Histochemical and Cytochemical Studies on the Succinic Dehydrogenase in the Developing Cerebellum.*

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Histochemical or cytochemical demonstration of succinic dehydrogenase activity (SDA) in the mammalian brain tissues has been made by many investigators, such as LEDUC and WISLOCKI (1952), PADYKULA (1952), MALATY and BOURNE (1953), NEUMANN and KOCH (1953), RUTENBURG et al. (1953), MUSTAKAL-LIO (1954), PEARSON and DEFENDI (1954), FRIEDE (1957), ORTMANN (1957), SHIMIZU et al. (1957), MORIKAWA (1958 a, b), FRIEDE (1959), ODA et al. (1959), POTANOS et al. (1959), WOLFGRAM and ROSE (1959), CHASON and PEARSE (1961), OGAWA and OKAMOTO (1961), THOMAS and PEARSE (1961), FRIEDE (1962), MOSSAKOWSKI (1962), TEWARI and BOURNE (1962), YONEZAWA et al. (1962), SCHIFFER and VESCO (1963).

The detailed study, however, on the changes of SDA in the developing cerebellum from the embryonic life to the adult has not yet been done. Only FRIEDE (1957) studied this in the developing cerebellum of the rat, while his data are dealt only with the activity from newborn to adult.

The present investigation was undertaken to demonstrate the distribution of SDA in the developing cerebella of cats and mice from the middle fetal life to adult at the cytological level, employing the technique modified by the present author.

I. Historical Remarks.

Based upon the idea postulated by THUNBERG, SEMENOFF first demonstrated SDA in 1934 in the tissue sections, by use of methylene blue under anaerobic condition. He described that the activity was lost by the use of fixative, or in paraffin sections. In 1951, ditetrazolium chloride (blue tetrazolium = BT) was first introduced by SELIGMAN and RUTENBURG. They found that the results were dependent upon the thickness of sections, very thin sections (8 μ) failed to stain altogether whereas thicker sections (30—40 μ) showed uniformly a deep-blue stain, and sections of 20 μ thickness were optimum. PADYKULA (1952) investigated various tissues of the rats using BT, and found that the best results were obtained with sections 5 to 20 μ in thickness in case of the tissue with high enzymatic activity, but in tissues of lower or moderate activity, it was necessary to use thicker sections such as 40 to 20 μ. She

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also mentioned the usefulness of cryostat sections in the reactions, and the enhancement of diformazan formation under the strict anaerobic condition.

For the demonstration of SDA in the tissue which had low activity (thyroid), GOODARD and SELIGMAN (1952) devised a supravital technique. The tissues were cut with the razor blade into slices thinner than 0.1 mm, washed in phosphate buffer, stained, dissociated with dissecting needles after removal from the medium and mounted in agar which contained the incubation medium in it.

In the screening tests of various tetrazolium salts, SHELTON and SCHNEIDER (1952) found that evenly distributed formazan precipitation was consistently obtained in 30 μ sections when neotetrazolium (NT), tetrazolium violet (TV), or triphenyl tetrazolium chloride (TPT = TTC) were used, and then when BT was used, partially blank sections were found even at 75 μ. MALATY and BOURNE (1953) investigated the reduction of BT in various tissues of guinea pig, rabbit, mouse and rat, and obtained poor results in sections of 20 μ thickness. The excellent and consistent results were obtained in sections of 50 — 100 μ thickness.

RUTFNBURG et al. (1953) published the improved method for thin sections such as 10 μ thickness. According to their method, the sections were stained after mounting on a clean glass and dried in the air, or stained during floating in the incubation medium which contained activators such as Ca++, Mg++, Al+++ and HCO3-. It was noted that the sections mounted on slides stained less intensely than those stained floating in the medium, and that the anaerobic condition and the activators greatly accelerated the velocity of reaction.

PEARSON and DEFENDI (1954) described that they encountered two difficulties, i.e. in getting thin sections of approximately 5 μ and the unevenness of the reaction. They overcame these difficulties by rapid freezing of tissues using dry ice at −70°C, and by the use of INT.

ROSA and VELARDO (1954), utilizing 10—20 μ fresh frozen sections, which were mounted upon slide glasses and dried in the air, found that the use of cyanide in the incubating medium afforded equally good reduction of NT to diformazan, and that the use of phosphate buffer at higher pH (8.2) showed the enhancement of the tetrazolium reduction than at pH 7.6.

In 1956, FARBER and LOUVIERE found that the rate of stainability was greatly increased by the use of a tetrazolium salt together with a suitable oxidation-reduction dye such as methylene blue or azure dye.

