Acta histochem. Bd. 22, S. 40—45 (1965)



Department of Anatomy and Histology, University Medical School Szeged, Hungary
(Head: Prof. Dr. A. GELLÉRT)

# A new modification of the Koelle-Friedenwald method for the histochemical demonstration of cholinesterase activity

By F. JOÓ, G. SÁVAY and B. CSILLIK

With 8 figures in the text
(Received January 19, 1965)

The acetylthiocholine method described originally by KOELLE and FRIEDENWALD (1949; 6) as well as its numerous modifications (see PEARSE 1961) are widely used for the histochemical demonstration of cholinesterase activity. In the dimensions of light microscopy this method is undoubtedly the most suitable for the cytological localization of this enzyme. However, as apparent from COUTEAUX's (2) studies the weak electron density of the end product (copper sulphate) is a major disadvantage in the direct applications of this method for electronhistochemical purposes. To obtain a more electron opaque end product BIRKS and BROWN (1) attempted to substitute silver sulphate for copper in the incubation solution. Although the electron density of this precipitate in the junctional area proved to be enhanced, yet the cytological localization was incomplete. KARNOVSKY and ROOTS (4) introduced potassium ferricyanide into the incubation solution to obtain HATCHETT's brown (copperferricyanide precipitate) as an end product of the reaction, to make it suitable for electronhistochemical purposes.

In our first experiments we studied the light microscopic resolving power of the KARNOVSKY modification. The acetylcholinesterase activity of the motor end plates, as revealed by this method, shows a very fine cytological localization. Therefore we attempted further modifications of the KOELLE technique by means of introducing lead ions that might result at least theoretically in the most electron dense reaction product.

Samples were fixed in 4% neutral formalin at 4°C for 3 hours. The best results were obtained (in the motor end plates) whith the following incubation solution:

| | | |
|---|---|---|
| Acetylthiocholine | | 5 mg |
| Sodium acetate | 0,1 N | 2,5 *ml* |
| Acetic acid | 0,1 N | 0,3 *ml* |
| Lead nitrate | 0,03 M | 0,5 *ml* |

Frozen sections were incubated at 4°C for 5—10 minutes.

The cholinesterase activity of the subneural apparatus of the motor end plates incubated in this solution appeared in a fine cytological localization (Fig. 1). Unfortun-

ately when dehydrating the sections, the end product has been dissolved. Therefore, this procedure seemed unsuitable for electron microscopic purposes. It was found however that, by precipitating the sections in a 2% ammonium polysulphide solution the end product could be stabilized to withstand dehydration (Fig. 2). Similar results were obtained in frog muscles, too; here the precipitate outlined the brushes of KÜHNE i. e. the postsynaptic membrane of the junctions (Fig. 3).

When using this incubation solution for the demonstration of cholinesterase activity of the rat cerebellum the activity was confined to archicerebellar areas (nodule and lower uvula), albeit in the form of a very rough precipitate. Therefore we tried to substitute lead nitrate with lead acetate (in the amount of 0.5 m*l*, 0.013 M). Sections incubated in this solution showed not only the regional differentiation (3) but also a sufficient localization of the enzyme, confined to the parenchyma islets of the granular layer (Fig. 4). Also in the cerebellar cortex of the cat where, according to our previous studies (5) the parenchyma islets of the granular layer show an overall uniform cholinesterase activity, this activity could be demonstrated in a fine cytological localization (Fig. 5 and 6). Furthermore, also the ontogenetical variations of acetylcholinesterase activity in PURKINJE cells demonstrated by KÁSA and collaborators in our laboratory could succesfully be demonstrated by means of this technique (Fig. 7). The enzyme activity could be inhibited with eserine ($10^{-4}$ M) or by a preincubation in BW 284 C 51 ($10^{-4}$ M).

Finally, also the pseudocholinesterase activity of the capillaries of the rat central nervous system could be shown by means of this technique when using butyrylthiocholine as substrate (Fig. 8). Enzyme activity appears in a periodical manner on the capillary wall. Preincubation with Mipafox ($10^{-6}$ M) inhibits this enzyme activity.

