

Studies with Electron Microscopic Autoradiography of Thyroxine ¹²⁵I in Organotypic Cultures of the CNS II. Sites of Cellular Localization of Thyroxine ¹²⁵I¹

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INTRODUCTION

Thyroxine, important in the metamorphosis of amphibians, as well as in the differentiation of mammalian tissues, exerts its effects on such diverse tissues as cartilage, liver and brain(1). Effects of thyroxine at the cellular level have been variously ascribed to its uncoupling of oxidative phosphorylation(2), its direct interaction with enzymes(3,4), and its stimulation of mitochondrial dependent protein synthesis(5); it has also been implicated in the transcription of both mDNA and rDNA(6-8), and it has been assumed to have a role in nuclear transport(9) and attachment of ribosomes to membranes(10).

Perhaps one of the most fascinating controversies centers on the "primary action" of thyroxine(5); Sokoloff *et al.*, have claimed that the mitochondria is the first and crucial focus of its effects; Tata has claimed the hormone's primary, albeit tardy thrust is in the nucleus(9). More specifically, Sokoloff has demonstrated that the early enhancement of protein synthesis by thyroxine (2 h) requires mitochondria(5), and he postulated that thyroxine may not even participate directly in the delayed response of increased nuclear RNA that occurs at 15 to 30 h. Tata on the other hand has considered the mitochondrial effects to be "toxic" or "catabolic" manifestations of high doses of the hormone, that may be entirely separable from its actions on growth and development(9,11).

The present experiments are concerned with the ultrastructural localization of thyroxine in the cell, which has not heretofore been described. More specifically, cells of the developing nervous system known to be responsive to thyroid hormones(12-14) were studied. The localization of thyroxine ¹²⁵I at early (15 min)

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and late (22 h) times after its administration might give some additional insight into the possible site or sites of its action with respect to the arguments presented above. Whether or not increased thyroxine accumulated in cells at different stages of morphological differentiation(15) was also studied. In previous studies the similarities of localization both of triiodothyronine ^{125}I and thyroxine ^{125}I in organotypic cultures of immature brain in different species of mammals has been demonstrated(16). Experiments with chase and nonchase solutions and with various methods of fixation have been recorded(16). The present methods of fixation and dehydration localize thyroxine ^{125}I associated with nonextractable cell sites, and these tissues after fixation retain no free iodine(17).

METHODS

Spinal cord-dorsal root ganglion combinations from 13-day mouse embryos were cultivated according to the method of Peterson, Crain and Murray(18) for 19 days before adding radioactive thyroxine; slices of cerebellum from 2-day-old newborn mice were cultured as described by Bornstein and Murray(19), and kept for 11 days *in vitro* before addition of thyroxine ^{125}I . Horse serum containing less thyroxine ($0.8 \mu\text{g}\%$) than other sera for CNS culture ($12 \mu\text{g}\%$) was utilized in order to make feeding media for both spinal cord and cerebellar cultures(19).

Immediately prior to use, L-thyroxine ^{125}I (sp act $69 \mu\text{C}/\mu\text{g}$) was dried, and added to feeding media as described(16,17) to give a final concentration of $3.5 \times 10^{-7} M$ thyroxine ^{125}I ; chromatography and G-25 Sephadex analysis of this media revealed that 90% of the radioactive hormone was bound to serum proteins, and 10% or $3.5 \times 10^{-8} M$ was present as free thyroxine ^{125}I . It should be noted here that the total concentration of thyroxine in the media was in the physiologic range as judged by three criteria: (a) it was less than two times the concentration of the usual feeding medium (*vide supra*), (b) it was significantly less than amounts used to uncouple oxidative phosphorylation in immature brain(20), and (c) it was significantly less than amounts used to hasten differentiated function in these tissue cultures(13).

Groups of spinal cord and cerebellar cultures fed with the media containing thyroxine ^{125}I were incubated for 15 and 30 min, 2, 4 and 22 h prior to fixation. At least three cultures were examined at every time interval. Cultures so incubated were also examined in the living state with a $\times 40$ oil immersion objective. Techniques of fixation and autoradiography have been described(15,17). The autoradiographic exposures of 8 weeks and processing in all cases were identical.

Analysis of grain localization was done from a large number of electron photomicrographs. Autoradiographic background was virtually zero(17). The localization of grains at the earlier times after administration, i.e., 15 and 30 min in cerebellar cultures and 15 min in spinal cord cultures was assessed qualitatively, since fewer than 100 grains per culture cross section were photographed. At least 100 grains per cross section were counted at other times after exposure to thyroxine ^{125}I . Counting was performed in two ways: (a) all organelles within a

1400 Å diameter from the center of the grain were counted. These results were expressed as frequency of distribution of grains for each time after exposure to thyroxine ¹²⁵I. The organelle with the greatest number of grains was assigned the number 100 and the frequency of grains over other organelles was expressed as a number of relative to this (for example, if the cell membranes were counted most frequently, e.g., 300 times, and the mitochondria were counted 100 times, the cell membrane would have a frequency of 100 and the mitochondria a relative frequency of 33); (b) grains were also counted with respect only to cell organelles on which they directly lay. This was calculated to give an absolute percent of grains over a given cell organelle at the different times after exposure to thyroxine ¹²⁵I. The total was slightly higher than 100% because some grains directly overlay two structures. Frequency and percent analysis closely correlated with each other. Increase or decrease of grains over certain structures with increasing time after exposure to thyroxine ¹²⁵I was noted only if they were at least 5% more or less than the composite value of grains for that structure, and also, if there were an increase or decrease of a frequency of 20 compared to the composite frequency. Spinal cord and cerebellar cultures were analyzed as separate groups.

RESULTS

Spinal cord cultures. Living spinal cord cultures exposed to thyroxine ¹²⁵I at all times showed no "toxic" changes(21) and appeared as others have described(18).

Light microscopic autoradiography revealed that the localization of grains at all times after exposure to hormone was the same; longer exposures to thyroxine produced only more grains at the same loci. This quantitative difference in grains was thought to reflect the increased binding of thyroxine by the cells with the longer exposures(17). As early as 15 min after thyroxine ¹²⁵I application, grains were seen over the nuclei of both neurons and glial cells. Some of these grains lay over the nuclear envelope; less frequently they were observed over the nucleolus. Cytoplasmic grains in general outnumbered nuclear grains by a ratio of about 3:1. Regions with a high density of cytoplasmic membranes such as the myelin sheath and myelin-neurite interface sometimes were layered with grains.

A slight predominance of grains over neurons as compared to glial cells by light microscopy was always seen. This did not shift with different exposure times. Two further observations were made: oligodendroglial cells closely associated with myelin sheaths rarely displayed central nuclear grains, and some neurons had as many nuclear as cytoplasmic grains.