In the recent year, special attention has been payed to the development of enzyme activities in the immature animals. FRIEDE (1957, 1959) studied first the distribution of SDA in the developing rat brain using 60 μ unfixed frozen sections which were cut at a temperature ranging from −3°C to −2°C. The tetrazolium salt used was TV. On the other hand, MORIKAWA (1958 b) investigated the SDA in the brain of rat from late fetal life to adult, using 20 μ unfixed frozen sections which were mounted on slide glasses in a cryostat (SHIMIZU et al. 1956) and put by drying in the air at room temperature. The tetrazolium salt used by him was NT. But, there are conspicuous discrepancies in the results obtained by both workers. In cerebellum, FRIEDE demonstrated strong SDA from the 2nd day after birth, while MORIKAWA demonstrated it from the 7th day. The differences were presumably derived from the
differences of the sections used and in part from the tetrazolium salts utilized.

The introduction of Nitro-BT by NACHLAS et al. (1957) brought forth a new era for the histochemistry of succinic dehydrogenase. They realized the use of paraffin sections for the demonstration of SDA. Of course, they also prepared frozen sections. In their method, sections of 2 to 8 μ thickness were cut at −20°C in a cryostat following rapid freezing of the tissue blocks of 3–5 mm thick by isopentane, attached upon untreated glass slides and stained. Owing to this new tetrazolinm salt, they could obtain the excellent staining preparations which facilitate the investigation to the cytological level.

An attempt has also been made with an aim to improve intracellular localization of succinic dehydrogenase by the use of 7.5% polyvinylpyrrolidone sucrose medium (NOVIKOFF 1957, PEARSE 1961, YONEZAWA et al. 1962). The usage, however, was soon criticized by THOMAS and PEARSE (1961) because of the possible interference with penetration of the reagents into nervous tissues.

In 1959, POTANOS et al. found a new method which had no freezing procedure and thus avoided the possibility of damaging mitochondria. The method consisted of the following procedures, i.e., after sectioning the tissues by hand into blocks of 0.5–1.0 mm thickness, the blocks were submitted for incubation at 5°C for 24 hours, fixed with 10% neutral formal saline for ca. 24 hours, dehydrated, embedded in paraffin and sectioned at 10 to 15 μ.

More recently, MIZUTANI et al. (1961) published the improved technique for the demonstration of various dehydrogenase in paraffin sections.

OGAWA and SHINONAGA (1961), using cryostat sections, investigated the role of intermediators in the chain of electron transfer system in the tetrazolium dehydrogenases. They observed that phenazine methosulphate and vitamine K3 play a role in enhancement of the reactions.

In 1962, MAEDA et al. suggested the use of carbowax (polyethylene glycol) embedded tissues for the demonstration of various dehydrogenases. Their method was as follows, i.e., tissue slices were directly immersed in carbowax 300 for one hour at 0–10°C, carbowax 600 for 30 minutes at 25°C and carbowax 1,000 for 15 minutes at 38–40°C. They were solidified in a dessicator which was kept in a refrigerator, embeded in paraffin (M. P. 50°C) and cut and stained in a usual fashion.

The present author has elaborated simple method for the demonstration of SDA in the immature soft brain, as will be described in the 'materials and methods' in this paper. By this method, sections of 5–14 μ thickness were easily obtained and incubation medium gave a good color reaction even in such a thin section. The morphological integrity of the stained preparation was well preserved even in the immature soft brain, also.

II. Materials and Methods.

Eighteen cats were used in the present investigation. They were classified as follows: a fetus at the middle stage of embryonic life (ca. 55 g); a fetus at the late stage of embryonic life (near term) (ca. 80 g); a newborn within 10 hours after birth; a newborn within 12–24 hours after birth; 2, 4, 6, 7, 9, 12, 14, 15, 21, 34, 45,
56, 65 day-old kitten and an adult cat (2.5 years old).

Webster-Swiss strain mice of various ages were also used. They were; newborn within several hours after birth, 2, 4, 5, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 19, 20, 22, 23, 25, 28 day-old, 2 month- and 6 month-old.

All the animals were decapitated without anesthesia. The cerebellar hemispheres were removed dropping the cold acetone onto them, transferred into the chilled acetone, diced as quickly as possible to the small blocks of ca. 3–5 mm³ large in size, and the fixation was further continued for 45–60 minutes in a refrigerator.

Following 30 minutes of carbowax (polyethylene glycol) infiltration (M.W. 1,500 or 1,000 was used) at 37°C, the blocks were immersed briefly in distilled water, and sagittal sections of 5–14 μ thickness and 60 μ were cut by a cryostat or a electro-freezing microtome (OKAMOTO and MIZUNO 1963). Those thin as well as thick sections were immediately transferred into the 60 % acetone, 30 % acetone and distilled water successively. Each of the Procedure had 5–3 minutes immersion. By this procedure, sections were stretched over the surface of water and became flat by virtue of the differences of the surface tension between the fluids. Those thin as well as thick sections were picked up and incubated in open vessels or tightly closed vessels for 60–120 minutes at 37°C. Only in the case of fetus at the middle stage of embryonic life, incubation was continued for 12 hours.

