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THE ACTIVITY OF OXIDATIVE ENZYMES IN NEUROGLIA CULTIVATED IN VITRO *

I. DEHYDROGENASES LINKED WITH COENZYME I AND SUCCINIC DEHYDROGENASE

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Histochemical studies were carries out with tissue cultures of glia from newborn rats. Cultures aged 1, 2, 3, 4, 5, 6, 7, 10, 11, 13, 14—19, 21, 25, 28 and 30 days were studied. Activity of the following enzymes was investigated: dehydrogenase of coenzyme I, succinic, lactic, glutamic, malic and ethyl alcohol dehydrogenases.

Activity of all the studied enzymes, with the exception of the dehydrogenase of coenzyme I, was lower in the early stages of growth (1—10 days) than in later stages. Tetrazolium reductase, which exhibited the highest activity, was not affected by the age of the culture.

Significant differences in the distribution of enzymatic activity were found in relation to the type of glial cells and age of the culture.

The dehydrogenase of coenzyme I and lactic dehydrogenase showed the highest activity. Glutamic, succinic and malic dehydrogenases showed lower activity, and alcohol dehydrogenase the lowest activity, which increased in older cultures.

Marked individual differences in intensity of enzymatic activity of different cells of the same type were noted, presumably in connection with the functional state of the cells.

The intensity of enzymatic activity in glial cell *in vitro* is similar to that in reactive glia *in vivo*.

This communication represents the first part of an extensive study, concerning the variability of the enzymatic reactions linked with the intracellular oxidative processes in neuroglia cultured *in vitro*, under various environmental conditions (hypoxia, anoxia, varying pH of the tissue culture medium, presence of various chemical compounds altering the cell membrane permeability). The subject of the present study consisted of an estimation of the activity of certain enzymes, pertaining to the group of anearobic dehydrogenase, in the culture of normal neuroglia.

The enzymatic activity of dehydrogenases linked with coenzyme I and II in neuroglia, has been the subject of relatively numerous communications, re-

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ported in recent years (*Friede* 1958, 1961, 1962; *Friede, Fleming* and *Knoller* 1963; *Rubinstein, Klatzo* and *Miquel* 1962; *Smith* and *Rubinstein* 1962; *Osterberg* and *Wattenbers* 1962; *Adams, Davison* and *Gregson* 1963; *Ibrahim* and *Adams* 1963; *Mossakowski* 1963; *Chason, Gonzales* and *Landers*, 1963; *Meyer* and *Meyer*, 1964). In spite of differences in the obtained results, concerning the activity of particular dehydrogenases, it has been generally accepted, that a relatively low enzymatic activity of normal glia rises significantly following reactive growth and hyperplasia of glia, connected with such processes as demyelization (*Friede*, 1961; *Ibrahim* and *Adams*, 1963), tissue injuries of vascular origin (*Friede*, 1961; *Osterberg* and *Wattenberg*, 1962; *Chason, Landers* and *Gonzales*, 1963), brain edema (*Rubinstein, Klatzo* and *Miquel*, 1962) and healing of nervous tissue injuries, induced by mechanical and thermic factors (*Osterberg* and *Wattenberg*, 1962; *Mossakowski*, 1963). Quite important changes in the intensity of enzymatic processes in glia, were observed also during maturation of the nervous system (*Meyer* and *Meyer*, 1964), particularly in the course of the myelinization process (*Friede*, 1961). *Yonezawa, Borstein, Peterson* and *Murray* (1962) performed a histochemical study of the oxidative enzymes in cultures of the central nervous tissue, *in vitro*.

The culture of neuroglia was considered as a most convenient model, particularly for the investigation of changes in enzyme activity during the process of developing and maturation of tissue. There are also other advantages, such as the possibility to create various experimental conditions, difficult to attain *in vivo*; to perform histochemical estimations on fresh tissue, without previous fixation or freezing of the material, which induces more or less marked damage of the tissue. It was also assumed, that it may be of interest to compare the results of the histochemical study performed on glia in tissue culture, with those, obtained by other authors in neuroglia within nervous tissue.

MATERIAL AND METHODS

The tissue culture of glia from the cerebellum of newborn (6 to 24 hours old) albino Wistar rats was used in this study.

Tissue culture technique. Full technical details of the glia culture applied in the Laboratory for Tissue Culture of our Department, were reported in a separate communication (*Kraśnicka* and *Mossakowski*, 1965).

In this communication we shall described only fundamental technical details. The cultures were carried out in Carrel's flasks on narrow coverslips. The solid medium for tissue culture consisted of a reconstitued rat tail collagen, prepared according to *Borstein* (1958). The liquid medium was composed of an inactivated human serum, 10 percent extracts of 9-day chick embryos in Earl's buffered solution and 5 percent glucose, in proportions of 5:4:1. The histochemical studies were performed on tissue cultures aged: 1, 2, 3, 4, 5, 6, 7, 10, 11, 13, 14,

15—19, 21, 25, 28, and 30 days. The activity of following enzymes was investigated: succinic dehydrogenase, dehydrogenase of coenzyme I, and also dehydrogenases linked with coenzyme I (NAD) such as: lactic (LDH), malic (MDH), glutaminic (GDH), ethyl alcohol (ADH) dehydrogenases.

Histochemical technique. The coverslips with fresh, non-fixed cultures were transfered from the medium directly to the incubation fluid. The incubation time in incubator at 37°C was 1 hour and 30 minutes for dehydrogenase of coenzyme I, and 2 hours for the remaining enzymes. After the incubation period, the slides were stained with 3 percent aqueous eosin solution, dehydrated with alcohol, cleared in xylene and then mounted in Permount. The incubation fluids for particular dehydrogenases were prepared as follows: for succinic dehydrogenase — 0·2 M aqueous solution of natrium succinate 0·25 ml, 0·2 M phosphate buffer at pH 7·4, 0·25 ml and aqueous solution of Nitro BT (1 mg/1 ml) 0·5 ml (according to *Potanos* and *Cowen*, 1959);

for dehydrogenase of coenzyme I (according to *Farber*, 1958) — an aqueous solution of NADH (reduced form of nicotinamid-adenine-dinucleotide) 0·3 ml (10 mg/1 ml), 0·2 M phosphate buffer at pH 7·4, 0·3 ml, and aqueous solution of Nitro BT (1 mg/1 ml) 0·3 ml;

for dehydrogenases linked with coenzyme I NAD (according to the modified technique of Hess, Scarpelli and Pearse, 1958): the substrate in aqueous solution (0·1 to 1·0 M)... 0·1 ml, an aqueous solution of nicotinamid-adenine-dinucleotide NAD (0·1 M) — 0·1 ml, 0·2 M phosphate buffer at pH 7·4 — 0·25 ml, kalium cyanide (0·1 M) — 0·1 ml and aqueous solution of Nitro BT (1 mg/1 ml) — 0·25 ml.

As a substrate for particular dehycrogenases, the following substances were used, respectively: natrium lactate (PPH Polskie Odczynniki Chemiczne — Polish Chemical Reagents), natrium glutamate (Xenon, Łódź), l-malic acid (Light, London), neutralized KOH to pH 7·4 and 1·0 M solution of ethyl alcohol. The hydrogen ion concentration in all incubation fluids was maintained at the standard level of pH 7·2 by means of a buffer solution.

RESULTS

A basic pattern of the distribution of enzymatic activity was common for all estimated dehydrogenases. Nevertheless, a distinct difference in the intensity of enzymatic reactions occurred in some of them. Therefore, in the first group there will be described in detail the common features for all dehydrogenases, and then the results of the consecutive groups will be referred to it, with special emphasis on the detected differences.

