phorylated intermediate in the enzyme reaction, its existence and its response to various transport variables can hardly be questioned.

ARTHUR KAHLENBERG
PETER R. GALSWORTHY
LOWELL E. HOKIN
Department of Physiological Chemistry, University of Wisconsin, Madison

References and Notes

1. J. C. Skou, *Physiol. Rev.* **45**, 596 (1965).
2. R. L. Post, A. K. Sen, A. S. Rosenthal, *J. Biol. Chem.* **240**, 1437 (1965); R. W. Albers, S. Fahn, G. J. Koval, *Proc. Nat. Acad. Sci. U.S.* **50**, 474 (1963).
3. L. E. Hokin, P. S. Sastry, P. R. Galsworthy, A. Yoda, *Proc. Nat. Acad. Sci. U.S.* **54**, 177 (1965).
4. K. Nagano, T. Kanazawa, N. Mizuno, Y. Tashima, T. Nakao, M. Nakao, *Biochem. Biophys. Res. Commun.* **19**, 759 (1965); H. Bader, A. K. Sen, R. L. Post, *Biochim. Biophys. Acta* **118**, 106 (1966); D. A. Hems and R. Rodnight, *Biochem. J.* **101**, 516 (1966).
5. A. Kahlenberg, A. Yoda, P. R. Galsworthy, N. Dulak, L. E. Hokin, *Fed. Proc.* **26**, 394 (1966); A. Yoda, A. Kahlenberg, P. R. Galsworthy, N. C. Dulak, L. E. Hokin, *Biochemistry*, in press.
6. A. Kahlenberg, P. R. Galsworthy, L. E. Hokin, in preparation.
7. C. F. Chignell and E. Titus, *Proc. Nat. Acad. Sci. U.S.* **56**, 1620 (1966); W. Schoner, R. Kramer, W. Seubert, *Biochem. Biophys. Res. Commun.* **23**, 403 (1966).
8. E. Santiago-Calvo, S. Mulé, C. M. Redman, M. R. Hokin, L. E. Hokin, *Biochim. Biophys. Acta* **84**, 550 (1964).
9. Supported by research grants from NIH (NB-01730) and the Wisconsin Alumni Research Foundation. L.E.H. is a research career awardee, NIH (No. 5-K6-GM-1347).

1 May 1967

Tritiated Thymidine: Effect of Decomposition by Self-Radiolysis on Specificity as a Tracer for DNA Synthesis

Abstract. *Tritiated thymidine undergoes decomposition by self-radiolysis. As shown by beta liquid-scintillation measurement and by autoradiography, the resultant breakdown products did not label DNA, but entered macromolecules, other than nucleic acid, contained in the cytoplasm. Caution should be exercised in using aged tritiated thymidine solutions.*

The use of thymidine labeled with tritium at a high molar specific activity as a tracer in biological research, and especially as a specific label for DNA in cytological investigations, is currently widespread (*1*, *2*). The need to use radiochemically pure thymidine for such research has been stressed (*2*). However, in the case of tritiated thymidine it had not been demonstrated that the products of self-radiation could give misleading results if such products are present in the solutions of the tracer compound. We now report a study of the products of self-radiolysis of tritiated thymidine when it is used as a tracer in cytological investigations.

Two series of experiments were carried out with the following solutions (*3*): (i) thymidine-(*methyl*-^{3}H) freshly prepared at a specific activity of 1000 mc/mmole (radioactive concentration 1 mc/ml); (ii) thymidine-(*methyl*-^{3}H) decomposed by gamma irradiation (2.5 megarad during 1 hour) and having a specific activity of 1000 mc/mmole (radioactive concentration 1 mc/ml); (iii) thymidine-6-^{3}H impurities isolated by preparative chromatography on paper from a solution of the tritiated compound which had been allowed to undergo self-radiolysis over a period of about 3 months. The impurities were separated from the thymidine-6-^{3}H on Whatman No. 1 paper, first in *n*-butanol saturated with water and then in ethyl acetate saturated with phosphate buffer at *p*H 6.0. All impurities were combined, and the solution (radioactive concentration at 0.37 mc/ml) was used in the experiments described below. The specific activity of the tritiated thymidine was 12,000 mc/mmole.

