THE VIABILITY OF THE ANUCLEATE CYTOPLASM OF AMOEBA PROTEUS

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SUMMARY

The viability of Amoeba proteus cytoplasm was tested at 1- to 2-day intervals throughout the 14 days following enucleation. Viability was demonstrated by renucleating the anucleate cytoplasms and observing whether the resultant amoebae could resume normal activities such as moving, feeding and dividing. Four lines of A. proteus were studied, approximately 200 transfers being made on amoebae of each line. The introduction of a nucleus into anucleate cytoplasm initiated a temporary reactivation followed by a period of relative inactivity before either death by cytolysis or permanent reactivation with feeding, growth and division. Though approximately 70% of anucleate cytoplasms were still viable 6 days after enucleation, temporary reactivation disappeared about this time and the period of relative inactivity which generally followed it increased in duration from a few days to several weeks. Few amoebae which were able to divide failed to form clones. Anucleate cytoplasms of wheat-cultured amoebae remained viable for longer periods than anucleate cytoplasms of tetrahymena-fed amoebae.

A number of studies has been made on the cytoplasm of enucleated amoebae to determine what activities it is capable of in the absence of the nucleus (Brachet, 1961; Hirshfield, 1959; Plaut, 1959). Since some activities are found to be hardly impaired after periods of a week or more without the nucleus it is important to know whether the cytoplasm remains viable. The most effective way of demonstrating viability of the cytoplasm is to renucleate it and then to observe whether the resultant amoeba can resume normal activities such as moving, feeding and dividing. The viability of Amoeba proteus cytoplasm was tested in this way at one to two day intervals throughout the 14 days following enucleation.

MATERIAL AND METHODS

The lines of A. proteus used were:

- PDa a strain of A. proteus obtained from J. A. Dawson (New York), grown in wheat culture from 1940 to 1965 at King's College, London, and since 1965 at Southampton University.
- PDa X65 a single from a PDa wheat clone cultured for 18 months using the tetrahymena-feeding method (Griffin, 1960; Prescott & James, 1955).
- PDa NY66 a single from a PDa wheat clone cultured as for PDa X65.

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a strain of *A. proteus* obtained from Sister Monica Taylor (Glasgow), grown in wheat culture from 1950 to 1965 at King's College, London, and since 1965 at Southampton University.

*a line derived from 100 \( P_{T1} \) wheat-cultured amoebae but fed on tetra-

Cytolysis was used as the criterion for death. Micrurgy was done using a de Fonbrune micromanipulator and the technique of Comandon & de Fonbrune (1939). Membrane was considered fragile if a puncture or small tear was followed by leaking out of cytoplasm, this generally forming a plug and preventing complete cytolysis. Amoebae were kept during the anucleate period in Chalkley's medium without food. Renucleated amoebae were kept singly in small dishes, fed tetrahymena and changed to clean dishes with fresh food twice weekly. Volume measurements were made by drawing amoebae into a straight walled pipette, diameter 55 \( \mu \). Binucleate amoebae were obtained by exposing division spheres to Chalkley's medium adjusted to pH 4 using \( 1 \) N HCl.

**RESULTS**

Figure 1 gives the survival times after enucleation for four of the lines of amoebae. The tetrahymena-fed \( P_{Dn}X65 \) amoebae survived for a shorter time after enucleation than the wheat-cultured \( P_{Dn} \) amoebae. A second line of tetrahymena-fed \( P_{Dn} \) amoebae, \( NY66 \), behaved in a similar manner. At the time of the first experiments \( P_{T1}A66 \) amoebae, fed on tetrahymena for 4 months, were similar in survival time to the wheat-cultured \( P_{T1} \) amoebae, but these amoebae had decreased in average volume from \( 28 \times 10^6 \mu^3 \) to \( 14 \times 10^6 \mu^3 \) after a further 4 months of tetrahymena-feeding, and their average survival after enucleation had decreased from 13 to 9 \( \frac{1}{2} \) days.

