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THE ACTIVITY OF OXIDATIVE ENZYMES IN NEUROGLIA CULTIVATED *IN VITRO* *

II. DEHYDROGENASES LINKED WITH COENZYME II, α-GLYCEROPHOSPHATE DEHYDROGENASE, AND ISOCITRIC DEHYDROGENASE LINKED WITH COENZYME I

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A histochemical study was carried out on the activity of the dehydrogenases linked with coenzyme II (diaphorase of coenzyme II, isocitric and glucose-6-phosphatase dehydrogenase) and with coenzyme I (isocitric and α-glycerophosphatase dehydrogenase) in neuroglia cultivated *in vitro*.

Glucose-6-phosphate, α-glycerophosphate and isocitric dehydrogenase linked with coenzyme II showed the greatest intensity of the enzymatic reaction; less intensive reactions were obtained with isocitric dehydrogenase linked with coenzyme I; and least intensive with diaphorase of coenzyme II.

Astrocytes were characterized by uniform distribution of the formazan granules in the cytoplasm and cell processes already in the earliest phases of growth of the cultures. In oligodendrocytes enzymatic activity appeared later and was localized only in the perinuclear zone of protoplasm at first, and after 10—11 days of growth in the processes. Glucose-6-phosphate dehydrogenase activity was the first to appear in the oligodendroglia, followed by the remaining dehydrogenase activities.

The high activities of the studied dehydrogenases, which represent the enzymatic systems involved in the aerobic and anaerobic metabolism of glucose, indicate intensive metabolic processes in growing glial tissue, in contrast to the low level of metabolism in glial tissue *in vivo*.

This communication represents the second part of an extensive study concerning the activity of oxidative enzymes in the culture of neuroglia *in vitro*. The direct aim of the present study was to investigate the enzymatic changes in growing and adult glia, in the glial tissue culture carried out in standard conditions, considered as normal. The results of numerous investigations performed on neuroglia *in situ* and *in vitro*, point to a great variability in the activity of dehydrogenases in various types of glial cells, conditioned by their functional state, altered either by various pathologic processes in the nervous tissue (*Friede*, 1961, a, b; *Rubinstein et al.*, 1962, *Chason et al.*, 1963, *Mossa-*

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kowski, 1963), or changes in the culture medium (*Friede*, 1964). *Meyer* and *Meyer* (1964) found a very high glucose-6-phosphate dehydrogenase activity in the glial tissue, particularly in oligodendroglia during the process of nervous tissue development. According to these authors, a high activity of this enzyme is an indicator of active aerobic carbohydrate metabolism, connected with the myelinization process in the nervous system. The observations concerning the potentiation of the oxidative enzyme activity in glial tissue in connection with the myelinization of nervous fibers, have been already reported by *Friede* (1961) in the nervous tissue *in situ*, and by *Yonezawa et al.* (1962) in the tissue culture *in vitro*. Similar conclusions derived from our own observation, have been presented in the first part of this study (*Mossakowski et al.*, 1965).

The subject of the present study is the investigation of the activity of dehydrogenases linked with coenzyme II, NADP (glucose-6-phosphate dehydrogenase, isocitric dehydrogenase, triphosphoridine nucleotide diaphorase or coenzyme II dehydrogenase), α-glycerophosphate dehydrogenase and isocitric dehydrogenase linked with coenzyme I, NAD.

These enzymes represent the enzymatic links of various patways and phases in the intracellular carbohydrate metabolism. It was therefore assumed, that the activity of these enzymes may be indicative of the intensity of glucose metabolism in glia, as main source of energy for growing tissue. The changes in the enzyme activity could be therefore considered as an exponent of the metabolic process variations in the course of the development of neuroglia.

METHODS

The investigations were carried out on a culture of neuroglia, derived from the cerebellum of newborn albino Wistar rats (6 to 24 hours after birth). The experimental conditions applied in this tissue culture were exactly the same, as in the study reported by *Kraśnicka* and *Mossakowski* (1965). Histochemical estimations were performed on cultures aged: 1, 2, 3, 4, 5, 6, 7, 10 to 11, 12 to 13, 14, 15 to 19, 21, 25, 28 and 30 days. The activity of the following dehydrogenases was estimated: coenzyme II dehydrogenase, glucoso-6-phosphate dehydrogenase, isocitric NADP linked dehydrogenase, α glycero-phosphate dehydrogenase and isocitric coenzyme I linked dehydrogenase. The histochemical estimations were carried out by means of the techniques reported by *Pearse* (1959), *Hess et al.* (1958) and *Nachlas et al.* (1958). A precise description of the applied histochemical methods has been given in the previous communication (*Mossakowski et al.*, 1965).

As a substrate for particular dehydrogenases, a reduced form of triphosphopyridine nucleotide (Sigma), isocitric acid (Fluke A. G. Chemische Fabrik Bichs S. G.), glucose-6-phosphate (C. F. Boehringer, Mannheim) and natrium α-glycero-phosphate (Sigma) were used respectively.

