INCORPORATION OF TRITIATED THYMIDINE IN THE CELLS OF *CAENORHABDITIS BRIGGSAE* (NEMATODA) REARED IN AXENIC CULTURE

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ABSTRACT

In the rhabditid nematode *Caenorhabditis briggsae* the incorporation of thymidine-H^3 has been studied by autoradiography after Feulgen staining, with animals maintained under axenic conditions in a medium of only partly defined composition. Labeling has been followed in adults left in the presence of thymidine-H^3 for periods of from ½ to 24 hours, as well as in adults reared from larvae in the presence of the tritiated nucleoside. A massive incorporation is found in the nuclei of the gonads and intestine; also a less intense particulate cytoplasmic incorporation is clear in certain cells, especially those of the intestine. In general, all labeling has proved to be sensitive to DNase, but resistant to RNase. The label's stability has been tested by the transfer of adults into a medium containing "cold" thymidine. They remain there for up to 48 hours. A transfer for 24 hours results in a considerable decrease in the intensity of nuclear and cytoplasmic labeling; a stay of 48 hours leads to its complete disappearance from non-dividing (intestinal) as well as dividing (gonadal) nuclei. A phenomenon of DNA turnover is envisaged and discussed as a possible physiological attribute of *C. briggsae*.

INTRODUCTION

Within the class Nematoda certain rhabditoids constitute particularly valuable subjects for the study of the metabolism of labeled molecules, for the following reasons: (*a*) these organisms can be maintained indefinitely in axenic culture (4, 5, 7, 10–12, 26); (*b*) media whose chemical composition is almost entirely known (6, 7, 12, 24) and in which one can vary only one (or a few) component(s) at will can be used in axenic cultures; and (*c*) experiments can be performed with isolated individuals (7) or with pooled populations of a million or more (26).

So far, radioisotopic studies with nematodes in sustained axenic culture have been restricted to the species *Caenorhabditis briggsae*. This organism is of particular interest as it belongs to a group (superfamily Rhabditoidea) that is characterized by *eutely* (1, 27); *i.e.*, in somatic tissues the number of cells and nuclei are fixed, from varying larval stages on, depending upon the organ; this constancy does not apply, of course, to the gonads. Most members of a population of *C. briggsae* are self-fertilizing, protandrous hermaphrodites (15), resembling the more thoroughly studied *Caenorhabditis elegans* (14) and many other rhabditoids.

In order to compare, in *C. briggsae*, the metabolic activities of somatic and gonadal cells, it was decided to follow the incorporation of a labeled

precursor of DNA. Accordingly the work was performed with axenic cultures, each containing a small number of individuals, and the behavior of tritiated thymidine (thymidine-H^3), a specific DNA precursor (9, 21), was investigated. A brief abstract on preliminary phases of these studies has been published (16).

MATERIAL AND METHODS

C. briggsae was reared axenically. In most respects the basic cultivation techniques employed followed those described by Dougherty and his collaborators (7). Early studies (16) were performed with adults or larvae from mass cultures of varying ages, but most of the work reported here depended upon a standardized procedure of initiating each culture, when destined for experimental use, with 10 larvae. Stock culture tubes contained 0.15 ml of a medium consisting of a base of vitamins, nucleotides, and mineral salts supplemented with casein peptone at 4 per cent (by weight) a heated liver extract (7, 11, 24) and at 10 per cent (by volume). The only difference between this medium and the one used earlier by Rothstein and Tomlinson (22 is that, in this study, casein peptone was substituted for the original soybean peptone.

0.05 ml of either labeled or "cold" thymidine was directly added to each tube containing 0.15 ml of the medium. The thymidine-H^3 solution (New England Nuclear Corporation, Boston) had an activity of 6.4 c/mmole (1 mc/ml) and a concentration of 0.038 mg/ml. The thymidine-H^3 uptake was investigated by using the nematode preparation technique, originally developed by Nigon (13), and the autoradiographic technique of Pelc (17). After fixation in acetic sublimate, the slides were stained by the Feulgen technique and covered for a 2 week period with a photographic emulsion (Kodak stripping film AR 10).