The three samples of tritiated material were analyzed by paper and thin-layer chromatography (*4*), the products being as follows: (i) more than 98 percent thymidine-(*methyl*-^{3}H), and less than 1 percent each of labeled thymine and other decomposition products; (ii) about 5 percent thymidine (*methyl*-^{3}H), about 20-percent-labeled thymine, and about 75 percent other decomposition products; (iii) less than 2 percent thymidine (*methyl*-^{3}H), about 25-percent-labeled thymine, and greater than 73 percent other decomposition products.

At least six major decomposition products were present in each of the solutions ii and iii, but because of the complexity of the mixture and the difficulty in separating the products, their quantitative estimation and that of the thymidine and thymine present is only approximate. However, the products of the self-radiolysis and gamma radiolysis of thymidine in aqueous solution have been fully investigated (*5*).

Tetrahymena pyriformis (amicronucleate strain GL) was grown in 2 percent proteose-peptone and 0.1 percent liver extract; the cultures were synchronized for cell division by a series of heat shocks (*6*). Radioactivity in the amount of 20 μc was added to 20-ml (50,000 cells per milliliter) cultures which were agitated in a 100-ml erlenmeyer flask at 28°C. Label was added 100 minutes after the end of the heat shocks, about 25 minutes after the first maximum division activity (Fig. 1). At this time, a maximum (60 percent) of cells has just entered DNA-synthesis. During the ensuing 150 minutes, 1-ml samples were taken from each flask at 20- or 30-minute intervals. Then, 1 ml of 10 percent trichloroacetic acid (TCA) at 0°C was immediately added, and the samples were kept in an ice bath until suction-filtered onto Whatman No. 3 paper filters. The filters had been previously treated with 5 ml of growth medium mixed with an equal volume of 10 percent TCA

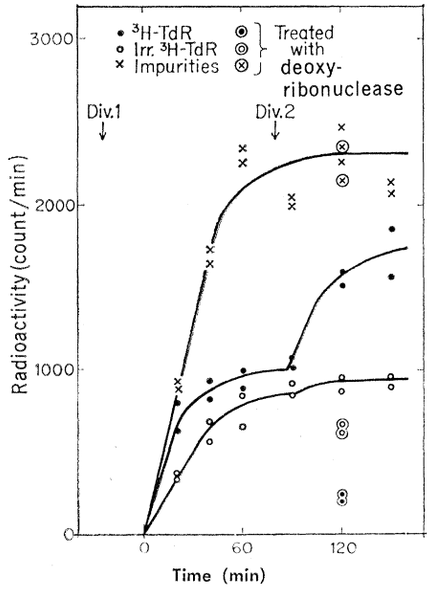

Fig. 1. Beta liquid-scintillation measurements of label incorporation. Freshly made ^{3}H-thymidine gives rapid label incorporation with a leveling off toward 90 minutes; at the maximum of division 2, another period of label incorporation begins. Treatment with deoxyribonuclease at 120 minutes removes almost all the label. Irradiated ^{3}H-thymidine gives a similar, but lower, curve for label incorporation for the first 90 minutes, after which there may be a slight acceleration of new label incorporation. Deoxyribonuclease treatment removes only 30 percent of the label. The impurities give very rapid incorporation for the first 40 minutes and no further incorporation. Deoxyribonuclease treatment did not remove any of this label. With both the irradiated ^{3}H-thymidine and its impurities there is apparently leveling off of incorporation, probably due to the depletion of the label. The counts recorded have been corrected for quenching and background.

(both 0°C), and precipitates were washed with 10 ml of this mixture; with this procedure the background count was very low. The filters were put into scintillation bottles, dried at 80°C for 1 hour, and the bottles were then filled with 10 ml of scintillation fluid [4 g of PPO (2,5-diphenyloxazole) and 50 mg of POPOP (phenyloxazolyl-phenyloxazolyl-phenyl) per liter of toluene]. The samples were counted in a Beckman beta liquid-scintillation spectrometer.

Exactly 120 minutes after the label was added, parallel samples (1 ml) were removed for treatment with deoxyribonuclease as follows. The samples were rapidly frozen and thawed twice to stop the incorporation and to disrupt the cells; then we added 1 ml of 0.03 percent deoxyribonuclease (Worthington) solution in a phosphate buffer at *p*H 7, and incubated the mixture at 34°C for 2 hours. Two milliliters of 10 percent TCA at 0°C were added to each sample, which was then treated as described above. (Fig. 1).