The activity of dehydrogenase coenzyme I (diaphorase of diphosphopyridine nucleotide). In all examined cultures the glial cells showed high enzymatic activity, manifested as abundant darkblue deposits

of formazans. Marked differences in the distribution of enzyme activity was observed in relation to the type of glial cells and the age of culture. The distribution of enzyme activity in astrocytes is practically constant. The formazan grains fill uniformly the protoplasm and the cell processes up to their furthest extremities. The accumulation of formazan grains within the cell protoplasm was sometimes so large, that it screened the cell nucleus, free of enzyme activity. No difference in the intensity of enzyme activity between particular parts of the cell was observed. This type of enzymatic activity distribution is manifested not only by astrocytes, but also by their less differentiated forms, such as spongioblasts. In the older cultures (25 to 30 days) the appearance of hyperplastic forms of astrocytes, sometimes with several nuclei, was observed. These cells show a high enzymatic activity, even higher than normal astrocytes.

Relatively high activity was found even in cells undergoing fatty degeneration. The formazan deposits in protoplasm form a network with meshes filled by enzymatically negative lipids. No significant difference concerning the distribution and intensity of enzyme activity were detected in the astrocytes, in relation to the age of tissue culture, whereas, in oligodendrocytes this difference was found to be significant. In young cultures (2 to 7 days old) the number of oligodendrocytes is small, usually smaller than that of astrocytes.

Part of the oligodendrocytes is grouped alongside of a thin glossy fiber, corresponding to the axons growing from explantates of the neurons. At that period of time, the enzyme activity in oligodendrocytes is very low and may be detected as a scanty, perinuclear accumulation of the formazan grains. In the other cells of oligodendroglia a bigger accumulation of formazan at one pole of the cell, creating perinuclear "enzymatic cap", was observed. Simultaneously, no enzyme activity in the oligodendrocytes processes was detected. A slight symptom of the enzymatic activity in the processes appeared only about the 6th or 7th day of culture. Concomitantly, an increase of the enzymatic activity in the protoplasm, particularly in the perinuclear region, was observed. The intensity of the enzymatic activity in the protoplasm and in the cell processes rose gradually, in concordance with the age of the culture. The highest activity was attained on the 21st day or so of the tissue culture development and maintained further on the same level. Some individual differences in enzyme activity of particular cells were found. This concerned both astrocytes and oligodendroglia, but they were much more distinct in oligodendroglia. In the 2 to 3 weeks old cultures, besides large cells with a small number of processes showing a rich deposit in cytoplasm and cell processes, a numerous aggregation of oligodendroglia with a narrow perinuclear rim of enzyme activity were detected, which correspond to the early form of activity in young oligodendroglia.

The activity of succinic dehydrogenase. During the whole course of the observations, the activity of the succinic dehydrogenase in the culture was much lower, than that of coenzyme I dehydrogenase. In the astrocytes,

the distribution of the enzyme activity was identical to that evaluated for tetrazolium reductase. However, in the process of the culture development, the activity of this enzyme gradually increased, which could be demonstrated by a growing accumulation of the formazan grains in the protoplasm and cell processes of the astrocytes. This process was found to be steady and continuous, without any marked spontaneous start of growth. The distribution of enzyme activity in oligodendrocytes did not significantly differ from that, observed in the previous group, being, however, much lower. Similarly as in the previous group, two types of the enzyme activity distribution, related to the age of culture, were observed. At the early stage of growth, only a very narrow perinuclear rim of the enzyme activity, without any activity in the cell processes is observed, whereas at the late stage of tissue culture an increased activity is present in the entire cell protoplasm, as well as in the cell processes. The earlier described phenomenon, concerning the individual differences in enzyme activity in particular cells, as well as the state of this activity in the hyperplastic glial cells and in degenerated or degenerating cell elements, was found to be identical to a typical picture for NAD diaphorase, with much lower activity, however.

The activity of dehydrogenases linked with coenzyme I (lactic dehydrogenase, malic dehydrogenase, glutamic acid dehydrogeanase and alcohol dehydrogenase). A fundamental pattern of the enzymatic process in both, astrocytes and oligodendrocytes, did not significantly differ from those, reported as typical for tetrazolium reductase and succinic dehydrogenase. The differences concerned only the intensity of enzymatic processes, the rate of their potentiation and the morphology of the formazan grains. The distribution of the activity of all enzymes estimated in this group was found to be uniform in the protoplasm and the cell processes of the astrocytes, whereas in oligodendrocytes the highest activity appeared in the perinuclear region, often in its polar configuration. In the processes the enzyme activity appeared only at a later period. Among enzymes estimated in this group, the lactic dehydrogenase showed the highest activity, second only to that exhibited by coenzyme I dehydrogenase. The glutamic acid dehydrogenase came next, in regard to the potency of exhibited enzyme activity, while malic dehydrogenase was third. The alcohol dehydrogenase showed the lowest activity. At the early phase of the tissue culture (up to 5 days) the activity of alcohol dehydrogenase is represented only by a few formazan grains, situated perinuclearly, in both astrocytes and oligodendroglia. In cultures more than 25 days old, the activity of the alcohol dehydrogenase becomes much higher, attaining the level of dehydrogenase activity. The activity of all dehydrogenases estimated in this group increases along with the progressing age of the culture. A particularly pronounced rise was observed during the second week of the culture growth. At the same period of time the appearance of enzyme activity in the oligodendroglia processes and its rise in the protoplasm of oligodendro-

cytes was observed. The enzyme activity in the processes of oligodendroglia appears as a rule on the 7th day of the culture growth. The activity of malic and glutamic acid dehydrogenases present a certain delay; the first signs of their activity in oligodendrocytes processes were detected after 10 days of the culture growth. The enzyme activity of estimated dehydrogenases varied significantly in hyperplastic cells and degenerating ones. In the hyperplastic cells this activity was high, whereas it was much lower in degenerating cells.

The evaluated difference in the morphology of the formazan grains, designating the site of the activity of particular dehydrogenases, should be emphasized. In case of malic and glutamic acid dehydrogenases, the formazan forms coarse, loosely spread grains in contrast to the delicate, fine-grained formazan deposits of the succinic and alcohol dehydrogenase. In case of lactic dehydrogenase, the formazan is bacilliform.

DISCUSSION

The results of our study indicate the difference of enzyme activity present in astrocytes and oligodendrocytes. This difference concerns first of all the spatial distribution of the enzyme activity within the cells, and much less the intensity of enzymatic reactions. In astrocytes, the enzyme activity is distributed evenly in the protoplasm and the cell processes, showing only slight changes during the whole course of the observation. In the process of tissue culture development, the activity of all estimated dehydrogenases, with the exception of coenzyme I dehydrogenase, increases markedly. The activity of the coenzyme I dehydrogenase was found to be very high already during the first few days of culture growth. In oligodendroglia a biphasic enzyme activity was observed. At the early phase, the enzyme activity is very low, noticeable only in the perinuclear region and completely absent in the cell processes. In a later phase, starting with the 7th to 10th day of the culture growth, a potentation of the enzyme activity in the protoplasm and its appearance in the processes of oligodendrocytes was observed. During the following several days, the enzyme activity in oligodendrocytes increases further and then about the third week of the culture growth, becomes stabilized on the same level as in astrocytes, or on a higher one. However, it should be emphasized that during the whole period of observation, the highest enzymatic activity in oligodendroglia was distributed in the perinuclear region, or in the form of the characteristic unipolar aggregations, as a typical "perinuclear cap". It was established that the biphasic type of the enzyme activity and its typical distribution within oligodendrocytes is a constant and reproductible phenomenon, for it was observed in all the estimated cultures, including all dehydrogenases. It is therefore postulated that this phenomenon may be considered as one of the characteristic features in differentiation of astrocytes from oligodendrocytes. The differences in enzyme

