A Quantitative Study of the Osmium Impregnation of the Contractile Vacuole of *Chilomonas parameecium* (Cryptomonadina)

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With two Text-figures

INTRODUCTION

THE application of the metazoan Golgi techniques of Weigl (Mann-Kopsch) and Kolatchev to Protozoa has shown that the region immediately surrounding the contractile vacuole in many organisms is very osmiophilic. This was first shown by Nassonov (1924, 1925), who demonstrated the presence of osmiophil cortices around or associated with the contractile vacuoles of *Paramecium caudatum*, *Lionotus folium*, *Nassula laterita*, *Dogielella* sp., *Childon* sp., *Campanella umbellaria*, *Epistylis gallea*, *Zoothamnium arbuscula*, *Vorticella* sp., and *Chilomonas paramecium*. Nassonov attempted to homologize the osmiophil material and the contractile vacuole in these forms with the Golgi apparatus in metazoan cells—a hypothesis which was only accepted in part by later workers.

The osmium techniques have since been applied to a great many different Protozoa, and a vast amount of literature has been published on the whole question of the homology of the Golgi apparatus in Protozoa; this problem has recently been reviewed in detail (Smyth, 1944) and will not be discussed further here.

Apart from the question of homology of the Golgi apparatus, the fact that there is in many Protozoa a ring of osmiophil material surrounding the contractile vacuole is in itself of interest. There is much evidence from the literature of the problem to show that in the hands of different workers the osmium techniques have produced very different results—even in work on the same organism. Any cytologist who has worked constantly with the osmium technique is aware of the fact that it is very inconsistent in its results, sometimes giving beautiful preparations, at others failing to impregnate completely. Gatenby (1941) writes: 'As regards the technique, it must be at once admitted that it can be capricious. The reason or reasons for this are unknown to cytological technicians.'

It is not surprising then to find, that with work based on such a fallible technique, discrepancies in the results of different workers are common. For example, in the case of the Cryptomonad, *Chilomonas paramecium*, Nassonov (1924) impregnated the contractile vacuole with osmium, but did

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not mention whether his preparations were always successful; in the same organism Hall (1930) described the contractile vacuole wall blackened in only 54 per cent. of the specimens; Gatenby and Smyth (1940) stated that in normal undividing cultures of *Chilomonas* the contractile vacuole was impregnated in 99 per cent. of the organisms examined; Patten and Beams, however, were unable to impregnate the contractile vacuole in this organism, though specimens of *Euglena* in the same culture solution were successfully blackened. Similarly, in *Colpidium colpoda* the present writer (1941) showed that the contractile vacuole in this form has a very well-marked osmiophilic cortex, the presence of which had been previously denied by Hall and Alvey (1933) using essentially the same technique.

Numerous other examples could be quoted from the literature of protozoan cytology, but those given above suffice to show that there is a need for an investigation into the factors governing osmium impregnation, and that until these factors are fully understood it seems likely that further confusion will only arise in future work on this problem.

In the present paper a preliminary investigation into some of the possible factors governing impregnation of the contractile vacuole in *Chilomonas paramecium* is described. This organism has the advantage that it has been investigated cytologically by several workers and its general morphology is consequently well known; it is easy to obtain in almost pure cultures which can be maintained for some considerable time without difficulty, and it is sufficiently large to enable its contractile vacuole and related structures to be observed without difficulty.

Previous Work

Only one attempt has been made to throw light on the osmic impregnation of Protozoa by a quantitative investigation. MacLennan (1940) investigated the impregnation of the contractile vacuoles of *Actinosphaerium eichhorni*, *Epidinium caudatum*, *Eudiplodinium maggii*, *Haptophrya michiganensis*, *Ichthyophthirius multifiliis*, *Metadinium medium*, and *Ostracodinium monolobum*. Only in *Haptophrya* and *Metadinium* was the contractile vacuole impregnated in 100 per cent. of the organisms examined. In the remaining ciliates the percentage impregnation was considerably lower, varying between 33 and 64 per cent. MacLennan attempted to explain the inconsistency of impregnation of the contractile vacuole in these forms as being due to the fact that the osmiophily varies with the phase of the vacuole. He states that 'the impregnation of the contractile vacuoles is consistent *when like functional stages are compared* and the apparent inconsistency in impregnation shown in Table 1 (MacLennan's paper) is due to lack of analysis of the data'. He claims that the quantitative variation as shown by his results is correlated with the 'cyclic granular aggregations demonstrated in living specimens'. The 'granular aggregations' referred to by MacLennan when concentrated around the contractile vacuole are considered to represent the so-called 'osmiophil cortex' of Protozoa; he considers there is no true osmiophil

vacuolar membrane in any of the forms studied except *Haptophrya*. Although MacLennan states, 'The slides were searched systematically with the aid of a mechanical stage and all the individuals of the species in question were studied and the impregnation recorded', no details of the numbers of organisms studied were given, nor does he give any statistical evidence as to the accuracy of his results.

