USE OF MICROGASOMETRIC TECHNIQUES IN PHARMACOLOGICAL STUDIES

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CONVENTIONAL histochemical techniques for enzyme localization based on the staining of tissue sections are generally limited by certain sources of error such as: staining nonspecificity, difficulty in localizing the staining product and destruction or partial enzyme inactivation, due to the fixation and imbedding procedure. Moreover they are mostly unsatisfactory for quantitative comparative purposes.

For this reason microquantitative techniques seem to be better adapted to fine metabolic studies at tissue or cellular level. These latter techniques, however, imply a certain degree of technical difficulty. A relatively simple gasometric microtechnique for analysis at the cellular level is the Cartesian diver of Linderstrøm-Lang[1,2] which has been employed during the past 20 years for different biological studies.

This presentation will be limited to illustrations of some of the applications of this technique in the field of neuropharmacology at the cellular level and a brief discussion of the results obtained.

The paper is divided into 6 short sections each one of which summarizes a particular aspect of the employment of the Cartesian diver technique.

1. LOCALIZATION OF ENZYMES IN DIFFERENT TYPES OF CELLS

A detailed study of the acetylcholinesterase (AChE) activity of different types of cells isolated by fresh preparations of the nervous system of the rat[3] demonstrated that the concentration of this enzyme and its specific localization may vary from one cell type to another.

Enzyme activity in anterior horn cell bodies, expressed in $\mu l\ CO_2/\mu^3$, is the same or greater than in the axon, while in spinal ganglion cells the axon has activity 10-100 times greater than the cell body (Fig. 1 parts 1 and 2).

In sympathetic cells (see Fig. 1 part 3) the activity of the cell body is found to be from 1 to 10 times that of the axon. In other words we find in these three types of cells two different distribution patterns: one which we may call type A (higher concentration of the enzyme in the axon) as in the spinal ganglion cells; and the other, type B, (similar

concentration in the cell body and axon or higher in the cell body) as in the sympathetic and anterior horn cells. The parasympathetic cells fit type B.

We tried to explain these differences in terms of functional activity postulating that the enzyme may be present in higher concentrations in those cell bodies which form part of the reflex arc (e. g. motoneurons) and lower in concentration in those which are not integrally in the arc (e. g. spinal ganglion).

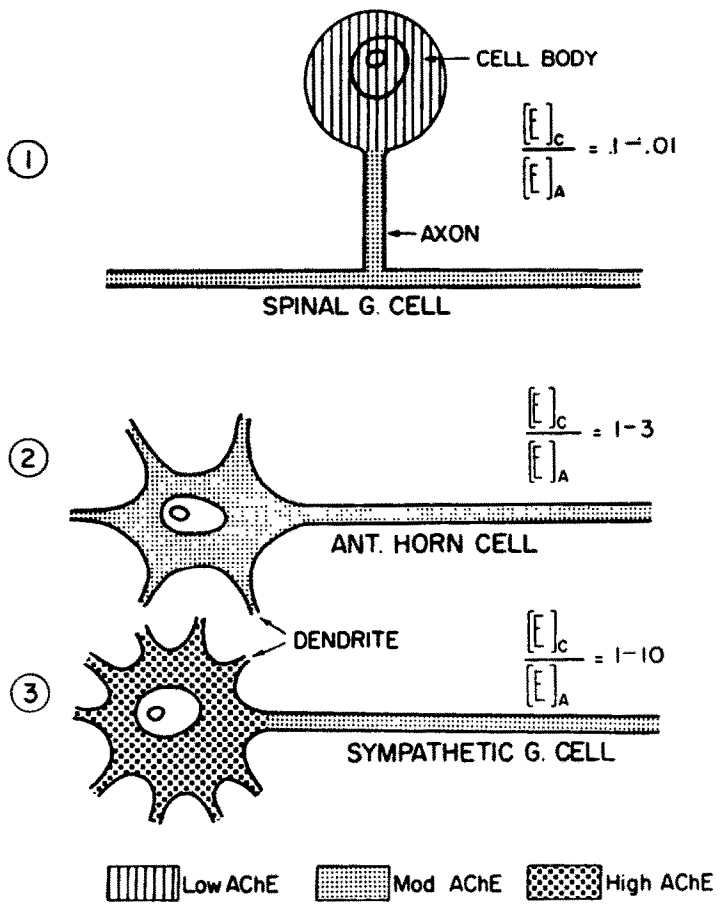


FIG. 1. Schematic representation of the intracellular distribution of AChE in three different types of nerve cells. $[E]_C/[E]_A$ = AChE concentriation in the cell body/AChE concentration in the axon.

