Intracellular Localization of Compounds Labeled with Tritium, H^3, By Radioautography.* (18731)

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(Introduced by C. P. Rhoads.)

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The ultimate goal of radioautography is the localization of an element or compound to small intercellular areas or to intracellular structures. The demonstration of radioactivity in human or animal organs or parts of an organ has often been achieved(1) and the concentration of an isotope in individual isolated cells demonstrated(2). Attempts at finer localization have been limited by two factors—lack of emulsion resolution and the long average path in emulsion of many isotopes. Resolution of emulsions has been recently increased by the nuclear track emulsions so that lines of 2.5 μ width have been resolved(3). This suggests the possibility of intracellular localization. However, the maximum paths in the nuclear emulsions of even such weak beta particle emitters as carbon^{14} and sulfur^{35} are approximately 90 and 100 μ, respectively. Under-exposure and long development of emulsions considerably reduce these disadvantages by making visible individual beta particle tracks(4). The initial high energy of beta emitters causes wide grain spacing at the beginning of the track and this detracts from efforts towards the microscopic localization of a radioactive particle to its site of emission. Alpha particle emitters are foreign to most biological organisms and no extensive use of them as a label has been reported.

We have employed tritium, H^3, whose average beta particle traverses less than 1 μ in nuclear track emulsions, to give radioautographic images of individual yeast cells and intracellular paramecium structures (Fig. 1, 2).

Experimental. Paramecium aurelia were grown for 7 days in a Timothy hay infusion medium in which *Aerobacter aerogenes* had been incubated at 27°C for a 48-hour prior period. The medium included aqueous solution of sodium acetate containing tritium. The activity of the water was 2×10^7 disintegrations per min. per mg and the sodium acetate, labeled in the methyl groups with tritium, had an activity of 4×10^7 disintegration per min. per mg. After the growth period paramecia were fixed in 2% aqueous osmic acid solution. The mixture was centrifuged and the organisms washed with distilled water and fixed in 2% of U.S.P. formalin solution for 2½ hours. After being washed in distilled water and dehydrated in 70, 95, and 100% alcohol, the organisms were placed in an ether-alcohol solution, embedded over a period of 2 days in celloidin and finally in 2 changes of paraffin to give a rigidly embedded block. Sections were cut at 1 μ on a wedge type microtome(5). Whole organisms were put through the same initial procedures but after being fixed, were washed, centrifuged, and kept in distilled water. A control group of organisms was processed simultaneously under similar circumstances with the exception that the medium contained no radioactivity.

Torula utilis, a saprophytic yeast, was grown in a salt solution(6) in which the only source of nitrogen was ammonium ion and the

