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Abstract

With the purpose to elucidate the relation between the enzyme activity and the morphology of mitochondria the author carried out histochemical and biochemical investigations of cytochrome oxidase and succinic dehydrogenase activities of liver cells obtained at various intervals after the oral administration of CCl4, to male rats. And the data were compared with those reported in the first report. In the normal liver histochemically demonstrable cytochrome c oxidase activity and succinic dehydrogenase activity can be seen in parenchymal cells. In both cases the cells lying in the peripheral area show a more intense activities than those in the central part of liver lobules. The activity of cytochrome c oxidase falls markedly 5 to 6 hours after the CCl4, administration, while the activity of succinic dehydrogenase is retained almost at normal level for about 20 hours. Quantitative estimation of the succinic dehydrogenase activity of tissue homogenate revealed a transient rise in the activity 90 minutes after the CCl4, administration, and thereafter the values have been kept in almost normal level by 20 hours though a gradually fall has been seen in this period with a marked degree at 22nd hour. Taking the changes of minute structure occurring at each stage into consideration, which have been reported in the previous paper, the author concludes that the activity of succinic dehydrogenase is closely correlated with the maintenance of double membraneous structure of mitochondria, but the activity of cytochrome c oxidase is reduced by the swelling of mitochondria.

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MORPHOLOGY OF MITOCHONDRIA AND CELL RESPIRATION

II. HISTOCHEMICAL STUDY ON THE LIVER WITH EXPERIMENTAL CARBON TETRACHLORIDE POISONING*

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Recent stupendous progress in cytochemistry and biochemistry has made it possible to analyse the function of some cell organellae on the basement of morphology. Biochemical observations of mitochondria isolated by improved fractionation method have led to the assumption that in a mitochondrion numerous enzymes should form a definite reaction unit by linking with each other by which the electron transport for respiration proceeds (GREEN[1]). Studies on the disintegrating process of mitochondria under electron microscope, correlating with the loss of their enzyme activities. Green proposed the sensational opinion that the double membraneous structures of mitochondria are composed of the respiratory enzyme units arranged in the lipid membrane sandwiching the materials required for energy source and related enzymes. ODA and OKAZAKI[2] have demonstrated that most of succinic dehydrogenase exist in the cristae of mitochondria in their electron microscope study on the cells of animals injected with potassium tellurite and fixed with osmic acid. According to CLAUDE[3] a single mitochondrion contains as many as 2000 groups of such enzymes, each of which is composed of 25 different kinds of enzymes and takes part in the terminal stage of aerobic oxidation[4].

By the histochemical studies on the liver of the animals with carbon tetrachloride poisoning WAHI[5] and STOWELL[6] described that the activities of alkaline phosphatase, acid phosphatase and esterase in the central part of lobules are decreased; LEDUC[7] pointed succinic dehydrogenase is altered in the same manner. And by the biochemical studies TSUBOI[8] and THOMSON[9] observed the activities of respiratory enzymes such as succinic oxidase, cytochrome oxidase and acid phosphomonoesterase diminish in parallel with the degree of the liver distur-

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bances.

These observations seem to surpport CHRISTIE and JUDAH's opinion[10] that the primary action of carbon tetrachloride on the hepatic cells is a direct one, affecting mitochondria and there is a disorganization of the chain of enzymes correlating with the tricarboxylic acid cycle and the further oxidation.

In the observation of fine structures in the liver with carbon tetrachloride poisoning the author also found that mitochondria and endoplasmic reticulum were greatly affected as reported in the previous paper[11], and in the present paper the results of histochemical investigations on respiratory enzymes, that are so closely related to mitochondria, are presented.

MATERIALS AND METHODS

The same as in Part I, forty-two male rats weighing about 100—150 g. were used. Twenty-eight animals of them received oral administration of carbon tetrachloride once, 0.25 ml. of carbon tetrachloride per 100 g. of body weight, which was introduced by using a fine gum catheter. Fresh livers were obtained 1.5, 5, 6, 10, 17, 20, and 22 hours after the administration, killing the animals by decapitation. For each group four animals were respectively fed as their controls.