Another fact which emerged from this study was that the typical distribution pattern of AChE is constant for a single type of nerve cell. The consequence of these findings is that the ratio $[E]_C/[E]_A$ (enzyme concentration in the cell body/enzyme concentration in the axon) in Fig. 1 may be an expression of functional factors specific for a given type of cell rather than an indication of the site of synthesis of the

enzyme. Furthermore, it can be said that the distribution of the AChE present in the types of cells considered above, seems to support the hypothesis of a somato-axonal convection of the enzyme.

2. DISTRIBUTION OF ENZYMES IN DIFFERENT REGIONS OF THE NERVOUS SYSTEM

The Cartesian diver technique offers the possibility of studying the distribution of an enzyme in very specific regions of the nervous system and to determine whether such an enzyme is equally distributed in the different cells and to detect its variation in the individual cells. We studied, therefore, three different regions of the nervous system of the rat in respect to the content of AChE in the individual cells according to the following plan:

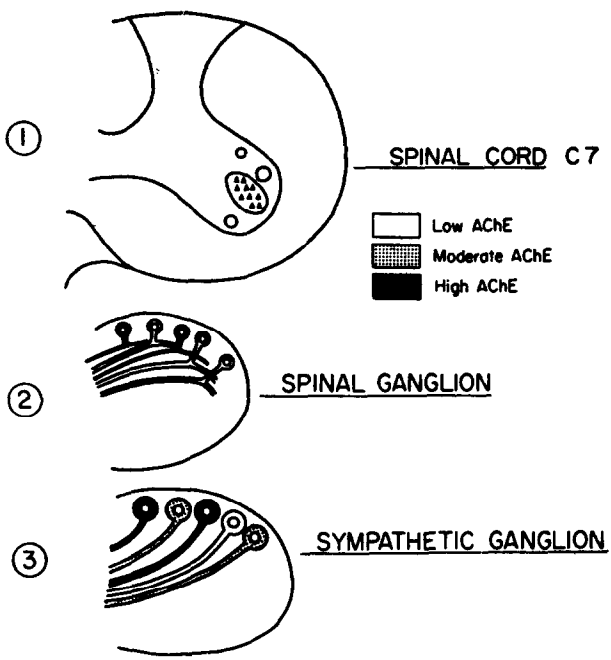


FIG. 2 Schematic representation of the distribution of AChE in three different regions of the nervous system.

(a) We selected a limited group of cells (motoneurons) from the cervical cord of the rat (see Fig. 2 part 1) whose neurites innervate the extensor muscles of the fore limb and found[4] that on the basis of their AChE activity two significantly different groups of cells could be demonstrated. One group of cells showed approximately four times the activity of the other.

Among the different possibilities for explaining these two enzyme levels the most likely seems to be that these neurons may correspond to the two groups of motoneurons: the slowly discharging tonic ones innervating red muscle fibres and the rapidly discharging phasic ones,

innervating white muscle fibres according to the findings of Granit, Henatsch and Steg[5] and Eccles *et al.*,[6] in different muscles of the cat. This could, therefore, represent an example of how a different level of enzyme may be correlated to the functional activity of a cell.

(b) In an earlier histochemical study[7] applied to single cell preparations it was found that in the superior cervical ganglion of the rat the cells could be divided into three groups on the basis of their AChE activity. One group (about 10 per cent) showed high AChE activity while another group (approximately 40 per cent) showed moderate activity and finally the remaining cells (about 40-50 per cent) showed very slight activity or no activity at all (see Fig. 2 part 3). When the activity of similar sympathetic cells was studied quantitatively by means of the Cartesian diver technique they showed wide variations in their AChE activity but they could not be classified into any distinct groups. It could be confirmed, however, that no enzyme activity existed in a certain number of these cells.

