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Studies on the Incorporation of Isotopically Labeled Nucleotides and Amino Acids in Planaria [1]

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ABSTRACT Neither intact nor regenerating planaria incorporated any significant amount of tritiated thymidine, tritiated L-leucine, or C^{14}-L-leucine from free solution even over long periods of exposure. Tritiated thymidine was incorporated into regenerating rat liver and fed to planaria, but tritium hydrogen exchange rendered this method useless. A large incorporation of C^{14}-uracil and C^{14}-L-leucine into planaria was obtained by growing baker's yeast in a defined medium containing the labeled compound and then feeding the acetone dried yeast to the planaria. C^{14} assay of the amino acid components of planaria fed on C^{14}-L-leucine labeled yeast showed C^{14} in only the L-leucine component and yielded a minimum estimate for protein turnover of 46% in seven weeks. Radioautographs of planaria fed on C^{14}-uracil labeled yeast exhibited specific incorporation into ribonucleic acid. Previous radioisotopic studies on planaria are evaluated and criticized. The amino acid composition of planarian protein was determined.

Although planaria have been used extensively for studies on regeneration and morphogenesis (Brondsted, '55; Wolff and Dubois, '47; Wolff, '47, '61, '62; Dubois, '49, '50; Flickinger, '59, '64) and on the mechanisms of learning and memory (McConnell et al., 59, '62; Best and Rubinstein, '62; Corning and John, '61; Jacobson, '63; Barnes and Katzung, '63; Westerman, '63; Van Deventer and Ratner, '64; Halas et al., '62) only limited information has been available on the underlying cytochemical mechanisms. Lindh ('57) and Clement ('44) have studied differences in nuclei acid composition and content during regeneration of planaria. Pedersen ('59) has conducted histochemical studies on them. Henderson and Eakin ('61) have studied the effect of purine analogues on differentiation.

A salient gap in the information needed to interpret the results of these many studies is that there has been no really satisfactory method of estimating the rates of renewal and transformation of the various kinds of cells in planaria, nor the rates of turnover of such constituents as ribonucleic acid (RNA) and protein in these cells. High resolution radioautographic procedures (e.g., Everett et al., '60; Cronkite et al., '60) using tritium or C^{14} for labeling of appropriate precursor molecules would seem to provide a suitable experimental method to furnish this needed information. Utilization of these methods assumes as a necessary condition that one can obtain sufficient incorporation of the isotopically tagged precursor to obtain a radioautograph. There are technical difficulties in obtaining this incorporation. The present study is concerned with the development of a successful method of obtaining such isotopic incorporation and subsequent high resolution radioautographs.

METHODS — GENERAL

Prior to their use in the experiments the planaria were maintained in enameled dish pans in aged tap water and fed twice weekly on raw beef liver. Preliminary to their regimen of experimental treatments or feedings they were placed in glass fingerbowls and fasted for one week.

Planaria were prepared for radioautography by fixing in 10% formalin, dehydrating in graded concentrations of ethanol, embedding in paraffin, and cutting serial sections 5 μ thick. Following deparaffinization and rehydration, the sections were dipped in Eastman NTB-2 emulsion which had been warmed to 42°C. After harden-

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ing, the sections were placed in the dark at 5°C with a silica gel dessicant, usually for 14 days.[2] The sections were developed, washed, and then stained with methyl green pyronin or Giemsa stains. The radioautographic procedure was tested on a smear of tritium labeled ascites tumor cells (provided through the courtesy of Dr. Theodore Puck of the Department of Biophysics, University of Colorado) as well as on unlabeled control sections.

Tissues (liver, planaria, or yeast) were assayed for tritium or C^{14} in a liquid scintillation counter (Tri-Carb — Packard Instrument Co.) using either of two different procedures. In one (I) the tissue was digested in 1 N NaOH and this digest absorbed *onto* Cab-o-sil which was then dried and dispersed in the toluene containing the scintillator. In another (II) the tissue was homogenized in a glass tissue grinder in the cold, precipitated with 7% perchloric acid (PCA) and the precipitate hydrolyzed with 6N HCl in a sealed tube at 110 C for 22 hours. A small aliquot of this hydrolysate, or supernate, was then added to a mixture of ethanol, toluene, and scintillator for counting.

