# MICRURGICAL STUDIES IN CELL PHYSIOLOGY.

## IV. Colorimetric Determination of the Nuclear and Cytoplasmic pH in the Starfish Egg.*

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An essential feature in determining the hydrogen ion concentration of protoplasm is the maintenance of a normal condition of the protoplasm during the procedure. Results obtained by immersing cells in solutions of dyes have been inadequate owing to the lack of satisfactory indicators to which living cells are freely permeable (1). Attempts have been made to overcome this difficulty by artificially altering the permeability of living cells (2). Any procedure, however, which exposes the cells to abnormal conditions may seriously affect the results obtained. The existence of natural dyes in the tissues has been utilized (3, 4) but has not given definite results. Some investigators have made both potentiometric and colorimetric determinations of cellular extracts (5, 6).

Recently, Vlès and his coworkers (7–10) have introduced a method (méthode microscopique d'écrasement) by means of which echinoderm eggs, immersed in an indicator solution, are carefully crushed between the cover slip and slide of a compressorium. As soon as the egg bursts pressure is released whereupon the dye passes in through the breaks over the surface of the egg. The objection that the pH of crushed cells may be quite different from that of the living protoplasm has already been considered by the Needhams (11). The results obtained by the micrurgical technique have brought out the importance of the plasma membrane for the maintenance of protoplasm (12–14). If a cell is crushed so that the plasma membrane disintegrates, the

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exuding material either merges with the environing medium and is destroyed or forms spherules of normal appearing protoplasm walled off by new surface membranes. From experiments described in this paper these spherules are no more permeable than the protoplasm in its original condition. The disintegrated material scatters or rounds up into droplets which swell and burst.

A potentiometric determination of the pH with the aid of the micrurgical apparatus has been made possible recently by the development of the micro electrodes of Ettisch and Péterfi (15) and Taylor (16). However, the use of micro electrodes thus far has not met with success in the determination of the pH of the protoplasm.

The micro injection of indicator dyes into the protoplasm of cells has met with considerable success. Kite (17) was probably the first to inject dyes into the protoplasm of a cell. With a meagre supply of indicators at his disposal he concluded that the interior of the ameba is faintly alkaline. More recently Schmidtmann (18, 19) introduced solid particles of dyes into mammalian tissue cells. He obtained values of pH varying from 5.9 to 7.8 in different cells. The Needhams used aqueous solutions of the Clark and Lubs series of indicator dyes and determined the internal pH of a number of marine ova to be $6.6 \pm 0.1$.

The investigations described in this paper were carried out principally because of the discrepancy between the results obtained by the Needhams and those of other investigators—notably Vlès, Reiss, and Vellinger (7–10)—and because of the desirability of also determining the pH of the nucleus.

### *A. Methods and Material.*

The experiments were performed on the eggs of the echinoderm *Asterias forbesii.* The eggs to be injected with indicator solutions were immersed in hanging drops of normal or of acidified sea water. The pH of normal sea water when determined colorimetrically is 8.4 and, potentiometrically, 8.2 (20). To obtain acid sea water, $KH_2PO_4$ was added.

The dyes used were neutral red and those of the Clark and Lubs series of pH indicators covering the range from 4.4 to 8.4 (Sörensen units), *viz.*, methyl red, brom cresol purple,[1] brom thymol blue, phenol red, and cresol red. The neutral

[1] A peculiar feature of brom cresol purple is that the color of its alkaline range under the microscope appears distinctly blue rather than purple.

red was made up in a saturated aqueous solution and NaOH was added until the solution changed from a red color to a deep orange red with no sign of a precipitate. All the Clark and Lubs' indicators used were obtained from Hynson, Westcott, and Dunning, Baltimore. The dyes were prepared according to Clark (20) in 0.4 per cent aqueous solution with a molecular equivalent of NaOH. The brom thymol blue was found to be decidedly toxic upon injection into the eggs. All the other dyes were relatively non-toxic except brom cresol purple which gave evidence of toxicity only when injected into the cell nucleus. The Needhams, who used dyes from the British Drug Houses, did not find brom thymol blue to be especially toxic. On the other hand, they reported that the brom cresol purple produced cytolysis with considerable ease.

Fortunately, all the dyes in Clark's series for determining the pH are used as sodium salts and do not cause coagulation but quickly spread through the protoplasm and give it an even, diffuse color. This feature has already been noted for certain other acid dyes (21).

