer (1951) in their investigations on cell fixation. The cells migrated out of the tissue in exactly the same manner as in Hédon-Fleig's saline and the preparations were in all other respects indistinguishable in appearance.)

It was, of course, necessary to establish that 0.7 per cent. NaCl solution was in fact isotonic, and to do this it was thought advisable to find the limits of these cells' osmotic tolerance by measuring them in a range of salt solutions of different strengths. The results of this are shown in fig. 2. It will be seen that no significant shrinkage or swelling of either the whole cell or the nucleus occurs until the salinity reaches 0.9 per cent. and 0.4 per cent. respectively. Over a remarkably wide range, 0.5 per cent.—0.8 per cent., the modes remain constant, and these modes (15 eyepiece divisions (19μ) for the whole cell, and 10 eyepiece divisions (12μ) for the nucleus) were taken as datum lines from which shrinkages could be estimated. Incidentally, the wide tolerance is of interest in that it indicates a very efficient osmotic compensating mechanism inherent in the cell's metabolism.

The only other difficulty with the living cells was that they might be partially squashed by the pressure of the coverslip and appear larger than they really were; this was overcome by including a small piece of tissue debris in each preparation, thus raising the coverslip slightly at one point, and only cells in this region—which were often seen to be slowly moving and so were definitely free—were measured.

The living spermatocytes were nearly always practically spherical, but in the fixed preparations some degree of distortion often occurred, although the

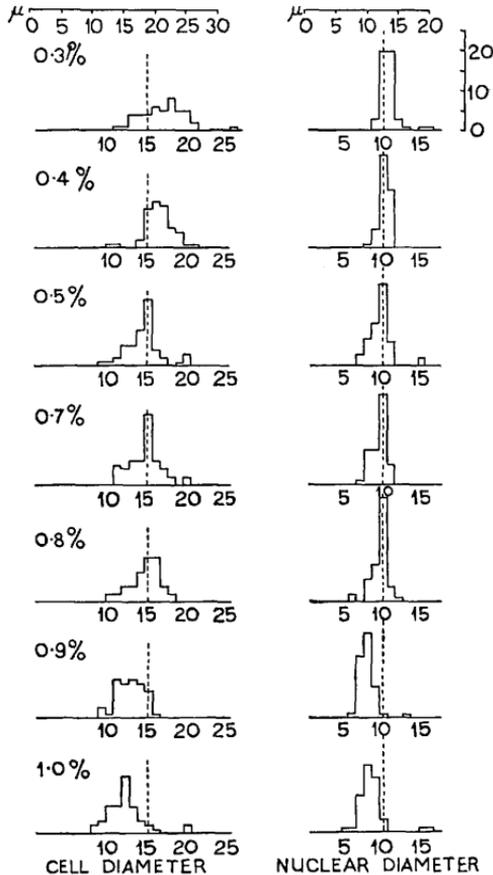


FIG. 2. Frequency polygons showing the size-distribution of living *Helix* primary spermatocytes in sodium chloride solutions at various concentrations. (CaCl_2 was present in each case at 0.02 per cent.). Fifty cell measurements and fifty nuclear measurements from each preparation.

maximum and minimum diameters seldom differed by more than about 10 per cent. Instead of measuring two diameters in these cases, the eyepiece micrometer scale was kept stationary throughout the examination, and diameters were measured as the cells crossed this, regardless of where their axis lay. This was obviously justifiable with loose, unorientated cells.

There is one factor, however, that could affect orientation. It was Mr. H. K. Pusey who drew my attention to the possibility of distortion occurring from

the shearing action of the microtome blade. Aumonier (1938) found that sections of paraffin wax, without embedded material, of the same thickness (8μ), were quite appreciably narrower than their block even after being expanded by flattening; and so, to check this, the following test was carried out on one of the blocks of fixed material. Several consecutive sections were cut, mounted on a slide, and flattened in the ordinary way. The block was then removed from the microtome chuck, and the back of the block was carefully pared down until it was of approximately the same thickness as a microscope slide. It was then mounted on a slide with the surface cut by the microtome uppermost. Its width in the direction of the sweep of the blade and the width of the embedded tissue were then measured with an eyepiece micrometer and a 3-inch objective. These dimensions were then compared with those of the adjacent sections, which were placed on two slides to bring them into the same plane of focus. The results, given in table 1, show that, although the block itself may be distorted by as much as 8 per cent. (which lies just within the limits of accuracy of the cell-measuring technique), the tissue only changes by some 6 per cent., and one can probably safely assume the cells themselves are even less affected by this factor. So this is really more troublesome to the histologist and the micro-anatomist than to the cytologist.

