THE EFFECT OF ENUCLEATION ON THE DPN LEVEL OF AMEBA

By ADOLPH I. COHEN,*‡ Ph.D.

(From the Department of Pharmacology, Washington University School of Medicine, St. Louis)

(Received for publication, September 12, 1955)

INTRODUCTION

Brachet (1) has postulated that the nucleus may exercise control over oxidative phosphorylation in the cell by regulating the synthesis of diphosphopyridine nucleotide (DPN). This conjecture has been strengthened by reports that, relative to the cytoplasm, the cell nucleus has a high specific activity of an enzyme synthesizing DPN from nicotinamide nucleotide (NMN) and adenosinetriphosphate (ATP) (2–4). Brachet's conjecture was, in fact, based in part on the finding of Mazia and Hirshfield (5) that enucleate amebae had about one-third the radiophosphate uptake of nucleate amebae.

The most direct way of approaching the question of the relation of the cell nucleus to cell's DPN is to deprive a cell of its nucleus and to follow the cell's total and oxidized pyridine nucleotide levels. For this purpose, the ordinary ameba, *A. proteus*, is ideal, since it may be cut in two and comparative studies made on the halves. As a mononucleate cell lacking symmetrical organization, such halves may be assumed to differ only in the presence or absence of a nucleus. Within minutes after cutting, the enucleate half becomes spheroid, irresponsive to stimuli, and ceases to feed. A sudden change in the level of any molecule of general importance could be involved. Since the nucleus is but a small fraction of the cell's mass, a comparative study of nucleate and enucleate halves, while dramatically capable of showing in an artifact-free manner any entity as being exclusively or largely nuclear, is less useful where the material is both nucleoplasmic and cytoplasmic. In this instance, the cytoplasmic background could cover up a nuclear concentration several fold that of the cytoplasm. In such instances isolation studies could be more useful, but these are subject to absorption and elution problems. Even nonaqueous methods do not control possible intracellular rearrangements during lyophilization. This is particularly true for small molecules such as DPN.

* Current address: Department of Anatomy, Washington University School of Medicine, St. Louis 10, Missouri.

‡ This research was performed during tenure of a Post-doctoral Fellowship of the U. S. Public Health Service.

In the case at hand, measurement of DPN at the level of amebae has not been possible in the past because of the small amounts of tissue involved. Recently, however, a new, highly specific, and ultrasensitive method of DPN assay has been developed by Lowry and Roberts[1]. This procedure has generously been made available to us for this purpose.

Experimental

The strain of *A. proteus* employed was obtained from Dr. Daniel Mazia of the University of California (Berkeley) and derives from a Turtox Biological Supplies strain. It differs from other *A. proteus* strains (*e.g.* North Carolina Biological Supply) in its larger size, ability to feed on the ciliate *Tetrahymena*, and in minor characteristics. The amebae were raised directly on the glass surface of finger bowls (cleaned with 50 per cent nitric acid) in a simple salt medium containing KCl, 4 mg. per liter, $MgSO_4$, 2 mg. per liter, and saturated with $CaHPO_4$. *Tetrahymena gelei* raised in sterile proteose peptone cultures and washed twice with ameba medium by centrifuging were used as the sole food source. However, inasmuch as ameba cultures are unsterile, bacterial growth may have provided a source of additional nutrition. An effort was made to adjust the density of amebae and food so as to keep the cultures in logarithmic growth, but complete success has not been achieved in this regard.

Cells to be enucleated were first freed of most contamination by stirring the cultures, allowing the relatively dense amebae to settle, and siphoning off the supernatant debris and ciliates. After a number of such siphonings, washing was completed by transferring the cells with the aid of braking pipettes through a number of dishes of medium.

In general, cells used in experiments were allowed to starve for at least 12 hours before enucleation or other experimental processing in order to preclude the possibility that ingested food organisms contributed to the measurements.

Cells to be enucleated were transferred to small Petri dishes containing ameba medium over a thin base of washed agar. The cells were then induced to become monopodial by placing them in the path of a horizontal beam from a microscope lamp (universal AO Spencer). While monopodial, cells were cut in two using a hand-held rod with a fine tungsten wire loop at one end. A low power dissecting microscope provided the magnification required and cutting was begun at the side of the ameba group closest to the light source. Although an experiment to be reported indicated no effect of light on the DPN level, cells that were not cut were kept under illumination as controls. Whole amebae or nucleate halves migrate away from the light source, whereas enucleate halves, although tending to elongate in a manner minimizing the surface exposed to the light source, cannot successfully migrate. After a few hours, separation of nucleate and enucleate halves is achieved with a success of 95 to 100 per cent, as determined on stained samplings. On removal from illumination, enucleates immediately round up, thus providing a check on the light induced separation. Since enucleate amebae cannot feed, nucleate and whole ameba controls are kept under starvation conditions.