The incubation medium (OGAWA and SHINONAGA 1961) was consisted of 1.5 ml 0.1 M sodium succinate, 1.2 ml 0.1 M Sörensen’s phosphate buffer (pH 7.6), 0.3 ml 250 mg % Nitro-BT and 0.1 ml 100 mg % phenazine methosulfate or 0.1 ml 200 mg % vitamine K₃. After the incubation, the sections were washed in distilled water carefully and postfixation was carried out with 10 % formalin for 15 minutes at room temperature. Those sections were embedded in glycerin jelly or balsam after dehydration with alcohol.

For the controls, substrate-free media and the incubation media containing sodium malonate as competitive inhibitor, in addition to sodium succinate, in the final concentration of 0.1 M were used (pH was adjusted with NaOH). The sections were also heated at 80°C for one hour and incubated in the experimental media for 2 hours.

Besides those controls, some sections were incubated in the experimental media which contained respectively, monoiidoacetic acid, oxalacetic acid, selenic acid (DAS 1937, FRIEDE 1958) in the final concentration of 0.01 M.

For the references, hematoxilin-eosin preparations, BIELSCHOWSKY’s silver impregnated preparations and FEULGEN preparations were made, and electron microscopic observations were also done in some cases.

Carbowaxes used in the present study were manufactured by Carbide & Carbon Chemicals Corporation (U.S. A.).

III. Results.

Sections incubated in the experimental media presented blue or dark blue color due to the reduction of tetrazolium salt, while those in the control media presented pink or red. Therefore, pink or red color was regarded as SDA negative in the present study.

Between phenazine methosulfate and vitamine K₃ which were used as intermedi-
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ators, no differences were found on the distribution of enzyme activity and the intensity of the reaction. No nuclei were stained in any of the cells examined, SDA was recognized exclusively in the cytoplasm and the neuropile.

a) External granule layer.

Enzyme activity, though extremely weak, was already recognized at the middle

Figs. 1 and 2. SDA in the cerebellar cortex of a fetus of a cat at the middle stage of embryonic life (Fig. 1), ×240, and of a fetus of a cat at the late stage of embryonic life (near term) (Fig. 2), ×140. eg external granule layer, ig inner granule layer, P pia mater, W white matter. For the detailed explanation, see in the text.
stage of embryonic life (Fig. 1). The intensity of the reaction ranked next to that of
the PURKINJE cell layer. At the late stage of embryonic life (near term) the activity
increased remarkably (Fig. 2). In the preparations of new borns within 10 hours a.
b. (after birth), the activity was a little more intensified (Fig. 3). Between new borns
and the 2nd day a. b., there were no remarkable differences about the intensity of
reaction. The maximum activity of this layer was seen between 6 and 15th day a. b.

![Image of cerebellar cortex](image.png)

Figs. 3 and 4. SDA in the cerebellar cortex of a new born kitten within 10 hours after
birth (Fig. 3), ×140 and of a kitten on the 6th day after birth (Fig. 4), ×180. Division
into two zones are recognizable, i. e. superficial zone (S) and deeper zone (D). In the deeper
zone, a narrow band (arrows) which showed a relatively strong activity is seen. P pia mater,
ig inner granule layer.

(mouse 5—12th day) (Figs. 4, 5, 7 and 8). During this period, the activity of each
cells which compose this layer was also maximum.

Under a low magnification (Fig. 8) this layer appeared as the blue meshes of a
net, and division into two zones were also recognized, i. e., the superficial zone adja-
cent to the pia mater and the deeper zone. The former exhibited always a little
stronger activity than that of the latter (Fig. 4). This division could be seen already
on the day of birth. The cells possessed, under a higher magnification, large nuclei
and thin rim of the cytoplasm, in which a small number of fine, blue formazan
granules were seen to be scattered (Fig. 5). The distinction of neural or glial cells
from those external granule cells could not be made in the present study.

Observations on the 21st day a. b., however, revealed that the superficial zone
had decreased greatly in thickness. In the deeper zone, it was observed a narrow band
which showed a relatively strong activity (Figs. 3, 4, and 9).

