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Studies with Electron Microscopic Autoradiography of Thyroxine ^{125}I in Organotypic Cultures of the CNS[1] I. Fixation of Thyroxine ^{125}I

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INTRODUCTION

In previous experiments with ^{125}I thyroxine and triiodothyronine in organotypic cultures of the nervous system, it was found that alcohol fixatives (absolute methyl alcohol alone, and absolute methyl alcohol, chloroform and glacial acetic acid, 6:3:1) leached the tissue almost entirely of its radioactivity; in contrast, using glutaraldehyde fixation with postosmification, the tissue retained enough radioactivity to make autoradiographic localization in these tissue cultures feasible(1). However, in view of the criticisms that steroid hormones and other "soluble" compounds are removed from, or translocated in tissue that has been fixed, dehydrated and embedded(2), it was important to investigate such possible artifacts with thyroxine. Additionally the validity of using ^{125}I for light microscopic autoradiography with 0.8-μ sections was assessed since this isotope has been reported to produce a "halo" of grains around a source(3). The following is a report of an analysis of radioactive components lost during fixation, dehydration and embedding of explant tissue cultures of embryonic spinal cord and cerebellum treated with thyroxine ^{125}I. Light microscopic autoradiography was compared with the localization of grains using the higher resolution of electron microscopic autoradiography.

MATERIAL AND METHODS

Tissue cultures of spinal cord-dorsal root ganglia combinations(4) from 13-day mouse embryos, and of newborn mouse cerebellum(5) were maintained for 19

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and 11 days before addition of thyroxine ^{125}I. Several sera were kindly assayed for thyroxine content by H. Seligson and a horse serum containing less total thyroxine [10^{-8} *M* (0.8 μg%)] than most sera used for CNS cultures [1.6×10^{-7} *M* (12 μg%)] was chosen to make feeding medium for both maintenance and experimental periods.

Thyroxine ^{125}I (Amersham-Searle, specific activity 69 μc/μg) was shown to be greater than 99% pure on receipt by chromatography, and it was used within 24 hr for these experiments. Propylene glycol was removed(1) by heating in a water bath at 43° while drying via a lyophilizer for 35 min. The dried thyroxine was rapidly dissolved in Earle's saline with 0.1 *N* NaOH (corrected to 306 mosm with H_2O) and appropriate amounts of hormone were added to the feeding media to give a final concentration of 3.5×10^{-7} *M* thyroxine ^{125}I, which was slightly less than 1 μc/drop/culture.

Cultures were fed immediately with this solution and were incubated for 15 min, 30 min, 2, 4, or 22 hr prior to fixation. Light microscopic autoradiography of cultures exposed to thyroxine ^{125}I and triiodothyronine ^{125}I in saline or in medium, with and without a chase solution, showed previously no differences in localization of grains(1). Therefore all cultures were briefly dipped in saline before being fixed in Millonig's buffered glutaraldehyde(6) (pH 7.1, 400 mosm) rinsed in buffer, postosmified, dehydrated, oriented and embedded in Epon in the usual manner(1). One additional group of cultures, treated with radioactive hormone for 22 hr was fixed only in *s*-collidine buffered(7) osmium tetroxide and embedded as above. All fixing and dehydrating solutions from the cultures incubated for 15 and 30 min were collected as one group, and the solutions of the cultures exposed for 22 hr were kept as another separate group. Aliquots of all solutions from both groups were counted on a Nuclear Chicago γ counter. Counts of each aliquot were multiplied by the total volume of that solution collected, and divided by the number of cultures fixed in that solution, to give an estimate of counts/average culture for each solution. Single cultures embedded in Epon were also counted using the same equipment. In order to compare the amount of radioactivity lost during processing of tissue, to that bound by cultures in Epon, the counts/average culture of all solutions plus the counts/average culture in Epon for each exposure (i.e., 15/30 min and 22 hr) were summed. This value was considered to represent the total radioactivity present in these cultures prior to fixation.

To assess possible fixation of free iodine in the cells, saline and Eagle's medium (1:1), with greater than 1 μc/drop/culture of K^{125}I, was applied to another set of cord ganglia and cerebellar cultures for 15 min and 2 hr. Fixation with glutaraldehyde, dehydration, embedding and counting were done as above. Furthermore, other spinal cord and cerebellar cultures that had not been exposed to thyroxine ^{125}I in the living state were fixed in Millonig's buffered glutaraldehyde containing thyroxine ^{125}I to see if the latter could be incorporated in the tissue during the process of fixation. These cultures were dehydrated, embedded and counted as above. A minimum of three separate cultures were included in every experimental group described above.