In order to check the specificity of the thymidine-H^3 labeling, before application of the stripping film, control slides were subjected to the nucleases of RNA and/or DNA. Both RNase and DNase (California Corporation for Biochemical Research, Inc., Los Angeles, 10 mg in 100 ml of buffer solution) were applied at 37°C, the first for 2½ hours and the second for 7 hours. Certain slides were subjected to both nucleases, in succession.

Finally, in certain experiments *C. briggsae* individuals, incubated for various periods, in the presence of thymidine-H^3, were transferred to tubes containing the medium supplemented with "cold" thymidine (Nutritional Biochemical Corporation, Cleveland). This transfer was carried out after several washings of the animals in 1/15 M K-phosphate buffer (pH 7). The concentration of the "cold" thymidine solution was equal to that of the radioactive thymidine.

Three major types of experiments have been performed and repeated several times:

1. 6-day-old *C. briggsae* adults reared in stock cultures were transferred to a medium supplemented with thymidine-H^3. At first, they were sacrificed after an incubation time that varied from ½ hr to 48 hours, but, in most experiments, the incubation time was held to 24 hours.

2. Larvae of *C. briggsae* were transferred to a medium containing thymidine-H^3. They were hatched from eggs laid by 6-day-old adults that were kept in 1/15 M K-phosphate buffer (pH 7) for 24 hours. The larvae remained in the labeled medium throughout their period of growth and were sacrificed on the 7th day, when they had clearly reached the adult stage.

3. Animals derived from a second generation of *C. briggsae* raised in the presence of thymidine-H^3 were studied. These were obtained in two steps. First, nematodes, having matured (as in the second type of experiment) in tubes containing thymidine-H^3, laid eggs, which hatched to give larvae during the course of the 7th day. Second, these larvae were removed and were transferred into new tubes, which also contained thymidine-H^3; after 7 more days of incubation in the labeled medium, they became second generation *C. briggsae* adults. The latter were then the objects of study.

In fact, in each of the foregoing three major types of experiments, only half of the *C. briggsae* adults were as a rule sacrificed immediately at the end of their stay in the tritiated medium; the other half were transferred further, for periods of between 24 and 48 hours, into tubes in which the thymidine-H^3 was replaced with equimolar amounts of "cold" thymidine.

RESULTS

The experiments carried out according to the procedures described above show, in most instances, a clear labeling of the cells of *C. briggsae*. Only once did the labeling fail (16), and that occurred in an experiment done with animals treated according to the first two of the three major procedures just described. However, numerous experiments, in which labeling was observed regularly, have yielded many results concerning the localization, specificity, and stability of thymidine-H^3 uptake.

Localization of the Thymidine-H^3 Labeling

Adults of *C. briggsae*, incubated in the presence of thymidine-H^3 (first type of experiment), show labeling in the nuclei only. If the incubation lasts less than 24 hours, the uptake is found only in the

gonads. After a very short period of incubation (½ hour), incorporation affects only the apical region of each gonad (Fig. 1). As the time of incubation is increased, the number of labeled nuclei grows rapidly from the proximal toward the distal end, and, after 24 hours of incubation, each gonad appears labeled heavily along its full length (Fig. 2). If incubation in thymidine-H^3 lasts 24 hours or longer, nuclear uptake is seen not only in the reproductive cells, but also in the intestine and in the wall of the genital tract (Fig. 2).