Material and Methods

An account of the Weigl method as used for Protozoa has already been given in a previous paper (Gatenby and Smyth, 1940), but a slight variation of the usual technique is used in the present work and will be described here in some detail.

The organisms were obtained from an infusion of soil and leaves to which some boiled hay solution was added. Unlike most flagellates common in hay infusions, *Chilomonas paramecium* will remain in large numbers in such a culture—apparently being able to withstand quite large changes in pH. A good culture will remain in a flourishing condition for several months, provided a little fresh tap-water is added to keep up the water-level, and the culture is covered to keep bacterial pollution at a low level. The organisms were concentrated by gentle centrifuging in a hand centrifuge. After concentration the flagellates were shaken up to break up any clumps and were allowed to remain in about $\frac{1}{4}$ in. of solution in the centrifuge tube for half an hour to allow any cytological disturbances caused by centrifuging to subside. Fixation with Mann's fluid followed. In order to keep uniformity throughout the series of experiments, the amount of fixative was kept constant—3 c.c. were used in every case. This was introduced into the centrifuge tube by means of a fine pipette—the fixative being squirted in very suddenly to produce as instantaneous and uniform a fixation as possible. The tube was then shaken further for a few minutes to complete the mixing. After fixation the organisms were washed in two changes of distilled water—15 minutes each—brought into 3 c.c. of pure osmium tetroxide solution, and transferred to an osmication tube and placed in an oven at 32° C. Details of the tubes and the fixation varied in different experiments and will be described under the various sections. After osmication was completed, or, in some experiments, during the process of osmication, the organisms were removed from the osmium tetroxide with a fine pipette and mounted in a drop of Farrants's medium. This latter mounting medium allows of surprisingly clear cytological observation of the osmicated Protozoa, and has the advantage that it enables a very small number of the flagellates to be removed and examined—a procedure which is difficult if they have to be dehydrated, cleared, and mounted in balsam. The percentage of impregnated organisms was counted carefully by observation under oil immersion, the field being moved uniformly by means of a mechanical stage. In each preparation the number of impregnated organisms in the first 500 observed were taken.

Morphology of Chilomonas paramecium

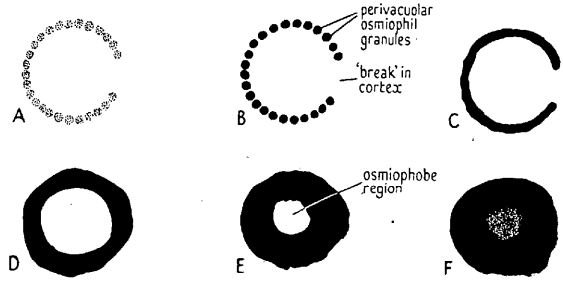
A detailed account of the morphology of this Cryptomonad has already been given in a previous paper (Gatenby and Smyth, 1940) and only a brief description will be included here. The organism is about 20 μ in length, but the size varies considerably with the state of nutrition. The nucleus is spherical and median with a large nucleolus. There are two anterior flagella. The gullet is deep and contains peripheral trichocysts. The single contractile vacuole is anteriorly placed, and lying between it and the nucleus are two (in old cultures) or one (in rapidly dividing cultures) large ovate endoplasmic bodies, which have been identified as pyrenoids. In addition to the osmiophil material associated with the contractile vacuole, a number of small scattered osmiophil granules are invariably found in the region between the vacuole and the pyrenoids.

The Stages of Impregnation

Examination of preparations made at intervals during the osmicating process showed that the impregnation of the contractile vacuole of *Chilomonas* follows a very definite course, which, for the sake of description, can be divided into a number of more or less well-defined stages. The earliest sign of impregnation is the appearance around the contractile vacuole of a ring of light greyish-coloured granules of almost uniform size (A, Text-fig. 1). These granules follow the perimeter of the contractile vacuole very closely, but as far as could be observed do not lie on or in any distinct vacuolar membrane. This ring of granules is complete except for a small break in its periphery, equal in length to about one-sixth of its circumference. The position of this gap is always the same, i.e. it lies on the side of the contractile vacuole nearest to the gullet. The area between the contractile vacuole and the pyrenoids is occupied by a few scattered greyish granules of approximately the same size as those surrounding the cortex.