The tritiated thymidine and tritiated D L-leucine used in experiments 1, 2, and 3 were obtained from Volk Radiochemical Co. All other isotopic compounds were obtained from New England Nuclear Corporation.

Experiment 1
Attempt to obtain incorporation of soluble tritiated thymidine into planaria

Planaria of the species *Dugesia dorotocephala* and *Cura Foremanni* were given a single feeding of an admixture of fresh heparinized horse blood and tritiated thymidine (2.5 μc/ml). Some were fixed 12 hours later. Others were transected at 12 and 40 hours and allowed to regenerate 0, 1, 24, 72, and 148 hours before fixation. Other groups of whole and transected *Dugesia dorotocephala* were kept in a solution of tritiated thymidine (1.0 μc/ml) in aged tap water for eight days and then fixed.

Radioautographs of these planaria gave no indication of labeling.

Experiment 2
Attempt to obtain incorporation of tritiated thymidine into planaria by preincorporating it into liver DNA which was then used as food

Approximately 3/5 of the liver of a 200 gm, Wistar rat was removed in a partial hepatectomy. Five intramuscular injections of 5 μc each of tritiated thymidine were administered during the first three days of hepatic regeneration. Six days after the operation the liver was removed and portions fixed for radioautography, assayed by method I, and the remainder frozen for subsequent feeding to planaria. The liver showed moderate tritium uptake (0.022 μc/gm wet weight) and characteristic grain clustering around a number of cell nuclei in the radioautographs. The tritiated liver was fed to a group of *Dugesia dorotocephala* twice a week for ten days, some fixed, some transected and fixed eight days later, and some continued on tritiated liver for 46 days before fixation. None of these planarian radioautographs exhibited high enough grain counts to be useful.

These negative results can be accounted for by the relatively low specific activity of the liver and by the extensive hydrogen exchange of tritium with water incurred by the necessarily long exposure to an aqueous phase.

Experiment 3
Attempt to obtain incorporation of a labeled amino acid from solution into planaria

Since the rate of cell division in planaria is unknown and thymidine would not, in any event, be incorporated in the absence of cell division, we decided to ascertain whether isotopically labeled amino acids would be incorporated, since nearly all tissues manifest some protein turnover.

Dugesia dorotocephala were placed in solutions of 0.1 μc/ml of tritiated DL-leucine in aged tap water for four days and

[2] This period was chosen as nearly optimal since the activity levels were too low to yield adequate grain counts in periods much less than a week while an increase to 28 days would only yield a doubling of the grain count and increase the labeling resolutions by $\sqrt{2}$. Periods greater than 28 days were considered impractically long.

then assayed by method I. Two other groups were maintained in 0.02 μc/ml of C^{14}-L-leucine. One of these was fasted and the other fed twice weekly on raw beef liver. A third group was fed twice weekly and maintained in 0.02 μc/ml of tritiated L-leucine. Planaria were sampled from these latter three groups after 1, 14, 16, 24, 31, 35, 38, and 98 days of such maintenance and assayed by method II.

There was no measurable C^{14} or tritium incorporation in the first 38 days by any group. The 98 day group showed 0.025 μc of tritium and 0.006 μc of C^{14} per gm wet weight. Virtually none of the isotopic L-leucine was incorporated. The relatively higher value for tritium labeling is probably due to hydrogen exchange. This failure to incorporate L-leucine from free solution cannot be ascribed to absence of L-leucine in the protein of planaria since the results of an amino acid analysis (table 1) show an ample fraction of planarian protein to consist of L-leucine.

Experiment 4

Histological studies on planarian digestion (Rosenbaum and Rolan, '60; Jennings, '62) indicate that food particles are incorporated into phagocytic cells lining the digestive cavity. Intracellular digestion of these particles then occurs. If food is normally processed this way, then conceivably the cells may be poorly adapted for the utilization of amino acids and nucleotides in solution. This would be consistent with the results of experiment 1 and 3. If the isotopically labeled amino acid or nucleotide were presented as part of a digestible particulate then it should be incorporated. An earlier observation, that planaria would feed on dried baker's yeast and that the attractive substance was not extracted by acetone (Miller and Johnson, '59), provided a clue to the manner in which this could be accomplished. Because of the tendency of tritium to exchange with the hydrogen of water, and thereby obscure the results of the experiment, C^{14} labeling was used.

