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LIGHT-SENSITIVE PIGMENT IN THE VISUAL CELLS OF THE FROG

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The first detailed study of the visual pigments was made by Kühne (1878), who worked with whole retinae. He also discovered a method of preparing solutions of visual pigments, and since it is easier to make accurate measurements upon such solutions, almost all later work has been performed with them.

It is usually supposed that visual pigments behave in solution as they do in the retina, though there is very little evidence that this is the case. Indeed, some of Kühne's observations on retinae have not been explained by more recent work on visual pigment solutions, which indicates that there may be differences.

It therefore seemed worth while to investigate the behaviour of visual pigments in an environment which approximated more closely to that of the living eye than does a purified solution. It is very difficult to carry out spectrophotometric examinations on whole retinae, but it is possible to make from them cell suspensions which are suitable for such analyses, and in which the visual end-organs are relatively undamaged.

In this paper such a method is described, and some preliminary experiments are compared with similar experiments on visual pigment solutions. The differences found will be analysed in subsequent papers.

Kühne's observations were all qualitative. He described colour changes in isolated retinae, and found that those taken from dark-adapted eyes were pink-coloured, owing to the presence in the rods of a pigment, visual purple. When such a retina was exposed to light, its colour changed to orange, and then to yellow. Kühne stated that the yellow colour was due to a breakdown product of visual purple, and he called this substance visual yellow. Visual yellow slowly faded, and as it did so the retina began to fluoresce green in ultra-violet light (quite apart from its usual blue fluorescence). Kühne explained this by supposing that visual yellow turned into another substance,

visual white. When the retina was returned to the dark, this sequence of changes could be reversed, and visual purple reformed. Kühne therefore suggested that in the living retina visual purple underwent a cyclical process of bleaching and regeneration.

Lythgoe, in 1937, analysed the bleaching of visual purple in solution. In solution, the product of bleaching is a yellow substance, which Lythgoe named indicator yellow, since he found that its absorption spectrum varied with pH. Indicator yellow was the end-product of bleaching, and no analogue to visual white was detected.

The chemistry of the visual pigments was investigated by Wald (1935). Visual purple is a chromoprotein, and Wald showed that its chromophore was a carotenoid, which he called retinene. Retinene could be easily extracted from the retina soon after exposure to light. Wald thought that the action of light on visual purple was merely to split the chromophore from its protein, and that the uncombined retinene was visual yellow. When the bleached retina had faded to Kühne's visual white stage, another carotenoid, vitamin A, could be extracted from the retina.

Morton & Goodwin (1944) showed that retinene was vitamin A aldehyde, and Wald (1949) then showed that the reduction of retinene to vitamin A could be accomplished in the retina by alcohol dehydrogenase, an enzyme which is linked to coenzyme I.

Morton, Collins & Love (1952) have now shown that indicator yellow is a retinene-protein conjugate. Pure retinene does not possess indicator properties.

The object of the experiments described below was to discover whether the behaviour of visual pigments was the same inside the visual receptors as it is in solution. Kühne's terminology is therefore used in the initial description of the results, since it refers to changes which occur in whole retinae and is non-committal. One exception has been made. Kühne knew of only one visual pigment, visual purple, but since his time others have been discovered. Throughout this paper reference is made to the 'visual pigment' contained in the frog outer limbs, in order to avoid the question (which is discussed in a later paper) whether it has the same properties as the visual purple which can be extracted from frog retinae.

APPARATUS AND METHODS

The end-organ suspensions were examined in a spectrophotometer very similar to that described by Dartnall (1952). The differences are due to the fact that the instrument used in the present experiments was designed for use in both the visible and ultra-violet parts of the spectrum. The beam of light from a car headlamp bulb was focused on the entry slit of a monochromator system, which consisted of two monochromators arranged in series. Both monochromators had front-aluminized mirrors and quartz prisms. The light beam which was dispersed by the first prism was ocused on the entry slit of the second monochromator. In this way stray light was reduced to a minimum.

