Journal of Neurocytology **17**, 639–648 (1988)

Schwann cell proliferation in the sciatic nerve of hypothyroid chick embryos studied by autoradiography and image analysis

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Received 7 July 1987; revised 30 November 1987 and 29 April 1988; accepted 10 May 1988

Summary

The proliferation of Schwann cells in the sciatic nerve of chick was studied from day 11 to day 27 of development in control and thyroid-deficient embryos. Hypothyroidism was induced by tetramethylthiourea injection on days 8 and 19 of incubation. The parameters of the cell cycle were determined using autoradiographs (tritiated thymidine) and by image analysis of Feulgen-stained nuclear smears. The duration of the cell cycle was lengthened and the growth fraction was reduced in hypothyroid animals, at 11 and 15 days of incubation. At later stages (days 21 and 27), these parameters were not significantly different from the controls as if the sensitivity of Schwann cells to thyroid hormones was scheduled to occur during a limited period of development. The total number of axons was the same in control and hypothyroid animals suggesting that the slowing down of Schwann cell proliferation is not a consequence of neuronal cell death. The consequence of that slowing down is a delay in the isolation of promyelin axons and a reduction in the proportion of myelinated axons at all the stages studied.

Introduction

The mechanisms by which Schwann cell proliferation is controlled have been studied mainly *in vitro*. Autoradiographic studies by Wood & Bunge (1975) have shown that the rate of division of cultured Schwann cells is low when they are grown in isolation but increases dramatically when sensory or sympathetic neurons are grown in the same culture. The addition of neurite membrane fractions to the culture medium is sufficient to stimulate the proliferation of the Schwann cells (Salzer & Bunge, 1980 a,b; Salzer *et al.*, 1980; Cassel *et al.*, 1982; Meador-Woodruff *et al.*, 1985). Immunofluorescence studies by Sobue & Pleasure (1985) have shown that the mitogenic signal is located on the membrane of the neurites. These results have been confirmed by observations *in vivo* of peripheral nerve development. Developmental studies of the rat sciatic nerve indicate that the differentiation of axons precedes Schwann cell proliferation (Peters & Muir, 1959; Martin & Webster, 1973; Webster *et al.*, 1973) and that satellite cell proliferation commences only after neuronal cell division is essentially completed (Lawson *et al.*, 1974).

Thyroid hormones are known strongly to influence the development of the CNS and their different effects have been reviewed by Balàzs *et al.* (1971), Swanson *et al.* (1981) and Legrand (1984). In particular, hypothyroidism delays the growth and maturation of neurons (Honegger & Lenoir, 1980; Romijn *et al.*, 1982). In the PNS, hypothyroidism slows the maturation of the bundles of embryonic fibres in the sciatic nerve of radiothyrectomized mice and slows down the growth of Schwann cells (Reier & Hughes, 1972). Saxod and Bouvet (1982a) have shown that cutaneous nerves of chick embryos incubated for 8 days injected with tetramethylthiourea show hypomyelination, and a delay in the isolation of non-myelinated axons. These authors suggest that thyroid hormones may control Schwann cell proliferation. Unfortunately, only a few works deal with *in vivo* Schwann cell proliferation (Asbury, 1967; Martin & Webster, 1973; Salzer & Bunge, 1980a) during normal development, and the effects of thyroid hormones on Schwann cell proliferation were only studied in the case of experimental peripheral nerve regeneration (Stelmack & Kiernan, 1977).

The aim of the present study is to determine whether thyroid hormones influence Schwann cell proliferation *in vivo*. This paper describes a quanti-

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tative analysis of Schwann cell proliferation in the sciatic nerve of chick embryos, during normal development and in the case of thyroid hormone deficiency.

Materials and methods

Chick embryos were made hypothyroid by depositing 0.1 ml of a solution of tetramethylthiourea (TMTU; Fluka, 20 mg ml^{-1} in Tyrode) on the embryonic vascular area on day 8 of incubation, that is, two days before the beginning of any secretion of thyroid hormone. A second dose of TMTU was given to the embryos on day 19 of incubation to maintain the hypothyroid state during late stages of development. The level of plasma thyroxine was determined by means of a competitive binding method adapted to birds by Astier *et al.* (1978), using human plasma as the source of thyroxine-binding globulin.

Electron microscopy

Sciatic nerve segments were excised from hip to knee and fixed in 5% glutaraldehyde in 0.07 M phosphate buffer (pH 7.6) at room temperature for 1 h. The specimens were then fixed in 2% osmium tetroxide in the same buffer for 1 h, dehydrated in a graded ethanol series and embedded in Euparal. Cross-sections were cut in the middle of the nerves and micrographs were taken. The final magnification was approximately 25 000 after printing. Photomontages were prepared and the total number of axons in the sciatic nerves was determined by extrapolating the density of axons measured on the micrographs to the actual cross-sectional area of the nerves. The numbers of unmyelinated, myelinated and promyelin axons as defined by Friede and Samorajski (1968), were counted on the photomontages.

Autoradiography

Because of the high death toll in treated embryos at late stages, it was not possible to have a large enough number of 27-day hypothyroid animals to perform a complete series of labelling at this stage. Consequently, three series of 20 control and 20 hypothyroid embryos were taken on days 11, 15 and 21, respectively. Every 3 h, 25 μCi doses of ^{3}H-methyl-thymidine (^{3}HT, specific activity 44 Ci mM^{-1}) were administered to embryos in order to obtain a continuous labelling (Cleaver, 1967). The doses were given to embryos by depositing 0.1 ml of a solution of ^{3}HT, 250 μCi ml^{-1} on the chorioallantoic membrane. Two embryos from each series were killed 0.5, 1, 2, 3, 4, 6, 8, 10 and 12 h respectively, after the first dose of ^{3}HT had been given. Segments of sciatic nerves (from hip to knee) were taken and fixed for 6 h in a solution of ethanol and acetic acid (3:1) at 4° C. The nerves were rinsed for 12 h in 95% ethanol and embedded in paraffin. Specimens were cut longitudinally (section thickness 7 μm) and stained with acetic orcein, and then dried in open air. Sections were coated with photographic emulsion (Ilford L4), and exposed in absolute dark for 3 weeks at a temperature of 12° C. Autoradiographs were developed with standard photographic developer (Ilford D19).

