THE CELL IN STARVATION: PHYSIOLOGICAL AND CHEMICAL OBSERVATIONS*

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In their extensive study "The Biology of Human Starvation," Keys, Brožek, Henschel, Mickelsen, and Taylor raise the question (1) as to how the proportional composition of single cells may be altered in inanition, and note the paucity of data on this question. Data obtained on starving metazoans, while abundant, consist of mean changes in parenchymal and stromal cells and is further complicated by hormonal effects and the inability to effect a total starvation or to determine what is available to the cells under partial inanition.

To circumvent these objections, a number of laboratories have carried out investigations on fresh water amebae (2–5), because despite the obvious specialization of this cell, it possesses a heterotrophic nutrition and is of a size permitting the application of certain micro methods. Moreover, it can be placed under inanition in an artificial pond water, lacking carbon and nitrogen, with confidence that the medium is "normal" for the animal.

Previous measurements on amebae have involved the use of (*a*) the Cartesian diver for respiration measurements (5), (*b*) the Cartesian diver balance of Zeuthen (6), giving "reduced" weight (dry weight less a buoyancy factor), (*c*) a starch solution density gradient method for density (7), and volume and density by weighing in starch solutions of known density (6). From these data and various deductions considering known specific gravities of typical proteins, lipide, and carbohydrate, an attempt was made to approximate ameba composition and its change during starvation (5).

Because methods now exist for direct measurement of dry weight, protein, lipide, and volume of cells as small as *Amoeba proteus*, a mononucleate ameba, it was decided to survey again the variation in the above materials, as a preliminary to following quantitative variation of particular molecules, such as enzymes, in starving amebae.

In the course of this investigation the question arose as to whether cell size may influence the resistance of a cell to inanition. More specifically considered, the question involves, in part, the extent to which a cell may consume for pur-

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poses of energy, molecules of itself which ordinarily subserve "structural" functions. Such an ability, if it both exists and can significantly extend survival of the cell, may be defined as "degrowth." If there is a minimal cell size, the question then arises as to whether a large cell has more opportunity for degrowth.

In addition, if a cell is to be capable of "degrowth" as herein defined, then some physiological arrangement must exist which determines which structure is consumed and which remains, for it is obvious that useful consumption of a structure requires the retention of a residuum of processing molecules and of structure (genetic and otherwise) for resuming function or growth when inanition ceases.

Material and Methods

The cell used in the investigations to be reported was a large mononucleate ameba, *Amoeba proteus*, of the race maintained by the General Biological Supply House (Chicago). These animals were raised in unsterile cultures with Tetrahymena gelei as the principal food source. Details of the method of culturing and of enucleation have been published (8). All culturing and experimentation was carried out at 18°C. The medium for growth and starvation was $MgSO_4$ 2 mg.; KCL 6 mg.; and $CaHPO_4$ to saturate per liter glass distilled water. The growth rate was one division every 3.4 days. No agar was employed in either the culture or maintenance dishes and glassware was chemically cleaned (hot 50 per cent nitric acid).

For experiments on starvation, cells were first carefully washed free of ciliates by transferring with braking pipettes. When individual cells were to be observed, each cell was kept in 0.5 ml. of medium and was transferred daily to fresh medium. Where populations were being followed, no more than 60 cells were kept in 10 ml. of medium in a small Petri dish and were counted and transferred to fresh medium daily.

For the measurements of dry weight, the amebae were lyophilized according to the following procedure. First the cells were placed in distilled water.[1] Each cell, in a small volume of distilled water, was then transferred to the conical base of a small, numbered Pyrex tube (0.5 mm. O.D. and 3.5 cm. length), and 50 of such tubes were bound together with a rubber band and lowered into a lyophilizing chamber. The chamber was then submerged in an acetone-solid CO_2 freezing bath.

