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Abstract

Cytochemical observation of the activities of diphosphopyridine nucleotide diaphorase (DPNH-D), triphosphopyridine nucleotide diaphorase (TPNH-D), succinic dehydrogenase (SDH) and a-glycerophosphate dehydrogenase (α-GDH) of Ehrlich ascites carcinoma cells were made and following results were obtained. The smeared cells showed moderate reactions and no marked difference in the intensity among the individual cells. The free floating cells were stained relatively faint but showed the differences in the staining intensity in individual cells. In the presence of benzalkonium, the reaction intensity proved to be intermediate between the smeared cells and free floating cells without benzalkonium and the differences in the staining intensity in individual cells were more marked. Observations revealed that the reaction intensity changes closely corelated with the stage of mitotic cycle of each cell. Namely, DPNH-D activity of the tumor cells, which generally hihger than that of leucocytes, increased remarkably in the end stage of interphase and decreases abruptly in mitotic stage reaching the lowest level in metaphase. After the metaphse the activity increased slightly and it is kept at almost the same level during the first half of interphase. This enzyme is localized mainly in the granules of the eytoplasm. The activity of TPNH-D showed the similar localizations as those of DPNH-D, though the reaction intensity is lower than that of DPNH-D. The activity of SDH of the tmnor cells is lower than that of leucocytes and its diformazan granules are localized in mitochondria. Its activity decreases in the mitotic stage the lowest level in metaphase and in the followed interphase it is kept in a almost constant low level. α-GDH activity of the tumor is lower than that of SDH but show the similar localizations as the latter.

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A CYTOCHEMICAL STUDY OF OXIDATIVE ENZYMES IN EHRLICH ASCITES TUMOR CELLS

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Since SELIGMAN and RUTENBERG[1] used the tetrazolium salts for the histochemical demonstration of succinic dehydrogenase, there have been documented many histochemical studies on oxidative enzymes in human and animal tumors, but there are only a few reports concerning cytochemical study of oxidative enzymes in ascites tumor cells.

In the present paper, the cytochemical demonstration of diphosphopyridine nucleotide diaphorase (DPNH-D), triphosphopyridine nucleotide diaphorase (TPNH-D), succinic dehydrogenase (SDH) and alpha-glycerophate dehydrogenase (α-GDH) is made in Ehrlich ascites carcinoma cells, and it is also demonstrated that these enzymatic activities of the cell change according to their mitotic cycles, and that the intensity of cytochemical reaction changes aceording to the methods employed for incubation.

MATERIALS AND METHODS

0.1 ml of Ehrlich carcinoma ascites which was obtained from the Cancer Institute of Okayama University Medical School and contained 5 million tumor cells were transplanted to dd-mice by intraperitoneal injection. After seven days the tumor cells grown in the ascites were used for the cytochemical observations.

Incubation medium for the demonstration of the dehydrogenase activties was prepared by the modified WATTENBERG's method[2], and besides the substrate it contained 0.11*M* NaCl, 0.003*M* KCl, 0.001 *M* $MgSO_4$, 0.03*M* phosphate buffer (pH 7.5), and 0.6 mg of nitroblue tetrazolium chloride or neotetrazolium chloride per ml. The substrate was DPNH (1.5 mg per ml), TPNH (1.5 mg per ml), sodium succinate (0.05 M) or sodium alpha-glycerophosphate (0.05 M). DPNH and TPNH were of the Sigma Chemical Co.

In the first series of observation Ehrlich ascites fluid was smeared on four object glasses, dried rapidely at room temperature, and each one smeared was mounted with 0.2 ml of each incubation medium, just described, and incubated at 37 °C for thirty minutes. Thereafter they were washed in water, dried, and fixed in formalin vapor. Two series of the samples were prepared for one obser-

vation. One series of the specimen was counterstained with Mayer's haemalum for 10 minutes, washed, dried and mounted in balsam.

