Sterol-Like Compound from Sensory Ganglia

EFFECT OF A NERVE GROWTH FACTOR AND INSULIN ON ITS BIOSYNTHESIS

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1. Sensory ganglia from 8-day-old chick embryos were incubated with a specific nerve growth factor and with insulin. 2. From the total lipid extract of the ganglia a compound with steroid characteristics was isolated. 3. The synthesis of this compound, measured spectrophotometrically, diminished after addition of the nerve growth factor and insulin to the incubation medium. 4. The incorporation of sodium $[2-^{14}\text{C}]$ acetate and DL- $[2-^{14}\text{C}]$ mevalonic acid into total lipids of the sensory ganglia was stimulated by the nerve growth factor and insulin, but the radioactivity of the sterol-like compound was slightly lower. The incorporation of labelled mevalonic acid either into total lipids or into the sterol-like compound was about 25% lower. 5. About 20% of the acetate incorporated into total lipids and about 87% of the mevalonic acid were recovered in the sterol-like compound.

The growth and differentiation of sensory and sympathetic nerve cells is stimulated by a specific protein, which can be isolated from mouse salivary glands (Levi-Montalcini & Cohen, 1960). The synthesis of this protein could be stimulated by injection of testosterone (Levi-Montalcini & Angeletti, 1964) and it has also been found in detectable amounts in human blood (Levi-Montalcini & Angeletti, 1961). The protein, referred to as NGF*, has a marked effect at a very low concentration on metabolic processes occurring in explanted sensory and sympathetic ganglia of 8- and 9-day-old chick embryos (Levi-Montalcini, 1966).

It has already been demonstrated that the NGF stimulates the synthesis of RNA (Toschi, Dore, Angeletti, Levi-Montalcini & de Haën, 1966); also, the synthesis of proteins (Angeletti, Attardi-Gandini, Toschi, Salvi & Levi-Montalcini, 1965) and of total lipids (Liuzzi, Angeletti & Levi-Montalcini, 1965) is increased in NGF-treated ganglia. Glucose metabolism is stimulated mainly through the hexose 6-phosphate pathway (Angeletti, Liuzzi, Levi-Montalcini & Attardi-Gandini, 1964). To gain a better insight into the mechanism of action of the NGF on the responsive nerve cells, a more detailed analysis of its effects on lipid metabolism has been undertaken.

In the present work we have isolated from the total lipid extract of embryonic sensory ganglia a compound with steroid characteristics. As steroids influence membrane permeability, the effect of NGF on the biosynthesis of this compound was

studied and the results were compared with data obtained by incubating embryonic sensory ganglia with insulin, an agent that shares in common with NGF some metabolic effects (Levi-Montalcini, 1966). Insulin in fact is active in altering membrane permeability (Fisher & Zachariah, 1961) and stimulates protein synthesis (Guidotti, Loreti, Gaja, Ragnotti & Foà, 1965), lipogenesis (Krahl, 1961) and RNA synthesis (Wool & Munroe, 1963).

MATERIALS AND METHODS

Chemicals. The solvents used were obtained from Carlo Erba S.p.A., Milan, Italy, and were all of chromatographic grade. The steroids were obtained from Calbiochem, Los Angeles, Calif., U.S.A.; squalene was from L. Light and Co. Ltd., Colnbrook, Bucks.; Eagle basal medium was from the Microbiological Associates, Bethesda, Md., U.S.A. (catalogue no. 12-105); insulin was from the Sigma Chemical Co., St Louis, Mo., U.S.A.; sodium $[2-^{14}\text{C}]$ acetate (specific radioactivity 27.4 mc/m-mole) and DL- $[2-^{14}\text{C}]$ mevalonic acid (specific activity 4.82 mc/m-mole) were from The Radiochemical Centre, Amersham, Bucks.; silica gel G and HF₂₅₄ were from E. Merck A.-G., Darmstadt, Germany. The purified NGF was a gift from the Wellcome Research Laboratories, Beckenham, Kent.

Preparation of the ganglia. Sensory ganglia were explanted from 8-day-old chick embryos and pooled in groups of 60 ganglia. They were incubated in Maximow dishes containing 1.0 ml. of Eagle basal medium to which 0.2 µg. of NGF/ml. or 0.5 unit of insulin/ml. was added. For the determination of the incorporation of labelled acetate or mevalonate, 2.0 µC of labelled compound was added/ml. of incubation mixture. The incubations were carried out in a CO₂-conditioned incubator at 37°. At the end of the

* Abbreviation: NGF, nerve growth factor.

incubation period the ganglia were immediately removed from the culture fluid and washed three times with unlabelled 0.1M-sodium acetate for the acetate experiments and with unlabelled 0.1M-sodium mevalonate for the mevalonic acid experiments.