The light which left the second monochromator passed through an aperture in a cell holder identical with the one described by Dartnall. It consisted of a brass block which slid to and fro on its base so that one of two slots cut in it intercepted the light beam. These slots held the quartz absorption cells. Holes were bored in the block so that water could be pumped round the absorption cells. The water was kept at a constant temperature by a 'Sunvic' thermostat so that the cell holder was always at $20{\cdot}0 \pm 0{\cdot}1°$ C.

After the light had passed through one of the absorption cells it fell on the photocathode of a RCA 931 A photomultiplier. The output from the photomultiplier was fed, via a variable dropping resistance, into a critically damped Cambridge galvanometer of the lantern and scale type.

The suspensions were bleached by exposing them for 10 min to the focused light spot coming from the galvanometer lantern.

Calibrations. The band-width of the monochromator was determined by measuring the displacement of the wave-length drum necessary to move a spectral line across the exit slit. It varied from 1·7 mμ at 405 mμ to 7·6 mμ at 579 mμ.

The photocell was carefully checked to see whether the relationship between its output and the light intensity falling on the photocathode was linear. It was found to be so within 1% over the range of intensities used in the experiments. The relationship of galvanometer deflexion to applied current was also found to be linear to within 1%. Wave-length calibrations were performed every fortnight with mercury and cadmium metal vapour lamps. Twelve spectral lines were used for calibration, from 324 to 579 mμ. The wave-length calibration was found not to vary. The performance of the apparatus was compared with an entirely different spectrophotometer, the Unicam S.P. 500. Both gave the same experimental results, indicating that they were free from systematic errors.

Material. Suspensions were prepared from the retinae of dark-adapted frogs, *Rana temporaria*. The suspensions were made in a dark-room illuminated by a deep red safe-light. The frogs were decapitated and the heads washed clean of blood. The retinae were removed by the method described by Lythgoe (1937). A cut was made in the cornea, and both lens and retina squeezed through it by pressing on the roof of the frog's mouth. The retinae were dropped into a centrifuge tube which contained frog Ringer-Locke solution, and shaken. This detached the outer limbs of the end-organs from their cell bodies. The tube was then spun for 20 min and the supernatant fluid, which contained some blood, was removed. The outer limbs were separated from the other cells of the retina by Saito's method (Saito, 1938). The compacted mass in the tube was shaken up in 35% (w/v) sucrose solution, and centrifuged once again. The outer limbs floated in the sucrose, while the rest of the retina sank to the bottom of the tube. The sucrose was pipetted off and kept. Three or four washings with sucrose removed almost all the outer limbs. The retinae of six small frogs provided 2 ml. of a suspension in which the density of visual pigment was 0·5 for a 1 cm path-length.

RESULTS

Microscopical appearance of the suspensions

The appearance of outer limbs suspended in sucrose was compared with the appearance of those which had been detached from the retina by shaking in Ringer-Locke solution.

In the first 2 hr after removal of the retina the cells in both sorts of suspension appeared the same. Many rod outer limbs could be seen (see Pl. 1 *a*, *b*). They were cylindrical, refractile, and had smooth outlines. Their lengths varied between 14 and 83 μ, and the diameter between 3·5 and 8 μ. The longest rods had complete outer limbs. One end, which *in situ* lay next to the choroid, was rounded. The other ended in the inner limb. Most of the rods were shorter, and had incomplete outer limbs, which had broken off a few μ from their

proximal ends. Smaller fragments were also present. The interior of some of the rods appeared homogeneous, but most showed cross-striations, whether they were complete or not.

There appeared to be two kinds of rod; thin ones (diameter 4·5 μ) and thick ones (diameter 7·5 μ). The frequency-distribution curve of diameters was skewed towards the shorter diameters, and was possibly bimodal, but it was impossible to be sure of this, partly owing to the difficulty of measuring the diameters.

Two kinds of cone outer limb were also seen. One, the most common, had a large, highly refractile ellipsoid, to which was attached a very small outer limb. The other, less frequently seen, had a longer (10 μ) outer limb, which was thin and tapering. These two kinds of cone correspond to cell types which can be seen in fixed and stained preparations.

Storage properties of the suspensions

When the suspensions were stored, changes occurred in the structure of the outer limbs, which are illustrated by the photomicrographs.

