A METHOD FOR STUDYING INTRACELLULAR MOVEMENT OF WATER-SOLUBLE ISOTOPES PRIOR TO RADIOAUTOGRAPHY

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INTRODUCTION

The compartmentalization of cell structure as revealed by electron microscopy has been shown to have a striking parallelism with the segregation of enzyme systems as revealed by differential centrifugation. The biochemical anatomy of the cell is now of great interest, and a powerful technique for its investigation has been provided by the recent application of radioautography at the ultrastructural level (1–3).

At present, this technique has been most successful in the localization of proteins (1) and nucleoproteins (4). However, one might also expect smaller compounds and ions to show a pattern of differential localization similar to that of the enzymes mediating the reactions with which they are concerned.

Though of great interest, the intracellular localization of diffusible compounds has been a problem of exceptional difficulty, as the careful studies of D. K. Hill (5, 6) have clearly demonstrated. While investigating such a problem, it is essential to have prior knowledge of the movement of the compound, both under the experimental conditions and during the steps of histological preparation.

While studying the cellular localization of a small organic compound, serotonin, and a divalent cation, calcium, the authors found that the curves of the washout of radioactivity from the tissues of serotonin-C^{14} and calcium-45 provided a useful tool for investigating intracellular movement and finding a means of limiting it.

MATERIALS AND METHODS

Serotonin

Mice, averaging 20 gm in weight, were injected intravenously with 5-hydroxytryptophan-3 C^{14} (5-HTP-C^{14}) 1 hour after an intraperitoneal injection of β-phenylisopropylhydrazine (PIH), a monoamine

oxidase inhibitor. This procedure was shown to result in the production of radioactive serotonin in the gut and will be dealt with in detail in a subsequent publication. Segments of duodenum, 25 to 40 mg in weight, were removed from the animal 4 hours after the injection of 5-HTP-C^{14}. The lumen was opened by a longitudinal slit and the duodenal strips were quickly weighed and then transferred at various time intervals through a succession of tubes containing physiological saline. The remainder of the experimental procedure is described in the legend of Fig. 1.

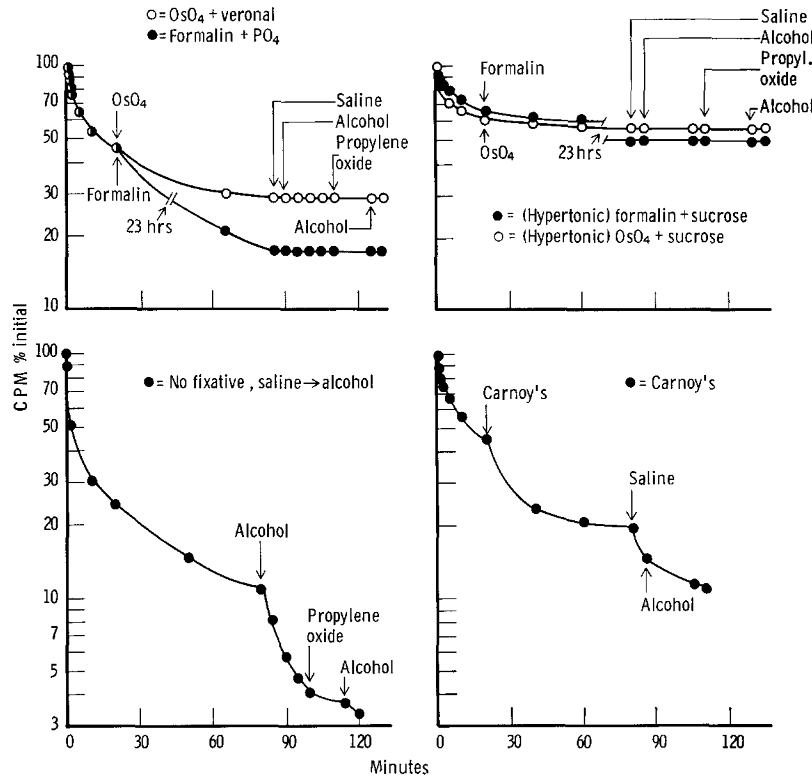

FIGURE 1 The washout of radioactive 5-OH indoles from duodenal strips in physiological saline and various experimental solutions. Temperature 20–22°C. After washout, the duodenal strips were dissolved in 12 N HCl and the residual radioactivity measured. Initial radioactivity was obtained by adding that which left the strips in saline or experimental solution to the residual radioactivity. The amount of radioactivity remaining in the strip, as per cent of the initial radioactivity, was then plotted semilogarithmically against time.

