

ELECTRON MICROSCOPE HISTOCHEMICAL EVIDENCE FOR A PARTIAL OR TOTAL BLOCK OF THE TRICARBOXYLIC ACID CYCLE IN THE MITOCHONDRIA OF PRESYNAPTIC AXON TERMINALS

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ABSTRACT

Respiration-linked, massive accumulation of Sr^{2+} is used to reveal the coupled oxidation of pyruvate, α -oxoglutarate, succinate, and malate by *in situ* mitochondria. All of these substrates were actively oxidized in the dendritic and perikaryal mitochondria, but no α -oxoglutarate or succinate utilization could be demonstrated in the mitochondria of the presynaptic axon terminals. A block at an early step of α -oxoglutarate and succinate oxidation is proposed to account for the negative histochemical results, since the positive reaction with pyruvate and malate proves that these mitochondria possess an intact respiratory chain and energy-coupling mechanism essential for Sr^{2+} accumulation. This indicates that the mitochondria in the axon terminals would be able to generate energy for synaptic function with at least some of the respiratory substrates. With regard to the block in the tricarboxylic acid cycle, the oxaloacetate necessary for citrate formation is suggested to be provided by fixation of CO_2 into some of the pyruvate.

INTRODUCTION

Current attempts at elucidating enzymatic functions and other metabolic properties of the different structural elements of neural tissue (neuron perikarya, dendrites, axons and their synaptic terminals, specific postsynaptic sites, glia, etc.) are seriously limited by thorough entanglement of these elements in a three-dimensional network. A relatively unexplored approach to this problem is opened through electron microscope histochemistry. Although they have the undeniable drawback of giving only qualitative information, histochemical experiments have in their favor the significant advantage of direct visualization of metabolic activities in the various tissue elements.

A histochemical procedure for the demonstration under the electron microscope of the coupled

oxidation of various respiratory substrates by *in situ* mitochondria has been developed recently (15) with the use of respiratory-linked accumulation of Sr^{2+} . The present paper reports results obtained by this method, which show substantial metabolic differences between mitochondria located in the presynaptic axon terminals and those located in other neural elements.

MATERIALS AND METHODS

Respiration-linked, massive accumulation of Sr^{2+} , giving an electron-opaque reaction product (8), was applied as described previously (15), in order to demonstrate the coupled oxidation of the different respiratory substrates by *in situ* mitochondria.

Small tissue blocks 1–2 mm³ were excised from the

cerebral and cerebellar cortex, thalamus, caudate nucleus, the spinal gray matter, and were incubated immediately for 45 min at 37°C, in open glass dishes. The standard incubation medium contained 20 mM Tris-HCl buffer (pH 7.0), 80 mM NaCl, 10 mM respiratory substrate (sodium salts, pH 7.0), 4 mM $\text{Sr}(\text{NO}_3)_2$ (containing as contamination 0.01 mM CaSO_4) and 4 mM sodium-potassium phosphate buffer (pH 7.0). Pyruvate, α -oxoglutarate, succinate, and malate were tested as respiratory substrates. Following incubation, the blocks were fixed in 1% osmium tetroxide buffered at pH 7.4, dehydrated in a graded ethanol series, and embedded in Durcupan (Fluka AG, Basel, Switzerland). The blocks were trimmed as described previously (13), for facilitating the detection of the 30–200 μ thick reacted surface layer under the electron microscope. Sections were cut with an LKB Ultratome (LKB Produkter, Stockholm, Sweden), and stained with lead citrate (Reynolds, 20) for 6–8 min. Electron micrographs were made with the aid of the Tesla BS 413 electron microscope.

RESULTS

Sr^{2+} uptake is demonstrated routinely in the so-called glomeruli (or cerebellar islands) of the cerebellar cortex granular layer, a complex synaptic apparatus of well known ultrastructure (3, 6, 7, 11). Perikarya, axons, and other tissue elements are always found nearby. It has to be emphasized, however, that the choice of the cerebellar cortex to represent the central nervous system in general is based upon the observation of entirely similar Sr^{2+} accumulation pattern in all other—central and peripheral—neural regions studied.