Untill 12th day a. b. (mouse 7th day) exernal granule layer showed stronger ac-
tivity than that of inner granule layer, and thereafter the activity of inner granule
layer became predominant. In the preparation of a cat on the 56th day a. b. (mouse
Fig. 5. A higher magnification of the external granule layer as presented in Fig. 4. *P* pia mater. \( \times 1,100 \)

Fig. 6. A higher magnification of the inner granule layer as presented in Fig. 4. *G* a large cell, presumably the astrocyte, *g* inner granule cells, *gC* glomerulus cerebelli, in which a concentration of dark blue, fine formazan granules are seen. \( \times 1,100 \)
15th day), the thickness of external granule layer decreased to 2 or 3 rows of cells and the enzyme activity, if any, a little decreased also (Fig. 13). On the 65th day, this layer decreased to a single row of cell or partially disappeared (mouse 19th day).

b) Molecular layer.

Molecular layer was not clearly identified in the fetuses. Appearance of a narrow band of a weak activity, just above the PURKINJE cell layer was the first indication of this layer. It was seen on the day of birth (mouse 5th day) and continued to develop upwards, situating between external granule layer and the PURKINJE cell layer, increasing its activity (Figs. 3 and 4). The strong activity of the dendrites of PURKINJE cells in the molecular layer was constantly seen from the 12th day a. b. on (mouse 10th day) (Figs. 7, 8 and 10). Comparing with mice, the kitten dendrites were fairly well developed. Following this stage, the lower end of molecular layer showed as strong activity as that of the PURKINJE cell bodies, and further increased its thickness towards the pia mater. It was also observed that the enzyme distribution of molecular layer was not uniform throughout the whole life, i.e., the upper most part always exhibited a weak activity as compared with the rest part of this layer.

![Fig. 7. SDA in the cerebellar cortex of a kitten on the 12th day after birth. Growth of the dendritic trunks are clearly seen. P pia mater, eg external granule layer. ×250](image-url)
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Fig. 8. SDA in the cerebellar cortex of a kitten on the 14th day after birth. Growth of the dendritic branches (arrows) and strong activities in the neuropile of molecular layer (M) are seen. P pia mater, eg external granule layer, C capillary. ×250

Fig. 9. SDA in the cerebellar cortex of a kitten on the 21st day after birth. A number of cells (arrows) are seen in the molecular layer (M). Divisions of superficial (s) and deeper zone (d) in the external granule layer (eg) are still recognizable. P pia mater, ig inner granule layer. ×240
and that the lowest part showed the most intense activity (Figs. 7, 8 and 9). In the
extension of this layer, many cells were seen scattered at the different levels of
heights from ca. 12th day a. b. on (mouse 10th day), whose cytoplasm were so nar-
row that the distinction from the molecular layer itself was impossible in the most
cases.

It was observed that the development of this layer was completed when the ex-
ternal granule layer disappeared.

c) PURKINJE cell layer.

The enzyme activity was already recognized in the fetus at the middle stage of
embryonic life, and it was always stronger than any of the other layers. At the late
stage of embryonic life, prominent increase in the activity was seen (Figs. 1 and 2).
On the day of birth a little increase of the activity was seen in both animals. There
were no remarkable differences on the intensity of reaction between newborn and the
animals of 2 day-old. Intracellular localization of the activity of immature PUR-
KINJE cells was peculiar, i.e., about the 6th day a. b. (mouse 8th day) the nuclei
were located towards the bottom of cell
bodies. The enzymatic activity was conspic-
uous in the area of dendritic origin (Fig.
11). Approximately, from the 7th day on
(mouse 10th day), the dendrites in the mo-
lecular layer began to show as prominent
activities as that of the PURKINJE cell
bodies, and their branches began to develop
too. On the 12th day (mouse 10th day),
PURKINJE cell bodies came to line up far
beneath the molecular layer, due to the
growth of dendritic trunks (Figs. 7, 9, 10
and 14), and the nuclei were located in the
center of racket-shaped cytoplasm and the
deeply stained perinuclear area was seen
(Fig. 7). It was also recognized that the
heavy deposition of the formazan granules
occurred in the endoplasmic region, but
lesser amount of deposition in the ecto-
plasmic region. On the 21st day a. b. spiny
feature of the dendrites were already seen
(Fig. 10).

In the adult animals, it was some-
times observed that in the row of PUR-
KINJE cells or in the region just beneath the PURKINJE cell bodies, there were
cells which showed strong activities both in the cytoplasm and their processes. These
cells were interpreted to be the BERGMANN cells. Besides these, the basket fibers
and satellite cells were clearly seen with moderately strong activities (Figs. 14 and
15).
d) **Inner granule layer.**

During the embryonic life, the greater part of this layer did not stained except for the narrow region adjacent to the PURKINJE cell layer (Fig. 2). It was recognized that the region showed the weak activity with the gradual decrease towards the white matter. At this stage, a boundary between the inner granule layer and the white matter was not clear as in the adult.

