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ON THE INCORPORATION OF THYMIDINE IN THE CYTOPLASM OF *AMOEBA PROTEUS**

W. PLAUT

University of Wisconsin, Madison

Résumé—L'incorporation de la thymidine tritiée par le cytoplasme a été étudiée dans plusieurs souches différentes d'*Amoeba proteus*. La majeure partie sinon la totalité du matériel radioactif non soluble dans les acides est localisée dans une molécule similaire à celle de l'ADN, comme l'indiquent les résultats de l'incubation de préparations fixées, en présence de ribonucléase ou de désoxyribonucléase. L'incorporation dans le cytoplasme a lieu aussi bien en présence qu'en absence du noyau et est relativement indépendante de la nutrition préalable des cellules. L'aspect de l'image autoradiographique des cellules anuclées ainsi que des cellules intactes soumises à un jeûne prolongé suggère que la matériel sensible à la DNase est associé à un constituant cytoplasmique particulaire dont la distribution se modifie au cours du jeûne et en l'absence du noyau. Rien ne permet de préciser le rôle physiologique éventuel de ce matériel qui ne peut être caractérisé jusqu'à présent que par sa sensibilité à l'action de la DNase et son insensibilité à l'action de la RNase. Des études préliminaires au microscope électronique ne permettent pas d'attribuer l'incorporation d'isotopes à la présence de corps étrangers dans le cytoplasme amibien. A titre d'hypothèse, on peut imaginer que dans les conditions de ces expériences, des désoxyribonucléotides s'accumulent et se polymérisent dans le cytoplasme alors que normalement ils le feraient dans le noyau.

IN THE course of a series of studies designed to elucidate the ribonucleic acid metabolism of *Amoeba proteus* it was thought desirable to obtain some information on the nuclear deoxyribonucleic acid (DNA) metabolism of this organism. The weak Feulgen reaction exhibited by the nucleus and the absence of incorporation of ^{32}P, adenine-^{14}C and orotic acid-^{14}C into amoebal DNA in previous experiments (PLAUT, 1959) suggested that DNA was present in low concentration and that the use of labelled precursors with high specific activity would lead to more reliable data. We therefore incubated amoebae with tritiated thymidine and studied the incorporation of this precursor in autoradiographic preparations. In early experiments (PLAUT and SAGAN, 1958) nuclear incorporation could be detected in a proportion of the cells, as was to be expected, but we also found incorporation of radioactivity into a cytoplasmic component in every cell examined. Moreover, the incorporated radioactivity was not affected by digestion of the fixed cells with ribonuclease but completely absent from both nucleus and cytoplasm in cells incubated, after fixation, with desoxyribonuclease. The nature of the precursor, the acid insolubility of the labelled entity in the cells, and the behaviour of this entity toward

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ribonuclease (RNase) and desoxyribonuclease (DNase) digestion suggested that we were dealing with a DNA and not a thymine containing ribonucleic acid (RNA) as had been reported in some organisms (LITTLEFIELD and DUNN, 1958). These early observations permitted no insight into the nature of the cytoplasmic DNA, its possible function in the metabolism of the cell, and its generality. More recent experiments have furnished some additional information which permits the drawing of some limited conclusions and possibly the establishment of a preliminary working hypothesis which can serve as a basis for more precise experimental probing.

The first question we have examined is that of the possible nuclear origin of the cytoplasmic DNA. When mixtures of nucleate and anucleate fragments of *Amoeba* (obtained by cutting whole cells with fine glass needles) were analysed after incubation with tritiated thymidine under conditions similar to those used previously (10 μc/ml., specific activity 360 μc/μmole, food-free medium, 40 hr or more of incubation, 70–80 days of autoradiographic exposure) it was found that DNase-sensitive radioactivity occurred in the cytoplasm of both types of half-cells. In fact, anucleates were generally somewhat more heavily labelled than nucleates and exhibited a greater tendency toward clustering of grains in the autoradiographic image as though the radioactivity were associated with a particulate cytoplasmic component whose distribution is less homogeneous in the anucleate than in the nucleate cell. Quite apart from these differences, the experiment indicated clearly that the nucleus is not necessary for the cytoplasmic incorporation of thymidine and suggests that the cytoplasmic radioactivity in whole cells is of cytoplasmic and not nuclear origin.