Histochemical investigations were conducted on two enzymes, cytochrome c oxidase and succinic dehydrogenase. For the detection of cytochrome c oxidase activity Nadi's reaction (unstable Nadi reaction) was employed following GRÄFF's technics[12]. Namely, fresh liver tissue is frozen and sliced, about 10 μ thick immediately after extirpation, and the slices are immersed in the reagent at 37 °C for 10 minutes exactly. The reagent is prepared just before use by mixing 25 ml. of 0.1 % α-Naphtol, 25 ml. 0.12 % dimethyl-p-phenylendiamine solution, 4 ml. 0.1 N sodium hydroxide, and 6 ml. 0.75 % glycocol (pH, 8.2). After washing with physiologic saline solution for a little while the slices are mounted with glycerin and examined under microscope. For the detection of the activity of succinic dehydrogenase Oda's method was employed[13]. Just as in the case of cytochrome c oxidase reaction, unfixed fresh livers are frozen and sliced, and the slices are immersed in the reagent, and incubated at 37 °C for 30 minutes. The reagent is prepared just before use by mixing 0.2 ml. of 0.2 % sodium neotetrazolium solution, 0.2 ml. 0.2 M sodium succinate and 0.2 ml. 0.1 M phosphate buffer, pH. 7.6 (per 10 mg of the tissue). At the end of incubation 0.4 ml. 10 % formalin is added to stop the reaction. Then the slices are examined under microscope by mounting with glycerin.

For the quantitative estimation of succinic dehydrogenase activity, the method devised by ODA is employed[13]. Namely, 100 mg. of the fresh liver tissue

is homogenized in a glass homogenizer by adding 2ml. of 0.1 M phosphate buffer solution (pH, 7.6). Then 0.2ml. of the homogenate is put into the reagent consisting of 0.2ml. 0.2 M sodium succinate as the substrate and 0.2ml. sodium neotetrazolium as hydrogen accepter. Incubated at 37°C for 30 minutes.

At the end of incubation the reaction mixture is added with 0.4ml. of 10% formalin solution to stop the reaction. The colored reduction product of neotetrazolium salt, di-formazan is extracted by adding ether-aceton, a mixture in equal volumes. Ether-aceton is added shaking the mixture until the color of the tissue homogenate fades completely. Then the absorption density of 1ml. of the extract is estimated at the wave length of 520 mμ by using Beckman type spectrophotometer, Shimazu Co.. The activity of succinic dehydrogenase is represented as $E \times V$, where V is the volume of the extract used and E is the optical density in arbitrary unit. By this method the data obtained show the activities of succino-oxidase system superimposed with the cytochrome c-cytochrome oxidase system[13,14].

RESULTS

Histochemical findings: In the control group G-Nadi granules generally appear positive in the entire liver lobules, more markedly in the peripheral area than in the central part of the lobules, and especially marked in the area surrounding the portal vein in Glisson's capsule. Each parenchymal cell gives a positive reaction in cytoplasm but not in the nucleus. The picture of the histochemically demonstrable activity of succinic dehydrogenase behaves similarly (Fig. 5).

Ninety minutes after the CCl_4 administration the G-Nadi positive granules in cytoplasma decrease in number, and the decrease is rather marked in the central part of lobules, often giving a negative reaction. The reaction in the peripheral area of the lobules remains positive, especially distinct in those liver cell bundles surrounding portal veins (Fig. 3).

Indophenol blue formed by Nadi's reaction stains fat droplets, too[15], which appear rich in the cells showing fatty degeneration by CCl_4 intoxication but they give a clear purple color and can be distinguished from G-granules of a deep blue color.

The decreased activity of succinic dehydrogenase is likewise seen in the central part of lobules already 90 minutes after the CCl_4 administration but the decrease is not so striking as in the G-Nadi reaction.

By 5—6 hours after the administration of CCl_4 G-Nadi granules further decrease in number with the increased number of fat droplets (Fig. 4), and the central part of lobules gives actually negative reactions being clearly bordered with the Nadi positive peripheral areas. The Nadi positive peripheral areas

present almost the same enzyme activity as in the corresponding areas of the liver from control animals. In this stage the activity of succinic dehydrogenase also tends to decrease, giving a markedly diminished activity in the central part of liver lobules (Fig. 6).

By 10 hours after the CCl_4 administration Nadi positive granules in parenchymal cells decrease markedly whose cytoplasms are filled with a number of fat droplets. In this stage the activity of succinic dehydrogenase in cytoplasm grows irregular, though generally the central parts of lobules still show a lowered activity (Fig. 7).

In the liver tissues taken 17 hours after the CCl_4 administration the histologic picture of the G-Nadi reactions are almost the same as those of samples taken 10 hours after the CCl_4 administration. The activities of succinic dehydrogenase observed histochemically also showed no actual difference between the samples from these two groups showing still a considerable high activity after 17 hours.

By 20—22 hours after the administration no recovery of the Nadi positive reaction can be seen but in some area the histochemically demonstrable activity of succinic dehydrogenase recovers nearly to the original level as can be seen in the controls (Fig. 8). However, in the parts where a marked fatty degeneration occurs, the activity is still low, though there appear a number of di-formazan crystals and many fat droplets stained a light purple by di-formazan.