Pl. 1*a*, *b* were taken of suspensions which were less than half an hour old. All the cell types mentioned above can be distinguished. The outer limbs in Pl. 1*a* are suspended in Ringer-Locke solution, while those in Pl. 1*b* are suspended in sucrose. In the latter, the cells are floating throughout the depth of the Thoma counting chamber in which they were photographed, and not all are in focus.

After 24 hr storage in the dark at 2° C, samples of the suspensions were again photographed (Pl. 2*a*, *b*). In the sucrose suspension the cross-striations are more marked, but the cells are not otherwise altered, while those rods which were suspended in Ringer-Locke solution have become curved and swollen. This swelling occurs equally if the rods are stored in a Ringer's solution whose sodium has been replaced by potassium (Pl. 2*c*). It is therefore unlikely to be an osmotic phenomenon.

After 48 hr storage, there is little further change in the sucrose suspension, but the rods in Ringer-Locke solution have split up into the platelets of which they are composed (Pl. 3*a*, *b*).

If sucrose is added to a suspension of swollen outer limbs in saline till the suspending medium is 10 M, the swelling is not reduced (Pl. 3*c*). If the same experiment is performed after the platelets have separated, they are completely destroyed.

These experiments suggest that rod outer limbs are protected from damage by the rod sheath. The sheath imbibes water if the rod is stored in saline, swells and finally ruptures. When this happens, the platelets, which form the interior of the rod (Sjöstrand, 1949), float unchanged in the saline, but they are now unprotected and can be destroyed by the addition of sucrose.

Optical properties of the suspensions

Text-fig. 1 shows the absorption spectrum of a suspension, both before and 1 hr after it had been exposed to light. Wave-length is represented along the abscissa in mμ, while the ordinate represents the optical density (D) of the suspension with reference to a sucrose blank, i.e.

$$D = -\left[\log_{10} \frac{\text{light intensity transmitted by the cell suspension}}{\text{light intensity transmitted by a sucrose blank}}\right].$$

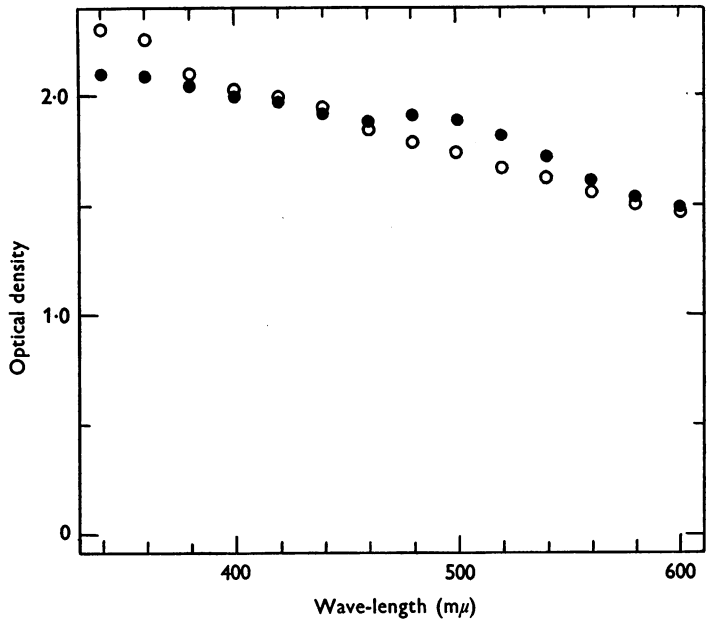
The exposure to light causes a small change in the density of the suspension. In the visible spectrum there is an increase of light transmission, while there is a decrease in the ultra-violet.

These changes suggest that a visual pigment has been destroyed and visual white has been formed.