When the contents of the tubes had frozen, evacuation was initiated, the chamber was removed from the bath, and lyophilization was allowed to proceed with an acetone-solid CO_2 trap in the system to collect the water. The chamber with the cells under vacuum was stored at −10°C. until use. At this time it was allowed to come to the temperature of the weighing room and the vacuum was carefully broken. (The weighing room was dehumidified as required.) In weighing, a cell was removed from the tube with a sharpened hair affixed to a glass rod and transferred to the pan of a fish-pole type quartz fiber balance as devised by Lowry (9). Each cell was replaced in its tube after weighing. The weighed cells were then extracted

[1] Amebae do not cytolyze in distilled water, nor do they seem to increase in volume. Indeed, contrary to what might be predicted, they increase their surface. Exposure of cells to distilled water (unlike exposure to unbalanced salt solutions) for as long as tested (1 day) appears not to harm the cells which seem perfectly active and normal on return to normal media. Despite the possibility that some unbound ions and metabolites may be extracted, passage through distilled water was undertaken, to avoid salt crystal encrustations on the surface of the dried cells.

successively with hexane (15 minutes), absolute alcohol (30 minutes), and briefly with hexane again, from which they were allowed to dry. This last step was necessitated by the fact that cells dried from alcohol tend to stick to the glass. The cells were then reweighed. The difference in weights was referred to as the weight of extractable lipide. Hexane extraction removes about one-fourth of the total loss after both solvents. Doubtlessly alcohol removes some non-lipide. It was felt, however, that this error could not be too serious since pilot experiments on fed animals gave values for extractable lipide which, when added to protein values, gave combined weights that were a reasonable proportion of the total dry weight, *i.e.* 87 per cent, and reasonable lipide protein ratios as cited in Table 3 of Holter and Zeuthen's paper (5), *i. e.* 40 per cent.

Volume measurements on the living amebae were made by two methods, neither of which is completely satisfactory. The first is based on the chamber method of Lumsden and Robinson (10), but employed a Petroff-Hauser bacterial counting chamber as a compression chamber of known depth, and a microscope, a camera lucida, and a planimeter for measuring the area instead of photographic apparatus. The counting squares on the chamber were employed for calibrating the camera lucida and planimeter. Each animal was placed on the chamber in a small water drop and the coverglass carefully lowered. Since the chamber depth may vary An effort was made to keep the animal in the cross-marked areas. Amebae of any size, particularly if they are recently fed, may rupture and heal when compressed; therefore, any cell seen to rupture was no longer employed in the experiment.

Because it was felt that the compression might cause a response in the ameba involving a volume change, it was decided to employ a second method for size determination. In this, advantage was taken of the marked light sensitivity of this race of *Amoeba proteus*. Animals were placed in a rectangular chamber which contained agar and ameba medium and exposed to a horizontal light beam from a microscope lamp (Universal A. O.). Cells in the beam become more or less monopodal. With the use of an ocular micrometer, the length and width of the animals could be estimated in arbitrary units. The product of these values was then employed as a crude estimate of relative size.

The value of both methods was that it permitted within a short time interval, the measurement of the large number of animals required to obtain meaningful survival data. These animals had to be obtained from the same culture at the same time, since ameba nutrition is not under precise control.

During the early period of starvation, *i.e.* the 1st week at 18°C., some divisions still occur in the population. This is in accord with Prescott's (11) observations on this ameba. Information on cell division was employed as a measure of population nutrition, in the sense that any group of 100 cells failing to show at least a 4 per cent increase during this time was rejected. As a further device to insure uniformity in the material used, populations being prepared for an experiment were removed from the mass cultures and cultivated on a smaller scale (with weekly counting) in an effort to force maximal growth.

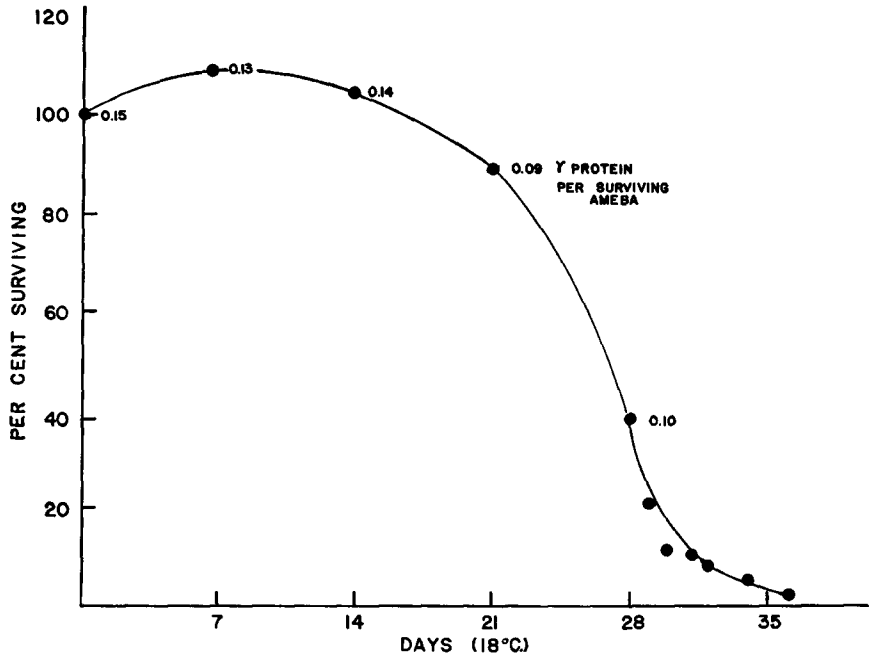
The protein data presented were obtained by the method of Lowry *et al.* (12), with crystalline bovine serum albumin as a standard. In addition to the peptide linkage, this method possesses some reactivity with tyrosine.