Calcium

Paired sartorius muscles, dissected from pithed frogs weighing approximately 20 gm, were placed in Ringer's solution containing Ca^{45} (specific activity 2 μc/ml) at 6°C for 16 hours. The muscles were then removed from the isotope, rinsed in a non-radioactive Ringer's solution for 1 hour, during which time they were mounted at resting length on Pyrex glass racks by means of cotton ties. The remainder of the procedure is described in the legend of Fig. 2.

Measurements of Radioactivity

Radioactivity was assayed by means of a windowless gas flow counter. Care was taken to assure even spreading on aluminum or glass planchets, and samples were corrected for self-absorption. The coefficient of variation of the counts of five pipettings of standard solutions containing 1000 CPM was 1.42 per cent.

Experimental Solutions

(1) Formalin and PO_4: 10 per cent formalin; 90 per cent 0.1 M Sorenson's phosphate buffer, pH 7.4. (2) Formalin and PO_4 and sucrose: As (1) with 9 per cent w/v sucrose. (3) Formalin and PO_4 and oxalate: As (1) with sodium oxalate added to give a final oxalate concentration of 0.02 M. (4) OsO_4:

Palade's fixative. (5) OsO_4 and sucrose: As (4) with 9 per cent w/v sucrose. (6) OsO_4 and PO_4: 50 per cent v/v of 2 per cent OsO_4; 50 per cent v/v of 0.1 M Sorenson's phosphate buffer, pH 7.4. (7) OsO_4 and PO_4 and oxalate: As (6) with sodium oxalate added to give a final oxalate concentration of 0.02 M. (8) Carnoy's solution: 10 per cent glacial acetic acid; 30 per cent chloroform; 60 per cent absolute ethanol.

RESULTS

Serotonin

Radioactive 5-OH indoles were allowed to wash out of duodenal strips in physiological saline, and, when the slow component of the washout curve had been reached, the strips were transferred to one of the experimental solutions. The curves of washout of radioactivity are shown in Fig. 1. The rate of washout of radioactivity was moderately increased over that in saline by the hypotonic fixatives, OsO_4, and formalin and PO_4, but was markedly increased by ethanol (70 to 100 per cent) alone and by Carnoy's solution. In contrast, the rate of washout was not increased when the OsO_4 and formalin were made hypertonic by the addition of sucrose. All of the fixatives except Carnoy's solution prevent the subsequent extraction of radioactivity by saline, alcohol, or clearing agent.

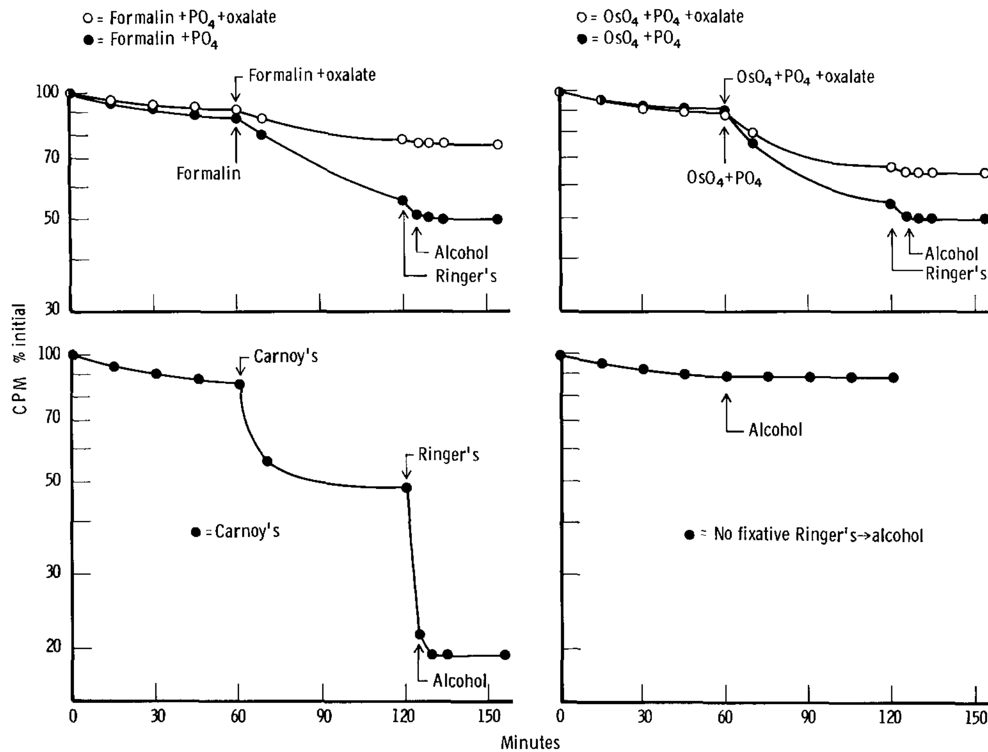

FIGURE 2 The washout of radiocalcium from frog sartorius muscles in Ringer's and experimental solutions. Temperature 6°C. After washout, the muscles were ashed at 600°C for 24 hours and the residual radioactivity measured. The rapid phase of Ca^{45} release has not been included in the graphs. One hundred per cent has been arbitrarily set as that amount of radioactivity present in the muscle after 1 hour in non-radioactive Ringer's solution.