In order to analyze whether radioactive components in the fixing and dehydration solutions were iodine, thyroxine or larger molecules (e.g., protein-bound thyroxine), ascending thin-layer chromatography using the solvent system described by Weinert *et al.*(8) was employed. $K^{125}I$ and thyroxine ^{125}I were run as markers parallel to the solutions to be tested. Strips were cut and counted on a γ counter as there was too little material to be detected by ninhydrin or other dyes. Sephadex G-25 columns were run on these same test solutions to confirm the results of chromatography. A small amount of blue dextran and NaCl were added to the test solutions, or run on a separate column using the same volume of fluid, at the same speed, in order to mark the fractions containing high and low molecular weight substances. The fractions containing NaCl were detected after γ counting by precipitation with $AgNO_3$. Although 100% of radioactivity was recovered from runs with high total radioactivity, no more than 80% of total counts was recovered from solutions with little radioactivity, and this was probably due to nonspecific binding of thyroxine by the Sephadex. An estimate of large molecular weight substances by trichloracetic acid precipitation for proteins gave unreliable results as the ultrafiltrate when passed again through a second Millipore filter lost the same percentage of counts as it had during the first filtration. Results obtained within a few days of fixation were considered to be the most accurate since later runs tended to show increasing breakdown of molecules in the solutions. In all instances where there was a low level of counts (see Fig. 3) samples were counted for 10 min to avoid statistical variations in counting that would be reflected at 1 min.

For light microscopy, 0.8-μ sections were coated with Ilford K5, exposed for 2 weeks and stained after development with toluidine blue(9). For electron microscopy, uncoated grids with silver sections, stained with uranyl acetate, were looped with a monolayer of Ilford L4(10), exposed for 8 weeks, developed in Microdol-X, and poststained with lead citrate(9). The localization of grains by light microscopy was compared to that found by electron microscopy. Background of Epon immediately adjacent to tissue in the electron microscope was also counted. Furthermore, in order to see if there was displacement of radioactive material with different penetration of fixative, the localization of grains in cells at the top of the tissue culture was compared to that from the deepest portions.

The number of thyroxine molecules incorporated per cell was calculated by counting known amounts of thyroxine ^{125}I (sp act 69 μc/ug) and comparing this to counts of cultures embedded in Epon. There were 6×10^{10} molecules per culture or a rough estimate of 8×10^4 molecules per cell.

RESULTS

Feeding media, diluted with saline, examined by thin-layer chromatography showed that most of the counts remained at the origin and did not migrate (Fig. 1). Another smaller peak, about 10% of total counts was identified as free thyroxine since pure thyroxine ^{125}I peaked in this same fraction. No counts were

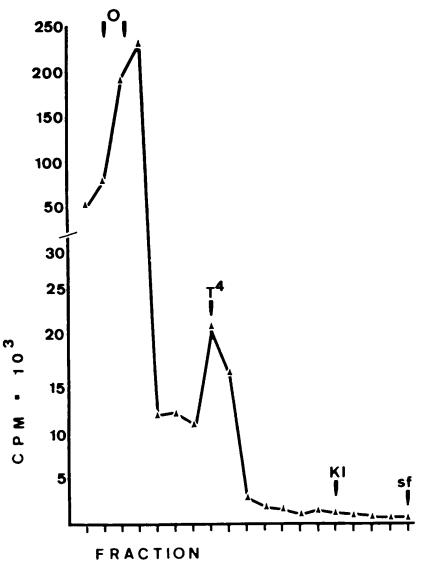

FIG. 1. Thin-layer chromatography of feeding media with added thyroxine ^{125}I. The largest fraction remained at the origin (0), a smaller peak (note the change in scale) migrated with thyroxine (T^4). No free iodine ^{125}I is present (KI); sf is solvent front.