For each nerve, four microscopic fields were randomly chosen along the longitudinal sections avoiding zones with perineurium or blood vessels. The number of labelled nuclei and the total number of nuclei were counted in those fields, amounting to a total of about 800 nuclei per nerve. Superposed nuclei were not taken into account. The background was 1–1.5 grains per cell. A cell was considered to be labelled if the number of grains per nucleus was greater than six. The index of labelling (IL) was given by the ratio of labelled nuclei to the total number of nuclei. Saturation curves of IL as a function of the duration of labelling were established for each series. The cell cycle parameters were deduced from the curves (Wimber, 1963; Cleaver, 1967) as follows. Figure 4 shows a typical saturation curve of IL for an exponentially growing cell population. This curve is composed of three different parts: (i) a shallow slope whose length corresponds to the cumulative duration of phase G2 and of mitosis M; (ii) the steeply ascending portion which corresponds to the duration of phase G1; and (iii) a saturation region with a shallow slope, when all proliferating cells have incorporated ^{3}HT (Wimber, 1963). The inflexion point between the steep region and the saturation region gives the value of the growth fraction GF, that is, the proportion of proliferating cells. The intercept of the first region corresponds to a quantity k which is directly related to GF and the durations of phase S and of the cell cycle T. These durations are obtained by solving the following system of equations:

$$\begin{cases} k = \frac{T_s}{T_c} \mathrm{Ln}\,(GF + 1) \\ T_c = T_s + T_{G1} + T_{G2+M} \end{cases}$$

where T_c, T_s, T_{G1} and T_{G2+M} are the durations of the cell cycle T and of phases S, G1 and G2+M, respectively.

Image analysis

On days 15, 21 and 27 of development, respectively, the left and right sciatic nerves of three embryos were taken and lightly fixed for 1 h in a solution of ethanol and acetic acid (3:1) and then rinsed for 1 min in 45% acetic acid. The perineural sheaths of the nerves were removed. The nerves were suspended in 1 ml of 45% acetic acid in a Potter Thomas AA47 tube. The tissues were crushed using a Teflon piston. The gap between the piston and the tube wall was 0.09 mm, so that cells were broken without damaging the nuclei. The suspensions of nuclei were spread out on gelatine-coated slides and covered with greased coverslips. After the preparations had been frozen for 30 min at − 30° C, the coverslips were rapidly removed in order to keep a maximum number of nuclei on the slides. After postfixation for 1 h in Smith fixative (0.5 mg potassium bichromate, 10 ml formaldehyde 45%, 2.5 ml pure acetic acid, 87.5 ml distilled water) the preparations were stained with Feulgen (Schulte, 1986), and then dehydrated and mounted in Euparal.

The Feulgen-stained nuclear smears were analysed using an automatic cell image analyser SAMBA 200 (TITN, Le Trident, Grenoble, France). The system allows the acquisition and analysis of 100 cell images per minute. Chick erythrocytes have very small and condensed nuclei; thus these cells are automatically eliminated from the analysis by selecting adequate nuclear surface area and optical density thresholds. The mathematical processing of the nuclear image provides 16 parameters. The parameters are grouped in three families: (i) morphological parameters which give

information on the size and the shape of nuclei; (ii) photometric parameters such as integrated optical density (IOD) of nucleus; and (iii) texture parameters which express the state of condensation of nuclear chromatin. The detailed list of parameters and their computation have been described by Brugal (1984). Because Feulgen staining is specific and stoichiometric for nucleic acids, the photometric parameter IOD can be directly related to the quantity of nucleic acid in a nucleus.

Nuclei were classified into different groups as a function of their similarities which were determined with an automatic multivariate classification method (Al Nachawati, 1985). These groups were identified in terms of cell cycle phases by analysing their projections in two-dimensional planes respectively defined by pairs of parameters (e.g. IOD) whose biological significance had been assessed (Giroud, 1982; Brugal, 1984; Brugal *et al.*, 1985). Autoradiography and image analysis did not provide means of distinguishing fibroblasts from Schwann cells. Therefore, the measurements of the growth fraction were global values. However, the Schwann cell proliferation could be assessed from the global measures within error limits by considering the percentage of fibroblasts in the sciatic nerves. The proportion of fibroblasts was determined for each nerve by counting the number of fibroblasts and Schwann cells, respectively, on the electron micrographs used for counting axons. The two cell types were easily distinguished by the presence of a basal lamina around Schwann cells. Proliferation of Schwann cells alone, excluding fibroblasts, was assessed as follows:

$$GF_s = \frac{GF_g - (F_f GF_f)}{F_s}$$

where GF_s is the Schwann cell growth fraction, GF_g is the global growth fraction obtained by means of autoradiography and image analysis, GF_f is the assumed growth fraction of fibroblasts, F_s is the proportion of Schwann cells and F_f the proportion of fibroblasts. Extreme error limits were obtained by assuming GF_f to be 0 (no fibroblasts proliferating) and 1 (all fibroblasts proliferating), respectively.

Results

Control of hypothyroidism

During normal development the concentration of thyroxine in blood increased from a value of 4.3 ng ml^{-1} on day 15 of incubation to reach a maximum value of 11 ng ml^{-1} on day 21 (Fig. 1a). During the later stages of development this level was between 6 and 8 ng ml^{-1}. In treated embryos, the concentration of thyroxine remained significantly lower than in control embryos. On day 15 it was 0.8 ng ml^{-1}, rising to a maximum of 2 ng ml^{-1} during the later stages. Other controls for hypothyroidism were provided by the relative weight of the thyroid glands (Fig. 1b) and their histology (Fig. 2). In control embryos the ratio of the weight of the thyroid glands to the body weight remained constant (0.15 mg g^{-1}) as a consequence of a parallel increase of the body weight and of the thyroid weight (Table 1).

On day 15, the thyroid follicles were differentiated and filled with colloid and their appearance was similar to that observed in adult animals (Kameda, 1984). In contrast to this, the relative weights of the thyroids of treated embryos increased dramatically during development, from a value of 0.23 mg g^{-1} on day 11 to a value of 2.62 mg g^{-1} on day 27; that is, 17 times the control value. On day 15 the thyroid follicles of hypothyroid embryos were depleted, with an appearance similar to that observed on day 9 of normal development when no thyroid secretory activity was detected either by measuring the thyroxine concentration in blood (Daugeras-Bernard *et al.*, 1976) or by an immunocytochemical analysis of the thyroid glands (Kameda, 1984). Side-effects of the treatment were a 4–6-day delay in hatching and a high death toll. These effects are the results of the absence of the rise in T4 concentration which normally occurs on day 20