In the tissues for which nitro-neotetrazolium is employed as the hydrogen acceptor instead of neotetrazolium an extremely sensitive reaction can be seen. In this case the fat droplets appear blue black and mitochondria deep purple. The results are actually the same as in the cases incubated with neotetrazolium.

Summarizing the above, in the normal liver histologically demonstrable activities of cytochrome c oxidase and succinic dehydrogenase appear marked in the parenchymal cells with a stronger reaction in the peripheral area of lobules. Carbon tetrachloride administration results in a noticeable reduction both in the peripheral and the central parts of lobules, the decrease being specially marked in the central part. These disturbances grow more prominent with the lapse of time reaching a severe low activity already after 6 hours. No recovery of the cytochrome c oxidase activity can be seen, but a partial recovery of the succinic dehydrogenase activity can be recognized by 22 hours afterwards.

Estimation of the succinic dehydrogenase activity in tissue homogenate:

In the estimation of the succinic dehydrogenase activity in 10 mg. of the liver tissue homogenate at various intervals after the CCl_4 administration by the methods described the intensity of the activity, presented by the value $E \times V$, gives some different tendency from those demonstrated histochemically (Table I).

Table 1. Alterations of Reduced NT Absorption Density in 10 mg Rat Liver Homogenate at Various Intervals after CCl_4 Administration.

Hours after CCl_4 administration	(V) Volume of ether-aceton extract (ml)	(E) Absorption density of 1 ml extract (520 mμ)	E×V
1.5	22.0	0.175	3.762
5	20.5	0.095	1.948
6	6.60	0.151	0.997
10	4.92	0.194	0.995
17	5.40	0.171	0.932
20	4.88	0.179	0.874
22	4.75	0.023	0.109
Control	11.0	0.115	1.265

The succinic dehydrogenase activity shows a considerable rise at 90 minutes after the CCl_4 administration but it falls somewhat 5 hours afterwards. By 6 hours afterwards the activity is already below the normal level, and this is maintained up to 20 hours and it shows more marked fall at 22 hours. No recovery can be seen differing from the supposition on the histochemical pictures.

DISCUSSION

The observations just described clearly show that the histochemically demonstrable activities of cytochrome oxidase and succinic dehydrogenase in the normal liver are more intense in the peripheral areas than in the central part of lobules. These data coincide with the description in the past report[5, 7, 16]. It is also well established that these enzymes are contained in mitochondria[2]. Since the mitochondria in rat liver are equally distributed both in the cells lying in the central and peripheral areas of the lobules[17], the above-mentioned differences do not mean the difference in the population of mitochondria but they do signify that the enzymatic activity of mitochondria differs according to different sites. This suggests that the function of parenchymal cells in the centrolobular area may differ from those lying in the periphery. In the case of CCl_4 intoxication the degeneration occurs especially severe in the central parts. This may be induced secondarily by the anoxiaemia in this area, the capillary lumen being compressed by the swollen cells, but there is a great possibility that the cells lying in the centrolobular area are more susceptible to CCl_4. As has been reported in the first report[11], the author observed the swelling and disintegration of mitochondria under electron microscope in the case administered with CCl_4, it is well understood that the activities of their respiratory enzymes are decreased, but failed to compare the changes in the mitochondria in the cells lying in the

central to those in peripheral area. Histochemically, the cytochrome oxidase activity is markedly decreased or eliminated in the areas showing fatty degeneration or hydropic degeneration, demonstrating distinctly the degenerated parts as colorless or slightly colored area. But even in the cells containing a number of fat droplets mitochondria were observed, though swollen but not obliterated. The activity of succinic dehydrogenase which is contained in mitochondria, behaves somewhat similarly as the cytochrome oxidase activity, but the degree of the fall in the activity is less than that of cytochrome oxidase, showing a considerable activity even at 22 hours after the CCl_4 administration. This will show that the loss of cytochrome oxidase activity does not necessarily mean the complete degradation of mitochondria but the cytochrome oxidase is more susceptible than succinic dehydrogenase to CCl_4.