Neutral red, a basic dye (either the chloride or iodide of the color base), tends to coagulate protoplasm when it is injected (13, 21). If very little is introduced, the coagulating effect is localized at the spot of puncture and the dye diffuses slowly and evenly through the rest of the protoplasm. The diffuseness disappears after some time when the color accumulates in or on the cytoplasmic granules. Regions which are thickly beset with granules then appear more deeply colored than regions where the granules are sparse. In the following series of experiments the tints were recorded while the color was still in the optically homogeneous cytoplasm.

The dyes were injected both in their alkaline and acid states and in varied quantities. As long as the injection produced no visible signs of irreversible injury to the protoplasm the color always turned to that characteristic of a constant pH value. There was, therefore, no danger of masking or swamping out the cytoplasmic pH by the possible introduction of an excessive amount of the indicator solution.

The use of a completely overlapping series of indicators which show actual changes in tint rather than intensity differences were depended upon for determining the pH. Comparisons of the colors were made with the indicators in Clark and Lubs' standard buffer solutions. Direct comparisons on the stage of the microscope were also made by means of capillary glass tubes filled with the dye and by Pantin's method (22) of projecting the image of a series of colored test-tubes into the microscopic field.

The source of illumination was a 100-Watt nitrogen-filled, tungsten (Mazda C) bulb the magnified image of which was cast on the plane mirror of the microscope by means of a glass globe filled with water. Between the globe and bulb was inserted a ground "Daylite Glass," a color screen devised by Gage (23) for producing daylight artificially.

A Leitz aplanatic-achromatic, N.A. 1.40, condenser was used with its top lens removed (24). For critical reading the Leitz, 3 mm. apochromatic objective with an 8 X, periplan ocular was used.