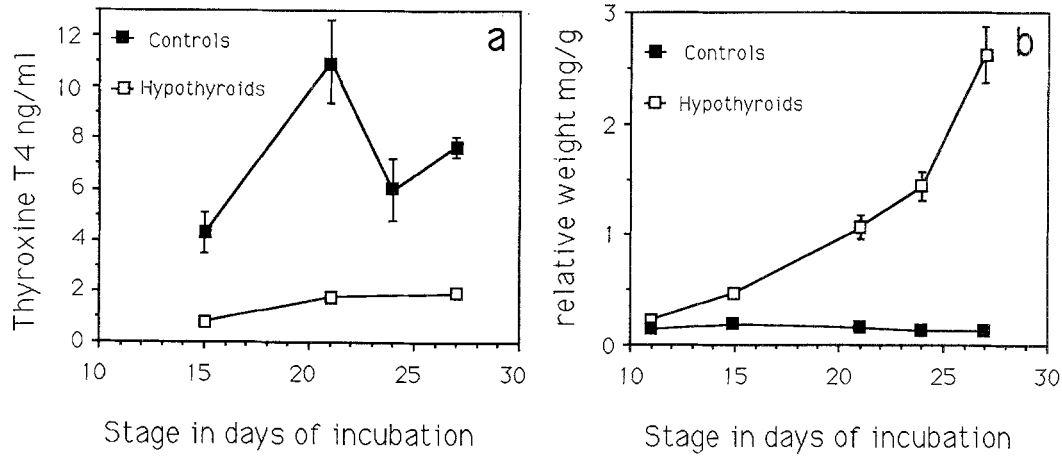
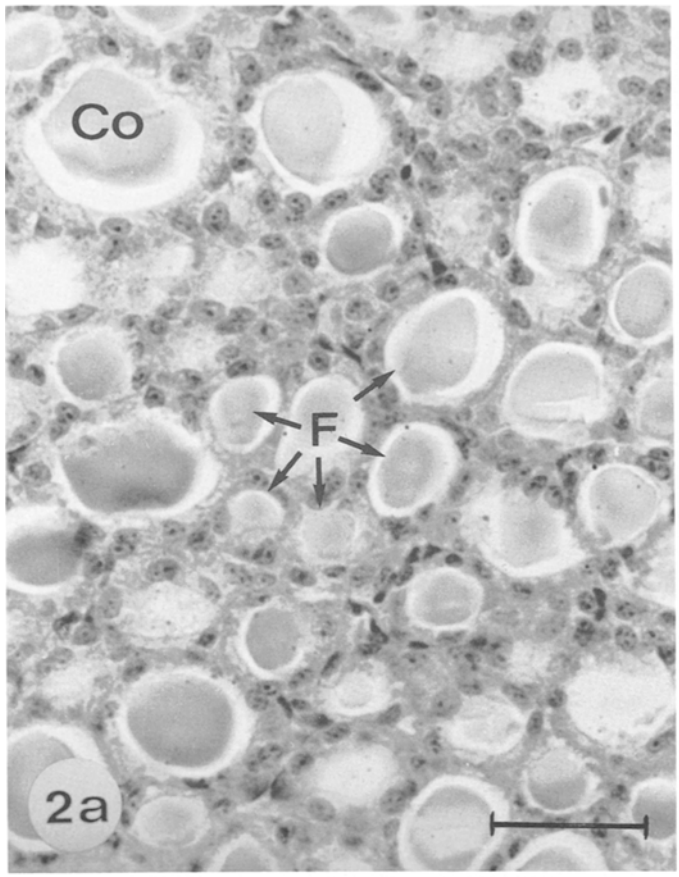


Fig. 1. (a) Curves of the plasma concentration of T4 hormone (ng ml^{-1}) as a function of developmental stage (in days of incubation). (b) Curves of the relative weight of thyroid glands (in mg of thyroid per g of body weight) as a function of development (in days of incubation). Hypothyroid embryos received tetramethylthiourea (TMTU) on days 8 and 19 of incubation. Bars represent standard deviations.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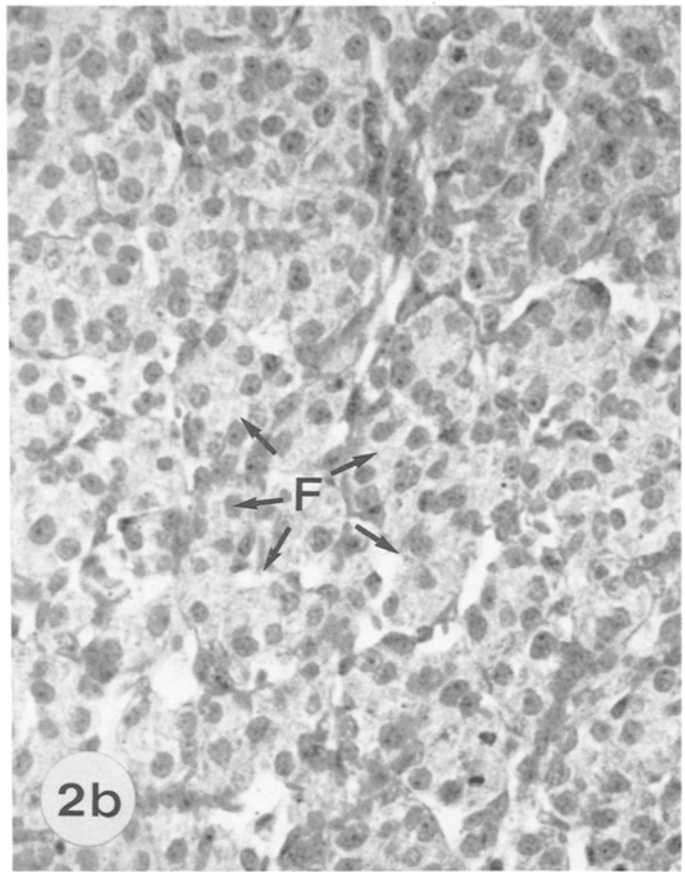
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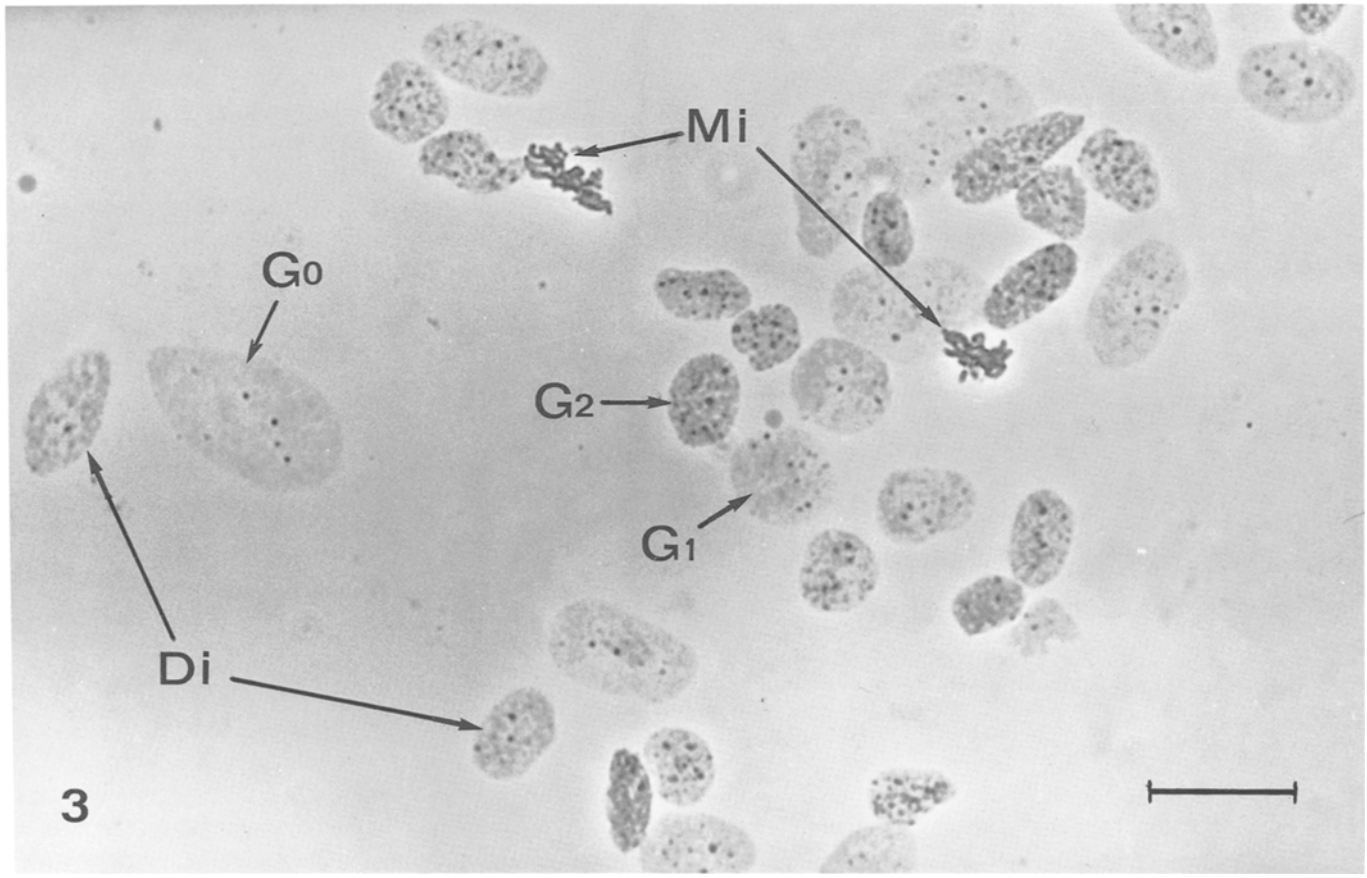
Mi
G0
G2
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Di
3