The stock DPN^+ and DPNH (sigma) were assayed by the cyanide method of Colowick (6) and by 340 mμ absorption respectively, and diluted to the required concentrations. DPN^+ solutions in distilled water were highly stable in the deep freeze. DPNH solutions were used fresh.

The assay method of Lowry and Roberts involves the use of DPN^+ or DPNH (indistinguishably) as a cycling electron carrier in a two-enzyme system generating malate from oxalacetate. The malate formed is then measured fluorimetrically with a modified Farrand

[1] To be published.

photofluorometer (7) by the β-naphthol procedure of Lowry *et al.* (8). The substrates employed are oxaloacetic acid (jansonsal) and ethanol, respectively. The enzymes employed are alcohol dehydrogenase (sigma) and a partially purified preparation of malic dehydrogenase (gift of Dr. O. H. Lowry). DPNH is oxidized *via* malate formation and the DPN^+ formed is reduced *via* acetaldehyde formation. Details of the method wil be published elsewhere by Lowry and Roberts.

For extracting amebae, the appropriate number of animals (or halves) were placed, with the aid of a braking pipette, in a 3 mm. diameter conical bottom tube in a small volume of ameba medium. A small volume of tris (hydroxymethyl) amino methane (tris) buffer (M/10; pH 8.2) was added and the capped tubes heated in a boiling water bath for 30 seconds and then placed in an ice bath. The pH employed was based on studies made by Greengard *et al.* (9) on the subject of pH *versus* stability of DPN^+ and DPNH. Additional heating did not increase the extraction and was avoided to minimize the danger of DPNH oxidation and destruction of either form. Shorter periods of heating gave lower DPN levels. The other ingredients were then added to the extraction tubes which were then used for incubation. Controls of various levels of DPN^+ and malate were carried through the identical procedure. The relationship between DPN level and malate formed was invariably linear within the periods of time employed and DPN levels were calculated on the basis of a standard curve run with each experiment.

If DPNH levels were to be studied, some tubes were brought to pH 1.0 by addition of acid, and neutralized after a few minutes. Control extract tubes received equivalent saline. Since DPNH is acid labile, the decrement in DPN level as compared to unacidified controls represents the DPNH content.

RESULTS

In the absence of the complete system, ameba tissue makes no contribution to fluorescence in the β-naphthol procedure. The addition of Neurospora DPNase, kindly donated by Dr. Sidney Colowick, also prevents the activity of the complete system. Since this enzyme and also the alcohol dehydrogenase are highly DPN specific, it seems reasonable to conclude that DPN is being measured.

When DPN is added to the extracts, quantitative recovery is made. This indicates that ameba DPNase activity is not a problem. Unheated amebae give about 50 per cent of the DPN level of tubes heated for 30 seconds. This may be due to DPNase, but is more likely due to failure to extract bound DPN.

The size variation in a growing ameba population called for large samples of animals in order to get low mass variation. However, a compromise had to be made with the number of halves that could be produced. Table I gives the DPN content of amebae, based on assays employing different numbers of animals. It is seen that DPN content is proportional to the amount of tissue and that an average of five samples of 9 to 11 animals each (or of 20 halves) is satisfactory.

Differences in the level after 24 hours of starvation of ameba DPN^+ in sampling different cultures suggest that the DPN^+ content can be influenced nutritionally. This suggests that flexibility in the concentration of this im-

portant cofactor is compatible with cell function. Levels ranging from 1 to 4×10^{-13} moles per ameba were obtained. All comparative studies, accordingly, were made on animals taken from the same culture at the same time.

TABLE I

The Effect of Sample Size in DPN Assay

No. of ameba per sample	Average DPN per ameba
1	0.83
3	1.00
5	0.99
7	1.14
9	1.02
11	1.04

DPN as moles $\times 10^{13}$, average of 5 samples.

TABLE II

The Effect of Illumination on Ameba DPN Level

Time illuminated	DPN
hrs.	
0	2.48
2	3.10
4	2.80
6	2.50
8	3.20
8 (plus DPNase)	0.30

DPN as moles $\times 10^{13}$, average 4 samplings, 10 animals each.

TABLE III

Comparison of DPN Levels of Whole and Half Amebae

Time after cutting	DPN (moles $\times 10^{13}$)		
	Whole	Nucleate × 2	Enucleate × 2
hrs.			
24	1.48 ± 0.20	1.71 ± 0.38	1.71 ± 0.21
168	1.90 ± 0.41	2.19 ± 0.13	1.72 ± 0.33

Average and standard deviation of 10 whole or 20 half amebae.

Table II shows that prolonged illumination is without effect on the DPN level of amebae. Tables III and IV show comparative studies of DPN content of whole and half amebae. Table III shows experiments involving large numbers of animals and clearly establishes that the DPN level of nucleate and enucleate amebae is the same and remains so over the period studied. Table

IV, based on fewer animals (each figure representing an average of two tubes of ten animals each), confirms the findings of Table III and also shows that acidification of the extracts gives no significant decrement in DPN level. Accordingly, almost all the DPN of the ameba must be oxidized.