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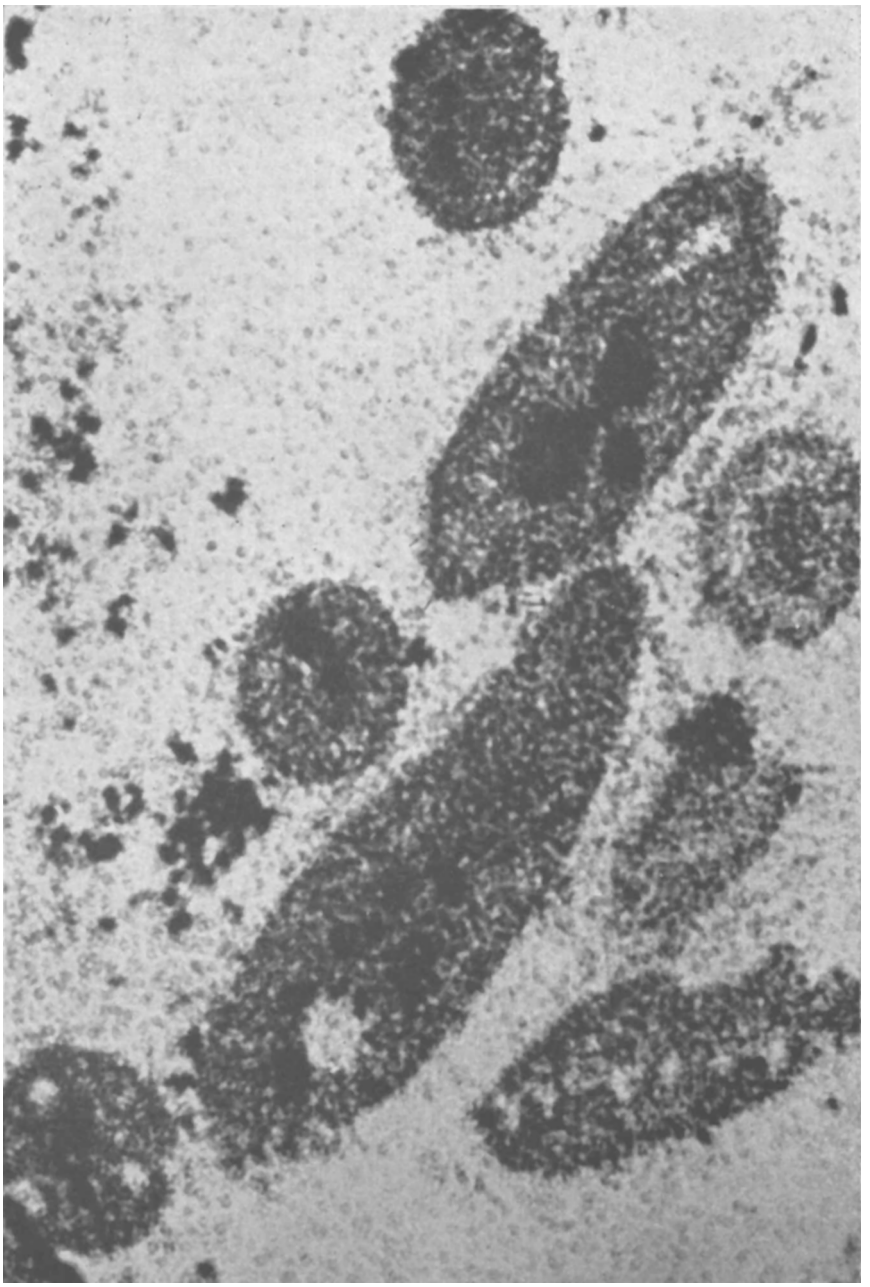

FIG. 1. Radioautograph (#51-24-K). One μ section of many paramecia. Diffuse activity with endoplasmic concentrations. Some areas of activity between organism (radioactive organisms or debris). Exposure two weeks, E.K. NTB_3, 10 μ, emulsion. Unstained. Original magnification 460.

only source of carbon was sodium acetate present in a concentration of 2%. The test organisms were grown in 2 ml of medium in a shaken culture for 4 days at 25°C. The activity of the sodium acetate was the same as used for the paramecium but the activity of the water was 2×10^6 disintegrations per min. per mg. After growth in the medium

