Mitochondria in Animal and Plant Cells as Revealed by LBC Reaction (leuco-brilliant cresyl blue reaction)

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Abstract. The aqueous solution of certain quinoneimine dyes, including oxazins and thiazins; e. g. brilliant cresyl blue, toluidine blue, methylene blue, were reduced by Zn powder. These leuco-dye solution and 2·4 dinitrophenol (or trinitrophenol, i. e. picric acid) were added to the fresh materials. New red colored dyes are produced on mitochondria. The color of such vitally stained mitochondria fades gradually in a short time. The fixation of this vital staining was achieved by using saturated aqueous solution of corrossive sublimate as a fixative. When the solution is replaced by gycerine or euparal, semi-permanent preparations are obtaninable. The detailed descriptions of the new technique and the results will be given in the following prapraphs.

Introduction

Reviewing the history of the investigation on the mitochondria, only several methods of demonstration can be enumerated. The most commonly employed methods are the following two. Other great many are the modifications from these two. The one is the combination of Regaud's fixative-Heidenhain's iron hematoxylin. These methods are, however, by no means successfull with every material. Especially, the materials of invertebrates and plants are difficult to work with. Moreover, the staining is rather obscure, and is not specific to the mitochondria. The other defect of these methods is that these

procedures are very time consuming and troublesome. The duration of rinsing in the chrome salts takes generally several days.

Besides these methods with the fixed materials, Michaelis (1900) found that Janus green B stained the mitochondria in living cells. This vital staining has afforded many advantages in the cell reserch. But the staining with Janus green B is not always successful. We have frequently experienced that the mitochondria in certain tissue cells do not stain or sometimes stain capriciously according to their physiological conditions. With the object of elucidating toward this question, Lazarow and Cooperstein (1953) made experiments, and presented a hypothesis on the processes of reduction and oxidation of the dye in the living cells. But some sceptism concerning this capricious staining still remains. Indeed, there are many materials in which mitochondria fail to be stained with Janus green B, and it is quite impossible to explain the mechanism of such an indifferent feature of the dye adequately.

With the purpose of obtaining better results of the vital staining of mitochondria, the writer have made various attempts, using various dyes. Among these attempts, the method "LBC reaction" named by the present author gave the best results.

Method

As stated in the forgoing abstract, several dyes belonging to the oxazin and the thiazin group were employed; viz. brilliant cresyl blue, toluidine blue, methylene blue. Of these three dyes, brilliant cresyl blue gave the most steady results.

Preparation of dye solution: 0.5g of dyes were dissolved respectively in 100 ml. of phosphate buffer solutions with the pH ranging between 4 and 8. The buffer solutions were prepared after McIlvain with several proportions of citric acid and secondary sodium phosphate. Practically, it is convenient to prepare the dye solutions with the pH 4.0, 6.0, 7.0, 8.0. About 3 ml. of each dye solution was poured into the test tube to which about 0.45g of Zn powder was added. Then it

was boiled over the flame until the blue color become colorless. The time of the reduction varies according to the pH of each solution. The Zn powder has scarecely any influence upon the pH of the solution. The more alkalic the solution is, the more time it takes to reduce. At pH 8, the reduction takes about three minutes, while at pH 4 it takes a little time after the boiling begins. The leuco-dye solution is not entirely colorless, but it often stains slightly pinkish. Then, the surface of the leuco-dye solution was covered with the liquid paraffin in order to prevent the oxidation by the air (Fig. 1). The solution was then cooled by leaving aside or by washing in the running water. The leuco-dye solution does not keep for a long time, so it should be prepared anew for every observation.