Chromatography. Thin-layer chromatography was carried out on 5 cm. $\times$ 20 cm. glass plates coated with a layer of 0.25 mm. of silica gel G or silica gel HF₂₅₄ activated at 110° for 30 min. The solvent systems used were: (1) hexane-acetone (22:3, v/v); (2) light petroleum (b.p. 40–70°)-acetone (9:1, v/v); (3) light petroleum (b.p. 40–70°)-benzene (1:1, v/v); (4) cyclohexane-ethyl acetate (1:1, v/v); (5) chloroform. The developed chromatograms on silica gel G layers were stained with: (a) iodine vapour; (b) SbCl₃ in chloroform; (c) phosphomolybdic acid in ethanol; or (d) Liebermann-Burchard reagent. Spots on silica gel HF₂₅₄ were revealed by their fluorescence under an ultraviolet lamp.

Gas-liquid chromatography was carried out on a Perkin-Elmer 801 gas chromatograph provided with a column 1.80 m. long $\times$ 3.0 mm. internal diam. packed with Chromosorb G (80–100 mesh) impregnated with SE-30 (2%). Injector and column were made of glass; injection temperature was 270°; the flow of N₂ was 40 cm.³/min. The column temperature was programmed from 125° up to 275° with a constant temperature increase of 8.33°/min.

Isolation and purification of the sterol-like compound. After freeze-drying, a batch of 100 sensory ganglia of 8-day-old chick embryos was weighed and then extracted three times in a mortar with 5 ml. of chloroform. The chloroform extract was concentrated and separated by thin-layer chromatography. Normally solvent system (1) was used together with SbCl₃ as spray reagent, revealing only one spot with *R_F* approx. 0.3. This compound, which appeared as a dark-blue spot under the ultraviolet lamp, was scraped from the plate and the silica gel was extracted with chloroform. To assess the purity of the SbCl₃-positive compound the chloroform extracts were run in the other solvent systems mentioned above; again only one spot resulted. On thin-layers the compound gave the following colour reactions with various reagents: light blue with SbCl₃; blue-green with phosphomolybdic acid; black with conc. H₂SO₄-water (1:1, v/v); brown with iodine vapour. To characterize the material further co-chromatography was carried out with some common steroids and with squalene in different solvent systems. These steroids are listed below with their relative migration rates in solvent system (5) with cholesterol as reference (= 1.00). Also, the retention times by gas-liquid-chromatographic analyses of the same compounds were determined. The first number in parentheses is the relative movement and the second number indicates the retention time (min.): cholesterol (1.00; 20.6); 7-dehydrocholesterol (0.96; 20.9); desmosterol (0.83; 21.1); 7-dehydrodesmosterol (0.91; 21.3); ergosterol (1.10; 21.7); squalene (11.0; -); androstane (14.1; 10.1); cholestane (13.3; 18.4); testosterone (0.7; 15.4); our compound (5.8; 10.8). The SbCl₃-positive material eluted from the thin-layer plates gave only one peak on gas-liquid chromatography. Its identity with the compound obtained after incubation of the ganglia with sodium [2-¹⁴C]acetate and DL-[2-¹⁴C]mevalonic acid was confirmed by their identical retention times on the gas-liquid-chromatography column.

It was not possible to detect cholesterol on the thin-layer

plates with the given spray reagents, but gas-liquid-chromatographic analyses demonstrated the presence of traces of this compound. Using stigmasterol as internal standard we measured about 5 μ g. of cholesterol and about 25 μ g. of our compound/100 freeze-dried ganglia.

Our sterol-like compound was not saponified by boiling it overnight in 2N-KOH solution in aq. 50% (v/v) methanol. It did not change its chromatographic behaviour after treatment with diazomethane in diethyl ether, before and after saponification. The extraction and separation were carried out 5° in diffuse daylight, because an ethanolic solution of the compound became yellow within a day.

Spectrophotometric determinations. The washed ganglia were freeze-dried, weighed and then homogenized with 5 ml. of chloroform. The extract was separated by thin-layer chromatography on silica gel HF₂₅₄ layers with solvent system (1). The band of our compound revealed at *R_F* approx. 0.3 under the ultraviolet lamp was cut out and extracted with three 2.5 ml. portions of aq. 95% (v/v) ethanol. The ultraviolet spectrum of this extract was determined in a Perkin-Elmer model 137 ultraviolet spectrophotometer.