With all respiratory substrates tested, the needle-like or granular deposits are localized exclusively in the inner compartment of the more or less swollen mitochondria. Pyruvate (Fig. 1) supports Sr^{2+} accumulation in all neuronal mitochondria. However, in the mitochondria of the large mossy fiber terminal, large, scattered granules about 400–600 Å in diameter are visible, whereas in those localized in the surrounding terminal “digits” of the granule cell dendrites, diffuse, needle-like deposits are observed. Malate (Fig. 2) brings about the same accumulation pattern as does pyruvate, but only a reaction product of granules about 200 Å in diameter can be observed.

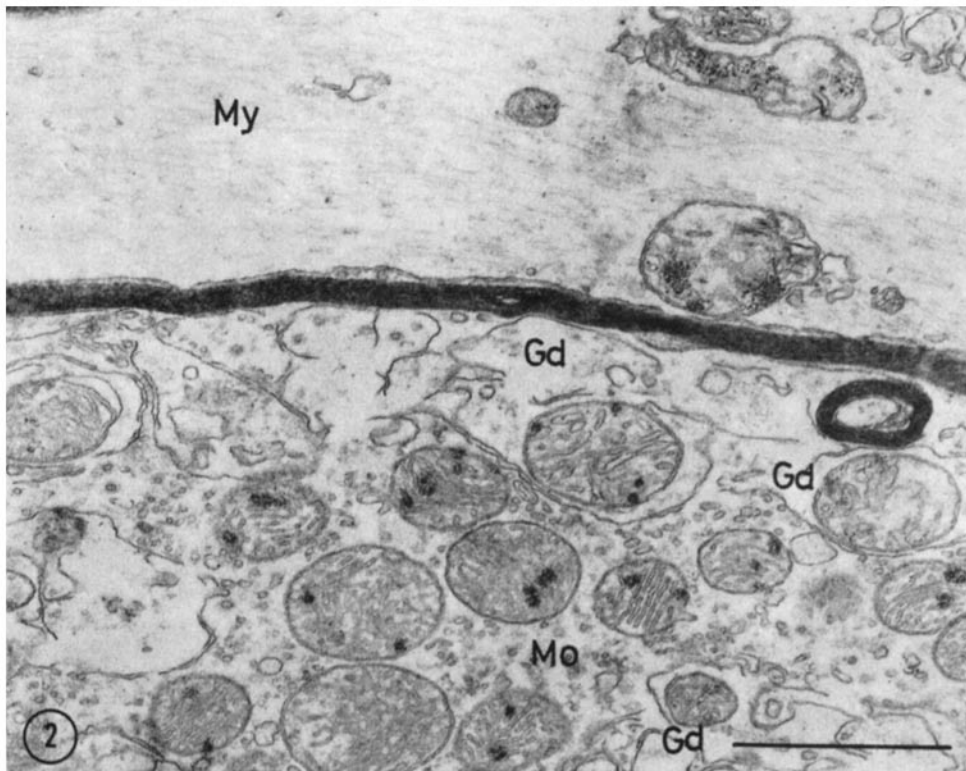
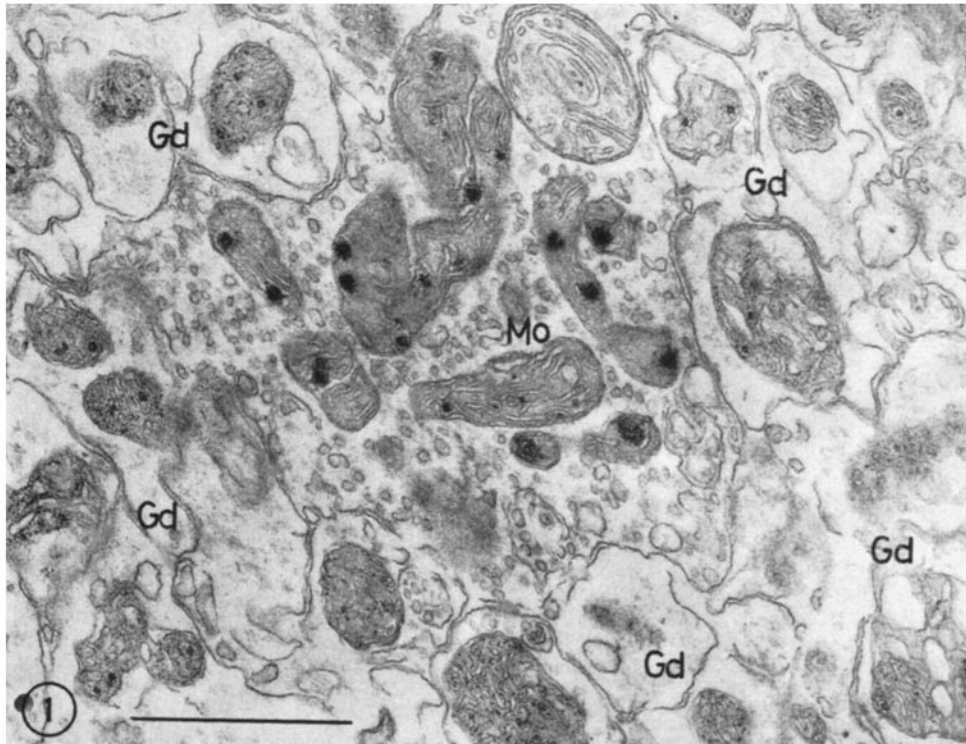
With the use of α -oxoglutarate (Fig. 3) and succinate (Fig. 4) as respiratory substrates, the mitochondria in the axon terminals are always devoid of precipitate. Abundant needle-shaped or