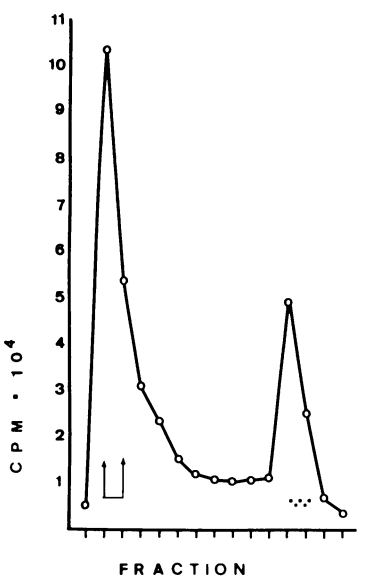

FIG. 2. A Sephadex fractionation of feeding media several days after its preparation. Most material is released with high molecular weight marker (arrows, blue dextran added to column). A smaller fraction is found near the low molecular weight fractions (dots, $AgCl_2$ from a separate column); 100% of radioactive material recovered.

observed in fractions on or near the iodine peak (K ^{125}I marker). An insignificant percentage of counts remained at the origin with the control markers, therefore it was concluded that almost 90% of the added thyroxine ^{125}I was bound to serum proteins, such as albumins and globulins in the feeding media, that could not migrate by virtue of their high molecular weights. From these results it was calculated that 3×10^{-8} *M* free thyroxine ^{125}I was present in the feeding media at the time of application to the cultures.

Feeding media analyzed by G-25 Sephadex a few days later showed similar results in that a large fraction of the radioactivity was present in the high molecular weight fractions, marked by blue dextran, and fewer counts were present in the low molecular weight fractions (Fig. 2). An estimate of free thyroxine by Sephadex fractionation (30%) was higher than that derived from chromatography (10%) since it was done several days after preparation of the feeding media, and with increasing time significantly greater amounts both of free thyroxine ^{125}I and iodine ^{125}I were seen both with chromatographic and Sephadex analysis.

Counts of the glutaraldehyde solution from tissue fixed after exposure of thyroxine ^{125}I for 15 and 30 min showed a higher number of counts per culture than counts of the glutaraldehyde solution from tissue fixed after 22 hr of exposure. This increased number of counts at the early times after exposure were probably due to nonspecific sequestration of labeled thyroxine in the feeding medium still left in the explants. Chromatography and Sephadex analysis of glutaraldehyde were not done because of the artefacts caused by glutaraldehyde in these systems. Sephadex analysis of the buffer rinse after glutaraldehyde fixation of these cultures exposed to thyroxine ^{125}I revealed a profile similar to that obtained from the feeding medium; most counts were in the high molecular weight fractions (Fig. 3). Analysis of the buffer solution by chromatography supported the findings of Sephadex fractionation, since most counts remained at the origin. These results would be compatible with the above idea of sequestration of serum thyroxine since this fraction would be of high molecular weight and would not migrate.

It had been stated in a study of the thyroid gland with ^{125}I that extraction during alcohol dehydration would remove free iodine(11). In order to determine what types of radioactive molecules were being extracted by alcohol, Sephadex and chromatographic analysis of these solutions were performed. A percentage of the total radioactivity was extracted in the graded alcohol solution with peaks in counts at 50 and 65% for tissue exposed for 22 hr and for 15/30 min, respectively (Fig. 4). No more labeled material could be extracted with further rinses in the same grade of ethanol and furthermore with higher concentrations of ethanol little or no radioactivity was found in the supernatant. Therefore extraction was considered complete. As much as 21% (the highest value from chromatography and Sephadex analysis) of the total radioactivity of the various alcohol solutions was shown to be of larger molecular weight. For example, chromatography of the 65% alcohol solution from tissue exposed for 15/30 min showed a peak at the origin (high molecular weight) and two other peaks that migrated beside thyroxine ^{125}I and K^{125}I, respectively (Fig. 5). Chromatography

of the 50% alcohol supernatant from tissue exposed for 22 hr was similar, and Sephadex fractionation of these alcohol supernatants showed an equivalent peak of radioactivity in the larger molecular weight fractions.

No radioactive counts were found either in the propylene oxide or Epon mixtures.

Counts of Epon-embedded cultures revealed that more radioactive hormone was bound in the tissue with increasing time after administration of thyroxine (Fig. 6). Thus, at 15 min there was 9000 cpm/average culture, and at 22 hr, cultures retained an average of 22,000 cpm. Cultures fixed with osmium tetroxide only at 22 hr after hormone application contained significantly less radioactivity (13,300 cpm) than their counterparts first fixed with glutaraldehyde.