Table 1. Average body weights and average thyroid gland weights of chick embryos from day 11 to day 27 of development.

	C11	*H11*	*C15*	*H15*	*C21*	*H21*	*C24*	*H24*	*C27*	*H27*
Body	2.7	2.8	10.1	8.2	31.5	14.3	32.3	14.4	49.2	18.3
Glands	0.4	0.6	1.9	4.0	5.1	14.7	4.2	20.8	6.4	48.8

C, control animals; H, hypothyroid animals. Body weights in g, thyroid gland weights in mg.

Table 2. Proportions of axons in the sciatic nerve of chick embryos from day 15 to day 27 of development.

	C15	*H15*	*C21*	*H21*	*C24*	*H24*	*C27*	*H27*
Non-myelinated (%)	80	84	64	80	56	67	55	67
Promyelin (%)	5	10	2	4	2	3	1	3
Myelinated (%)	15	6	34	16	42	30	44	30
Axons (no.)	1007	1287	1108	1061	809	625	732	810
Total no.	52000	51500	30000	32000	—	—	—	—

C, control animals; H, hypothyroid animals. The number of axons is the actual number of axons counted in each case to establish the percentages of axons. The total number of axons is extrapolated from the number of axons measured on the photomontages.

(Bacou *et al.*, 1979) and which has been proved to trigger the embryo activity (peeping, twitching and extension of the limbs) preceding and facilitating hatching on day 21 of development (Ockleford & Vince, 1980).

Proportions of the types of axons

The total number of axons in the sciatic nerves decreased during normal development from 52 000 ± 1000 on day 15 to 30 000 ± 2000 on day 21 (Table 2). A similar decrease of the number of axons from 51 500 ± 950 on day 15 to 32 000 ± 2000 on day 21 was observed in hypothyroid animals (Table 2). Student's *t* test showed that the differences in total number of axons between controls and hypothyroid embryos were not significant ($\alpha > 0.05$). The results show that during development the proportions of non-myelinated and myelinated axons varied inversely.

The percentage of myelinated axons was increased to the detriment of the percentage of non-myelinated axons. In control animals, the proportion of promyelin axons was 5% on day 15 of development. In hypothyroid animals, there was a significant deficit (Student's *t* test $\alpha < 0.001$) in the number of myelinated axons at all stages studied. In contrast to this, the percentage of promyelin axons doubled in comparison with the controls, suggesting that the promyelin phase, between the isolation of a fibre and the appearance of the first myelin lamellae, was lengthened in the hypothyroid animals.

Autoradiography

During normal development, the duration of the cell cycle lengthened regularly from 8 h 20 min on day 11 to 15 h on day 21 (Table 3). This lengthening is due to the progressive increase in the duration of the phases S and G2 + M. The duraton of phase G1 remained steady at all the stages studied (Table 3). The growth fraction was reduced from 0.36 on day 11 to 0.15 on day 21. In hypothyroid embryos the duration of the cell cycle was dramatically lengthened to a value of 17 h 40 min on day 11, that is, a 212% increase in comparison to control embryos at the same stage. At later stages the duration of the cell cycle oscillated around a value of 19 h and exceeded the values

Fig. 2. Histology of thyroid glands of (a) a control embryo and (b) a hypothyroid embryo of 15 days' incubation. F, follicles; Co, colloid. Scale bar: 40 μm.

Fig. 3. Nuclear smear stained with Feulgen from sciatic nerves of 15-day-old control embryos. The size, staining intensity and chromatin texture of the nuclei are very different. Some nuclei are large and ovoid with scattered chromatin in which 4–11 granules can be counted. Other nuclei are smaller and darker with dense chromatin and shapes ranging from circular to oval. Some nuclei are labelled with their cell cycle phases as identified by image analysis. Mi, mitosis; Di, nuclei of differentiated cells; G0, nuclei of cells leaving the cycle; G1, nuclei of cells in phase G1; G2, nuclei of cells in phase G2. Scale bar: 5 μm.