The numbers of cells which constitute this layer seemed to increase from about

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**Fig. 11.** SDA in the PURKINJE cells of a new born kitten. The nuclei are located eccentrically in the bottom of cell bodies (arrows), and the activities are seen mainly in the area of dendritic origin, though dendrites are poorly developed yet. ×540

Compare with the Fig. 10.

**Fig. 12.** SDA of the cells in the white matter (W) and of the neighboring inner granule layer (ig) of a kitten on the 56th day after birth. The activities are localized in their cytoplasm as well as the processes. ×140
the 7th day a. b. On (mouse 5th day). At the same time, it was observed that var-
guely demarkated speckes of a weak activity appeared, hear and there, among the
tightly packed inner granule cells, and those speckes contained a number of deposition
of formazan granules (Figs. 4 and 6) under a higher magnification. Those findings
were considered to correspond to the beginning of formation of glomerulus cerebelli.
The speckes increased rapidly in their number and the activities. On the 14th day a.
b. (mouse 8th day), the activities of inner granule layer were more intensified and
the distribution pattern was nearly the same in the adult (Fig. 14). Observations on

the 34th day (mouse 10th day) revealed that it became almost equivalent to that of
the adult. The preparations on the 45th day (mouse 13th day) showed the adult level
(Figs. 12 and 14).

The inner granule cells had also large nuclei and narrow rim of the cytoplasm
containing a small number of fine formazan granules scattered in it (Figs. 6, 14 and
16). With a higher magnification, it was always recognizable that along the circum-
ference of round nuclei, a small number of fine formazan granules were seen to be
scattered (Fig. 16). Sometimes, there appeared the deeply stained cells of relatively
large size in the different parts of this layer. The cytoplasm contained much more
formazan granules than the other inner granule cells (Fig. 6). These cells were dis-
distinctly seen during the period when the activities of glomerulus cerebelli were not
yet strong. They were considered to be the astrocytes.

Activities of glomerulus cerebelli were so intense, especially in the adult ani-
mals, that the intra-glomerular details were obscured in many cases (Figs. 6 and 16),
and their activities represented the activities of the whole inner granule layer as the inner granule cells were weak in activities.

Many fibers were stained, especially in the adult animals. Most of them were weakly stained, and no formazan granules were contained within them. But, there were a few fibers which showed relatively strong activities.
e) White matter.

In the fetuses, white matter was completely negative. Approximately on the 6th day a. b. (mouse 8th day) there appeared a small number of cells which showed a weak activities in their cytoplasm. These cells were lined up in a row, sometimes linked together in the direction of the fibers. In the preparations of the 21st day a. b. and the 45th day, increase in number of cells as well as the enzyme activities was seen (mouse 10—16th day). Some times, long fibers were clearly stained at this stage with a moderately strong activities. On the 56th day (mouse 19th day), the activities of the cells were, still, well sustained, and it was seen that their cytoplasm were heavily loaded with dense, dark blue formazan granules (Fig. 12). Following this stage, the rapid diminution of the enzymatic activities was observed, i. e., with the preparations on the 65th day a. b. (mouse 19th day), the activities of cells in the white matter were no longer recognizable. Thus, the white matter attained the adult pattern, i. e., in this study, it was observed that the white matter of the adult animals were stained weakly when stained anaerobically, while they were negativ
IV. Discussion.

1. Preparation of the sections using carbowax.

For the determination of the most appropriate carbowax to be used, and of the best time for infiltration, the present author checked the relations between the molecular weight of carbowaxes and the preservation of morphological integrity of the stained preparations in various infiltration time, and reached the following conclusions; the carbowax 1,500 or the 1,000 was the best for preservation of both enzyme activity and morphological integrity of the stained preparations, and the best results were always obtained when the infiltration time was set within 20—40 minutes. When the infiltration was continued longer than 60 minutes, decrease of the activity was seen. Carbowaxes of greater molecular weight than 1,500 were not used because of their high melting point above 37°C, in order to avoid the possibility of destroying when stained aerobically.
the SDA by high temperature.