In the second series of observation 0.02 ml ascites fluid was added to 0.2 ml incubation medium in a small test tube, and incubated for thirty minutes at 37°C being stirred every ten minutes or shaken continuously with Warburg's apparatus. Thereafter the packed ascites cells were obtained by centrifugation at 600 g for three minutes. One half of the cells was mounted in glycerine after fixing with 10 per cent formalin or without fixation. The other cells were smeared, dried and fixed with formalin vapor and mounted in balsam. Some smears were fixed with formalin vapor, counterstained with Mayer's haemalum and mounted in balsam.

The third series of experiment were carried out for checking the changes of permeability of the ascites carcinoma cell menbrane for the staining of oxidative enzymes. 0.02 ml benzalkonium chloride solution (0.00061 per cent to 0.625 per cent) in physiological saline was added to the same quantity of ascites fluid, then the mixture was stirred up and added with 0.2 ml of the incubation medium immediately after stirring, and treated with the same cytochemical procedure as in the 2nd series of experiment.

In the fourth series of experiment the cells were observed being suspended in the ascites fluid. The ascites fluid by centrifugation at 600 g for five minutes. More than 0.6 mg of NT or Nitro-BT was added to 1 ml of the ascites fluid being dissolved almost completely. Each substrate was added to 0.2 ml of this ascites fluid dye mixture in each test tube respectively. 0.02 ml of carcinoma ascites was added to it and incubated at 37°C for thirty minutes while stirring every ten minutes. The cells were separated by centrifugation at 600 g for five minutes and washed with physiological saline solution, then fixed and mounted as in the case of 2nd series of experiment. All procedures were performed rapidly and under aseptic condition. The controls were made in two samples, one excluding the carcinoma cells and another the substrate. The concentration of substrates was the same as that of 1st series of experiment.

In the fifth series of experiment observations were made on the transplantation of the carcinoma cells previously treated with cytochemical procedures. Five million carcinoma cells, stained by the techniques in thc 1st to 4th series of experiments, were washed with physiological saline solution just after the incubation and transplanted into the intraperitoneal cavity of dd-mice. All the procedures were performed under aseptic condition.

RESULTS

In the reactions observed on smeared cells the intensities appeared almost the same level in each cell for the same enzyme. The activity of DPNH-D of

Ehrlich ascites careinoma cells appeared intensely, especially in large cells. The difromazan formed was found to be localized in the cytoplasmic granules. In most cells, a large number of the intensely stained granules were disseminated in the cytoplasm. In the cells with the nucleus situated in the one side of the cell, the granules were rather densely observed in the other side of the nucleus and in the cells with nucleus having indentation on one side or both sides, the granules were rather densely distributed around the indentation.

The abundant deposition of diformazan made it difficult to observe the detailed structure of the nucleus and only the mitotic figures from anaphase to telophase was distinguished. In these phases, the granules appeared densely around two divided chromosome groups (Fig. 1).

The activity and localization of TPNH-D showed almost the same as those of DPNH-D, but the staining intensity was slightly weak in comparison with that in DPNH-D (Fig. 2).

Concerning SDH, all the cells in interphase showed a moderate activity and diformazan appeared being localized in the cytoplasmic granules. The granules showing the positive reaction were densely distributed around the nucleus, especially the indentations of it or in the area with relatively large mounts of cytoplasm. The nucleus and the nucleolus never showed the positive reaction. In the mitotic cells, the granules were concentrated around the chromosome groups. In the anaphase cells, the number of stained granules was not so different as that of other stage cells and the granules were observed between both sides of divided chromosome groups (Fig. 3).

The localization of α-GDH were almost the same as in SDH. But in comparing with SDH, the staining intensity of the cells was sligtly weak (Fig. 4).

The reaction intensities of the cells floated in the incubation media showed an ununiformity, some cells are rather strong in the intensity and some others are rather weak.

The activity of DPNH-D appeared moderately or intensely in the cells of about 10 per cent, and faintly or not stained in another 90 per cent.

