ON THE SMALLEST PARTS OF STENTOR CAPABLE OF REGENERATION; A CONTRIBUTION ON THE LIMITS OF DIVISIBILITY OF LIVING MATTER.

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In experiments on the power of multiple development of the ovum the result has been reached, that a portion of less volume than one-fourth that of the normal ovum does not possess the capacity of producing an *embryo* or *larva*, though it may a gastrula (see postscript); while a portion equal to one-fourth or more of the volume of the normal ovum may, under suitable conditions, produce a gastrula and finally a larva of corresponding relative bulk. This result has been attained by Loeb,[1] Wilson,[2] Driesch,[3] Morgan,[4] and Zoja.[5] Wilson found only a single larva of Amphioxus of one-fourth the normal size and that showed several defects; and Driesch has not, so far as I am aware, mentioned any pluteus of less than one-quarter size. Morgan has recently published the results of his studies on the power of multiple development of the echinoderm ovum. In this he shows that "the volume of the smallest gastrula which can be produced from fragments of the egg falls below $\frac{1}{64}$ the volume of normal gastrulae. The volume of the fragments of the egg which produced such gastrulae, varies between $\frac{1}{40}$ and $\frac{1}{50}$ of the volume of the ovum." (Summary p. 124 *loc. cit.*) But these smallest gastrulae were unable to

[1] Jacques Loeb. "On the Limits of Divisibility of Living Matter." In *Biol. Lectures of Marine Biol. Lab. for 1894*. Boston, Ginn & Co. Also in *Archiv für Ges. Physiologie von Pflüger*. Vol. LIX. 1894.

[2] E. B. Wilson. "Amphioxus and the Mosaic Theory of Development." Journal of Morphology. Vol. VIII, No. 3. 1893.

[3] Driesch. "Entwicklungsmechanische Studien." III–VI. *Zeitschr. f. wiss. Zool.* Bd. LV, p. 9. 1893.

[4] "Studies of the Partial Larvae of Sphaerechinus," by T. H. Morgan, in Roux's *Archiv für Entwicklungsmechanik der Organismen*. Bd. II, H. 1. 1895.

[5] Sullo sviluppo dei blastomeri isolati dalle uova di alcune meduse (e di altri organismi) per il Dr. Raffaello Zoja; in Roux's *Archiv für Entwicklungsmechanik der Organismen*. Bd. II, H. 1. 1895.

develop further. Morgan himself says (p. 117): "The smallest pluteus which I have found measured 7 × 8, and the normal form in the same dish 12 × 15. Another larva at the beginning of the pluteus stage measured 6 × 7. The larvae have apparently one-eighth the volume of the normal, and correspond in size very nearly to the pluteus figured by Loeb. If, however, we compare these small larvae with the larvae derived from isolated blastomeres, the conclusion is forced upon one that these plutei have in all probability come from fragments of the egg having only about *one-half to one-fourth of the volume of the egg*." Inasmuch as the test proposed for the limits of divisibility rests upon the capacity for complete development to an *embryo*, or *larva* properly so-called, it is only these last figures of Morgan's that demand consideration. It would seem from these that Loeb (*loc. cit.*) has made his figure, *one-eighth*, too low, not having taken in account the fact, emphasized by Morgan, that the growth of the small blastulae, gastrulae and plutei is less rapid than that of the normal.

Zoja's results on the separation of the blastomeres of the ova of certain medusae must also be considered. In the summary, p. 32, we find the following remarks: "Medusae; Die Entwicklung der getrennten Blastomeren ($\frac{1}{2}$ und $\frac{1}{4}$ Ei von Liriope mucronata, Geryonia proboscidalis, und Mitrocoma annae; $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, Ei von Clytia flavidula und Laodice cruciata) ist ganz genau in allen ihren Phasen wie diejenige des ganzen Eies." — "Es bildet sich endlich immer eine schwimmende Larva, aus zwei Gewebe bestehend, die von jener, welche aus $\frac{1}{1}$ Ei hervorgeht, nicht unterscheidbar ist, ausser in den Dimensionen."