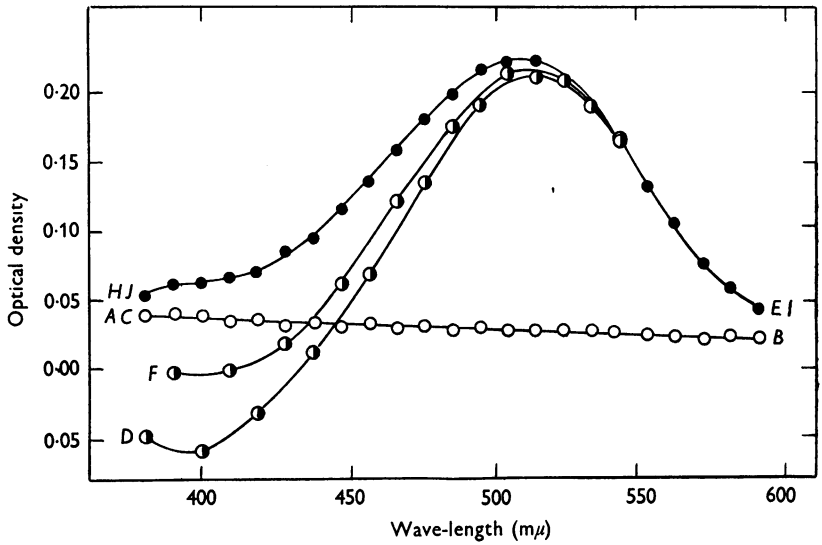
However, the changes in light transmission are very small in comparison with the total density of the suspension, and the method cannot yield accurate results. In most experiments, therefore, a different procedure was adopted, illustrated by Text-fig. 2. Here the abscissa represents wave-length (in mμ), and the ordinate optical density.

Two absorption cells were filled with samples of the same suspension, and the difference in the light transmitted by them was measured in 20 mμ steps from short to long wave-lengths (A–B) and then back again in interlacing 20 mμ steps (B–C). The 'cell-difference' is small and neutral, and is due to the difference in the light transmitting properties of the quartz walls of the cells. One absorption cell was then taken out of its holder, and exposed for 10 min to the bleaching light. Immediately after the measurements D–E and E–F were taken. After a pause of 3 hr, the measurements represented by the curves H–I and I–J were made. Since, before exposure to light, the two sets of measurements A–B and B–C fell on the same curve, the suspension must have been stable. Three hours afterwards, the suspension was stable once more. The changes which occurred in between must therefore be due to the action of light on the suspension.

Since the change in the density difference between the two cells is due to the presence of unbleached pigment in one, and bleached pigment in the other, the curves are analogous to difference spectra with a base-line of ABC. The difference spectrum of the suspension is similar to the difference spectrum of visual purple in solution. The increase in light transmission is due to the bleaching of the visual pigment in the outer limbs, while at shorter wave-lengths there is a decrease in light transmission due to the formation of visual yellow. There are, however, several differences between the bleaching of visual purple in solution and the visual pigment of the cell suspensions. The difference spectrum maximum of the product of bleaching is at 390 mμ, which is unlike



Text-fig. 1. The absorption spectrum of a suspension before (●) and after (○) exposure to light. The optical density is measured with reference to a sucrose blank.



Text-fig. 2. The absorption spectrum of a suspension. Optical density measured with reference to another portion of the same suspension. *A–B* and *B–C*, two sets of interlaced measurements made before the suspension was exposed to light. *D–E* and *E–F*, measurements made immediately after the suspension had been exposed to light. *H–I* and *I–J*, measurements made 3 hr later.

the maximum of indicator yellow (365 mμ). Moreover, the visual yellow of the suspensions is unstable and rapidly disappears.

Another difference is in the wave-length of maximum absorption. The pigment present in the suspensions has a difference spectrum maximum at 511 mμ, significantly different from the 504 mμ maximum of visual purple in solution.

DISCUSSION

When a retina is gently teased out on a microscope slide, the rod outer limbs which are detached appear quite structureless, and are almost all complete. The slightest trauma causes cross-striations to appear, and breaks the rods perpendicularly to their long axis. They then appear as they do in the suspensions. The sucrose used as a suspending medium causes no damage by itself, since the frog outer limbs are protected by their thick sheaths, as Kühne first demonstrated.

This is not true of all species. The rods of tench and carp shrink when placed in sucrose, and the surface of the rod becomes crinkled. 35% (w/v) sucrose destroys visual pigments, and Dartnall has found that if sucrose suspensions of fish outer limbs are kept in the refrigerator overnight, all visual pigment disappears from them. It is very likely that the sucrose can enter the outer limbs and attack the pigment. In suspensions of frog outer limbs, on the other hand, the pigment is stable for about 12 days, after which it quickly disappears. Intact rods cannot be found in such a suspension. It is therefore probable that sucrose cannot enter the outer limbs of the frog. Experiments described in a later paper give further support to this idea.