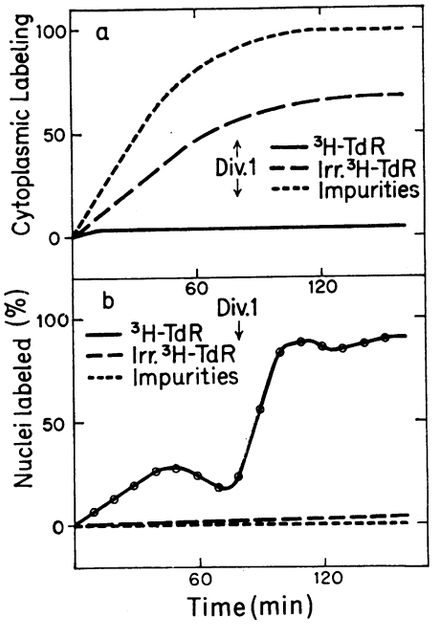

Fig. 2. Autoradiographic measurements of label incorporation. (a) Relative estimates of cytoplasmic incorporation. Freshly made ^{3}H-thymidine shows little or no cytoplasmic labeling. Irradiated ^{3}H-thymidine shows moderate cytoplasmic labeling which increases with time. The impurities show heavy cytoplasmic labeling which increases with time. Deoxyribonuclease and ribonuclease do not remove any cytoplasmic labeling. (b) Percent nuclei labeled. Freshly made ^{3}H-thymidine shows a pattern of incorporation already mentioned (7). Deoxyribonuclease treatment at any time removes the label completely. Irradiated ^{3}H-thymidine shows very little nuclear labeling. Deoxyribonuclease treatment removes this label. The impurities show no nuclear incorporation at all. The nucleus referred to is the macronucleus. This strain has no micronucleus.

Qualitative data on the location of the label incorporation was obtained by autoradiography. Cultures were grown and synchronized as described. The labels were added at the end of the heat shocks. For the next 150 minutes, at 10-minute intervals, 20-μl samples of the cell suspensions were taken. The samples were streaked onto slides coated with a thin layer of gelatin; the slide preparations were air-dried, fixed for 5 minutes in a mixture of ethanol and acetic acid (3 : 1), washed in tap water for 15 minutes and is distilled water for 5 minutes, and air-dried again. The slides were then dipped in Ilford Nuclear Research Emulsion L4, exposed with desiccant for 3 days, developed, and stained with methylene blue (Figs. 2 and 3).

Radiolysis of ^{3}H-thymidine in aqueous solution either by self-irradiation or by external gamma irradiation produces *cis*- and *trans*-thymidine glycols and peroxides, *cis*- and *trans*-thymine glycols, thymine and minor amounts of other, unknown compounds (*2*, *5*). These products are produced by attack at the carbon-carbon double bond of the pyrimidine ring by hydroxyl radicals. The results presented indicate that in *Tetrahymena* one or more of these labeled compounds are incorporated into TCA-precipitable compounds, which are exclusively or mostly cytoplasmic.

Just how these tritiated impurities are incorporated and bind so strongly to the macromolecular structures of the cytoplasm is not known. But the fact is that they do and cannot be removed with deoxyribonuclease or ribonuclease. The tests made with these enzymes in the autoradiographic experiments indicate that the incorporation is mostly in cytoplasmic macromolecules which remain unidentified, but which are neither RNA nor DNA. Absence of incorporation of the impurities into DNA is further supported by the results of digestion with deoxyribonuclease on the autoradiograms and of the homogenates prior to scintillation counting. These tests are negative for the impurities, but strongly positive for the pure ^{3}H-thymidine.

Only in the experiments with the gamma irradiated ^{3}H-thymidine are the results not clear. The autoradiographic studies show only little nuclear incorporation (< 1 percent). By other criteria (the almost exclusive cytoplasmic location of the incorporated label and its stability to deoxyuribonuclease), these studies show that the irradiated ^{3}H-thymidine gets into compounds other than nucleic acid. However, in the scintillation experiments, some label was removed by deoxyribonuclease treatment. This may be due to DNA labeling from the 5 percent ^{3}H-thymidine present in the irradiated sample, an amount so small that it is at the threshold of detection by our microscopic autoradiographic technique.