From this it appears that a $\frac{1}{16}$ blastomere of Clytia and Laodice is able to develop into a swimming larva. But from the next statement I judge that the development cannot go farther; this is: "Bei Clytia zeigten $\frac{1}{2}$ und $\frac{1}{4}$ Ei auch die vollständig entwickelte idroide Form, und bei Liriope gab $\frac{1}{2}$ eine kleine runde Medusa, in welcher die vier primären Tentaculi normalerweise im Kreuz angeordnet waren." Thus a fourth blastomere is the smallest portion capable of complete development.

There are three possible explanations of this failure of such small parts to develop:

1. That the whole organization of the species cannot be included in so small a space. Briefly, *deficient organization.*

2. That so small a volume of matter cannot fulfill the mechanical conditions consequent on cell-division, formation of a segmentation cavity, invagination, and so forth, owing perhaps to increased surface tension (Driesch), not to mention other conceivable alterations of the extrinsic factors of development.

3. That such a small part "is not able to set free that amount of energy which would be required for its transformation into a gastrula or a pluteus." (Loeb.) [1]

The third explanation seems to me inadequate; because such masses may continue to live for a considerable period of time and display an amount of energy in *atypical* form changes and rapid ciliary movement, which would suffice to produce the phenomena of normal development, did not other factors (included in the first or second of the above alternatives) prevent. Moreover, it is well known that exceedingly minute protoplasmic bodies, very much smaller than one-eighth the echinoderm ovum, may produce a relatively enormous amount of energy: *e.g.* bacteria and spermatozoa. Finally we do not know how much of developmental energy is of intrinsic and how much of extrinsic origin. *We are limited, then, to the first two alternatives.*

Now, in the regeneration of a unicellular organism those mechanical conditions consequent on cell-division, formation of a segmentation cavity, invagination, and so forth, are not required to be fulfilled. Surface tension and other extrinsic factors of development of Metazoa have not been shown to exercise a controlling influence in the regeneration of such an organism.[2] It occured to me, therefore, that the *ciliate Infusoria*

[1] Morgan's explanation, that the failure to develop is due to inability to produce a sufficient number of cells for the next ontagenetic stage, will come under the first or second of these alternatives, according to the general point of view.

[2] Of course it is possible for any one to maintain that extrinsic forces do control the regeneration. But the burden of proof rests upon the maker of such an assumption.

offer conditions for decision between these two alternatives. If it should be shown that a nucleated portion of the body below a certain minimal size is incapable of regeneration, the first alternative would receive support. If, on the other hand, the smallest nucleated fragments of the body are capable of regeneration with restoration of the normal form, the first hypothesis would fall, and the second tend to be established.

My material consisted of two species of Stentor, viz.: *S. polymorphus* and *S. coeruleus*. The former species occurred in immense profusion on decaying leaves of the water-lily in a small pond near Ann Arbor, the latter appeared in considerable numbers in a small aquarium which had stood in the laboratory for six or seven weeks, and contained gatherings from a swamp. This species is more favorable for experimental work than the former, because the protoplasm is transparent, enabling one to see the nucleus readily in the living animal. In *S. polymorphus* the body is rendered almost perfectly opaque by the presence of immense numbers of symbiotic unicellular Algae, the so-called zoochlorellae, which either hide the nucleus completely from view, or permit mere momentary glimpses of it. On account of the ease of procuring any desired supply of *S. polymorphus* my work was done chiefly on this form; but the results were checked on *S. coeruleus*, and were practically the same for both species.