Optical properties of the suspensions

Though the suspensions transmitted only a small fraction of the light incident upon them, accurate difference spectra could always be obtained by measuring the difference in the light transmitted by two samples of the same suspension, only one of which had been exposed to the bleaching light.

This method has the additional advantage that instabilities in the suspensions not due to the bleaching of visual pigment do not affect the final result. For example, if the outer limbs in the suspension tend to settle, this will happen equally in both samples and, though the light transmitted by both will increase, the difference between them will remain constant.

A disadvantage of this method is that, in the ultra-violet part of the spectrum where the intensity of the light source is small, and the suspensions are optically very dense, so little light is transmitted by them that no measurements can be made at all. The ultra-violet measurements had therefore to be made with reference to a sucrose blank, and all the difficulties of measuring small changes in high densities were encountered.

Comparison of results with visual purple difference spectrum

Since visual purple is the only pigment which has so far been extracted from the outer limbs of frog retinae, the present results should be compared with its difference spectrum.

The most striking difference in such a comparison is that the product of bleaching (visual yellow) is unstable, and apparently is transformed into visual white. The changes observed in the present experiments confirm Kühne's observations. Another significant, though less striking, difference is the wave-length of maximum absorption of the visual yellow, 390 mμ. This is very near to the wave-length of maximum absorption of uncombined retinene, and suggests that inside the visual cells, indicator yellow is not formed. These details of the visual purple cycle are discussed in a later paper.

A third difference between the difference spectra of the suspensions and of visual purple in solution is the position of the wave-length of maximum absorption (511 and 502 mμ). It is unlikely that the true maximum of visual purple is at 511 mμ, and that the pigment is altered by being brought into solution, since the frog's scotopic sensitivity curve has a maximum at 502 mμ (Granit, 1947). It is more likely that the 511 mμ maximum is an artifact. There is, however, a third possibility, that the outer limbs contain another light-sensitive pigment, which is destroyed, or not extracted, by the agents used to make solutions of visual purple. Such a pigment would have to have a maximum absorption at longer wave-lengths than visual purple in order to produce the difference spectrum shown in Fig. 2. This problem is investigated in a third paper.

SUMMARY

1. A method is described for making suspensions of the outer limbs of the rods and cones of *Rana temporaria*.

2. The difference spectrum of the pigment inside the cells is not the same as that of visual purple in solution.

3. The yellow coloured product of bleaching is not indicator yellow, and is unstable.

4. The difference spectrum maximum of the pigment present in the suspensions is at a longer wave-length than that of visual purple in solution.