Electron microscopic studies supported and amplified all the light microscopic findings noted above. The quantitative evaluation of grain distribution with regard to different cell organelles in spinal cord cultures is listed in Tables 1 and 2. Both percent and frequency analyses showed a high degree of correlation with respect to the magnitude of distribution over each organelle.

It can be seen both from percent (Table 1) and frequency (Table 2) analyses that the cell membrane had the greatest number of grains. After numerous observations it became apparent that more of these grains were localized on the membranes of small unmyelinated neuritic processes in the neuropil than on the perikaryal membranes of nerve or glial cells (Fig. 1).

The mitochondria showing the second order of labeling were distributed in all types of neuroepithelial cells. In these spinal cord cultures the number of mitochondrial grains increased above the average at 4 hr after exposure by both percent and frequency analyses. There was no correlation between mitochondrial swelling and the presence of grains over the mitochondria. In fact almost all labeled mitochondria had dense matrices and showed no swelling (see Fig. 2). "Free" ribosomes and "mixed" endoplasmic reticulum (membranes only partially

TABLE 1
SPINAL CORD: PERCENTAGE OF DISTRIBUTION OF GRAINS OVER DIFFERENT CELLULAR ORGANELLES.
AVERAGE AT ALL TIMES AFTER EXPOSURE TO THYROXINE ^{125}I OF ALL CELL TYPES

A. Structure	B. Average % of all cultures	C. >5% Change (hr)
Cell membrane	34	↓(2), ↑(22)
Mitochondria	22	↑(4)
Ribosomes-mixed ER	15.5	—
Nucleus	12	↑(4)
Synapse	11	↑(4)
Nuclear membrane	5	—
Tubules	5	—
Rough ER	2.5	—
Coated vesicles	1	—
Golgi	1	—
Nucleolus	1	—

TABLE 2
SPINAL CORD: FREQUENCY OF GRAINS RELATIVE TO THE CELL ORGANELLE WITH THE
HIGHEST GRAIN COUNT. AVERAGE AT ALL TIMES AFTER EXPOSURE TO
THYROXINE ^{125}I OF ALL CELL TYPES

A. Structure	B. Average frequency of all cultures	C. Change >20 (hr)
Cell membrane	100	—
Mitochondria	61.5	↑(4)
Ribosomes	52	↓(2), ↓(22)
Mixed ER	47	↓(22)
Nucleus	37	↑(4)
Synapse	36	↓(1/2), ↑(4)
Tubules	26	—
Nuclear membrane	13	—
Rough ER	8	—
Coated vesicles	4	—
Golgi	3.5	—
Nucleolus	1	—

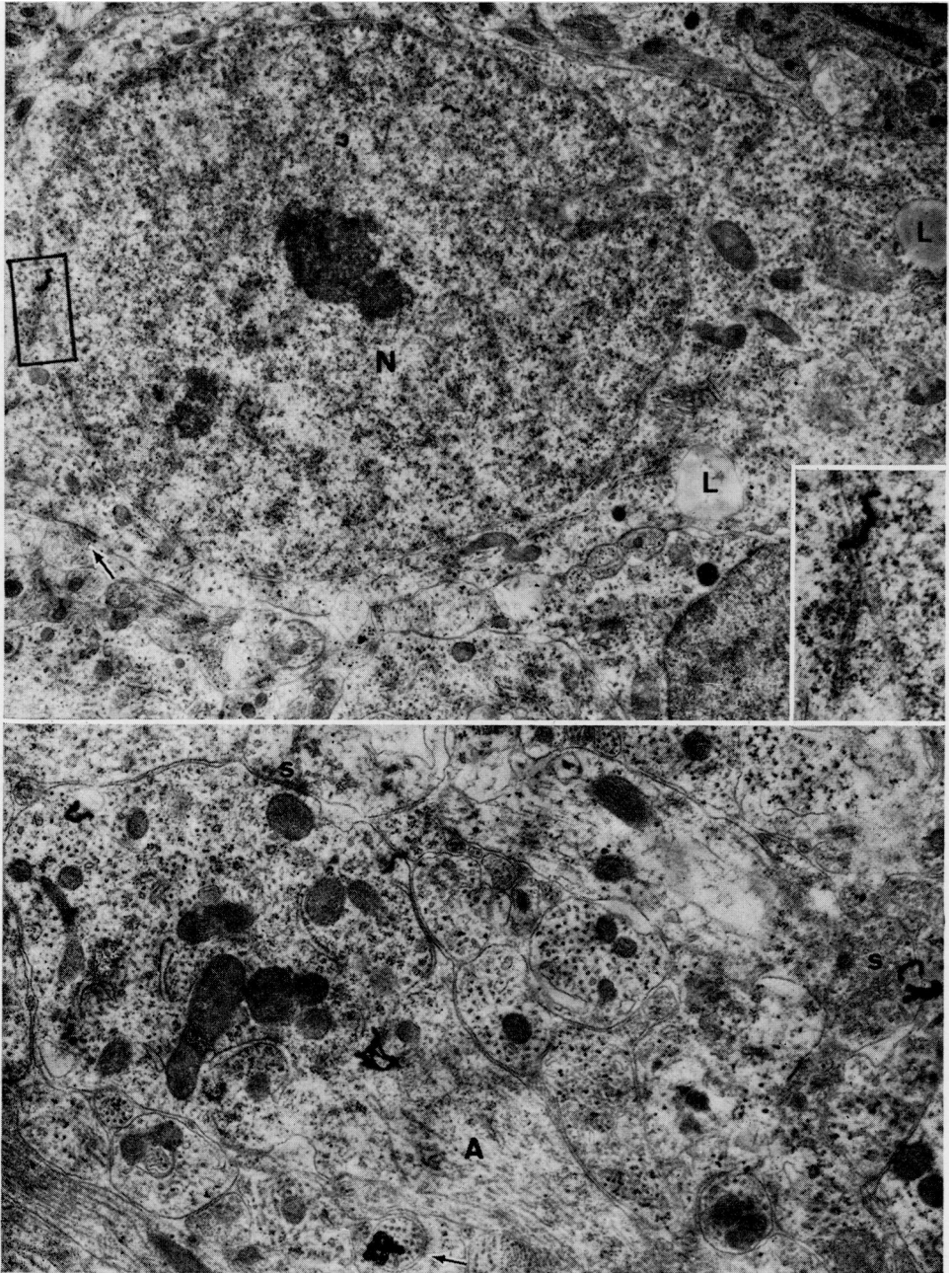


FIG. 1. Top: Spinal cord culture exposed to thyroxine ^{125}I for 30 min. Three grains are seen in the nucleus (N), and there are no cytoplasmic grains. Lipid droplets (L) are seen. Arrow points to the edge of a possible synapse ending on the cell soma. $\times 5,520$. Inset shows enlargement of grain on nuclear membrane in close proximity to offshoot of endoplasmic reticulum. $\times 20,700$. Bottom: A neural process (A) from the same culture, exposed for 30 min, exhibits cytoplasmic grains; grains are not seen over the tubules in this process. Cross section of a small neuritic process (arrow), and one region with synaptic vesicles (s) also display grains. $\times 15,860$.

studded with ribosomes) showed third order in both percent and frequency distribution of label, and were considered as one group since frequency analysis did not suggest any apparent difference between them (Table 2). All neuroepithelial cell types displayed ribosomal grains. No clear-cut increase or decrease in numbers of grains over "the ribosomes-mixed endoplasmic reticulum" occurred with different exposure times to thyroxine ^{125}I , since no consistent variations were found by both percent and frequency analyses (Tables 1C and 2C).