2. Evaluation of the results obtained in the present study.

As regards the cytochrome oxidase and succinic dehydrogenase in the cerebral cortex and liver of fetal guinea pig during the last half of gestation, FLEXNER et al. (1953) and FLEXNER (1954) found that these enzymes appear to have a relatively low and constant level of activity up to the period between 40th and 45th day, the activities then begin to increase, and reach to the two- or three-fold greater after the 55th day of gestation. In the brain of chick embryos also, cytochrome oxidase and succinic dehydrogenase were more active in 19 day-old embryo than those of 11 or 14 day-old embryos. Thus, the amounts of the enzymes increase 2.5 times in this period (LESLIE and YARNELL 1960), and the SDA during the embryonic life changes parallel to that of the cytochrome oxidase activity (DAVIDSON 1957). The facts mentioned above seem to indicate that the metabolism of embryonic tissues is not exclusively anaerobic, but a aerobic metabolism, although in lesser degree, is presumed to exist, and that the respiratory activity does not come into prominence untill late stage of embryonic life. In accordance with the predominance of anaerobic respiration in immature brain, HIMWICH et al. (1941), FAZEKAS et al. (1941) demonstrated experimentally the extraordinary tolerance of those brain to anoxia. KOBAT (1940) also reported that the brain of new borns were much more resistant to arrest of their blood circulation than the adult.

Admitting the existence of a positive correlation between the activities of aerobic metabolism and the distribution of SDA, low SDA in the embryos and infant animals will implies the low rate of aerobic metabolism and low oxygen consumption. In connection with this, HIMWICH et al. (1939), HIMWICH and FAZEKAS (1941), TYLER and HARREVELD (1942) investigated the oxygen consumption of the brains of new born animals, and reported that it was smaller than that of the adult.

As for the postnatal development of enzymes, POTTER et al. (1945) reported that, in the rat brain, cytochrome oxidase, succinic dehydrogenase and adenosine triphosphatase remained constant from about 3 days until about 6 days after birth and then increase rapidly so that the adult level was reached by the 30th day of life. It has also been shown, from the investigation in developing cerebral cortex of the rat, that succinic dehydrogenase, cytochrome oxidase (HAMBURGH and FLEXNER 1957) and malic dehydrogenase (KUHLMAN and LOWRY 1956) began rapidly to increase in activities nearly on the 10th day a. b. with increase of oxygen consumption (GÖRNICK et al. 1963) and glucose utilization (TYLER and HARREVELD 1942).

In the present investigation, the pronounced increase in the SDA was seen between the 6th and 45th day a. b. in the case of kitten (mouse 5—13th day), and attained the adult value approximately on the 65th day (mouse 19th day).

According to DAHL et al. (1959) the acceleration of O₂ utilization with brain development can be accounted for the increase in mitochondrial protein concentration which rapidly occurred on about 10th day a. b. (rat).

The increase of SDA, therefore, is a parameter of an oxidative metabolism of the tissues (FRIEDE 1959). The results obtained from the fetal and newborn animals in the present investigation are in good agreement with those data as described above,
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indicating the presence of the same developing pattern of the enzymes in the cerebellum as in the cerebrum.

On the fate of external granule layer, it has been studied by many authors. Some worker claimed the degenerations of those cells (see CAJAL), the others claimed the proliferation and migration of those cells into the molecular layer and the inner granule layer, developing into the nerve cells (ADDISON 1911, CAJAL 1929, MIALE and SIDMAN 1961, etc.; see CALAL), or into both the nerve cells and the glial cells (OBERSTEINER 1883, LUGARO 1894, SCHAPER 1894, POPOFF 1896, KERSHMANN 1938, UZMAN 1960). The present investigation, however, revealed the presence of SDA in those cells from the time of embryonic life to the last stage of its disappearance. This is in good agreement with the report by FRIEDE (1957), and suggests that those cells do not degenerate until the last day of disappearance.

Electron microscopically, the external granule cells possessed several small mitochondria, extremely poorly developed endoplasmic reticulum, small amounts of RNP granules and a few small GOLGI complexes scattered in a thin rim of the cytoplasm. At the same time, developing nerve cells were seen among the external granule cells. It was also seen that the spaces among neighboring cells were packed with a number of cytoplastic processes of both glial and nerve cells which contained mitochondria (SHINONAGA unpublished data). The reaction of SDA in this layer may well be attributed to those structures.

As for the PURKINJE cells, they always exhibited the strong activity throughout the whole life. FRIEDE (1957) observed the SDA from the 2nd day a. b. on, while MORIKAWA (1958 b) observed it from the 7th day on. In the present study, however, the activity was observed already at the middle stage of embryonic life. In relation to those strong activities of the oxidative enzyme as observed in the PURKINJE cells, FRIEDE (1962) suggested, studying with human brain, a correlation between functional activity, oxidative enzyme activity and deposition of lipofuscin in the nerve cells. Although FRIEDE found no lipofuscin in the PURKINJE cells, the present author found it in the PURKINJE cells of old rat (SCHINONAGA 1961). Such correlations, therefore, may exist in the case of the PURKINJE cells, as have been postulated by FRIEDE for the other nerve cells.