RESULTS

Significant differences in the activity of particular dehydrogenases, connected with the age of the culture, as well as with the type of glial cells — astrocytes and oligodendrocytes — were detected in our experimental material. Moreover, distinct differences in the morphology of the formazan grains, which represent the histochemical exponent of the enzyme activity of particular dehydrogenases, were observed. Independently of these differences, which will be discussed later on with the results obtained for particular dehydrogenases, a common pattern in the distribution of the enzymatic reaction, irrespective of the estimated enzyme, but significantly distinct for astrocytes and oligodendrocytes was found.

In the astrocytes, whether they were large or small, with a great or small number of cell processes, as well as in the polynuclear cell elements originating from astrocytes, the distribution of the formazan grains was found to be characterictically uniform, in both cytoplasm and cell processes. In the later period of the culture growth (third week) some slight local condensation of the formazan grains observed at the perinuclear zone and at the outset sites of the processes (Fig. 1). During the whole course of the culture growth and independently of the estimated dehydrogenase, the evaluated enzyme activity in the astrocyte processes was equal to that in the cell cytoplasm.

The pattern of the enzyme activity in oligodendroglia was different from that in the astrocytes. In the period of the first 7 days of culture growth, there were only few oligodendrocytes present in the culture. The enzyme activity was observed only in the protoplasm of oligodendrocytes in form of dispersed single formazan grains. No activity in the oligodendrocyte processes could be detected. In the later period of the culture growth, the formazan grains in the cytoplasm aggregate at one pole of the cell nucleus, forming a characteristic for this type of glia "perinuclear cap". Such distribution of the enzyme activity is very typical for oligodendroglia and, therefore may be considered as a test in differentiation of oligodendrocytes from astrocytes (Fig. 2).

The enzyme activity in the oligodendrocytic processes occurs between the 7th and 8th day of the culture growth and then gradually increases. During the period from the 14th to the 19th day, the enzyme activity in oligodendrocytes, including their processes, exceeds the enzyme activity in astrocytes.

In the astrocytes the morphology of the formazan grains shows variations related to the investigated enzyme, whereas, in oligodendroglia, especially after two weeks of culture growth, the formazan deposits form a compact mass, which does not permit to distinguish single grains and estimate their morphology.

The following results of histochemical investigations, on the activity of particular dehydrogenases, were obtained:

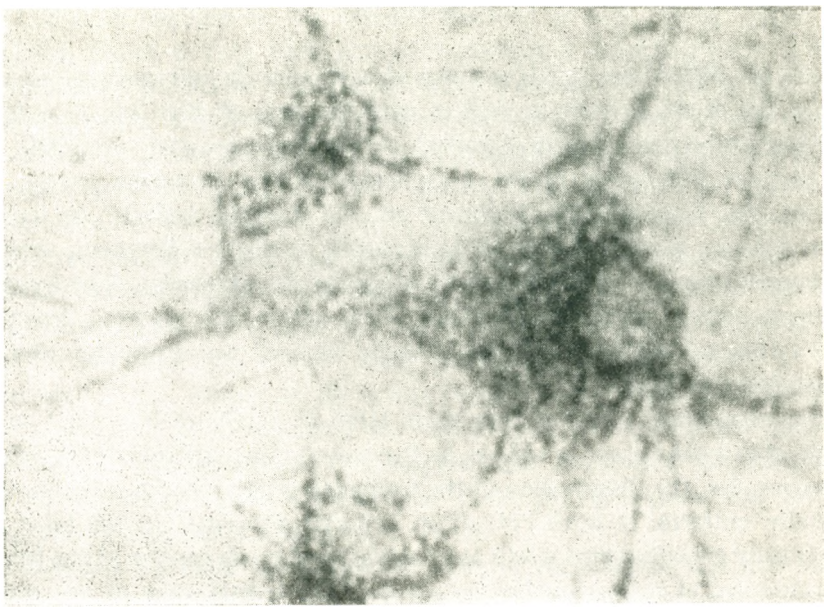

Fig. 1. Distribution of formazan grains marking the sites of enzymatic activity characteristic of astrocytes. The formazan grains are uniformly scattered in the protoplasm and in the cell processes and slightly more concentrated in the perinuclear zones. Magn. oc. × 15, obj. × 40.