To reach the desired result it was necessary to find or devise a method by which nucleated fragments of every possible size, beginning with a portion not much larger than a single node of the nucleus, could be produced in large numbers; for reliable quantitative results can be reached only by observation of a large number of cases of regeneration. For this purpose I tried the method of *shaking* which has yielded such admirable results with the animal ovum in the hands of Wilson, Driesch, Morgan, and others, and found it to succeed to perfection. If a number of Stentors are put in a small vial about one-third filled with water and shaken quite violently from five to twenty times (*S. coeruleus* requires to be shaken only about five times; *S. polymorphus* ten to twenty times), and then examined under a low power of the microscope, one sees that the animals have been broken into numerous fragments of every possible size and shape. In the field of the microscope there are present at the same time naked nodes of the nucleus either single or united in groups of two or three, and parts of the body, both nucleated and unnucleated, ranging in size through every possible gradation from 25μ in diameter to about 200μ. Most of the latter are being driven hither and thither by the action of the cilia with which they are covered; and many of them are of the most bizarre and curious shapes: T-shaped, Y-shaped, or provided with other arm-like processes, or of forms impossible to describe; but most of them are of more regular form, triangular, quadrilateral, oval, and spherical.

The moniliform character of the nucleus in these species of Stentor insures that a large proportion even of the smallest pieces receive at least some part of the nucleus. In order to satisfy myself that such is the case, I killed and stained the whole of one lot of *S. polymorphus*, which had been shaken as described, about fifteen minutes after the operation. The stained material was then mounted in balsam and measurements were made of the smallest nucleated pieces. Some of the measurements were as follows:

1. Naked nodes of nucleus, spherical or oval; 20–25μ.

2. A spherical piece 31μ in diameter containing a single node of the nucleus. Nucleus excentric. Protoplasm a thin cortex.

3. A spherical piece 37μ in diameter; contained a single node of the nucleus.

4. A spherical piece 37μ in diameter; contained two nodes of the nucleus.

5. A spherical piece 40μ in diameter; contained six nodes of the nucleus.

6. A spherical piece 50μ in diameter; contained one node of the nucleus.

7. A spherical piece 50μ in diameter; contained two nodes of the nucleus.

8. A spherical piece 50μ in diameter; contained four nodes of the nucleus.

9. A spherical piece 66μ in diameter; contained a single elongated node of the nucleus.

10. A spherical piece 69μ in diameter; contained seven nodes of the nucleus.

Other similar pieces containing one or more nodes of the nucleus were seen; some were of course not spherical, but I have given the spherical pieces as easier to compute the volume. Of larger nucleated pieces there was no lack; they were very numerous, as one would expect. There were, of course, numerous unnucleated masses of protoplasm of various sizes, but few large pieces. In fact, the majority of the pieces below 100μ in diameter were unnucleated; but the above list shows that a good many of such small pieces contained one or more nodes of the nucleus. I did not attempt to ascertain what was the proportion of nucleated to unnucleated pieces of such small size; but it must have been quite large, perhaps one to ten or even more.

One further remark as to my methods. When any doubt existed in my mind as to the presence of parts of the nucleus in examples noted, the specimen was killed and stained, generally in Schneider's aceto-carmine, and the actual condition of the nucleus thus determined with certainty. This of course involved the sacrifice of a great deal of material.

In consequence of the often curious and asymmetrical shapes of the pieces produced by shaking, I expected to obtain valuable results on the teratogeny of the Stentors for comparison with the results of Balbiani and Johnson, but I have been almost completely disappointed in this respect. When regeneration of a piece takes place at all, it almost always happens that a single more or less perfect animal of typical form results.

RESULTS.

In this paper I shall speak only of results obtained on the smallest parts capable of regeneration, leaving other questions suggested by the experiments for future consideration. From numerous experiments, involving many hundreds of *S. polymorphus*, it was found that the smallest parts capable of regeneration possess the volume of a sphere of about 80μ diameter. A lesser number of experiments on a smaller number of *S. coeruleus* yielded results almost identical. In the following list I give measurements of some of the smallest Stentors found. After measuring in a more or less expanded condition the animals were made to contract, when they assumed almost the form of a sphere; the diameter of the sphere was then measured, and this measurement was used for comparison.

1. *Stentor coeruleus.* 45½ hours after shaking. Regeneration was complete or nearly so. I could see the adoral spiral sinking into the oesophagus, the mouth, and contractile vacuole. When expanded the form was quite typical. There were two *separated nodes* of the nucleus present.

Measurements. None were made of the expanded animal. Diameter of contracted animal (spherical) 90μ.