The chief differences noted when wheat-cultured amoebae were changed to the tetrahymena-feeding method were a decrease in size and a greater fragility of the
Viability of anucleate cytoplasm

The average volume of the tetrahymena-fed X65 and NY66 amoebae was little more than half that of the wheat-grown PDa line, $15 \times 10^6 \mu^3$ as compared with $27 \times 10^6 \mu^3$. Determination of the survival times of X65 amoebae enucleated at 1, 4, 24 and 48 h after division did not show any increase in survival times coincidental with volume increases. Binucleate X65 amoebae (with much greater cell volumes) enucleated at 4, 24 and 48 h after nuclear division had far shorter survival times (Table 1). Neither the two-fold volume increase within the cell cycle, nor the four-fold volume increase obtained by using binucleate amoebae, gave any indication that

Table 1. Survival times after enucleation of X65 amoebae over a four-fold volume range

<table>
<thead>
<tr>
<th>Age when enucleated, h</th>
<th>Mononucleate amoebae</th>
<th>Binucleate amoebae</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>11 9 6½ 9½</td>
<td>4 24 48</td>
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<tr>
<td>Average survival after enucleation, days</td>
<td></td>
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<td>Average volume at time of enucleation, $\mu^3$</td>
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<tr>
<td>$9 \times 10^5$</td>
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<td>$10 \times 10^5$</td>
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<td>$28 \times 10^5$</td>
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<td>$35 \times 10^5$</td>
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<tr>
<td>Number enucleated</td>
<td></td>
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<tr>
<td>40 161 132 100 27 25 152</td>
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</tr>
</tbody>
</table>

Fig. 2. Percentage of anucleate amoebae able to form clones after the reintroduction of a nucleus. Experiments used four lines of *A. proteus*: PDa, ○ ○ ○ (228 transfers); PDaX65, × × × (242 transfers); PT, × × × (252 transfers); and PTA66, □ □ □ (190 transfers).

Increased size was responsible for the longer survival time of the larger wheat-grown amoebae. The time in the cell cycle when amoebae are enucleated appears to be an important factor, the period following division and possibly the period just prior to division giving anucleate cytoplasms with longer survival times. Fragility of the membrane affected survival, cytology frequently following enucleation of X65.
amoebae. The wheat-grown lines showed membrane weakness only after they had been anucleate for long periods.

Figure 2 gives the viability of anucleate amoebae as indicated by their ability to form clones when renucleated. In general the viability measured by renucleation was directly related to the length of time enucleated cytoplasm could survive before cytolyzing (see Fig. 1); the time at which renucleated amoebae were no longer able to form clones corresponding for $P_{Da}$ and $P_{Da}X65$ amoebae to the time when 75–80% of the enucleated amoebae had cytolyzed, but to the time when only 50% of $P_{Ta}$ and

![Graph showing relative durations of periods of temporary reactivation, inactivity, and permanent reactivation for $P_{Da}$ amoebae.](image)

Fig. 3. Diagram showing the relative durations of the periods of temporary reactivation (A), inactivity (B) and permanent reactivation (C,) for $P_{Da}$ amoebae. The curve $\bullet\bullet\bullet$ represents the average times for the first divisions of these renucleated amoebae when the interval between enucleation and reintroduction of a nucleus was varied from 3–12 days.

20% of $P_{Ta}A66$ enucleated amoebae had cytolyzed. Non-viability of anucleate amoebae was generally accompanied by a lack of movement, a tendency to forms with a minimum surface/volume ratio, membrane fragility, and a reduction in the density of crystals and other cytoplasmic inclusions. In relating success of renucleation experiments to cytoplasmic viability two factors must be borne in mind: first, renucleation experiments selected only the best amoebae surviving at any time and therefore give an abnormally high result for viability; and secondly, operational damage which caused a loss of approximately 10% at 0 days became greater as the anucleate period increased, so that by 3 days for the small $P_{Da}X65$ line, and 7 days for the
larger $P_{Da}$ and $P_{T1}$ lines, operational damage was probably responsible for at least 20% of the non-viable renucleated amoebae.