At a slightly later stage (B, Text-fig. 1) the granules around the vacuole become more heavily impregnated and losing their greyish colour become now a very dense black. At the next stage (C, Text-fig. 1), the material around the vacuole loses its granular nature and appears as a definite osmiophilic ring, which, however, still shows the peripheral break in most cases. Its granular origin is evident from the wavy irregularity of its outline, and the fact that stages intermediate between B and C are easily found in all preparations. As osmication proceeds, the impregnation of the vacuole becomes increasingly heavier and the thin wall found in stage B becomes thickened to form a dense osmiophilic cortex with a small osmiophobic area in its centre (D and E, Text-fig. 1). In preparations osmicated for sufficient time to show both these stages, the peripheral break is seldom visible; there is little doubt from the appearance of the earlier stages that this gap in the cortex becomes obliterated by the over-impregnation of the osmium. Prolonged osmication results in the complete impregnation of the whole contractile vacuole region, and an apparently solid mass of osmiophil material showing an irregular outline is

obtained (F, Text-fig. 1). The impregnation of the central (osmiophobe in earlier stages) area of the vacuole varies quite considerably in this final stage, and is seldom actually as heavy as the cortical region, though this appears to be the case at first sight. By using a very strong and concentrated source of illumination, this central region in some cases can be seen to be made up of a thin osmiophil layer which is in the nature of a membrane lying *within* the more heavily osmiophilic outer layer. In other cases, however, the central region appears to be as heavily impregnated as the outer cortex, and is equally opaque even with very intense light.



TEXT-FIG. 1. Stages in the impregnation of the osmiophil cortex of *Chilomonas paramecium*. Somewhat diagrammatic.

Although the course of impregnation is relatively easy to follow and interpret, it must be emphasized that the various stages outlined above by no means complete the picture, for since the process of impregnation is a continuous one, as is to be expected, a number of stages intermediate between those described above are found in all preparations. Moreover, the degree of impregnation in any culture after a given time of incubation is not by any means uniform, and a number of different stages can be seen in any one preparation.

An analysis of the percentage of different stages found in a typical culture (using sample A OsO_4) showing 70 per cent. maximum impregnation provides some interesting results. After 16 hours' incubation the first five stages A to E are all visible; about 64 per cent. show stages B or C and only some 17 per cent. are in the earliest impregnation phase. Approximately 16 per cent. have progressed to stage D, and a very small number (3 per cent.) show stage E. The complete impregnation of the vacuole (stage F) is never shown in organisms osmicated for less than 30 hours. After 27 hours' incubation the distribution of the stages follows the course expected, namely, there is a decrease in A, B, and C, which is compensated by a marked increase in D and E. After 66 hours stage E reaches its peak and large numbers have reached the total impregnation stage F, while the earlier stages A to D are much less frequent. Further osmication shows that numbers of organisms previously

at stage E have been converted to F, and in the final observation (162 hours) two-thirds of the impregnated organisms are at the F stage, 21 per cent. are at stage E, and only a very small proportion of the earlier stages (total 12 per cent.) can still be found.

At all stages of impregnation the scattered osmiophil granules—the first appearance of which was noted in the earliest stage—were present. Apart from becoming more densely blackened, their appearance changed little during the later period of impregnation.

Impregnation under normal Conditions

Normal conditions are those described under 'Methods' in the earlier sections. The first set of experiments were designed to determine whether under uniform conditions of fixation and osmication the percentage of organisms that are impregnated is constant.

The tubes used to carry out the osmication in this series of experiments were of ordinary drawn glass with measurements of 5×1 cm. Before use they were carefully cleaned first with soap and water, secondly with chromic acid, and finally rinsed in tap-water followed by distilled water. Fixation time was kept constant at 60 minutes. In one series of experiments a number of organisms were fixed simultaneously in the same centrifuge tube, and, after washing, were divided into three parts and transferred to three separate tubes for osmication. A second series of organisms was treated in *separate* centrifuge tubes, and after washing transferred to three separate tubes. Two samples of osmium tetroxide, A and C, obtained from different manufacturers, were used. In previous cytological studies on Protozoa, A had been found to give good impregnation results and C poor results, but the samples had not been tested quantitatively.