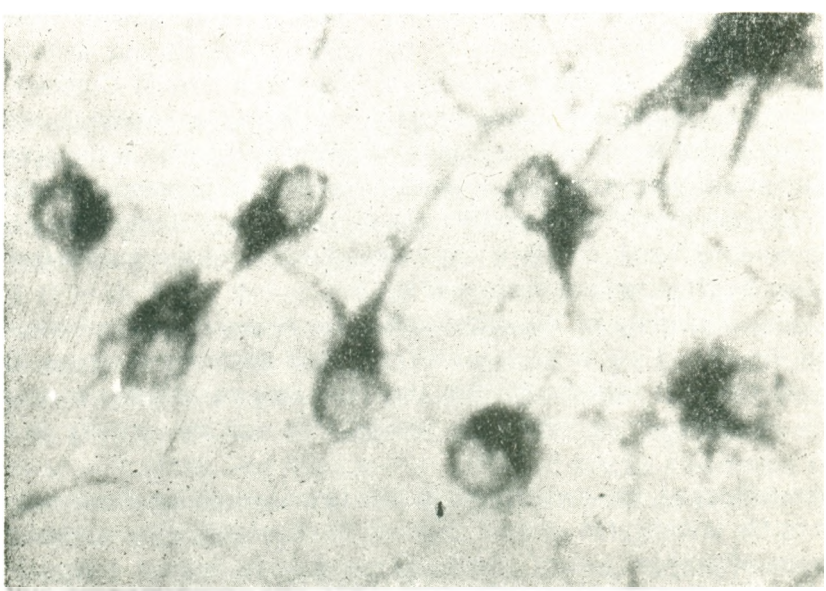

Fig. 2. Distribution of formazan grains typical of oligodendroglia. Compact masses of formazan grains from characteristic perinuclear caps. Magn. oc. × 15, obj. × 20.

Triphosphopyridine nucleotide diaphorase. In the astrocytes a very low activity of this enzyme was detected already on the first day of the culture growth. After the 6th day this activity increases, and undergoes further gradual potentiation in the following period. The formazan grains are very small scattered uniformly in the protoplasm and the cell processes. In the oligodendrocytes, the enzyme activity appeared during the second week of the culture, and could be observed in both the protoplasm and the cell processes (Fig. 3, 4).

Glucose-6-phosphate dehydrogenase. From the first days of the tissue growth, the activity of this enzyme in the astrocytes was very high. The coarse-grained deposits of formazan fill entirely the cell protoplasm, leaving only a free space, occupied by the nucleus. Beginning with the third day of the culture growth, a very high activity in the astrocytic processes, similar to that in the protoplasm was determined. In oligodendroglia, the activity of this enzyme was detected on the 5th day, while in the processes only on the 7th day of the culture growth. A very rapid potentiation of the enzyme activity, up to the third week of growth, was observed in oligodendrocytes. During the following period the activity is maintained on the same or sometimes slightly reduced level (Fig. 5, 6).

Isocitric, NADP linked dehydrogenase. A high activity of this enzyme, only slightly lower than that of glucose-6-phosphate dehydrogenase, appears in the astrocytes during the first days, and in oligodendrocytes on the 6th day of the tissue growth. During the whole course of the tissue growth the activity of this enzyme is maintained in a very high level. Tiny formazan grains are uniformly scattered in the protoplasm and the cell processes in both astrocytes and oligodendrocytes, with a typical unipolar perinuclear condensation in the latter (Fig. 7, 8).

α-Glycerophosphate dehydrogenase. A potentiation of the enzyme activity parallel to the tissue culture growth was observed. About the 11th day the activity of this enzyme became as high as that of the glucose-6-phosphate dehydrogenase. In the astrocytes, coarse formazan grains uniformly scattered in the protoplasm form slight aggregations close to the nuclear membrane and at the outset sites of the processes. In oligodendrocytes, the enzyme activity appears on the 6th day of the culture growth, showing a typical distribution in the protoplasm (Fig. 9, 10).

Isocitric, NAD linked dehydrogenase. The intensity of tetrazolium reaction was rather moderate, much lower than that of isocitric NADP linked dehydrogenase. In the course of the culture growth the enzyme activity increased, attaining its maximal level after 14 days. In the astrocytes, tiny formazan grains uniformly filled the protoplasm and the processes. In oligodendroglia, the activity of this enzyme appeared in the cytoplasm on the 6th day, and in the processes on the 11th day (Fig. 11 and 12).

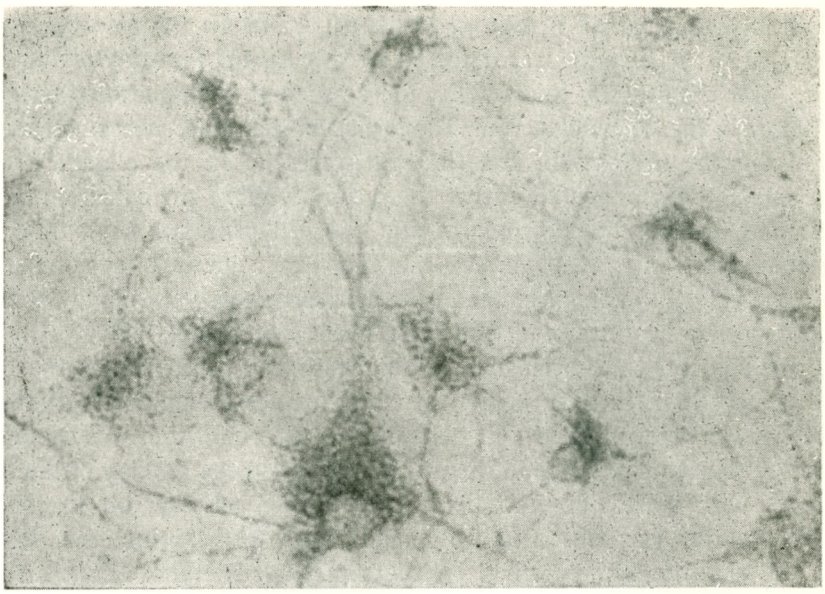

Fig. 3. Dehydrogenase of coenzyme II. Four-day culture. Numerous astrocytes with typical distribution of enzymatic activity. Low activity. Magn. oc. × 15, obj. × 40.

