eosin. Others were fixed for 1 h in half strength Karnovsky's[14] fixative, thoroughly rinsed in 0.08 M cacodylate buffer, and postfixed for 1 h in 1% osmium tetroxide. After fixation, specimens were stained en bloc with 1% uranyl acetate for 1 h, dehydrated in graded ethanol series, and embedded in Spurr's[15] resin. Sections (silver/pale gold) were contrasted with uranyl acetate and lead citrate and examined with a JEM 100B electron microscope.

Results. Our previous studies[8,9,11] showed that PNPs treated with various inducing agents always had the most complicated and well-defined structures (neural tube, notochord, nephric tubules, somite mesoderm, and pulsatile tissue) after 4 days of incubation. Thereafter, many structures apparently underwent dedifferentiation and/or disintegration. By the 10th day only neural tissue and nephric tubules were histologically identifiable. Thus the results described below are based on the examination of PNPs cultured for 4 days.

Of the 42 control PNPs, 41 remained undifferentiated (figure 1). Here, ectodermal cells were more or less rounded with highly vacuolated cytoplasm and a large nucleus compared to the overall cell size (figure 2). The cell surface was relatively smooth; microtubules were few and randomly arranged; microfilaments were not well developed (figure 3). By contrast, 27 out of 58 experimental PNPs acquired the capacity to undergo differentiation in the form of neural tissue (figures 4 and 5). Pulsatile tissue and notochord were observed in 4 of these PNPs. The induced neuroepithelium exhibited morphological characteristics identical to that of stage 10 chick embryos[12]. Briefly, the wall of the neural tube consisted of a pseudostratified columnar epithelium. Adjacent cell membranes were interdigitated; microtubules were numerous and were usually arranged parallel to the cell's long axis; microfilaments were associated into dense bundles (figure 5).

Discussion. The present study showed that cyclic AMP (0.5 mM) could induce differentiation in PNPs which was always manifest by the formation of neural tissue. Deshpande and Siddiqui[10] reported that cyclic AMP, at the same concentration, induced heart-like pulsatile tissues in 74% of the PNPs. Identical structures were also noted in this study, but the frequency of appearance was much lower (about 7%). This discrepancy may be due to difference in culture techniques and/or methods of treatment. Our results, however, were not surprising and agreed with many investigators who reported that cells of the presumptive epidermis or of neuroectodermal origin were relatively sensitive to cyclic AMP as an inducer of cell differentiation[16–18]. Furthermore, PNPs with recognizable structures were usually more compact than those showing no differentiation, suggesting that cyclic AMP induced differentiation in PNPs, at least in part, by promoting cell movements and tissue condensation, events that are considered essential to differentiation in early chick blastoderms[5,19]. Since microtubules are known to be responsible for cell elongation during neurulation[20], the possibility exists that cyclic AMP induces neural differentiation in the presumptive chick epidermis also by stimulating the assembly of microtubules.

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Effect of cholinesterase inhibitors on differentiation of cultured sympathicoblasts

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Summary. The 2 cholinesterase inhibitors, eserine and BW 284 C 51, inhibited the nerve fibre growth and differentiation of immature sympathicoblasts at low concentrations. The effect was nerve cell-specific and appeared in the course of the in vitro development, indicating that cholinesterases might play an important role in the early differentiation of sympathicoblasts.

In addition to acetylcholinesterase activity in the plasmalemma, probably directly connected with nervous transmission, sympathetic neurons also show intracellular acetylcholinesterase, the role of which is not fully understood[1]. The appearance of cholinesterases in the sympathicoblasts early during the differentiation[2–4] suggests that cholinesterases might play a role in the maturation of the sympathetic neurons.

Tissue culture of immature sympathetic ganglia[5] provides an opportunity to evaluate the significance of the cholinesterases by studying the effect of cholinesterase inhibition on the differentiation of sympathicoblasts.

