SYNTHESIS OF CHOLESTEROL AND FATTY ACIDS IN FRACTIONS OF PERIPHERAL NERVE * †

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Embryological and histological investigations have indicated that both axons and Schwann cells are necessary in the growth and maintenance of the myelin sheaths (2–4). Ben Geren (5) found that the myelin lamellae are formed as a result of winding around the axon layer by layer, the ends remaining attached on the inside to the axolemma and on the outside to the Schwann cell membrane. The Schwann cells have also been observed to deposit a fibrous layer binding them tightly to the nerve axons before the onset of myelination (4). The remainder of the nerve connective tissue is produced from fibroblasts (6).

The chemical composition of the lipid components of the myelin layers has been studied in some detail (7, 8), and the principal components are thought to be free cholesterol, cerebroside, sphingomyelin and phosphatidyl serine with a molecular ratio of 2 cholesterol: 2 phospholipid: 1 cerebroside. The biochemical processes involved in myelinization have been shown to include synthesis of cholesterol and phospholipids (9). The site of production of these lipids is uncertain. The fact that myelinization does not take place until Nissl substance maturation has occurred indicates that myelin formation depends on axon development. When myelinization takes place, it occurs simultaneously in several different areas. These are the areas in which Schwann cells are in contact with the nerve (4).

It was the purpose of this investigation to explore the relative roles of axons versus connective tissue elements and Schwann cells in the synthesis of cholesterol and fatty acids in adult peripheral nerve *in vitro*.

METHODS

Fractionation. Sciatic nerve segments 3 to 4 mm. long were removed from cats, some saved as controls and the others gently homogenized with 2 cc. Krebs-Ringer bicarbonate buffer in Dounce homogenizers with loose fitting plungers. This and the following procedures were carried out at 4° C. Two fractions were obtained: a milky homogenate, and an insoluble residue called the sheath fraction. These fractions were separated by simple decantation of the homogenate. The homogenate was centrifuged at 800 G for 10 minutes.

Incubation. Control nerves were placed in one flask modified according to Baruch and Chaikoff (10), the homogenate in another, and into a third the sheath was placed. The sheath and homogenate were recombined and placed in a fourth flask. The substrates used were added in the following concentrations: glucose, 9×10^{-3} M; potassium acetate, 1×10^{-3} M; and acetate-1-C^{14}, 8×10^{-5} M, in a total volume of 2.45 ml. The flasks were gassed with 95 per cent oxygen and 5 per cent carbon dioxide and stoppered with serum caps. The flasks were incubated in a Dubnoff shaker for two hours at 37° C.

ANALYTICAL METHODS

Deoxyribonucleic acid (DNA) analysis. The composition of the two fractions was studied with analysis of DNA and microscopic smears. Extraction of the nerves was based on Ceriotti's modification (11) of Ogur and Rosen's technique (12). After two extractions with absolute alcohol and two with ether, acid soluble components were removed with 2 per cent perchloric acid. The nucleic acids were then extracted from the residue with hot 10 per cent perchloric acid. The DNA in the perchloric extract was determined with Ceriotti's indole method (13). Spleen DNA was used for standard solution. All determinations were carried out in duplicate and the results averaged.

$C^{14}O_2$. At the end of the incubation, the flasks were placed in an ice bath. One ml. of alkaline Hyamine® 10X [1] in methanol-toluene 1:1, prepared as described by Passman, Radin and Cooper, (14) was injected through the serum cap into the center well, and the carbon dioxide was collected as previously described (15). The Hyamine®-$C^{14}O_2$ solution was next transferred quantitatively

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[1] Hyamine® 10X is the trade name for *p*-diisobutyl cresoxy ethoxy ethyl dimethyl benzyl ammonium chloride monohydrate (The Rohm and Haas Company).

with toluene into a 10 ml. volumetric flask. A 2 ml. aliquot was placed in 5 ml. of a 0.4 per cent solution of diphenyloxazole in toluene, and the C^{14} assayed in a Packard Liquid Scintillation Counter.

Cholesterol-C^{14} determination. The nerve and incubation mixture was saponified by adding 1 ml. of 90 per cent (weight per volume) potassium hydroxide and heating in an autoclave at 15 pounds' pressure in 120°C. for one hour (15). The saponified mixture was then transferred into a 250 ml. Erlenmeyer flask and ethanol was added to give a 50 per cent solution. The nonsaponifiable material was next extracted with petroleum ether, and the cholesterol fraction was isolated as the digitonide by the procedure of Sperry and Webb (16). This fraction also contains other 3β-sterols, which in the following are included under the term cholesterol. The digitonide was dissolved with heating in 2.2 ml. of methanol and 1 ml. of the solution was added to 16 ml. of the liquid scintillation solution for C^{14} determination.