In the cytoplasms of moderately or intensely stained cells, the positive granules were densely observed in the entire cytoplasm and the ground substances of cytoplasms were also diffusely stained in a lesser degree. Some cells showed a marked diformazan deposition so that nuclei were not observed, but most of the cells showed their nuclei often situated towards one side with or without indentation. And the cell in the mitotic phase was never observed. The granules were recognized all over the cytoplasm, especially in the area of relatively large amounts of cytoplasm, around the nucleus or indentation of it, and between the nuclei in the giant cells with high density of the granules. The cells unstained or showing a faint reaction had nuclei, which were swollen and faintly stained with haemato-

xylin, and also were of spherical or indented shapes. In these cells the nuclei were observed clearly and a small number of slightly or moderately stained granules were also observed in the cytoplasm in the outer aspect of the nuclear membrane. The cells in mitosis, from later prophase to early telophase, were distinguished distinctly from those in interphase. In the cells in prophase to anaphases, two or three faintly stained granules were observed near the cell membrane. The granules increased from the stage of anaphase in number but stained more faintly showing the same staining intensity as in interphase (Fig. 5). Two daughter cells connected with cytoplasmic bridge were also stained slightly.

The cytochemically demonstrated activity of TPNH-D was almost the same as in the case of DPNH-D (Fig. 6).

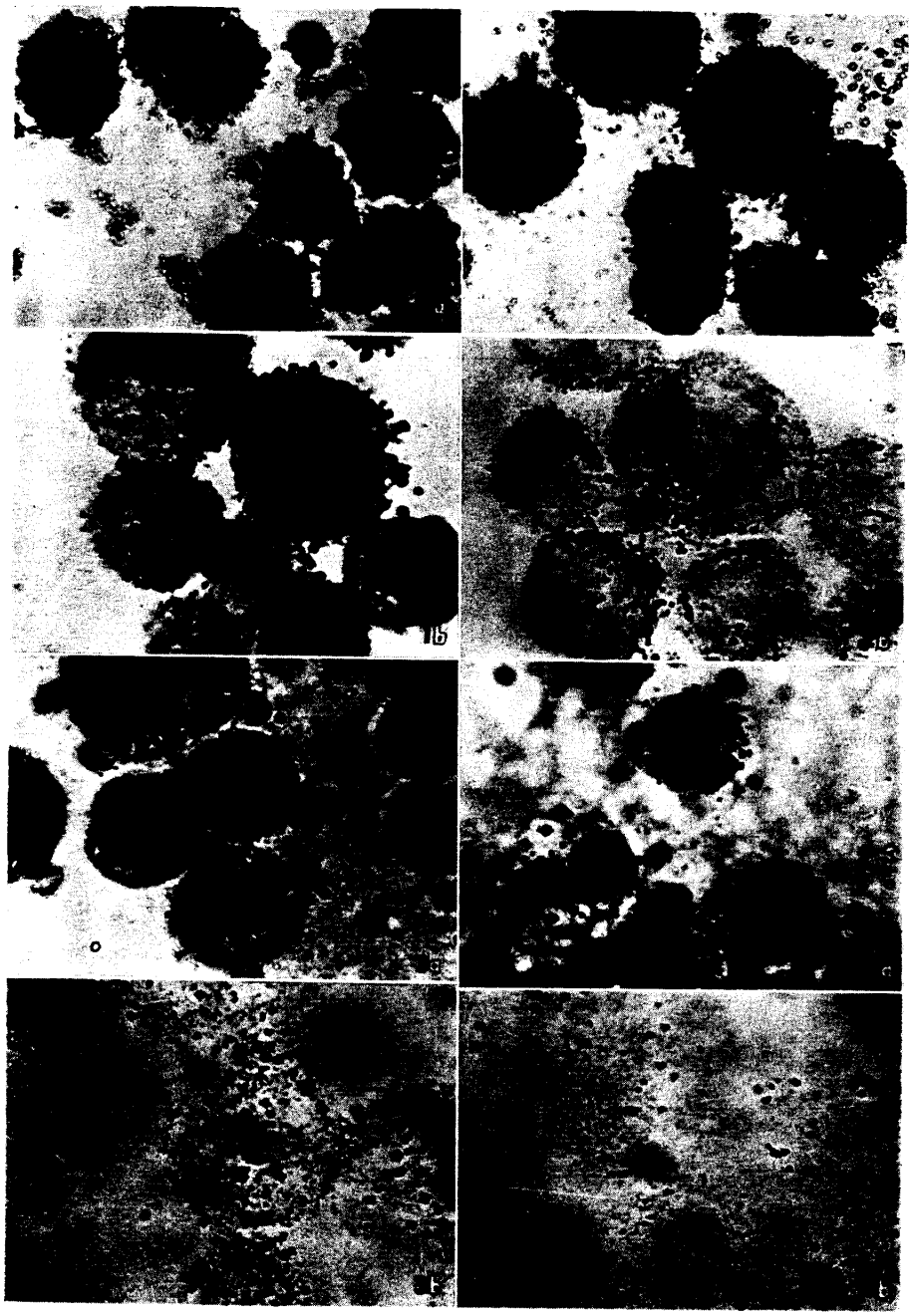
In the case of SDH, most of the cells showed a faint reaction being stained in the similar intensity in all cells. Neither nucleus nor nucleolus gave positive reaction, but only the cytoplasmic granules near the nucleus were faintly stained. The granules were observed rich in the indented area of the nuclei and in the wider area of cytoplasm. The mitotic cells were stained in a specific pattern quite different from the cells at interphase; from the middle prophase to metaphase the granules decreased near the cell membrane, especially in the metaphase, where the granules were very slightly. In anaphase these granules were oberved between the divided chromosome group; and in telophase these were increased around the two divided chromosome groups (Fig. 7).