The strategy was to first incorporate the C^{14} labeled amino acid or nucleotide into baker's yeast and then to feed the acetone dried yeast cells to the planaria. To provide the planaria with nutritionally complete and attractive food, the acetone dried yeast powder was supplemented with a powder of acetone dried liver.

To obtain an incorporation of C^{14}-L-leucine into the baker's yeast the following procedure was employed. Baker's yeast (Fleischmann) was grown aerobically at 25°C in a culture medium (modified Schultz and Atkin) consisting of:

50 ml	1% casein hydrolysate (Vitamin free) adjusted to pH 5.5
50gm	glucose
0.55 gm	KH_2PO_4
0.42 gm	KCL
0.12 gm	Ca C12·$5H_2O$
0.12 gm	$MgSO_4 \cdot 7H_2O$
2.5 mg	$FeCl_3 \cdot 6H_2O$
2.5 mg	$MnSO_4 \cdot 4H_2O$
50 ml	0.4 M potassium citrate at pH 5.5
25 mg	inositol
2.5 mg	calcium pantothenate
0.025 mg	biotin
0.5 mg	vitamin B_1
0.5 mg	pyridoxine HCl per liter of solution.

To 500 ml of the above medium was added 50 μc of C^{14}-L-leucine having (according to the supplier) a specific activity of 1,830 μc/mg.

When the yeast population had attained a plateau density the cells were harvested by centrifugation and washed by resuspending in distilled water. Following recentrifugation and decantation of the wash water the cells were resuspended in acetone (− 15 C), recovered by centrifugation, the acetone decanted off, and the residual acetone allowed to evaporate from the yeast cells in a dessicator.

The same procedure was used for C^{14}-uracil except that, instead of adding C^{14}-L-leucine to the yeast growth medium, 50 μc of C^{14}-uracil having (according to the supplier) a specific activity of 248 μc/mg was added to 500 ml of the medium.

The yeast prepared in this way was assayed for C^{14} by suspending a measured sample in an ethanol-toluene-PPO scintillator solution and counting in a scintillation counter (Tri-Carb).

Two parts of the dried C^{14} labeled yeast, one part of the liver powder, and one part of powdered glycogen were mixed together

with just enough water to make a thick paste. A "feeder" was constructed by drilling a series of holes 1/16 inch in diameter and 1/9 inch deep in a small plate of 1/4 inch thick acrylic plastic. A small amount of the paste was packed into each of these holes leaving enough margin so that when the paste expanded, as it will do upon hydration, it would not extrude out of the hole. When this feeder plate was placed in the bowl containing the planaria they would feed by extruding their pharynx into the hole containing the paste.

The planaria were fed twice weekly on the paste. After feeding the feeder plate was removed, the habitat bowl rinsed with aged tap water, and fresh aged tap water added. At various times after the feeding regimen on the C^{14} labeled medium was initiated, samples of planaria from the groups were assayed for their C^{14} content. The planaria were gently blotted to remove excess water, weighed on the microbalance, and assayed by Method II.

The yeast cells were found to contain an average of 1.97 μc/gm dry weight in the case of the C^{14}-L-leucine medium and 20 μc/gm dry weight for the C^{14}-uracil medium.

Dugesia dorotocephala fed on C^{14}-L-leucine yeast paste attained an overall specific activity of 2 μc/gm dry weight in the first two weeks of the feeding regimen. The overall specific activity of the *Cura formanii* fed on C^{14}-L-leucine yeast paste rose more slowly, requiring about six weeks to attain a level of 1.2 μc/gm of dry weight. The overall specific activity of the *Dugesia dorotocephala* fed on C^{14}-uracil yeast paste rose to 4 μc/gm of dry weight by the end of five weeks of the feeding regimen.

An average of 23% of the total radioactivity of the group fed on C^{14}-L-leucine yeast paste did not precipitate from the tissue homogenate with PCA, remaining in the supernate instead. An average of 53% of the total radioactivity of the group fed on C^{14}-uracil yeast paste remained in the supernate after PCA precipitation.

Experiment 5

To ascertain the degree of specificity of the incorporation of the C^{14} labeled compounds and obtained an estimate of the total amino acid "pool" in the planaria, the following procedure was carried out for the *Dugesia dorotocephala* fed on C^{14}-L-leucine yeast paste.

