nicotine with a short latency and then a second cell which presumably had a higher threshold commenced to discharge. Dihydro-β-erythroidine (DHE) was then applied by a current of 120 nA for 90 sec. The Figure (B) recorded shortly after the cessation of this application demonstrates that while glutamate excitation was unimpaired the cell was now no longer discharging spontaneously, and was not excited by either acetylcholine or nicotine. 15 min later (Figure C) the cell with the smaller spike had resumed discharging spontaneously and could be excited by acetylcholine and nicotine. The second cell remained in an inexcitable state for a further 60 min.

Atropine and to a lesser extent gallamine, mecamylamine and *d*-tubocurarine also antagonized the excitant actions of acetylcholine. The anticholinesterases, neostigmine and BW. 284C 51 exhibited powerful excitatory actions, in many instances being almost as active as acetylcholine. Such a marked excitant action was not shown by these compounds on either Renshaw or Betz cells[7,9] and it is tempting to suggest that this action may indeed have been due to inactivation of cholinesterase, rather than a direct effect.

In conclusion, therefore, it is clear that acetylcholine has a marked excitant action on cells in the cerebellar cortex of the cat. It seems likely that these cells belong to the granule cell layer. The pharmacological findings described indicate the possibility that acetylcholine may be mimicking the action of a naturally occurring synaptic transmitter. That acetylcholine or related compound may have a functional role in synaptic transmission is suggested by the pronounced potentiating action of anticholinesterase drugs and depressing action of DHE on spontaneous firing.

Résumé. Nous avons examiné directement 562 cellules du cervelet du chat par la méthode iontophorétique. Dans les chats anesthétisés par Dial, 9% des cellules étaient excitées par l'acétylcholine ou les substances cholinomimétiques, mais dans les chats anesthétisés par pentobarbital, la proportion était de 39%. La distribution des cellules responsives correspondait à la distribution des cellules de la couche granulaire. L'action de l'acétylcholine et l'activité spontanée étaient bloquées par la dihydro-β-erythroïdine.

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[9] D. R. CURTIS, J. W. PHILLIS, and J. C. WATKINS, J. Physiol. *158*, 296 (1961).

The Distribution of Cholinesterase in Cat Cerebellar Cortex

The experiments described in the preceeding paper[1] have made it necessary to extend previous studies on cerebellar cholinesterase[2–4], which dealt with vertebrate species other than the cat. Moreover, histochemical studies may be used as valuable adjuncts to such pharmacological studies, in localizing both cells and fibre tracts which may be involved in cholinergic synaptic transmission[5,6]. A detailed study of the distribution of cholinesterase in the rabbit, rat and guinea-pig[4] has led to the postulate that transmission at synaptic junctions between granule cell axons, Purkinje cell dendrites and stellate cells in the molecular layer is mediated by acetylcholine, as is transmission between granule cells and Golgi cells in the granule cell layer. Possession of similar detailed information for the feline cerebellar cortex would obviously be of considerable value in any pharmacological investigation.

Preliminary experiments were carried out on homogenized cerebellar cortical tissue to ascertain the relative proportions of acetylcholinesterase and pseudocholinesterase and also to determine what concentration of the specific inhibitors for the two enzymes should be used in the histochemical procedures. The cats were prepared by opening up the thoracic cavity after anaesthetization with pentobarbital sodium and perfusing 400 ml of 0.9% saline solution through the ascending aorta after occlusion of the descending aorta. In cats that were to be used for histochemical procedures this was followed by 400 ml of 10% formol saline to fix the brain *in situ*. A cerebellum was only accepted for further study if the perfusion had been completely successful in removing all blood from blood vessels in the tissue, thus avoiding any complications arising from erythrocyte and plasma cholinesterase.

Table illustrates the relative rates of hydrolysis of four substrates by cat cerebellar cortex cholinesterase and the degree of inhibition of this enzyme by three inhibitors. The enzyme hydrolyses acetyl-β-methylcholine but not

Substrate	Relative activity	Inhibitor	Pre-incubation time (min)	I_{50} (μM)
Acetylcholine	100	284 C 51	–	1.8×10^{-2}
Acetyl-β-methylcholine	50	*iso* ompa	45	10^{3}
Butyrylcholine	20	DFP	120	6.3×10^{-1}
Benzoylcholine	2.5			

Substrate and inhibitor specificity of cat cerebellar cholinesterase. The cortex from cat cerebellum was homogenized in Krebs bicarbonate buffer and diluted to 4 mg wet weight per ml. The homogenate was pre-incubated at room temperature with *iso* ompa and DFP for the times indicated. All subsequent measurements were carried out manometrically at 37°C, using acetylcholine as substrate.

[1] I. McCANCE and J. W. PHILLIS, Exper. *20*, 000 (1964).
[2] A. S. V. BURGEN and L. M. CHIPMAN, J. Physiol. *114*, 296 (1951).
[3] G. B. KOELLE, J. comp. Neurol. *100*, 211 (1954).
[4] M. A. GEREBTZOFF, in *Cholinesterases, a Histochemical Contribution to the Solution of Some Functional Problems* (Pergamon Press, London 1959).
[5] C. C. D. SHUTE and P. R. LEWIS, in *Histochemistry of Cholinesterase*, Symposium Basel (1960); Bibl. anat. *2*, 34 (1961).
[6] K. KRNJEVIC and J. W. PHILLIS, J. Physiol. *166*, 296 (1963).

appreciably butyryl- or benzoyl-choline and it is strongly inhibited by BW 284 C51, a specific inhibitor of acetylcholinesterase. In addition it is not markedly inhibited by *iso* ompa, a specific inhibitor of pseudocholinesterase[7]. Using acetylcholine as substrate at $6 \times 10^{-2} M$ the activity is only 55% of that at $6 \times 10^{-3} M$ showing that the enzyme is inhibited by excess substrate.