Table 3. Durations of the phases of the cell cycle in the sciatic nerve of chick embryos from day 11 to day 21 of development.

	C11	*H11*	*C15*	*H15*	*C21*	*H21*
G1	4:15	2:40	4:20	4:30	4:00	6:10
S	2:50	9:40	4:15	11:30	8:00	9:00
G2 + M	1:15	5:20	2:00	4:00	3:00	3:20
Cell cycle	8:20	17:40	10:30	20:00	15:00	18:30
GF	0.36	0.34	0.33	0.21	0.15	0.11

Duration in hours and minutes (i.e. 8:20 is 8 h 20 min). C, control animals; H, hypothyroid animals; GF, growth fraction.

Table 4. Proportions of cells engaged in phases of the cell cycle in the sciatic nerve of chick embryos from day 15 to day 27 of development.

	C15	*H15*	*C21*	*H21*	*C27*	*H27*
Di (%)	21	11	45	41	66	59
G0 (%)	43	69	37	47	20	29
G1 (%)	14	5	7	4	4	2
S (%)	10	11	7	5	7	7
G2 +M (%)	12	4	4	3	3	3
Total	232	321	355	327	289	307
GF	0.36	0.20	0.18	0.12	0.14	0.13

C, control animals; H, hypothyroid animals; GF, growth fraction; Di, differentiated cells, total is the total number of nuclei analysed in each case.

observed in control animals at all stages. On day 11 the growth fraction was slightly smaller in hypothyroids than in control nerves (Fig. 5) but this difference was not statistically significant. This can be explained by the fact that hypothyroidism was effective only from day 10, that is, for only 24 h. With respect to the duration of the cell cycle, the delay is too short to measure a significant decrease of the growth fraction. Conversely, on day 15 the growth fractions were significantly different (a decrease of 36%). On day 21 the value of the growth fraction of hypothyroid embryos also was smaller than the controls, but this difference was not statistically significant. At later stages the cell cycle duration and the growth fraction are comparable to those measured in cases of thyroid deficiency.

Image analysis

Figure 3 shows an example of a nuclear smear prepared from sciatic nerves of control embryos at 15 days of incubation. Nuclei were classified using an unsupervised iterative clustering algorithm proceeding by successive separation and fusion processes as a

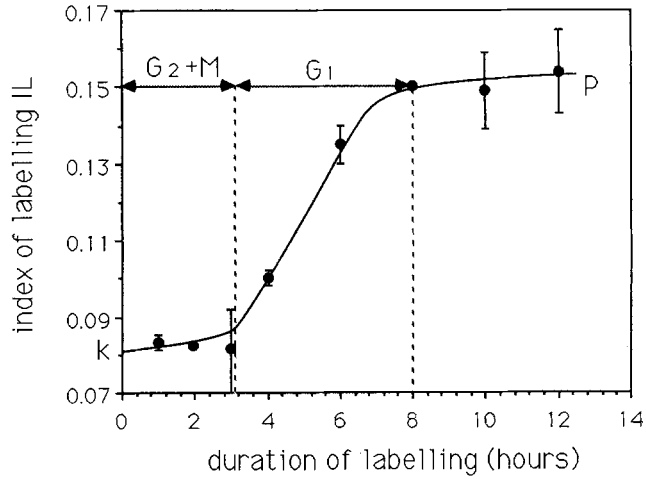

Fig. 4. Saturation curve of the index of labelling (IL) in the sciatic nerves of 21-day-old chick embryos as a function of the duration (in hours) of injection of tritiated thymidine. Cell cycle parameters including the growth fraction (GF) and the duration of phases G2 + M and G1 can be directly assessed from the curve. Durations of phase S and of the cell cycle are deduced from the intercept k extrapolated from the curve.

function of criteria of similarity and likelihood (Al Nachawati, 1985). The different groups were identified with respect to cell cycle phases according to Diamond *et al.* (1981) and Moustafa & Brugal (1984). The results of the classification are shown in Table 4 which regroups the values of the proportions of cells engaged in the different phases of the cell cycle.

Although the values of the growth fraction obtained by image analysis were slightly greater than those obtained at the same stages using the autoradiographic method, they confirmed the tendencies observed previously (Fig. 6). The slowing down of thyroid deficiency was maximal at 15 days of incubation when the growth fraction (GF) was 0.20 in hypothyroid embryos versus 0.36 in controls. On day 21, the reduction of GF in thyroid deficient embryos was greater than that observed by autoradiography and was significant (Student t test $\alpha < 0.05$). At the late stage of 27 days, the differences between hypothyroid and control embryos were no longer significant and the value of GF was around 0.135. If the proliferating cells only are taken into account, that is cells engaged in phases G1, S and G2 + M, it appears that during normal development the percentage of cells engaged in phase S increased from 28% on day 15 to 50% on day 27. In hypothyroid animals, the percentage of cells in phase S was already 55% on day 15 showing a slowing down of the kinetics of DNA synthesis.

In parallel with this study, cell proliferation was measured in the livers of the embryos. We observed that in 15-day hypothyroid animals there was a decrease of hepatic cell proliferation from a value of 0.60 to a value 0.51, that is a reduction of 15%.

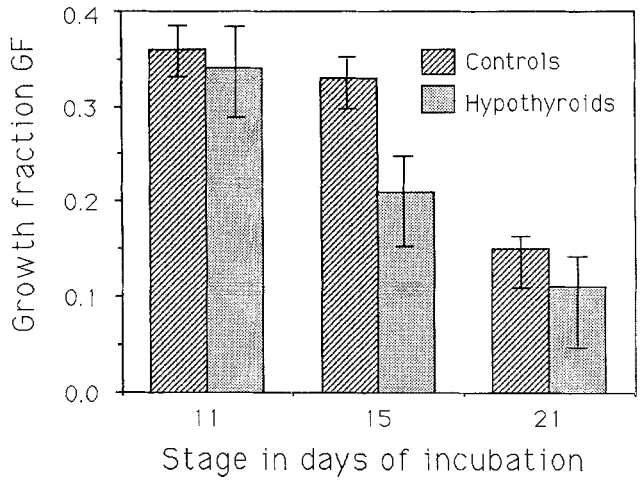

Fig. 5. Plots of the growth fraction (GF) in control and hypothyroid embryos obtained by means of autoradiography. Bars represent error limits depending on the proportion of proliferating fibroblasts. The error bars were calculated as explained in Materials and methods. Upper bar = no fibroblast proliferates, lower bar = all fibroblasts proliferate. The proportions of contaminating fibroblasts were 8% in control animals and 12% in hypothyroid animals. Consequently, the error of estimation was greater in hypothyroids than in control embryos. Examination of these errors shows that they are not symmetrical. This is an expected consequence of the calculation formula for the error. This error is symmetrical only for a growth fraction value of 0.5 and the negative error increases to the detriment of the positive error as the value of proliferation decreases.