TABLE IV

Total versus Oxidized DPN Levels in Amebae

Exp. No.	Time after cutting	No acid treatment (total DPN)				Acid-treated (DPN+)			
		W	N	E	N+E	W	N	E	N+E
	hrs.								
1	5	2.14	1.89	1.67	3.56	2.59	1.35	1.18	2.53
2	24	3.39	1.92	2.05	3.97	3.56	1.78	1.92	3.70
2	144	2.48	1.15	0.99	2.14	2.24	1.41	1.10	2.51
3	24	3.52	2.10	1.78	3.88	2.96	2.00	1.58	3.58
3	72	2.58	1.59	1.52	3.11	3.10	1.07	1.32	2.39
3	96	2.90	1.48	1.60	3.08	2.20	1.71	1.32	3.03
4	24	3.45	1.75	1.79	3.54	3.75	1.77	1.47	3.24
4	96	2.87	1.87	1.98	3.85	3.26	1.50	1.66	3.10
4	144	4.00	1.73	1.80	3.56	3.05	1.83	2.10	3.93
4	168	3.46	1.73	1.64	3.37	3.16	1.86	1.79	3.45

DPN as moles $\times 10^{13}$ per whole or half; W, whole; N, nucleate half; E, enucleate half.

DISCUSSION

It is at once clear that no change in total or in oxidized DPN correlates with the sudden appearance of the enucleation syndrome. Since this situation does not parallel the observed change in phosphate uptake (5), it would appear that no justified hypothetical leaps can be made from the possible general finding of high nuclear concentration of DPN synthesizing systems to an explanation of low phosphate uptake in enucleate cells. Thus the first half of Brachet's hypothesis, the postulation of the existence of a more or less direct dependence of the cell DPN level on the cell nucleus is not supported by the data. The higher concentrations of nuclear DPN synthesizing systems may be tentatively interpreted as reflecting either greater need for DPN by the nucleus or origin of the cell's DPN synthesizing enzyme in the nucleus. The present investigation does not apply to the relation of cell DPN to cell phosphorous metabolism. Since cytoplasmic DPN may be highly stable, in the absence of turnover data, remote nuclear DPN origin cannot be rigorously disproven by these findings.

The finding that practically all of this cell's DPN is oxidized, cannot be rigorously interpreted on the basis of the steady state data herein presented. Since oxidation of DPNH probably constitutes an energy source for ATP production (10), the finding that the ratio of oxidized to reduced DPN strongly

favors the oxidized form, could mean that this source of energy was not limited by the cell's capacity to oxidize DPNH.

The metabolic effects of enucleation in ameba have not, as yet, provided any clues to the origin of the few objective symptoms. From the rapid onset of the symptoms of enucleation and from the finding of Comandon and deFonbrune (11) that quick recovery follows insertion of a fresh nucleus into a cell which had been enucleate for 72 hours, there is suggested a close linkage of nuclear activity to cell metabolism. Brachet (1) found no change in the oxygen uptake for at least 24 hours after enucleation, unlike Clark (12) who found a fall in cyanide-sensitive respiration. This important point of information is therefore in need of reinvestigation. Mazia and Hirshfield (5) found a decreased uptake of radiophosphate by enucleate cells. This probably reflects decreased phosphate turnover which may in turn be due to the lower cell mobility and probable cessation of some synthetic activity. Brachet (13) found equal levels of ATP in aerobic halves of both types but this does not conflict with possible turnover differences. On the other hand, when anaerobic, the enucleate halves had a lower ATP level. Although glycolysis has not been demonstrated in this ameba, which is an aerobe, the above finding, taken together with some work of Linet and Brachet (14) suggesting a decreased utilization of glycogen by enucleates, could indicate a defect in glycolysis. Brachet (13), in the only study on a glycolytic enzyme to date, found no difference in triose phosphate dehydrogenase between nucleates and enucleates. Nuclei have been reported as being rich in glycolytic enzymes (15) but cytoplasmic levels are not inconsiderable (16) and no suggestion has been made that these are more nuclear than cytoplasmic. Although the above anaerobic difference may be a significant clue, it must be recalled that the symptoms of enucleation appear under aerobic conditions when ATP levels are equal in both types of half.

Because of the acuteness of the appearance of the symptoms of enucleation in amebae and because of the ease with which enucleate halves and controls may be obtained, further studies on this organism may cast important light on nuclear-cytoplasmic relationships.

SUMMARY

1. Amebae contain DPN at levels of from 1 to 4 $\times$ 10^{-13} moles per cell.

2. Following enucleation, nucleate and enucleate halves continue to have equal DPN contents over the six day period studied. Similarly, starving whole amebae maintain their DPN level over this period.

3. No reduced DPN could be detected in these aerobic animals. This remained true for whole amebae and for nucleate and enucleate halves over 6 days of starvation.

4. A method is described for the preparation and rapid separation of nucleate and enucleate ameba halves, based on a response of amebae to light.

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