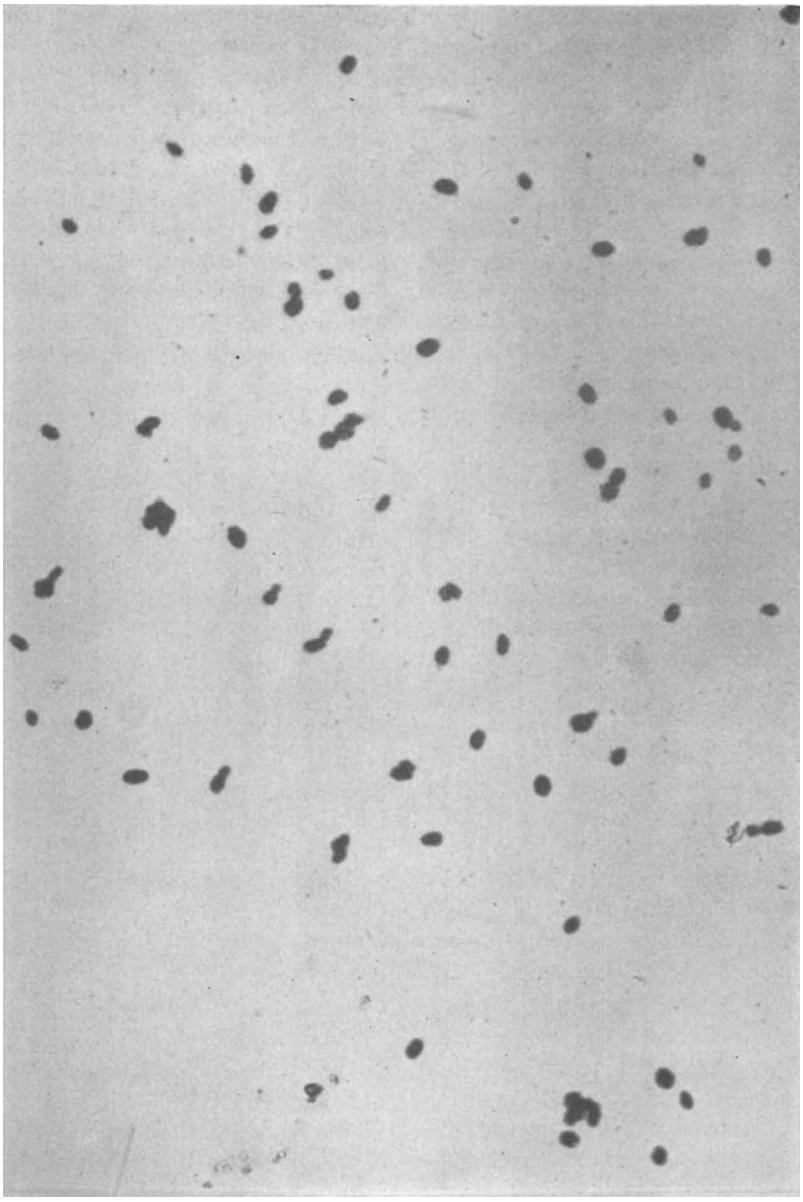

FIG. 2. Radioautograph (#51-35) of yeast cells obtained by placing Kodak Limited NTB stripping film over smear of dried organisms. Three weeks exposure. Unstained. Original magnification 492.

containing tritium, the yeast were washed with distilled water and centrifuged 6 times and kept in distilled water. Control organisms were simultaneously processed with the exception that the medium contained no radioactivity.

Radioautographic technic. Only the nuclear track types of emulsion were used. Eastman Kodak NTB, NTB_2, NTB_3 emulsions and a Kodak Limited stripping film from autoradiographic plates were used. A drop of distilled water containing the radioactive yeast or paramecia was placed on these emulsions. Drops of organisms were placed on glass slides, dried, and the slides were apposed to the emulsion surfaces. The stripping film was

placed over dried organisms on the slide. Sections of the paraffin-celloidin embedded organisms were transferred to a water bath, floated on to photographic emulsions, deparaffinized, dried and exposed. Deparaffinized sections in a glass slide were covered with Kodak Limited stripping film or apposed to emulsions. Organisms grown in a non-radioactive medium but otherwise identical, were processed simultaneously, placed on similar emulsions and exposed for equal time periods. Formvar coated emulsions were used to prevent direct contact of specimen and emulsion as a control to rule out pseudophotographic effects. Specimens were exposed for periods varying from 1 to 4 weeks, in black, light-tight, plastic boxes and kept in a refrigerator at about 5°C. Development was in D-19 solution for 4 minutes at 20°C. An acid stop bath of 1% acetic acid was used, and fixation was accomplished with 28% sodium thiosulfate. The specimens were washed, dried, and viewed unstained.

Results. Paramecia grown in the medium containing tritium gave well defined radioautographic images. The whole organism lying on the emulsion was sufficiently opaque to hide the underlying radioautographic image. If the organisms were removed, it was seen that a sharply defined radioautographic image was present. Emulsions apposed to organisms on a glass slide also gave sharp images.

Sections of paramecia cut at 1 μ gave the best autographs (Fig. 1). The sections were thin enough to permit the passage of light through the organism and to reveal the underlying emulsion image. Radioactivity was evident throughout the organisms both in endoplasm and ectoplasm. Many discrete round areas of concentration of tritium appear in the endoplasm. Most correspond to food vacuoles but a few very large areas resembled the macronucleus. The mouth was sharply outlined in some organisms. An exposure of one week gave good images. All controls were negative.