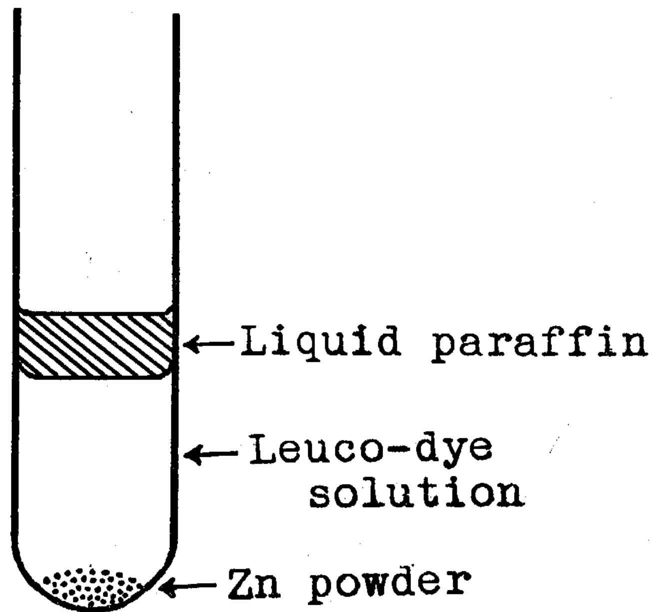


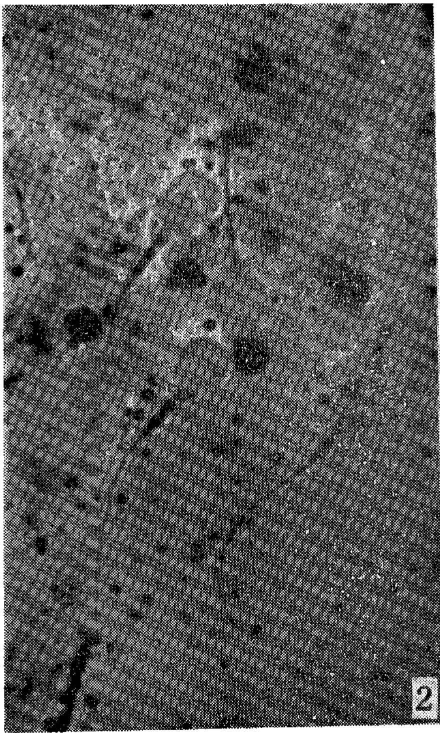
Fig. 1
Schematic sketch of the leuco-dye solution in a test tube (natural size).

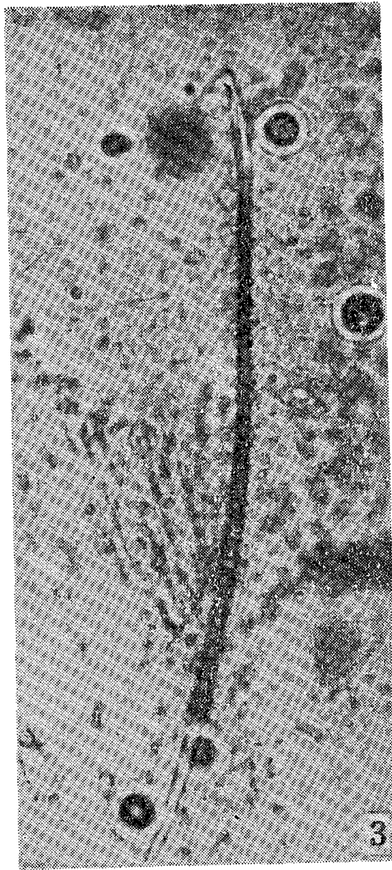
Preparation of DNP solution: lg. of DNP (2·4 dinitrophenol) was dissolved in 100 ml. of distilled water, to which 0.5g. of $NaHCO_3$ was then added. DNP is very slowly soluble in water, so it becomes solution on standing overnight. Then one drop of 10% NaOH was droped on the solution so that the pH of the solution became 7.0. When the materials are marine, the sea water should be employed as the solvent.

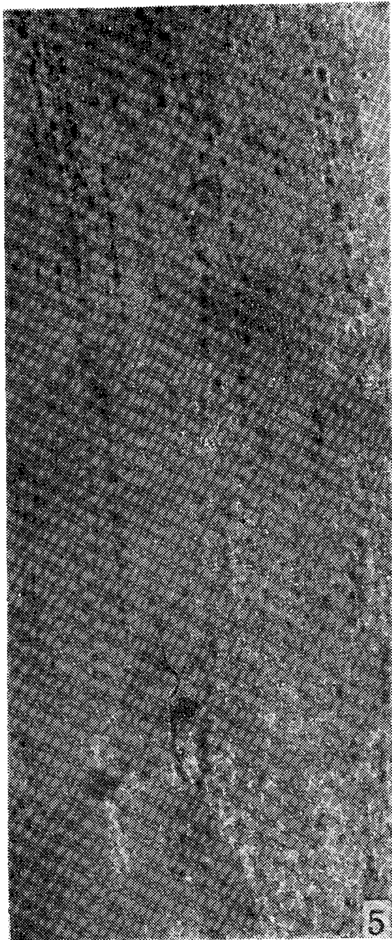
The procedure of the reaction: As the reaction is always applied to living cells, the materials should be thin or small so as to be easily observable under microscope. For this reason, the "vivotome" is highly recommended to obtain thin slices (cf. Tarao '62). The teasing of the tissue fragments will also applicable for this purpose. Muscle fibers, gill plates, pancreas, epidermis of leaves etc. *in toto* are also