The number of radioactive counts bound by cultures embedded in Epon was compared to the total radioactivity of cultures prior to fixation (see Methods). Cultures exposed for 15/30 min retained only 4% of the total radioactivity prior to fixation. More than 90% of their radioactivity was lost during glutaraldehyde fixation. By comparison, cultures exposed for 22 hr retained in Epon as much

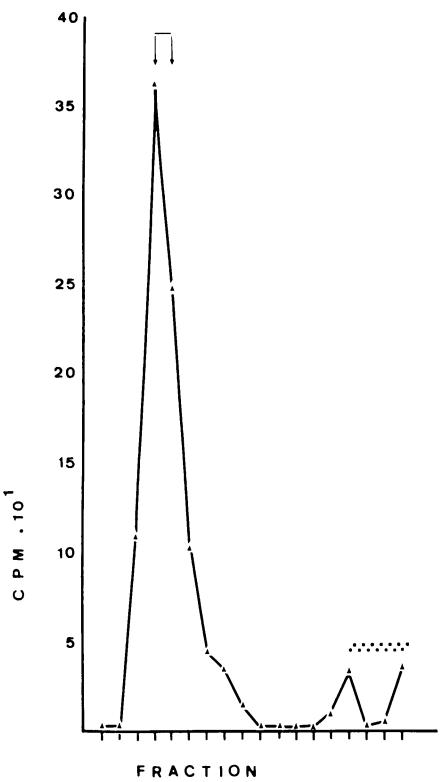

Fig. 3. Sephadex analysis of buffer rinse after glutaraldehyde fixation. Most material was found in high molecular weight fractions marked by blue dextran (arrows). Smaller molecules marked by silver precipitation of chloride (dots). Above run represents 75% of radioactivity added to column. The cpm are derived from 10-min counts.

as 25% of the total radioactivity. Again, most of the radioactivity was lost in the glutaraldehyde solution. Furthermore, the labeled high molecular weight substances leached in the alcohol extracts, (20% of the counts, see Fig. 4), accounted for less than 1.5% of the total radioactivity prior to processing, or at 22 hr no more than 5% of hormone bound and retained in Epon. (At this time 1000 cpm/average culture of the alcohol extract was of high molecular weight and the hormone bound in Epon-embedded cultures was 21,000 cpm/average culture.)

From the previous data, no free iodine (see Fig. 5), as an example of a soluble component, would be fixed by these methods. In order to prove this, other spinal cord and cerebellar cultures were treated with $K^{125}I$ for 15 min and 2 hr, and processed in glutaraldehyde as cited. As expected, and in contrast to the thyroxine ^{125}I experiments, counts from these Epon-embedded cultures were within the range of background counts. Furthermore, other control cultures, unexposed to thyroxine ^{125}I in the living state, and fixed in glutaraldehyde containing radioactive hormone, also showed no counts above background. Thus, no hormone could be artificially bound by fixative and retained by the cells if it were not first incorporated in the living state.

The isotope ^{125}I is generally considered to be a weak β or Auger electron emitter. However, as it also emits γ rays, the possibility existed that higher energy β particles, created by collision of γ rays with orbital electrons (Compton scatter-

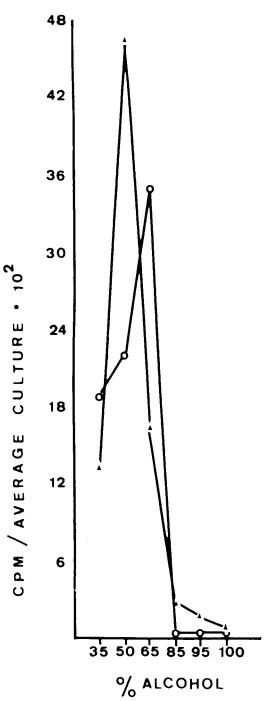

FIG. 4. Radioactivity recovered from alcohol solutions after exposure to thyroxine ^{125}I for 15 and 30 min (-0-) and 22 hr (-▲-). Peaks are at 65 and 50% stages, respectively.

ing), would significantly decrease the autoradiographic resolution. Nuclear grains observed by light microscopy (Figs. 7a and 8a) were also seen with the higher resolving power of electron microscopic autoradiography(12) (Figs. 7b, 8b, 8c). Furthermore, their frequency could not be accounted for either by nonspecific background, or background created by scatter, since in most cases Epon adjacent to radioactive cells had no grains (Figs. 8c and 9). Autoradiography as well as counting, showed no grains leached into the Epon. It is also of interest to note in terms of fixation, that cells on top of the culture showed the same localization of grains as those several layers deep, implying that fixation of the hormone was equivalent in both places, and that there was little or no diffusion of fixed hormone.