Fatty acid-C^{14} determination. After removing the nonsaponifiable material as described above, the 250 ml. Erlenmeyer flask was heated on a steam bath to remove the ethanol. Water was then added to give a volume of about 5 to 6 ml. and the mixture was acidified below pH 3.0 with concentrated sulfuric acid and chilled. The fatty acids were then extracted into a measured volume of petroleum ether by vigorous shaking for 15 minutes (15) at room temperature. An aliquot was washed with water and assayed for C^{14}.

Calculations. All determinations were carried out in duplicate. Because the values obtained agreed within 10 per cent, they were averaged and given in the tables as averages. Carbon dioxide-C^{14} samples counted from 1,770 to 133,115 cpm. Fatty acid samples contained 900 to 24,660 cpm. The cholesterol fractions had from 5,880 cpm to the same level as background. The background counts varied from 6.5 to 14.7 cpm. All aliquots with counts lower than 100 cpm were counted for 10 minutes, samples between 100 and 200 cpm for 5 minutes. Values over 200 cpm were averaged from four one minute counts. Background was always counted in comparable fashion and checked against every fourth sample of the same counting time. The maximal 0.95 counting error in carbon dioxide and fatty acid analysis is ± 0.4 mμMole. In cholesterol synthesis a 0.95 counting error of 33 per cent appears in samples with counts lower than 36 cpm and these are therefore listed as lower than 1 mμMole × 10^{-2}.

TABLE I

Deoxyribonucleic acid (DNA) in cat nerve homogenate and residue

Expt. no.	μg. DNA/100 mg. homogenate	μg. DNA/100 mg. residue (sheath fraction)
1	<5	68
2	10	41
3	<5	86
4	<5	54
5	6	59
6	<5	82
7	8	64
8	<5	78

RESULTS

DNA analysis

The results of the DNA analysis are seen in Table I. Eight nerves were analyzed. The amounts of DNA found in the homogenate fractions from five nerves was less than 5 μg. per 100 mg. substrate. This is the lower limit for the analysis method. In the other three, small amounts of DNA were detected. Microscopic analysis of the homogenate showed fragments of nerve fibers with or without myelin sheath. A few Schwann cells could also be seen.

In contrast, the residue fraction contained remnants of the endo- and perineurium, Schwann cells and some nerve axon bundles. The DNA contents varied from 41 to 86 μg. DNA per 100 mg. with residue. These amounts approximate the figure given by Logan, Mannell and Rossiter (17) for total nerve, *i.e.*, 60 μg. DNA per 100 mg. wet nerve tissue.

The results of the DNA analysis indicated that little nuclear material was left in the homogenate fraction. This fraction consequently contained mostly nerve axons and myelin. The residue fraction included the majority of the Schwann cells as well as the connective tissue from the peripheral nerve.

TABLE II

Incorporation of acetate-1-C^{14} into cholesterol in millimicromoles × 10^{-2}. Cat sciatic nerve

Expt. no.	1	2	3	4	5	6	7	8
Control (unhomogenized)	11	61	2	7	6	130	49	36
Homogenate	2	2	<1	<1	<1	<1	<1	1
Sheath	2	9	6	6	10	6	2	1
Homogenate and sheath	3	3	<1	7	34	2	4	1

Incorporation of radioactive acetate

Cholesterol synthesis from acetate-1-C^{14} in the unhomogenized controlled nerve ranged from 2 to 130 mμMoles × 10^{-2} (Table II). After separation into fractions, the sheath fraction retained the

ability to synthesize cholesterol whereas five out of eight homogenate fractions incorporated only a negligible amount of radioacetate into cholesterol. Recombination of the two fractions did not appreciably increase cholesterol synthesis (with the exception of Experiment 5).

The synthesis of fatty acids differed in two aspects from that of cholesterol. First, the amounts synthesized in the homogenate were larger than those obtained from the sheath fraction in six out of eight experiments (Table III). Second, the synthesis in the homogenate was higher than that in the control nerve in half of the experiments. Again, recombination of the two fractions seemed to have no stimulatory action on the fatty acid synthesis.

Production of carbon dioxide-C^{14} varied in the control nerve from 16 to 153 mμMoles (Table IV). After homogenization the homogenate fraction evolved smaller amounts of radioactive carbon dioxide (3 to 51 mμMoles) than the sheath fraction (6 to 219 mμMoles).