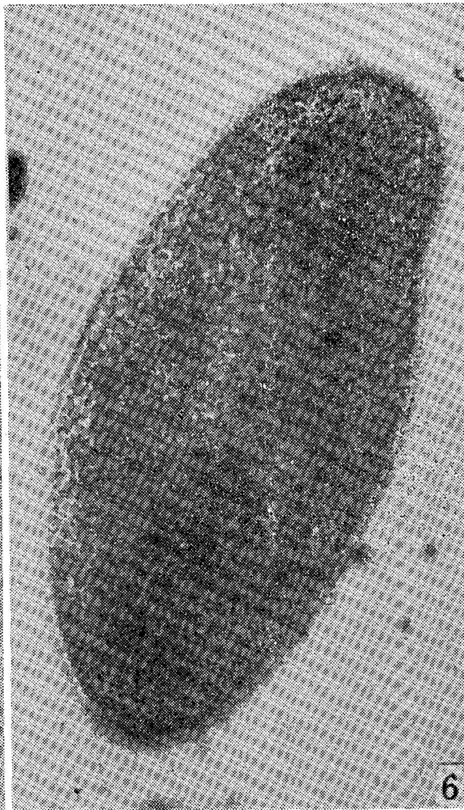
useful for the reaction.

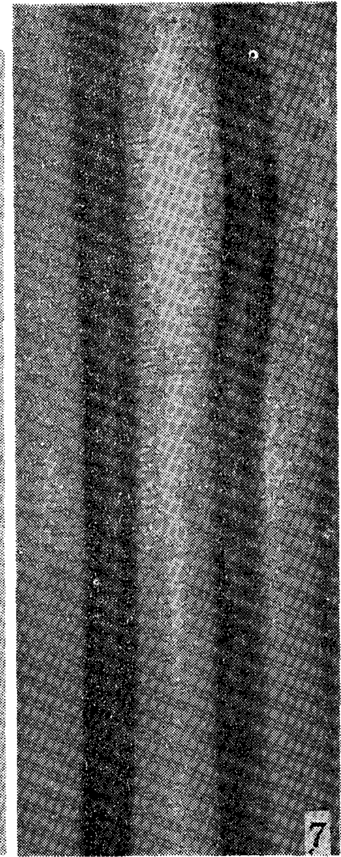
Previous to the reaction procedure, add several drops of a 10% $MgSO_4$ solution (in distilled water) to these fresh materials and leave them for 10 minutes. Then the solution is drained off with a mouth capillary pippette, and one drop of DNP solution and three drops of leuco-dye solution are added successively (almost at the same time). They are then exposed to the air for one-and-a-half minutes, and are covered with cover slips. Mitochondria stain red intensely. An attention should be paid that mitochondria of the tissue cells are apparentry very capricious in staining. Usually, the greater part of the tissue cells are negative and only a few parts are positive to the reaction. The most definitely positive are the cases when the cells are injured. In such cases, the reagents intrude into the cytoplasm, and the supply of oxygen is abundant. Myofibrils or sperms, in which the mitochondria are almost exposed on the surface or separated from the outer medium with an extremely thin membranes, the mitochondria are always positive to the reaction. The staining in such cases is very stable and keeps for a long time. The present author has termed such a staining "intrusion staining" (IS). On the other hand, there is another mode of staining, viz. when the cells are intact. In such cases, the staining keeps for only about 10 minutes. This type of staining is termed by the present author "vital staining" (VS). VS is, however, limited only to certain loci, where the full conditions for the reaction are saticefactory. Generally speaking, cytochemical reactions on the living cells are by no means evenly positive in the tissues. For instance, the oxidase reaction needs the following conditons; the penetration of reagents, direct contact with the reagents to the enzymes which may be often masked by lipids, mucus or else, sufficient volume of oxygen in the cytoplasm, optimal pH and so on. Even only one of these factors lacks, the reaction becomes negative. Above all these inhibiting factors, the tough cell membrane or cell cortex (cellulose, hyaluronic acid, chitin, or myelinated covering etc.) are difficult to overcome. To overcome this obstacle 10% of $MgSO_4$ was successfully employed. $MgCl_2$ also