Our conclusion is that little or none of the irradiated (self or gamma) ^{3}H-thymidine enters DNA. Thus, the use of freshly made ^{3}H-thymidine in all tracer experiments is desirable. With

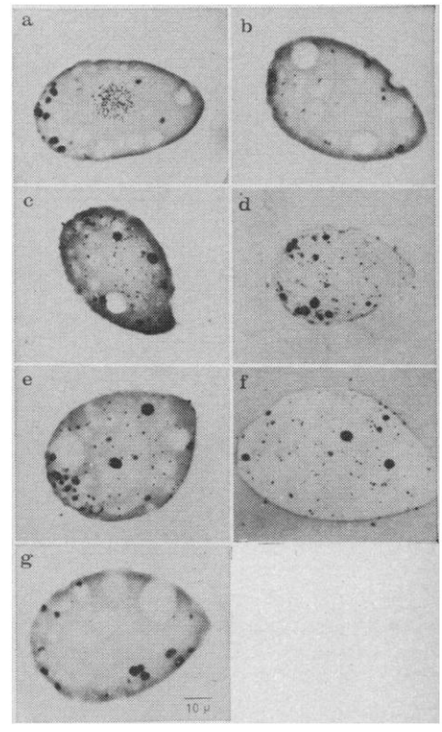

Fig. 3. Autoradiographs showing incorporation of the three labels. (a and b) Cells of *T. pyriformis* 2 hours after addition of freshly made ^{3}H-thymidine. (a) Typical nuclear labeling; (b) deoxyribonuclease treatment removes all the macronuclear labeling. (c and d) Irradiated ^{3}H-thymidine at 2 hours after addition of label. (c) Some cytoplasmic, but no nuclear labeling; (d) deoxyribonuclease plus ribonuclease treatment does not remove any of the cytoplasmic labeling. (e and f) Impurities at 2 hours after addition of label. (e) Heavy cytoplasmic, but no nuclear labeling; (f) deoxyribonuclease plus ribonuclease treatment does not remove any of the cytoplasmic labeling. (g) Control autoradiograph without any label added. The large black spots in the cytoplasm are probably food vacuoles containing condensed ingested material, and they are not to be confused with the smaller black spots which in the other pictures represent cytoplasmic and macronuclear label incorporation.

quantitative studies, such as those with beta liquid-scintillation measurements, aged ^{3}H-thymidine (as exemplified here by the irradiated ^{3}H-thymidine) could give spurious results. In all such studies, parallel samples must be digested with deoxyribonuclease to rule out a count from unspecific incorporation. With qualitative studies, such as those with autoradiography, the results could be equally misleading. Where just macronuclear labeling is being observed, the use of aged ^{3}H-thymidine might merely decrease the sensitivity of the autoradiographic method. We have used ^{3}H-thymidine with a storage life of 1 year in our autoradiographic experiments and there was only slight change in sensitivity and overall results (*9*). Where cytoplasmic incorporation is being observed, as with many recent studies on cytoplasmic DNA (*10*), the importance of using freshly made ^{3}H-thymidine and digestion controls is stressed.

Although our observations emphasize the possible misleading results which could be obtained with radiochemically impure tritiated thymidine as a tracer, it is equally true that similar erroneous interpretations are possible by the use of other radiochemically impure, labeled compounds. Such misinterpretations are perhaps especially possible in cytological investigations where the results are visualized by autoradiography, and where only a small fraction of the label used is actually incorporated into the cellular structures. Any selectivity of uptake of the impurities by the cell could indeed produce some very misleading results.

MARTIN WAND*
ERIK ZEUTHEN
Biological Institute of the Carlsberg Foundation, 16 Tagensvej, Copenhagen N., Denmark
E. ANTHONY EVANS
Organic Department, Radiochemical Centre, Amersham, Buckinghamshire, England

References and Notes

1. L. F. Belanger, in *The Use of Radioautography in Investigating Protein Synthesis*, C. P. Leblond and K. B. Warren, Eds. (Academic Press, New York, 1965), pp. 1–6.
2. E. A. Evans, *Tritium and Its Compounds* (Butterworths, London, 1966).
3. Prepared and supplied to us by the Radiochemical Centre, Amersham, England.
4. Paper chromatography on Whatman No. 1 in ethyl acetate saturated with phosphate buffer at *p*H 6.0. Thin-layer chromatography on silica gel in a system composed of *n*-butanol saturated with water and ethylacetate : methanol : acetic acid (8 : 1 : 1).
5. S. Apelgot, B. Ekert, A. Bouyat, *J. Chim. Phys.* **60**, 505 (1963); E. A. Evans and F. G. Stanford, *Nature* **199**, 762 (1963).
6. P. Plesner, L. Rasmussen, E. Zeuthen, in *Synchrony in Cell Division and Growth*, E. Zeuthen, Ed. (Interscience, New York, 1964), pp. 543–563.
7. K. K. Hjelm and E. Zeuthen, *Compt. Rend. Trav. Lab. Carlsberg* **36**, No. 8. (1967).
8. I. Agrell, *Pathol. Biol.* **9**, 775 (1961).
9. K. K. Hjelm, M. Wand, E. Zeuthen, in preparation.
10. R. R. Meyer, *Biochem. Biophys. Res. Commun.* **25**, 549 (1966).