Calcium

As in the case of the duodenal strips, Ca^{45} was allowed to wash out of the muscles until the slow component of the washout curve had been reached. In both cases, it was presumed that the slow rate of washout predominantly reflected loss from an intracellular depot (see Discussion). The results are presented in Fig. 2. When the muscles fixed in

formalin or OsO_4 with or without oxalate are compared, it is seen that more Ca^{45} is retained in the muscles fixed in the presence of oxalate. Furthermore, the loss of isotope when the muscles are returned to the Ringer's solution is significantly decreased if oxalate had been included in the fixative. Carnoy's solution caused the most striking release of calcium both in the fixative and after returning to Ringer's solution. In all cases ethanol (60 to 100 per cent) abruptly stopped calcium release. Alcohol will prevent the release of calcium even in the absence of prior fixation (1 per cent picric acid in 2-propanol also prevented calcium release).

DISCUSSION

By means of the relatively simple washout procedure described above, approximations of both the rate and quantity of isotope movement from the cells can be made. The speed of fixation can be estimated from changes in the rate of isotope release, and an indication obtained as to not only how much intracellular isotope has been retained, but also how quickly its movement has been halted. This estimation rests on the assumption that minimal release from the cells indicates minimal intracellular diffusion. The technique gives no information, however, as to whether a small amount of movement which may take place is selectively critical to a particular radioautographic problem. It is also possible that the alterations in isotope release with fixation reflect changes in membrane permeability to the molecules studied rather than limitations to their diffusion within the cell cytoplasm. If this were the case, then it would seem that after treatment with alcohol the membrane permeability changes selectively for different molecules. Ethanol abruptly halts Ca^{45} release yet increases the rate of loss of serotonin. It would also seem likely that if the cell membrane were acting as a barrier to diffusion after fixation, then intracellular membranes would also act as similar barriers to the diffusion of molecules located within membrane-limited compartments of the cell. In either case, therefore, the rate of loss of isotope from the cells would still provide useful information as to the extent of intracellular movement during and after fixation.

In order to obtain information about intracellular movement of the radioisotopically labeled material from the curves of washout, isotope released from within cells should be distinguishable from that of extracellular origin. In the case of serotonin, the serotonin-C^{14} was synthesized within cells from its administered precursor, 5-HTP-C^{14}. Four hours after administration, little radioactivity was found in a muscular organ, the heart, in the connective tissue or muscles of the extremities, or free in the blood plasma. Consequently, it was assumed that the slow component of the washout curves represented serotonin-C^{14} from an intracellular depot.

In the case of calcium, binding to connective tissue fibers (7) and cell surfaces (8) complicates the analysis. However, it has been shown (7) that, while at the beginning of the washout period significant amounts of radiocalcium in the sartorius muscle are bound to cell surfaces and connective tissue, after 90 minutes of washout 95 per cent of the remaining Ca^{45} is intracellular. After 120 minutes of washout (at which time the muscles were fixed, in the present experiments) almost all of the radiocalcium leaving the muscles is from intracellular stores. The amount of Ca^{45} still bound to connective tissue and cell surfaces is inadequate to explain the magnitude of the effects produced by immersion in fixatives.

The authors, therefore, conclude that the alterations in the washout curves of serotonin-C^{14} and Ca^{45} produced by the fixatives reflected events occurring at or inside the cells of the respective tissues. The curves of washout can thus be used to obtain useful information on which the radioautographic localization of these compounds can be based.

SUMMARY

Washout curves of serotonin-C^{14} from the mouse duodenum and Ca^{45} from frog skeletal muscle have been studied before, during, and after treatment of the respective tissues with various fixatives.

The release of tissue serotonin during fixation can be markedly reduced by making the fixative hypertonic. Alcohol causes a marked extraction of serotonin, but this extraction does not occur if fixation precedes exposure to alcohol.

The addition of calcium precipitant, sodium oxalate, to OsO_4 or formalin causes a substantial reduction in calcium release from muscle during fixation. Alcohol abruptly stops calcium efflux whether or not the addition of alcohol precedes or follows fixation.

It is felt that this washout technique may have wide applicability as a method for investigating

diffusion problems prior to radioautographic studies of water-soluble intracellular compounds.

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