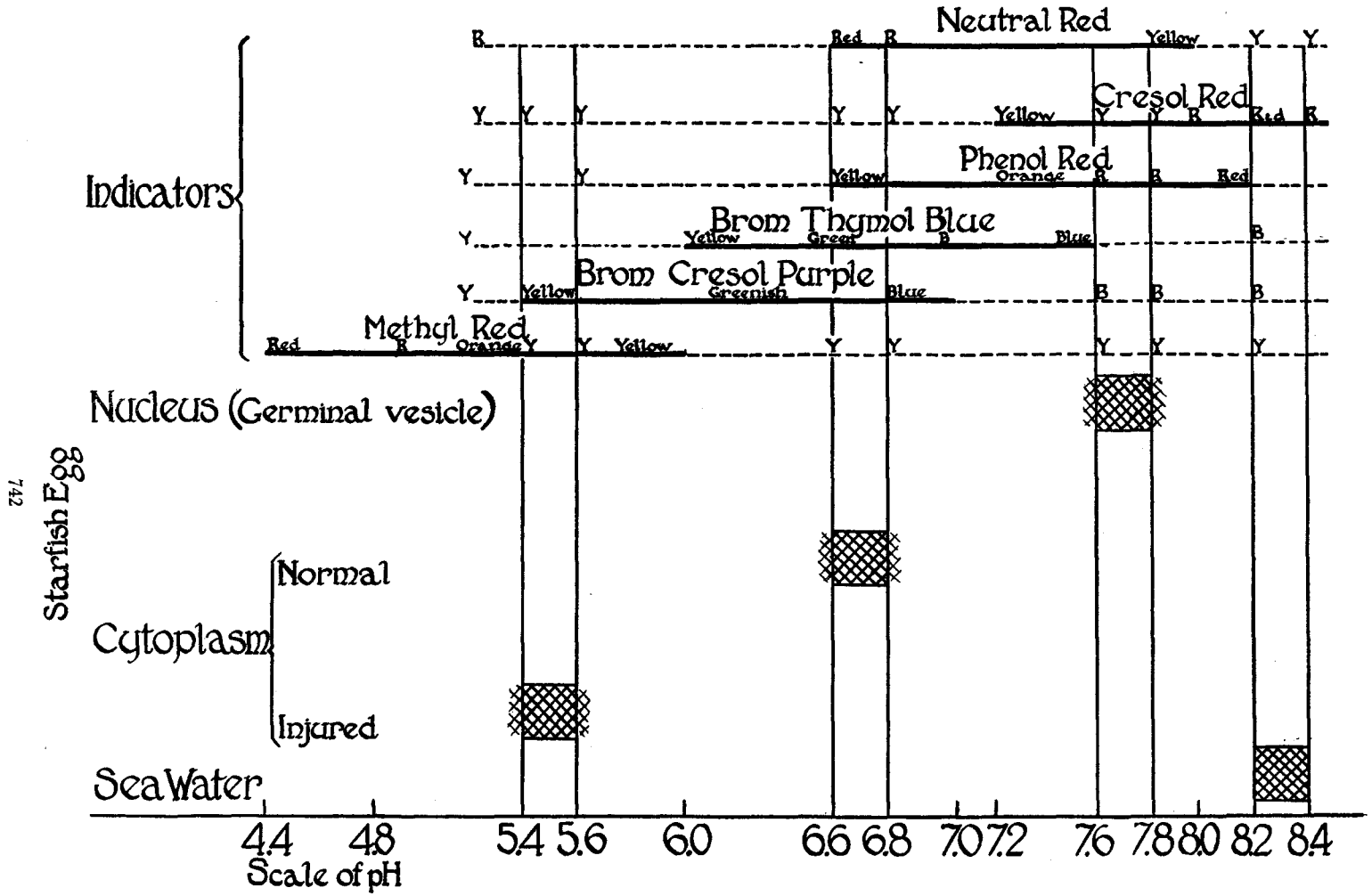


FIG. 1. Tabular representation of the pH determined for the normal and injured cytoplasm, and the interior of the germinal vesicle of the starfish egg.

### *B. Experiments.*

#### *1. The Cytoplasm.*

The dyes, when injected into the cytoplasm of the starfish egg, give colors which indicate a pH of 6.7 ± 0.1, Fig. 1. This value was determined from the injection of phenol red which gave a yellow color with no appreciable red tinge. The true colorimetric value may be one or two decimal points above this figure owing to the fact that any tinge of red would be obscured by the faint yellow pigment present in the normal cytoplasm. The same value

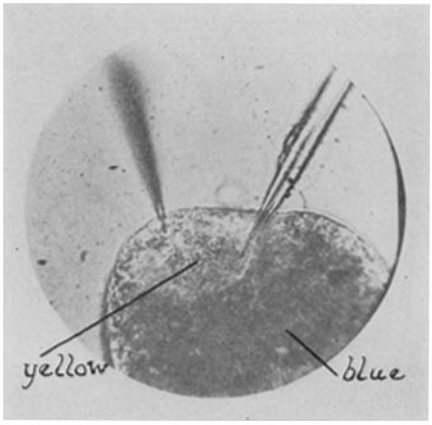


FIG. 2. Photograph of mature, unfertilized egg of the starfish injected with brom cresol purple and locally injured by a thrust of a micro needle. The region cytolyzed by the injury is yellow, the healthy cytoplasm is blue.

was obtained for the cytoplasm of eggs in the unfertilized, fertilized, and the first and second cleavage stages. Our results, therefore, closely approximate those of the Needhams.

#### *2. Effect of Injury on the Cytoplasm.*

(*a*) *Injury Accompanied by Visible Disintegration.*—A rapid tear of the cytoplasm of an egg induces cytolysis which spreads from the spot of injury (12, 13). Frequently, the spread of the cytolysis is stopped by the formation of a new membrane between the healthy and cytolyzing cytoplasm. Such a case is illustrated in Fig. 2.

An unfertilized starfish egg, colored blue by injecting brom cresol purple, was injured by repeated thrusts of a micro needle. The photograph was taken after the cytolysis had been localized. The immediate change in color of the cytolyzing region from blue to yellow shows that there is a rapid production of an acid due to injury which changes the pH to 5.6 or lower. A similar treatment of an egg colored yellow with injected methyl red results in no color change. This shows, *cf.* Fig. 1, that the pH of cytolysis is between 5.4 and 5.6. The same value was obtained when eggs were cytolyzed in sea water as acid as is consistent with viable conditions (pH 6.0). The cytolyzed region keeps its pH for several minutes until the seeping in of the sea water shifts the pH to that of the surrounding medium.