2. *S. polymorphus.* 67 hours after shaking. Regeneration complete.

Measurements. *a.* Expanded, 257μ in length; 80μ across frontal field. *b.* Diameter of contracted animal (spherical) 80μ.

3. *S. polymorphus.* 70 hours after shaking. Regeneration complete.

Measurements. *a.* Expanded, 257μ in length; 84μ across frontal field. *b.* Diameter of contracted specimen (spherical) 87μ.

4. *S. polymorphus.* 96 hours after shaking. Regeneration complete. The animal was sluggish and did not expand well.

Measurement. Diameter of contracted specimen 75μ.[1]

I have measurements of a number of Stentors of sligntly larger size than the ones given; but the smallest Stentors were very scarce. By far the greater number of nucleated parts, which possessed a spherical diameter of less than 100μ, were incapable of regeneration, or at any rate did not regenerate. However, but a single example is sufficient to show that a portion of the volume of the example in question is capable of regeneration.

[1] My note-book expresses a little doubt about this specimen, but it was certainly under 80μ.

The volume of the smallest Stentor found was thus equal to a sphere of somewhat less than 80μ in diameter. Not one of the hundreds of smaller nucleated parts regenerated, though I found one part, 71μ in diameter in spherical form, which had assumed a fairly typical form of semi-contraction and possessed a single bead of the nucleus; anterior and posterior ends (or foot) were thus recognizable, but there was neither oesophagus nor adoral membranellae present. Even if we admit this as regenerated, which I do not, it does not essentially alter the final result.

My conclusion is, therefore, that nucleated parts of *Stentor polymorphus* of less volume than a sphere of 80μ (approximately) in diameter are incapable of regeneration; nucleated parts of greater volume are capable under favorable conditions of complete regeneration.

The main results hitherto reached on the merotomy of the Protozoa can be summarized as follows:

1. Cytoplasm without nucleus is incapable of regeneration (Nussbaum, Gruber, Verworn, Balbiani, and others). This I can confirm. (Verworn has shown that the isolated central capsule of *Thalassicola nucleata* from which the nucleus has been removed is capable of partial regeneration, but it soon goes to pieces. Gruber has shown that if a Stentor in process of fission be transversely divided so that the posterior part receives no nucleus, this part is nevertheless able to regenerate.)

2. Nucleus without cytoplasm is incapable of regeneration. (Verworn, Balbiani.) This also I can confirm.

3. Portions of the body consisting of nucleus and cytoplasm are capable of regeneration. *To this I must add: provided that the amount of cytoplasm exceed a certain minimal volume* (which in the case of Stentor at any rate is quite considerable).

This amounts to a demonstration of Verworn's view that regeneration in the Protozoa is due to the reciprocal interaction of nucleus and cytoplasm. Organization resides in the cytoplasm as well as in the nucleus. How otherwise are we to explain the fact that a difference in the amount of cytoplasm alone (equivalent to 'the difference in volume of two spheres of 80 and 70 *micromillimeters respectively) determines the occurrence of regeneration?*

As regards the bearing of the results on the limits of divisibility of living matter: we are not concerned here with the question of the ultimate constitution of protoplasm, its composition of any ultimate vital elements whatsoever, but merely with the question propounded by Loeb, "What is the order of magnitude of the smallest particle that can show all the phenomena of life?" In the case of the animal ovum as already noted this is about one-fourth of its volume, if we include development as one of the phenomena of life. Certainly development includes all the phenomena of life. In the case of Stentor the volume is relatively considerably less as the following calculation will show:

The volume of the smallest perfect *Stentor polymorphus*, which I was able to produce, was equal to that of a sphere of about 80μ diameter; the average volume of the Stentors used in the experiments was equal to that of a sphere of about 230μ, as I determined from a series of measurements of animals killed in a weak killing-fluid, and thus completely contracted. That is, the ratio of the diameters of the smallest and the average Stentor is about 1:3; or the ratio of volume to volume is about 1:27. That is to say that the smallest Stentor which can be produced is about one twenty-seventh of the volume of the average Stentor.[1] This number is of course a mere approximation, but it certainly will not be made any greater by subsequent investigation, though it may be lessened somewhat.