The presence of cells in a sympathetic ganglion having different AChE activities (see Fig. 2 part 3) indicates in our opinion two things: first that the cell population of a sympathetic ganglion is probably not so homogeneous from a pharmacological point of view as is generally thought; and second that cholinergic postganglionic fibers may be present in this ganglion as it has been suspected for many years since the first observation of Dale and Feldberg in 1934, regarding the vasodilator nerves of the facial muscles of the cat[8]. More precise and conclusive evidence on this point is lacking however.

(c) In contrast with earlier histochemical findings[9] the Cartesian diver technique showed that most of the cells belonging to the spinal ganglia have, in fact, measurable AChE activity. In a certain number of cells, about 10-15 per cent, however, it was not possible to demonstrate any AChE at all (see Fig. 2 part 2).

These findings may not only explain the small amount of acetylcholine and acetylcholine synthesis which has been found in the dorsal roots but also suggest the possibility that sensory cells of cholinergic type may exist in the spinal ganglia. (For further discussion of this point see section 3).

3. LOCALIZATION OF ENZYMES IN UNITS OF FUNCTIONAL IMPORTANCE (monosynaptic reflex arc)

Fig. 3 shows schematically the components of the reflex arc which can be examined by dissecting each one separately under the microscope and than determining the AChE activity by means of the Cartesian diver technique.

From Fig. 3 it can be seen that the concentration of the enzyme varies in the different constituents of the arc. The anterior horn cells

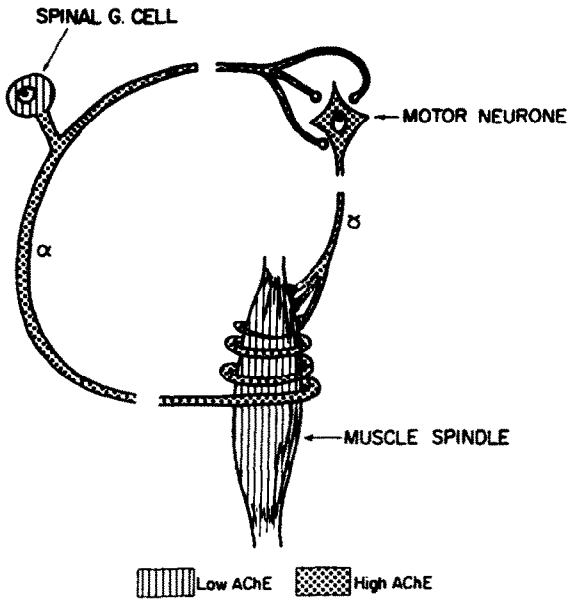


FIG. 3. Schematic representation of the distribution of AChE in a reflex arc.

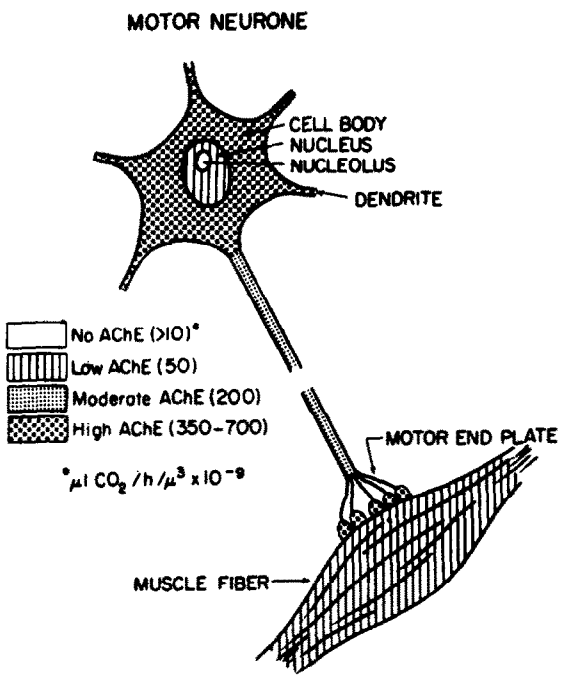


FIG. 4. Diagram of the intracellular localization of AChE in a motoneurone of rat.