Yeast grown in the medium containing tritium and exposed for one week gave positive radioautographs on the most sensitive emulsions. Clusters of yeast showed an image in the radioautograph that corresponded identically to their pattern of arrangement on the slide apposed to emulsion. Usually a complete silhouette of each organism was given in the radioautograph when organisms were distinct (Fig. 2). Individual budding yeast cells were defined. Control yeasts did not cause reduction of emulsion when placed directly on emulsions, when placed on a Formvar coat overlying emulsions, or when apposed to emulsion.

Measurement of the smallest yeast cells by a filar micrometer eye piece using a calibrated stage micrometer showed sharp radioautographic images on the stripping film that were 3 μ in diameter. Sections of paramecium cut at 1 μ showed well resolved endoplasmic concentrations of radioactivity as small as 3 μ in the stripping film. Even smaller areas of radioactivity scattered between the paramecia were discernible but it could not be determined whether they were radioactive organisms or debris, or photographic artefact.

Discussion. Tritium, a radioactive isotope of hydrogen, emits beta particles having a maximum energy of 0.0179 Mev. and has a half life of 12.1 $\pm$ 0.5 years(7,8). Ninety per cent of its beta particles would be absorbed by one grain of silver bromide in nuclear track emulsions. It approaches the ideal for intercellular or intracellular localization. It is available at relatively little cost from the Atomic Energy Commission.

Its biological use as a label will depend on the particular compound under study. Tritium atoms bonded stably to carbon atoms can be used to label the latter just as the inactive hydrogen isotope deuterium has been used(9). In some compounds it may be easier to use tritium than to use radiocarbon.

Summary. Tritium, H^3, the radioactive isotope of hydrogen has been used for radioautography and given radioautographic images after a week's exposure of individual yeast

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cells and paramecia grown in media including sodium acetate containing tritium. Measured radioautographic images of endoplasmic concentrations of radioactivity in paramecium and of individual yeast cells of 3 μ have been delineated. Since the average beta particle from tritium is absorbed by a micron or less of nuclear track emulsion, from the standpoint of its range, this isotope approaches the ideal for radioautographic localization.

Yeast grown in tritium-containing medium as described and exposed for a shorter time have shown differential localization of tritium in polar and peripheral extranuclear areas. Some of these foci showed only one or two grains of reduced silver (each about 0.3 μ to 0.4 μ) in the emulsion. This probably represents the limit of resolution of radioautography with the present emulsions available for biological experiments.

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Apparent Discrepancies in Evaluation of Adrenocorticotrophic Hormone (ACTH) Activity by Two Assay Methods. (18732)

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The availability of a rapid and sensitive method for the assay of ACTH preparations by the adrenal ascorbic acid depletion test in the hypophysectomized rat(1) has made it possible to carry out comparative assays to an extent not allowed by the more time consuming procedure of assay by the adrenal maintenance test in the hypophysectomized rat(2). The lack of internationally accepted test procedures and preparations has, however, prevented the direct comparison of the level of activity of different samples of ACTH. Further than this, no detailed study has been made to date of: (a) the relationship between the potency of preparations in terms of ability to deplete ascorbic acid in the adrenal of the hypophysectomized rat, as compared with various morphological assay methods (adrenal repair and maintenance in the hypophysectomized rat), and (b) the relationship between the ascorbic acid depleting potency and the actual secretory activity of the adrenal gland in terms of the many and known experimental and clinical effects.

It is also known that the criteria (electrophoresis, solubility curves) for establishing the chemical homogeneity of hormone preparations are not strict enough to rule out trace contaminants (either anterior or posterior lobe) which may be pharmacologically active. Under such circumstances, it is obligatory that comparisons be made between various methods of assay, in order that the potency of various preparations can be evaluated in terms of types of responses, which may not necessarily be directly related to each other quantitatively. The present communication describes three series of experiments in which various types of ACTH preparations assayed by the ascorbic acid depletion test did not manifest a proportionally equivalent response in the adrenal maintenance test, or in secondarily manifested effects on the thymus and lymph nodes.

Methods and materials. Five preparations of ACTH were employed: A. ACTH protein prepared according to the method described

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