A phenomenon which may be of significance in a study of cytolysis occurs if a starfish egg, injected with brom cresol purple, is injured so as to produce extensive cytolysis. The yellow, disintegrating material gradually separates into two constituents: a loose, granular coagulum, colored yellow, and an oil-like, free flowing liquid, colored blue. On standing, the latter becomes semi solid.

The acid due to mechanical injury can also be detected in the environment of the egg. This is shown in the following experiment. An immature starfish egg, immersed in sea water colored with brom cresol purple was injured with a needle. Prior to visible cytolysis of the egg, the sea water immediately around it turned yellow and, after a few seconds, reverted to the original blue color.

(*b*) *Injury Unaccompanied by Visible Disintegration.*—The mere fact that a slight tear or puncture of an egg causes no morphological changes characteristic of cytolysis does not indicate that no injury has resulted. The two following experiments offer evidence that an acid due to injury is produced with no consequent visible cytolysis when a micro pipette punctures an egg in the course of an injection or when the egg is slowly torn. A micro pipette, having an aperture of half a micron, was filled with brom cresol purple in its blue state. The pipette was then thrust into an egg and the dye immediately injected. The region of the puncture at once took on a distinctly yellow color in contrast to the blue which slowly spread throughout the rest of the cytoplasm. 1 or 2 seconds after the injection the yellow color, at the spot where the puncture had been made, changed to a

blue. In the other experiment a starfish egg, previously injected with brom cresol purple, was carefully punctured and slowly torn with a needle. A flash of yellow appeared in the immediate vicinity of the puncture quickly followed by a return to the blue.

In both of the above experiments the loss of the yellow color resulted in a disappearance of the only evidence that the cytoplasm had ever been punctured or torn.

### *3. The Nucleus of the Immature Egg.*

The susceptibility to mechanical injury of the germinal vesicle or nucleus of the immature starfish egg has been previously demonstrated (12, 13).

By taking special precautions it was possible to insert a pipette into the germinal vesicle and to inject indicator dyes into it with no visible sign of injury. The most serviceable pipette for this purpose is one with a tip which tapers rapidly and then extends as a hollow, rigid hair 8 or 10 micra long and a little over 1 micron in diameter at its base. The aperture at the hair tip is less than half a micron in diameter. Pipettes of hard or Pyrex glass are brittle and too easily broken. Soft glass pipettes are more satisfactory and can be rendered sufficiently free of alkali for the period of the experiment by rinsing before use.

An egg was held with a micro needle against the edge of a hanging drop of sea water. The pipette was then thrust into the egg and slowly pushed against the nucleus which it indented. The tip of the pipette finally broke through the nuclear membrane without causing visible injury. After a small amount of the indicator had been injected, the pipette was slowly withdrawn and the minute puncture closed as the indentation of the nuclear membrane flattened out. The egg was then pushed into the deeper region of the hanging drop where the nucleus resumed its normal shape and appearance except for the color of the injected dye. In this way all the dyes indicated in Fig. 1 except brom thymol blue and methyl red were successfully injected. Brom thymol blue was omitted because of its toxicity and methyl red because its useful range is too low.

The colors assumed by the dyes indicate an intranuclear pH between 7.4 and 7.6 (*cf.* Fig. 1). Brom cresol purple, in addition to coloring

the nuclear sap blue, fixes the nucleolus and stains it an intense purplish blue. Phenol red and neutral red are the least toxic and it was after the injection of these two dyes that a maturation of the injected germinal vesicle was observed. Fig. 3 shows three photographs of an egg whose germinal vesicle was injected with phenol red. In Fig. 3, 1, the tip of the pipette can be seen at *a* in the germinal vesicle of the egg which is flattened by being brought into the shallow part of the hanging drop. After the injection the germinal vesicle was colored diffusely rose red. Some of the dye passed into the cytoplasm either directly or through the nuclear membrane. The yellow color of the cytoplasm and the red of the nucleus offered a striking contrast. Fig. 3, 2, is a photograph of the egg 1 hour later when it had been returned to the deeper region of the hanging drop. The onset of a typical maturation is to be noted. The germinal vesicle has begun to collapse and its membrane to wrinkle and fade. The red nuclear sap streamed in several radial paths into the yellow cytoplasm which took on an everdeepening orange tint. After several minutes the orange color changed back to the original yellow. The last photograph, 3, was taken 1 hour later and shows the diminutive pronucleus in the state which precedes polar body formation. In the four cases in which this phenomenon was observed (three after the injection of phenol red and one after that of neutral red) no polar bodies were formed.