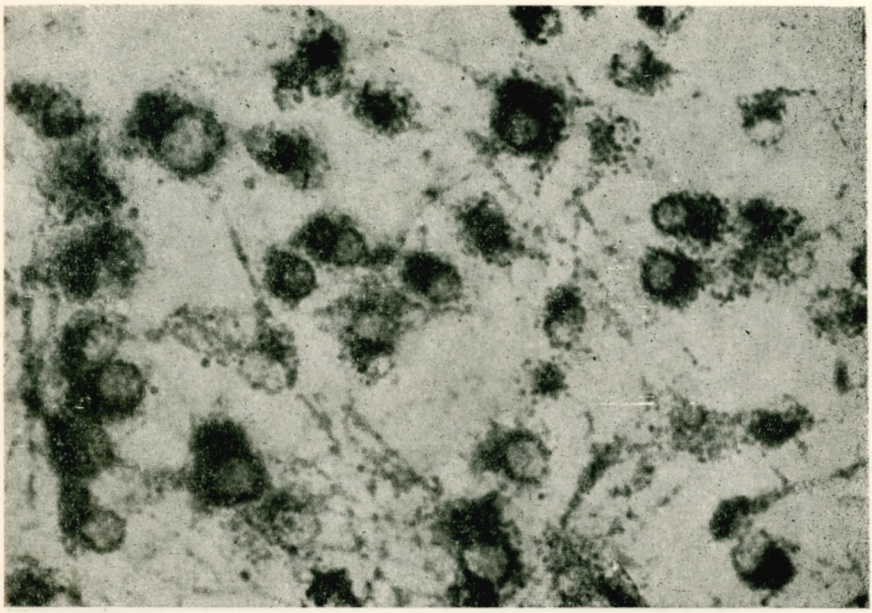

Fig. 1. Activity of dehydrogenase of coenzyme I. Fourteen-day culture. High enzymatic, activity in astrocytes and oligodendrocytes. Typical polar distribution of enzymatic activity in the oligodendroglia. Nitro-BT. Magn. oc. × 15, obj. × 40.

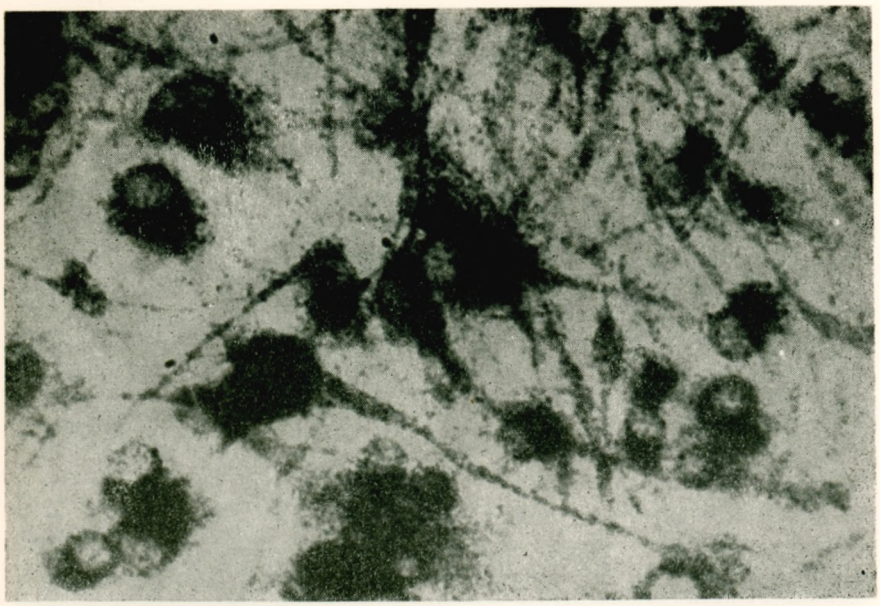

Fig. 2. Activity of dehydrogenase of coenzyme I. Fourteen-day culture. Formazan grains fill the protoplasm and processes of the astrocytes. High enzymatic activity in the protoplasm of oligodendroglia; cell processes still absent in most of the cells. Nitro-BT. Magn. oc. × 15, obj. × 40.

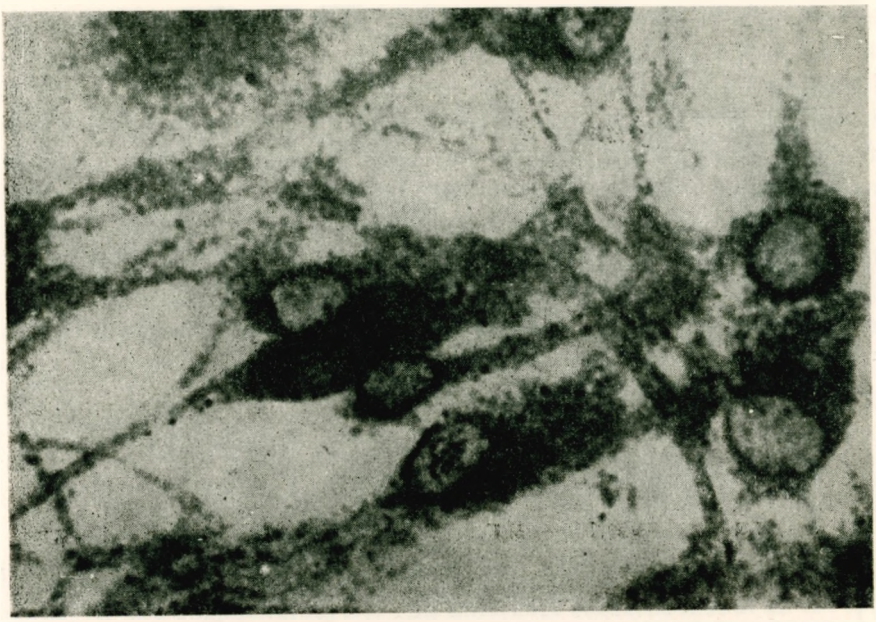

Fig. 3. Activity of dehydrogenase of coenzyme I. Twenty-five day culture. Group of astrocytes with activity in the cytoplasm and cell processes. Nitro-BT. Magn. oc. × 15, obj. × 40.

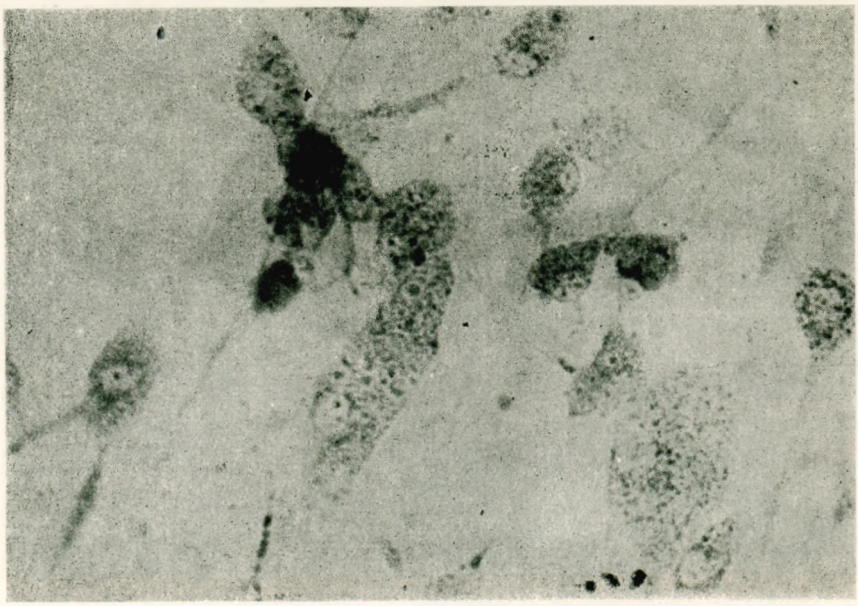

Fig. 4. Succinic dehydrogenase. Seven-day culture. Low enzymatic activity in the protoplasm and processes of the astrocytes. Perinuclear condensation of formazan grains. Nitro-BT. Magn. × 15, obj. × 40.