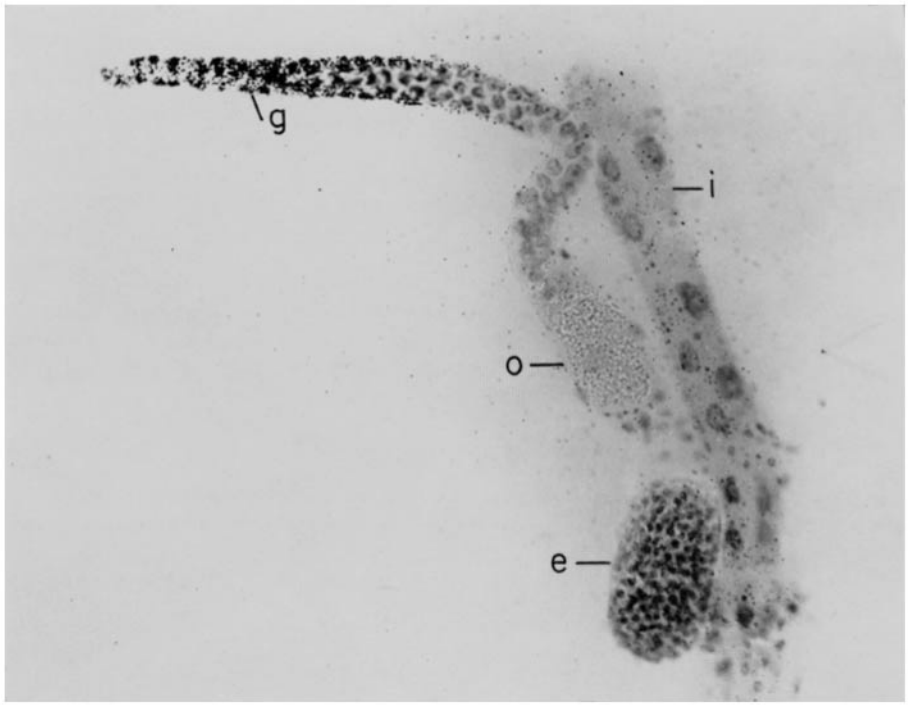

FIGURE 1 Autoradiograph of a gonad and a portion of the intestine in *Caenorhabditis briggsae*; the organism had remained, as an adult, less than 24 hours (*viz.*, 16 hours) in the presence of thymidine-H^3. Nuclear labeling is clearly visible in the apical half of the gonad (*g*). The oöcyte (*o*), embryonating egg (*e*), and intestine (*i*) show no labeling. Feulgen stain. × 500.

C. briggsae, incubated from an early larval stage (second type of experiment), ultimately shows uptake in both the nuclei and the cytoplasm. Nuclear labeling occurs in intestinal cells and in the wall of the genital tract, as in the preceding experiments. In addition, the nuclei of different organs (muscles, esophagus, hypodermis), which are not ejected outside the body during the dissection, are labeled. The reproductive cells show a clearly visible nuclear labeling up to the pachytene stage only (Fig. 3). More advanced stages (oöcytes in diakinesis and spermatozoa) show an irregular labeling from one slide to another and between different cells of the same slide. Cytoplasmic labeling is always less intense than nuclear labeling, but the former is particularly obvious in the intestine. Fertilized eggs, whose development has started, also show considerable uptake, which is, however, difficult to localize accurately.

C. briggsae reared in the presence of thymidine-H^3 for almost two generations (*i.e.* according to the third type of experiment) show a distribution and an intensity of labeling comparable to those found in animals reared in the second type of experiment.

Specificity of the Thymidine-H^3 Labeling

The specific nucleases exhibit identical effects in all the animals. RNase does not decrease the intensity of nuclear or cytoplasmic labeling observed in the different types of experiments. By contrast, DNase

causes almost total disappearance of labeling in all instances. The labeling remains clearly only in three posterior nuclei (probably of the rectal glands) and sometimes diffusely, *i.e.* in non-particulate form, in the posterior region of the body. The successive action of the two nucleases introduces no greater decrease of labeling than does DNase action alone. Furthermore, incubation in DNase buffer and/or RNase buffer never shows an effect on the labeling.