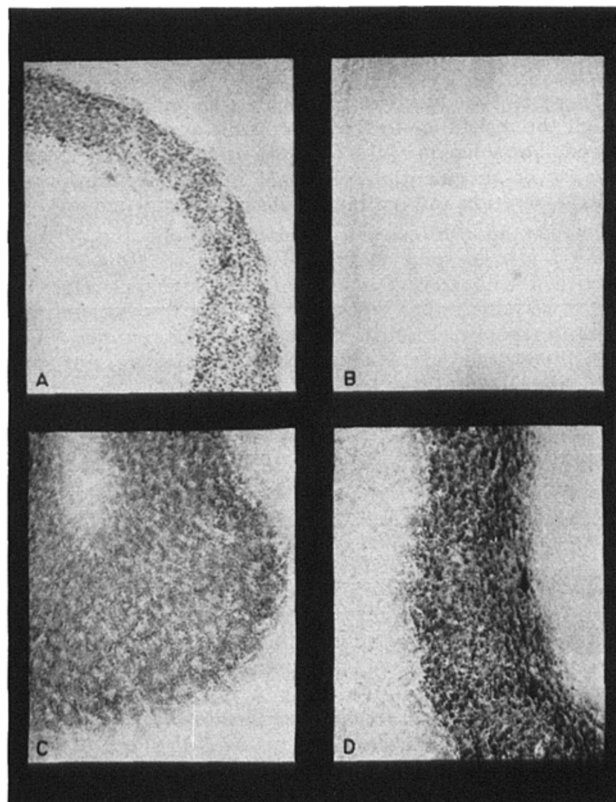
The relative rates of hydrolysis of the substrates, the degree of inhibition by the inhibitors and the inhibition by excess substrate all indicate that the enzyme present is almost all acetylcholinesterase.

This finding was confirmed by the histochemical investigations, using acetylthiocholine and butyrylthiocholine as substrates and di*iso*propyl-phosphorofluoridate (DFP) and BW. 284C51 as specific inhibitors. After perfusion with 10% neutralized formol saline solution the brains were removed and placed immediately in a refrigerator. After storage for 6–8 h in 10% neutralized formol saline solution to allow a more thorough fixation, specimens were selected from each cerebellum and 25 μ sections cut on the freezing microtome. Staining was then accomplished using the routine histochemical techniques[8,9]. DFP-treated sections were placed in a solution containing inhibitor at the appropriate concentration for 2 h and then processed routinely. When the reversible inhibitor of acetylcholinesterase, BW.284C51, was used the inhibitor was also included in the incubation solution. The Figure (A) illustrates a section of cerebellar cortex incubated after inhibition with DFP (5.4 μ*M*). This concentration of inhibitor was used to ensure the complete inhibition of any pseudocholinesterase present (it would also have caused a 70% inhibition of the acetylcholinesterase); however, cells in the granule cell layer continued to stain fairly densely. After treatment with both DFP (5.4 μ*M*) and BW.284C51 (18 μ*M*) all activity had disappeared (Figure B). Previous biochemical investigations indicated that this concentration of the specific inhibitor caused a 94% inhibition of acetylcholinesterase activity.

Sections incubated in a medium containing butyrylthiocholine failed to demonstrate any evidence of deposits.

The Figures (C and D) demonstrate another interesting finding. The granule cell layer in C was in close approximation to the surface of the cerebellum and stained only lightly whereas that in D which was located several millimeters deeper in the cerebellum stained very densely. This finding may bear a considerable relationship to the discovery reported in the previous paper that acetylcholine-sensitive neurones are more frequently encountered in the deeper layers of the cortex. Sections from the anterior, posterior, paramedian, lateral and nodular lobes of the cerebellar cortex all demonstrated a similar pattern of staining. In no area were we able to demonstrate an organized pattern of cholinesterase staining cells or fibres other than in the granule cell layer.

In summary therefore it has been shown that the feline cerebellar cortex contains little or no pseudocholinesterase, whilst its acetylcholinesterase is concentrated in the granule cell layer. There appears to be a relatively well-defined network of fibres travelling along the deep surface and extending into the granule cell layer especially in the case of the deeper layers. Such a network of fibres may well serve to synchronize the activity of cells within this layer.



Frozen (25 μ) sections of the cat cerebellar cortex, showing the acetylcholinesterase reaction given by cells in the granule cell layer. All sections demonstrate the full sequence of structural layers present in the cerebellar cortex. A: Section inhibited by DFP (5.4 μ*M*) to prevent any actions of pseudocholinesterase. B: Section treated with DFP (5.4 μ*M*) and BW. 284 C 51 (18 μ*M*) to inhibit all cholinesterase activity. C and D illustrate the differences in staining density between superficial (near surface) and deep granule cell layers. All sections were incubated for 2 h (× 100).

Résumé. Les types de cholinestérase et leur distribution dans le cervelet du chat ont été étudiés à l'aide d'un manomètre et d'une technique histochimique.

Nos recherches démontrent que l'enzyme est presque exclusivement l'acétylcholinestérase et qu'elle est restreinte dans sa distribution à la couche des cellules granulaires du cortex du cervelet. Il y a des indices qu'un réseau des fibres se trouve sur la surface profonde et s'étend par la couche des cellules granulaires.

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October 31, 1963.

[7] L. Austin and W. K. Berry, Biochem. J. *54*, 695 (1953).

[8] G. B. Koelle and J. S. Friedenwald, Proc. Soc. exp. Biol. Med. N.Y. *70*, 617 (1949).

[9] B. Holmstedt, Acta physiol. scand. *40*, 331 (1957).