DISCUSSION

It has been shown by Peters and Ashley that "free amino acids," blocked by puromycin from their incorporation into protein, can be fixed in the cell *in situ* by glutaraldehyde, presumably by binding their amino groups to nearby proteins, and it is further suggested that glutaraldehyde be used to "immobilize diffusible

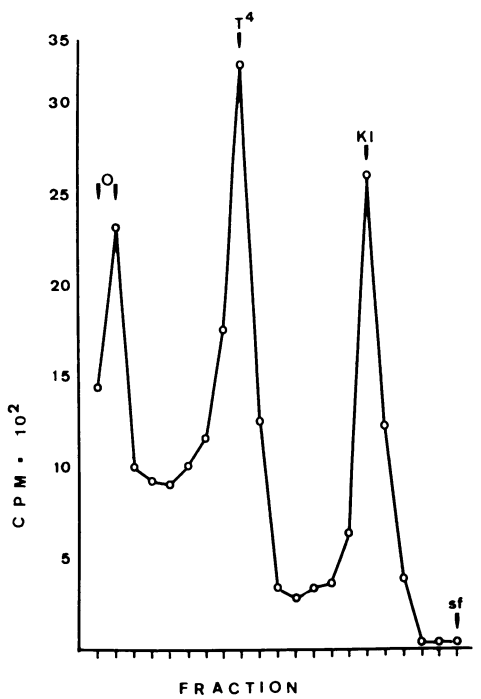

FIG. 5. Chromatography of 65% alcohol from tissue fixed at 15 and 30 min. Peaks are present at the origin (0), in the region of thyroxine (T^4) and in the region of the iodine marker (KI); sf is solvent front.

compounds containing amino groups"(13). Some of the "proteins" to which "free" amino acids were so fixed would be "soluble" and therefore lost during tissue processing, whereas others would be "insoluble" and would survive histological procedures(13).

The present results with thyroxine are most compatible with this proposed mechanism of fixation and immobilization of "free" amino acids(13). Thyroxine contains an alanine side chain, or amino group. It was found that thyroxine was associated with a fraction of large or high molecular weight "soluble" molecules after glutaraldehyde fixation that were lost during processing, i.e., the alcohol dehydration. This fraction removed in alcohol accounted for no more than 5% of the radioactivity in the cell retained after fixation, dehydration and embedding at 22 hr. The larger retained fraction (95%) would be equivalent to hormone fixed to insoluble tissue sites (*vide supra*). If the alanine group alone were being fixed by glutaraldehyde, due to some cleavage of the thyroxine molecule in the cell, no radioactivity would be demonstrable in the present studies since ^{125}I is attached to the ring structures of the thyroxine molecule.

Translocation of steroid hormones apparently occurs during liquid fixation and dehydration since these hormones can be shown in the embedding material(2). No such observations were made in the present experiments with thyroxine. Estradiol is not fixed in the cell by glutaraldehyde(14) and it is pertinent to recall that this steroid hormone has no free amino group. Peters and Ashley have shown that displacement or diffusion of molecules containing amino

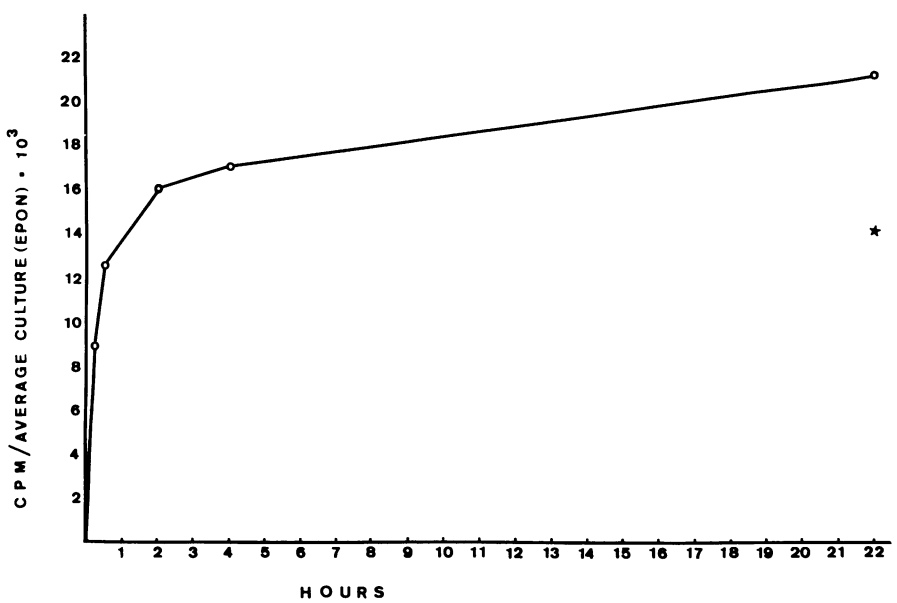

FIG. 6. Amount of radioactive hormone retained in cultures embedded in Epon with increasing time after hormone administration. Osmium fixation not preceded by glutaraldehyde (*) at 22 hr permits less retention of hormone.