The nucleus accounted for an average of 12% of the total grains. Nuclear grains were observed by electron microscopy as early as 15 min after exposure to thyroxine ^{125}I . Sometimes in such cases nuclear grains were seen in the absence of cytoplasmic grains (Fig. 1). The nuclear grains reached a maximum at 4 hr both by percent and frequency analyses and were observed most frequently over neurons and "immature" protoplasmic astrocytes(15). Nuclear grains were also occasionally observed over fibrous astrocytes but were infrequently observed over "young" oligodendrocytes closely associated with myelin sheaths. Somewhat less than half of the nuclear grains were found on or near the nuclear envelope. Sometimes these grains on the nuclear envelope were close to offshoots of endoplasmic reticulum (Fig. 1).

Grains over synapses were observed as often as nuclear grains (Tables 1 and 2). These grains were seen over both the synaptic vesicles and the mitochondria. Grains were occasionally seen over dense core vesicles close to synaptic regions as well as over the synaptic cleft, (see Fig. 2). There was an increase in synaptic grains above the average by both percent and frequency analyses at 4 hr.

The remaining structures listed in Tables 1 and 2 were labeled occasionally or infrequently and showed no real fluxes with increasing time of exposure to thyroxine ^{125}I . Of these structures, the high frequency of grains over the tubules is due in part to the overlap of grains over these structures and the plasma membranes of small neuritic processes. Tubules in the center of larger neuritic processes were rarely labeled (Fig. 1). It should be noted that well developed and extensive rough endoplasmic reticulum as was characteristically seen in neurons rarely displayed grains. Grains were not seen on or near coated vesicles at the cell membrane, but were occasionally observed on or near coated vesicles in the Golgi region.

The nucleolus was an infrequent site of grains (Tables 1B and 2B). This result might be somewhat misleading since the number of nucleoli sampled was by necessity small; only neurons and some developing glial cells have nucleoli, and furthermore relatively few sections through the cell nucleus would include this structure.

One spinal cord culture exposed to thyroxine ^{125}I for 22 hr, fixed with osmium but not preceded by glutaraldehyde fixation(17), showed the same localization over different cell organelles as listed in Tables 1 and 2. However, the nuclear grains were as numerous as the grains over the mitochondria and ribosomes-mixed ER. This increase in percentage of nuclear grains in the osmium-fixed culture was accompanied by slight decrease in the percentage of grains seen over the cell membrane and mitochondria.

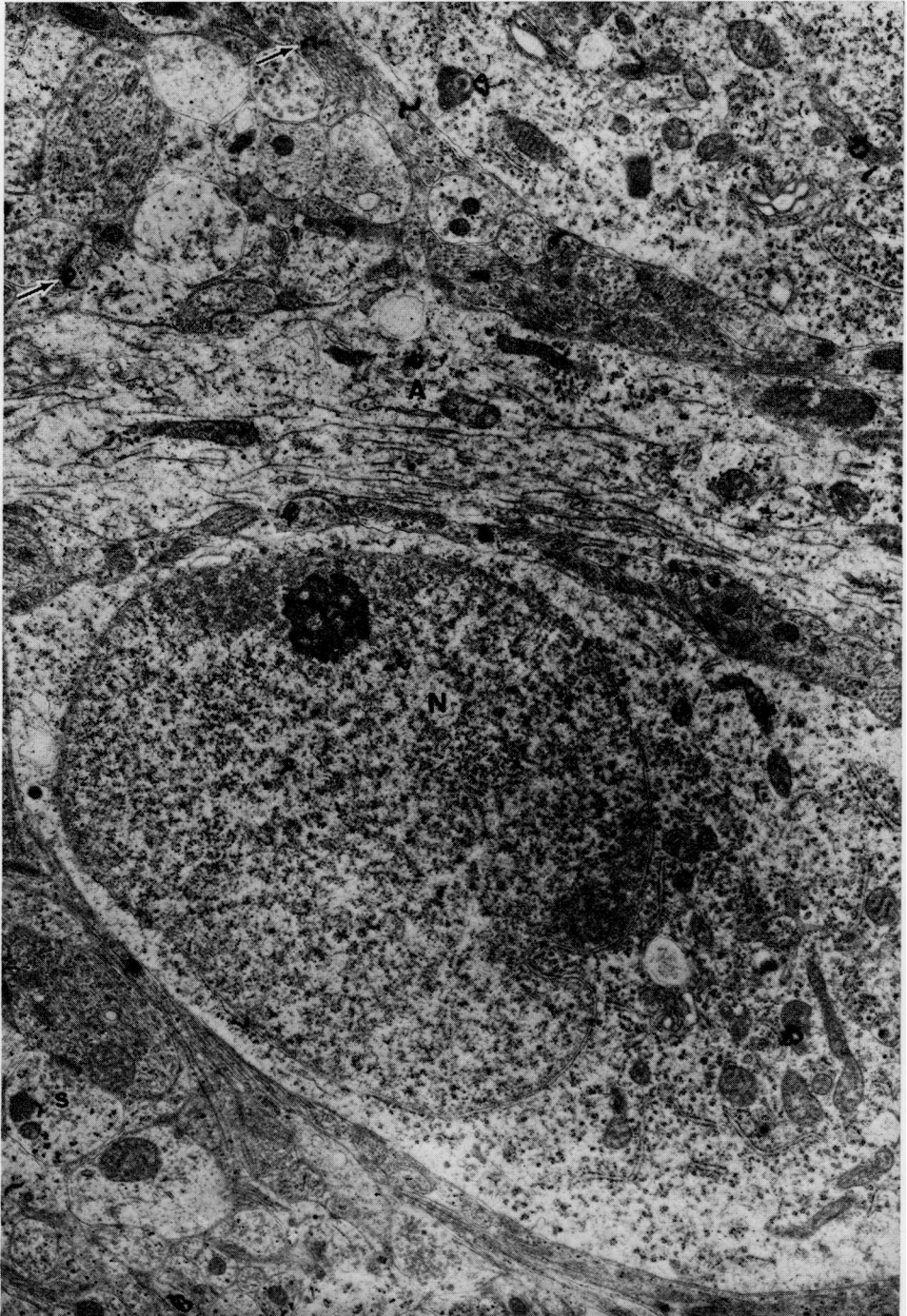


FIG. 2. A typical field from a spinal cord culture exposed to thyroxine ^{125}I for 4 hr. Grain over membranes partially studded with ribosomes in large neural process (A) is seen. Arrows point to grains over synaptic clefts, and grain is seen over presynaptic bouton (s). Many grains in field are over cell membranes, mitochondria and nucleus, but not over rough endoplasmic reticulum or Golgi apparatus; labeled mitochondria are not swollen. $\times 10,940$.