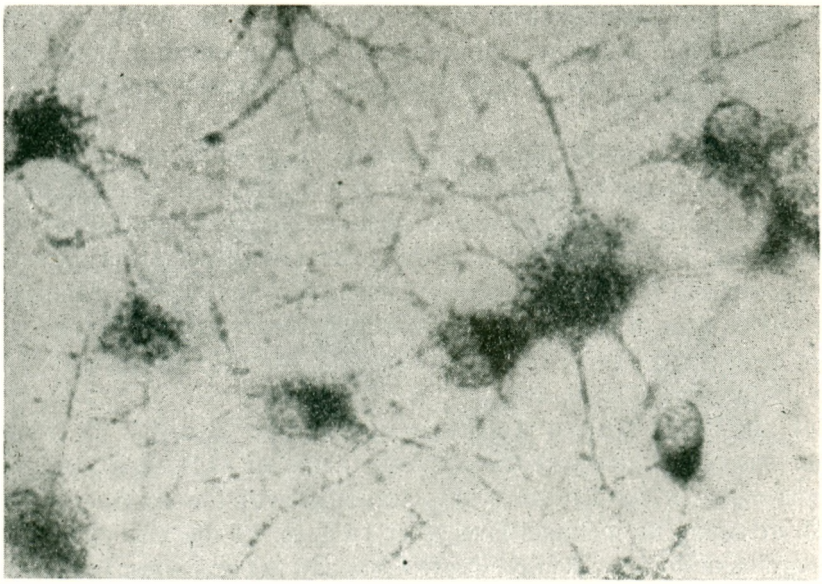

Fig. 4. Dehydrogenase of coenzyme II. Thirty-day culture. Astrocytes and oligodendrocytes with typical distribution of enzymatic activity. Compared with the preceding figure, enzymatic activity is much stronger. Magn. oc. × 15, obj. × 40.

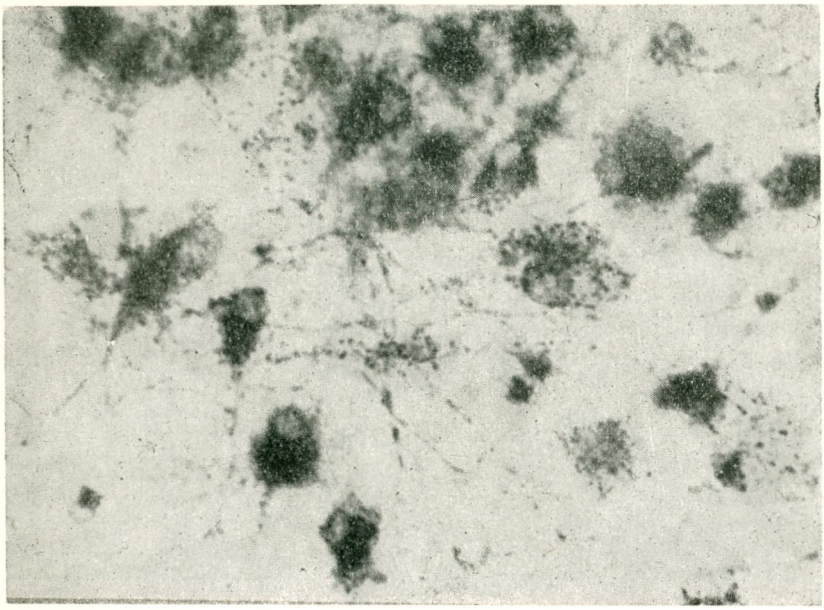

Fig. 5. Glucose-6-phosphate dehydrogenase. Seven-day growth. High enzymatic activity in astrocytes and oligodendrocytes, both in the cytoplasm and in the cell process. Magn. oc. × 15, obj. × 20.