* Yale University School of Medicine junior fellow, supported by the U.S. Public Health Service grant STO5 GM 1637-05.

22 May 1967

Morphological Changes in Human Scalp Hair Roots during Deprivation of Protein

Abstract. *Human subjects were deprived of protein for 15 days, after which time hair from the scalp of each subject was plucked and examined. Both the bulb and the external root sheath showed morphological changes. This technique may therefore be useful in diagnosing protein-calorie malnutrition.*

Human hair color (*1*) and texture (*2*) change during protein-calorie malnutrition. The cells of the hair matrix normally proliferate at a rate probably greater than any other tissue with the possible exception of bone marrow (*3*), an indication of the high protein-synthesizing activity of hair tissue. The majority of this synthesis is carried on in the hair bulb (*4*). Van Scott (*5*), using histogeometric analysis, estimated replacement time of the entire germinative matrix to be less than 1 day. Fiber growth rate of Merino sheep has been shown to vary with differences in protein quality as well as quantity (*6*). These facts, coupled with the known high mitotic activity of the hair bulb (*7*) and resultant high protein requirement, suggested that hair root activity may be useful in diagnosing protein-calorie malnutrition.

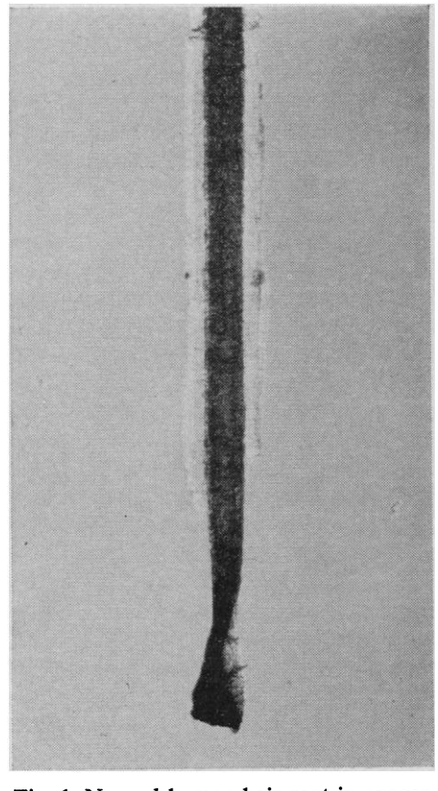

Fig. 1. Normal human hair root in anagen growth phase (× 75).

Eight male subjects in excellent physical condition, aged 24 to 29, were housed in a research ward for more than 3 months. Physical and biochemical examination showed that they remained in good health throughout the study. The subjects were fed a liquid formula diet (*8*) three times daily which provided either 75 g protein per man per day in the form of egg albumen, or no protein. In the latter case Dextri-maltose was substituted isocalorically for the egg albumen. The diet remained at 2800 calories per man per day throughout the study. None of the pathological factors causing telogen effluvium as reported by Kligman (*9*) or Van Scott (*10*) were present, nor were there histories of x-ray therapy or use of cancer therapeutic drugs. At the end of each 15-day experimental period approximately 100 hairs were manually plucked from the occiputal area of each subject with Tygon-coated needle-holding forceps (*11*) and examined in a dissecting microscope (× 45). Care was taken to use slightly different locations on the scalp in successive samples. The fast epilation technique described by Maguire and Kligman (*12*) was used to minimize dysplasia as a function of technique. To avoid bias, both protein-deprived and control subjects were sampled blind at the same time by the same investigator. The morphological changes were observed more clearly with suprastage cross light than with transmitted light. Bulb diameters were measured with an ocular micrometer. In most cases samples were taken during two complete dietary periods and represented a total of 21 samples (11 before, 10 after).

The 15-day periods of protein deprivation were too short to show changes in the ratio of the anagen (active) phase to the telogen (resting) phase. However, consistent morphological changes did occur after the subject was deprived of protein. (i) The hair