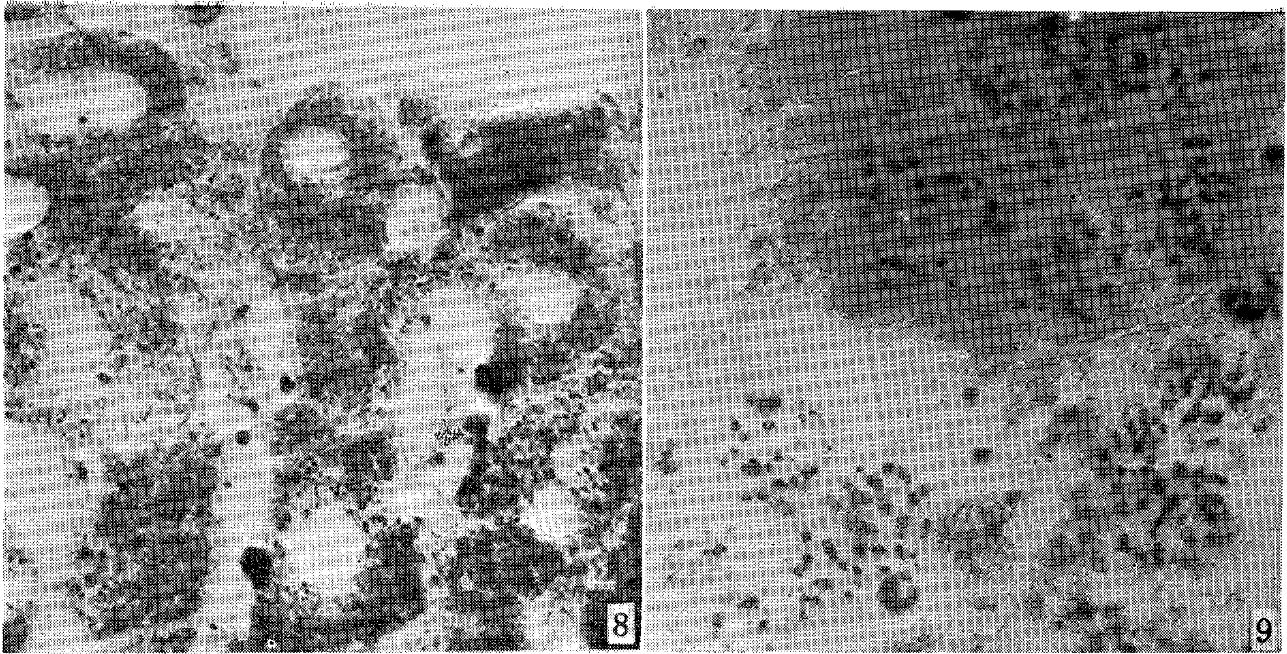


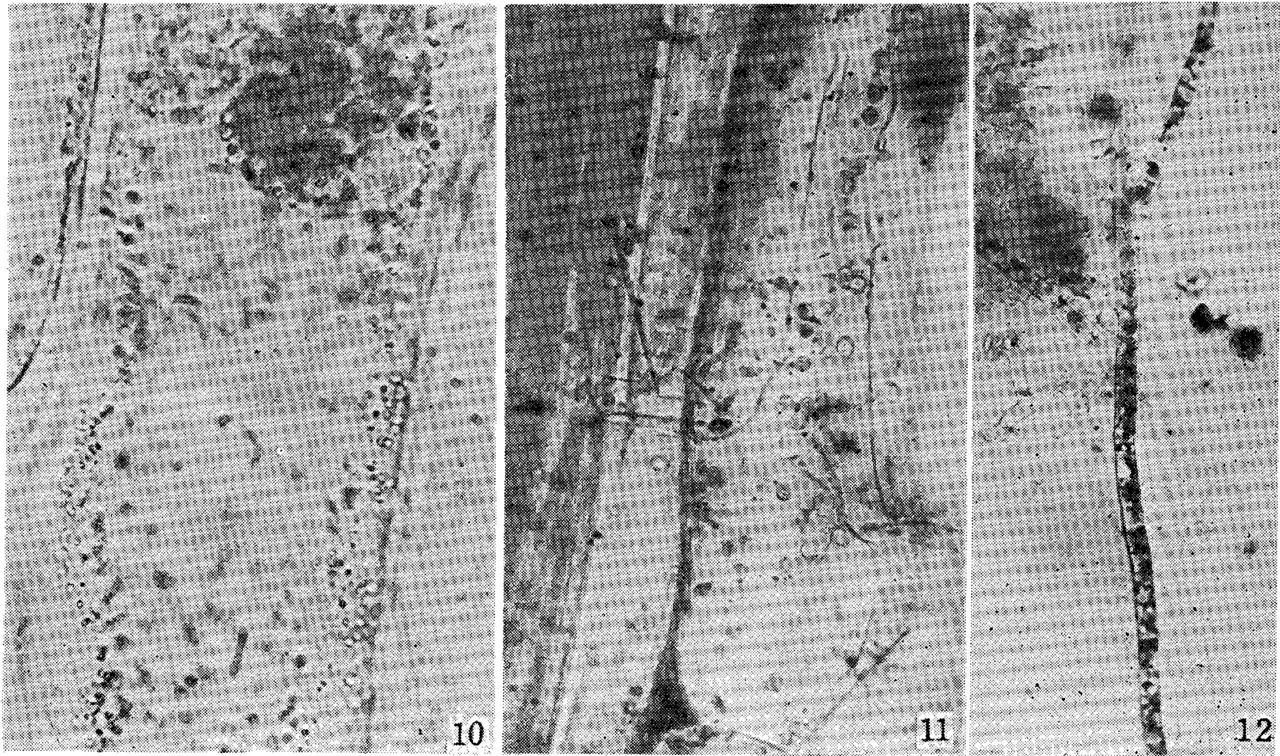






All figures are at the same magnification of X320. Fig. 2. Spermatozoa of Guinea pig. Fig 3. Spermatozoon of rat. Fig. 4. Flight muscle of bee, *Protichneumon* sp. Fig. 5. Leg muscle of cricket, *Gryllus* sp. Fig. 6. *Paramoecium caudatum* L. Fig. 7. Gill of fresh water clam, *Corbicula leana* Prime.





All figures are at the same magnification of X320. Fig. 8. Hepatic cells of rat. Fig. 9. Pancreatic acinar cells of rat. Fig. 10. A part of hair cell of stamen of *Tradescantia reflexa* Rafin. Fig. 11. Parenchymatous cells of petiol of *Phytolacca americana* L. Fig. 12. Hyphae of *Saprolegnia*.

did, but the sulphate gave far better results.

As it has been described above, the coloring of VS of mitochondria keeps for only 10 minutes, the fixation of produced colored substance on the mitochondria was desirable. For this purpose, the stained materials were fixed in the saturated aqueous solution of corrossive sublimate. By this fixation, however, the stained mitochondria seem to suffer slight fragmentation.

Results and Discussion