The results of a series of experiments of this nature are given in Table 1. The differences between the figures of the percentage osmication with the two samples of osmium tetroxide were in all cases highly significant, the impregnation with A being nearly twice that obtained with C. Organisms with common fixation and separate osmication showed a high degree of uniformity of impregnation, with standard deviations of 0·85 and 1·22 for A and 2·35 and 0·47 for C. It must be noted, however, that although the impregnation of any group of three tubes with a common fixation was fairly uniform, the difference in the figures for any two experiments of the same type with the same sample of OsO_4 was significant, i.e. the results of any one experiment were not reproducible. Cultures, both fixed and osmicated separately, showed standard deviations between the three tubes—in three out of the four experiments—that were significantly higher than cultures with a common fixation.

In order to investigate more fully the process of impregnation under normal conditions the rate of impregnation of vacuole of two cultures using the two samples of osmium tetroxide was determined. The procedure adopted was identical with that described previously for investigating the

stages of impregnation—namely, samples were taken at intervals during the osmication process and the percentage of impregnated organisms—in this case independently of their stages—were counted.

TABLE 1. *Percentage impregnation after common and separate fixation. Two different samples of osmium tetroxide used for osmication*

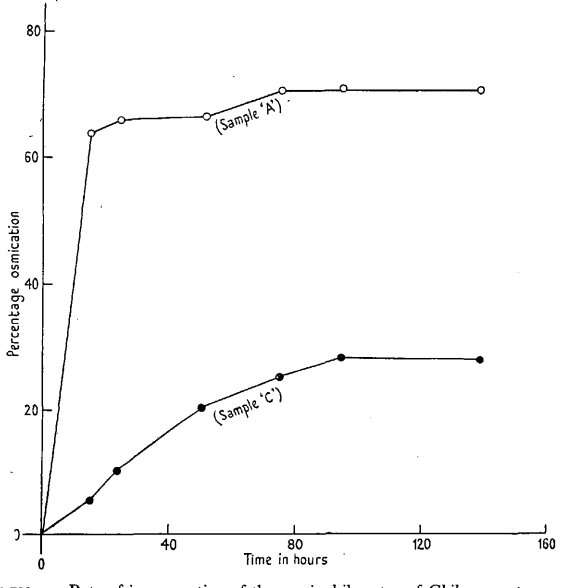
OsO_4 *sample*	*Fixation*	*Per cent. impregnation*	*Mean*	*Standard deviation*	*Standard error*
A	Common	84·0 82·5 84·0	83·67	0·85	0·49
A	Common	71·0 70·5 73·0	71·5	1·22	0·71
C	Common	30·0 25·0 25·0	26·66	2·35	1·36
C	Common	31·0 31·0 30·0	30·66	0·47	0·27
A	Separate	70·0 75·0 85·0	76·66	6·24	3·60
A	Separate	74·0 70·0 62·0	68·66	5·46	3·15
C	Separate	35·0 30·0 30·0	31·66	2·36	1·36
C	Separate	30·0 33·0 36·0	33·0	2·45	1·42

The results are shown graphically in Text-fig. 2. With sample A the maximum impregnation is almost reached within 16 hours, i.e. the majority of vacuoles which will be finally impregnated become impregnated within this time. With the poorer sample of osmium tetroxide—sample C—the initial impregnation rate is slow, but the percentage impregnation rises slowly with time, to settle to a steady figure in about the same time (approx. 100 hours) as sample A. From these curves it is at once evident that prolonged osmication cannot increase the percentage of impregnated organisms in any single culture.

EFFECT OF MIXING ON IMPREGNATION

During the course of examination and counting large numbers of preparations of osmicated *Chilomonas*, it was noted that in the case of very poorly impregnated cultures, i.e. cultures showing less than 30 per cent. impregnation, the impregnated organisms appeared sometimes in clumps in the field

of the microscope. This suggested the possibility that fixation or osmication, or both, was not uniform throughout the culture solution, and that some parts had been better fixed than others or were in closer contact with the osmium tetroxide solution during the incubation.



TEXT-FIG. 2. Rate of impregnation of the osmiophil cortex of *Chilomonas paramecium* with two samples of osmium tetroxide.

