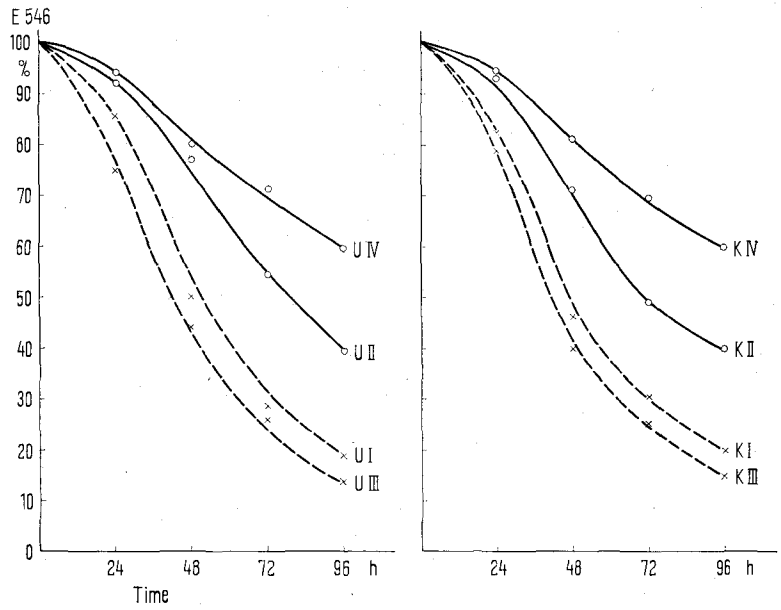

Decrease of an initial NBP-extinction of 8000/ml (=100%) in buffer solutions containing the major urine-metabolite of cyclophosphamide (U), respectively, the product of in-vitro oxidation (K). Incubation-temperature 37 °C. I and II ethylene-diamine-acetate buffer (I pH 6,15; II pH 7,15). III and IV: Sörensen phosphate buffer (III pH 6,2; IV pH 7,2).

R_{st}-values of the major urine metabolite of cyclophosphamide and a cyclophosphamide derivative received by $KMnO_4$-oxidation. Reference substance: Cyclophosphamide

System	I	II	III	IV
Urine metabolite	0.46	0.70	0.52	0.45
$KMnO_4$-product	0.46	0.70	0.52	0.44

Zusammenfassung. Nach Oxidation von Cyclophosphamid mit $KMnO_4$ lässt sich eine Verbindung gewinnen, die dünnschichtchromatographisch und reaktionskinetisch mit dem vorherrschenden Urinmetaboliten übereinstimmt. Es handelt sich um N,N-bis(2-chloräthyl)diamidophosphorsäure(2-carboxyäthylester). Da weder diese Verbindung noch das bereits als Nebenmetabolit bekannte 4-Keto-cyclophosphamid zelltoxisch wirkt, muss der Effekt des Cyclophosphamids von einem anderen Stoffwechselprodukt ausgehen. Durch modifizierte oxidative Umsetzung in vitro sollte es möglich sein, Cyclophosphamid auch in den wirksamen Metaboliten zu überführen.

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Choline Acetyltransferase and Acetylcholinesterase in Spinal Motor Neurons Cultured in vitro

Recent tissue culture studies on central nervous tissue have demonstrated the continued differentiation and maturation of the tissue in vitro evidenced by formation of synapses and myelin sheaths[1,2]. The development of synaptic connections in tissue culture was observed by silver impregnation method[3,4] and by electron microscopy[2,5]. The operation of functional synapses in such cultures was also demonstrated by the neurophysiological method[6]. Since the presence of acetylcholinesterase (AChE) in central nervous structure is regarded as an indication of cholinergic transmission in that area[7], we have studied previously the localization of AChE in neurons of mouse cerebellum and in motor neurons of chick spinal cord cultured in vitro[8,9]. The presence of acetylcholine (Ach) or choline acetyltransferase (ChAc) is generally regarded as being a more reliable indicator of cholinergic mechanisms than is the presence of AChE[7]; hence, it is more desirable to demonstrate ChAc activity in neuronal elements of the cultures in order to provide further evidence of the operation of cholinergic transmission in central nervous tissue maintained in vitro.

Cross sectioned explants from cervical level of 10–12-day chick embryo spinal cords were placed on collagen coated coverslips and maintained in Maximow's slides. The feeding medium composed of equal parts of horse serum, Hanks' balanced salt solution (BSS) and medium-199. Supplementary glucose was added in the final concentration of 600 mg percent. Cultures were incubated at 36 °C for 7–180 days. Twice a week the cultures were washed briefly in BSS and fed with fresh feeding medium.

For the histochemical demonstration of ChAc activity, Kása, Mann and Hebb's method[10] was used in modified conditions. The incubating mixture contained the following: acetyl CoA, 0.3 mM; choline chloride, 10.0 mM; lead nitrate, 0.3 mM; cacodylate buffer (pH6.0), 33.0 mM; and BW 284C51, 0.01 mM. The inclusion of BW 284C51, a specific inhibitor for AChE, is to prevent the hydrolysis

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of acetyl CoA by AChE in the tissue. Without fixation, cultures were incubated in substrate medium at 36 °C for 2 h, treated with weak ammonium sulfide solution, post-fixed in formol-calcium for 5 min and mounted on slides with gum syrup. Control cultures were incubated in medium in which substrates acetyl CoA and choline chloride were omitted and the enzyme reaction was always negative.