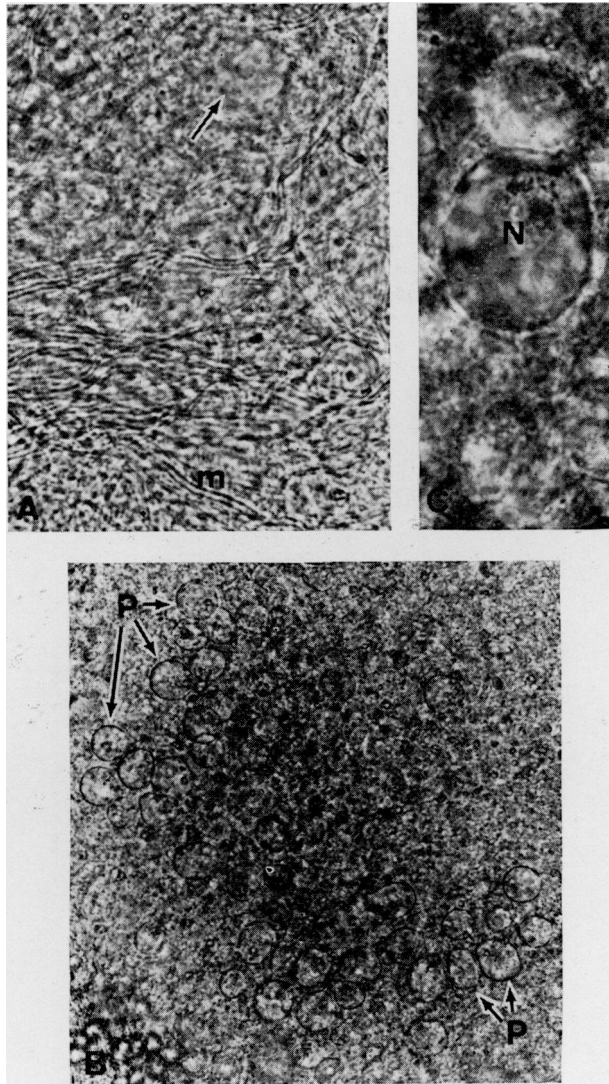


FIG. 3. A: Cerebellar culture, in the living state, after exposure to thyroxine ^{125}I for 30 min. Normal myelin (m) is seen criss-crossing the field. Arrow points to the nucleus of a Purkinje cell; note central nucleolus. The cytoplasmic membrane cannot be visualized. $\times 600$. B: After 2 hr of exposure same cerebellar culture shows marked swelling of Purkinje cells (P) that accentuate their organotypic arrangement; the cytoplasmic membranes are apparent even at lower magnification (arrows). $\times 150$. C: Higher magnification of Purkinje cell showing swelling of cytoplasm, but not of nucleus (N). $\times 800$.

Cerebellum cultures. Living cerebellar cultures exposed to thyroxine ^{125}I and examined in the living state at 30 and 45 min showed no degenerative changes; all morphological elements appeared as they had prior to exposure (Fig. 3A). However, at 2 hr after exposure a dramatic swelling of Purkinje cells was noted (Fig. 3B and C). This change was not accompanied by any changes in myelin or glial cells. No reversal of this swelling of Purkinje cells was noted at later times after hormone exposure. By 22 hr a few pyknotic glial or granule cell nuclei were also seen in the region of the swollen Purkinje cells but myelin and outgrowth of the explant were still normal at this time.

Light microscopy of thick sections, however, showed no changes in the morphology of any cells (including Purkinje cells) in the culture at any time after exposure to thyroxine ^{125}I . Interestingly, the distribution of grains was indistinguishable from that described by light microscopy for spinal cord cultures at all times after exposure to thyroxine ^{125}I . Indeed, nuclear grains were observed in increased numbers over some Purkinje cells with longer exposures (22 hr) to thyroxine ^{125}I .

In regard to "toxicity" observed in the living state in Purkinje cells, several pertinent electron microscopic observations can be made. First no ultrastructural changes were evident in any cells at 15 and 30 min and 2 or 4 hr after exposure to thyroxine ^{125}I . Mitochondria had dense matrices and still showed no swelling even at 4 hr after exposure (Fig. 4). At 22 hr after exposure however, some of the Purkinje cells had large swollen mitochondria that appeared to be degenerating (Fig. 5), but only some of these swollen mitochondria displayed grains. Other cell organelles in the affected Purkinje cells did not show obvious changes. Neurons other than Purkinje cells had normal ultrastructural features, and mitochondria although frequently labeled, showed no swelling (Fig. 5). Glial cells also revealed no toxic changes ultrastructurally. Because these mitochondrial changes in Purkinje cells occurred late, it is likely that they represent secondary changes to the cytoplasmic swelling evoked by thyroxine much earlier, and observed in the living state at 2 hr.

The localization of grains by frequency and percent analyses for the entire group of cerebellar cultures was similar to that observed in cord-ganglia cultures except that nuclear grains were more frequent in the cerebellar cultures (Tables 3 and 4). The mitochondrial grain concentration was decreased by both percent and frequency analyses (Tables 3C and 4C) at the time of swelling in the living state (i.e., 2 hr), but this apparently had no effect on the increase in nuclear grains seen at 4 hr which are comparable to that seen at 4 hr in spinal cord cultures. Furthermore, in the cerebellar cultures there was a rise in the number of synaptic grains at 4 hr both by percent and frequency distribution, although it was less marked than the rise seen in spinal cord cultures. This rise in nuclear and synaptic grains at 4 hr might suggest that uptake in these structures occurred independently of that of the mitochondria which showed a decrease.