We may also ask whether or not the labelled DNA in the cytoplasm will enter the nucleus. This question has been examined experimentally by incubating whole cells in tritiated thymidine and determining the percentage of cells with labelled nuclei in a group of cells fixed immediately after incubation and a similar group which was permitted to remain in non-radioactive medium for 2 to 3 days before fixation. Both groups of amoebae showed clear nuclear labelling in about 40 per cent of the cells. However, since it is difficult to establish the total absence of tritium label in a nucleus in a squash preparation of *Amoeba* the experiment is being repeated with sectioned material. The available data suggest that the labelled cytoplasmic DNA does not enter the nucleus under our experimental conditions.

A third possibility which must be considered is that the cytoplasmic DNA is attributable to the presence of foreign biochemical systems in the amoeba cytoplasm. The fact that *Amoeba proteus* requires other living systems for its nutrition makes this possibility very real. We have considered both *Tetrahymena* (the normal food organism in our cultures) and bacteria in this context. COHEN (1959) has suggested that about 12 days of starvation are required for the disappearance of *Tetrahymena* DNA from the amoeba. We have therefore incubated amoebae starved for 15 days with tritiated thymidine and compared their incorporation with that of cells fed just prior to incubation. The results show no major difference in the extent of isotope incorporation into a DNase-sensitive cytoplasmic component except a tendency toward labelling inhomogeneity in the cytoplasm of the starved cells resembling the labelling pattern in anucleate cells. There is thus no evidence

for the supposition that the cytoplasmic incorporation is dependent on *Tetrahymenae*. It is of some interest to note that about half of the starved cells showed extensive nuclear incorporation. This is consistent with earlier findings of increased Feulgen stain intensity in the nuclei of starving amoebae (BRACHET, 1950; HELLER and KOPAC, 1955) and suggests that starvation does not preclude the possibility of nuclear DNA synthesis in this organism. (The alternative possibility of incorporation via exchange has, however, not been ruled out since the increased Feulgen stain intensity could also be attributed to condensation without change in amount in the studies cited.)

The question of micro-organismal involvement in the cytoplasmic thymidine incorporation is less easily resolved. So far we have not been able to find any evidence for bacteria within the amoebae used by examination with the light microscope or by lysis of the cells on nutrient agar. Preliminary investigations with the electron microscope, carried out jointly with Professor HANS RIS*, have also failed to show recognizable bacteria. Bacteria are present in the medium in which the amoebae are grown and they are frequently associated with the amoebal surface. However, these surface contaminants, when found on amoebae after thymidine incubation, are invariably unlabelled. It is not possible at the present time to evaluate the possibility of a viral population within the amoeba. While we have not seen virus particles in our electron microscope preparations we have not as yet examined enough material for a finite conclusion.

In an attempt to check on the generality of cytoplasmic thymidine incorporation and the possibility of a unique infection in our stock cultures we have tested presumably unrelated strains of *Amoeba proteus* obtained from Dr. PRESCOTT at Los Angeles, Dr. ROSLANSKY at Princeton, and Dr. DANIELLI at London. The results with these cells did not differ significantly from those obtained with our own cultures, suggesting that the cytoplasmic incorporation is probably general with respect to *A. proteus*.

One of the technical difficulties in the experiments so far has been the length of time necessary for obtaining clear evidence for incorporation. The periods of incubation with the tritiated precursor have been 40–100 hr and the autoradiographic exposures from 70 to 120 days. We have tried to shorten these times by utilizing tritiated thymidine with a specific activity of approximately 3000 μc/μmole, at 100 μc/ml. At the higher level of isotope availability it is possible to obtain clear autoradiographic evidence for cytoplasmic incorporation with 6 hr of incubation and 20 to 30 day exposure periods. However, cell survival becomes a problem and the acid insoluble incorporated material is no longer completely removable with DNase. Caution is therefore necessary in the design and interpretation of experiments employing such extreme incubation media. One reassuring observation made on cells incubated with the high specific-activity solution for 24 hr is the following: the spotty, non-homogeneous distribution of cytoplasmic label, characteristic of starved and anucleate cells, was never found after DNase digestion even though a substantial label residue was present; the latter was invariable homogeneously distributed.