FIG. 3. Photograph of starfish egg undergoing maturation with its cytoplasm and germinal vesicle injected with phenol red. 1. Immature egg held by needle "*b*" in shallow region of hanging drop and with micro pipette "*a*" vertically inserted into the germinal vesicle. The cytoplasm is yellow, the germinal vesicle is red. 2. Egg, 1 hour later, in deeper region of hanging drop. The germinal vesicle has begun to shrivel and the cytoplasm is taking on an orange tint. 3. Egg 10 minutes later, with yellow cytoplasm. The diminutive pronucleus prior to polar body formation can be seen in the center of the egg.

FIG. 4. Sketches to show effect of mechanically injuring the germinal vesicle of a starfish egg. 1. Before injury. 2. Immediately after injuring the egg either by crushing or by puncturing the germinal vesicle. The remains of the germinal vesicle is to be seen as a hyaline sphere (nuclear remnant) and the cytoplasm around it has cytolyzed. The vitelline membrane is partially lifted, a phenomenon which frequently occurs when an egg is injured, *cf.* Chambers (12). 3. Completely cytolyzed egg within vitelline membrane.

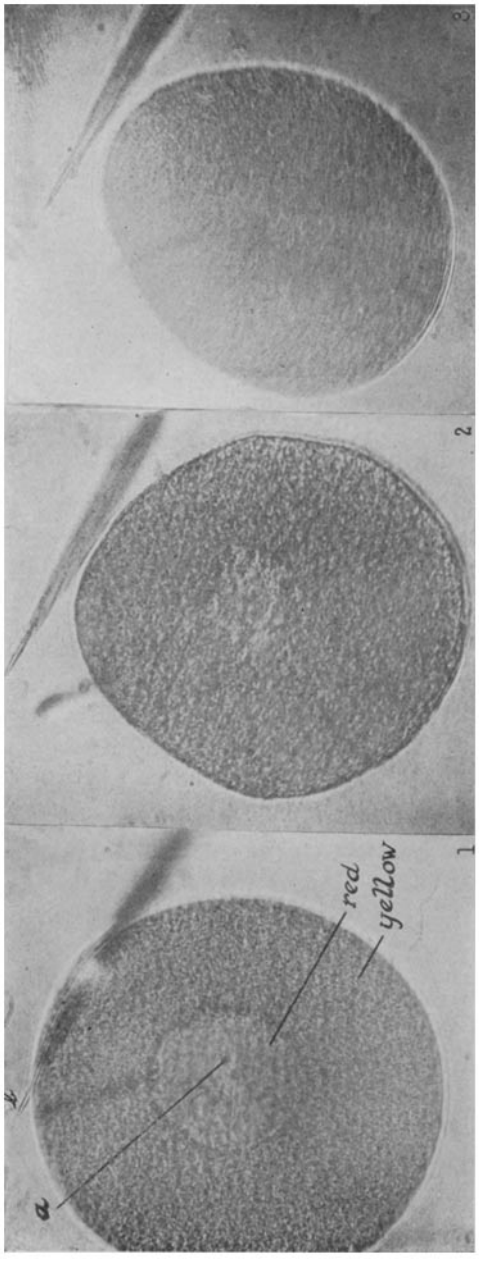


FIG. 3

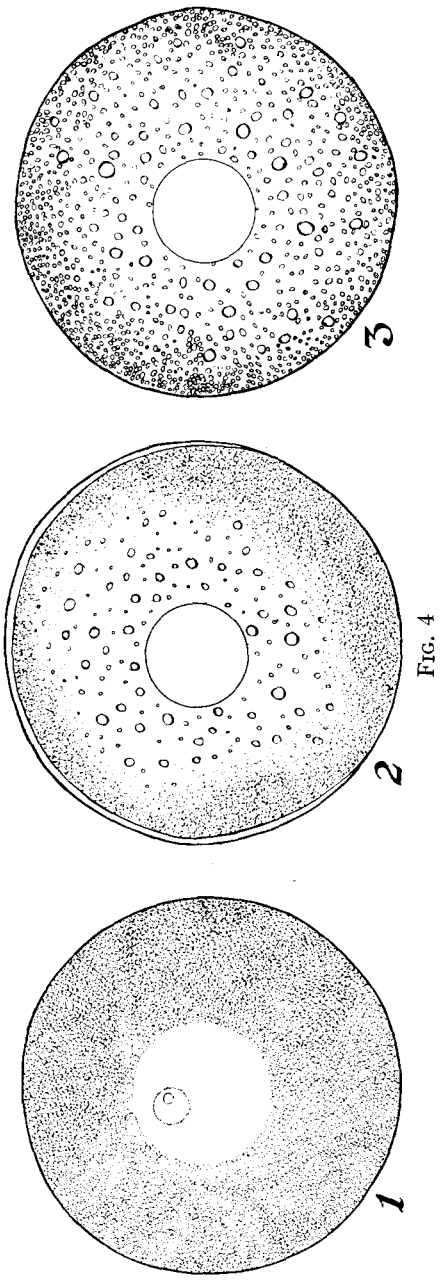


FIG. 4

The uniform orange tint in the germinal vesicle after an injection of neutral red was followed almost immediately by a deep, red zone in the bordering cytoplasm. The color in the nucleus rapidly became paler and almost completely faded while the cytoplasm tinged a rose red. Neutral red was the only dye which faded from the nucleus to such an appreciable extent.

The nucleolus is a more or less solid body and tends to become more intensely colored in time than the rest of the nucleus. When a stream of neutral red is directed against the nucleolus, the color of the dye spreads slowly through it from one side.

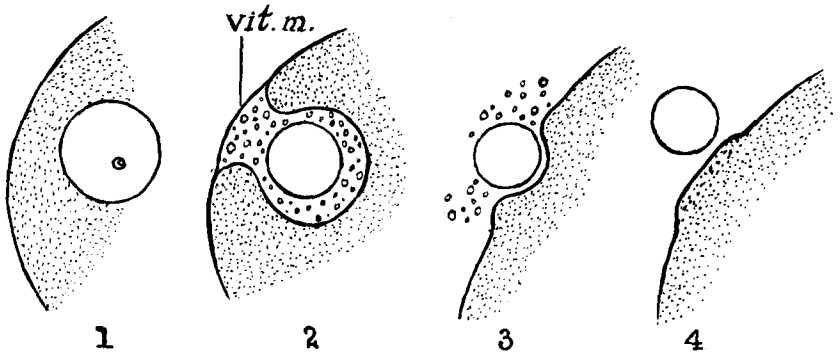


FIG. 5. Injury of germinal vesicle followed by extrusion of nuclear remnant. 1. Before injury. 2. Nuclear remnant in cytolyzed region which is walled off from healthy cytoplasm. vit. m. = vitelline membrane. 3 and 4. Nuclear remnant which is being extruded after breakdown of vitelline membrane.