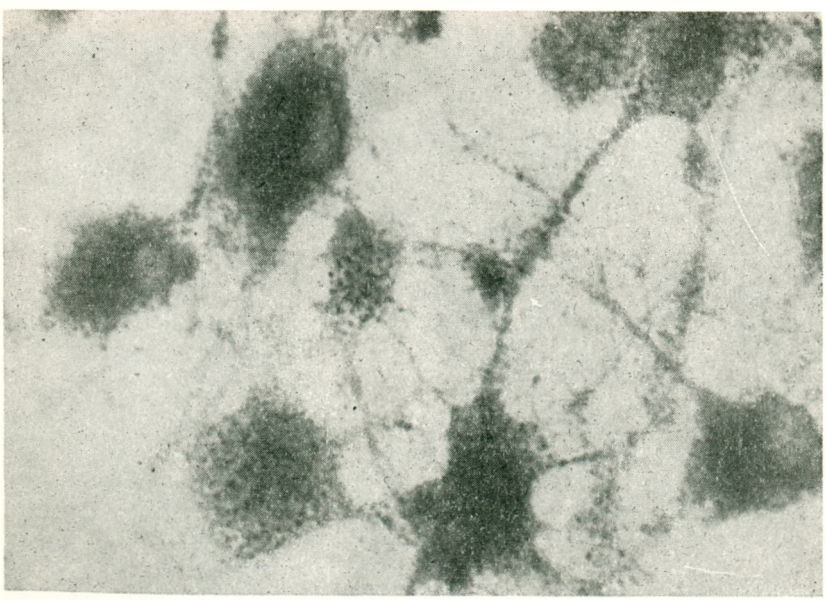

Fig. 6. Glucose-6-phosphate dehydrogenase. Culture on the 28th day. All the cells visible in the preparation exhibit high enzymatic activity in the cytoplasm and processes. Cytoplasmic deposits obscure the cell nucleus. Magn. oc. × 15, obj. × 40.

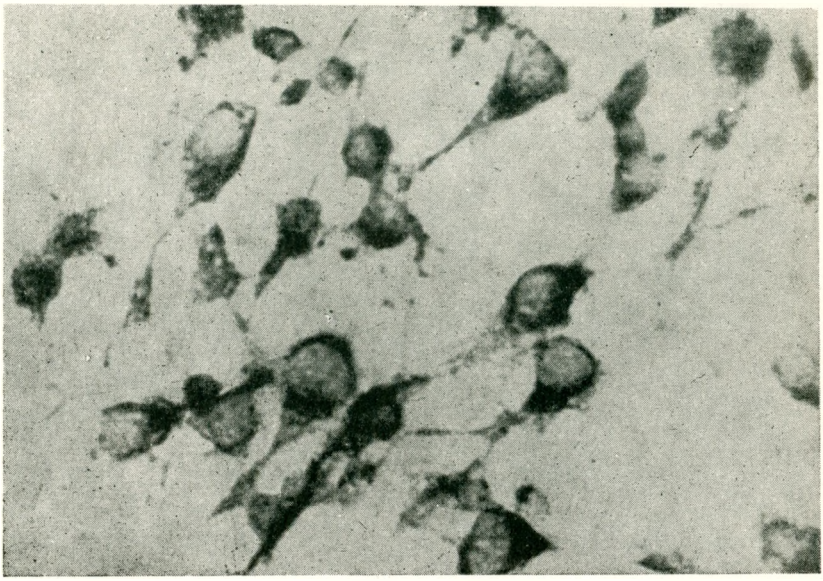

Fig. 7. Isocitric dehydrogenase bound with NADP. Two-day culture. Predominance of undifferentiated glial cells with high enzymatic activity. Magn. oc. × 15, obj. × 20.

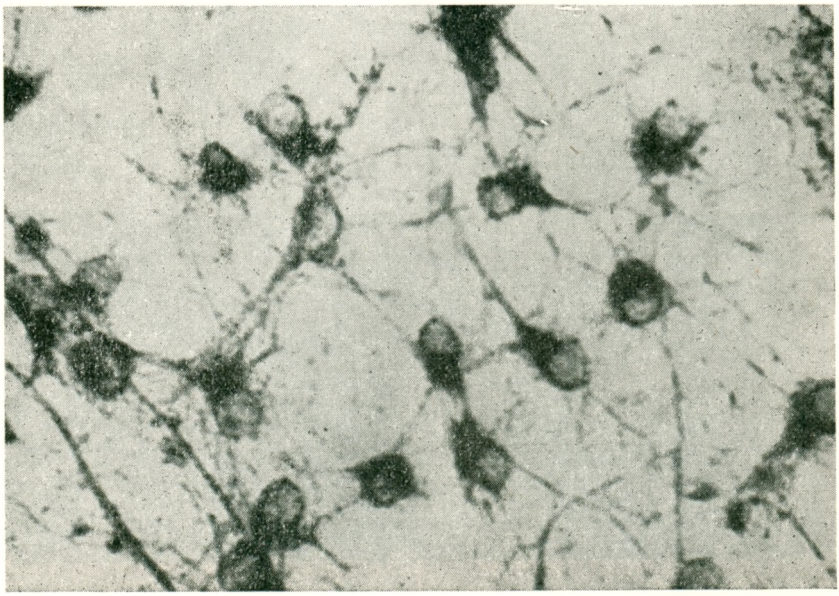

Fig. 8. Isocitric dehydrogenase bound with NADP. Culture on the 28th day. High enzymatic activity in astrocytes and oligodendrocytes. Magn. oc. × 15, obj. × 20.