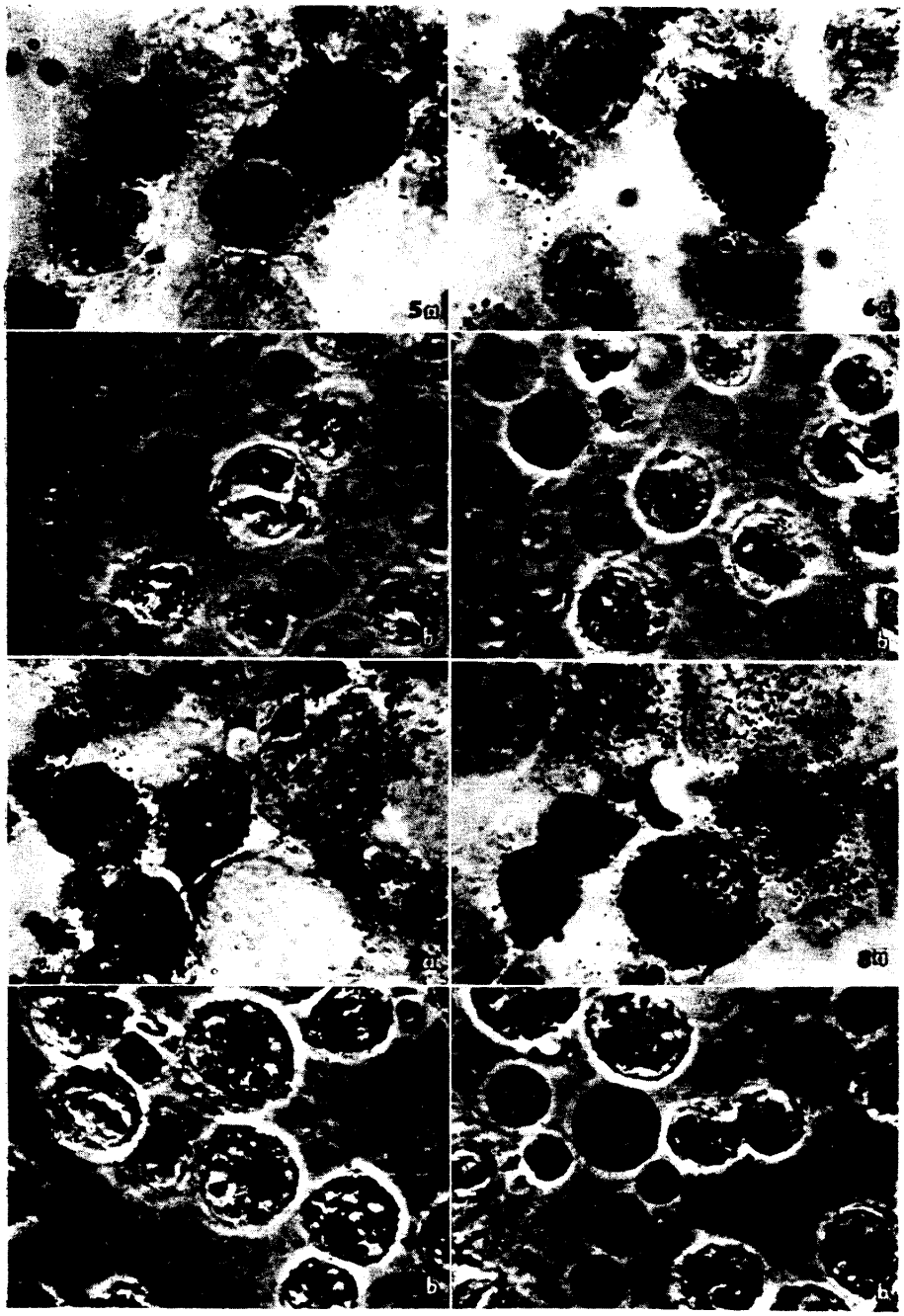
The activity of α-GDH was the same as SDH excepting that the reaction intensity is rather weak comparing to SDH (Fig. 8).