Materials and methods. Lumbar sympathetic ganglia of 7-day-old chick embryos were cultured for 2–7 days in a tricine-buffered medium containing 1 IU/ml nerve growth factor (Burroughs and Wellcome)[5] with and without cholinesterase inhibitors. Eserine (1′-methylpyrrolidino(2′:3′:2:3)1,3-dimethylindolin-5-yl N-methylcarbamate, Merck) or BW 284 C 51 (1:5-bis(4-allyldimethyl ammoniumphenyl)pentan-3-one dibromide, Wellcome Research Laboratories) were added to the cultures in 10^{-6}–10^{-3} M concentrations for the whole culture period beginning at the time of explantation. The acute effect of the inhibitors was tested by 24-h-treatment. Phase-

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contrasts examination and photography of the living cultures was performed with a Wild microscope and Kodak Tri-X-pan film. Altogether 179 cultures were used in the study.

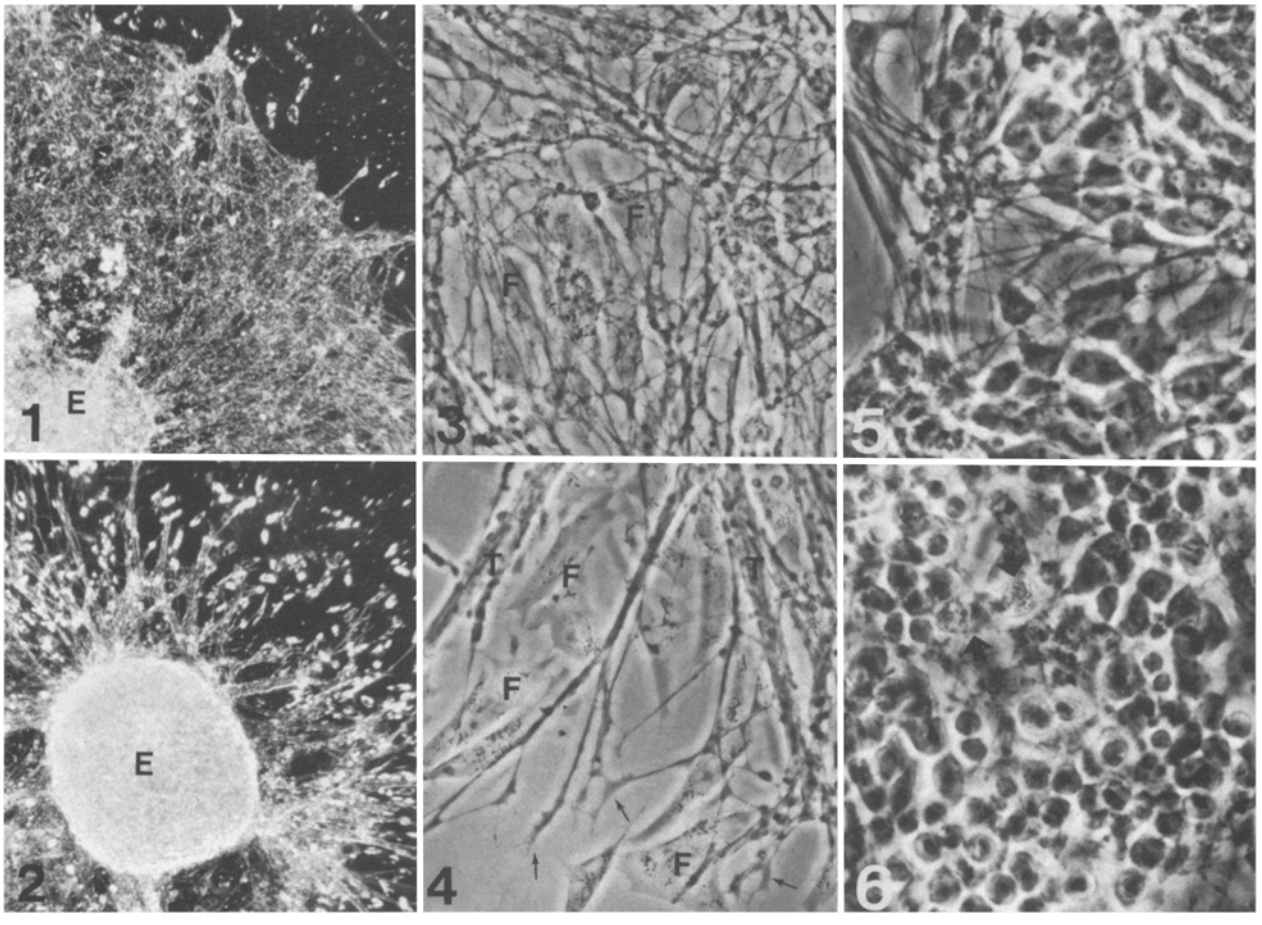
Results. Eserine at a concentration of 10^{-6} M had no effect on either fibre growth and formation of the nerve plexuses (figures 1 and 2) or on the increase in the diameter of the perikarya (figure 5). At concentrations of 10^{-5}–10^{-4} M a concentration-dependent decrease was observed in the number and extent of the nerve fibres. The growth of the nerve fibres was severaly inhibited by 10^{-4} M eserine (figure 2) as compared to the controls (figure 1), and the nerve plexuses showed abnormal morphology (figures 3 and 4). The growth of the sympathicoblast perikarya was also inhibited and signs of degeneration were widespread. Sympathicoblasts in small clusters were distinctly more severaly affected than those in the larger and thicker explants. Concentrations higher than 10^{-4} M of eserine proved to be lethal for the cultures within a few days.