Results : As it has been described in the forgoing paragraph, materials show diversity in reaction ; some are easily reactive or some are difficult. The causes are by no means easy to analyze. The optimal pH varies according to the kind of tissues or animals, yet pH 6.5 — 8.0 give generally good results. Some examples are chosen from the data, which are shown in the following photomicrographs. They are all at the same magnification of X 320. The black parts in the photographs represent red in natural color.

Explanations of figures : The middle pieces of sperms of Guinea pig (Fig. 2) are rather short. For sperms in general, the previous soaking in $MgSO_4$ solution is not necessary. The middle piece of sperm of rat shown in Fig. 3 is long. Here, the spiral formation of mitochondria is clearly seen. Head and tail remain unstained. Fig. 4 shows the so-called "sarcosomes" (mitochondria) on the surface of a flight muscle fiber of a certain kind of bee (*Protichneumon* sp). Exceedingly large mitochondria stained red are aligned in rows. They are liable to fall into the surrounding medium on touching the slide or cover glass. On the contrary, mitochondria of the leg muscle of cricket (Fig. 5) are small, rather radomly arranged in rows. Mitochondria in *Paramoecium* (Fig. 6) are very minute and are of allmost equal size. They are evenly distributed throughout the cell body. Fig. 7 shows the mitochondria of branchial cells of fresh water clam (*Corbicula leana* Prime). Mitochondria in cells of paired rows stain