granular deposits are characteristic, on the other hand, for mitochondria localized in other brain structures like the dendrites (Figs. 3, 4), perikarya (Fig. 3), and, most probably, glial cells. (Unequivocal identification of the glial elements meets, unfortunately, with considerable difficulty owing to their intense swelling.)

It is noteworthy that in the case of precipitation in scattered granules some mitochondria in all brain structures examined appear to be devoid of reaction product. This is probably caused by the fact that the deposit may easily be outside the plane of sectioning in part of the mitochondrion. This possibility decreases obviously with the increasing number of granules and does not occur in the case of needle-like precipitation. This does not, of course, interfere with the recognition of the consistent, complete negativity of the numerous mitochondria of the large mossy terminals.

DISCUSSION

With respect to the presence and the form of the reaction product, three kinds of mitochondria are observed: (a) some do not contain any precipitate, while in others deposits are encountered that may be either (b) needle-like and diffusely distributed all over the mitochondrial profile, or (c) in the form of scattered granules. These differences can be used as a semiquantitative estimate of reaction intensity, since, according to the observations of Greenawalt and Carafoli (8), needle-shaped deposition occurs only if large amounts of Sr^{2+} are accumulated, while in the case of more moderate uptake the deposits appear as large, scattered granules. It is striking that the mitochondria of presynaptic axon terminals never exhibit any reaction if α -oxoglutarate and succinate are used as substrates, and also that in pyruvate and malate oxidation only granular deposit appears. Conversely, with all substrates diffuse needle-like or abundant granular precipitate is seen in the mitochondria located in the perikarya and in the dendrites. These results indicate that the tricarboxylic acid cycle (TCAC) intermediates examined are less efficiently oxidized in the presynaptic mitochondria than in those located in perikarya, in dendritic, and, most probably, in glial structures. The demonstration of energy-dependent accumulation of Sr^{2+} with some TCAC intermediates proves, nevertheless, that the mitochondria in the axon terminals would be able to generate energy



for synaptic function with at least some of the respiratory substrates.

The positive reaction with pyruvate and malate indicates, furthermore, that neither the damage of the respiratory chain and energy-coupling mechanism necessary for Sr^{2+} accumulation nor the lack of essential cofactors could account for the negative result in the presynaptic mitochondria. The observations are, therefore, suggestive of a block in these mitochondria at an early step of α -oxoglutarate and succinate oxidation, probably at the level of the primary dehydrogenase itself (14). Lack in the presynaptic mitochondria of succinate oxidation demonstrable both with tetrazolium salt (22) and with ferricyanide (9) fits well into this picture. On the basis of these experiments, it cannot be decided whether the histochemical findings are due to the lack of the enzyme molecule or, conversely, only to blocked activity of the corresponding enzymes.