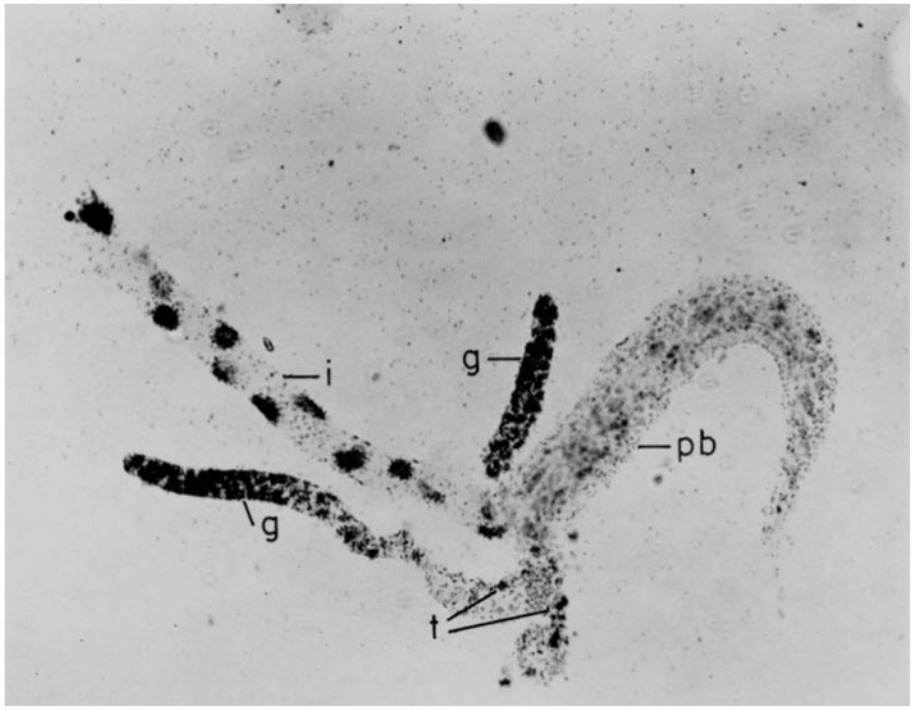

FIGURE 2 Autoradiograph of the gonads, intestine, and posterior half of the body in *C. briggsae*; the organism had remained, as an adult, 24 hours in the presence of thymidine-H^3. Intense nuclear labeling of the gonads (*g*), the intestine (*i*), and the wall of the genital tract (*t*) is to be noted, but none is visible in the posterior half of the body (*pb*). Feulgen stain. × 365.

Stability of the Thymidine-H^3 Labeling

The procedure whereby worms are transferred into "cold" medium has yielded particularly reproducible results. When the stay of *C. briggsae* in the presence of thymidine-H^3 is followed by a transfer into the medium containing "cold" thymidine, in which the worms are left for 24 hours, almost all the cytoplasmic labeling and a significant part of the nuclear labeling vanish (Fig. 4). As regards the latter, its disappearance is not homogeneous even in 2 neighboring nuclei in the same organ. But, when the stay in the cold medium lasts *48 hours*, all labeling, except that resistant to the successive action of RNase and DNase, vanishes uniformly and completely from somatic cells and from gametogonia (Fig. 5).

We call this disappearance *delabeling*.

DISCUSSION

Irregularity of Labeling in Adult Sex Cells

It was noted that, in some instances, labeling was not apparent in mature sex cells. This could be attributed to a limitation of the sensitivity of the autoradiographic technique. As oögenesis is accompanied by a considerable increase in the volume of the oöcyte nucleus (14), it can be imagined that, for a given quantity of thymidine-H^3 incorporated, the grain density is lower over the large nucleus of the mature oöcyte, in which