In order to eliminate any possible error due to this cause, experiments were carried out in which the solutions were kept in constant motion during the entire processes of fixation and osmication. The tubes containing the organisms were placed in a small box fitted to the axle of an electric motor, which rotated five times a second—a speed sufficient to mix the contents thoroughly and yet not centrifuge them to one end of the tube. The organisms were rotated during fixation, washing, and osmication—in the latter case the whole arrangement being fitted into the incubation oven.

The results obtained are shown in Table 2. The percentage impregnation showed a much greater variation with both specimens of osmium tetroxide than when osmicated under normal conditions. This was especially noticeable with sample A, when the impregnation figures in three experiments fell well below 30 per cent.—a low level never obtained in normal experiments with

this sample. In the first set of experiments of this nature ordinary corked tubes were used as before, and it was noted that since the corks came into contact with the osmium solution more frequently as a result of rotation, the former became more heavily blackened than was usual, and it was thought that this might possibly have some effect on the impregnation. In order to eliminate any factor introduced by this the experiments were repeated with tubes that had been sealed completely by means of a fine hot gas flame. Although some of the individual results were higher than those obtained with

TABLE 2. *Percentage impregnation after continual mixing during fixation and osmication, using corked and sealed tubes, with two specimens of osmium tetroxide*

	OsO_4 'A'		OsO_4 'C'	
	Corked tubes	*Sealed tubes*	*Corked tubes*	*Sealed tubes*
	78·0	41·0	40·0	4·0
	61·0	38·0	29·0	30·5
	25·0	85·0	30·0	26·5
	93·0	90·0	17·5	34·0
	20·0	17·0	15·5	16·0
	17·5	21·0	23·0	70·0
Standard deviation	30·0	25·4	8·3	20·4

corked tubes, the variation between the percentage impregnation for a given specimen of osmium tetroxide was still considerable and in no way compared with the more uniform results obtained by using stationary tubes. It is interesting to note, however, that in one case of osmication with C, the effect of rotation gave an impregnation figure of 70 per cent.—a result considerably higher than any previously obtained with this sample in stationary tubes, and comparable to that obtained with the better sample of the osmium tetroxide.

EFFECT OF FIXATION TIME ON IMPREGNATION

Organisms were treated as for normal osmication, but fixed for different periods of 5, 30, 60, and 90 minutes, both samples of osmium tetroxide being used.

Results of the four experiments carried out are shown in Table 3. It is at once evident that with either sample of the osmium tetroxide the impregnation shows no correlation with the fixation time, and that the variation between the impregnations obtained with either A or C for different fixation times is only that approximately to be expected under normal conditions as shown in Table 1, and as far as can be determined the period of fixation—within the limits of the above experiments—has no effect on the final impregnation.

TABLE 3. *Percentage impregnation for different fixation times, using two specimens of osmium tetroxide for osmication*

Fixation time in minutes	OsO_4 'A'		OsO_4 'C'	
	Exp. 1.1	*Exp. 1.2*	*Exp. 1.3*	*Exp. 1.4*
5	70·0	76·5	33·0	30·5
15	71·0	73·0	40·0	30·0
30	80·0	66·0	31·0	30·0
60	85·0	70·5	31·5	36·0
90	83·0	63·0	36·0	34·0

DISCUSSION

The cytological pictures presented by the contractile vacuole of *Chilomonas* during the various stages of its impregnation are difficult to interpret. The appearance of the osmium precipitate in the form of granules suggests that the so-called osmiophil cortex may be granular in origin. The occurrence of perivacuolar osmiophil 'granules' has been noted previously in a few other Protozoa. In the *Ophryoscolecidiae* MacLennan (1933) has claimed that the warm method of impregnation produced a thick osmiophil membrane around the contractile vacuole, whereas the longer method at room temperature demonstrated the same region to be granular. He also suggested that since the solid cortices around the contractile vacuoles of forms such as *Chilodon* and *Dogielella*, as figured by Nassonov (1924, 1925), showed a distinct granular roughening in the outer region, the solid structures described by him are really artifacts produced by over-osmication.

In *Plagiopyla* (Smyth, 1941) the impregnation of the contractile vacuole goes through a series of stages almost identical with those described in *Chilomonas*: 'The osmiophil cortex first makes its appearance as a thin ring of black beads. On further impregnation the beads coalesce, and the typical osmiophil cortex is produced. Further impregnation makes the ring so thick that the whole structure appears solid.' The phases in this form were not investigated in any great detail, and no figures are available. In *Lagenophrys* (quoted by Gatenby, 1941) Willis has described 'blackening of the accumulated granules which give the appearance of a distinctive Golgi cortex to the contractile vacuole'. Gatenby (1941) has shown that in *Vorticella* during binary fission the osmiophil cortex of the parent cell passes over completely to one daughter organism, whereas a new one is formed in the other by the accumulation of scattered osmiophil granules around the contractile vacuole.