Pretreatment with benzalkonium chloride solution prior to incubation resulted in the increase in the reaction intensity of the tumor cells as can be seen in Table 1. With benzalkonium chloride in the concentration of over 0.15625 per cent all cells were destroyed. The staining intensity in the DPNH-D and TPNH-D differed from cell to cell differently from the case of smeared cells where the cells are stained in the similar intensity. In the SDH and α-GDH, the staining intensity of all cells was found to be in the same degree but it was lesser the smeared cells. In concentrations from 0.07813 per cent to 0.03906 per cent the staining intensity of the four enzymes was highest. In concentrations below 0.07813 per cent the results, as already described, were about the same tendency in the four enzymes and their characteristic features of each enzyme of the staining were preserved. From these results, the cytochemical observations were conducted with the four

Explanation of Plates

Eigs. 1~4 Enzymatic activities demonatrated on the smeared cells by using nitro-BT, ×1000
Figs. 1a~b: DPNH-D, Figs. 2a~b: TPNH-D, Figs. 3a~b: SDH, Figs. 4a~b: α-GDH
a: counterstaining with Mayers haemalum. b: without counterstaining. Note the staining intensity decreased in following order: DPNH-D, TPNH-D, SDH, α-GDH.





Tab. 1

Name of enzymes / Benzalkonium chloride solution	DPNH-D	TPNH-D	SDH	α-GDH
0.00061 %	↓	↓	↓	↓
0.00244 %	→	→	↘	→
0.00977 %	↑	↑	→	↗
0.01953 %	↑↑	↑↑	↑	↑↑
0.03906 %	↑↑↑	↑↑↑	↑↑	↑↑
0.07g13 %	↑↑↑	↑↑↑	↑↑↑	↑↑↑
0.15625 %	↑↑	↑↑	↑↑	↑↑
0.625 %	↑↑	↑↑	↑↑	↑↑

The arrows (↑) show the staining intensity compared with the control experiment; ↓ slight decrease, ↘ intermediate between ↓ and →, → no change, ↗ intermediate between → and ↑, ↑ slight increase, ↑↑ moderate increase and ↑↑↑ high increase.

enzymes treated with 0.03906 per cent benzalkonium chloride solution.