The histochemical data are thus at variance with the data obtained by fractionation methods (see, for references, 25), reporting considerable succinic dehydrogenase (SDH) activity in the synaptosomal fraction attributed to the mitochondria entrapped in the nerve ending particles. However, in view of the results reported, this activity may be mainly or entirely due to the presence of mitochondria from sources (perikarya, dendrites, glia) other than axon terminals. We think, therefore, that the characterization of mitochondria located in the synaptic boutons on the basis of synaptosomal fraction studies would not seem justified with respect to the possibility of contaminating mitochondria having higher specific activities. At any rate, SDH cannot be considered to be a reliable mitochondrial marker in the brain, whereas cytochrome oxidase, which shows uniform activity in all brain mitochondria (13), would be a more suitable guide for indicating the distribution of mitochondria through the fractions.

The Validity of the Histochemical Results

In order to postulate absent or significantly diminished oxidation of a respiratory substrate, it is an essential requirement to show that the respiratory substrate had free access to the enzyme molecule. Extramitochondrial penetration barriers were ruled out by performing the histochemical assay on isolated mitochondria (10). But one has to consider, additionally, whether or not the mitochondria themselves are impermeable to the TCAC intermediates, which can cross the membrane only by the aid of specific permeases (1). It might well be assumed that the observations are due to the lack of substrate penetration. In other words, the presynaptic mitochondria might be able to utilize only the TCAC intermediates generated within them.

A possible way to rule out this objection would be to incubate frozen sections in which the mitochondrial membranes become disrupted to such an extent that even the soluble matrix enzymes are released into the incubation medium (4, 12, 24). Unfortunately, such treatment not only uncouples energy-linked processes but also destroys the fine structure of unfixed tissues so severely that the recognition under the electron microscope of the relevant tissue elements becomes virtually impossible. However, the pioneering work of Szentágothai (22) carried out on frozen sections of the ciliary ganglion of the chicken, in which the pre- and postsynaptic mitochondria are easily identifiable even at the light microscope level, has already shown a similar lack in SDH activity in the axon terminal mitochondria.

Another line of evidence can be derived from the fact that succinate and malate according to Chappell (1) require the same permease to enter the mitochondria. Since malate is actively oxidized in the presynaptic mitochondria, it is reasonable to assume that succinate, too, should be able to penetrate. Unfortunately, it cannot be

FIGURE 1 Cerebellar glomerulus of rat. Sr^{2+} accumulation is supported by pyruvate. Granular precipitation in the mitochondria of the mossy fiber terminal (*Mo*) is marked. The mitochondria of the granule cell dendritic "digits" (*Gd*) are filled with diffuse, very fine, needle-like precipitate. $\times 29,000$.

FIGURE 2 Cerebellar glomerulus of rat. Sr^{2+} accumulation is supported by malate. In the mitochondria of the mossy terminal (*Mo*) as in those of the granule cell dendritic "digits" (*Gd*), scattered precipitate granules indicate Sr^{2+} uptake. Mitochondria of the myelinated axon (*My*) contain needle-like precipitate. $\times 29,000$.