The survival time of an ameba was taken as the time when the cell could no longer be found. Cells dying in 1 week or less were presumed to be injured and were not included in the data. Cells that divided were kept, but were not included in the survival time computations (although this proved to be an unnecessary precaution).

EXPERIMENTAL

Fig. 1 summarizes the results of an experiment in which protein was measured during the decline of a starving population. The protein values given are the

average of those for two groups of thirty animals each. It will be noted that the population number shows an initial rise due to cell divisions occurring during the 1st week. On occasion, as many as 20 per cent of the cells have divided. Since measurements on cell samples drawn from such a population are used to infer the change of some entity in the total population, it follows that erroneous results will be obtained if suitable corrections are not applied for this population increase. In the experiments here reported, such population changes were noted and corrections applied. The protein is seen to decline from a level o



TEXT-FIG. 1. Protein level in starving amebae.

about 0.13 to 0.15 micrograms to 0.09 to 0.10 micrograms in the surviving, but dying members of the population. Measurements on protein standards, run with each determination, fell along a straight line. Accordingly, the variance in the measurements must be due to sample variance. The data do not permit drawing a curve for the decline, but the impression is that there was a stable period followed by a decline in population protein.

Table I gives the data for dry weight and dry weight after extraction with fat solvents, together with the calculated extractable lipide in both absolute amounts and in proportion of the dry weight. The initial dry weight is seen to have had a mean value of 0.23 μg. and the extracted component (E.L.) to have had a weight of 0.06 μg., which is 26 per cent of the dry weight. The dry weight

falls progressively, but at a declining rate. The extractable component, however, first falls, then rises to about initial levels. This has been the experience in two such experiments, differing only in that the one not reported was performed at a higher and less rigorously controlled temperature. Since a major proportion of the standard deviation (S.D.) is an unavoidable consequence of the variance in cell size in these random samples, and since this size variance is essentially maintained during starvation, it is felt that the differences are significant. This is particularly evident in the low standard error (S.E.) of the mean per cent weight loss at each interval.

If the hexane and alcohol extract all the fat including the lipide of lipoprotein, then the rise may be due to the extraction of some non-lipide component that accumulates during starvation and does not escape from the cell. In some previous work performed

TABLE I

Dry Weight and Extractable Lipide in Starvation

Length of time at 18°	No. of animals	Before extraction		After extraction				
		Average weight	S.D.	Average weight	S.D.	E.L.	E.L.	S.E.
days		*μg.*		*μg.*		*μg.*	Mean per cent	
0	41	0.23	0.09	0.17	0.04	0.06	22.6	1.6
8	34	0.18	0.07	0.13	0.04	0.05	25.0	2.3
15	38	0.15	0.04	0.13	0.04	0.02	16.1	1.2
22	39	0.13	0.04	0.10	0.03	0.03	27.8	1.3
39	24	0.10	0.04	0.06	0.04	0.04	32.5	2.9

at a considerably higher temperature, there was no loss of radiophosphate in starving amebae, measured while alive, up to the point of cytolysis (18). Such selective retentiveness is, therefore, a distinct possibility.

On the other hand, if not all lipoprotein fat is extracted by the hexane-alcohol, then perhaps unmasking of lipide is being observed. A third explanation, that lipide is being synthesized, seems unlikely. An investigation should be made with other solvents.