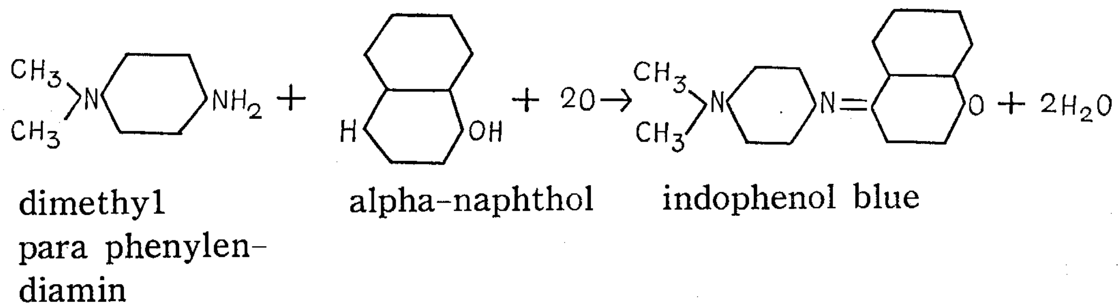
deeply red, and keep their color permanently when they are fixed with the corrossive sublimate, otherwise the color fades in 10 mimutes. Mitochondria in hepatic cells (Fig. 8) are minute and closely packed in the cytoplasm. Those of pancreatic acinar cells (Fig. 9) are large and swollen. Closer examination reveals that the reaction is positive on the covering membrnes of mitochondria. Mitochondria in plant cells are very difficult to demonstrate by the LBC reaction. The cause of the difficulty in reaction is not yet known. The positive cases are shown in Figs. 10 and 11. Fig. 10 shows the tiny mitochondria in the peripheral cytoplasm of hair cell of the stamen of *Tradescantia reflexa* Rafin. It was often observed that these colored mitochondria were drifting along the stream of cytoplasm. Fig. 11 shows the parenchymatous cells of the petiol of *Phytolacca americana* L. Mitochondria appear as small red particles which often gather around the chloroplasts. Mitochondria in the hypae of *Saprolegnia* are shown in Fig. 12. Inside the hypae mitochondria are packed densely.

Discussion : As described in the introductory paragraph, this technique is precious in the point that it saves much time in the studies of mitochondria. So, it is useful in the study of the behavior of mitochondria following physiological conditions. On the other hand, the reaction seems to be positive or negative according to the physiological conditions of the cytoplasm. The analysis of the causes of this negative reaction is by no means easy, because there are many factors which inhibit the reaction; e. g. the disturbance of the permeability of the reagents by cell mombranes, lack of oxygen, lack of certain oxidative enzymes which will be discussed later, and so on. Moreover, the masked substances may be suppoesd to present on the surface of the mitochondria; for instances, proteins, polysaccharides, or lipids. Generally speaking, the reactions on the living cells are usually very difficult. Even hydrochloric acid cannot penetrate the mucous covering. The red dye produced by the present reaction is soluble in fat solvents to some extent. So, the red color of mitochondria fades by washing with acetone. Therefore, the masking substance, if any, may