show high amounts of AChE in their cell bodies and neurites. This is true for both the large axons (α-efferents) connected to the muscle end plates (see Fig. 4) and for the thin fibers (γ-efferents) innervating the small intrafusal end plates (see Fig. 3). In the large afferents from the muscle spindles not only AChE is found but also non-specific cholinesterase (non spec. ChE). It seems reasonable to assume that these large afferent fibres from the muscle spindles which show high AChE activity are connected with the AChE containing ganglion cells previously described (see section 2).

In other words, the Cartesian diver results indicate that we are dealing with a AChE containing reflex arc in which the concentration of the enzyme varies in the different parts, the efferent part showing the highest concentration, the afferent one somewhat lower and finally the muscle fibre 100 times lower. The physiological significance of this specific distribution is still obscure.

4. INTRACELLULAR LOCALIZATION OF ENZYMES

The determination of enzyme activity at a subcellular level has so far been carried out exclusively by means of techniques which involve fragmentation of the cell and centrifugal fractionation. The main disadvantages of these techniques are: the difficulty of obtaining histologically homogeneous material and the difficulty of defining morphologically the different particles obtained. Other disadvantages are the alternation of the cellular structure produced during the separation process by mechanical manipulation and use of different solvents.

With the aim of obtaining a more exact tool for the study of the intracellular localization of enzymes a technique was developed[10] which permits determinations in μμl. samples of cytoplasm and nucleoplasm. This technique also allows the analysis of isolated nuclei and is therefore of special interest due to the fact that it is not yet possible to isolate an intact nuclear fraction by means of other techniques in a pure enough state to perform reliable determinations of enzyme activity.

When this technique was applied to the study of the AChE in a motoneuron it was possible to get an idea of the distribution of this enzyme in the different parts of a nerve cell.

Figure 4 shows that the cell body, and the dendrites exhibit the highest AChE activity in the cell, followed by the axon and finally by the nucleus, the latter having an activity 10–100 times lower than that of the cell body. The nucleolus did not show any activity at all. The end plate region shows the highest concentration of AChE (see Fig. 4).

5. ENZYMATIC RELATIONSHIP BETWEEN NERVE AND GLIAL CELLS

In spite of the fact that in the mammalian brain the ratio of glial cells to neurons as a whole is greater than 1 : 1 no present method

of homogenization and differential centrifugation produces sufficiently pure fractions of these cells. Microchemical methods of the Cartesian diver type seem, therefore, to be particularly suitable not only for studying enzyme activity in the different types of glial cells but also for providing new information on the functional relationships between glia and neurons.

In our study we have chosen two types of glial cells easily accessible to micromanipulation: first the oligodendrocytes which encapsulate the nerve cell bodies in spinal and sympathetic ganglia and those which surround the large neurons of the lateral vestibular nucleus of Deiters; and second, the protoplasmic astrocytes of the spinal cord. Two different enzymes (cholinesterase and carbonic anhydrase, CA) were studied in these cells. Figure 5 is a schematic illustration of the results obtained.