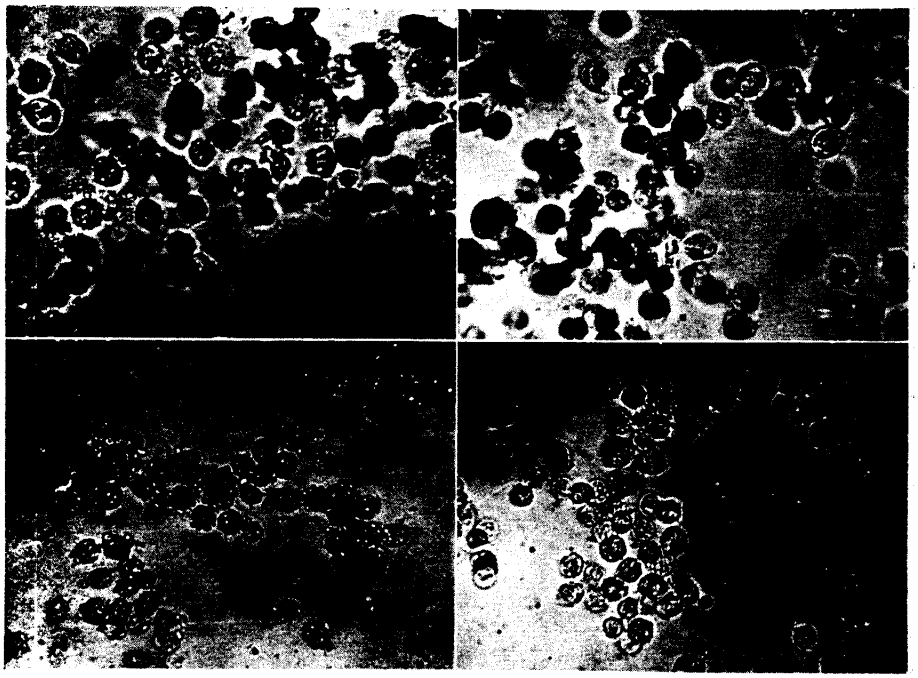
For the reaction to DPNH-D forty to fifty per cent of the carcinoma cells were stained moderately or intensely and the remaining cells were faintly stained. In the moderately or intensely stained cells nuclei could be seen being moderately stained with haematoxylin. Some of these cells revealed the similar morphological findings of nuclei to those of intensely stained cells in the 2nd series of experiment. But the other of intensely stained cells showed no morphological changes in their nuclei. Some of the faintly stained cells have swollen nuclei stained slightly with haematoxylin, and others have nuclei in early interphase stage stained moderately with haematoxylin. The cells in later prophase to metaphase showed the weakest activity (Fig. 9).

The activity ef TPNH-D showed the similar intensity as DPNH-D, but the cells moderately or intensely stained account for 30 to 40 per cent of all (Fig. 10).

By the reaction for SDH about 80 per cent of the cells were stained moderately, but the other 20 per cent faintly. The staining pattern of cytoplasm was the same as that in the free floating cells and was hardly related to the morphological change of the nuclei except for the change in mitotic phase.

In early prophase and metaphase the granules were scarcely stained or a few of faintly stained granules near the cell membrane, but in anaphase and telophase faintly or moderately stained granules increased in number around the two

Figs. 5~8 Enzymatic activities demonstrated on the free floating cells with nitro-BT, ×1000
Figs. 5a~b DPNH-D, Figs. 6a~b: TPNH-D, Figs. 7a~b: SDH, Figs. 8a~b: α-GDH a: counterstaining with Mayer's haemalum. b: a phase-contrast microscopic picture. Note the weak reaction of all dehydrogenases in the mitotic phase and the intense reaction of the DPNH-D and TPNH-D activities in the end stage of the interphase.



Figs. 9~12 Enzymatic activities on the free floating cells in the presence of benzalkonium. Nitro-BT was used as hydrogen-acceptor, ×400

Fig. 9: DPNH-D, Fig. 10: TPNH-D, Fig. 11: SDH, Fig. 12: α-GDH Note the intermediate intensity of the reaction between the smeared cells and free floating cells without benzalkonium. Method: see text.

chromosome groups (Fig. 11).

For α-GDH the similar findings as SDH were obtained, but in general the staining intensity of the enzyme was lower than that of SDH (Fig. 12).

Observations on the tumor cells incubated with the ascites fluid containing NT and substrate proved the same staining intensity of the four enzymes as that in those incubated with medium prepared with water instead of ascites. In the controls without the substrate, the carcinoma cells were not stained, and in the controls without carcinoma cell, the ascites fluid remained colorless through the experiment. In this epxeriment, NT only was used because the incubating medium was stained blue color with nitro-BT.