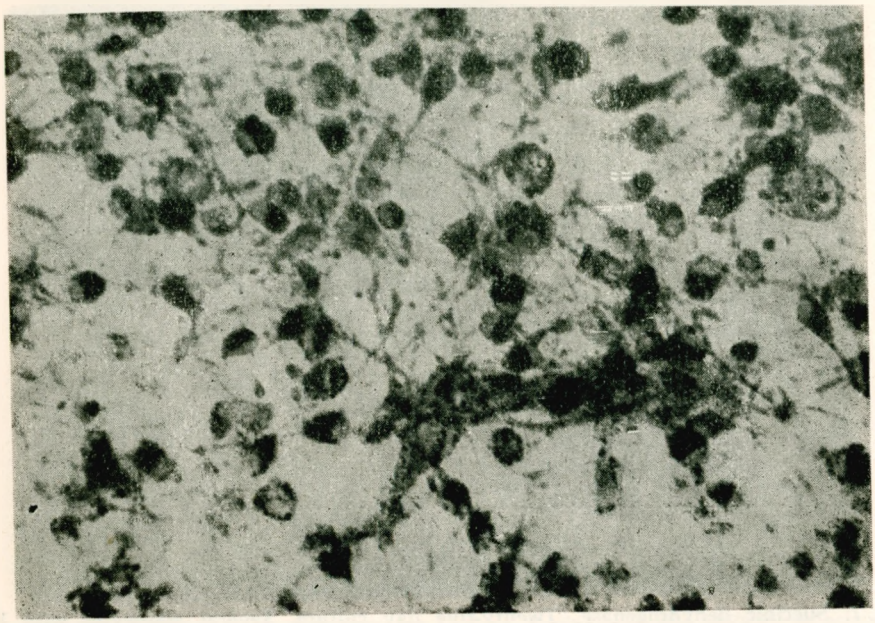

Fig. 5. Succinic dehydrogenase. Ten-day culture. Colony of astrocytes and oligodendrocytes. The oligodendrocytes exhibit polar distribution of activity. Short oligodendroglial processes contain formazan deposits. Nitro-BT. Magn. oc. × 15, obj. × 20.

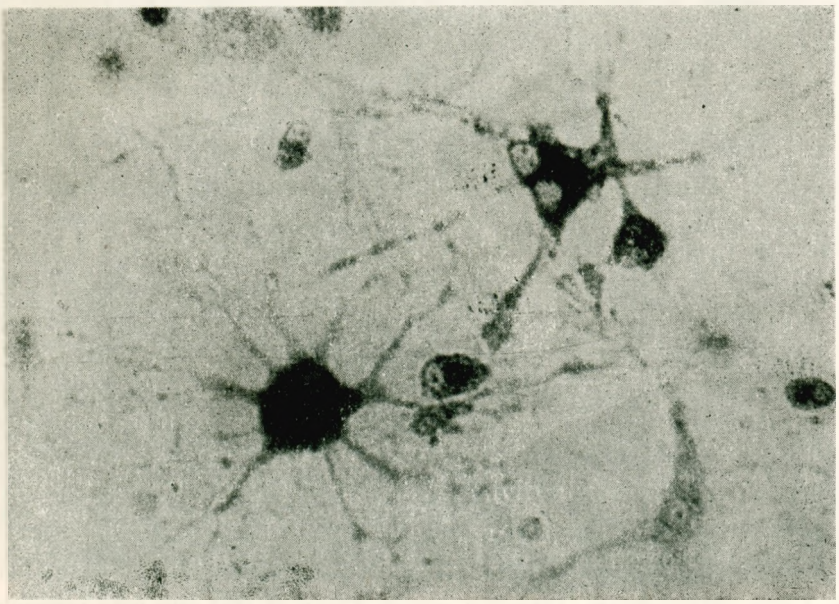

Fig. 6. Succinic dehydrogenase. Eleven-day culture. Astrocytes with high enzymatic activity. Individual differences in the activity of different cells may be noted. Nitro-BT. Magn. oc. × 15, obj. × 40.

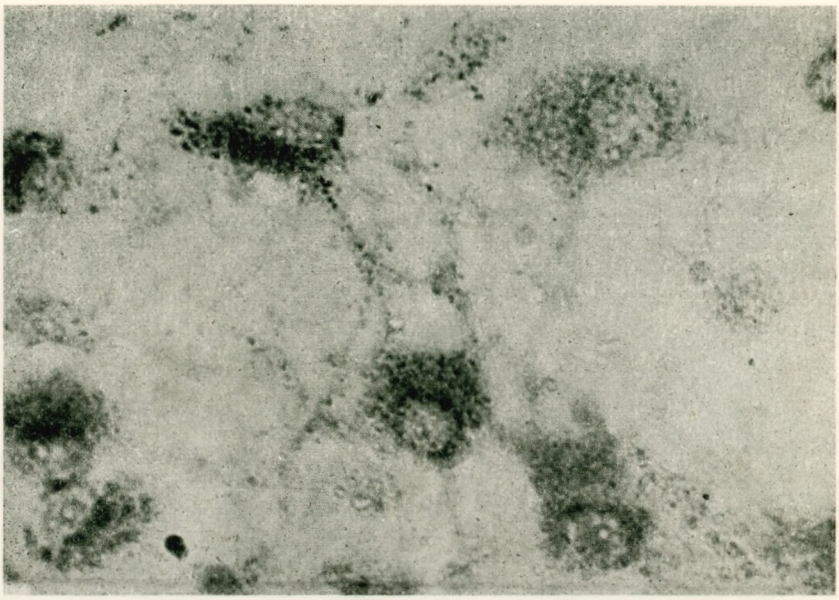

Fig. 7. Succinic dehydrogenase. Twenty-five day culture. Several astrocytes with high enzymatic activity. Nitro-BT. Magn. oc. × 15, obj. × 40.

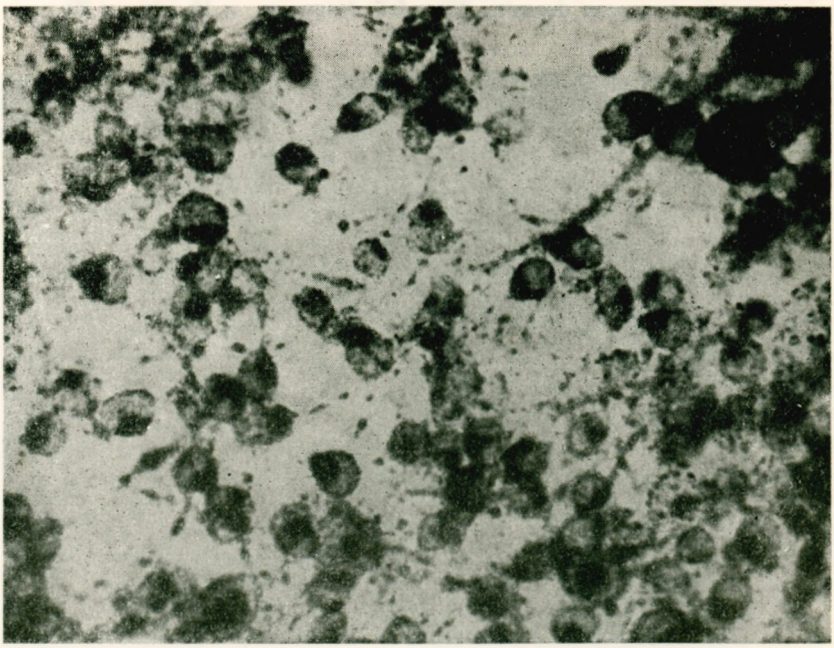

Fig. 8. Lactic dehydrogenase. Three-day culture. Group of glial cells with preponderance of oligodendrocytes. High enzymatic activity in the perinuclear and polar systems. Absence of enzymatic activity in the processes of the oligodendroglia. Nitro-BT. Magn. oc. × 15 obj. × 20.