The quantity of the sterol-like compound in the ganglia was determined by measuring its extinction at 286 m μ after elution of the spot from the chromatogram. As reference the absorption of 1 mg. of the purified compound dissolved in 100 ml. of aq. 95% ethanol was used. The extract of an equivalent blank area of a silica gel HF₂₅₄ layer was used for the correction necessary for the residue extracted.

Radioactive incorporation. The ganglia, washed as described above, were homogenized in a glass homogenizer with 1.0 ml. of chloroform-methanol (2:1, v/v) for the extraction of total lipids by the method of Folch, Lees & Sloane-Stanley (1957). The extract was washed and dried over anhydrous Na₂SO₄, and a sample (10%) was taken for determination of radioactivity incorporated into total lipids. The sample was first subjected to thin-layer chromatography with chloroform-methanol-water (14:6:1, by vol.) as solvent. The developed chromatogram was scanned with a model 7200 radiochromatogram scanner (Packard Instrument Co. Inc., Downers Grove, Ill., U.S.A.) with a slit width of 2.5 mm. and a scanning velocity of 0.5 cm./min. The efficiency of the apparatus for our chromatograms was 1.1%. The incorporated radioactivity was calculated by measuring the peak areas of the single spots of the radiochromatograms and the total radioactivity incorporated into the lipids was obtained by adding the radioactivity of the single spots. In this way we confirmed the validity of our technique as compared with the radiosintillation technique described by Liuzzi *et al.* (1965) for the incorporation of sodium [2-¹⁴C]acetate into total lipids of sensory ganglia.

The remainder of the extract (90%) was separated by thin-layer chromatography with solvent system (1) for measurement of the incorporation of radioactivity into our sterol-like compound. The incorporation of total radioactivity from DL-[2-¹⁴C]mevalonic acid into our compound was measured only with solvent system (1).

RESULTS

After separation of a chloroform extract of freeze-dried ganglia by thin-layer chromatography the chromatograms were stained with different

spray reagents to characterize the separated compounds. With antimonytrichloride in chloroform a single blue spot was revealed. The antimony trichloride-positive area, after elution as described above, gave a single ultraviolet-absorption maximum in aq. 95% ethanol at 286mμ (Fig. 1). The compound presumably has a steroid structure, since it incorporated radioactivity from labelled acetate and mevalonic acid. The high incorporation ratio of DL-[2-¹⁴C]mevalonic acid, 80–90%, compared with the 15–25% values for sodium [2-¹⁴C]acetate, favours this assumption.

It was observed that a chloroform extract of ganglia incubated with NGF, after separation and staining with antimony trichloride, gave a spot with a lower colour intensity than an extract of an equal amount of control ganglia. This qualitative observation was confirmed by determining the quantity of the compound by its absorption maximum in ultraviolet light. The results, ex-

pressed in μg. of steroid-like compound/100 ganglia, are shown in Table 1. The amount of the unknown material formed was about 20% lower for the NGF-treated ganglia as compared with control ganglia after 4hr. of incubation and did not change after 6 and 8hr. As shown in Table 1 a similar result was obtained with insulin instead of NGF. From these data it appears that the sterol-like compound normally accounts for about 5% of ganglia dry weight.

To study more specifically the effect of NGF and

Fig. 1. Ultraviolet spectrum in aq. 95% ethanol of the sterol-like compound extracted from the sensory ganglia of 8-day-old chick embryos.

Fig. 2. Scans for radioactivity of thin-layer chromatograms of the total lipid extract of sensory ganglia from 8-day-old chick embryos incubated with (a) sodium [2-¹⁴C]acetate or (c) DL-[2-¹⁴C]mevalonic acid, compared with (b) a control thin-layer chromatogram sprayed with SbCl₃ in chloroform.

Table 1. *Effect of NGF and insulin on formation of the sterol-like compound*

Sensory ganglia from 8-day-old chick embryos were incubated with or without NGF (0.2 μg./ml.) or insulin (0.5 unit/ml.) in 1 ml. of Eagle basal medium for 4, 6 and 8 hr. The results are the mean values (± s.e.m.) of five separate experiments carried out at the same time under identical conditions.