On the other hand, it must be emphasized that there is some evidence from metazoan cytology that granular precipitates of heavy metals—notably silver —may not necessarily indicate the presence of pre-formed granules. Barnett and Fisher (1943), using the acid silver nitrate method to demonstrate the presence of vitamin C in artificial mixtures of olive oil, ground glass, or kieselguhr in gelatine solutions, found that the form of the silver precipitate

had 'no bearing on the prior localisation of ascorbic acid' and concluded that 'it is unjustifiable to infer the whereabouts of ascorbic acid within the cell from the site of the silver precipitates by the silver nitrate method'. Bourne (1944), however, has criticized their results and has cited cases where exact correspondence was obtained between granular mitochondria stained in Janus green B and vitamin C granules in adjacent frozen sections of various tissues (Bourne, 1935; Leblond, 1934; Giroud, 1938). Thus, in some cases at least, metallic precipitates in the form of granules would seem to indicate the presence of pre-formed granules. Nevertheless, the perivacuolar 'granules' in *Chilomonas* have never been demonstrated by methods other than those based on the reduction of osmium tetroxide, and the possibility that these osmiophil granules merely represent the first stage in the reduction of the osmium tetroxide in a specialized area, and not necessarily pre-formed granules, must therefore not be overlooked.

Nassonov (1924) figures the 'broken-ring' type of osmiophil cortex in *Chilomonas* as representing that of a contractile vacuole in diastole, and considered the completely impregnated vacuole to represent the condition found at *systole* by the collapsing of the osmiophil cortex. From the fact that in the present experiments the very early impregnation stages (about 16 hours) showed only open rings, whereas the very prolonged stages showed mainly completely impregnated vacuolar areas, it must be concluded that the so-called 'systole' condition of Nassonov is simply due to over-impregnation, and that the osmiophil material in *Chilomonas* does not change during the vacuolar cycle.

Since the contractile vacuole itself undergoes collapse, whereas the osmiophil cortex does not, it is evident that the latter lies *outside* the region of the contractile vacuole proper. Some workers have taken the impregnation of the vacuolar region as being indicative of the presence of a vacuolar membrane, but if the osmiophilic area lies *outside* the vacuole, this provides no evidence for the existence of such a membrane.

The presence of a 'break' in the osmiophil ring in the early stages of impregnation suggests the presence of a permanent pore by means of which the contractile vacuole is enabled to discharge its contents to the exterior. According to MacLennan (1944), a permanent pore exists in the contractile vacuole region of the *Ophryoscolecidae* where a similar 'break' is found, whereas in *Amoeba proteus*—where there is no perivacuolar osmiophil material (Singh, 1938)—the vacuolar pore is lacking.

The results of the quantitative experiments on the degree of impregnation show that the percentage impregnation with the same specimen of osmium tetroxide and the same fixing fluid can vary quite considerably even under the most carefully controlled conditions. The fact that organisms fixed in the same tube and osmicated separately give much more uniform results than organisms fixed separately, indicates that there is some controlling factor introduced *at the time of fixation*. The only materials concerned in the fixation process are (*a*) the culture solution containing the organisms; (*b*) the fixing

fluid; (*c*) the centrifuge tube. Now since both (*a*) and (*c*) are identical in any series of experiments, the factor must be introduced by either the centrifuge tube itself or by the actual physical action of fixation. Each centrifuge tube used in the present experiments was approximately the same size and shape and had been treated by a similar cleaning process; it is difficult to imagine that there could be any difference between the tubes sufficient to cause such variation as is seen with specimen A (Table 1) where the percentage impregnation varied from a minimum of 62 per cent. to a maximum of 85 per cent.—a difference of 23 per cent.! It is always possible, however, that the process of impregnation is so delicate that the slightest trace of some impurity may upset it, as it is well known that in many chemical and physical reactions a trace of some impurity can act as a retarding agent which inhibits the normal working of the process (Bailey, 1937). It is always evident that the actual process of fixation can never be *identical* since a personal factor is always introduced, and it is possible that the speed and uniformity of the introduction of the fixative into the centrifuge tube plays a part in the determination of the maximum percentage impregnation. Since the controlling factor is introduced at the time of fixation, there is no evidence to suggest that with a given specimen of osmium tetroxide the later stages in the osmication play any part in the determination of the final impregnation figure. The fact that different specimens of osmium tetroxide give such widely different figures is further evidence that the impregnation process itself is a very delicate one that is easily inhibited by impurities; indeed it is difficult to account for the results of different samples by any other hypothesis. There is no evidence to indicate the nature of these impurities, but it is well known that the pH is an important factor in the reduction of metals from solutions, and it is possible that the impurities in different samples are such as to affect this factor.