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The results obtained to date can be summarized briefly. Cytoplasmic incorporation of tritiated thymidine occurs in at least four strains of *Amoeba proteus*. The effect of RNase and DNase on the retention of the acid insoluble cytoplasmic radioactivity suggests that most but not necessarily all of the label is in a DNA-like molecule. The cytoplasmic incorporation occurs in the absence as well as in the presence of the nucleus and is at least qualitatively independent of the previous feeding history of the cells. The autoradiographic pattern seen over anucleate cells or well-starved whole cells suggests an association of the DNase-sensitive radioactivity with a particulate cytoplasmic component which undergoes a change in distribution during starvation and in the absence of the nucleus. (The distribution of autoradiographic grains over the nucleus is always homogeneous and never shows any tendency toward clustering as occurs over the cytoplasm.) We have not been able to obtain any unequivocal evidence for the postulated cytoplasmic DNA with either the Feulgen reaction or with acridine orange. We have no information on the chemical nature of the labelled entity beyond its sensitivity to DNase; the base composition and the degree of polymerization of the material, if it in fact is DNA, are unknown. It must also be pointed out that we have so far examined only squash preparations and can exclude the possibility that the label is associated with the cell membrane rather than within the cytoplasm only by the fact that the grain pattern over the nuclei is often distinctly different from that over the cytoplasm.

One might well conclude that in view of these uncertainties our observations should not be regarded as a proper basis for speculation but filed away as due to idiosyncrasies of a cell which is hardly typical of cells in general in many respects. However, potentially similar data have been obtained in other biological systems. LOGAN (personal communication) has observed the presence of cytoplasmic labelling after *in vitro* incubation of ascites cells with tritiated thymidine. TAKATS (1959) has recently reported incorporation of the same precursor into presumptive DNA in the cytoplasm of the microsporocytes of *Lilium longiflorum*. BRACHET (1958) has noted incorporation of tritiated thymidine in anucleate *Acetabularia*; in this case, however, it appears that most of the radioactivity was recovered in an RNA fraction.) CHÈVREMONT *et al.* (1960) have observed Feulgen-positive material on mitochondria as well as cytoplasmic thymidine incorporation in tissue culture cells after treatment with acid DNase. In addition there have been numerous demonstrations of a DNA-like material in the cytoplasm of eggs (SOLOMON, 1957) and there is the work of CHAYEN (1954, 1959) which suggests the cytoplasmic location of much if not all of the DNA in some plant meristematic cells during interphase. While these various findings are not strictly comparable and subject to their own uncertainties one may conclude that there exists some evidence for the notion that the cytoplasm is involved in DNA anabolism. This notion derives considerable support from recent demonstrations of a DNA polymerase in the cytoplasmic fraction of metazoan cells (BOLLUM, 1958; MANTSAVINOS and CANNELAKIS, 1958; SMELLIE *et al.*, 1959; DAVIDSON, 1960). I would like to suggest, as a highly tentative working hypothesis, that the amoeba data here reported be regarded as evidence for a normal cytoplasmic polymerization of desoxyribonucleotides which has proceeded to the point of experimental detectability under the conditions of our experiments. This clearly speculative hypothesis

is based in part on the one common feature of several of the reported instances of cytoplasmic DNA or precursor incorporation reviewed above: the temporary cessation of cell division, either as a normal or an induced phenomenon, in cells which will normally divide again; one might postulate that under these conditions the nucleus is a poor receptor of small oligonucleotides for final polymerization and permits these to accumulate in the cytoplasm, possibly to a higher state of polymerization than would normally be the case.

SUMMARY

Cytoplasmic incorporation of tritiated thymidine has been observed in several unrelated strains of *Amoeba proteus*. The effects of incubation of the fixed cells with ribonuclease and desoxyribonuclease indicate that most if not all of the acid insoluble radioactivity is present in a DNA-like molecule. The cytoplasmic incorporation occurs in the presence as well as in the absence of the nucleus and is relatively independent of the previous feeding history of the cells. The autoradiographic pattern seen over anucleate cells and well-starved whole cells suggests an association of the DNase-sensitive radioactive entity with a particulate cytoplasmic component which undergoes a change in distribution during starvation and in the absence of the nucleus. There is no evidence so far for a possible physiological role of this material nor has it been defined in any way other than the aforementioned behaviour toward enzymes. Preliminary studies with the electron microscope have revealed no foreign agents in the amoeba cytoplasm to which the incorporation might be attributed. As a tentative working hypothesis it is suggested that under the conditions of these experiments there is an accumulation and polymerization of desoxyribonucleotides in the cytoplasm which would normally take place in the nucleus.

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DISCUSSION

P. ALEXANDER: Evidence is accumulating which shows that the enzymes necessary for the synthesis of DNA in the nucleus are only produced when the cell is stimulated to divide. The amoeba you used were under such unfavourable physiological conditions that they may not have these enzymes. As a result the DNA precursors may pile up and this could give rise to an atypical process in the cytoplasm which has no physiological significance.

W. PLAUT: The fact that a fair percentage of cells even under extreme starvation show nuclear incorporation of thymidine suggests that nuclear DNA synthesis can occur and hence that the necessary enzymes are present in at least some cells. Nonetheless, the physiological significance of the cytoplasmic incorporation process is by no means established at the present time.