TABLE 1. *Dimensions of flattened paraffin-wax sections compared with that of the block from which they were taken, showing the distortion from the action of the microtome blade. (Block and sections measured with eyepiece micrometer and 3-inch objective.) Paraffin block was approximately one slide thick, and the focus of the microscope was left unchanged when examining sections which were mounted on the two slides. 8- μ sections. 52/54° M.P. wax. (The figures represent arbitrary units.)*

Paraffin block	6.3	Embedded tissue in block	3.7
Section <i>a</i> (proximal)	6.0	" in section	3.5
<i>b</i>	5.8	" " " " " "	3.5
<i>c</i>	5.8	" " " " " "	3.5
<i>d</i>	5.7	" " " " " "	3.5
A gap of three or four sections			
<i>x</i>	5.7		
<i>y</i>	5.7		
<i>z</i>	5.7		
Distortion of whole section $\frac{6}{6.3} = 8$ per cent. approx.		Distortion of tissue $\frac{3.7}{3.5} = 6$ per cent. approx.	

PRESENTATION OF THE RESULTS

Of the fixatives tried, nine were simple substances comprising nearly all the ingredients normally used as constituents of fixative mixtures, while eight were chosen from the most commonly used fixing mixtures. Seven of these—Aoyama, Bouin, Flemming, mercuric-acetic, mercuric-formol, Helly, and Zenker—may perhaps be classified as 'rational' fixing mixtures inasmuch

as their action on protoplasm can, to a certain degree, be inferred from the chemical properties of their constituents; and they can be distinguished from the large numbers of (very often chemically unstable) mixtures in use, whose

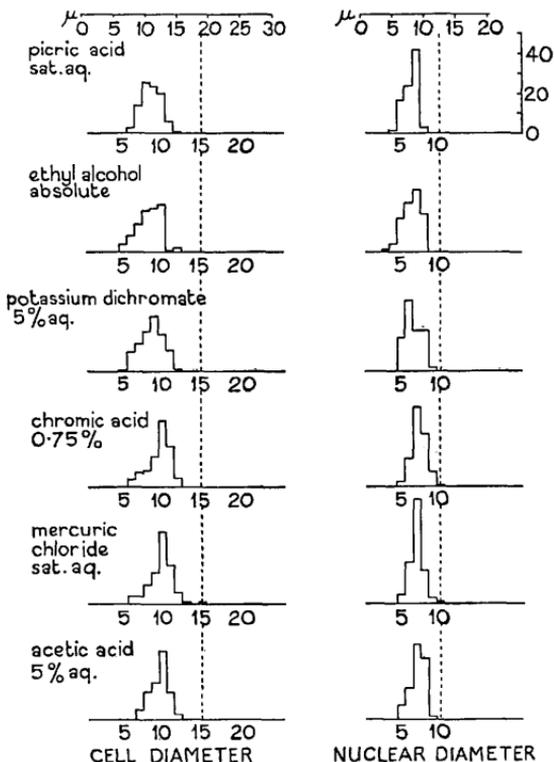


FIG. 3. Frequency polygons showing the size-distribution of *Helix* primary spermatocytes after fixation in various simple fixatives, embedding in paraffin, and mounting in Canada balsam. Measurements from two separate preparations are combined in each polygon (100 cell measurements and 100 nuclear measurements in all).

precise mode of action is by no means clear. It ought to be pointed out, however, that this designation need not necessarily imply condemnation in other respects, and an 'irrational' fixing mixture included here (Sanfelice's fluid) finds favour with many cytologists in giving clear-cut and consistent results. In the case of those mixtures in which the proportions of the ingredients are sometimes varied, a note on the composition used in these experiments accompanies the histograms (figs. 3-5).