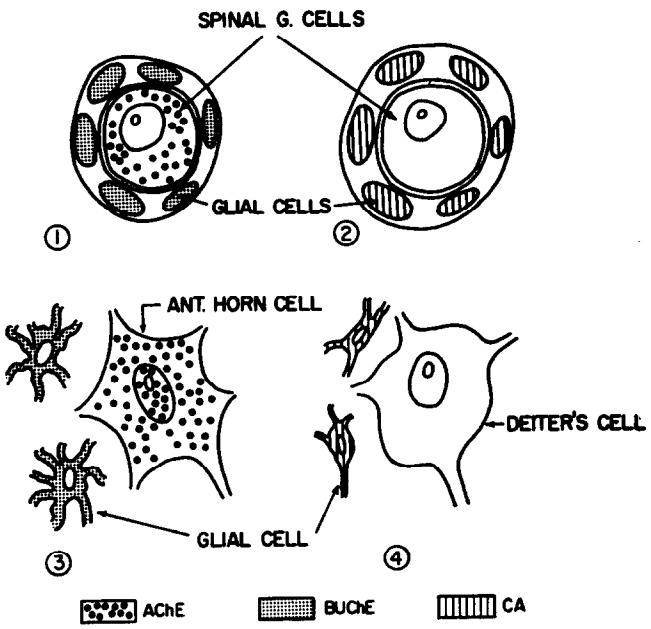
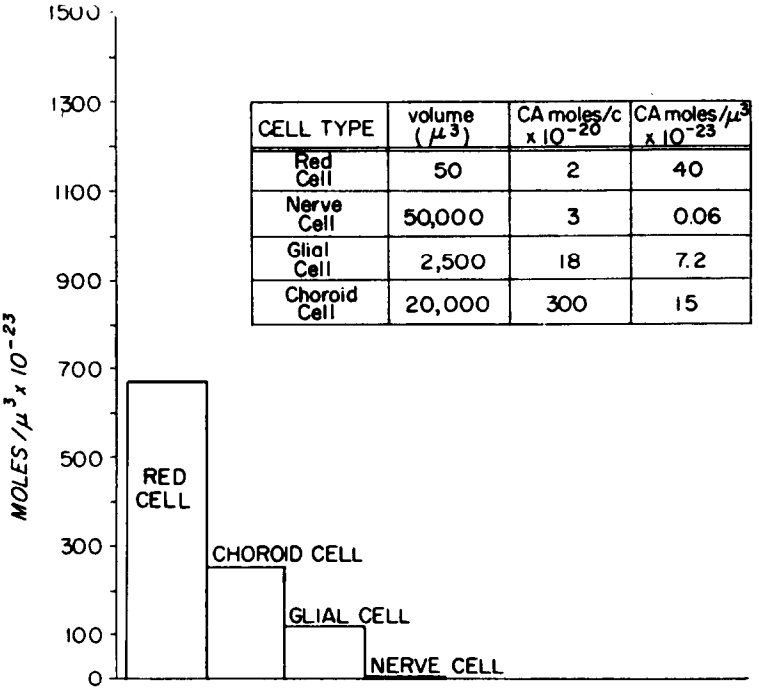


FIG. 5. Schematic representation of enzymatic relationships between glial and nerve cells.

Non spec. ChE (butyrylcholinesterase) was found in high concentration both in the oligodendrocytes of the spinal and sympathetic ganglia and in the astrocytes of the spinal cord of the rat (See Fig. 5, 1 and 3). It is of interest to remark that in the spinal and sympathetic ganglion neurons both cholinesterases are represented, AChE, being largely predominant.

In the Deiter's nucleus of the rat groups of glial cells (see Fig. 5 part 4) varying in volume between 18 and 50 $\times 10^3 \mu^3$, exhibited up to 120 times higher concentrations of CA (moles CA/unit volume), than equivalent volumes of nerve cells (see Fig. 6).

This specific and selective localization of CA supports the implication that the glial CA is involved in the mechanism for the active transport of chloride and sodium from the capillaries to the interstitial and cerebrospinal fluid[11,12].



CELL TYPE	volume (μ^3)	CA moles/c x 10^{-20}	CA moles/μ^3 x 10^{-23}
Red Cell	50	2	40
Nerve Cell	50,000	3	0.06
Glial Cell	2,500	18	7.2
Choroid Cell	20,000	300	15

FIG. 6. Concentration of CA in four different types of somatic cells of the rat.

6. POLYENZYMATIC DETERMINATIONS IN A SINGLE CELL AND COMPARISON OF ENZYME ACTIVITY IN CELLS OF DIFFERENT TISSUES

With the μμl diver technique previously described[10] it has been found possible to determine two or more enzymes in the cytoplasm of the same cell. Another possibility for performing such an experiment is to isolate a single axon and then to determine different types of enzyme activity in several segments subsequently isolated.

In preliminary experiments the following enzymes have so far been determined in the same neuron: cholinesterases (acetyl- and butyryl-), oxidases (cytochrome and succinic), anhydrases (carbonic anhydrase).