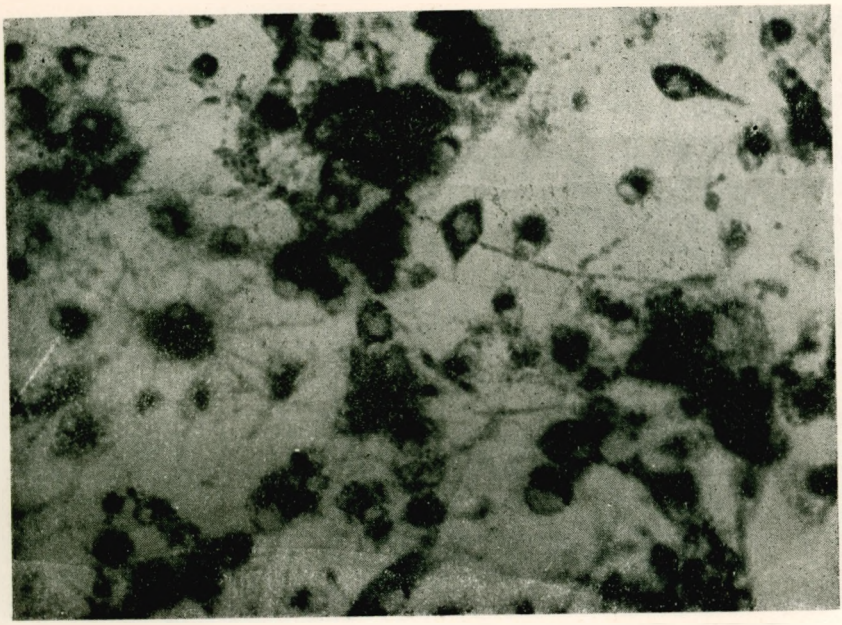

Fig. 9. Lactic dehydrogenase. Fourteen-day culture. High enzymatic activity in astrocytes and oligodendroglia. The oligodendrocytes have short processes. Nitro-BT. Magn. oc. × 15, obj. × 40.

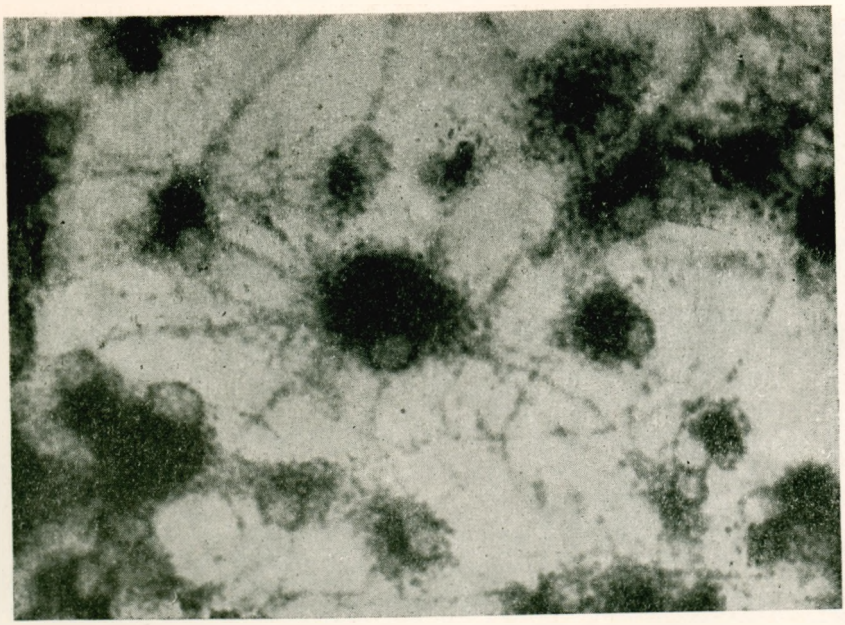

Fig. 10. Lactic dehydrogenase. Fourteen-day culture. Fragment of Fig. 9 in linear enlargement. Isolated formazan grains mark the site of enzymatic activity in the protoplasm and cell processes. Nitro-BT. Magn. oc. × 15, obj. × 40.

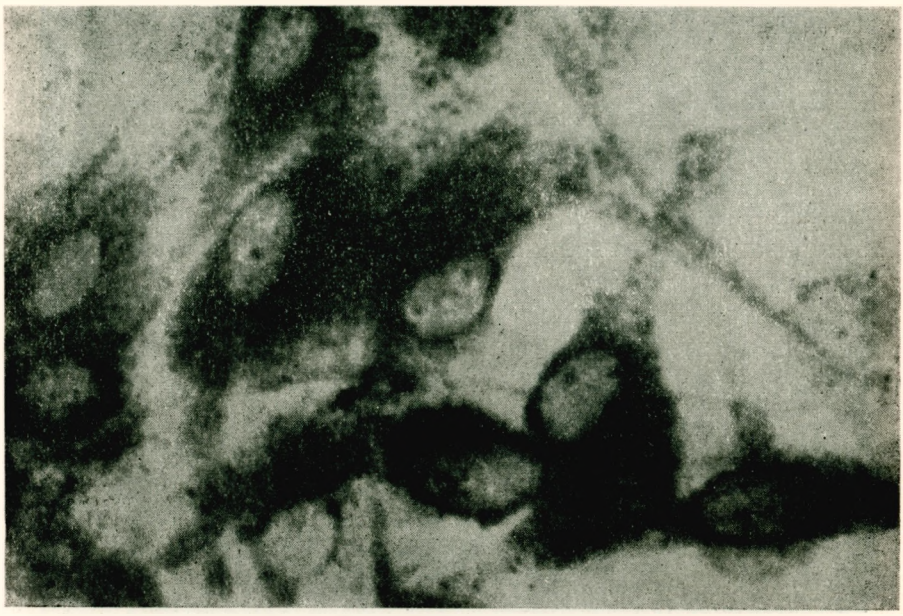

Fig. 11. Lactic dehydrogenase. Thirty-day culture. Group of glial cells with high enzymatic activity. Nitro-BT. Magn. oc. × 15, obj. × 20.

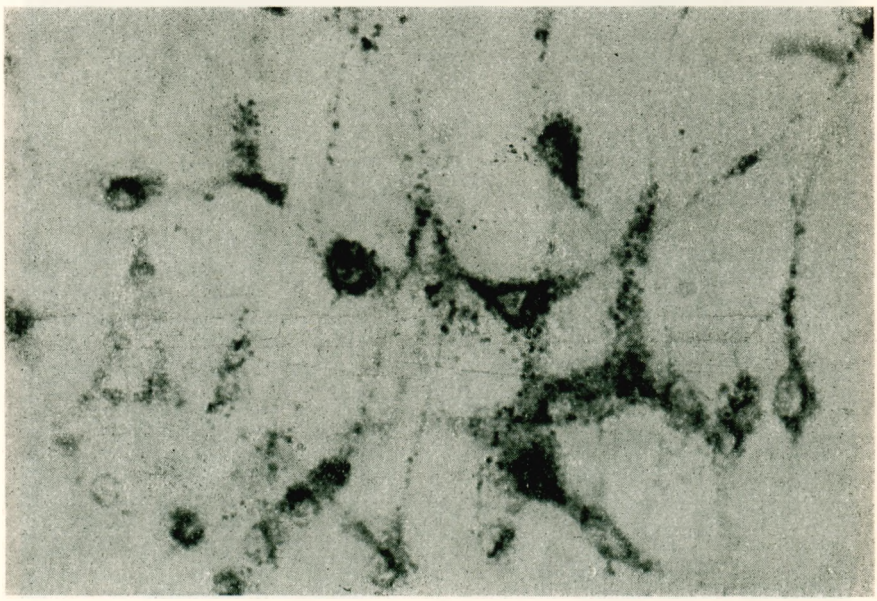

Fig. 12. Glutamic dehydrogenase. Two-day culture. Low enzymatic activity in young astrocytes and oligodendrocytes. No enzymatic activity in the processes of the oligodendroglia. Nitro-BT. Magn. oc. × 15, obj. × 20.