[8] In the case of *S. coeruleus* the figures are different: the smallest measurement which I have of this species is 90μ; the average is 280μ; thus the ratio of the smallest to the average is about 1:3 in terms of the diameter, or 1:27 in terms of volume. I believe, however, that it would be possible to produce a smaller *S. coeruleus* by working over a larger amount of material. I do not think that there is much difference in the *absolüte* size of the smallest Stentors which can be produced, whether one uses the largest or smallest normal specimens. If *e.g.* the average size of a lot of large Stentors were 320μ, the smallest specimen which could be produced would still be 80μ. The ratio of volumes would then be 1:64. Of course this does not necessarily mean that 64 Stentors could theoretically be produced at one time from a single one, for I doubt that the nucleus could undergo that amount of division.

In any case this relation forms a striking contrast to that found in the development of the animal ovum, where a portion of less volume than one-fourth that of the ovum does not develop into an embryo (see postscript). It has been very generally found that a portion (of the two- or four-cell stage) equal to one-eighth the volume of the ovum never develops farther than the gastrula stage.

In the case of the animal ovum, again, parts slightly smaller than the minimum necessary for the complete development may undergo partial development. In Stentor we have a parallel phenomenon: parts of less than 80μ spherical diameter may undergo partial regeneration, but are unable to complete it.

It seems to me that neither increased surface tension due to diminution of surface area, nor yet any other external factor, is responsible for this failure of small pieces of Stentor to regenerate. The cause lies within; and I do not believe that it is to be sought in an insufficient production of energy. For such small pieces may live for days, constantly producing and expending energy in the ordinary processes of metabolism. I am forced, therefore, to the conclusion that the organization of these parts is in some way deficient. *There is probably for each species of animals a minimal mass of definite size consisting of nucleus and cytoplasm within which the organization of the species can just find its latent expression.* This is the *minimal organization mass.*

In the case of the Protozoa the size of this minimal mass is that of the smallest part capable of complete regeneration. But I do not believe that in the Metazoa the minimal organization mass is that of the smallest part of the ovum capable of developing into a normal embryo; for undoubtedly the influence of external factors is of the greatest importance here. I would conceive then that in the Metazoa this hypothetical minimal organization mass is smaller than any part yet observed to develop into a normal embryo. Still, from my results on Stentor, I believe that it is of such a size as to be easily visible under a low power of the microscope.

ZOÖLOGICAL LABORATORY OF THE
UNIVERSITY OF MICHIGAN, Dec., 1895.

POSTSCRIPT. — After sending the above article to the editor I had access to Boveri's recent paper entitled "Ueber die Befruchtungs- und Entwickelungsfähigkeit kernloser Seeigeleier und über die Möglichkeit ihrer Bastardirung," published in Bd. II, Heft 3, of Roux's *Archiv für Entwickelungsmechanik der Organismen*, Oct. 22, 1895. Boveri states that the smallest dwarf larva which he obtained came from a fragment which could not have measured more than $\frac{1}{20}$ the volume of the intact ovum "bei ungünstiger Rechnung." His conclusion is: "Das Fragment des Seeigeleies bis herab zu einer Grösse von $\frac{1}{20}$ des ursprünglichen Eivolumens besitzt die formative Wertigkeit des ganzen Eies." This is in marked contrast to the results of the other authors quoted, none of whom have found a figure less than $\frac{1}{4}$. The difference may be due in part to the fact that Boveri shook the ova before fertilization, while the other experimenters performed this or an analogous operation after fertilization; although this does not seem very probable. If the exact proportion of the minimal organization mass to the whole ovum be a matter of any importance, very great care in the estimation of the volumes of dwarf larvae would seem to be necessary, taking into account the differences in thickness of the layers in dwarf and normal larvae, and also the relatively slow increase in volume of the former.

The figure which I have found for Stentor is but little lower than that of Boveri for the animal ovum, and this approximation suggests interesting comparisons.