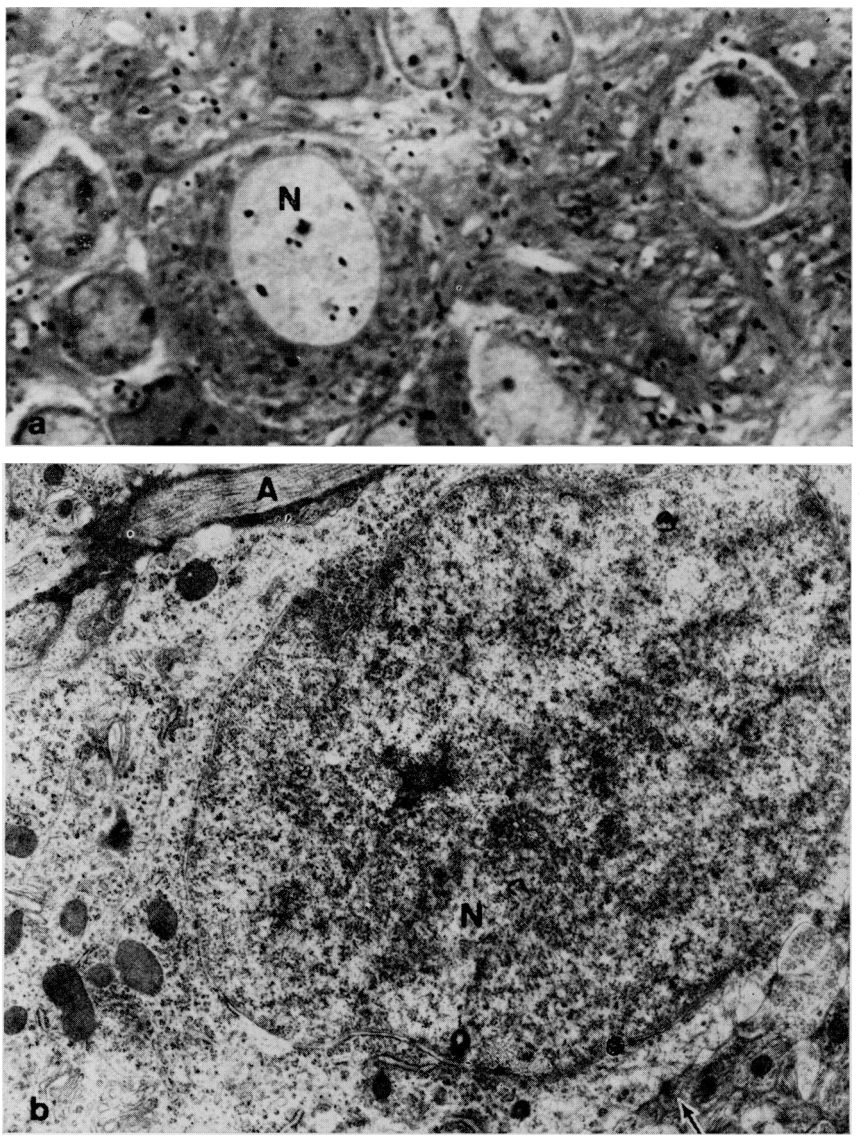

FIG. 7a. Spinal cord. Grains are seen in the nucleus of a neuron (N) by light microscopy. Exposed to thyroxine ^{125}I for 2 hr. About ×2000.

FIG. 7b. Spinal cord. Three grains are seen in the nucleus of a neuron (N) as early as 30 min after exposure to thyroxine ^{125}I. Arrow points to synapse on this cell's soma. A neuritic process showing early myelin formation is seen (A). About ×9200.