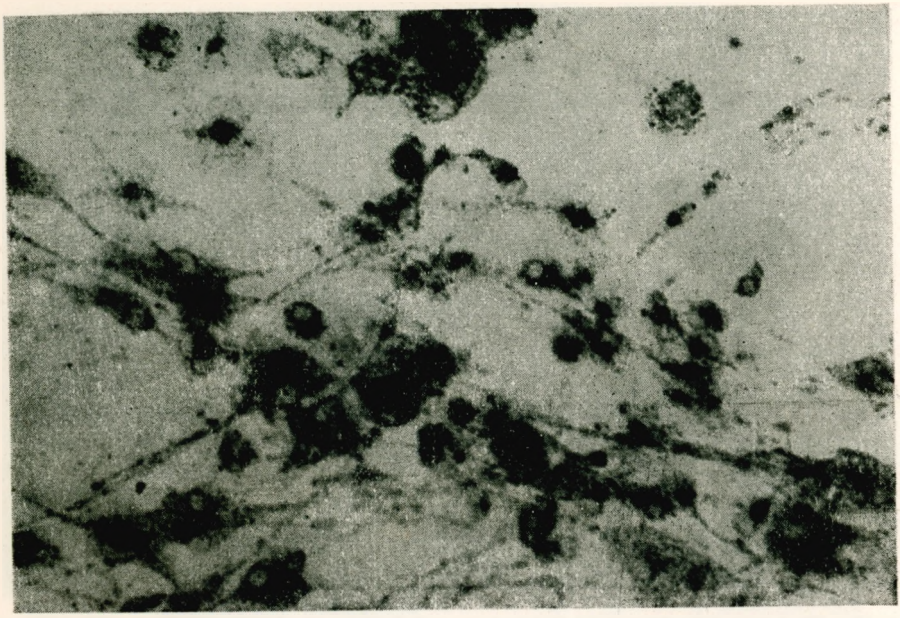

Fig. 13. Glutamic dehydrogenase. Seventeen-day culture. Distinct increase in enzymatic activity compared with Fig. 12, both in the astrocytes and in the oligodendroglia. Nitro-BT. Magn. oc. × 15, obj. × 20.

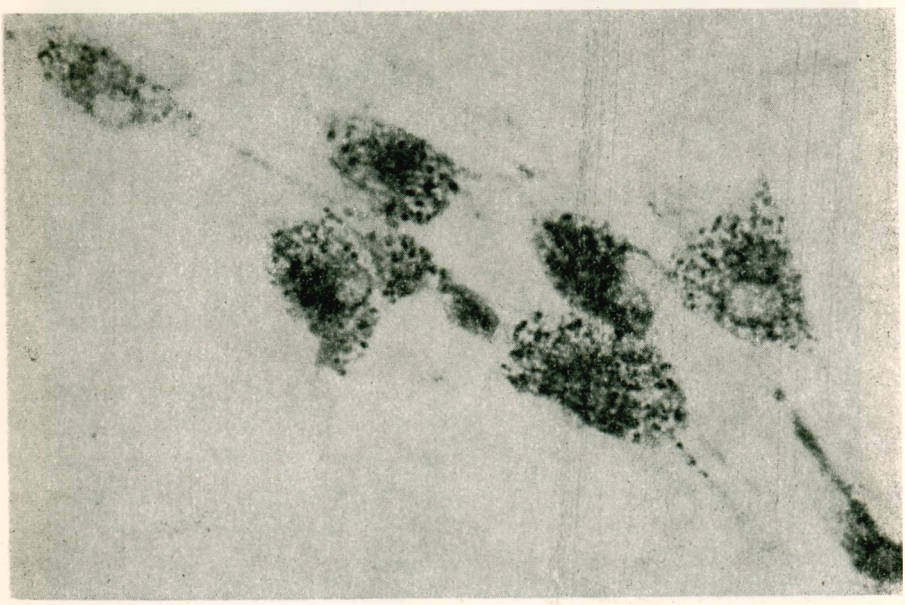

Fig. 14. Glutamic dehydrogenase. The same preparation as in Fig. 13. Group of astrocytes with marked enzymatic activity. Nitro-BT. Magn. oc. × 15, obj. × 40.

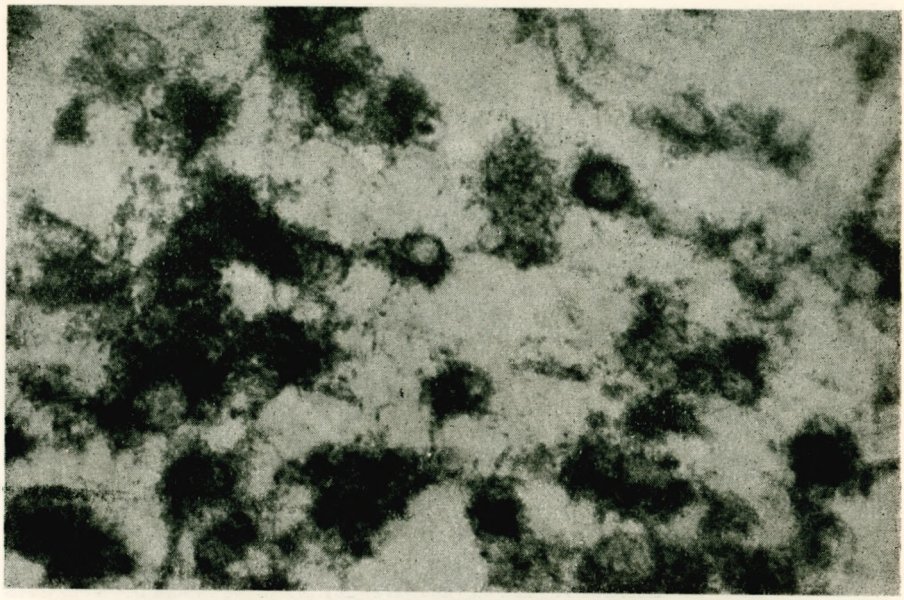

Fig. 15. Glutamic dehydrogenase. Twenty five-day culture. Astrocytic and oligodendroglia colonies with uniform distribution of enzymatic activity. Nitro-BT. Magn. oc. × 15, obj. × 20.

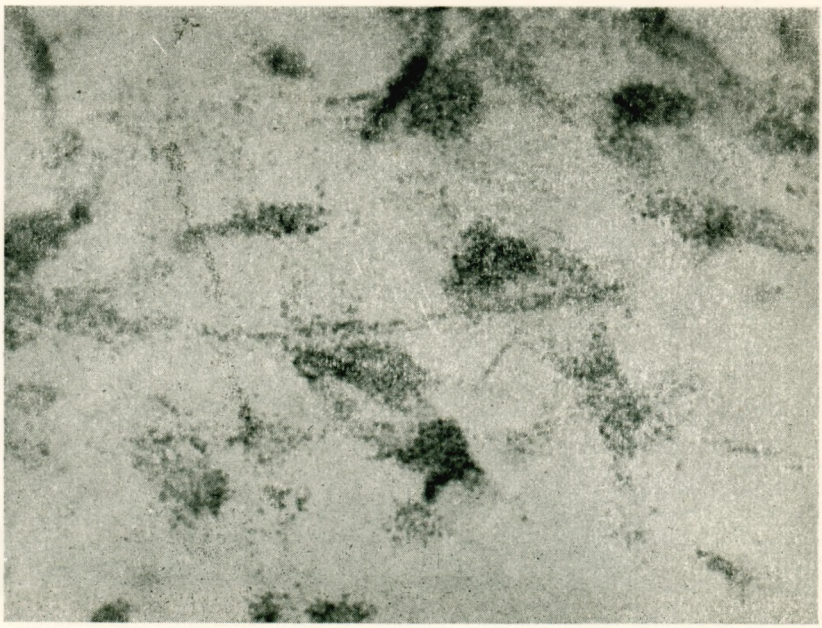

Fig. 16. Malic dehydrogenase. Four-day culture. Low enzymatic activity in the protoplasm and processes of astrocytes. Nitro-BT. Magn. oc. × 15, obj. × 20.