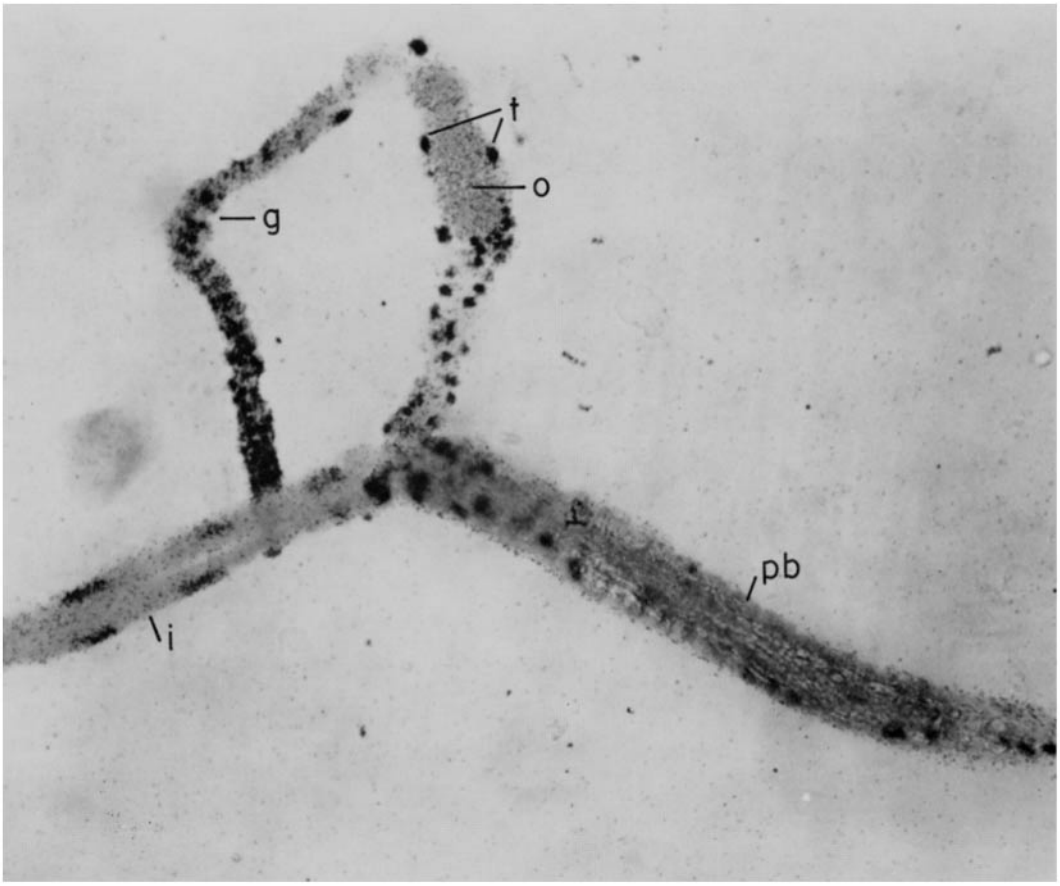

FIGURE 3 Autoradiograph of a gonad, the intestine, and the posterior half of the body in *C. briggsae* reared from an early larval stage to adulthood (7 days) in the presence of thymidine-H^3. Same localization of nuclear labeling as in Fig. 2, with heavy uptake by the gonad (*g*) and nuclei of the wall of the genital tract (*t*). In addition, the presence of nuclear labeling in the posterior half of the body (*pb*) and of cytoplasmic labeling in the intestine (*i*) is to be noted, also the absence of clear labeling in the oöcyte (*o*). Feulgen stain. × 420.

the chromatin is dispersed, than over the small nuclei of oögonia, in which the chromatin is condensed. Thus, mature oöcyte labeling can become difficult to distinguish from the background.

The lack of labeling in the nuclei of certain mature germ cells could also be explained by the presence of absorbing layers of cytoplasm. In fact, it was observed that the nuclei of the oöcytes, which did not appear to be labeled, are in general separated from the photographic emulsion by a significant thickness of cytoplasm. But, when the oöcytes are flattened, as when their membrane is burst during the preparation of the specimen, the nuclei show a well defined labeling. Similar observations were made for spermatozoa. Neither grain count estimates nor actual thickness measurements were performed to test either one of the above explanations. But this work is in progess now.

Labeling

CYTOPLASMIC LABELING: The development of cytoplasmic labeling observed after long incubation tends to align *C. briggsae* with certain protozoa such as *Amoeba* (19, 20), *Tetrahymena* (25), and *Euglena* (23), in which the incorporation of thymidine-H^3 into cytoplasmic inclusions has also been reported under specific circum-

stances. The RNase and DNase tests appear to demonstrate that the thymidine-H^3 is incorporated into cytoplasmic DNA in *C. briggsae*. However, the Feulgen-positive structures situated, as those described by Wessing (28), in the cytoplasm of oöcytes of *Rhabditis anomala* were never observed in *C. briggsae* slides.

nucleoside lasts 24 hours, the entire gonad becomes labeled. One can speculate that this extension of labeling with time corresponds to a progression of the nuclei, which, once labeled during their stay in the apical and midregions, advance along the gonad to participate ultimately in the formation of mature gametes. If, in fact, the majority