Following seven weeks of the feeding regime described in Experiment 4, the planaria were thoroughly homogenized and the aqueous homogenate precipitated with cold PCA as described previously. The washed precipitate was then placed with 6N HCL in an evacuated sealed glass tube and heated to 110°C for 20–22 hours to hydrolyze the protein into its constituent amino acids. This hydrolysate was then analyzed with a Spinco Amino Acid Analyzer equipped with a radioactivity counting head.

The amino acid composition of *Dugesia dorotocephala* is given in table 1. The only amino acid found to contain C^{14} was then the L-leucine fraction. The specific activity of this fraction was found to be 2.0 μc/mmole of L-leucine.

TABLE 1

Amino acid composition of the protein of the planarian dugesia dorotocephala

Amino acid	Micromoles/ worm
Lysine	0.120
Histidine	0.369
Arginine	0.266
Aspartic acid	0.087
Threonine	0.046
Serine	0.050
Glutamic acid	0.080
Proline	0.027
Alanine	0.051
Valine	0.036
Methionine	0.020
Isoleucine	0.036
Leucine	0.020
Tyrosine	0.013
Phenylalanine	0.029
Glycine	0.059

These findings strongly indicate that, at least under the conditions of culture chosen in Experiment 4, the conversion of L-leucine into other amino acids, by either the yeast or the planaria, is negligible. These results do not preclude the transformation of some of the L-leucine into compounds other than amino acids or nucleotides. That some amount of conversion into such other compounds may occur is suggested by the result of Experiment 4 in which

23% of the C^{14} in the planaria was not PCA precipitable.

Experiment 6

Dugesia dorotocephala which had been on the C^{14} yeast paste feeding regimen for 4 to 7 weeks were radioautographed.

Radioautographic sections of the *Dugesia dorotocephala* which had been on the C^{14}-L-leucine yeast paste feeding regime showed a uniform grain distribution over the regions occupied by tissue, but few grains elsewhere. The radioautographic sections of *Dugesia dorotocephala* which had been fed on C^{14}-uracil yeast paste exhibited a moderately dense grain distribution over the pyronin stained (pink coloration) regions, but few grains elsewhere.

The results of these radioautographs are in accord with the expected specificity of the labeling. Since protein is more or less uniformly distributed through the tissues one would anticipate a uniform labeling distribution over these for times of sufficient duration to allow turnover of all the various proteins. Most of the uracil of the cells is a constituent of their RNA. Although pyronin is not completely specific for RNA, RNA is the primary pyronin-staining material. Thus the grain distribution in the case of the sections from C^{14}-uracil yeast fed planaria indicates that most of the C^{14}-uracil present in the tissue section has been incorporated into RNA. The procedures used in paraffin embedding would, of course, tend to remove any C^{14} going into lipids, or into water soluble compounds that were not rendered insoluble by the formalin fixative. Such compounds would be lost from the section and would not, therefore, contribute to the grain distribution of the radioautograph.

DISCUSSION

Approximately 5% of the dry weight of baker's yeast is L-leucine (Spector, '61). Approximately 1.39% of the dry weight of beef liver is L-leucine (Spector, '61). If one assumes the C^{14} of the yeast grown on C^{14}-L-leucine is in the form of C^{14}-L-leucine, the specific activity of the L-leucine supplied to the planaria in the yeast-liver paste of Experiment 4 is 5.56 μc/mmole. If, as is likely, some of the C^{14} is in nonprotein components of the yeast, it will be less. The specific activity of the L-leucine of planaria, found in Experiment 5, is 2.0 μc/mmole for *Dugesia dorotocephala* sampled at the end of seven weeks. These values yield a minimum estimate for the turnover of the protein of 46% in seven weeks.

Flickinger ('59) used C^{14}-glycine incorporation to demonstrate an axial metabolic gradient of the kind postulated by Child ('41). To demonstrate this incorporation, planaria of the species *Dugesia dorotocephala* which had been fasted for a week were placed in a solution containing 27 μc of C^{14}-glycine (specific activity of 18.4 μc/mg) per ml for two hours. Assayed at the end of this period, the cold trichloracetic acid precipitate fraction of the homogenized planaria yielded an average of 200 counts/minute/mg of carbon, i.e., 9.1×100^{-5} μc/mg of carbon.