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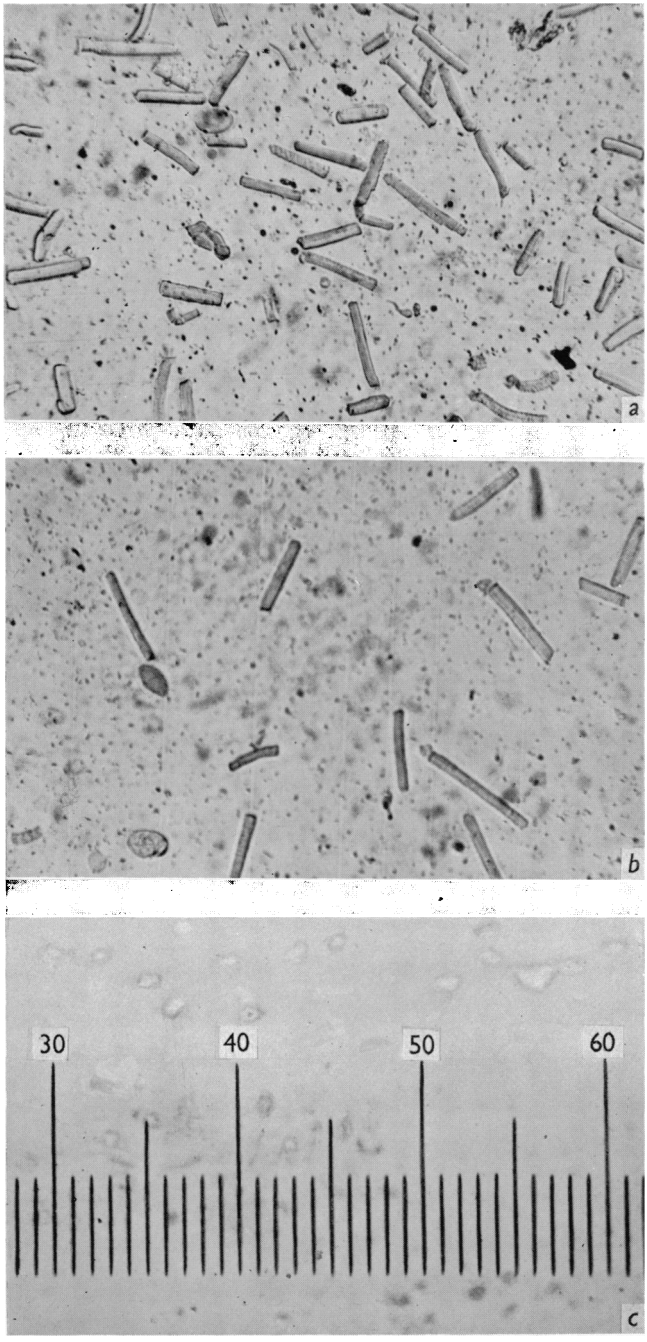