The frequency polygon serves as a concise method of presenting the whole collected data, that is to say the total cell-size distribution in each case. The amount the mode has shifted to the left of that of the living material (repre-

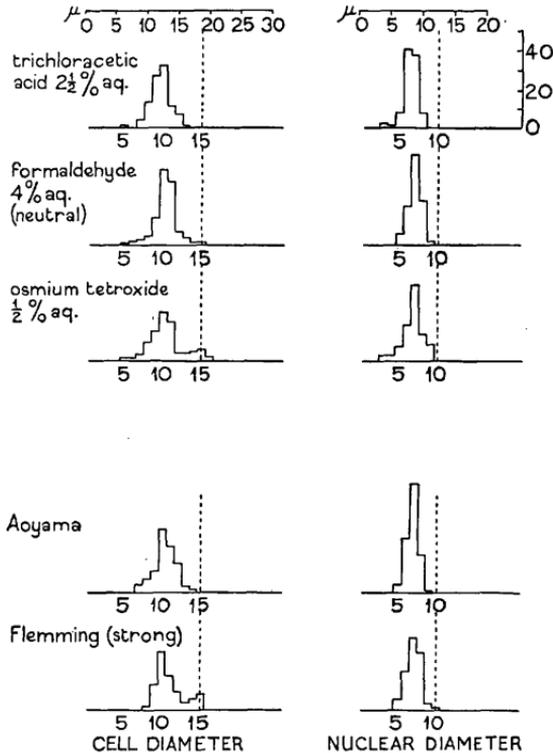


FIG. 4. Frequency polygons showing the size-distribution of *Helix* primary spermatocytes after fixation in various simple fixatives and fixing mixtures, embedding in paraffin, and mounting in Canada balsam. Measurements from two separate preparations are combined in each polygon (100 cell measurements and 100 nuclear measurements in all).

sented in every case by a dotted line) gives an indication of the shrinkage that has occurred. But as a basis for calculating actual figures for shrinkage the mode can be misleading, since the distributions are usually skew and may even be bimodal (as in the case of Sanfelice, fig. 5). It was therefore decided to use the median as the central tendency, and the medians (in μ) for the living material and the fixed are shown in tables 2 and 3 respectively.

As was mentioned above, the work on the fixed material was done a second

time, and in order to see just how closely the two sets of results corresponded, the medians were calculated separately for each set of measurements, and the two compared. It was found that, with respect to whole cell-diameters, with no

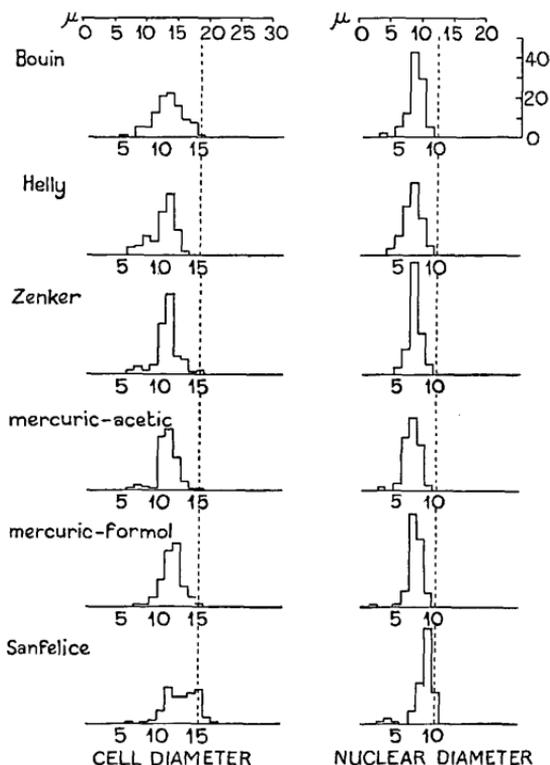


FIG. 5. Frequency polygons showing the size-distribution of *Helix* primary spermatocytes after fixation in various fixing mixtures, embedding in paraffin, and mounting in Canada balsam. Measurements from two separate preparations are combined in each polygon (100 cell measurements and 100 nuclear measurements in all).

Composition of the mercuric-acetic used:

Mercuric chloride, sat. aq.	95 c.c.
Acetic acid (glacial)	5 c.c.