Incubation time (hr.)	Control		NGF-treated		Insulin-treated	
	Dry wt. of 100 ganglia (μg.)	Amount of sterol-like compound/100 ganglia (μg.)	Dry wt. of 100 ganglia (μg.)	Amount of sterol-like compound/100 ganglia (μg.)	Dry wt. of 100 ganglia (μg.)	Amount of sterol-like compound/100 ganglia (μg.)
0	495 ± 31	27.2 ± 1.1	—	—	—	—
4	454 ± 25	25.2 ± 1.7	435 ± 20	20.5 ± 1.8	458 ± 30	17.2 ± 1.5
6	476 ± 26	20.7 ± 1.7	484 ± 27	18.5 ± 0.8	467 ± 21	17.1 ± 0.6
8	450 ± 24	20.5 ± 0.9	486 ± 25	17.8 ± 0.6	451 ± 24	12.4 ± 0.4

Table 2. *Effect of NGF and insulin on incorporation of [2-¹⁴C]acetate into total lipids*

Sensory ganglia from 8-day-old chick embryos were incubated with or without NGF (0.2 μ g./ml.) or insulin (0.5 units/ml.) in 1.0 ml. of Eagle basal medium, plus sodium [2-¹⁴C]acetate (2.0 μ C/ml.; 0.073 μ mole/ml.). The results are the mean values ($\pm$ s.e.m.) of four separate experiments expressed in counts/min. of acetate incorporated/60 ganglia after 4 and 8 hr. of incubation. The control, NGF and insulin experiments were carried out at the same time under identical conditions.

Sample ...	Control	NGF-treated		Insulin-treated	
	(counts/min./60 ganglia)	(counts/min./60 ganglia)	(% change)	(counts/min./60 ganglia)	(% change)
Incubation time (hr.)					
4	21886 $\pm$ 344	38102 $\pm$ 945	+71	27854 $\pm$ 512	+27
8	29824 $\pm$ 325	43809 $\pm$ 984	+49	35644 $\pm$ 793	+19

Table 3. *Effect of NGF and insulin on incorporation of [2-¹⁴C]acetate into the sterol-like compound*

Sensory ganglia from 8-day-old chick embryos were incubated in 1.0 ml. of Eagle basal medium with or without NGF (0.2 μ g./ml.) or insulin (0.5 unit/ml.), plus sodium [2-¹⁴C]acetate (2.0 μ C/ml.; 0.073 μ mole/ml.). The sterol-like compound was separated from the total lipids by thin-layer chromatography. The results are the mean values ($\pm$ s.e.m.) of four experiments expressed in counts/min. after 4 and 8 hr. of incubation. The control, NGF and insulin experiments were carried out at the same time under identical conditions.

Sample ...	Control		NGF-treated		Insulin-treated	
	(counts/min./60 ganglia)	(% of total incorporated acetate)	(counts/min./60 ganglia)	(% of total incorporated acetate)	(counts/min./60 ganglia)	(% of total incorporated acetate)
Incubation time (hr.)						
4	5184 $\pm$ 124	22	4801 $\pm$ 103	13	5012 $\pm$ 94	18
8	6496 $\pm$ 215	22	5412 $\pm$ 174	12	5500 $\pm$ 193	15

Table 4. *Effect of NGF and insulin on incorporation of [2-¹⁴C]mevalonic acid into total lipids*

Sensory ganglia from 8-day-old chick embryos were incubated in 1.0 ml. of Eagle basal medium with or without NGF (0.2 μ g./ml.) or insulin (0.5 unit/ml.), plus DL-[2-¹⁴C]mevalonic acid (2.0 μ C/ml.; 0.415 μ mole/ml.). The results are the mean values ($\pm$ s.e.m.) of four separate experiments expressed in counts/min. of mevalonic acid incorporated into the total lipids of 60 ganglia after incubation for 4, 6 and 8 hr. The control, NGF and insulin experiments were carried out at the same time under identical conditions.

Sample ...	Control	NGF-treated		Insulin-treated	
	(counts/min./60 ganglia)	(counts/min./60 ganglia)	(% change)	(counts/min./60 ganglia)	(% change)
Incubation time (hr.)					
4	486 $\pm$ 21	392 $\pm$ 29	-19	405 $\pm$ 21	-17
6	582 $\pm$ 79	407 $\pm$ 47	-30	451 $\pm$ 32	-22
8	848 $\pm$ 60	697 $\pm$ 45	-19	604 $\pm$ 51	-29

insulin on the synthesis of the antimony trichloride-positive compound a time-sequence study on the incorporation of isotopically labelled precursors was carried out. After extraction and separation by thin-layer chromatography of the total lipids of the treated ganglia the radiochromatograms showed three major peaks in solvent system (1) (Fig. 2). Staining with antimony trichloride demonstrated that the blue spot coincided with the peak with R_F approx. 0.3 with both acetate and mevalonic acid as precursors. Moreover, the identity of the

antimony trichloride-positive compound and the acetate- and the mevalonate-labelled material was confirmed by gas-liquid-chromatographic analyses after extraction from the radiochromatograms. The incorporation of sodium [2-¹⁴C]acetate into total lipids was about 40% higher in NGF-treated and in insulin-treated ganglia than in control ganglia after 4 and 8 hr. of incubation, as already noted by Angeletti, Liuzzi & Levi-Montalcini (1966) (Table 2). However, incorporation of radioactivity into the peak with R_F approx. 0.3 in NGF-treated and in