J. DANIELLI: If one is to take cytoplasmic DNA synthesis seriously, the cytoplasmic DNA must be isolated and identified. Otherwise other explanations of thymidine fixation, e.g. terminal addition of a few nucleotides to some other molecule, cannot be disregarded. Bacteria-free amoebae can probably by obtained by culture in penicillin, to which they are highly resistant. But the possibility of virus infection cannot be solved so readily. The question of the relationship between nuclear and cytoplasmic thymidine could be settled more readily by studying amoebae, at known intervals after division, in logarithmic growth.

W. PLAUT: (1) While we plan to attempt an isolation and characterization of the thymidine-containing cytoplasmic component it appears doubtful that we will be able to separate it from the small amounts of *Tetrahymena* DNA which may be present, unless the two differ significantly in composition. In the absence of such information, the behaviour of the labelled component with respect to DNase and RNase does suggest that it has considerable similarity to DNA.

(2) One of the experiments reported utilized cells which had been incubated with penicillin prior to incubation with tritiated thymidine. The results were identical to those obtained with untreated cells.

(3) We are currently using synchronized amoebae in incubation experiments and should be able to establish the relationship, if any, between cytoplasmic incorporation and stage in the division cycle.

J. DAVIDSON: These interesting observations raise the very important question of the intracellular location of the enzymes involved in DNA synthesis. BOLLUM and POTTER (1959 *J. Biol. Chem.* **233**, 478) have prepared from regenerating rat liver a cell-free extract which is essentially cytoplasmic in nature and which contains the enzymes necessary for the incorporation of thymidine into DNA. My colleagues and I have worked with a similar extract from Ehrlich ascites tumour cells which can bring about the synthesis of DNA *in vitro* (SMELLIE, KEIR and DAVIDSON (1959) *Biochim. Biophys. Acta* in press). It is prepared by the osmotic disruption of the ascites cells without breakage of the nuclei. If the nuclei are now separated and disrupted so as to produce a nuclear extract, the ratio of the activities of cytoplasmic to nuclear extracts is 100 to 33. These results do not necessarily mean, however, that the enzymes of DNA synthesis are essentially cytoplasmic for they might well be extracted from the nuclei during the preparation of the

cytoplasmic extract. Moreover, the extract is prepared from a population of cells in which a high proportion are dividing and nuclear enzymes might easily pass into the cytoplasm during mitosis when the nuclear membrane breaks up.

I should like to ask Dr. PLAUT whether he has ever met amoebae in which synthesis of nuclear DNA was active?

W. PLAUT: About 40% of our cells show nuclear as well as cytoplasmic incorporation.

P. FREDERICQ: Could the cytoplasmic synthesis of DNA in *Amoeba* be similar to the formation of kappa particles in *Paramecium*?

W. PLAUT: We hope to obtain an answer to this question by (a) subjecting amoebae to extensive electron microscope examination and (b) looking for cytoplasmic DNA synthesis in *Paramecium* without kappa particles.

F. KASTEN: (1) Have you tried using other stains for DNA besides the Feulgen test, such as methyl green, etc.?

(2) Have you determined whether the cytoplasmic DNA is affected by irradiation?

W. PLAUT: (1) We have tried acridine orange without clear positive results.

(2) An irradiation experiment is in progress.

H. FIRKET: (1) The nuclear incorporation must be particularly difficult to study with squashed amoebae. They are still a very thick object for tritium autoradiography as most of the tritium electrons are absorbed within 1 μ. So let us look forward to the sectioned amoebae, mentioned by Dr. PLAUT.

(2) Not all cytoplasmic DNA syntheses are as puzzling as the ones we have heard about from Prof. CHÈVREMONT and Dr. PLAUT, not even in Protozoa. With Dr. and Mrs. STEINERT (1958 *Exp. Cell Res.* **15**, 632), we have been able to show DNA synthesis by thymidine incorporation in the blepharoblasts of trypanosomes. This very small structure (1 $\mu \times 1{\cdot}5\,\mu$) is well known as an auto-reproducing organelle within the cell and has been suspected for a long time to contain DNA. We gave the final proof by the desoxyribonuclease test on the Feulgen reaction and the incorporation of tritiated thymidine. Synthesis of DNA occurs there only prior to duplication and with a slight delay after the beginning of the nuclear incorporation. So it is likely that it remains under nuclear control.

W. PLAUT: There is no evidence for any clear correlation between nuclear and cytoplasmic incorporation in the amoebae so far.