Electron microscopic studies on these starving cells were performed routinely and will be reported elsewhere. They show that dense droplets presumed to be lipide droplets tend to disappear by the 2nd week, and that the originally low density mitochondrial "core" is decidedly dense during terminal starvation, suggesting unmasking of lipide. Mitochondrial lipide alone, however, could not account for the rise indicated. If one totals the terminal protein of the cells of Fig. 1 and the terminal extractable lipide of Table I, one obtains a value which exceeds the terminal dry weight. This suggests that a retained non-lipide but hexane-alcohol extractable entity also contributed to the protein value. It should be noted, however, that these measurements were not obtained on the same group of cells and that the group of Fig. 1 showed a more rapid population decline.

No data are as yet available for carbohydrate on this race of ameba, but Linet and

Brachet (13) have given data for nucleate and enucleate glycogen levels for an *Amoeba proteus* measured after 24 hours of starvation. The sum of these weights, per ameba, is 0.04 μg. of glycogen.

Adding this value to 0.14 μg. of protein and 0.06 μg. of lipide, we obtain 0.24 μg. which corresponds well with our dry weight figure. In the manner of Holter and Zeuthen (5), we may calculate a volume for the dry weight components on the assumptions of typical specific gravities of 1.35 for protein, 1.55 for carbohydrate, and 0.9 for lipide. Since grams per cubic centimeter are equivalent to micrograms per 10^6 μ^3, this calculates to a volume of 2×10^5 μ^3. This is about 13.5 per cent of the total mean ameba volume as found with our chamber method, the later value being 14.8×10^5 μ^3 (based on 171 cells). If the remainder of the cell is water, the density then calculates to be 1.02. This compares favorably with the data on the giant ameba *Chaos chaos*, Løvtrup (7) finding a density of 1.0186, and Lumsden and Robinson (10) about the same value.

TABLE II

Cell Size and Inanition Resistance

Experiments carried at 18°C.

Method	Size classes	Survival
		days
1. Monopodal length (arbitrary units)	I. 7.5 ± 0.3 S.E.	25.1 ± 2.3 S.E.
	II. 12.0 ± 0.5	26.2 ± 2.0
	III. 18.5 ± 0.8	23.5 ± 1.4
	Total population	25.5 ± 1.4
2. Chamber volume	I. 12.1 ± 0.65 S.E. $\times 10^5 \mu^3$	34.6 ± 3.5 S.E.
	II. 16.2 ± 1.26	32.2 ± 3.3
	III. 25.0 ± 2.74	33.2 ± 4.2
	Total population	33.3 ± 2.0
	Nucleate "halves"	33.0 ± 1.3
	Enucleate "halves"	23.7 ± 0.8

The indicated loss in the dry weight with starvation is of the order of 0.13 μg. Of this, approximately 0.045 μg. would appear to be a protein loss, with the preliminary data suggesting that this occurs late in starvation. It is quite possible that this mean loss represents a greater loss for certain morbid cells and no loss or very little for premorbid cells. It is difficult to interpret the lipide changes, but certainly a loss of 0.04 μg. is a reasonable estimation, since this represented the extent of loss when the extractable fraction was minimal. Finally, if the glycogen levels of this race are comparable to those of the *A. proteus* strain studied by Linet and Brachet (13), then 0.043 μg. of glycogen is a maximal value for utilization. The sum of these values is close to 0.13 μg. Considered in terms of percentile loss, protein has fallen by 30 per cent and lipide by 66 per cent.

Prescott (11) has given values for the post-telophase volume of *fixed Amoeba proteus*

and for the reduced weight during growth. Considering that some shrinkage occurs during fixation, his volume value of $5 \times 10^5 \mu^3$ is in fair agreement with out volume range and his figures for reduced weights are of the order of one-tenth of our values. This is due to the cancellation of weight by buoyancy.

Table II gives the results of experiments on the relation between cell size and survival time under total inanition. In addition to the data presented, we have obtained similar results at higher temperatures and in numerous pilot experiments whose primary aim was to see whether the mean survival time could be standardized by nutritional control. These experiments did not produce this desired goal and, accordingly, it cannot be emphasized too strongly that comparative experiments (*e.g.* size, enucleation, etc.) must be made on cells taken from the same culture at the same time. The data do not permit any conclusion but that cell volume differences do not yield significant survival time differences Accordingly, "degrowth," if it occurs, does not appear to confer any advantage to the large cell.