### *4. Effect of Injury on the Germinal Vesicle.*

The usual sign of approaching disruption is the fading of the nucleolus. This is followed by a cytolysis which spreads from the surface of the injured nucleus. A spherical, optically homogeneous, nuclear remnant frequently persists in the disintegrated region, Fig. 4, and can be dragged out into the surrounding sea water. Brom cresol purple has the peculiar property of frequently fixing both the nucleolus and the nuclear membrane without hindering disintegration of the rest of the egg.

If an injected germinal vesicle is injured there is no change in color regardless of the indicator used. This is in striking contrast to the

immediate change which is produced in the cytoplasm upon injury. The germinal vesicle and cytoplasm of a starfish egg, Fig. 5, 1, were injected with phenol red and injured so as to produce cytolysis, Fig. 5, 2. The healthy cytoplasm and the débris resulting from cytolysis were yellow and the nuclear remnant red. In Fig. 5; 3 and 4, the healthy egg remnant has rounded up and the consequent flattening out of the bay has carried the nuclear remnant and the cytolyzed débris into the surrounding sea water. The débris turned red on coming into contact with the sea water the pH of which is 8.4. The nuclear remnant maintained its red color for several seconds until it collapsed and disappeared. This shows that injury to the nucleus causes no increase in acidity.

Cresol red was injected into the germinal vesicle of another egg. Upon injury the nuclear remnant retained the yellow color until it collapsed in the cytolyzed débris. This shows that injury to the nucleus causes no increase in alkalinity.

The ease with which the nuclear remnant assumes the color of its environing medium is seen from the following. If brought into sea water colored with phenol red it turns red if the sea water has a pH of 8.4 and yellow if it has a pH of 6.0.