Introduction of a nucleus into anucleate cytoplasm produced a temporary reactivation not necessarily indicative of recovery. This reactivation was observed in the operating chamber immediately after the transplantation of a nucleus, but it was more reliably characterized by the ability of renucleated amoebae to attach and to move when placed in small dishes. This reactivation generally followed within 15 min of renucleation, but in amoebae in which the anucleate period had been longer than 3 days it was sometimes delayed for up to 4 h and in such cases lasted for less than 24 h. Reactivation was seldom observed in $X65$ renucleated amoebae when the cytoplasm had been without a nucleus for more than 3 days, but in the other lines it was frequently observed when the cytoplasm had been without a nucleus for up to 6 days.

Except in the case of immediate renucleation temporary reactivation was generally followed by an inactive period during which little movement or feeding occurred. Increasing the period between enucleation and renucleation increased this period of inactivity so that in renucleated amoebae with cytoplasm which had been without a nucleus for 6 or more days this period lasted a week or more before a return, if cytoplasm was still viable, to normal feeding, growth and division; this has been represented diagrammatically for $P_{Da}$ amoebae in Fig. 3. Once renucleated amoebae divided subsequent divisions were normal and few of such dividing amoebae failed to form clones.

**DISCUSSION**

The introduction of a nucleus into anucleate cytoplasm initiates the following sequence of events:

\[ \text{A} \quad \text{B} \quad C_{1} \quad C_{2} \]

\[ \text{Renucleation} \rightarrow \text{Temporary reactivation} \rightarrow \text{Period of inactivity} \rightarrow \text{Death by cytolysis} \]

\[ \text{Permanent reactivation followed by feeding, growth and division} \]

Temporary reactivation (A) decreased in duration with increase in the length of the anucleate period and seldom occurred when the cytoplasm had been without a nucleus for more than 6 days. The shorter the period of temporary reactivation, the longer was the period of inactivity (B). Once amoebae became active again (C$_{2}$) feeding and growth began and division soon followed.

In view of the role of the mitochondria in generating ATP for the energy cycles of the cell, it is of interest to consider the way in which reintroduction of a nucleus initiates energy-consuming activities such as movement and attachment. Brachet (1961) comparing nucleate and anucleate halves of amoebae found that the nucleus exerted little effect on the respiratory rate for periods of at least a week and concluded
that the 'mitochondrial enzymes were remarkably independent of nuclear control'.
Yet the rapidity with which activities such as movement and attachment decline on
the removal of the nucleus and the return of these activities on the reintroduction
of the nucleus, suggests that the nucleus plays a key role in the utilization of energy.
Further, it is worth noting that even a foreign nucleus, that is one from a different
species of amoeba, is able to initiate similar activities in the host cytoplasm though
such heterotransfers are unable to carry on other activities which would lead to
successful clone formation (Lorch & Danielli, 1950, 1953).

It must be borne in mind that when a nucleus is pushed from one amoeba to
another a small amount of cytoplasm inevitably accompanies it, and the possibility
that ATP or some other substrate is conveyed to the anucleate cytoplasm in this
manner cannot be excluded. However, since reintroduction of a nucleus causes
reactivation in cytoplasm which has been deprived of a nucleus for only a very short
time and so would not have depleted its energy stores, accompanying cytoplasm
would not appear to be the factor responsible for reactivation. Brachet (1961) has
shown that ATP content in the anucleate halves of amoebae decreases only very
slowly, remaining higher than that found in starved nucleate halves. The cytoplasm
then has energy stores as well as an energy-generating system, yet the nucleus must
still hold the key for allowing the utilization of its energy for at least some activities.