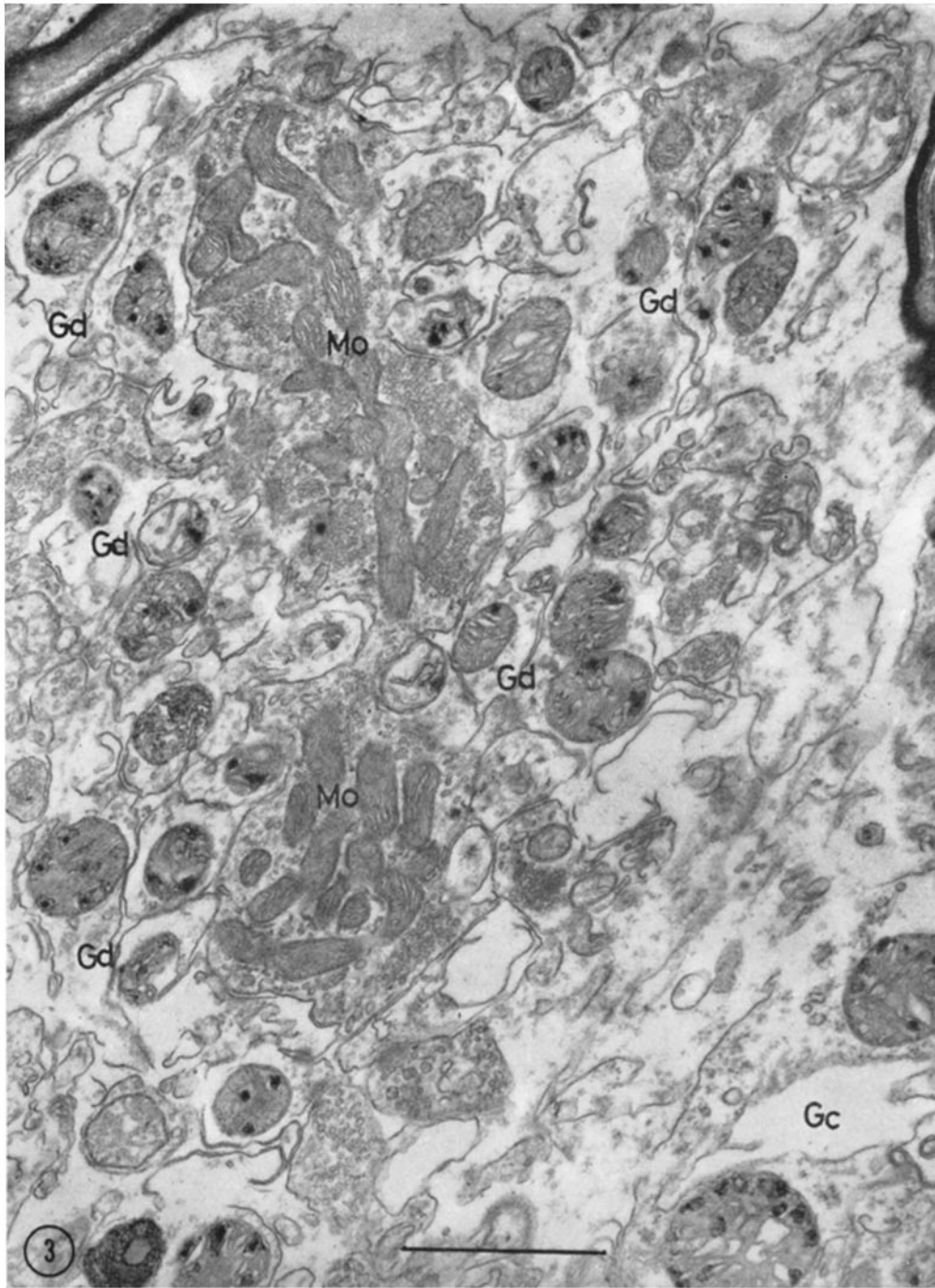


FIGURE 3 Cerebellar glomerulus of the rat. Sr^{2+} accumulation is supported by α -oxoglutarate. No reaction product is visible in the mitochondria of the large mossy "rosette" (Mo), whereas the mitochondria in the granule cell dendrites (Gd) and granule cell perikarya (Gc) contain numerous precipitate granules. $\times 29,000$.