TABLE III

Incorporation of acetate-1-C^{14} into fatty acids in millimicromoles. Cat sciatic nerve

Expt. no.	1	2	3	4	5	6	7	8
Control (unhomogenized)	5.4	14.0	16.5	1.9	46.7	4.2	10.0	11.1
Homogenate	2.4	17.8	6.7	9.2	52.8	3.8	3.7	19.9
Sheath	2.7	10.9	1.9	6.5	33.8	2.1	7.6	8.6
Homogenate and sheath	4.3	8.7	5.9	6.6	11.5	32.6	3.4	9.4

TABLE IV

Acetate-1-C^{14} to carbon dioxide in millimicromoles. Cat sciatic nerve

Expt. no.	1	2	3	4	5	6	7	8
Control (unhomogenized)	70	45	102	63	153	72	43	16
Homogenate	3	4	3	4	51	36	4	7
Sheath	10	16	34	34	90	219	6	9
Homogenate and sheath	18	13	20	66	226	90	5	6

DISCUSSION

The homogenate fraction obtained with our technique contained axon and myelin fragments as well as soluble components from disrupted Schwann cells and connective tissue. The sheath fraction included the majority of the Schwann cells and the connective tissue of the peripheral nerve. In the axon fractions, fatty acids were synthesized but little or no cholesterol was formed; in the sheath fractions, definite amounts of cholesterol were synthesized and, in addition, some fatty acids.

It would appear that cholesterol synthesis from acetate could not take place in axon and myelin fragments *in vitro*. Neither did the necessary enzyme systems seem to be soluble enough to be extracted from the total nerve, with our technique. It has been stated that cholesterol cannot be synthesized by adult brain tissue (18). If we assume that the immature nerve tissue can synthesize cholesterol, then one or more of the enzyme systems used disappears. The possibility also remains that the nerve cell nucleus is necessary for this synthesis. It seems more likely that in the peripheral nerve, cholesterol synthesis from acetate takes place in the Schwann cells or fibroblasts which surround the nerve axon. It has been shown that fibroblasts in culture can produce cholesterol (19). In view of the previously stated morphological evidence that Schwann cells participate in myelination, it seems more attractive to assume that they supply the cholesterol necessary for the myelin sheath. Additional evidence to support their role in lipid synthesis has been brought out by the demonstration of an increase of Schwann cells in response to injury (20), an increase which parallels the increase in cholesterol synthesis (21). Furthermore, if the amount of C^{14} incorporated into lipid by a single Schwann cell is calculated (22), the greatest activity occurs at peak level of myelinization or about 20 days after birth.

Fatty acid synthesis must play a major role in the maintenance of the myelin sheath. The synthesis of sphingomyelin and glycerol phospholipid requires fatty acids of probably different chain lengths. But, also, the axon lipids contain fatty acids. It is therefore not remarkable that fatty acid synthesis would take place in the axon fraction. The homogenization procedure used does not exclude the possibility that soluble cofactors

from the sheath fraction pass over to the homogenate. The additional increase of such cofactors as would occur on recombination of the two fractions does not seem to stimulate fatty acid synthesis any further. However, maximal stimulation may have already occurred. The fact that the homogenized nerve occasionally incorporates more acetate-1-C^{14} into fatty acid than does the intact nerve could be explained on the basis of a higher penetration of substrate into the axon fragment. On the other hand, occasionally lower rates of synthesis of fatty acids are observed in the homogenate; this could be explained on the basis of liberation of nucleotidases during the homogenization procedure. It has been shown that triphosphopyridine nucleotide (TPN) is rate limiting in fatty acid synthesis in liver (23). We have found that fatty acid and cholesterol synthesis in normal rat nerve is stimulated by addition of the reduced triphosphopyridine nucleotide (TPNH) generating system (24). An excessive destruction of TPN may therefore limit lipogenesis in the axon. Because the sheath fraction could never be completely freed of axon tissue, the question of how much fatty acid synthesis normally takes place in the Schwann cell could not be answered.

The CO_2 production does not seem related to either the amounts of cholesterol or fatty acids produced. Neither does there seem to be any competition between oxidative processes and lipogenesis. The higher carbon dioxide synthesis in the sheath fraction is thought to reflect simply the larger amount of cellular material.

The findings stress the fact often pointed out by histologists that myelinization is dependent on the presence of both sheath elements and nerve fibers. According to Speidel (3), a nerve sprout coming from a myelinated nerve differs from a nonmyelin emergent fiber. The former in combination with a primitive Schwann cell leads to the formation of a new myelinated segment; the latter ordinarily does not. This would indicate that there is a basic difference in the structure of myelinated and nonmyelinated nerve fibers which influences the degree of myelination. The presence of Schwann cells around both types of nerves could be taken to indicate that the limiting factor in myelinization may be the quantity of specific fatty acids supplied by the axon.

SUMMARY

Gentle homogenization of cat sciatic nerves resulted in the separation of two fractions. One is a homogenate containing chiefly axons and myelin, the other is a sheath fraction, containing fibrous tissue and Schwann cell nuclei.

In vitro cholesterol synthesis from acetate occurs almost exclusively in the sheath fraction.

In vitro synthesis of fatty acids often is more active in the homogenate fraction.

The possibility is discussed that such a separation in synthetic properties may reflect the relative role of the axons and Schwann cells in peripheral myelinization.

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