As in the spinal cord cultures, grains were present over all types of cells. "Immature" protoplasmic astrocytes or "inactive" astrocytes(15), as well as granule cell neurons, showed the greatest concentration of nuclear grains. The localiza-

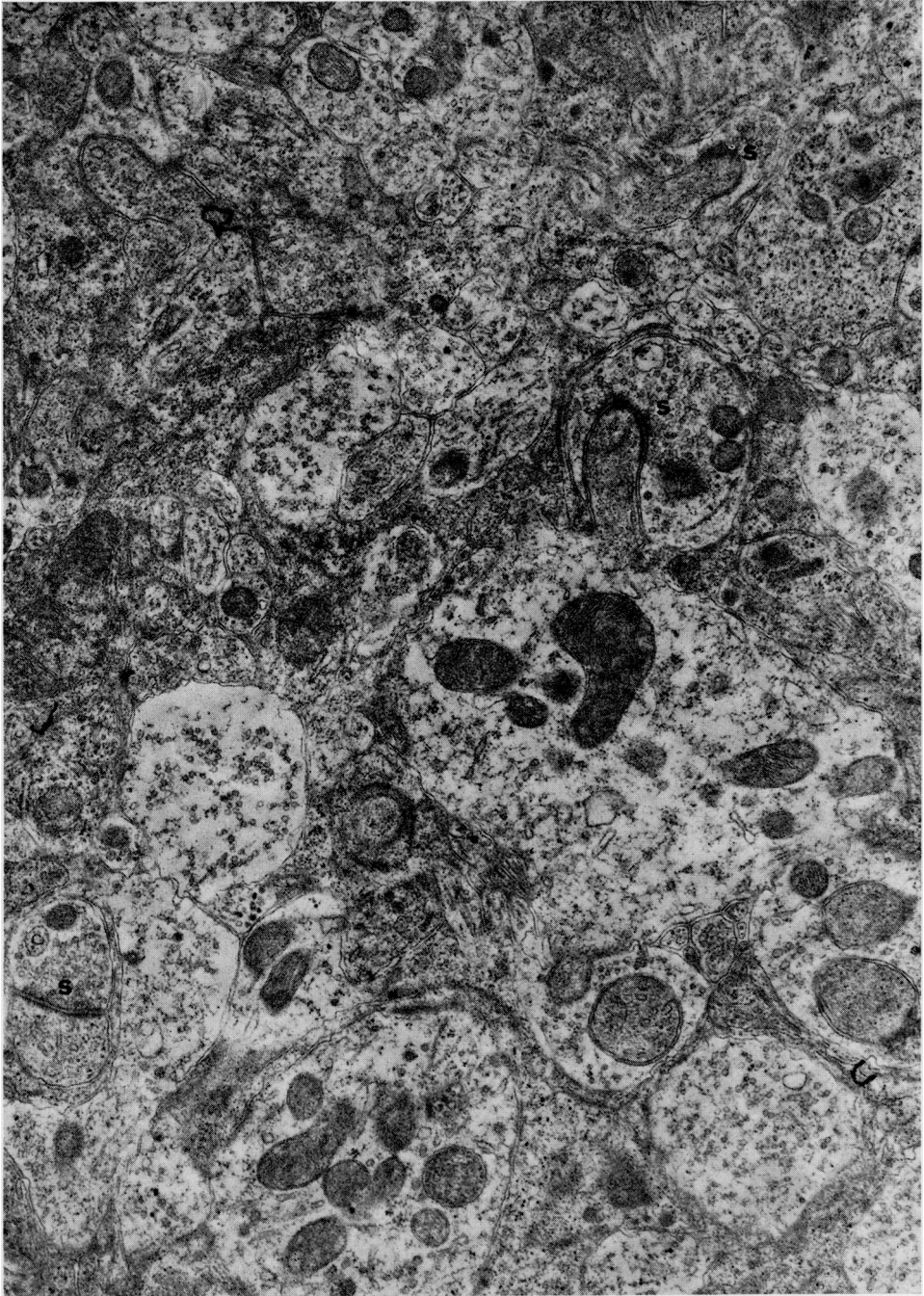


FIG. 4. Typical field from a cerebellar culture exposed to thyroxine ^{125}I for 4 hr. Mitochondria in the neuropil have dense cores and show no swelling. Three grains are seen over cytoplasmic membranes. Several synapses (s) are seen; two of these end on dendritic spines. $\times 19,920$.

tion of cytoplasmic grains in different glial cell types was similar with the exception that in the ependymal cells grains more frequently overlay the Golgi apparatus which in other cells was infrequently labeled. It might also be noted here that no cilia, containing a typical array of tubules, were labeled. Grains over membranes of a developing myelin sheath are seen in Fig. 6.

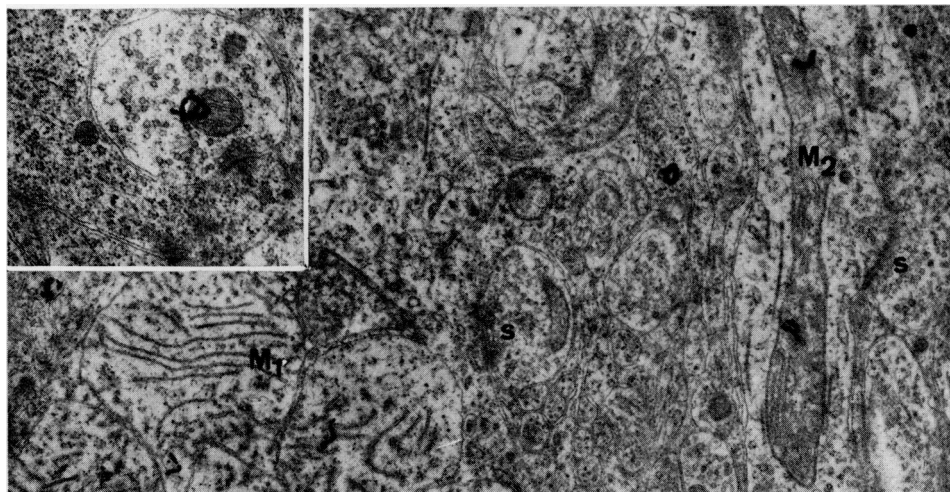


FIG. 5. Cerebellar culture exposed to thyroxine ¹²⁵I for 22 hr. Several markedly swollen mitochondria (M₁) are seen, grains are present over two of these. This cell receives a synapse (s) nearby. Another mitochondrion (M₂) displays three grains and shows no swelling although also in a neural process and innervated by a synapse (s). $\times 16,100$. Inset shows labeled mitochondrion with no swelling in a synaptic bouton ending on a dendritic thorn. Also exposed for 22 hr. $\times 19,800$.

TABLE 3

CEREBELLUM. PERCENTAGE OF DISTRIBUTION OF GRAINS OVER DIFFERENT CELLULAR ORGANELLES. AVERAGE AT ALL TIMES AFTER EXPOSURE TO THYROXINE ¹²⁵I OF ALL CELL TYPES

A. Structure	B. Average % of all cultures	C. >5% Change (hr)
Cell membrane	33	↑(2) ↓(4)
Mitochondria	19	↓(2)
Ribosomes-mixed ER	19	—
Nucleus	15.5	↑(4)
Synapse	7	—
Nuclear membrane	4	—
Tubules	2	—
Golgi	2	—
Rough ER	1.5	—
Nucleolus	1	—
Coated vesicles	1	—

DISCUSSION

The present autoradiographic results suggest that thyroxine is bound by the cell at multiple sites including such diverse structures as the cell membrane, mitochondria, ribosomes, synapse and nucleus. This distribution is entirely consistent with biochemical assays of thyroxine localization in different experimental arrangements, e.g., subcellular fractionations. Three hours or more after an injection of thyroxine ^{131}I or thyroxine ^{14}C to rats, the hormone was found in all liver cell fractions including the nucleus, ribosomes and mitochondria(22). In studies of the developing CNS(23), thyroxine ^{131}I was evenly distributed in particles