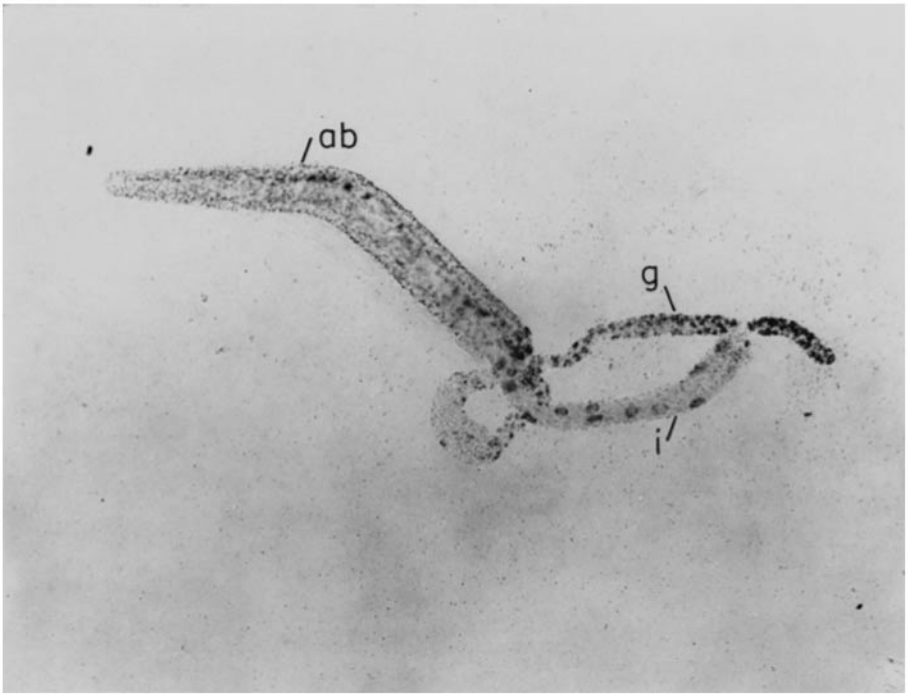

FIGURE 4 Autoradiograph of the gonads, the intestine, and the anterior half of the body in *C. briggsae* reared for 7 days in the presence of thymidine-H^3 before being placed for 24 hours in the presence of "cold" thymidine. By comparison with Fig. 3 it is evident that much of the nuclear and cytoplasmic labeling has disappeared and that the intensity of the residual labeling is feeble (*g*, gonad; *i*, intestine; *ab*, anterior half of the body). Feulgen stain. × 420.

NUCLEAR LABELING: As regards the gonads, one expects to find labeling in two places: (*a*) In the apical zone of multiplication of the gametogonia (germinal zone) where a synthesis of DNA is doubtless linked to mitosis. (*b*) In a short zone directly behind the apical zone where a synthesis of DNA may be associated with the onset of meiotic prophase. Indeed, it was noted that the adult individuals, put in the presence of thymidine-H^3 for half an hour, show an intense incorporation limited to the apical and midparts of the gonad (*i.e.* before the synapsis stage zone). But, when the stay in the presence of the labeled

of nuclei progressed along the entire length of the gonad in 24 hours, each nematode should lay, according to Delavault (3) and Nigon (13), approximately 300 zygotes in that time. However, under axenic conditions and at the incubation temperature used for *C. briggsae*, both gonads appear capable of producing only from 25 to 50 zygotes. Therefore, all the nuclei labeled during their stay in the apical and midregions certainly do not reach the end part of the gonad. Thus, the labeling found after a stay of 24 hours in thymidine-H^3 cannot result exclusively from an outgrowth of the apical zone. Hence, one must