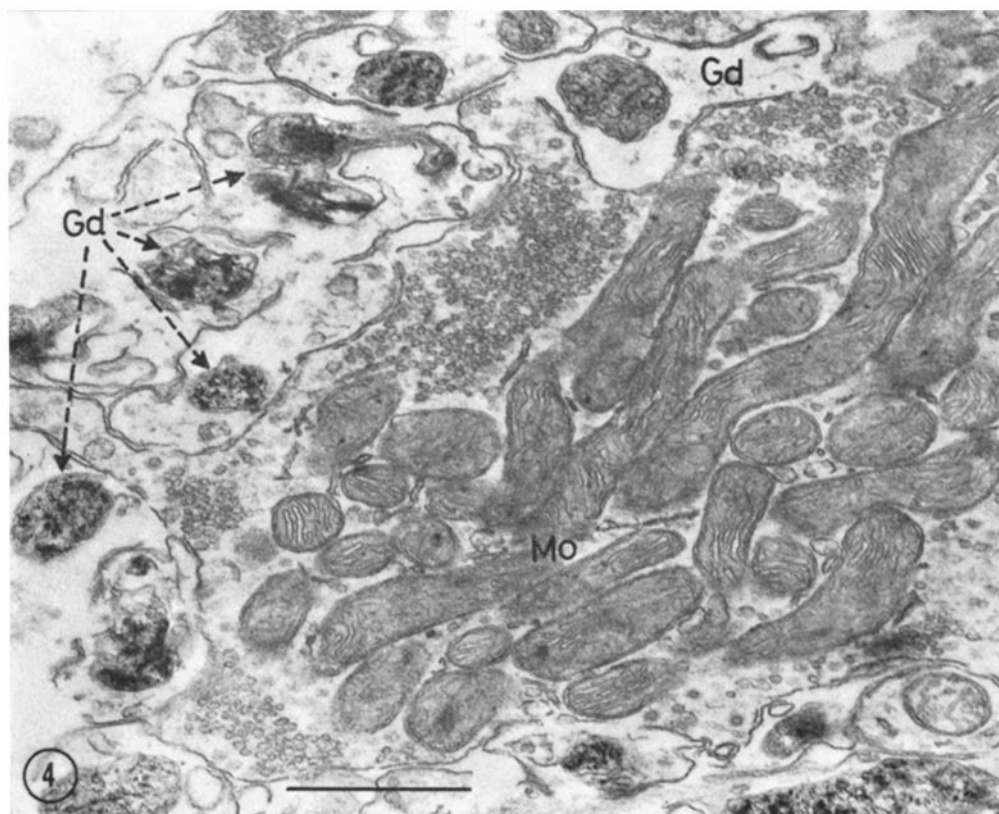


FIGURE 4 Cerebellar glomerulus of the rat. Sr^{2+} accumulation is supported by succinate. The mitochondria localized in the presynaptic ending of the mossy fiber (*Mo*) do not exhibit any reaction deposit. An abundant needle-form precipitate is, on the other hand, characteristic for those situated in the granule cell dendrites (*Gd*). $\times 24,000$.

excluded that the accumulation of Sr^{2+} is due to the oxidation of pyruvate generated from malate outside the mitochondria.

But even if the difference in the histochemical reactions of presynaptic and other mitochondria were not to be merely a permeability phenomenon, one might also think of some specific inactivation process occurring in the presynaptic axon terminals. Moreover, the apparent block of the TCAC might happen only under *in vitro* conditions and might not reflect *in vivo* circumstances. Although no satisfactory answer may be given to these questions, for the time being, the problem might be further elucidated by looking more closely at the oxidation of pyruvate (as is done in the following paragraph) and by doing further, similar studies involving other respiratory substrates (presented elsewhere, 16).

The Oxidation of Pyruvate

A block in the TCAC would bring the whole cycle rapidly to a standstill since no oxaloacetate, essential for the entrance of pyruvate, would be generated. However, the oxaloacetate may be provided by fixation into pyruvate of CO_2 , generating oxaloacetate either directly through pyruvate carboxylase or over malate by the malic enzyme. Since in the nervous system the equilibrium of the latter favors the production of pyruvate and CO_2 , this path is less likely. On the other hand, the activity of pyruvate carboxylase could easily account for the CO_2 fixation observed in the nervous system (5, 21). Indeed, in the isolated nonmyelinated lobster nerve the bulk of the oxaloacetate, used for citrate synthesis, is generated through CO_2 fixation into pyruvate (2). The

high level of CO₂ fixation is, according to those authors, a compensation mechanism to replace the net loss of TCAC intermediate observed in the isolated axons.

In slices of the corpus striatum, however, only about 10–15% of oxaloacetate is produced by CO₂ fixation, according to the same authors, showing the predominance of the TCAC pathway generating this compound in the gray matter. This is also supported by the observation by McMillan and Mortensen (19), showing that around 10–15% of the total activity of the carbon skeleton of glutamate, after the intracisternal injection of (2-¹⁴C) pyruvate, is contained in the second and third carbon atoms. Although the amount of pyruvate consumed in this way may not be too large, its significance would be difficult to deny. The importance of CO₂ coupled to the function of the TCAC has been stressed already by Waelsch et al. (23), particularly in view of its importance in the maintenance of nerve function (17, 18). One is tempted, on the basis of our data, to propose that the CO₂ fixed by the nervous tissue is used predominantly for the maintenance of the synaptic metabolism.

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