Composition of the mercuric-formol used:

Mercuric chloride, sat. aq.	90 c.c.
Commercial formalin	10 c.c.

less than 11 out of the 17 fixatives tried the values obtained for the first and second experiments differed significantly. With respect to nuclear diameters the correspondence appeared to be rather closer, a significant difference occur-

ring in only seven cases; but as the nuclei are smaller, the measuring is here less critical.

This (at first sight) rather disturbing discovery might be held to cast doubt on the precision of the technique, although it is certainly true that considerable trouble was taken to make the conditions the same for the two sets of experiments. Alternatively, it might be taken to suggest that there may, after all, be an appreciable variation between individual snails with respect to their cell-size, but it can be seen in table 2 that the six living preparations measured differ from each other considerably less with respect to their medians than many of the fixed, and in only one case is this difference large enough to be significant.

TABLE 2. Medians in μ (absolute size) of size-distributions of living cells in saline media.

Snail number	Cell-diameter	Nuclear diameter
34 (0.7 per cent. NaCl) . .	18.4	12.1
28 " . .	18.9	12.2
51 " . .	18.7	..
52 " . .	17.3	..
28 (Hédon-Fleig's saline) . .	19.2	12.0
40 " . .	18.4	11.3
Combined medians . . .	18.5	12.0

On the other hand, when one considers the complexity and individual variability of the chemical substances in an animal cell and the continual changes they undergo during active metabolism, they can hardly be expected to react in precisely the same manner each time they are treated with a given reagent, like two simple chemicals in a test tube. From this viewpoint, it seems less unreasonable that there should be a certain variability of action when the same fixative is used more than once on the same kind of cell. On this contention one might expect a still greater variability to result when the fixative also is not a simple substance, and this is, indeed, suggested by the fact that in most cases the difference between the medians is greater in the case of the fixing mixtures than with the simple fixatives.

Nevertheless, it must be emphasized that although in the majority of cases these differences are large enough to be of statistical significance, they are not in any instance very large; and it is obvious that, even admitting the variability, *approximate* values for shrinkage can be assigned: the crucial question being the limit of accuracy that can be allowed.

On examination of table 3, it can be seen that, with respect to whole cell diameters, the fixatives can be classified into six fairly distinct groups, which can be arranged in order of decreasing shrinkage as follows:

The greatest shrinkage is shown after using certain simple fixatives:

1. Picric acid, ethyl alcohol, and potassium dichromate.
2. The remaining simple fixatives except neutral formalin and osmium tetroxide.

3. Neutral formalin and Aoyama's fluid.
4. A markedly variable group comprising osmium tetroxide, and all the 'rational' fixing mixtures except mercuric-formol.
5. Mercuric-formol.
6. Sanfelice's fluid, which very noticeably shrinks least of all.

TABLE 3. Medians in μ (absolute size) of size distributions of cells in fixed preparations

Fixative	Cell-diameters		Nuclear diameters	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
picric acid	11.5	10.2 S	8.5	7.4 S
ethyl alcohol	10.2	11.2 S	8.1	8.4
potassium dichromate	10.8	11.9 S	7.4	9.1 S
chromic acid	12.5	12.1	9.9	8.5 S
mercuric chloride	12.6	12.1	8.7	8.5
acetic acid	12.5	11.8 S	8.6	9.1
trichloroacetic acid	12.2	11.9	8.2	7.9
neutral formalin	13.4	12.5	8.7	8.7
osmium tetroxide	12.4	14.6 S	8.7	8.3
Aoyama	12.6	13.1	8.0	9.0 S
Flemming	12.5	14.9 S	8.4	9.4 S
Bouin	12.6	14.6 S	8.9	9.0
Helly	12.1	13.8 S	7.6	9.4 S
Zenker	12.8	13.9 S	8.6	9.1
mercuric-acetic	13.2	14.4 S	8.1	9.1 S
mercuric-formol	14.0	14.8	9.3	9.1
Sanfelice	16.6	15.5 S	11.1	10.9

The letter S in the third and fifth columns indicates a significant difference between the medians given by the two sets of measurements.

With respect to nuclear diameters, there is considerable overlap, and classification is a rather more arbitrary affair, but five main groups can be made out which broadly correspond to those for whole cell-size.