Biochemical estimation of the succinic dehydrogenase activity of liver homogenate showed a transient but remarkable rise 90 minutes after the CCl_4 administration falling to the normal level by 5 to 6 hours after the administration, which were kept up to 20 hours at a fairly fixed level, though it again falls markedly 22 hours after the administration. The transient rise of the enzyme activity observed 90 minutes after the CCl_4 administration and the marked fall after 22 hours could not be revealed by the histochemical method. In this case the effect of homogenization on enzyme activity need not be considered as it is reported by HOGEBOOM[18] that the destruction of fractionated mitochondria acts as to suppress the succinic acid oxidation, though my result is different from the report of CHRISTIE and JUDAH[10] who mention the increased activity of succinic dehydrogenase 5 hours after the CCl_4 administration and decreased activity 10—15 hours later. Therefore, the transient rise of the enzyme activity should be occurring *in vivo*. In this point the submicroscopic structure of mitochondria will give an information. The electron microscope picture at 90 minutes after the CCl_4 administration, which corresponds to the stage of raised activation of enzyme, appears approximately normal, though some rearrangement and change in shape of endoplasmic reticulum are observed. These findings will show that the activity of the enzyme is raised actually in this stage with the elevated function of mitochondria. AMANO[19] is of the opinion that such a transient rise in the enzymatic activity observable in the injured cells is due to the disturbances of the mechanism regulating the enzymatic activity, and MÖLBERT[20] attributes it to the influence of nucleoprotein discharged from the nucleus. The author is of the opinion that the raised activity of this mitochondrial enzyme will probably be due to the adaptation phenomena to the disturbed function of cytoplasm, as the mitochondrial structure is retained normal. However, the actual mechanisms involved remain still obscure. The visible swelling of mitochondria and the structural change can be observed only 5—6 hours after the CCl_4 administration.

This will partially explain the decreased activity of the enzyme at this stage comparing to that at 90 minutes. But in this stage the activity is maintained nearly normal and this state continues for a rather long time till 20 hours after the CCl_4 administration. Clearly swollen mitochondria still have their normal enzymatic activity. The regenerated mitochondria may show the activity but the retained double membraneous structure seems to be responsible for the maintenance of activity, as the enzyme activities are retained *in vitro* so long as the double membraneous structure is preserved. The fall in the enzymatic activity 22 hours after the CCl_4 administration seems to suggest then destruction of minute structures of mitochondria, though electron microscopically, some double membraneous structure of cristae is still maintained. Differing from succinic dehydrogenase, the cytochrome oxidase activity seems to be lost by the swelling of mitochondria even in the state where the double membraneous structures of cristae are retained.

CONCLUSION

With the purpose to elucidate the relation between the enzyme activity and the morphology of mitochondria the author carried out histochemical and biochemical investigations of cytochrome oxidase and succinic dehydrogenase activities of liver cells obtained at various intervals after the oral administration of CCl_4 to male rats. And the data were compared with those reported in the first report.

In the normal liver histochemically demonstrable cytochrome c oxidase activity and succinic dehydrogenase activity can be seen in parenchymal cells. In both cases the cells lying in the peripheral area show a more intense activities than those in the central part of liver lobules. The activity of cytochrome c oxidase falls markedly 5 to 6 hours after the CCl_4 administration, while the activity of succinic dehydrogenase is retained almost at normal level for about 20 hours.

Quantitative estimation of the succinic dehydrogenase activity of tissue homogenate revealed a transient rise in the activity 90 minutes after the CCl_4 administration, and thereafter the values have been kept in almost normal level by 20 hours though a gradually fall has been seen in this period with a marked degree at 22nd hour.

Taking the changes of minute structure occurring at each stage into consideration, which have been reported in the previous paper, the author concludes that the activity of succinic dehydrogenase is closely correlated with the maintenance of double membraneous structure of mitochondria, but the activity of cytochrome c oxidase is reduced by the swelling of mitochondria.

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EXPLANATION OF FIGURES

Fig. 1. Rat liver 17 hours after the oral administration of 0.25 cc/100 g body weight of carbon tetrachloride (the same in the following cases). Hematoxylin-eosin stain (10×10).
The nuclei are picnotic and there are many balloon cells possessing transparent cytoplasm in the intermediate zone of the liver lobules.

Fig. 2. Rat liver 20 hours after the CCl_4 administration. Sudan IV stain (10×10). Fatty degeneration is marked.

Fig. 3. Rat liver 90 minutes after the CCl_4 administration. G-Nadi reaction (15×40).
The activity of cytochrome c oxidase can be recognized uniformly in the cytoplasm of liver cells. In some places fat shows secondary staining.

Fig. 4. Rat liver 5 hours afterwards. G-Nadi reaction (15×40). The activity of cytochrome c oxidase is diminished and fat is markedly increased.

Fig. 5. Normal rat liver showing the succinic dehydrogenase activity (NT method) (10×10).
The activity is marked in the peripheral area of lobules.

Fig. 6. The succinic dehydrogenase activity of the rat liver 5 hours after the CCl_4 administration (10×10).

Fig. 7. The succinic dehydrogenase activity of the rat liver 10 hours afterwards (10×10).

Fig. 8. The same activity of the rat liver 20 hours after the CCl_4 administration (10×10).
The activity is decreased slightly in the descending order of Figs. 6—8, but the degree of the fall is not so marked.