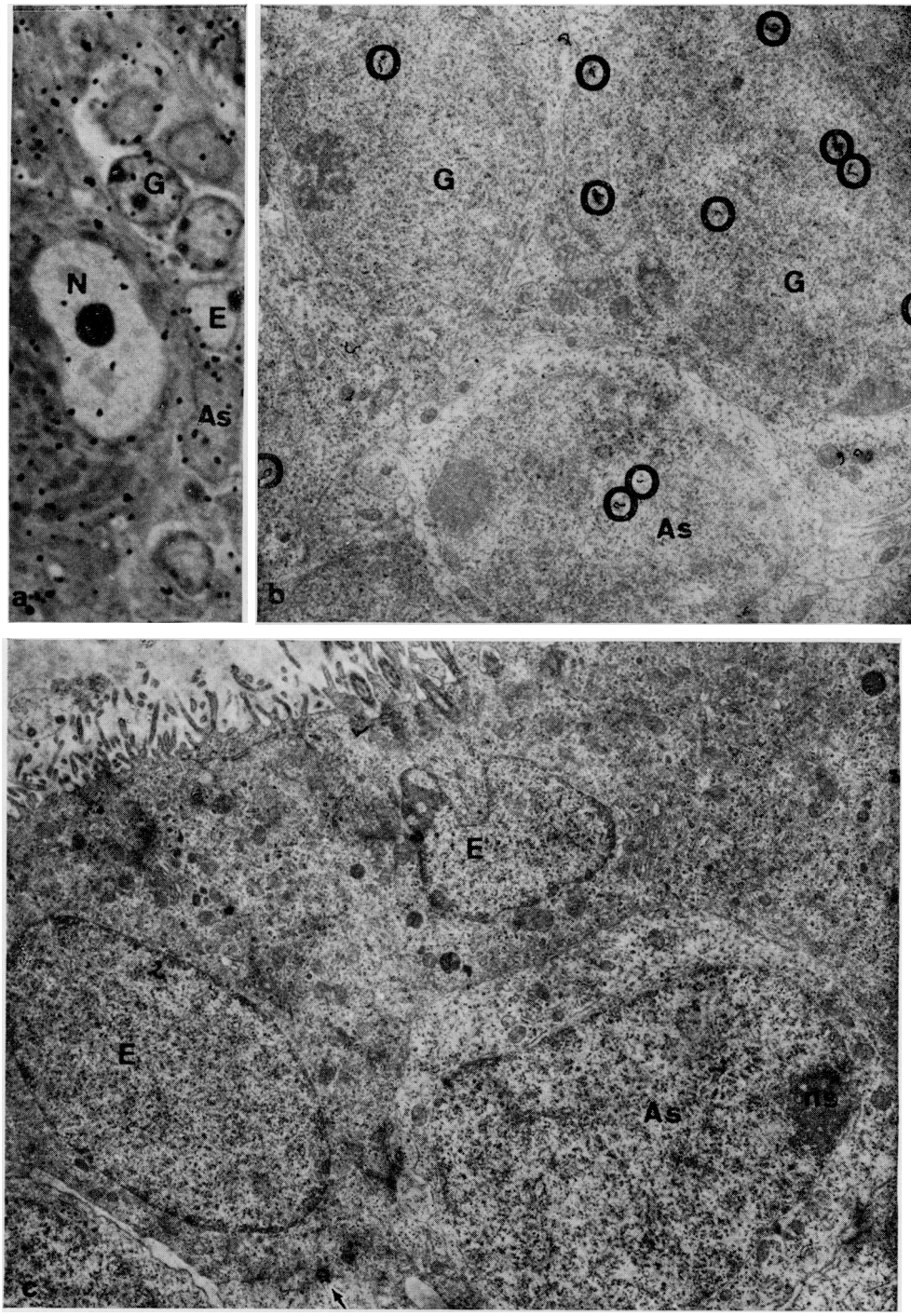

FIG. 8a. Cerebellum. A culture exposed for 4 hr to thyroxine ^{125}I shows nuclear grains over a large neuron (N), a granule cell (G), and an astrocyte (As). Note the grains on the nuclear membrane of what is probably an ependymal cell (E). About ×1650.

FIG. 8b. Cerebellum. Culture exposed to thyroxine ^{125}I for 22 hr. Numerous grains are seen over the nuclei (circled) of granule cells (G) and an astrocyte (As). Cytoplasmic grains are over mitochondria and ribosomes or rough endoplasmic reticulum. About ×7300.