In transplantaion test the Ehrlich ascites carcinoma cells proved to be not transplantable to dd-mice, when the cells are smeared and staind, while the carcinoma cells treated in the floating state were transplantable to another dd-mice. Thus, the carcinoma cells were killed by the cytochemical procedures or smearing, but they survived through the treatment in free fleating state. Ascites cells

from the mouse implanted the treated cells revealed completely the same characteristics as those of controls both in morphological picture and enzymatic activity.

DISCUSSION

Since Straus et al[3]. in 1948 reported that tumor cells reduced triphenyltetrazolium chloride, tetrazolium salts have been widely used in the hitstochemical demonstration of oxidative enzyme in normal and tumor tissues. And the ctytochemical studies of ascites tumor cells using tetrazolium salts have been reported by Birns[4], Quaglino et al[5]. Woodliff et al[6]. and others. In these studies, however, no precise finding concerning the relationship between the activity of oxidative enzyme and mitotic cycle has ever been described.

In the present study, two different staining methods, namely, the smear and the free floating have been employed. And very different results can be obtained by these two methods, and an assumption has been given on the changes of enzymatic activities according to mitotic cycle.

In the 1st experiment, ascites carcinoma cells are smeared on slide glass and dried at room temperatur the specimens are incubated in the incubation method, all cells are stained uniformely. On the other hand, in the 2nd experiment the ascites cells incubated without drying the staining intensity is different in individual cells, for instance, some of the cells are stained moderately or intensely but most of cells are slightly in the DPNH-D and TPNH-D reaction.

In order to calrify the cause of this difference, some experiments have been carried out in the present study. At first, in the smear, the contact of cells to incubation media is uniform. In the cells incubated with floating method, there is a possibility that the carcinoma cells are not uniformely in contact with incubation medium. But both of the results obtained on the cell stirred every ten minutes and those stirred continuously with Warburg's apparatus gave the same results. This shows that the ununiformity of contact is not the cause of the ununiformity of the reaction. The difference in the reaction intensity from cell in the 2nd experiment is due to the changes in the stage of mitotic cycle of each cell. In the smear, no difference of intensity in the staining is noticed during entire mitotic phases. The former, but not the latter is in agreement with the reports of Takeda[7] and Osato et al.[8] that the mitochondria are decreased in number in metaphase of the cell division. In the free floating cells, it has been thought that enzymatic activities in the cells are demonstrated in a more natural state than in the smeared cell, because this incubation is performed in a condition capable of successive transplantation to another mouse after the cytochemical reaction in this method. In addition, the observation on ascites cell floating clarified that no significant difference can be found in the results between the cell floating in

ascites and those in the artificial medium. Here, the effect of nitroblue tetrazolium chloride on the cells has been ignored. Normal cells, such as ovum cells of the sea urchin, are immediately killed in the medium used in this experiement, but Ehrlich ascites carcinoma cells survived through the incubtaion with the reaction medium retaining the transplantability. So it might be reasonably thought that for cytochemical reaction the test on free floating cells is the suitabl one for the detection of dehydrogenase activity of ascites carcinoma cells.

The exact mechanisms of increase in the staining intensity in the smeared cells have not been elucidated, but it might be assumed that some inhibitory mechanism for the enzymatic activity exists in the cells in some connection with the mitotic cycle, and the destruction of cells such as drying suppress the inhibitory mechanism and staining intensity is thus increased.

This difference in the reaction intensity between the smeared cells and free floating cells is an important problem for the cytochemistry of free cells. Because most histochemical and cytochemical studies of ascites tumor cells have been performd on the smeared cells, so that the change of enzymatic activity of the cells according to the stage of mitotic cycle is not demonstrated. The precise knowledge of relationship between the enzymatic activity and mitotic cycle are obatined by the observation of the free floating cells. In order to clarify this relationship, phase contrast microscopy and the observation of formazan-haematoxylin counterstained cells are helpful.