Finally, Fig. 6 records schematically the results of a further application of the Cartesian diver technique to a comparative study of CA activity in single somatic cells of four different types taken from the rat[12].

The amount of CA calculated per single intact red cell was approximately 2×10^{-20} moles, in a single nerve cell 3×10^{-20} moles, in a glial

cell 18×10^{-20} moles and in a single cell of the choroid plex 300×10^{-20} moles.

On the basis of the concentration of CA per unit volume it can thus be established that a red cell has 670 times, a choroid cell 250 times and a glial cell 120 times the CA concentration of a nerve cell of Deiters nucleus[12].

CONCLUSIONS AND PROSPECTIVES FOR FUTURE WORK

This brief presentation attempts to illustrate the applicability of the Cartesian diver technique to different neuropharmacological problems.

The review demonstrates that the technique can be adapted to many different histochemical problems: it is not a substitute for the current histochemical staining techniques but it may give more precise indication on the actual localization and on the concentration of an enzyme in a tissue. If used together with conventional techniques of homogenization and centrifugation it may prove to be a valuable help for integration and interpretation of results from these methods by defining more exactly the morphological substrate to which the enzyme activity is related. This is particularly true for a tissue like nervous tissue where at least four different types of cells are present.

We believe that this technique will prove itself even more useful in the future for investigation in the field of neuropharmacology at the cellular level and presently we are trying to develop it in two different ways: first by extending the number of the enzymes which may be determined and second by improving the method (see part 6) for simultaneous analysis of several enzymes in the same cell. Finally we are carrying on preliminary experiments with a Cartesian diver technique which allows us to work with a living nerve cell preparation with which we can not only record the action potential before and after the enzyme estimation but also selectively stimulate the cell during the analysis. We hope to be able to use this preparation for correlating the variations of enzyme activity with the functional changes in the cell.

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DISCUSSION

F. HOBBIGER: How much of the acetylcholinesterase activity which you estimate is attributable to functional AChE and how much to reserve AChE?

E. GIACOBINI: If Dr. Hobbiger means by the word "functional" the AChE which is localized on the cell membrane, no experiments have been so far carried out by us to estimate quantitatively this part of the AChE of the neurone. There is evidence however both from studies performed with the Cartesian diver technique (Giacobini, E. *Arch. Ital. Biol.* **99** 163–177, 1961) and with centrifugal fractionation (Toschi. G. *Exp. Cell. Res.* **16**, 232–255, 1959) which supports the view that the largest part of AChE is localized in the cytoplasm of the neurone. The function of this cytoplasmic AChE is however still obscure.

DONALD C. KROEGER: Would Dr. Giacobini wish to elaborate on the techniques for preparation of tissue samples?

E. GIACOBINI: A technique described by us (Giacobini E. *Acta Physiol. Scand.* **36** 276–290, 1956) was employed, according to which the microdissection is carried out under a binocular dissection microscope with the aid of thin metal micro-needles, either free hand or by means of a very simple micromanipulator. The complete isolation of a single nerve cell can usually be performed within 10–15 min. The cell diameters are measured by means of a micrometer eyepiece at 450 ×.

E. G. ERDÖS: What part of the muscle was your cholinesterase activity associated with?

E. GIACOBINI: The cholinesterase activity of the *rectus abdominis* of the rat was studied histochemcally and quantitatively with the Cartesian diver technique in collaboration with Dr. B. Holmstedt (Giacobini E. and Holmstedt B., *Acta pharmacol. et toxicol.* **17** 94–105, 1960). AChE alone was detected in the large axons connected to the muscle end plates. Both AChE and BuChE were demonstrated in the muscle end plates. The thick afferent fibres from the muscle spindles contained both AChE and BuChE: AChE however was largely predominant. Low amounts of AChE and BuChE were also found in the intrafusal and extrafusal muscle fibres. The intrafusal muscle fibres showed an activity three times greater than that of the extrafusal fibres. These findings leave open the question whether the enzyme in the muscle fibre is localized in the sarcolemmal structures or in the myofibrils.