Sister cultures were also prepared for the histochemical demonstration of AChE using the method of KARNOVSKY and ROOTS[11]. Tetraisopropyl pyrophosphoramide (Iso-OMPA) was included in the incubating mixture in the concentration of 0.001 m*M*. After fixation in ice-cold formol-calcium for 5 min and subsequent rinsing in distilled water, cultures were incubated in the medium for 2 h at 36 °C.

In order to provide morphological evidence of the presence of synapses, sister cultures were also stained with a modification of Bodian's protargol method[12], and other cultures embedded in Epon were examined with Philips 200 electron microscope[5].

The general account of chick spinal cord cultures has been described previously[13, 14]. Myelin formation usually starts 6–8 days in vitro and reaches its peak by 14–16 days in vitro. Large neurons are found consistently in presumptive anterior and lateral horn regions. After silver impregnation numerous multipolar neurons are found in those areas with fine neuro-fibrillary networks extending throughout the perikarya, dendrites and axons (Figure A). Terminal boutons as fine neurofibrillary rings are also seen on or near the neuronal perikarya and dendrites (Figure B). Electron microscopic examination reveals the first occurrence of immature synaptic structures in 3-day-old cultures, and by 12–14 days in vitro there are numerous well developed synapses with typical junction contacts and synaptic vesicles (Figure C). Both axosomatic and axodendritic synapses are observed.

ChAc activity is observed mainly in large motor neurons (Figure D). The reaction products are demonstrated as fine granules located throughout the perikarya while dendrites have no reactions. Neuroglia cells are always negative for enzyme activity. Enzyme activity in axons is also indicated by linear alignment of fine reaction-positive granules near the neurons (Figure D).

AChE activity, as reported previously[8], is demonstrated both in large anterior horn neurons and smaller dorsal horn neurons. Large anterior motor neurons reveal intense granular reaction in the perikarya and less intense reactions in dendritic processes (Figure E). Sometimes moderate reaction is encountered in axonal processes. Two variations of enzyme stain are observed among the large motor neurons. In most of the motor neurons AChE activity is localized intracellularly. In some others, however, the enzyme activity is found lining the surface of perikarya and dendrites devoid of any intracellular enzyme reaction. We tentatively suggest that the former may represent cholinergic neurons and the latter cholinoceptive neurons[7].

Histochemical demonstration of ChAc and AChE activity in spinal neurons thus provides a favorable evidence for the operation of cholinergic transmission mechanism in neurons of central nervous tissue cultured in vitro.

Zusammenfassung. Die Enzymaktivität der Cholinacetyltransferase und Acetylcholinesterase wurde an Nervenzellen des Rückenmarks von Hühnerembryonen in Gewebekultur mit histochemischer Methode untersucht, wobei die Enzymaktivität sowohl an der Zelloberfläche wie auch an den Dentriten nachzuweisen ist.

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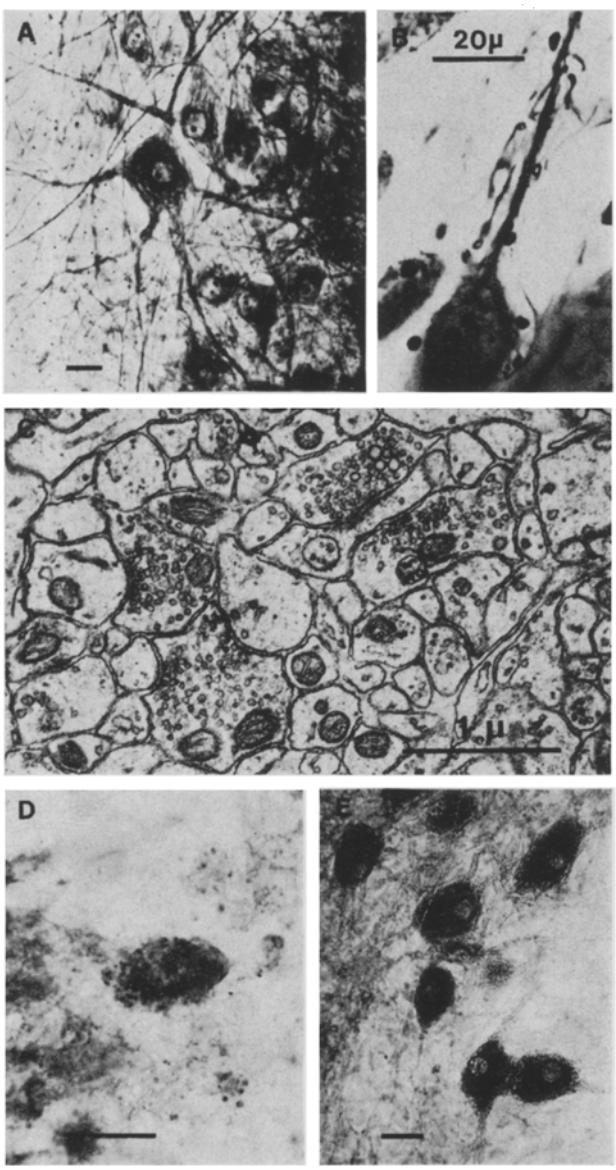

A) Large motor neurons in a presumptive anterior horn region. Bodian stain. 14 days in vitro. B) Numerous terminal boutons surrounding a neuronal perikaryon and its main dendrite. Bodian stain. 14 days in vitro. C) Several axodendritic synapses are shown in this electronmicrograph. 14 days in vitro. D) Choline acetyltransferase activity is demonstrated in fine granular reactions in perikaryon of motor neuron. Nucleolus has intense reaction. 2 h incubation. 14 days in vitro. E) Acetylcholinesterase activity in motor neurons in a presumptive anterior horn region. 2 h incubation. 14 days in vitro. Bars in Figures A), B), D) and E) represent 20 μm.

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