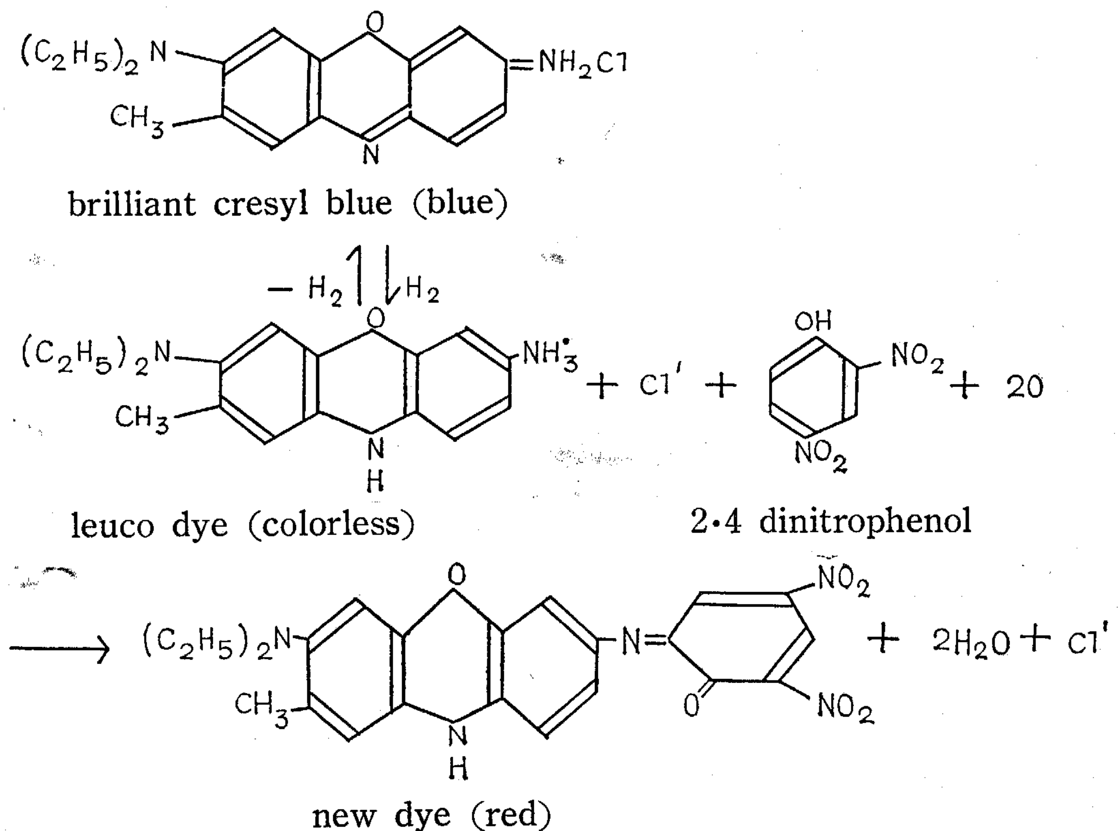
be lipinous.

Next, the specificity of the reaction should be discussed. The selective staining on the mitochondria may suggest that the reaction is specific to a certain enzyme within the mitochondria. Among enzymes in the mitochondria, respiratory ones are most probable to concern this reaction. Though it is not yet verified, there is some parallelism on chemical equations between the Nadi reaction (indophenol blue reaction) and the LBC reaction.

Nadi reaction : The formula is generally believed as follows.



LBC reaction : The formula is postulated likewise as follows.



The interaction between amino bond of the leuco dye of brilliant cresyl-blue and hydroxyl bond of dinitrophenol makes us remind of the similar case between dimethyl paraphenylendiamin and alpha-naphthol of Nadi reaction. In both cases, the dyes newly produced may be supposed to comprise commonly the linkage of -N=, and 2 oxygen atoms are demanded respectively in their reactions, giving as the products a new colored dye and 2 water molecules.

As mentioned in the technical paragraph, the optimal pH is respectively exist for each material employed. VS is far more susceptible than IS. Yet both have almost the same optimal pH ranging between 6.5 and 8.0.

It has been thought that the Nadi reaction is brought about by the cytochrome oxidase. It is inhibited by the addition of KCN, NaN_3 or hydroxylamin previously to the reaction. The LBC reaction is likewise inhibited by the previous soaking in these solutions. KCN is not suitable for the reaction, because Mg $(OH)_2$ arises as a flocculent precipitation by the reaction between KCN and $MgSO_4$. In this case, therefore, the process of soaking in the $MgSO_4$ solution should be omitted. As the materials of the experiments about the influences of these inhibitors, sperms and ciliated epithelial cells of gill of clams are most suitable. Though there are data described above, this supposition is not yet settled. The most regrettable defect of this reaction is the appearance of a surplus red dye on leaving for a long time, unless the reaction is stopped by fixing with the corrossive sublimate. This artefact is supposed that the red dye is autocatalytically generated upon itself. This surplus product of red dye makes up suspect the validity of the LBC reaction on cytochrome oxidase.* But from the above mentioned reasons the cytochrome oxidase on mitochondria must be one of the principle catalizers of the production of the new dye. Therefore, the reaction is not strictly specific as the cytochemistry aims.

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*The same phenomenon has been experienced in G-Nadi reaction. Even when the alpha-naphthol solution and the dimethyl paraphenylendiamin only are mixed (without any living material), the blue color arises on standing for a while.

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