With regards to the specificity of this modification the question arises whether or not the lead salts in the incubation solution might interfere with the so-called "lead-reactive material" described by SÁVAY and CSILLIK (7). Since however, there can no "lead-reactive material" be demonstrated after formalin fixation, the reaction found in our sections, obtained from formalin fixed material, can not be looked upon as a "lead reactivity". This is evident also from the fact that in sections incubated without substrate (acetylthiocholine) there could no lead sulphide precipitate be found even after prolonged incubation. Finally the studies performed with specific enzyme inhibitors prove unequivocally that the reaction obtained by the modified KOELLE technique actually corresponds to cholinesterase activity.

It appears, therefore, that the lead combination of the KOELLE-FRIEDENWALD method is capable of demonstrating the tissue localization of cholinesterase in light microscopic dimensions. The presence of a highly electron dense ion in the end product is promising in the terms of electronhistochemistry. Studies for this purpose are in progress.

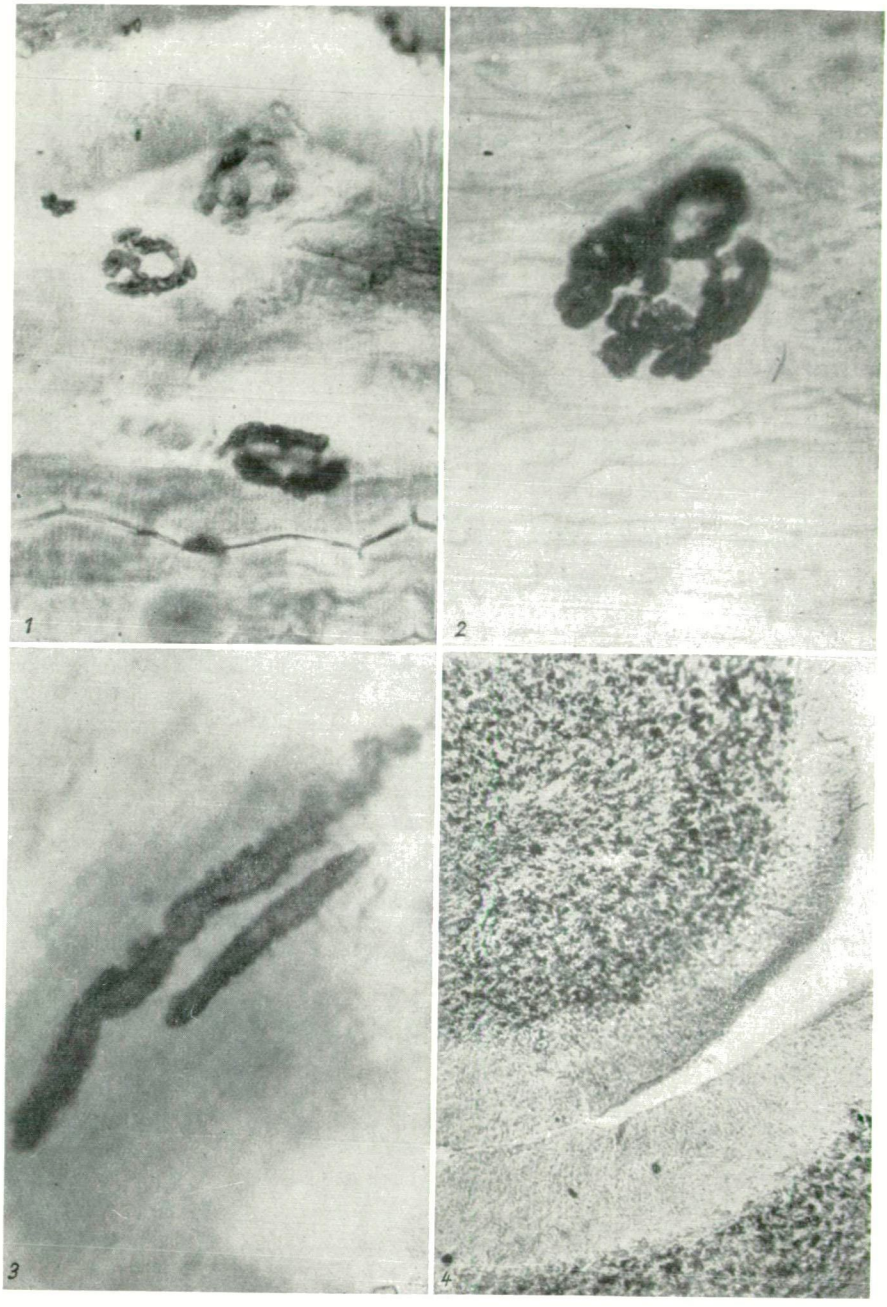


Fig. 1—4

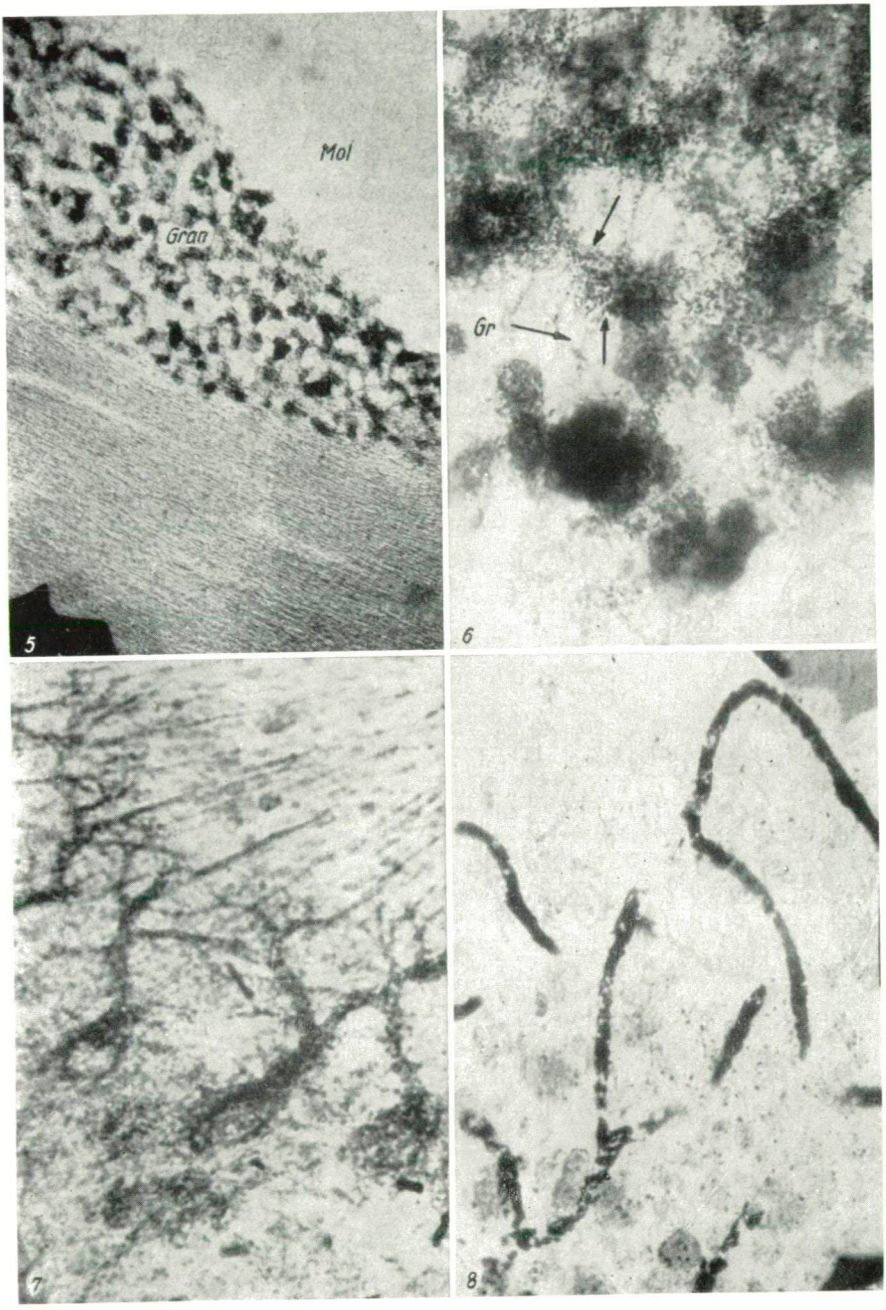


Fig. 5—8

Fig. 1. Acetylcholinesterase (AChE) activity of the subneural apparatus of the motor end plates. Frozen section of the rat's diaphragm, incubated in the lead-substituted acetylthiocholine solution. No post-treatment with polysulphide. ×360.