According to these data, 2.19% of the protein carbon is in glycine. Considering the trichloracetic acid precipitate to be protein and the C^{14} in this precipitate to be the result of glycine incorporation into protein, the anticipated value would be 1.25 μc/mg of carbon for 100% turnover with C^{14}-glycine having a specific activity of 18.5 μc/mg. The value of 9.1×10^{-5} μc/mg of carbon that was actually found would thus correspond to a 0.00725% turnover in two hours or 4.3% in seven weeks. This is less than one-tenth the minimum estimate calculated from the data of Experiments 6 and 7 of the present study. The apparent discrepancy between the two results strongly indicates that the C^{14}-glycine in the solution in which the planaria were placed in the Flickinger experiment interchanged with their "metabolic pool" at a rate that was much slower than the rate of protein turnover. Significant amounts of labeling were achieved only as a result of the inordinately high C^{14} concentration employed. This is consistent with our own finding that L-leucine in free solution is incorporated by planaria to only a negligible extent. Thus Flickinger's data are consistent with our own in regard to incorporation of amino acid from free solution. In view of these considerations the axial gradient Flickinger found in the rate of C^{14}-glycine in-

corporation may thus reflect a gradient in permeability to glycine rather than a gradient in the rate of protein synthesis.

In another study Flickinger ('64) has reported radioisotopic studies in which he used 143 μc/ml of $C^{14}O_2$ to secure labeling of a planarian which was used as donor of a window graft to an unlabeled host. Subsequent decapitation of the recipient of the window graft resulted in no evidence of migration of the C^{14} labeled cells from the graft. This finding was considered contradictory to the findings of Wolff and Dubois ('47) indicating mobilization of neoblasts to form the regeneration balstema. Such high levels of C^{14}, necessitated by the inefficient route of incorporation, are apt to produce radiation injury effects. It is, therefore, of interest to compute the roentgen equivalent to be anticipated from the C^{14} levels used in the Flickinger experiment.

The energy of the C^{14} beta particles is approximately 0.15 Mev and a microcurie will result in 2.2×10^6 disintegrations/minute. Thus, 143 μc will result in 1.4×10^{11} Mev/ml over the 51 hours representing the minimum period of such exposure in this experiment. A roentgen of x-rays results in about 6×10^7 Mev/ml in water. On this basis the cells of the labeled graft would have received the equivalent of 2,300 roentgens of x-irradiation; however, soft beta rays are more damaging because of their high linear energy transfer. Thus the roentgen equivalent of the dose received would more probably be of the order of 3,000 roentgens. This value is very near to the x-ray dose used by Wolff and Dubois to inactivate the neoblasts. It is, therefore, quite probable that the C^{14} labeled cells in the transplant in the Flickinger experiment did not migrate because they had been rendered nonviable by radiation injury.

These experiments of Flickinger need to be repeated using lower levels of C^{14}/ml and more efficient routes of incorporation such as those indicated here.

It should be noted that another laboratory (Bennett, '64), in agreement with the findings reported here, found little or no incorporation of amino acids or nucleotides from solution by planaria.

CONCLUSIONS

1. Amino acids and nucleotides in solution are incorporated by planaria only very slightly or not at all.

2. A large incorporation of amino acids or nucleotides is obtained when these are ingested as part of protein or nucleic acid in the form of digestible particles. An experimentally useful way of obtaining incorporation of C^{14}-amino acids or of C^{14}-nucleotides into planaria is first to incorporate these into baker's yeast grown in a defined medium and then to feed the isotopically labeled yeast to the planaria.

3. Use of baker's yeast as a vehicle of incorporation for C^{14}-amino acids and nucleotides into planaria is specific enough to be useful for histochemical investigation of protein and nucleic acid turnover rates.

4. Protein turnover of the planarian *Dugesia dorotocephala* is at least 46% in seven weeks.

5. Earlier results of Flickinger ('59) on C^{14}-glycine incorporation yield deceptively low estimates of the rate of protein turnover in planaria because of the slow rate of exchange of amino acids from solution with the "metabolic pool." The axial gradient in C^{14}-glycine incorporation rates observed by Flickinger may reflect an axial gradient in permeability rather than rate of protein synthesis.

6. The high levels of C^{14} used by Flickinger ('63, '64) to label planarian cells with $C^{14}O_2$ in order to verify whether they migrate during regeneration probably inflicted sufficient radiation injury to render them nonviable. This may account for the apparent discrepancy between his results and those of Wolff and Dubois ('47, '61, '62, '49, '50).

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