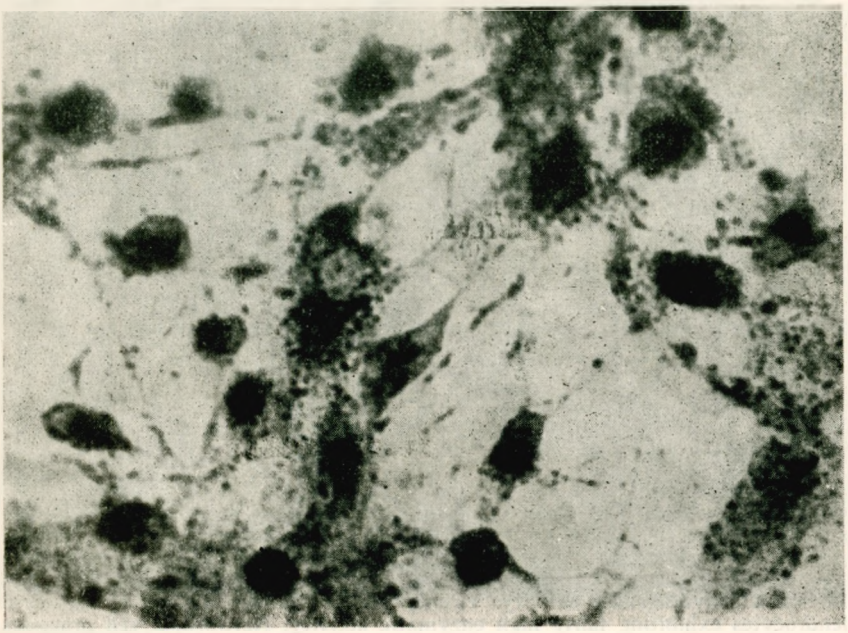

Fig. 17. Malic dehydrogenase. Ten-day culture. Distinct increase in enzymatic activity, especially in oligodendrocytes. Activity in their processes. Nitro-BT. Magn. oc. × 15, obj. × 20.

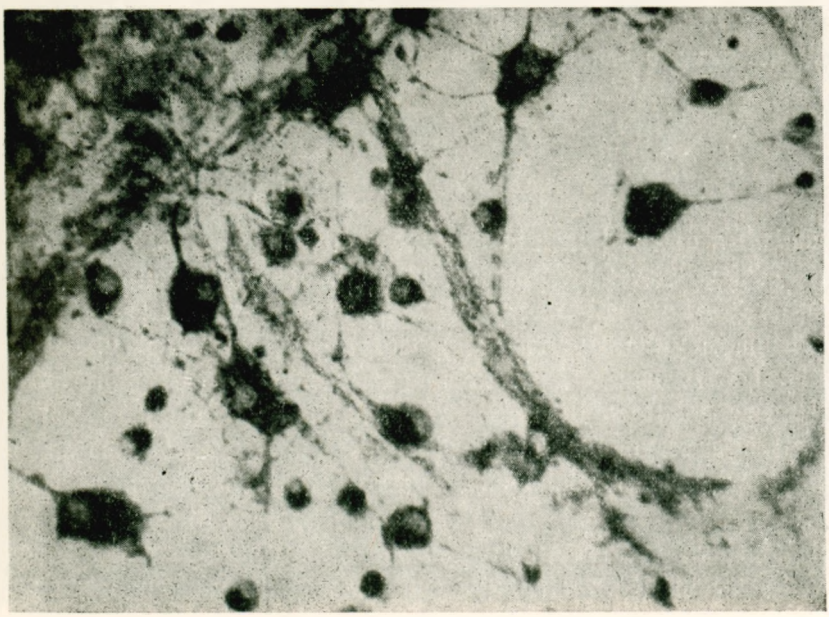

Fig. 18. Malic dehydrogenase. Twenty-five-day culture. Fairly high enzymatic activity in astrocytes and oligodendrocytes. Nitro-BT. Magn. oc. × 15, obj. × 40.

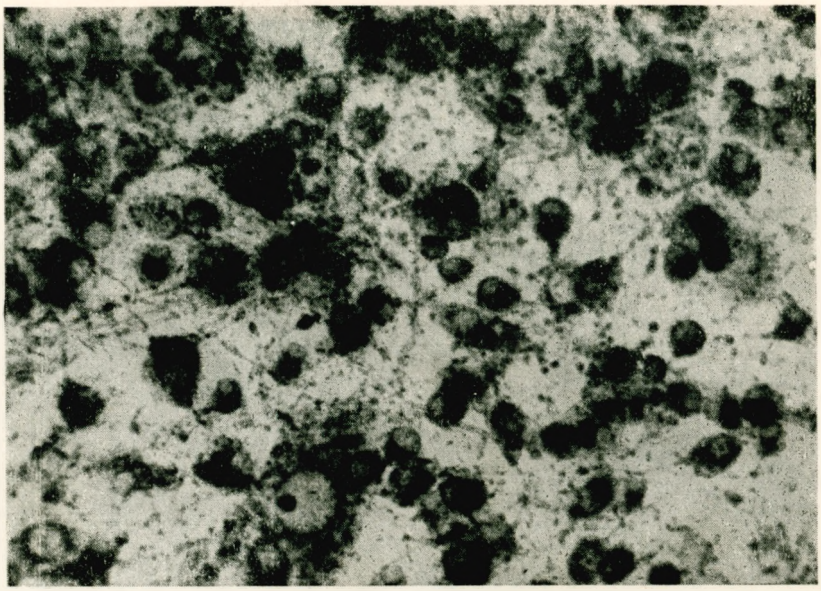

Fig. 19. Malic dehydrogenase activity. Twenty-five-day culture. Colony of astrocytes and oligodendrocytes exhibiting fairly high enzymatic activity. Network of cell processes with formazan deposits. Nitro-BT. Magn. oc. × 15, obj. × 20.

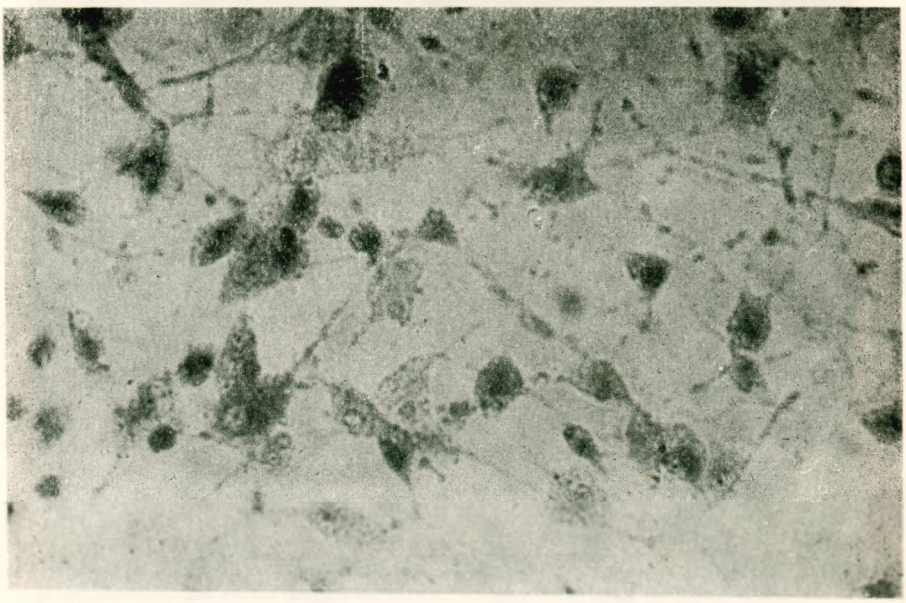

Fig. 20. Alcohol dehydrogenase. Seven-day culture. Very low enzymatic activity. Only some of the cells contain formazan grains around the nuclei. Nitro-BT. Magn. oc. × 15, obj × 20.