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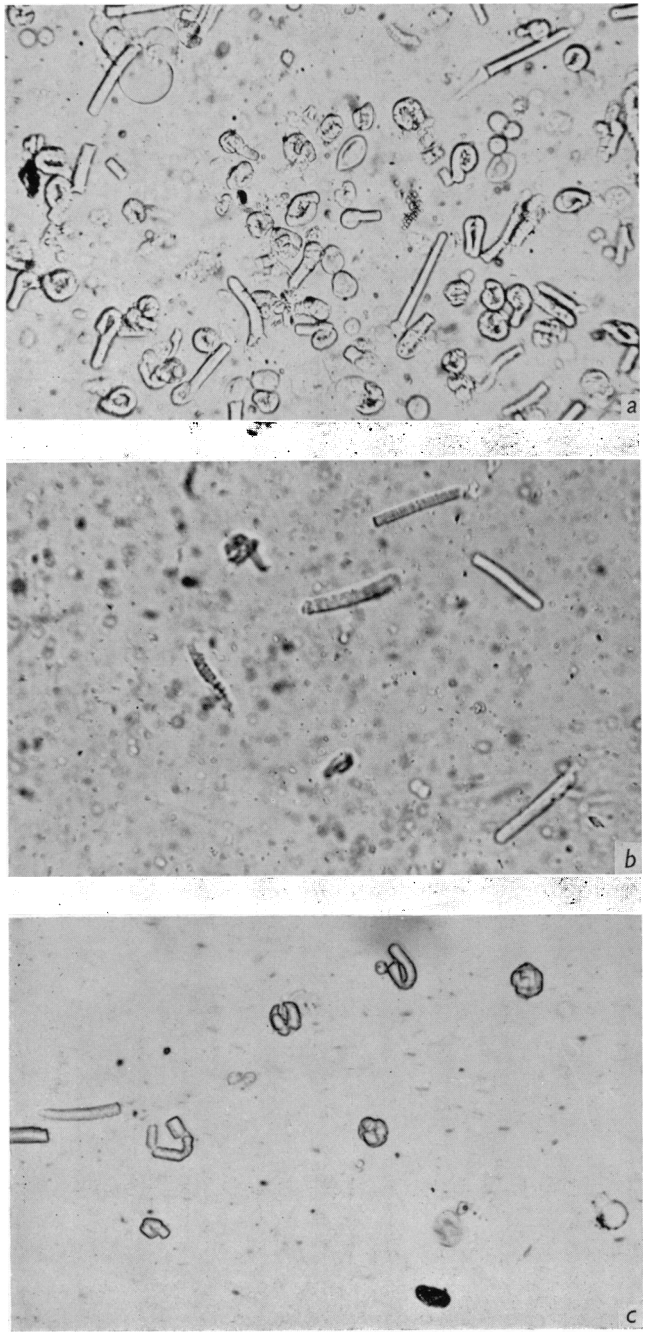
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b
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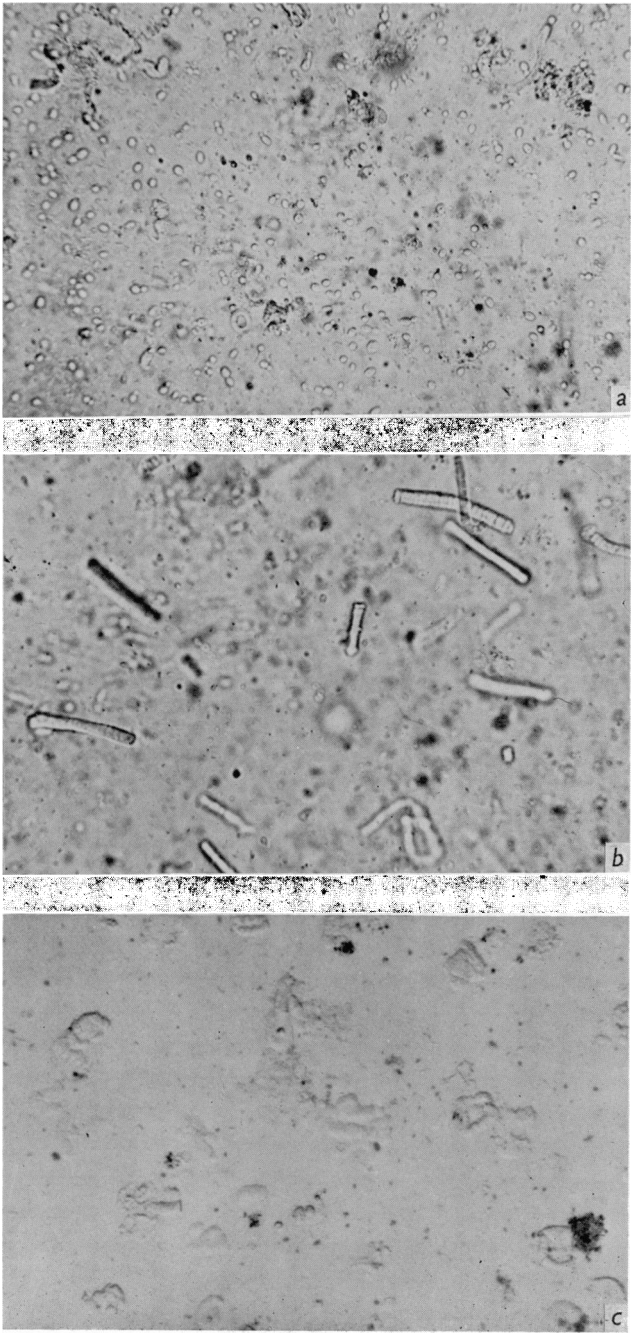
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EXPLANATION OF PLATES

PLATE 1. Photomicrographs of cell suspensions

a Outer limbs of rods and cones suspended in Ringer-Locke solution.

b Outer limbs of rods and cones suspended in 35% sucrose. Photomicrographs taken 30 min after the suspensions had been prepared.

c Calibration for Pls. 1–3. Each division is $\frac{1}{100}$ mm.

PLATE 2. Photomicrographs of cell suspensions

a Outer limbs of rods and cones suspended in Ringer-Locke solution.

b Outer limbs of rods and cones suspended in 35% sucrose.

c Outer limbs of rods and cones suspended in potassium-Ringer.

Photomicrographs taken 24 hr after the suspensions had been prepared.

PLATE 3. Photomicrographs of cell suspensions

a Outer limbs of rods and cones suspended in Ringer-Locke solution.

b Outer limbs of rods and cones suspended in 35% sucrose.

c The suspension of *a*, 1 hr later. Sucrose has been added to the suspension to a concentration of 10 M.

Photomicrographs taken 48 hr after the suspensions had been prepared.