It may be argued that the time of disappearance of a cell from its container may not correspond to the time of cell death. This raises the question of the definition of cell death. Certainly the loss of reproductive capacity, while of interest, is not held to be a sign of death. The time of loss of function may be of interest for certain cells where function has established criteria, but even such a sign does not imply total decay. Accordingly, we feel that the activation of autolytic phenomena can be the only useful sign of morbidity and the most non-subjective sign of these events is disappearance of the cell.

The data then suggest that the survival of a nucleated cell is primarily a function of the concentration of cytoplasmic factors. What is more interesting is that the nucleate half showed no detectable survival difference. On the other hand, the absence of the nucleus produced a significant loss of survival ability. In the experiment presented, the nucleate and enucleate portions derived from a particular animal could be followed. One nucleate portion preceded its corresponding enucleate fragment in cytolysis by 1 day. One pair vanished on the same day. All the remaining enucleates (28 cells) were gone before the nucleate portions.

It is of interest to note that the enucleate animals had a mean survival of 23.7 days (S.E. mean 0.8) at 18°C. in the experiment cited. This is a typical period. Brachet (14) has stated that enucleate amebae of his *A. proteus* race survive about 10 to 15 days and nucleate animals, about 3 weeks. E. Baltus (personal communication) has informed me that experiments in the Brachet laboratory are conducted with the animals at 16°C. It is not clear as to whether the differences observed are due to technical or racial factors. A suggestion that Brachet's cells may be deteriorating more rapidly than our animals is seen in the fact that where protein levels have been followed in nucleate and enucleate cells, Brachet finds little or no loss for nucleate animals, but

losses of considerable magnitude in 2 or 3 days on the part of enucleate cells. Thus, one publication (16) gives nucleate to enucleate protein ratios of 1.29 and 1.50 after 2 days and another (15) 1.32 in 3 days. In view of these disparities for protein, it is hard to see how cause and effect conjectures can be made for pyridine nucleotide losses of equal magnitude (17) without reported protein levels. In fact, by a more sensitive method, over a longer interval, and at higher temperature, statistically treated data showed no significant DPN losses in enucleate animals (8). It may be noted that the maximal protein loss of our surviving animals in a rapidly declining population was of the order of 30 per cent.

DISCUSSION

The data presented in this paper are in agreement with the data obtained for *Chaos chaos* by Holter and Zeuthen (5). The densities calculated to virtually the same figure, and there was an indication of lipide consumption during starvation and of a considerable quantity of lipide in feeding animals. Since a populational study has cells in varying degrees of starvation, we cannot comment on the variations observed in the patterns of starvation exhibited by individual cells.

The principal source of variation in amebae is size and this apparently, in nucleate animals, does not produce a detectable survival effect. For animals of a given size, however, the principal source of variation is undoubtably the variation in the number of food vacuoles. In particulate feeders, such as amebae, this is exceedingly difficult to control.

It also raises the question of how much of an ameba is vacuole content. Our electron microscopic studies reveal readily detectable Tetrahymena remnants even after 21 days of starvation (18°C.). Accordingly, whenever any entity is measured in this animal, the decision as to what extent one is measuring amebae becomes rather difficult.

The place of the ameba crystals in the measurements of weight and components is also a difficult problem. These survive osmic fixation and subsequent dehydration through alcohols to methacrylate. They survive starvation. They seem to constitute a non-negligible mass of the cell, yet their composition remains essentially unknown.

It is for these reasons that much of the data on these animals must be regarded as approximations.

SUMMARY

Observations made on *Amoeba proteus* during total inanition revealed the following changes:

Dry weight declined progressively, but at a decreasing rate to about 45 per cent of the initial levels when determined in surviving members of a dying population. Protein fell to about 70 per cent of the initial level. A hexane-alco-

hol extractable component fell during early starvation then rose to about its initial absolute level in the dying cells. While initially most of this component is probably lipide, it is not certain that other materials are not extracted during cell degeneration.

Survival as a function of cell size was studied. No advantage in survival was apparent for any size class. Nucleate cell "halves" likewise showed no survival time differential, unlike a highly significant decrease in the survival of enucleate portions. The maintenance of the initial variance about the mean population weight (after hexane-alcohol extraction) during starvation, likewise supports the idea that survival depends largely on concentration parameters.

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