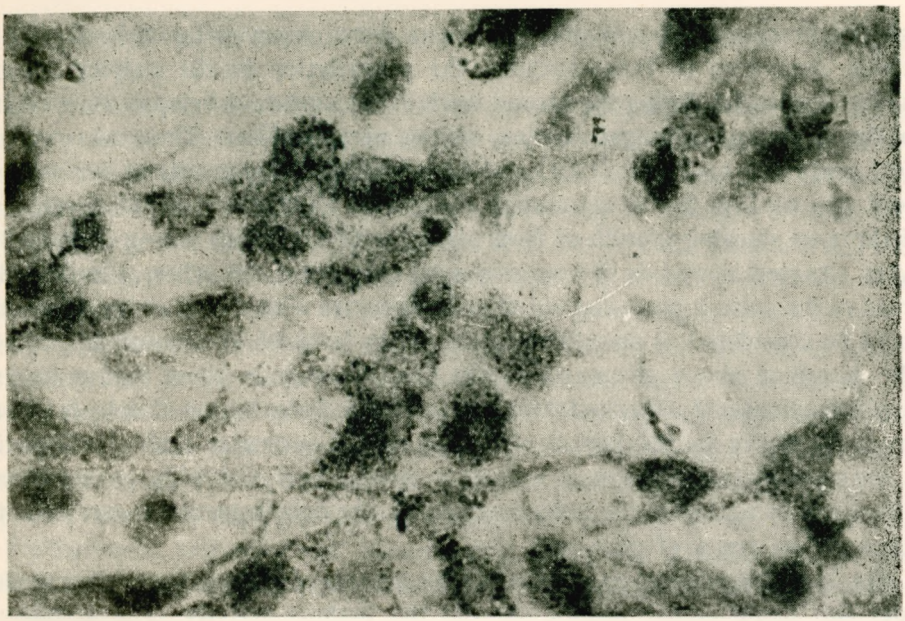

Fig. 21. Alcohol dehydrogenase. Ten-day culture. Increasing enzymatic activity. Individual differences in the activity of different cells. Nitro-BT. Magn. oc. × 15, obj. × 20.

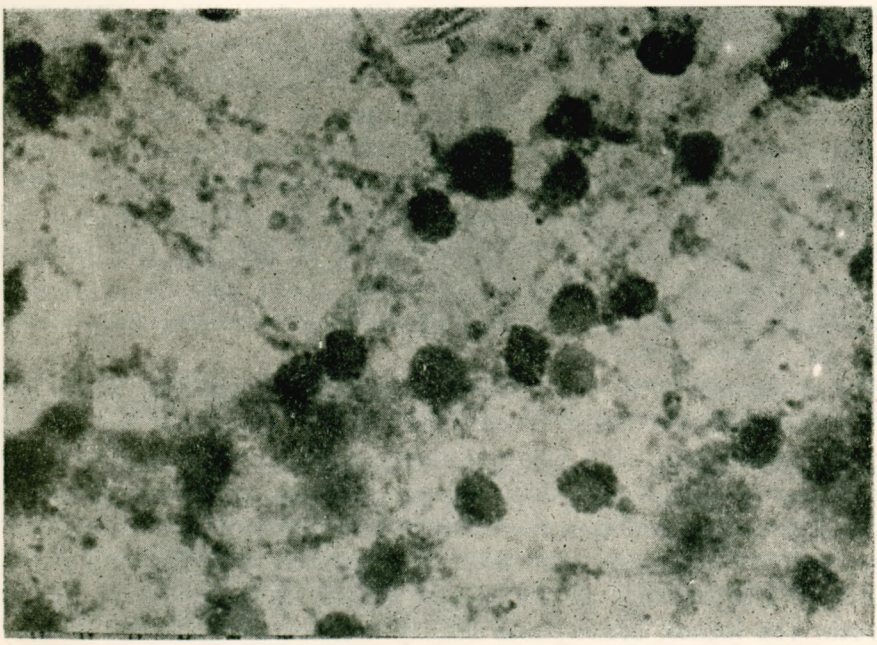

Fig. 22. Alcohol dehydrogenase. Twenty-one-day culture. Further increase in enzymatic activity. Group of oligodendrocytes. Nitro-BT. Magn. oc. × 15, obj. × 20.

activity in astrocytes and oligodendroglia were observed by *Yonezawa et al.* (1962). According to these authors, the potentiation of the enzyme activity in oligodendroglia during the second week of the culture development, is related to the beginning of myelinization of the nervous fiber. These observations were confirmed by *Friede* (1961) and *Meyers* (1964). The results of our observations also confirm the interpretation offered by *Yonezawa et al.* The evaluation of the area showing growth of fresh tissue, with numerous young oligodendrocytes, situated along side of processes a growing nerve cell, whereas in the other areas of the culture prevailed old oligodendrocytes with a high enzyme activity, could be considered as an additional reason in favor of *Yonezawa's* hypothesis. The described above phenomenon was detected only in those "old cultures", in which colonies of nerve cells were present. The potentiation of dehydrogenase enzyme activity in the period of myelinization, indicates their participation in the myelinization process according to *Yonezawa et al.* (1962). No significant differences in regard to the activity of particular dehydrogenases were detected between two types of glial cells.

The dehydrogenase of coenzyme I (a diaphorase of diphosphopyridine nucleotide) and lactic dehydrogenase showed the highest activity. The activity of glutamic, succinic and malic dehydrogenases was lower. The lowest activity characterized the alcohol dehydrogenase. Our observations, concerning the evaluation of the alcohol dehydrogenase activity in the tissue culture of the glial cells, differ from those reported by *Chason et al.* (1963). These authors, examining the tissue *in vivo*, could not detect the activity of this enzyme neither in normal and reactive glia, nor in gliomas. The behavior of this enzyme activity in our study, was found to be quite significant. At the earliest period of tissue growth its activity is very low, but then becomes gradually potentiated, parallely to culture growth. The highest activity was found in the old cultures. The presence of the alcohol dehydrogenase indicates the existence in glial cells of metabolic processes leading to the production of alcohols. The oxidation of alcohols results in production of aldehydes, which may, in turn, produce a damaging effect of the glial cell. This process becomes evidently intensified with the growth of the culture.

The evaluation of the high activity of lactic dehydrogenase, which remains in agreement with the observations of other authors (*Chason et al.*, 1963; *Ostenberg* and *Wattenberg*, 1962) following their investigations of the tissue *in vivo*, concomitantly with a high dehydrogenase activity, catalyzing the reactions in the Krebs cycle, indicate the coexistence in the glial cells of both aerobic glycolysis. All dehydrogenases, with the exception of tetrazolium reductase, presented a potentiation on the enzyme activity in the course of the culture growth. The highest activity was usually attained after about 3 weeks. The initial level of the dehydrogenase coenzyme I activity was so high, that it was

practically impossible to establish whether this activity increased during the culture growth, or not.

It should be emphasized, that as far as enzyme activity is concerned, the glia in tissue culture behave much more like reactive glia, than neuroglia *in vivo*. As has been already mentioned, the majority of authors emphasize a low enzyme activity of dehydrogenases in normal glia, especially in astroglia (*Friede*, 1961, 1962; *Rubinstein et al.*, 1962; *Osterberg* and *Wattenberg*, 1962; *Chason et al.*, 1963; *Mossakowski*, 1963). Their activity became potentiated in the processes of the glia reaction. In our study, the enzyme activity in astrocytes cultured *in vitro* was similar to that detected in reactive glia. Moreover, the activity of succinic dehydrogenase in the astrocytes cultured *in vitro* was higher than the activity of this enzyme detected in the reactive glia by *Ostenberg* and *Wattenberg* (1962). It should be also mentioned that their observations disagree with those of *Rubinstein et al.* (1962); *Chason et al.* (1963) and *Mossakowski* (1963), who reported relatively high succinic dehydrogenase activity in the reactive glia.

Furthermore, following our experimental study we established significant individual differences, concerning the intensity of enzymatic reactions in particular glial cells, in the same tissue culture. These differences are most probably related to the functional state of the cells, because no corresponding changes in the morphology of cells could be detected. The cells characterized by a similar morphology exhibited a different degree of the enzyme activity and *vice versa*: the same enzyme activity was present in cells of different morphologic shape.

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