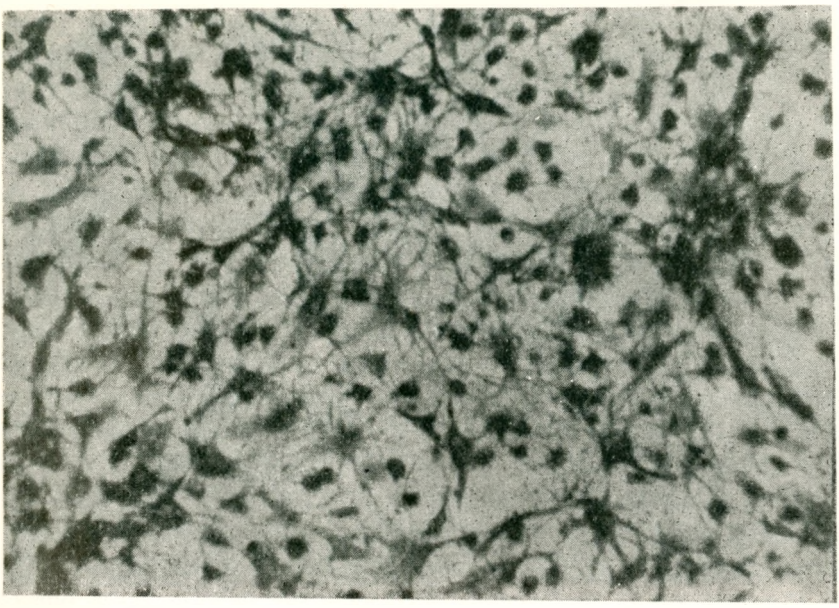

Fig. 9. α-Glycerophosphatase dehydrogenase. Twenty-one-day culture. General view of the culture which contains various types of glial cells. Differences in the intensity of the formazan reaction in different cells, with a generally high level of enzymatic activity. Magn. oc. × 10, obj. × 10.

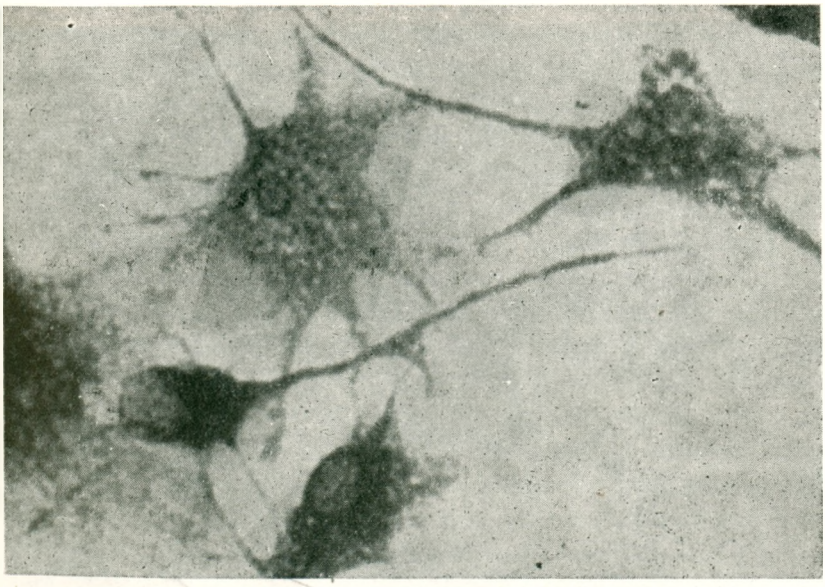

Fig. 10. α-Glicerophosphate dehydrogenase. Twenty-eight-day. Various intensity and distribution of enzymatic activity in astrocytes and oligodendroglial cells. Magn. oc. × 15, obj. × 40.

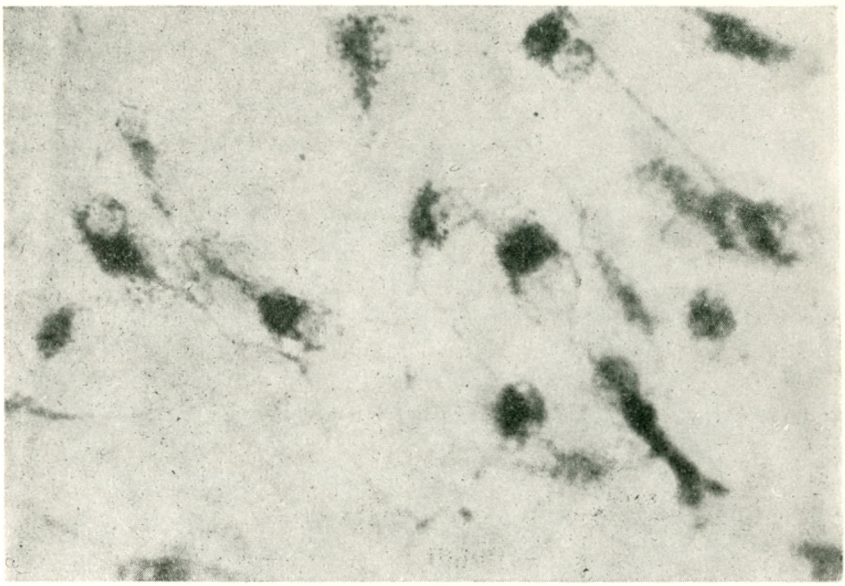

Fig. 11. Isocitric dehydrogenase bound with NAD. Ten-day culture. Predominance of oligodendrocytes with relatively high enzymatic activity. Magn. oc. × 15, obj. × 40.