Table 5. *Effect of NGF and insulin on incorporation of [2-¹⁴C]mevalonate acid into the sterol-like compound*

Sensory ganglia from 8-day-old chick embryos were incubated in 1.0 ml. of Eagle basal medium with or without NGF (0.2 μ g./ml.) or insulin (0.5 unit/ml.), plus DL-[2-¹⁴C]mevalonic acid (2.0 μ C/ml.; 0.415 μ mole/ml.). The sterol-like compound was separated from the total lipids by thin-layer chromatography. The columns headed '% of total' indicate mevalonic acid incorporated into the sterol-like compound as percentages of the total incorporation. The results are the mean values ($\pm$ s.e.m.) of four separate experiments expressed in counts/min. after 4, 6 and 8 hr. of incubation. The control, NGF and insulin experiments were carried out at the same time under identical conditions.

Incubation time (hr.)	Sample ... Control		NGF-treated		Insulin-treated	
	(counts/min./60 ganglia)	(% of total)	(counts/min./60 ganglia)	(% of total)	(counts/min./60 ganglia)	(% of total)
4	405 $\pm$ 27	83	317 $\pm$ 11	81	278 $\pm$ 10	70
6	437 $\pm$ 21	87	352 $\pm$ 14	91	282 $\pm$ 10	63
8	662 $\pm$ 41	88	617 $\pm$ 39	89	437 $\pm$ 20	72

insulin-treated ganglia after 4 and 8 hr. of incubation was slightly lower (3–16%) than in the control ganglia (Table 3). The incorporation of DL-[2-¹⁴C]-mevalonic acid into total lipids of NGF-treated and insulin-treated ganglia was markedly lower than into control ganglia after 4, 6 and 8 hr. of incubation (Table 4). The radioactivity incorporated into the sterol-like compound was also diminished by about 25% in the presence of NGF and insulin, but almost all (about 90%) of the incorporated mevalonic acid was present in the sterol-like compound in control and NGF-treated ganglia, whereas the insulin-treated ganglia showed a slightly lower rate of incorporation, about 70%. These incorporation percentages remained at the same level during the three periods of incubation investigated (Table 5).

DISCUSSION

Our results describe the effect of NGF and insulin on a compound that belongs to the non-saponifiable fraction of the lipids. From its colour reactions a steroid structure is postulated for this compound. This hypothesis is substantiated by the observation that 80–90% of the mevalonic acid, a known precursor of steroids (Lynen, Eggerer, Henning & Kessel, 1958), incorporated into the total lipids was found in the sterol-like compound. The effect of NGF in decreasing the incorporation of DL-[2-¹⁴C]mevalonic acid into total lipids by about 25% was paralleled by a similar decrease of incorporation into the sterol-like compound. It must be pointed out, however, that the percentage of the total incorporated mevalonic acid found in this compound was not affected, remaining at 80–90%. This is the first time that an inhibitory rather than stimulatory effect of NGF on the metabolic processes occurring in ganglia has been observed. The effect of insulin is less pronounced, but a decrease of the incorporation of mevalonic acid into

total lipids and into the sterol-like compound was also observed. Earlier Hotta & Chaikoff (1952) reported that the insulin inhibited the synthesis of cholesterol from sodium [2-¹⁴C]acetate in rat liver slices.

It is rather surprising that two proteins like NGF and insulin, which are so different in their chemical and biological properties, elicit similar biochemical effects on ganglia explanted and cultured *in vitro*. This may be accounted for by the fact that the two substances exert their action on different cell types; many observations suggest that NGF acts on the neurons, stimulating the growth of fibres, whereas insulin would be active on the satellite cells of the ganglia, as demonstrated by experiments *in vitro* with explanted ganglia cultured for a long period with this hormone (Levi-Montalcini, 1966).

Our data show that the synthesis of the sterol-like compound was inhibited by NGF and insulin. It is not yet known where this compound is localized in the ganglionic cells, but the fact that 5–10% of the dry weight of the ganglia can be attributed to the compound suggests that it might be an important cell membrane constituent. It is known that the embryonic cells, of which the sensory ganglia are an example, can double their quantity of membranes/hr. (Gross, 1967). The decrease of the sterol-like compound, probably localized in the cell membrane, might be linked to changes of cell permeability or to the differentiation process of the ganglia accelerated by NGF.

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