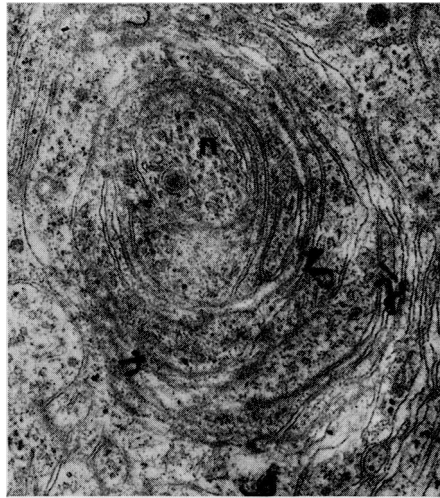


FIG. 6. Cerebellar culture exposed to thyroxine ^{125}I for 22 hr. Grains are seen over membranes of a developing myelin sheath that surrounds a neuritic process (n). $\times 19,800$.

TABLE 4

CEREBELLUM. FREQUENCY OF GRAINS RELATIVE TO THE CELL ORGANELLE WITH THE HIGHEST GRAIN COUNT. AVERAGE AT ALL TIMES AFTER EXPOSURE TO THYROXINE ^{125}I OF ALL CELL TYPES

A. Structure	B. Average frequency of all cultures	C. Change >20 (hr) ,
Cell membrane	100	—
Mitochondria	63	↓(2) ↑(4)
Ribosomes	64	—
Mixed ER	60	↓(22)
Nucleus	55	↑(4)
Synapse	22	—
Nuclear membrane	17	—
Tubules	6.5	—
Golgi	6.5	—
Rough ER	3	—
Nucleolus	2	—
Coated vesicles	2	—

sedimenting at 800, 20,000 and 100,000g as well as in the particle-free sediment 4 hr after injection. Since thyroxine is found in several cellular organelles, the question arises whether it acts on all, or only one of these sites(24). To that end, we discuss the present results and that of other workers.

Two lines of evidence favor an action at the cell membrane in addition to absorption prior to entry. First, the present experiments demonstrate an accumulation of hormone at this site with increasing time. Second, after thyroxine administration, altered functions associated with the cell membrane have been described by others. There is, for example, some evidence that thyroxine influences ion distribution in the CNS(25), and it has been suggested in other epithelial cells, that thyroxine may have an early effect on cell permeability(24,26). Indeed, in the present investigation the Purkinje cells showed acute swelling with no concomitant mitochondrial swelling; the concentration of thyroxine used in our studies was well below that needed to uncouple oxidative phosphorylation (toxic effect) *in vitro* in mitochondria from immature brain(20). Thus this swelling of Purkinje cells seen here might represent permeability changes occurring via the cell membrane. Although thyroxine-induced swelling of mammalian Purkinje cells may not be an entirely physiological effect, that is, it is exaggerated in tissue culture but does not occur *in vivo*, it is worth noting that there is thyroxine-induced involution of specific neurons (Mauthner's neurons) in amphibian metamorphosis(27). Such involution might be based in part on such an action of thyroxine at the cell membrane in sensitive cells as observed here.

Furthermore, thyroxine may act on the cell membrane in some cases in the developing nervous tissue. Thyroidectomy of newborn rats has been shown to result in a diminished growth of the axodendritic neuropil of the cerebral cortex(12), and as well the aborizations of cerebellar Purkinje cells, under similar conditions, are permanently dwarfed(14). In this context, the frequent observation of grains in the neuropil, over the membranes of small neuritic processes is of interest. Furthermore, in organotypic cerebellar cultures, increased thyroxine in the feeding medium has been reported to speed the onset of myelination(13). It is then of some interest that in the present studies grains were seen over developing myelin sheaths (Fig. 6).

The presence of grains at the synapse leads to the speculation that increased or decreased levels of thyroxine at that site might cause neurological disturbances. Thus, the clinical observations that Parkinsonian tremors are increased in hyperthyroidism(28), and that delayed and sluggish reflexes are associated with myxedema, are recalled. In fact there is some electrophysiological evidence that thyroxine alters synaptic transmission(29) and it has been suggested that thyroxine modulates central catecholamine synapses(30), but further investigation of the effect of thyroxine at the synaptic locus is needed.

Mitochondria have been implicated in the action of thyroxine by several workers, in such capacities as protein synthesis(1,5,31), oxidative phosphorylation(2) or respiratory control(32), and more recently in mitochondrial turnover(33). In the present experiments there was obvious localization of hormone

to the mitochondria with doses in the physiological range (see Methods). Furthermore, there were no toxic effects of this hormone on the spinal cord cultures; cerebellar cultures showed only alterations in Purkinje cell neurons (*vide supra*).

Thyroxine has been shown to increase protein synthesis in a mixture containing ribosomes with no mitochondria(34,35), and a 100-fold amplification of ribosomal translation has been reported in thyroxine-treated tadpoles(36). In addition, as thyroxine induced amphibian metamorphosis progresses, there is increasing ribosomal attachment to membranes(10). In this context the observation of grains over free ribosomes, and over membranes only partially studded with ribosomes is of interest. Abundant well developed rough endoplasmic reticulum, as was often seen in large neurons, contained little label.

In the present studies the accumulation of thyroxine ^{125}I was not obviously dependent on a previous sequential accumulation at one of the other cellular sites; there was a concomitant thyroxine accumulation at all sites. Again, biochemical studies would be entirely compatible with this observation since the thyroxine stimulated amino acid incorporating activity of isolated mitochondria is elevated simultaneously with, but independent of, that of the ribosomes(37).

In the present investigation the localization of thyroid hormone in the nucleus of cells as early as 15 min after its administration leads away from the notion that the nuclear effects of thyroxine such as increased nuclear RNA(8) are entirely secondary to its actions elsewhere(5). Indeed thyroxine has been considered to have a role in transcription of the genes(6,38). In an evolutionary context, and also in view of the fact that in different cells of the same species thyroxine is associated with increased levels of vastly different enzymes(1,39), it would be difficult to envision that thyroxine could itself recognize specific gene sequences. More likely in some way it "amplifies" or "stabilizes" the genetic message being read at that time. In the nervous system thyroxine affects the level of certain enzymes(39), and this finding might be predicated upon such a "genetic" role for the hormone. Furthermore, lack of thyroxine in immature rats temporarily leads to an abnormal migration of the granule cells of the cerebellum(14); similar but permanent abnormal migration of granule cells has also been described as a genetic variant(40). The present frequent observation of grains in the nuclei of developing granule cells might be in keeping with the notion that thyroxine influences the expression of such a genetic factor. Although it is possible that thyroxine in the nucleus might be associated with a replicative rather than a transcriptional role, low and normal thyroid hormone concentrations in cerebellar cultures did not lead to an appreciable difference in mitotic activity(41).