Tables 4 and 5 show percentage shrinkages worked out for each of these groups, with the approximate error in each case. The error here is not solely defined by the maximum difference between the medians but is taken to extend beyond them by at least 1 per cent. in each direction. While no claims are made that they are above criticism, the values given are probably essentially correct.

It must be stressed that the shrinkage referred to here is the final shrinkage due to *all* the treatment received by the cell up to and including mounting in balsam. Some fixatives that do not shrink (e.g. acetic acid) permit shrinkage by subsequent procedures.

CONCLUSIONS

For reasons already fully discussed, it is certainly inadvisable to attempt to relate the shrinkages found to the chemical properties of the fixatives, and it

TABLE 4. *Approximate shrinkages of whole cells (per cent.)*

<i>Fixative</i>	<i>Per cent. shrinkage in diameter</i>	<i>Per cent. shrinkage in volume</i>
(1) picric acid ethyl alcohol potassium dichromate	40±6	78±6
(2) chromic acid mercuric chloride acetic acid trichloroacetic acid	34±4	71±5
(3) neutral formalin Aoyama	30±4	66±6
(4) osmium tetroxide Zenker Flemming Bouin Helly mercuric-acetic	27±9	61±14
(5) mercuric-formol	22±4	53±7
(6) Sanfelice	13±5	34±11

TABLE 5. *Approximate shrinkages of cell nuclei (per cent.)*

<i>Fixative</i>	<i>Per cent. shrinkage in diameter</i>	<i>Per cent. shrinkage in volume</i>
(1) picric acid ethyl alcohol trichloroacetic acid	34±6	71±8
(2) potassium dichromate	31±9	67±13
(3) osmium tetroxide mercuric chloride Aoyama Helly mercuric-acetic	28±9	63±14
(4) chromic acid neutral formalin acetic acid Zenker Flemming Bouin mercuric-formol	23±7	54±12
(5) Sanfelice	9±3	25±7

would be unwise to try to draw too many conclusions. Nevertheless, a certain number of points seem to have emerged during the course of the work which can be summarized briefly as follows:

(1) The living snail primary spermatocytes will tolerate quite a wide range of salinity (0.5 per cent. NaCl-0.8 per cent. NaCl inclusive) without any detectable change in size.

(2) Almost without exception, cells embedded in paraffin wax are shrunk more after being fixed with simple fixatives than when fixed with fixing mixtures.

(3) Nuclear shrinkage seems, for the most part, to be fairly consistently related to whole cell shrinkage, but the cytoplasm nearly always shrinks more than the nucleus.

(4) Sanfelice's fluid is remarkable in giving considerably less shrinkage than any other reagent. The mixture contains acetic acid, which, used alone, often initially swells cells, and it might be thought that its use here in conjunction with a protein precipitant (chromic acid) enables the cells to be fixed in a distended condition and shrink relatively less in the subsequent embedding; but this cannot be the whole story, since mercuric-acetic, Zenker's fluid, &c., also contain acetic acid and a protein precipitant, and are in no way remarkable in this respect.

(5) Excepting Sanfelice's fluid, there is no very great difference between the shrinkages given by any of the reagents here tried; and the cytologist who, as a rough approximation, assumes that the cells in a paraffin-wax preparation have shrunk linearly by about *one third* is unlikely to be very far wrong.

This last conclusion is certainly the most important since, in emphasizing the general similarity between all the preparations, it suggests that of the two factors responsible for cell shrinkage mentioned in the introduction—the fixation and the subsequent embedding—the embedding process, which was kept as an invariable in these experiments, is a very important factor in determining the final cell size; and in many cases the individual fixatives may play only a subordinate role. All the reagents used in paraffin-wax embedding, alcohol, xylene or toluene, and molten paraffin-wax tend to shrink cells and, generally speaking, the effect of these reagents together with the fixative is to reduce the *volume* of the cell to about 30 per cent. of what it was in life.

It is necessary to add one final word of caution: all the work described was done with very small pieces of loosely organized tissue, and the reagents, to all practical intents and purposes, had immediate access to every cell. It is by no means certain whether the same results would be obtained in the case of cells deeply embedded in relatively large pieces of compact organs, such as often form the basis of standard histological preparations (as opposed to cytological preparations). This particularly applies in the case of fixing mixtures, for it is known that one ingredient often penetrates tissues faster than another.

ACKNOWLEDGEMENTS

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