In some cells stained intensely, no nuclei can be observed in detail because of the heavy formazan deposition. The identification of these intensely stained cells is important problem. Some nontumor cells which are stained intensely i. e., some mast cells and leucocytes observable in about 10 per cent of whole cells in Ehrlich ascites[9] can be distinguished from the tumor cells their morphological features. According to Kaltenbach[10], a few killed cells were found in Ehrlich carcinoma cells floating in Ringer's solution. But such killed cells were not observed in the present experiment, because no similar variations of individual cell seen in the staining of DPNH-D and TPNH-D would be noticed in the staining of SDH and α-GDH. Furthermore, hyper-matured carcinoma cells with enlarged nuclei are not stained intensely. And cells with high enzymatic activity cannot be observed in the mature leucocytes which divide no longer.

Therefore, it has been presumed that intensely-stained cells are living carcinoma cells themselves. Thus the observations indicate that the activities of the DPNH-D and TPNH-D are remarkably increased in the end stage of interphase and followed by mitotic phase. In the mitotic phase, the activities of the enzymes can be abruptly decrease into almost negative and the lowest activity can be obseved in metaphase. In telophase the activity is increased somewhat higher and is kept in the same level during the first half of interphase. The enzymatic

activity of cells, which no longer divide, cannot be observed to increase in the end stage of interphase. In general, the activity of TPNH-D is slightly lower than that of DPNH-D.

Those cells showing high enzymatic activities are stained slightly in the entire cytoplasms and the cytoplasmic granules are stained most intensely. It might be suggested, as reported by Mellons[11] and Birns[4], that enzymatic reaction occures outside the mitochondria. But in cells showing the low enzymatic activities the formazan deposition is noticed only in the granules.

The activity of SDH and α-GDH decreases in the mitotic phase and it is lowest in metaphase, while in other phases the activities are found to keep an almost low level throughout. Ogawa et al.[12] reported that SDH activity was decreased in prophase and metaphase, and their results is in good accord with the present results. According to Birns[4], Nachalas et al.[13], Hess et al.[14], Hudack et al.[15] and Ogawa et al.[12] the SDH and α-GDH are contained in mitochondria. In the present study, it has also been observed that diformazan depoistions on the granules are presumed to be mitochondria.

When the enzymatic activity of Ehrlich ascites carcinoma cells is compared with that of the leucocytes, the DPNH-D and TPNH-D activities are remarkably high, while the SDH and α-GDH activities are low. These results also agree with those from other carcinoma cells.

SUMMARY

Cytochemical observation of the activities of diphosphopyridine nucleotide diaphorase (DPNH-D), triphosphopyridine nucleotide diaphorase (TPNH-D), succinic dehydrogenase (SDH) and α-glycerophosphate dehydrogenase (α-GDH) of Ehrlich ascites carcinoma cells were made and following results were obtained.

The smeared cells showed moderate reactions and no marked difference in the intensity among the individual cells. The free floating cells were stained relatively faint but showed the differences in the staining intensity in individual cells. In the presence of benzalkonium, the reaction intensity proved to be intermediate between the smeared cells and free floating cells without benzalkonium and the differences in the staining intensity in individual cells were more marked.

Observations revealed that the reaction intensity changes closely corelated with the stage of mitotic cycle of each cell.

Namely, DPNH-D activity of the tumor cells, which generally hihger than that of leucocytes, increased remarkably in the end stage of interphase and decreases abruptly in mitotic stage reaching the lowest level in metaphase. After the metaphse the activity increased slightly and it is kept at almost the same level during the first half of interphase. This enzyme is localized mainly in the

granules of the eytoplasm. The activity of TPNH-D showed the similar localizations as those of DPNH-D, though the reaction intensity is lower than that of DPNH-D. The activity of SDH of the tumor cells is lower than that of leucocytes and its diformazan granules are localized in mitochondria. Its activity decreases in the mitotic stage the lowest level in metaphase and in the followed interphase it is kept in a almost constant low level. α-GDH activity of the tumor is lower than that of SDH but show the similar localizations as the latter.

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