In the present study there were few obvious differences in localization of thyroxine in different neuroepithelial cells. In previous experiments(16) more grains were found over nucleoli under circumstances in which the tissue cultures had been exposed to thyroxine at about a week earlier *in vitro*, and this change in timing might explain the difference. In the present studies the cerebellar cells showed a higher frequency of nuclear grains than the spinal cord cells, and this might suggest some developmental differences with respect to uptake or binding of thyroxine by the nucleus. Furthermore, nuclear grains were very rare in

oligodendroglia cells closely associated with myelin sheaths. It is also of interest to note that in spinal cord cultures, glial cells actively synthesizing DNA did not show increased cytoplasmic uptake of ^{125}I thyroxine(42).

It has been reported by others that the increase in nuclear RNA stimulated by thyroxine is delayed, and occurs at 12–15 hr after hormone administration(7,37). By comparison, the increase in nuclear RNA stimulated by another hormone, estradiol, occurs at 1 hr(37). In our experiments thyroxine accumulates in the nucleus as early as 15 min after administration, thus, if thyroxine acts in the nucleus to enhance certain genetic messages, the delayed increase of nuclear RNA under its aegis must be explained. It is possible that a significant increase in nuclear RNA might in part be dependent on the action of thyroxine at other loci in the cell. To this end the following action of thyroxine on the cell is tentatively proposed. As schematically depicted in Fig. 7, under the influence of thyroxine (*) the mitochondrial population M might “turnover”(33) to M' and thus give rise to a molecule *k* which would enhance genetic message B being produced by the nucleus (N) at that time. Additionally the ribosomes (R and MER), influenced by thyroxine at that as well as other sites, might also produce more of molecule *b* which would also stimulate an increase in nuclear RNA. Furthermore, such nuclear functions might be increased only by the accumulation of both *b* and *k* together in the nucleus with thyroxine. In such a way the nuclear effect might be vastly influenced by the state of the mitochondria and ribosomes at that time. Furthermore, the sequential genetic message being read in the nucleus would again be translated on the ribosomes. Thus, the whole sequence of events could be repeated and amplified, if the nuclear message read on the ribosomes was a permissive one. In such a scheme the specificity of thyroxine in different cells would be dependent on the interaction of its effects at

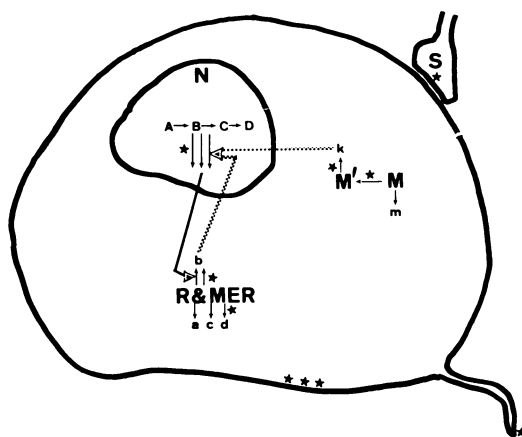


FIG. 7. A tentative model for the sites at which thyroxine may act in the developing CNS. Key: Site of action of thyroxine: (*); S: synapse; M: mitochondria; M': newly synthesized mitochondria; k, m: molecules produced by the mitochondria; R and MER: ribosomes and mixed endoplasmic reticulum producing molecules a,b,c,d; N: nucleus where sequential genes A,B,C,D are being transcribed.

several loci, and the degree of differentiation of each of these loci. Both of these factors are probably necessary to explain the effect of thyroxine on the differentiation of the nervous system at one time and that time only. Furthermore, this model, where the influence of thyroxine on cytoplasmic organelles subsequently influences its action in the nucleus, might help to explain the lack of effect of thyroxine on isolated chromatin or nuclear preparations *in vitro*(6).

ADDENDUM

While this paper was in press, M. Griswold and P. Cohen (*J. Biol. Chem.* **247**, 353–359 (1972)) reported an increase in RNA polymerase in thyroxine-treated tadpoles, but not in isolated nuclei, and they postulated that “thyroxine has an effect at some other level of cellular function before RNA polymerase activity is stimulated.” However, unlike the above hypothesis, they suggested only an initial extranuclear role for thyroxine.

SUMMARY

Organotypic cultures of developing spinal cord and cerebellum were exposed to thyroxine ^{125}I for 15 min to 22 hr. Electron microscopic autoradiography revealed that the major sites of hormone binding were the cell membrane, mitochondria, ribosomes—“mixed” endoplasmic reticulum, nucleus and synapse in that order of decreasing intensity. These organelles were all labeled as early as 15 min after hormone exposure, and there was increasing accumulation of hormone at all these sites with longer exposures. Both glial cells and neurons were labeled at all times. The present evidence is used to support the notion that thyroxine acts at multiple cell sites.

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REFERENCES

1. Rall, J. E., Robbins, J. and Lewallen, C. G. The thyroid. In *The Hormones* (G. Pincus, K. Thimann, and E. Astwood, Eds.), Vol. 5, p. 159–439. Academic Press, New York, 1964.
2. Hoch, F. L. and Lipmann, F. The uncoupling of respiration and phosphorylation by thyroid hormones. *Proc. Nat. Acad. Sci. U.S.A.* **40**, 909–921 (1954).
3. Litwack, G. Interactions of thyroid hormone and enzyme systems *in vitro*. In *Actions of Hormones on Molecular Processes* (G. Litwack and D. Kritchevsky, Eds.), p. 132–153. John Wiley and Sons, New York, 1964.
4. Snyder, L. and Reddy, W. Mechanism of action of thyroid hormones on erythrocyte 2,3-diphosphoglyceric acid synthesis. *J. Clin. Invest.* **49**, 1993–1998 (1970).