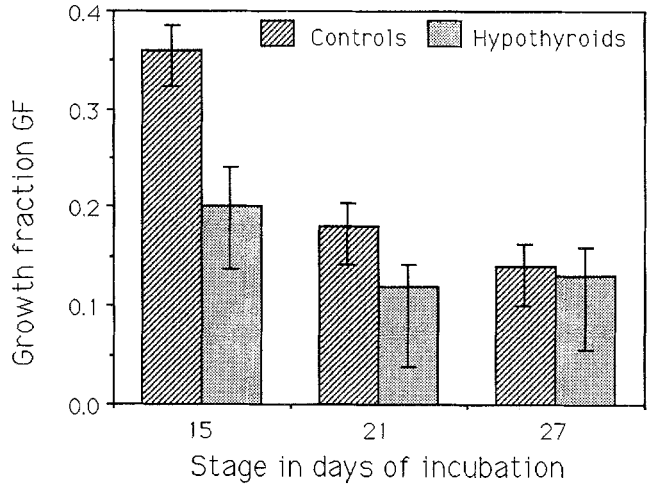

Fig. 6. Plots of the growth fraction (P) in control and hypothyroid embryos obtained by means of image analysis. Bars represent error limits depending on the proportion of proliferating fibroblasts (see Materials and methods). Upper bar = no fibroblast proliferates, lower bar = all fibroblasts proliferate.

Discussion

During the normal development of peripheral nerves many morphogenetic phenomena occur that exert opposite constraints on the rhythm of Schwann cell proliferation. The rhythm of division must be fast to permit the isolation of the axons destined to become myelinated but at the same time Schwann cells must leave the mitotic cycle to differentiate and produce myelin. Furthermore, maturation of the non-myelinated fibre bundles leads to their progressive fragmentation into groups by invasion of lamellar processes of the Schwann cells (Allt, 1969; Verna & Saxod, 1979). In the sciatic nerve of the neonatal rat, Asbury (1967) showed that 48 h after birth 27% of the Schwann cells were dividing and a quarter of these were leaving the mitotic cycle to differentiate. In the sciatic nerve of chick embryos, the study of the cell cycle and of the proportions of the different types of axons (non-myelinated and myelinated) shows that there are three stages in development during which the balance between proliferation and maturation is modified: (i) from day 11 to day 15 of incubation, the growth fraction of Schwann cells remained at a value of 0.34. It is during this period that promyelin axons become isolated. On day 13 the first signs of maturation of the Schwann cells are visible with electron microscopy. These include the first myelin lamellae (Uyemura *et al.*, 1979) and the appearance of tight junctions at the level of the internal mesaxons (Tezlaff, 1978). (ii) From day 15 to day 24 of development the growth fraction decreased to a value of 0.17. During this period, the proportion of promyelin axons decreased and the balance between proliferation and maturation was gradually modified in favour of maturation. (iii) After day 24 the rhythm of division of Schwann cells was reduced (growth fraction = 0.13) and the proportions of the different types of axons were stabilized. The present study shows that thyroid deficiency resulted in a clear reduction of the growth fraction of the Schwann cells, particularly on day 15 of development (average reduction of 40%) during the critical period when axons become isolated. At the same stage, the growth fraction in another tissue (hepatic cells) was reduced, but by a smaller proportion (15%). Considering the mitogenic influence of axons on Schwann cells, we wonder whether this reduction of proliferation might be a consequence of increased nerve cell death or of a growth deficiency of the nerve fibres. In the CNS, it has been shown *in vitro* that triiodothyronine (T3) improved the life duration of neurons of the cerebellar cortex in the rat (Honegger & Lenoir, 1980; Puymirat *et al.*, 1983) and increased the growth of neurites (Romijn *et al.*, 1982). Similar observations *in vivo* have been reported showing that hypothyroidism, if established before the critical period of cerebellar development, was responsible for

a reduction of the growth of neurites and dendrites (Clos, 1980; Nunez, 1984). In the PNS, hypothyroidism has more limited effects on the growth of neurons and the innervation of peripheral territories. Using the technique of cholinesterase staining (Saxod, 1978) to study the dorsal cutaneous nervous network of chick embryos, Bouvet and Saxod (1984) showed that there were no differences either qualitative or quantitative between hypothyroid and control embryos. Furthermore, in the present work it was shown that the total number of axons was similar in hypothyroid and control embryos. Consequently, the hypothesis that a reduction in the growth fraction of Schwann cells is a consequence of a deficiency in nerve fibres must be excluded. However, as thyroid hormone deficiency delays the growth of the animals one may think that it also delays the growth of axons and consequently reduces the proportion of the axonal membrane mitogenic factor. Ultrastructural morphometric analysis of non-myelinated and myelinated fibres showed no reduction of the axonal diameter in hypothyroid embryos (Usson, 1985). On the contrary, except on day 15 of incubation the mean axonal diameter was greater as a consequence of a delayed ensheathment by the Schwann cells. To the best of our knowledge, there is no evidence that hypothyroidism reduces the mitogenicity of the membrane surface of the axons.

Concerning the long-term effects of the absence or higher levels of thyroxine, most of the studies have shown that thyroid hormones appeared to act mainly on the schedule of maturation of the myelin, but that final lipid composition was unaffected (Balàzs *et al.*, 1969). Thus in rats made hypothyroid at birth, the specific activity of enzymes associated with myelination is significantly reduced at 30 days of age (Matthieu *et al.*, 1975), and increased after T3 administration (Wysacki & Segal, 1972). In 10-day neonatally thyroidectomized rats, the composition in myelin lipids corresponded to that of normal 5-day rats, while in rats receiving thyroxine daily from birth the myelin composition was similar to that of normal 20-day rats (Dalal *et al.*, 1971). Results obtained *in vitro* (Almazan *et al.*, 1985) emphasize the direct effect of T3 on myelination. Therefore, in hypothyroid young animals hypomyelination appears to be transitory and due to a significant delay in timing of myelin component synthesis and expression.