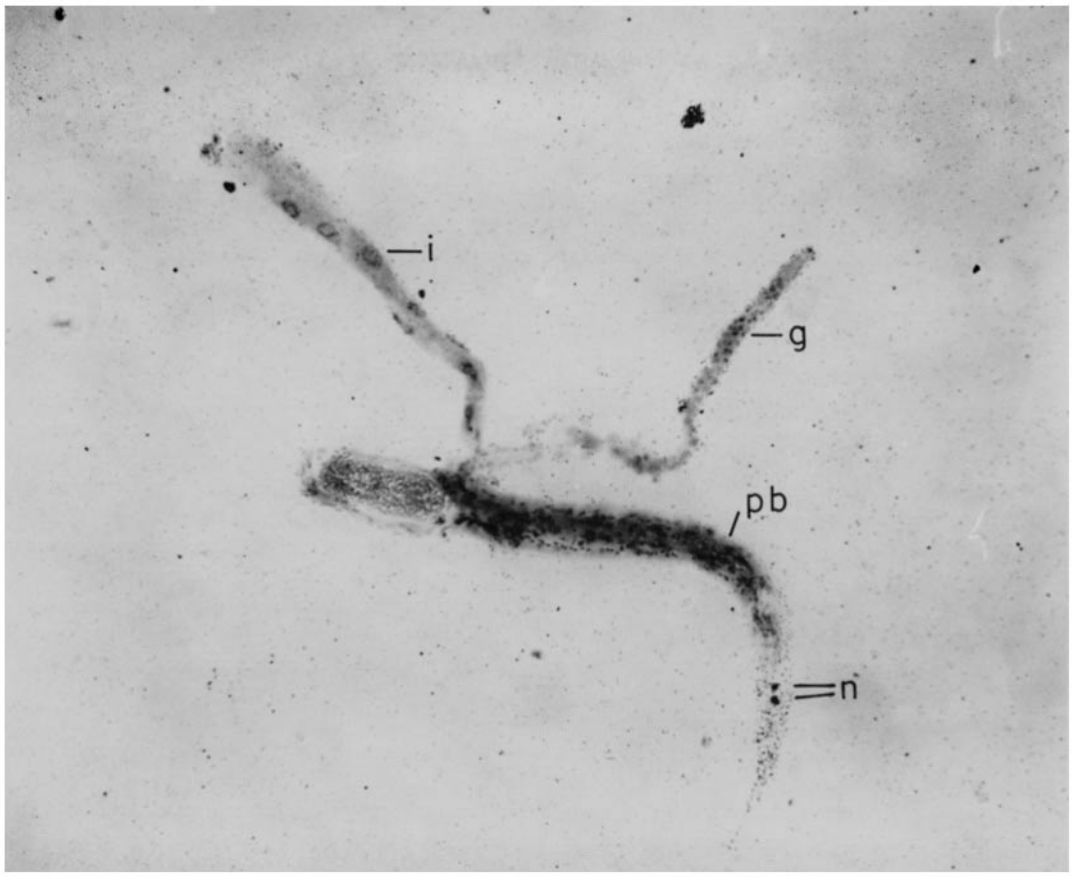

FIGURE 5 Autoradiograph of a gonad, the intestine, and the posterior half of the body in *C. briggsae* reared for 7 days in the presence of thymidine-H^3 before being placed for 48 hours in the presence of "cold" thymidine. The complete disappearance of labeling is to be noted except for that of two nuclei (*n*) situated in the tail and resistant to DNase (*g*, gonad; *i*, intestine; *pb*, posterior half of the body). Feulgen stain. × 350.

assume that this labeling reflects a DNA synthesis in a region of the gonad that corresponds essentially to the pachytene stage.

In short, one is led to conclude that:

(*a*) A rapid synthesis of DNA is obviously accomplished in the apical part of the gonad of *C. briggsae* and is linked to cellular multiplication and to the first stages of meiosis. It is possible that labeling acquired in this zone extends in 24 hours, as a result of the growth of labeled cells, to part of the rest of the gonad.

(*b*) A somewhat slower synthesis of DNA appears to occur in the rest of the gonad.

As regards somatic structures, the significance of thymidine-H^3 incorporation can only be studied reliably with the cells of the intestine. The nuclei of other organs (hypodermis, muscles, esophagus) are so small and the structure of their chromatin so difficult to analyze that one can scarcely formulate valid conclusions based on their study. A constant number of giant nuclei are found in the intestinal cells of *C. briggsae* adults. The massive incorporation of thymidine-H^3 in these nuclei shows that an important DNA synthesis takes place in them.

Delabeling

The unanticipated phenomenon of delabeling surely constitutes a surprising experimental finding. Its existence brings to mind the work of

several authors showing that a thymidine-H^3–labeling decrease is observed in cells of mammals, plants (18), and regenerating liver (8). Results obtained here differ, however, from the observations of those authors with respect to the speed and completeness of delabeling. Thus, in the cells of *C. briggsae*, the intensity of labeling decreases to such an extent that after 24 hours about half of it has disappeared, while after 48 hours the totality has vanished.