5. Sokoloff, L., Roberts, P., Januska, M. and Kline, J. Mechanisms of stimulation of protein synthesis by thyroid hormones *in vivo*. *Proc. Nat. Acad. Sci. U.S.A.* **60**, 652-659 (1968).
6. Cohen, P. Biochemical differentiation during amphibian metamorphosis. *Science* **168**, 533-543 (1970).
7. Eaton, J. and Frieden, E. Molecular changes during anuran metamorphosis; early effects of triiodothyronine on nucleotide and RNA metabolism in the bullfrog tadpole liver. In *Molecular Basis of Endocrinology*, Gunma Symposia on Endocrinology (N. Ui, Ed.), Vol. 5, p. 43. Tokyo Press, Japan, 1968.
8. Tata, J. R. Hormonal control of metamorphosis. In *Control Processes in Multicellular Organisms*, A Ciba Symposium, edited by (G. Wolstenholme and J. Knight, Eds.), p. 131-150. J. and A. Churchill, London, 1970.
9. Tata, J. R. Ribosomes and thyroid hormones. In *Wirkungsmechanismen Der Hormone*, 18. Colloquium der Gesellschaft für Physiologische Chem (P. Karlson, Ed.), p. 87-103. Springer-Verlag, Berlin, 1967.
10. Tata, J. The formation, distribution and function of ribosomes and microsomal membranes during induced amphibian metamorphosis. *Biochem. J.* **105**, 783-801 (1967).
11. Tata, J. Biological action of thyroid hormones at the cellular and molecular levels. In *Actions of Hormones on Molecular Processes* (G. Litwack and D. Kritchevsky, Eds.), p. 58-131. John Wiley and Sons, New York, 1964.
12. Eayrs, J. Effects of thyroid hormones on brain differentiation. In *Brain-Thyroid Relationships* (M. Cameron and O'Connor, Eds.), p. 60-74. Little Brown, Boston, 1964.
13. Hamburgh, M. and Bunge, R. P. Evidence for a direct effect of thyroid hormone on maturation of nervous tissue grown *in vitro*. *Life Sci.* **3**, 1423-1430 (1962).
14. Legrand, J. Analyse de l'action morphogénétique des hormones thyroïdiennes sur le cervelet du jeune rat. *Arch. Anat. Micr. Morphol. Exp.* **56**, 205-244 (1967).
15. Manuelidis, L. and Manuelidis, E. E. An autoradiographic study of the proliferation and differentiation of glial cells *in vitro*. *Acta Neuropathol.* **18**, 193-213 (1971).
16. Manuelidis, L. and Bornstein, M. I-125 labelled thyroid hormones in cultured mammalian nerve tissue. *Z. Zellforsch.* **106**, 189-199 (1970).
17. Manuelidis, L. and Manuelidis, E. E. (1971). Studies with electron microscopic autoradiography of thyroxine ¹²⁵I in organotypic cultures of the CNS. I. Fixation of thyroxine ¹²⁵I. *Yale J. Biol. Med.* **45** (1972).
18. Peterson, E., Crain, S. and Murray, M. Differentiation and prolonged maintenance of bioelectrically active spinal cord cultures (rat, chick, human). *Z. Zellforsch.* **66**, 130-154 (1965).
19. Bornstein, M. B. and Murray, M. Serial observations on patterns of growth, myelin formation, maintenance and degeneration in cultures of newborn rat and kitten cerebellum. *J. Biophys. Biochem. Cytol.* **4**, 499-504 (1958).
20. Sokoloff, L. Action of thyroid hormones and cerebral development. *Amer. J. Dis. Child.* **114**, 498-506 (1967).
21. Bornstein, M. Tissue culture studies of structural and functional alterations of the nervous system related to the demyelinating disorders. In *The Central Nervous System; Int. Acad. Pathol. Monogr.* **9**, 71-86 (1968).
22. Lipner, H., Barker, S. and Winnick, T. The distribution of thyroxine in rat liver cell fractions. *Endocrinology* **51**, 406-411 (1952).
23. Peterson, N. A., Nataf, B. M., Chaikoff, I. L. and Ragupathy, E. Uptake of injected ¹³¹I-labelled thyroxine, triiodothyronine and iodide by rat brain during various stages of development. *J. Neurochem.* **13**, 933-943 (1966).
24. Tata, J. R. Hormone regulation of growth and protein synthesis. *Nature (London)* **219**, 331-337 (1968).
25. Geel, S., Valcana, T. and Timiras, P. Effect of neonatal hypothyroidism and of thyroxine on L-(C¹⁴) leucine incorporation in protein *in vivo* and the relationship to ionic levels in the developing brain of the rat. *Brain Res.* **4**, 143-150 (1967).
26. Green, K. and Matty, A. Effect of thyroxine on the permeability of the isolated toad bladder. *Nature (London)* **194**, 1191-1192 (1962).

27. Weiss, P. and Rossetti, F. Growth responses of opposite sign among different neuron types exposed to thyroid hormone. *Proc. Nat. Acad. Sci. U.S.A.* **37**, 540-566 (1951).
28. Bartels, E. and Rohart, R. The relationship of hyperthyroidism and parkinsonism. *AMA Arch. Int. Med.* **101**, 562-568 (1958).
29. Ignatkov, V. Y. Presynaptic inhibition and dorsal root potentials in cats with experimental thyrotoxicosis. *Bull. Exp. Biol. Med.* **70**, 985-987 (1970).
30. Emlen, W., Segal, D., and Mandell, A. Thyroid State: Effects on Pre- and Postsynaptic Central Noradrenergic Mechanisms. *Science* **175**, 79-82 (1971).
31. Klee, C. and Sokoloff, L. Mitochondrial differences in mature and immature brain. *J. Neurochem.* **11**, 709-716 (1964).
32. Hoch, F. Rapid effects of subcalorigenic dose of *L*-thyroxine on mitochondria. *J. Biol. Chem.* **241**, 524-525 (1966).
33. Gross, N. Control of mitochondrial turnover under the influence of thyroid hormone. *J. Cell Biol.* **48**, 29-40 (1971).
34. Tata, J. R., Ernster, L., Lindberg, E., Arrhenius, E., Pedersen, S. and Hedman, R. The action of thyroid hormones at the cell level. *Biochem. J.* **86**, 408-428 (1963).
35. Carter, W. J., Faas, F. H. and Wynn, J. Thyroxine Stimulation of Protein Synthesis *in Vitro* in the Absence of Mitochondria. *J. Biol. Chem.* **246**, 4973-4977 (1971).
36. Unsworth, B. and Cohen, P. Effect of thyroxine treatment on the transfer of amino acids from aminoacyl transfer ribonucleic acid into protein by cell-free extracts from tadpole liver. *Biochemistry* **7**, 2581-2588 (1968).
37. Tata, J. Hormones and the synthesis and utilization of ribonucleic acids. *Progr. Nucl. Acid Res. Mol. Biol.* **5**, 191-250 (1966).
38. Siegel, E. and Tobias, C. End-organ effects of thyroid hormones: Subcellular interactions in cultured cells. **153**, 763-765 (1966).
39. Hamburgh, M. and Flexner, L. B. Effect of hypothyroidism and hormone therapy on enzyme activities. *J. Neurochem.* **1**, 279-288 (1957).
40. Wolf, M. Anatomy of cultured mouse cerebellum II. Organotypic migration of granule cells demonstrated by silver impregnation of normal and mutant cultures. *J. Comp. Neurol.* **140**, 281-297 (1970).
41. Manuelidis, L. and Manuelidis, E. E. An autoradiographic study of the proliferation and differentiation of glial cells *in vitro*. (Abs.) *J. Neuropathol. Exp. Neurol.* **31**, 193 (1972).
42. Manuelidis, L. Localization of thyroxine ¹²⁵I in developing CNS tissue in culture. Electron microscopic autoradiography. (Abs.) *Soc. Neurosci.* **1**, 13.23 (1971).