A germinal vesicle was injected with brom cresol purple. Upon injury to the nucleus, extensive cytolysis of the egg ensued. The acid débris and the blue nuclear remnant were retained within the confines of the persisting vitelline membrane. The nuclear remnant gradually changed from a blue to a yellow color, indicating that it had assumed the pH of its immediate environment of cytolyzed material.

### *5. The Rate of Surface Membrane Formation in Its Relation to the Entrance of Dyes through a Torn Surface of a Starfish Egg.*

Frequently, if cytolysis occurs when too large a puncture is made in injecting a plasmalemma quickly forms about the cytolyzed area and the fluid ejected from the pipette simply lies in a pocket sharply marked off from the healthy cytoplasm. When this occurs with eggs immersed in normal sea water, the newly formed membrane serves as an effective barrier against the passage of the dye into the cytoplasm. Evidently, the membrane must form with extreme rapidity. If, however, the same procedure is carried out on eggs in sea

water having a pH of 6.0, the dye frequently penetrates into the cytoplasm. This indicates that this new surface membrane in an environment which is more acid than normal, either has a different permeability, or is retarded long enough in its formation to allow the dye to enter. It is possible that both factors are operative.

## DISCUSSION.

Of the considerable number of papers which have recently been published on intracellular pH, only those can be specially mentioned

TABLE I.

*Differences in Dissociation of Acid and of Basic Dyes in Their Acid and Alkaline Ranges.*

| Dyes | | Acid range | Alkaline range |
|---|---|---|---|
| Acidic | Brom cresol purple | Low dissociation Yellow | High dissociation with salt formation **Blue*** |
| | Phenol red | Low dissociation **Yellow*** | High dissociation with salt formation Red |
| Basic | Methyl red | High dissociation with salt formation Red | Low dissociation **Yellow*** |
| | Neutral red | High dissociation with salt formation **Red*** | Low dissociation Yellow |

* Color assumed by the cytoplasm when the dye is injected into it.

which deal with marine ova. Our results on the pH of normal cytoplasm of starfish eggs are in close accord with those of the Needhams. We place the pH of normal cytoplasm of the eggs between 6.6 and 6.8, the Needhams place it at $6.6 \pm 0.1$.

In answer to the possible objection that the errors are too great to permit a determination of the protoplasmic pH, it may be pointed out that all the dyes give consistent indications toward the same pH irrespective of their chemical constitution, Table I. For ex-

ample, brom cresol purple gives to the cytoplasm the blue color of the salt of its alkaline range while phenol red imparts to the cytoplasm the yellow, non-dissociated color of its acid range. The same principle also holds true for the two basic dyes used: *viz.*, neutral red and methyl red.[2] Both are yellow in their alkaline ranges where they are in the state of lowest dissociation and least salt formation. In their acid ranges, where their dissociation is greatest and, consequently, where salt formation predominates, both of the dyes are red in color. The fact that methyl red gives a yellow while neutral red a rose red color when injected into the cytoplasm is added evidence that the hydrogen ion concentration is a prime factor in the formation of the colors.

The production of an acid associated with injury or death of cellular tissues has been frequently reported in the literature (3, 4, 19, 25).

Concerning the pH of the injured cytoplasm of echinoderm eggs our results differ somewhat from that of the Needhams. In the eggs of *Paracentrotus lividus* they placed the value below 5.0 and above 4.0 because of the results obtained with methyl red and brom phenol blue. The methyl red we used was the sodium salt while they used the saturated aqueous solution.

The difference in reaction of the egg to a slow and to a rapid tear is probably due to the amount of acid produced by the injury. With a slow tear very little acid results at any given moment and it is presumably neutralized as fast as it is formed. With a rapid tear a considerable amount of acid is produced which cannot be taken care of by the cytoplasmic buffers upon which cytolysis sets in. With the spread of the disintegration more acid accumulates and the cytolysis continues. It is also significant that mechanical injury occasions an increase in acidity both outside the egg and within its cytoplasm, before there is any visible sign of cytolysis.