The other inhibitor, BW 284 C 51, caused changes in the maturation in culture parallel to those induced by eserine, but was effective at somewhat lower concentrations. At 10^{-5} M concentration only a few nerve fibres were observed between the nerve cell perikarya, which themselves were markedly smaller (figure 6) than those in the control cultures (figure 5). The 10^{-4} M concentration of BW 284 C 551 caused total inhibition of fibre growth and degeneration of the cultures within 3 days.

The 24-h-treatment of mature cultures with 10^{-5} or 10^{-4} M of either inhibitor had no acute effect on the sympathetic neurons as observed by light microscopy. Neither of the inhibitors, even at the highest concentration, seemed to affect sympathicoblasts during the first 12 h after explantation.

The non-neural, mesenchymal elements in the cultures showed neither vacualization nor degenerative granulation when cultured with the inhibitors (figure 4).

Discussion. The possibility cannot be disregarded that some previously unknown effect of the inhibitors not related to cholinesterase inhibition might be responsible for the changes observed in the present study. However, although the concentration, at which eserine distinctly interfered with the growth and differentiation of the sympathicoblasts is about 10 times higher than that needed for total inhibition of cholinesterases in fixed tissues or in tissue homogenates[1] it is still about $^1/_{100}$ of that



Figures 1 and 2 are dark-field photomicrographs (×55) and figures 3–6 are phase-contrast photomicrographs (×275) showing 3-day-old cultures of sympathetic ganglia of 7-day-old chick embryos.

Fig. 1. Nerve fibre growth from an explant (E) in a control culture.

Fig. 2. Poor and uneven fibre growth from a large explant (E) in a culture with 10^{-4} M of eserine.

Fig. 3. Plexus of nerve fibres in a control culture. Flat mesenchymal cells (F) are observed underneath the nerve fibres.

Fig. 4. Nerve fibres form short and thick trunks (T) with only a few individual fibres with growth cones (arrows) in a culture with 10^{-4} M of eserine. Note the normal morphology of the flat mesenchymal cells (F).

Fig. 5. Sympathicoblasts and nerve fibres in a control culture.

Fig. 6. Sympathicoblasts in a culture with 10^{-5} M of BW 284 C 51. The perikarya are rounded and smaller than in controls. Degenerative signs are observed (arrows). Nerve fibres are not as noticeable as in control cultures.

causing inhibition of non-choline esterases[6,7]. This indicates that, at a concentration of 10^{-4} M, it is specifically cholinesterase that is inhibited, suggesting that the higher concentration would be needed for the penetration of the inhibitor through the intact cell membrane and for the maintenance of an effective intracellular concentration of the inhibitor broken down by cholinesterases[8].

The 2 inhibitors used in the present study caused identical effects in the differentiating sympathicoblasts, thus further emphasizing the role of acetylcholinesterase inhibition as the basic cause for the changes.