The results of experiments with the solutions in constant rotation during fixation and osmication indicate that there is nothing to be gained by this technique which only produces extremely variable results. In some individual cases these are higher than those of normal fixation, but on the whole give very poor impregnation. It is difficult to account for the irregular results produced by the mixing, and there is no evidence to indicate what factor is thereby introduced. Considering the small size of *Chilomonas paramecium*, it is not surprising to find that the length of fixation time has no effect in determining the impregnation percentage; but it is interesting to note that the power of impregnation with a poor specimen of the osmium tetroxide is not increased by prolonging the fixation time, as was first thought might be the case.

MacLennan (1940), from work on a number of Protozoa, stated that the irregularity of impregnation was due to a variation in the osmiophily of the vacuolar region with the phase of the contractile vacuole, and that 'impregnation is consistent, *when like functional stages are compared*' (the italics are MacLennan's). Thus he considered his results to be uniform, and correlated

his findings with the fact that, as opposed to metazoan tissues, we are dealing with organisms so small that each part is in close touch with the fluids concerned in the technique. He writes:

'In the Protozoa, the individual cells are separate and the distance of any granule from the free surface is measured in microns rather than millimeters. Thus, fixation, washing, impregnation and every other stage of the Golgi techniques are uniform with respect to every protozoan in the lot, rather than uniform only with respect to a narrow layer of cells equi-distant from the surface. Because of these advantages which are inherent in the Protozoa, it is possible to achieve uniformity of conditions both with respect to conditions of fixation and impregnation and also with respect to conditions within the cell. With these uniform conditions the Golgi impregnations give uniform results.'

These conclusions are open to serious criticism on the grounds that, as far as can be inferred from the data given in his paper, they are based on the results of a *single* series of experiments. Moreover, he does not take into account irregularities which may arise due to the specimen of osmium tetroxide used. It has been shown in *Chilomonas* that this is a factor of major importance, and it is unlikely that with the Protozoa used by MacLennan the same fact does not apply. It seems more likely that the irregularity of his results is due either to the osmium tetroxide used or to some inhibitory agent rather than to a variation in the osmiophily during the vacuolar phases. It is impossible in *Chilomonas* that the osmiophily varies with the vacuolar phases, for although in the present experiments the maximum impregnation reached with normal osmication was only 85 per cent., in a previous paper (Gatenby and Smyth, 1940)—where a better sample of osmium tetroxide was available—consistent impregnations of 99 per cent. were obtained. Since the vacuolar phase must have varied greatly in the large number of organisms used it is evident that in *Chilomonas* there is no relation between the percentage impregnation and the vacuolar phase.

Summary

1. Examination of the contractile vacuole of *Chilomonas paramecium* during progressive impregnation by the Weigl osmic technique revealed that the so-called 'osmiophil cortex' appeared first as a number of perivacuolar osmiophilic granules. Prolonged impregnation caused these to fuse to form a closed ring which, after very prolonged incubation, became a solid osmiophilic mass.

2. Cultures fixed together and osmicated in separate tubes gave more consistent impregnations, in any one series, than those both fixed and osmicated separately.

3. With the two samples of osmium tetroxide used, after normal fixation, A consistently gave impregnations of 62–85 per cent., and C impregnations of 25–36 per cent.

4. Constant mixing during fixation and osmication was not advantageous, and gave more irregular results than normal methods.

5. Time of fixation, between the limits of 5 to 90 minutes, had no effect on the impregnation.

6. It was suggested that the irregularities of impregnation with a given sample of osmium tetroxide were due to the presence of a trace of some retarding agent, possibly introduced at the time of fixation.

7. It was shown that the osmiophily of the perivacuolar region in *Chilomonas* did not vary with the vacuolar phase.

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