Though the investigations of a number of workers have shown little immediate
change in cellular constituents or functions on the removal of the nucleus, phosphate
uptake and protein synthesis excepted, a number of important metabolic changes
take place during the 3- to 6-day period following enucleation (Brachet, 1955, 1959b;
Linet & Brachet, 1951; Mazia & Hirschfield, 1950; Prescott & Mazia, 1954). Of
particular note are disappearance of glycogenolysis followed by the utilization of
protein; a change in the structure of the 'ergoplasmic small granules'; and a
decrease of almost 50% in protein content. The 3- to 6-day period corresponds to
a time in the present experiments when cytoplasm was still 70% viable (except in the
case of X65 amoebae) but when it was fast losing its potential for reactivation by
nuclei and the relatively inactive period B was becoming established. It seems probable
that period B is a time during which the protein synthesizing system of the cell is
being restored; this could entail the repair of ribosomes, the synthesis of new
messenger RNA and, once protein synthesis has been restored, the replacement of
enzymes which have decreased in the absence of the nucleus, e.g. acid phosphatase
and esterase (Brachet, 1961). It would be expected that as the anucleate period was
increased so the time necessary to restore the system would increase.

Though the protein content of anucleate cytoplasm decreases rapidly in anucleate
halves of amoebae, the incorporation of [58S]methionine (Mazia & Prescott, 1955)
and [14C]phenylalanine shows that some protein is still being synthesized even 8 days
after enucleation (Brachet, 1959a; Brachet & Ficq, 1956; Ficq, 1956). On the other
hand, the incorporation of [14C]adenine and uracil shows RNA synthesis to be
negligible by 8 days though some synthesis does occur up to this time (Brachet,
1959a; James, 1954; Plaut & Rustad, 1956; Shreb-Guicher, 1961). Thus, RNA
synthesis occurs during the time, as found in these experiments, when cytoplasm is
still viable; its disappearance by 8 days, and very low protein synthesis, corresponding to the time when the cytoplasm is becoming non-viable. These results indicate that there is a level below which RNA and protein cannot fall without irreversible damage to the cytoplasm and its inclusions.

Survival times after enucleation of the tetrahymena-fed lines were much shorter than the wheat-fed A. proteus. Membrane fragility leading to operational damage was at least partly responsible for this shorter survival period. The reason for the membrane fragility of amoebae fed for long periods on tetrahymena is not known. Enucleation by chemical means gave survival times for the tetrahymena-fed X65 amoebae equivalent to those obtained for the wheat-grown amoebae after mechanical enucleation (12–14 days following exposure to actinomycin-D, 14–16 days following exposure to puromycin), providing always that these substances were used at a concentration sufficient to prevent all growth and division (M. J. Ord, unpublished observations). Apart from producing membrane fragility the tetrahymena-feeding method led to a decrease in volume of the amoebae. This decrease in volume was shown to have no direct relationship to decrease in survival time after enucleation.

Despite the quantity of work done on both enucleated amoebae and nudeate and anucleate halves of amoebae no serious attempt has been made to assess the viability of such anucleate cytoplasm over the periods studied. Comandon & de Fonbrune (1939) using Amoeba sphericnucleus found that two of five amoebae renucleated 2 days after enucleation survived, but had no success at renucleating 6-day anucleate cytoplasm; Lorch & Danielli (1953) found that two of eight Amoea proteus and three of six Amoea discoides renucleated 24–48 h after enucleation formed clones while reactivation was observed on the renucleation of 3-day anucleate cytoplasm. The experiments reported here show that cytoplasm is viable for a much longer period. The anucleate cytoplasm of wheat-cultured A. proteus could be used up to 8 days after enucleation for studies on nuclear/cytoplasmic relations or the action of chemicals and radiations in the absence of nuclear influence, though shorter periods would be advisable when using tetrahymena-fed amoebae and checks for viability throughout the experimental period by reintroduction of nuclei should be carried out. The absence of reactivation (A) on reintroduction of a nucleus is not a reliable criterion for viability.

REFERENCES

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