The mesenchymal elements in the cultures presented an interesting opportunity to compare the effects of the inhibitors on sympathicoblasts and non-neural cells in the same culture. The effects proved to be neuron-specific. Moreover, is seemed not to be a question of an acute toxic influence of the inhibitors on nerve cells, but of an inhibition of growth and differentiation of sympathicoblasts appearing in the course of development in culture. The results of the present study strongly suggest that cholinesterases play a significant role in the early differentiation of the sympathicoblasts.

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Lysosomal enzyme release associated with the invasion of rat liver by Novikoff hepatoma

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Summary. The lysosomes of both Novikoff hepatoma and liver from Novikoff hepatoma-bearing rats were found to be relatively intact structurally, lower in acid phosphatase activity, greatly depleted in number but with nearly normal membrane integrity when compared with normal liver.

Mounting evidence indicates that lysosomal enzyme release may accompany malignant growth and invasion. On the basis of enzyme activity studies performed on mouse interstitial tumor fluid, Sylvén has postulated that leakage of active lysosomal enzymes, from tumor and/or host, cells, may occur[2]. Pure lines of cultured tumor cells release or secrete enzymic proteins[3] and Carević et al. have demonstrated that acid phosphatase release accompanies the course of progressive infiltration of the liver by leukemia[4]. Our objective was to characterize release possibly associated with the solid form of Novikoff hepatoma (an extensively documented, highly invasive tumor[5]), by comparing the levels of the lysosomal marker, acid phosphatase, in control liver with the levels in tumor and tumor-invaded rat liver. These activities were correlated with lysosomal permeability, to provide a measure of membrane leakage. Finally, a histochemical approach enabled us to correlated the distribution of lysosomes in tumor, tumor-invaded liver and normal liver with the cellular distribution of acid phosphatase activity.

Materials and methods. Male Sprague-Dawley-Holtzman rats weighing 250–350 g were provided with Purina Lab Chow and water ad libitum. Over a period of 11 weeks, Novikoff hepatoma (kindly provided by Dr Ernest Borek,

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Free, total and unsedimentable acid phosphatase acrivities in whole liver homogenates of normal and Novikoff hapta-bearing rats and in whole tumor homogenates. Lysosomal membrane permeability to substrate (alpha-napthol acid phosphate) and to acid phosphatase for the same 3 tissues

Tissue homogenate	Acid phosphatase activity (μmoles P/15 min/g wet weight)			Lysosomal membrane permeability	
	Free[a]	Total[b]	Unsedimentable[c]	Enzyme[d] (acid P'ase)	Substrate[e] (γ-napthol P)
Control liver*	1580 ± 241 (44)	2070 ± 292 (44)	60.3 ± 6.9 (29)	2.91 ± 0.53	75.5 ± 16.3
Liver of rat bearing Novikoff hepatoma*	423 ± 38 (48)	552 ± 34 (49)	46.7 ± 12.1 (29)	8.5 ± 2.3	74.6 ± 9.6
Novikoff hepatoma**	60.5 ± 3.2 (51)	90.0 ± 4.5 (51)	9.76 ± 1.2 (26)	10.8 ± 1.4	63.2 ± 5.6

Values for enzyme activity are computed as means ± SEM. Numbers of experiments in parentheses. * 1 test animal utilized in each experiment. ** Duplicate experiments performed on each test animal. [a]) Free activity – enzyme activity in 0.25 M sucrose homogenate; [b]) total activity – enzyme activity following homogenization in 0.25 M sucrose containing 1% Trition X-100 detergent, to cause complete release of enzyme activity from the lysosomes; [c]) unsedimentable activity – the 0.25 M sucrose homogenate was centrifuged at 270,000 × g for 1 h, and the enzyme activity was measured in the supernatant fraction. [d]) The ratio of unsedimentable to total activity (expressed in percent) gives the lysosomal membrane permeability to acid phosphatase. [e]) The ratio of free minus unsedimentable activity to total minus unsedimentable activity (expressed in percent) gives the lysosomal membrane permeability to γ-napthol acid phosphate.