In conclusion, the results presented in this paper show that hypothyroidism dramatically reduces the growth fraction of Schwann cells (maximum reduction of 40% on day 15) by lengthening phases S and G2 of the cell cycle in the sciatic nerve of chick embryos during the early stages of myelination, without altering the total number of axons. The response of Schwann cells to thyroid hormone deficiency is greater than that of hepatocytes in the livers, indicating that the Schwann cells are particularly sensitive to the absence of thyroid hormones. The lower proliferation of Schwann cells in hypothyroid embryos could explain the delayed isolation of promyelin axons reported in this paper. Hypomyelination of peripheral nerves observed in ultrastructural morphometric studies (Bouvet & Saxod, 1984; Saxod & Bouvet, 1982b; Usson, 1985) seems to be due both to reduction in Schwann cell number and to retardation in myelin component synthesis. The present study suggests that during normal development of the chick embryo, the rise in plasma concentration of thyroid hormones from day 10 to day 20 of incubation allows the Schwann cells to maintain a high growth fraction (0.36) during the critical period when nerve fibres become isolated. At the later stages, the growth fraction of Schwann cells reaches a value comparable to that observed in thyroid deficiency as if the sensitivity of Schwann cells to thyroid hormones was scheduled to occur during a limited period of development.

Acknowledgements

This work was supported by a grant (CRE 836026) from the Institut National de la Santé et de la Recherche Médicale (INSERM). The assays of thyroxine were performed by Dr Jallageas, CNRS Laboratoire de Physiologie Animale de l'Université de Montpellier II. The authors wish to thank the members of the Equipe de Reconnaissance des Formes et Microscopie Quantitative, Université de Grenoble I, for helping them in the use of their image analysis facilities.

References

ALLT, G. (1969) Ultrastructural features of the immature peripheral nerve. *Journal of Anatomy* **105**, 283–93.

ALMAZAN, G., HONEGGER, P. & MATTHIEU, J. M. (1985) Triiodothyronine stimulation of oligodendroglial differentiation and myelination. A development study. *Developmental Neuroscience* **7**, 45–54.

AL NACHAWATI, I. (1985) Processus de classification séquentiels non arborescents pour l'aide au diagnostic. PhD thesis in Applied Mathematics. Université Scientifique Technologique et Médicale de Grenoble, France.

ASBURY, A. K. (1967) Schwann cell proliferation in developing mouse sciatic nerve. A radioautographic study. *Journal of Cell Biology* **34**, 735–43.

ASTIER, H. S., DANIEL, J. H. & JALLAGEAS, M. (1978) Estimation of plasma concentration in ducks in relation to different environmental and experimental conditions. *Experientia* **34**, 1228–9.

BACOU, F., JALLAGEAS, M., NOUGUES, J. & VIGNERON, P.

(1979) Différences sexuelles de l'évolution de la thyroxinémie chez l'embryon et le jeune poulet. *Comptes Rendus Académie des Sciences de Paris* **288**, 1228–9.

BALAZS, R., BROOKSBANK, B. W. L., DAVISON, A. N., EAYRS, J. T. & ZILSON, D. A. (1969) The effects of neonatal thyroidectomy on myelination in the rat brain. *Brain Research* **15**, 219–32.

BALAZS, R., KOVACS, S., COCKS, W. A., JOHNSON, A. L. & EAYRS, J. T. (1971) Effect of thyroid hormone on the biochemical maturation of rat brain. Postnatal cell formation. *Brain Research* **25**, 555–70.

BOUVET, J. & SAXOD, R. (1984) Analyse ultrastructurale quantitative du développement des nerfs cutanés chez le poulet hypothyroidien. *Archives d'Anatomie Microscopique et Morphologie Expérimentale* **73**, 27–43.

BRUGAL, G. (1984) Image analysis of microscopic preparations. In *Methods and Achievements in Experimental Pathology* (edited by JASMIN, G. & PROSCHECK, L.) **11**, pp. 1–33. Basel: Karger.

BRUGAL, G., GIROUD, F. & GABRIEL, A. (1985) Analysis of cell kinetics during planarian regeneration by means of SAMBA 200 cell image processor. *Roux's Archives of Developmental Biology* **194**, 148–54.

CASSEL, D., WOOD, P. M., BUNGE, R. P. & GLASER, L. (1982) Mitogenicity of brain axolemma membranes and soluble factors for dorsal root ganglion Schwann cells. *Journal of Cellular Biochemistry* **18**, 433–45.

CLEAVER, J. E. (1967) Thymidine metabolism and cell kinetics. In *Frontiers of Biology* (edited by NEUBERGER, A. & TATUM, E. L.) **6**, pp. 116–18. Amsterdam: North-Holland Publishing Company.

CLOS, J. (1980) Influence de l'état hypothyroïdien et de la sous-alimentation sur les interactions cellulaires au cours du développement du cervelet du rat. Analyse morphologique, histochimique et biochimique. Thesis, Doctorat ès-Sciences, Université de Montpellier II.

DALAL, K. B., VALCANA, T., TIMIRAS, P. S. & EINSTEIN, E. R. (1971) Regulatory role of thyroxine on myelinogenesis in the developing rat. *Neurobiology* **1**, 211–18.

DAUGERAS-BERNARD, N., LELOUP, J. & LACHIVER, F. (1976) Evolution de la thyroxinémie au cours du développement de l'embryon de poulet. Influence de l'hypophysectomie. *Comptes Rendus Académie des Sciences de Paris* **283**, 1325–7.

DIAMOND, L. W., DENNIS, D. W. & RAPPAPORT, H. (1981) The relationship between lymphocyte nuclear morphology and cell cycle stage in lymphoid neoplasia. *American Journal of Hematology* **11**, 165–73.

FRIEDE, R. L. & SAMORAJSKI, T. (1968) Myelin formation in the sciatic nerve of rat. A quantitative electron, histochemical and radioautographic study. *Journal of Neuropathology and Experimental Neurology* **27**, 546–70.

GIROUD, F. (1982) Cell nucleus pattern analysis. Geometric and densitometric featuring automatic cell phase identification. *Biology of the Cell* **44**, 177–88.

HONEGGER, P. & LENOIR, D. (1980) Triiodothyronine enhancement of neuronal differentiation in aggregating fetal rat brain cells cultured in a chemically defined medium. *Brain Research* **199**, 425–34.

KAMEDA, Y. (1984) Immunocytochemical studies on differentiation of the thyroid gland in rabbit fetuses and chick embryos. *Histochemistry* **80**, 23–9.

LAWSON, S., CADDY, K. & BISCOE, T. (1974) Development of rat dorsal root ganglion neurones: studies of cell birthdays and changes in mean cell diameter. *Cell and Tissue Research* **153**, 399–413.

LEGRAND, J. (1984) Effects of thyroid hormones on central nervous system development. In *Neurobehavioural Teratology* (edited by YANAI, J.), pp. 331–63. Amsterdam: Elsevier Science Publishers.