In this regard it is of interest to note the pH findings on echinoderm eggs by Vlès and his coworkers. They crush the eggs in the indicator and observe the resulting color. This method is open to several objections: first, there is the danger of mixing the intracellular fluids

[2] Methyl red has both an active acid and basic group in its molecular structure. The indicator is generally used as the sodium salt, but it shows the typical dissociation curve of a base. It is the basic group which is responsible for the color changes from red to yellow.

with the fluid which surrounds the cells; second, the cytoplasmic fluid may also mix with the fluids of intracellular vacuoles; and, third, the injury to the plasma membrane, which is a necessary consequence of crushing cells, almost always initiates disintegrative changes in the protoplasm. In addition to this the instantaneous production of an acid upon mechanical injury followed by the further development of acid concomitant with visible cytolysis must, to a considerable degree, modify the actual pH of normal, uninjured cytoplasm.[3]

After the publication of the Needhams' criticism of the crushing method Vellinger (27) checked the previous potentiometric determinations (9) of egg material procured by crushing the eggs in a chamber cooled to −60°C. Potentiometric readings were then made on the powder as it thawed. The first readings gave the highest pH. Subsequently, as the temperature rose, the pH dropped until it reached a constant value equal to that already recorded by Vlès, Reiss, and Vellinger (9) as the normal pH of the cytoplasm of the eggs. The fact that Vellinger's first readings give the highest pH, can be interpreted to mean that the excessively low temperature prevents or at least delays the production of the acid accompanying injury when the eggs are crushed. The first readings should then more nearly approach the pH of the normal cytoplasm. With the progressive thawing of the egg material more and more acid is produced and hence the pH falls till it reaches a level typical for cytolysis.

In this connection may be mentioned the recent result of Bodine (28) who obtained some fluid from the large yolk-laden *Fundulus* egg by pricking the dried surface of the egg. The exuding fluid was drawn into a dry glass capillary. The pH of the fluid, determined potentiometrically, was found to be 6.4.

The Needhams made no special investigation of the pH of the germinal vesicle but it is significant that they report it to give the alkaline color of brom cresol purple in both the *Echinocardium* and the *Asterias* egg even after cytolysis had occurred.

[3] Reiss (26) claims to have found by his crushing method that the pH of the *Paracentrotus* egg changes during the different stages of its development. The values which he gives lie between the extremes of 5.3 and 5.6 and are small enough to be considered within the limits of probable error. However, it is conceivable that difference in pH may occur in the disintegrated material obtained from cells in the different stages of their development.

The fact that the nucleus does not change in reaction after cytolysis of the egg, indicates why Reiss was able to report from his results on eggs crushed in indicator solutions that the nucleus is faintly alkaline. This is in accordance with the results we obtained with the nuclear remnants of crushed *Asterias* eggs.

In order, however, to determine the pH of the normal nucleus there must be a definite proof, as given in the experimental part of this paper, that both the egg and its nucleus are alive and active during the period of the determination.

The difference in the hydrogen ion concentration between the nucleus and cytoplasm of the living immature starfish egg is of considerable interest. It would, however, be premature to speculate from this on the interrelationships of the nucleus and cytoplasm of cells in general. On the other hand, it might well be pointed out that the immature egg, although it has a much enlarged nucleus, is, nevertheless, more truly to be compared with a somatic cell than the mature egg.

## SUMMARY.

I. *Cytoplasm.*

1. The normal cytoplasmic pH, colorimetrically determined, of the starfish eggs in the unfertilized, fertilized, and first and second cleavage stages is 6.7 $\pm$ 0.1.

2. Cytolysis lowers the pH to a value 5.5 $\pm$ 0.1.

3. The cytolyzed material in time assumes the pH of its environing sea water.

4. The acid due to mechanical injury can also be detected in the environment of the egg.

5. Injury to the cytoplasm unaccompanied by visible disintegration causes an increase in acidity which is quickly neutralized.

II. *Germinal Vesicle.*

6. The intranuclear pH, colorimetrically determined, of the immature *Asterias* egg is 7.5 $\pm$ 0.1.

7. Injury to the nucleus does not change its pH.

8. The spherical nuclear remnant which persists after injury gradually assumes the pH of its environment.

III. *Plasmalemma.*

9. A dye to which the cell is normally impermeable can penetrate through a tear in the surface from an environment more acid than normal. This may be due to a difference in the formation of the plasmalemma in a normal and an acid medium.

We take this opportunity of thanking Dr. Barnett Cohen of the Hygienic Laboratory, Washington, for valuable advice given in the preparation of this paper.

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