It was also revealed by means of electron microscope that the dendrites of PURKINJE cells contained a number of variously sized mitochondria, and the many processes of different cells with or without mitochondria were tightly packed around them (HERNDON 1961, SMITH 1963), as it was the case in the nerve cells of cerebral cortex (PAPPAS and PURPURA 1961). The intense SDA in the dendrites and the neuropile in the molecular layer are attributed to those structures, too, as SDA is associated with the mitochondria (De ROBERTIS 1960, CHANCE et al. 1961, OGAWA and SCHINONAGA 1962, LOVTRUP and SVENNERHOLM 1963, GREEN 1964).

In the inferior part of molecular layer and in the PURKINJE cell layer, SCHIFFER and VESCO (1963) observed the SDA, besides in the PURKINJE cells, in the BERGMANN fibers, oligodendrocytes and the other cells. In the present study, the BERGMANN cells and its processes were clearly demonstrated, showing the high SDA. The satellite cells exhibited moderate degree of the activity, the basket cell fibers
showed also the moderately strong activity. These data obtained in the present study are essentially in agreement with that of the SCHIFFER and VESCO, and with electron microscopic study of HERNDON (1964).

On the other hand, it has been known that the young mouse shows a rapidly developing EEG between 6th to 9th day a. b. and tends to become similar to the adult by 10—14th day of life (HIMWICH 1962). The time is, in the mouse cerebellum, exactly correspond to the rapid development of the molecular layer accompanied by a rapid increase in the SDA in the inner granule layer as observed in this study.

As for the activity of the inner granule layer, it was mainly represented by the enzymatic activity of the glomerulus because the activity of the cells of inner granule layer was very weak. Studying the adult animals, MALATY and BOURNE (1953), POTANOS et al. (1959), ODA et al. (1959) and SCHIFFER and VESCO (1963) recognized SDA in the inner granule cells or both inner granule cells and glial cells of this layer. ODA et al. observed two kinds of cells: one with a strong and the other with a weak activity. On the other hand, there are workers who observed no activity in the inner granule cells (TEWARI and BOURNE 1962, YONEZAWA et al. 1962, using the tissue culture). In the present investigation, the SDA of the inner granule cells were clearly seen from the stage in which the glomerular activities were not yet strong. Furthermore, electron microscopic studies also revealed the existence of several small mitochondria in the narrow rim of cytoplasm (GRAY 1961, PALAY et al. 1962, SHINONAGA unpublished data, SMITH 1963).

It is of interest that, in the early stage of development, the distribution of SDA of inner granule layer was restricted to the narrow region adjacent to the PURKINJE cell layer with a gradual decrease of the activity to the white matter, and that the distribution extended gradually to the deeper area and afterwards the same intensity was observed throughout the whole area. This evidence might have some relations to the cell migration which occurred from the external granule layer towards the inner granule layer as described by CAJAL and the other investigators.

Strong positive reaction of the fibers in the inner granule layer was considered to be due to unmyelinated fibers or long processes of glial cells. The weakly stained fibers observed were considered to be the mossy fibers, and it was, in part, a diffusion artefacts, because they were free from the formazan granules.

According to THOMAS and PEARSE (1961), high SDA was found in unmyelinated fibers and lower activity in the axons of myelinated fibers. While recent biochemical studies on the myelin by ADAMS et al. (1963) reported that enzyme inactivity of myelin in both CNS and peripheral nerves. They denied the report by TEWARI and BOURNE (1960) in which they described the presence of the SDA in the myelin sheath of peripheral nerves as a center of metabolic activities.

Though many of the histochemical studies of SDA in the nervous tissues coincide with each other in the point that it is not demonstrable in the white matter, this negative result, according to WOLFGRAM and ROSE (1950), is due to the lack of sufficiently sensitive tetrazolium salts and to the technical difficulties rather than to its true absence. Favoring of his view, recent worker observed the SDA in the white matter, i. e., POTANOS et al. (1959), MOSAKOWSKI (1962), and SCHIFFER and VESCO (1963) recognized it in the glial cells in the white matter of the adult animals.
ODA et al. also observed a weak activity in the white matter.

On the O₂ consumption of the white matter of a lam brain, KOREY et al. (1958) determined that one-third could be attributed to the respiratory activity of the axoplasmic structures coursing through the white matter and two-thirds to the cellular elements of the white matter. According to the report by KAWAKITA (1956), who estimated the succinic dehydrogenase in the brain of rabbit, the amount of this enzyme was highest in the cerebellum, and the amounts in the cerebral gray matter was 51—57% of the white matter. This evidence was remarked by CHASON and PEARSE (1961) that the preponderant activity of succinic dehydrogenase in the cerebral white matter is diametrically opposed to that seen with histochemical methods and can not be explained. In the present investigation, white matter of adult animals were stained weakly when incubated anaerobically, while they stained pink (negative) when incubated aerobically.