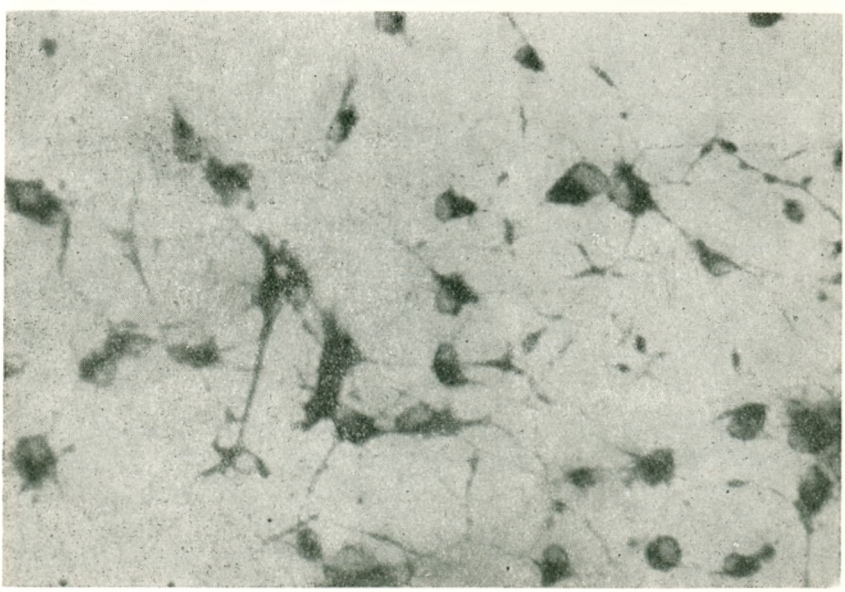

Fig. 12. Isocitric dehydrogenase bound with NAD. Thirty-day culture. Various types of glial exhibiting high enzymatic activity. Magn. oc. × 15, obj. × 10.

DISCUSSION

The results of our investigations showed a characteristic delay in the appearance of enzyme activity and a typical distribution of this activity in oligodendrocytes. The evident differences in the activity of practically all investigated dehydrogenases, have been already pointed out in our previous communication. It may be assumed, that they may represent a reliable histochemical criterion in the differentiation of the two types of glial cells in the tissue culture.

The intensity of enzymatic activity of all studied dehydrogenases, with the exception of that of glucose-6-phosphate dehydrogenase which showed a maximal activity from the very beginning, increased during the course of the tissue culture growth, attaining its maximal level during the second and third week.

Our observations concerning the enzyme activity in oligodendroglia seem to indicate an important increase of intracellular metabolism in this group of cells, which occurs the beginning of the myelinization process. A higher enzyme activity in oligodendroglia than in astrocytes, detected at this phase of tissue growth, represents an additional item in favor of the hypothesis put forward by *Yonezawa et al.* following their studies *in vitro* (1962), and *Friede* (1961) and *Meyers* (1964) following their observations of the neuroglia *in situ*.

The highest enzyme activity was exhibited by glucose-6-phosphate dehydrogenase, α-glycerophosphate dehydrogenase and isocitric NADP linked dehydrogenase. Isocitric NAD linked dehydrogenase presented a lower activity, while coenzyme 11 dehydrogenase (TPN diaphorase) the lowest.

A very high enzyme activity of both glucose-6-phosphate and α-glycerophosphate dehydrogenases, indicates that the carbohydrate metabolism, representing the main source of energy for growing glial cells is carried through the anaerobic phosphorylating glycolysis, as well as in the aerobic pentose-phosphate cycle. It seems that this fact is noteworthy and should be emphasized, especially in view of *Heller* and *Elliot's* (1955) findings, showing a low oxygen consumption by the glial cells, as well by astrocytes as oligodendrocytes, in the nervous tissue *in situ*. The potentiation of the aerobic glucose metabolism in the glia appears, according to *Meyer* and *Meyer* (1964) in the course of myelinization and concerns, first of all, oligodendrocytes. Similar biochemical observations were reported by *Lowry* (1955). In our study, a very high enzymatic activity of glucose-6-phosphate dehydrogenase was detected in both types of the glial cells. This may indicate a high activity of the aerobic glucose metabolism, carried through the pentose-phosphate pathway in both astrocytes and oligodendrocytes, even at the period preceding the myelinization process. This could represent a quite significant difference between neuroglia culture and neuroglia *in situ*. Moreover, it should be mentioned that the pentose-phosphate pathway of the glucose metabolism serves as a main source of ribose-5-phosphate, one of the most essential elements in the sythesis of nucleotides. A high mitotic index in the neuroglia culture *in vitro* may suggest the exi-

stence of a great demand for building material, required for nucleotide synthesis.