Fig. 2. The same as Fig. 1, but post-treated with yellow ammonium sulphide, dehydrated in an alcohol series and mounted in Canada balsam. ×265.

Fig. 3. AChE activity of the motor end plates in the iliofibular muscle of the frog. Technique as in Fig. 2. ×990.

Fig. 4. Archicerebellum of the rat. Frozen section incubated in the lead-substituted acetylthiocholine solution, but using lead acetate in lieu of lead nitrate. The activity is confined to the mossy fiber apparatuses. ×180.

Fig. 5. Cerebellar cortex of the cat. Technique as in Fig. 4. Mol: molecular layer (non reacting); Gran: granular layer. ×333.

Fig. 6. The same as Fig. 5. High power view of the granular layer. Note the fine cytological localization of the reaction product in the parenchyma islets (mossy fiber apparatuses). Gr: granule cells (non reacting); arrows point at the enzyme-active axonal expansions of the mossy fiber. ×810.

Fig. 7. AChE activity in the cerebellar cortex of a 6-days old kitten. The activity is confined to the somata and dendrites of Purkinje cells. ×675.

Fig. 8. Pseudocholinesterase activity in the cerebellar capillaries of the rat. Substrate: butyrylthiocholine. ×477.

## Summary

The Koelle thiocholine method has been modified by substituting copper for lead in the incubation solution. This modification yields succesful results in the motor end plates and in cerebellar structures. The modification gives new hope for a succesful electronhistochemical "staining" of cholinesterase activity in the nervous tissue.

## Zusammenfassung

Die Koellesche Thiocholin-Methode wurde dahingehend abgeändert, daß Blei bei der Inkubationslösung durch Kupfer ersetzt wurde. Diese Modifikation ergibt günstige Resultate bei den motorischen Endplatten und bei den Kleinhirn-Strukturen. Diese Abänderung läßt auch erfolreiche elektronen-histochemische „Färbung" der Cholinesteraseaktivität im Nervensystem erhoffen.

## Выводы

Тиохолиновый метод Кэлле модифицирован таким образом, что содержащееся в инкубационной смеси олово замещалось медью. Этой модификацией получают хорошие результаты, пользуясь ею при исследовании моторных концевых пластинок и структур мозжечка. Такое видоизменение позволяет ожидать также успешного электронно-гистохимического «окрашивания» холинэстеразной активности нервной системы.

## Résumé

Les auteurs ont modifié la méthode de thiocholine de Koelle de façon à substituer du cuivre au plomb dans la solution d'incubation. Cette modification donne de bons résultats sur les plaques motrices terminales et sur des structures cérébelleuses. On espère que, grâce à cette modification, on réussira une «coloration» électron-histochimique de l'activité cholinestérasique dans le tissu nerveux.

## Literature

1. Birks, R. I., and Brown, L. M., A method for locating the cholinesterase of a mammalian myoneural junction by electron microscopy. J. Physiology **152**, 5—7 (1960).
2. Couteaux, R., The differentiation of synaptic areas. Proc. Roy. Soc. **158**, 457—480 (1963).
3. Csillik, B., Joó, F., and Kása, P., Cholinesterase activity of archicerebellar mossy fibre apparatuses. J. Histochem. Cytochem. **11**, 1131 (1963).
4. Karnovsky, M. J., and Roots, L., A "direct coloring" thiocholine method for cholinesterases. J. Histochem. Cytochem. **12**, 219—221 (1964).
5. Kása, P., Joó, F., and Csillik, B., Histochemical localization of acetylcholinesterase in the cat cerebellar cortex. J. Neurochem. **12**, 31—35 (1965).
6. Koelle, G. B., and Friedenwald, J. S., A histochemical method for localizing cholinesterase activity. Proc. Soc. exp. Biol. (N. Y.) **70**, 617—622 (1949).
7. Sávay, Gy., and Csillik, B., Lead reactive substances in peripheral synapses. Experientia, **15**, 396 (1959).

Address: Dr. F. Joó, Doz. Dr. G. Sávay and Doz. Dr. B. Csillik, Szeged (Hungary), Department of Anatomy. Kossuth Lajos sugárút 40.