The delabeling can be explained in one of several ways,

First, if thymidine-H^3 incorporation *is not linked* to a true DNA synthesis, one could suggest three hypotheses:

1. The labeling observed in *C. briggsae* corresponds to a non-specific fixation of thymidine-H^3, —this would explain the speed of the delabeling. Such a hypothesis is difficult to maintain when one considers the very specific effects of the nucleases, which clearly indicate that thymidine-H^3 is incorporated into DNA.

2. The delabeling represents an exchange (of the methyl radical or of the thymidine molecule) between the thymidine-H^3 incorporated and the "cold" thymidine added at the end of the experiments. Neither the observations made in the course of our investigations nor the data from the literature justify the assumption of the existence of such a process.

3. Most of the thymidine-H^3 incorporation is the consequence of an irradiation effect. Since (*a*) the organisms were exposed to a relatively high concentration of thymidine-H^3 and (*b*) it is only upon prolonged incubation that incorporation was found in normally non–DNA-synthetizing nuclei, one could ask whether this incorporation is not the result of an artefactual production of "growing points" in the DNA molecules. However, the fact that *C. briggsae* is known to be highly radio-resistant goes against this third hypothesis. For instance, in the experiments in which *C. briggsae* were incubated for two generations in the thymidine-H^3 medium, no radiation effects were detected by studying the general metabolism and fecundity of the second generation.

Secondly, if thymidine-H^3 incorporation is *linked* to a true DNA synthesis, one could suggest the following phenomena to explain the delabeling in germ and intestinal cells:

1. In the apical and midpart of the gonad, the DNA synthesis is obviously linked to cellular multiplication and to the first stages of meiosis. This synthesis, which goes on in the absence of the labeled thymidine as soon as the animals are transferred into the "cold" medium, would naturally result in the appearance of unlabeled DNA and thus in a progressive decrease of the labeling. Thus in this part of the gonad, the DNA would follow the classic rules of stability and conservation that are generally attributed to it, the delabeling being only the result of a dilution process.

2. In the region of the gonad that corresponds to the pachytene stage, only a fraction of the delabeling can result from the migration (from the apical and midregion) of cells that have become less and less labeled. Furthermore, the delabeling cannot be the result of a dilution process, as could be assumed, because histophotometric studies have shown that the DNA content per nucleus does not increase during the pachytene stage (2). Hence one is led to assume that, in the pachytene nuclei, part of the DNA disappears while new DNA is synthetized and, therefore, that a DNA turnover occurs in the pachytene region of the gonad.

3. In the intestinal cells, the delabeling cannot be associated either with cellular multiplication (as these cells are fixed in number, reference 1) or with constant cell death (as the nematode *C. briggsae* shows no intestinal turnover, reference 1). Furthermore, no significant increase of DNA content has been observed, by qualitative histological procedures, in the intestinal nuclei. As a consequence, it appears that a DNA turnover occurring in the intestinal cells is the only hypothesis capable of explaining the delabeling.

CONCLUSION

The authors have been led to postulate that, in a specific region of the gonad and in the intestine, at least part of the nuclear DNA turns over.

A small number of researchers have advanced earlier the idea of a "metabolic" DNA to explain certain aspects of thymidine-H^3 incorporation. They have hypothesized that unstable DNA can be juxtaposed, in certain cells, with DNA endowed with genetic continuity (18). However, with the materials used by these researchers, the unstable DNA activity is very much less marked than with *C. briggsae*.

One could easily conceive that, in most organisms, genetic DNA and metabolic DNA could

co-mingle and thus make their individual discrimination difficult,—to such an extent that the metabolic would be completely obscured by the genetic. However, on the basis of the evidence presented here, one can assume that the metabolic dominates the genetic DNA in *C. briggsae*. Hence, *C. briggsae* proves to be valuable in revealing a DNA turnover that may exist and not be easily detectable in other organisms. One of the hypotheses that comes to mind to account for this peculiarity would consist in establishing a connection between it and other peculiarities (*i.e.* a very fast growth rate, a largely fixed number of somatic nuclei) shared by *C. briggsae* with related animals (*e.g.* other nematodes, gastrotrichs, rotifers, etc.)

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