MARTIN, J. R. & WEBSTER, H. DE F. (1973) Mitotic Schwann cells in developing nerve: their changes in shape, fine structure and axon relationships. *Developmental Biology* **32**, 417–31.

MATTHIEU, J. M., REIER, P. J. & SAWCHAK, J. A. (1975) Proteins of rat brain myelin in neonatal hypothyroidism. *Brain Research* **84**, 443–51.

MEADOR-WOODRUFF, J. H., YOSHINO, J. E., BIGBEE, J. W., LEWIS, B. L. & DEVRIES, G. H. (1985) Differential proliferative response of cultured Schwann cells to axolemma and myelin-enriched fractions. II. Morphological studies. *Journal of Neurocytology* **14**, 619–35.

MOUSTAFA, Y. & BRUGAL, G. (1984) Image analysis of cell proliferation and differentiation in the thymus of the newt *Pleurodeles waltlii* Michah. by SAMBA 200 cell image processing. *Roux's Archives of Developmental Biology* **193**, 139–48.

NUNEZ, J. (1984) Effects of thyroid hormones during brain differentiation. *Molecular and Cellular Endocrinology* **37**, 125–32.

OCKLEFORD, E. M. & VINCE, M. A. (1980) Effects of thyroxine on prehatching developmental rate and behaviour in the chick. *Journal of Comparative and Physiological Psychology* **94**, 280–8.

PETERS, A. & MUIR, A. R. (1959) The relationship between axons and Schwann cells during the development of peripheral nerves in the rat. *Quarterly Journal of Experimental Physiology and Cognate Medical Sciences* **44**, 117–90.

PUYMIRAT, J., BARRET, A., PICART, R., VIGAY, A., FAIVRE-BAUMAN, A. & TIXIER-VIDAL, A. (1983) Triiodothyronine enhances the morphological maturation of dopaminergic neurons from fetal mouse hypothalamus cultured in serum-free medium. *Neuroscience* **10**, 801–10.

REIER, P. J. & HUGHES, A. F. (1972) An effect of radiothyroidectomy upon non-myelinated axons and associated Schwann cells during maturation of the mouse sciatic nerve. *Brain Research* **41**, 263–82.

ROMIJN, H. J., HABETS, A. M. M. C., MUD, M. T. & WOLTERS, P. S. (1982) Nerve outgrowth, synaptogenesis and bioelectric activity in fetal rat cerebellar cortex cultured in serum-free, chemically defined medium. *Developmental Brain Research* **2**, 583–9.

SALZER, J. L. & BUNGE, R. P. (1980a) Studies of Schwann cell proliferation. I. An analysis of proliferation during development. Wallerian degeneration and direct injury. *Journal of Cell Biology* **84**, 739–52.

.LZER, J. L. & BUNGE, R. P. (1980b) Studies of Schwann cell proliferation. III. Evidence for the surface localization of the neurite mitogen. *Journal of Cell Biology* **84**, 767–78.

SALZER, J. L., WILLIAMS, A. K., GLASER, L. & BUNGE, R. P. (1980) Studies of Schwann cell proliferation. II. Characterization of the stimulation and specificity of the response to a neurite membrane fraction. *Journal of Cell Biology* **84**, 753–66.

SAXOD, R. (1978) Combination of cholinesterase staining of nerves and stereoscopic viewing for three-dimensional study of skin innervation on whole mounts. *Journal of Investigative Dermatology* **70**, 95–7.

SAXOD, R. & BOUVET, J. (1982a) Effets de la déficience thyroidienne sur le développement des nerfs cutanés du poulet. *Comptes Rendus Académie des Sciences de Paris* **294**, 19–24.

SAXOD, R. & BOUVET, J. (1982b) Quantitative analysis of growth and myelination of cutaneous nerve fibers in the chick. *Developmental Neuroscience* **5**, 143–55.

SCHULTE, E. (1986) Hematoxylin and Feulgen reagent in nuclear staining. In *Standardization and Quantitation of Diagnostic Staining in Cytology* (edited by BOON, M. E. & KOK, L. P.), pp. 15–26. Leyden: Coulomb Press Leyden.

SOBUE, G. & PLEASURE, D. (1985) Adhesion of axolemmal fragment to Schwann cells: a signal- and target-specific process closely linked to axolemmal induction of Schwann cell mitosis. *Journal of Neuroscience* **5**, 379–87.

STELMACK, B. M. & KIERNAN, J. A. (1977) Effects of triiodothyronine on the normal and regenerating facial nerve of the rat. *Acta Neuropathologica* **40**, 151–5.

SWANSON, J. W., KELLY, J. J. & MCCONAHEY, W. M. (1981) Neurologic aspects of thyroid dysfunction. *Mayo Clinic Proceedings* **56**, 504–12.

TEZLAFF, W. (1978) The development of zonula ocludens in peripheral myelin of the chick embryo. A freeze fracture study. *Cell and Tissue Research* **189**, 187–201.

USSON, Y. (1985) Prolifération des cellules de Schwann du nerf sciatique de l'embryon de poulet normal et hypothyroïdien: quantification par morphométrie ultrastructurale et analyse d'image. PhD thesis in Cellular and Molecular Biology, Université Scientifique Technologique et Médicale de Grenoble, France, 20 December 1985.

UYEMURA, K., HORIE, K., KITAMURA, K., SUZUKI, M. & UEHARA, S. (1979) Developmental changes of myelin proteins in the chick peripheral nerve. *Journal of Neurochemistry* **32**, 778–88.

VERNA, J. M. & SAXOD, R. (1979) Développement de l'innervation cutanée chez le poulet: analyse ultrastructurale et quantitative. *Archives d'Anatomie Microscopique et Morphologie Expérimentale* **68**, 1–16.

WEBSTER, H. DE F., MARTIN, J. R. & O'CONNELL, M. F. (1973) The relationships between interphase Schwann cells and axons before myelination: a quantitative electron microscopic study. *Developmental Biology* **32**, 401–16.

WIMBER, D. E. (1963) Methods for studying cell proliferation with emphasis on DNA labels. In *Cell Proliferation* (edited by LAMERTON, L. F. & FRY, R. J. M.), pp. 1–12. Oxford: Blackwell Scientific.

WOOD, P. M. & BUNGE, R. P. (1975) Evidence that sensory axons are mitogenic for Schwann cells. *Nature* **256**, 661–4.

WYSACKI, S. J. & SEGAL, W. (1972) Influence of thyroid hormones on enzyme activities of myelinating rat central nervous tissues. *European Journal of Biochemistry* **28**, 183–9.