FIG. 8c. Cerebellum. A culture exposed for 2 hr shows grains on or near the nuclear membrane of two ependymal cells (E) as in Fig. (8a) above. Grains are also seen over the nucleus and nucleolus (ns) of an astrocyte (As), and over a cell membrane (arrow). Projecting cilia are seen and no grains are present in the Epon (top left). About ×6900.

groups is not a problem for at least several cell layers with glutaraldehyde in tissue slices(13). As well, in the cultures used, which were several layers deep, no difference was seen in localization and amount of grains in cells at the top of the culture and those at the bottom, also suggesting that diffusion was not an important factor.

If thyroxine were being fixed by glutaraldehyde to a nearby or coincidental protein it is possible that some movement or displacement within the cell might occur. However, in view of the enormous number of cellular proteins this displacement would probably be small, and would be well below the resolution of electron microscopic autoradiography, e.g., displacement within a mitochondrion is immaterial if one can only localize a grain to the entire organelle(12). Freezing methods, sometimes employed for the localization of soluble compounds might offer an alternative control for the present studies on thyroxine. However, problems of cellular distortions secondary to ice crystal formation and migration of small molecules during freeze–drying have been raised(15); these displacements in fact might be far larger than the molecular displacements presumed to occur during glutaraldehyde fixation.

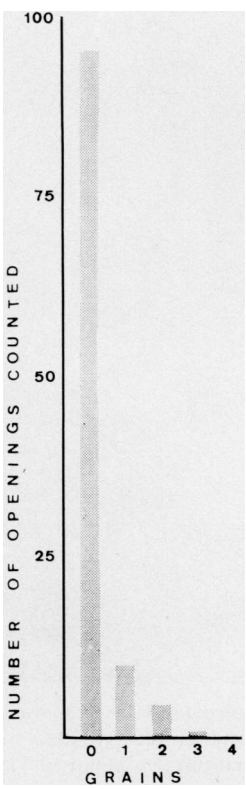

FIG. 9. The number of grains per grid opening over Epon adjacent to tissue, by electron microscopy, is plotted against the total number of grid openings counted. Usually more than 15 cells may be seen in a single opening.

Although after glutaraldehyde fixation, thyroxine is "bound" to larger molecular weight substances, some of which may be proteins, it is not known whether thyroxine is truly bound to these higher molecular weight substances, or even loosely associated with them in the living state. However, no "binding" of thyroxine occurred if it were not first taken up by the cell prior to fixation. It is of interest that more radioactivity per culture was extracted in glutaraldehyde solution after short (15/30 min) exposures to thyroxine ^{125}I than with longer exposures (22 hr). Furthermore after fixation, considerably more hormone was retained at insoluble tissue sites with the long exposures. This might suggest that these tissue sites were physiologically related to thyroxine. In this regard it is pertinent to note that "tight" binding of thyroxine to certain cell components, e.g., mitochondria, has been shown to occur in the absence of fixative(16), and in developing brain all subcellular components were found to actively take up and bind thyroxine, whereas iodide was confined predominantly to the soluble, nonparticulate fraction(17). More recently, concomitant with amphibian metamorphosis, an "intense capacity to bind thyroid hormones" by living cells has also been described(18).

In conclusion, iodine, free thyroxine, or thyroxine associated with large soluble molecules cannot be localized with the described methods; some of these, e.g., free iodine may be degradation products. These methods, however, can demonstrate a percentage of the thyroxine in the cell, that which is associated with nonextractable tissue sites, and the localization of this component to the various organelles or cell sites can be effectively investigated.

SUMMARY

Mammalian tissue cultures of spinal cord and cerebellum were exposed to thyroxine ^{125}I for 15 min to 22 hr. All solutions used for fixation, dehydration and embedding were counted, and appropriate ones were analyzed by G-25 Sephadex and thin-layer chromatography. Most of the radioactivity removed during fixation was found in the glutaraldehyde solution and this material was apparently sequestered labeled feeding medium. Hormone fixed in the cell by glutaraldehyde increased dramatically within the first 2 hr and by 22 hr this fraction accounted for 25% of the total radioactive hormone in the explant prior to fixation. No free ^{125}I was fixed by these methods, and thyroxine ^{125}I hormone could not be fixed in the cells if it were added to the fixative and not to living cells. None of the data suggested incomplete extraction of soluble components. Less hormone was fixed in the cell with osmium fixation not preceded by glutaraldehyde. The possibility of "translocation" of hormone was discussed. In addition, localization of grains was essentially the same by electron microscopic autoradiography as by light microscopic autoradiography; it was concluded that Compton scattering could not account for the nuclear grains noted with thyroxine by light microscopy.

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