On the SDA of the white matter, further histochemical studies associated with biochemical investigations and electron microscopic observations are needed.

As to the changes of enzyme activities of cells in the white matter during the development, observations in the present study confirmed the results of FRIEDE (1957).

V. Summary.

1. A study was done on the distribution of succinic dehydrogenase activity (SDA) in the developing cerebella of cats and mice, from the middle fetal life to the adult, using fresh frozen sections prepared by the use of carbowax (polyethylene glycol).

2. Although extremely weak, the SDA was already recognized at the middle stage of embryonic life. The activity was seen to be intensified at the later stage (near term). There was a remarkable difference on the distribution of SDA between the embryos and the adults.

3. The pronounced increase in the activities with the development of all the components of cerebellum were seen from about the 6th day after birth on again (mouse 5th day). Observations on the 65th day (mouse 19th day) revealed that they showed the adult pattern.

4. Cytologically, the SDA was beautifully demonstrated in the external granule cells, the cells in the molecular layer, the PURKINJE cells, and their satellite cells, basket cell fibers, the inner granule cells and other cells in the granule layer. The activities were, also, observed in the cells of the white matter during the development.

5. In the inner granule layer, the activities were at first restricted mainly to the region adjacent to the PURKINJE cell layer with the gradual decrease towards the white matter.

6. By the 6th day after birth (mouse 8th day), the PURKINJE cell nuclei were located towards the bottom of cell bodies showing the eccentric localization of the activity in the area of dendritic origin. Thereafter, they were located in the center of racket-shaped cytoplasm showing perinuclear staining of the cytoplasm.

7. Except for the above mentioned results, findings were essentially the same to
哺乳類の脳組織におけるコハク酸脱水素酵素の分布に関する組織化学的あるいは細胞化学的研究は1952年頃より急速に発展し、多くの研究者の強い関心を集め、明らかに脳組織は複雑で軟弱であるため、標本作成の途中で破壊されたり、溶解して消失するので、従来より細胞レベルにおける詳細な研究は困難であった。これは動物が若ければ若いほど、また切片が薄ければ薄いほど顕著であった。この障害を打開すべく多くの研究者によって様々な努力が試みられてきたけれども、染色標本の組織形態もよく保存され、また酵素活性の減弱をも招来しないという相反した条件を充たす優れた染色方法はいまだ確立されていない。

著者はこの困難を打開すべくいろいろと試みた末、カーポワックスを使用することにより染色標本の破壊が防止され、比較的良好な結果が得られることを確認した。そして、これを用いて軟弱で破壊されやすい幼若動物の脳組織のためのコハク酸脱水素酵素の染色法を確立し、種々の対照実験をも併せ行ない、その特異性を確認したので、胎生中期から成熟期まで各段階における家猫並びにマウスの小脳のコハク酸脱水素酵素の分布に関する研究を行なった。それによって若千の新知見を得ることができ、以下のごとき結論を得た。すなわち、

1. 非常に微弱ではあるが酵素活性はすでに胎生中期において認められ、胎生末期（出産日近く）には非常に増強される。さらにかかる胎児と成熟動物との間に酵素学的に著明な相違がみられる。

2. 小脳のすべての構成要素の発育に伴ない、猫では生後6日目（マウスで5日目）より著明な酵素活性の増強が再び認められ、以後浸次増強を続ける。そして猫では65日目、マウスでは19日目でほとんど成熟動物と同等の酵素活性が認められた。

3. 細胞化学的には本酵素は糸粒体の分布と一致して存在するのがよく判り、外顆粒細胞、分子層の中の諸細胞、Perkinje細胞およびその衛星細胞、網線維、Bergmann細胞、内顆粒細胞およびグリヤ細胞などに活性が認められた。また発育中の、白質中の諸細胞にも強い活性がみられた。とくに内顆粒細胞の酵素活性の有無については著者両論があるが、本研究においては明瞭に酵素活性の存することを観察した。

4. 内顆粒細胞層の酵素活性は、初期にはPerkinje細胞層に隣接した部分に主として認められ、後にになって次第に白質の方へひろがっていく。

5. 生後6日目までの猫（マウスで8日目）ではPerkinje細胞の核は扁平して細胞体の底部に位置しており、酵素活性は単なる樹状突起の起始部に認められる。しかしその後はラケット状を呈した細胞体の中心部に位置するようになる。

6. 本研究は、大要においてラット小脳において行なったFriede（1957）の報
Succinic Dehydrogenase in the Developing Cerebellum.

References.

Y. SHINONAGA:

Succinic Dehydrogenase in the Developing Cerebellum.