The morphology of cells, as well as the distribution of enzymatic activity of investigated dehydrogenases indicate that the biology of the culture of neuroglia *in vitro* is similar to that of the reactive glia, which appears in the adult neuroglia in the course of various pathologic processes affecting the central nervous system, as well as in the course of various types of the glia "functional mobilization" during the process of the development of nervous system. This could suggest, that the character of the cellular reaction of glia is similar and not necessarily connected wiht the kind of casual factor.

Another problem is connected with the evaluation of a low enzyme activity of the coenzyme II dehydrogenase (TPN diaphorase). It seems that this phenomenon could be explained by the applied histochemical technique. None of the incubation fluids applied in this study contained menadione, which is known to activate the reaction of so called coenzyme diaphorases. With the technique used in this study, when menadione is excluded, only a reaction with mitochondrial diaphorase can be observed (*Pearse*, 1961). Therefore, our slides show only mitochondrial coenzyme II diaphorase, which can be confirmed by the observation of fine-grained, and occasionaly even minute dusty formazan grains in the course of this reaction.

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REFERENCES

1. *Chason, I. L., Landers, I. W., Gonzales, I. E.*: Respiratory enzyme activity of human gliomas. A slide histochemical study. J. Neuropath. Exp. Neurology, 1963, 22, 471—478.
2. *Friede, R. L.*: A histochemical study of DPN-diaphorase in human white matter with some notes on myelination. J. Neurochem., 1961a, 8, 17—30.
3. *Friede, R. L.*: Enzyme histochemical studies in multiple sclerosis. Arch. Neurol., 1961b, 5, 433—443.
4. *Friede, R. L.*: A cytochemistry of normal and reactive astrocytes. J. Neuropath. Exp. Neurol., 1962, 21, 471—478.
5. *Friede, R. L.*: The enzymatic response of astrocytes to various ions *in vitro*. J. Cell Biology, 1964, 20, 5—15.
6. *Heller, I. H., Elliot, K. A. C.*: The metabolism of normal brain and human gliomas in relation to cell type and density. Canad. J. Biochem. Physiol., 1955, 33, 395—403.
7. *Hess, R., Scarpalli, D. G., Pearse, A. G. E.*: The cytochemical localization of oxidative enzymes. II. Pyridine nucleotide linked dehydrogenases. J. Biophys. Biochem. Cytol., 1958, 4, 753—760.
8. *Kraśnicka, Z., Mossakowski, M. J.*: Zmienność obrazu morfologicznego gleju w hodowli tkanek (The morphological variability of the culture of the neuroglia). Neuropatologia Polska, 1965, 3.
9. *Lowry, O. H.*: A study of nervous system with quantitative histochemical methods. Biochemistry of developing nervous system. Red. Waelsch, H., New York Academic Press, 1955, 350—357.

10. *Meyer, I., Meyer, P.*: Enzyme histochemistry of growing and adult oligodendroglia. Acta Neurologica Scandinavica, 1964, 40, 89—90.
11. *Mossakowski, M. J.*: The activity of succinic dehydrogenase in the reactive glia. Acta Neuropath., 1963, 2, 282—290.
12. *Mossakowski, M. J., Kraśnicka, Z., Renkawek, K.*: Aktywność enzymów oksydacyjnych w tkance glejowej hodowanej *in vitro*. Część I. Dehydrogenazy związane z koenzymem I i dehydrogenaza bursztynowa (The activity of oxidative enzymes in the neuroglia cultivated *in vitro*. I. Dehydrogenases linked with coenzyme I and succinic dehydrogenase). Neuropatologia Polska, 1965, 3, 245—258.
13. *Nachlas, M. M., Walker, D. G., Seligman, A. M.*: The histochemical localization of triphosphopyridine nucleotide diaphorase. J. Biophys. Biochem. Cytology, 1958, 4, 467—473.
14. *Pearse, A. G. E.*: Histochemistry theoretical and applied. Churchill, London 1959.
15. *Pearse, A. G. E.*: Properties of tetrazolium salt and alternative electron acceptor as factors in dehydrogenase histochemistry. Biochem. J., 1961, 78, 14.
16. *Rubinstein, L. J., Smith, B.*: Triphosphopyridine nucleotide (TPN) diaphorase and TPN-dependent dehydrogenase activity of reactive macrophages in tissue necrosis. Nature, 1962, 193, 895—896.
17. *Yonezawa, T., Borstein, M. B., Murray, M.*: A histochemical study of oxidative enzymes in cultures of central nervous tissue. J. Neuropath., Exp. Neurol. 1962, 21, 479—487.