EFFECT OF GAMMA IRRADIATION ON THE DESOXYRIBONUCLEASE II ACTIVITY OF ISOLATED MITOCHONDRIA*

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PLATES 65 AND 66

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In a previous communication, one of the authors (1) reported that x-ray exposure increased the desoxyribonuclease II (DNase II) activity of isolated liver mitochondria. Since the enzymatic activity was altered only to a moderate degree under the conditions of irradiation, further experiments were done with higher doses of radiation using gamma rays from a Co^{60} source. Electron microscopic evidence will be presented supporting the hypothesis (1) that the increase in DNase II activity after x-irradiation is causally related to structural damage to the irradiated mitochondria. This structural damage is accompanied by a release of substances, most likely nucleotide in nature, from the damaged mitochondria.

Methods

Preparation of Mitochondria.—Mitochondria were isolated from 1 to 3 gm. of rat liver by the method using 0.44 M sucrose and citric acid, described by Dounce *et al.* (2), and were suspended in 10 ml. of unbuffered 0.44 M sucrose.

Irradiation.—Aliquots of 10 ml. of mitochondrial suspension in 25 ml. Erlenmeyer flasks were irradiated at room temperature with gamma rays from a 670 curie cobalt^{60} source at a dose rate of 4,790 roentgens per minute.

Controls.—Since the time of exposure at the higher doses was fairly long (3 to 4 hours), the non-irradiated aliquots used for control studies (as well as the aliquots irradiated less than 4 hours) were kept at room temperature for the maximum irradiation time to avoid complications introduced by different degrees of aging.

Sonic Treatment.—A 9 kc./sec. Raytheon vibrator unit of the magnetostriction type was used as a source of sonic vibration. The mitochondrial preparations were treated at room temperature for 10 minutes.

DNase II Assay Method.—The method used for enzymatic assay was a slight modification of the incubation procedure described by Kowlessar and coworkers (3) combined with a spectrophotometric method of measurement of the decomposition products of DNA similar to that used by Schneider and Hogeboom (4). The method of assay will be described briefly.

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Aliquots of homogenate (0.5 ml.) of irradiated and control samples of mitochondria were diluted with an equal volume of 0.0032 per cent (weight/volume) ethylenediamine tetraacetate (versene) in 0.44 M sucrose to remove Mg^{++} and other divalent ions (3). The assay system contained 1 ml. of the versene-treated aliquot, 0.5 ml. of substrate solution containing 2 mg./ml. of highly polymerized sodium desoxyribonucleate, prepared by the method of Kay *et al.* (5), and 1.5 ml. of 0.1 M acetate buffer at pH 5.6 made up in 0.44 M sucrose instead of in distilled water as used in the Kowlessar procedure (3).

The mixture was incubated for from 30 minutes to 1 hour at 37°C. and then the reaction was stopped by adding 1 ml. of 2.88 M trichloracetic acid.

Control tubes or "tissue blanks" were run simultaneously with the test samples, and differed from test samples in that the DNA was added after the incubation instead of before.

Because sucrose interferes with the Dische diphenylamine reaction used by Kowlessar *et al.* (3), the depolymerization products of DNA were determined by measuring the optical absorption of the supernatant fluid obtained after centrifuging the samples for 20 minutes at 200 *g*. The supernatant fluid was placed in the 1 cm. cell of the Beckman spectrophotometer, and the optical density read at 300 mμ instead of at 260 mμ which Schneider and Hogeboom used (4). The optical density at 260 mμ is too high to measure at the concentrations used here, and it was found necessary to dilute the suspensions in order to use this wave length. Since a linear relationship was found between optical density at 300 mμ and at 260 mμ for serially diluted samples and since the undiluted samples gave readings at 300 mμ in the range where optical density was found to be linear with mitochondrial concentration (0.0–0.9), this wave length was used in this study. This eliminated the necessity of diluting the samples. At least five enzyme determinations were done for each sample of mitochondria, and the average of the values obtained used to compute the results.

Preparation of Mitochondria for Electron Microscopy.—Aliquots of irradiated and control mitochondria were taken from the samples after irradiation and centrifuged to form a pellet. This pellet was then resuspended in a fixative consisting of 2 per cent osmium tetroxide made up in 0.44 M sucrose and fixed in the cold (about 4°C.) for 18 hours. Dehydration in an ethanol-water series and replacement of the alcohol by *n*-butyl methacrylate monomer followed. The suspension was centrifuged in each solution to form a pellet which was then resuspended in the following solution. The final pellet was embedded in *n*-butyl methacrylate at 60°C. Sections were cut on a Porter-Blum microtome, mounted on carbon films (6), and examined in a modified RCA EMU-2A electron microscope.

RESULTS

A. Enzymatic Activity after Irradiation with High Doses of Gamma Rays.—Reference to curve *B* of Text-fig. 1 shows that the mitochondrial DNase II activity increases as the radiation dose is raised to 6×10^5 roentgens. The enzymatic activity does not increase further when the mitochondrial preparations are exposed to higher doses. Treatment of mitochondria with sonic vibration causes an elevation of DNase II activity to the level shown by curve *A* of Text-fig. 1. It is seen from curve *A* that the level to which the sonic treatment raises the enzymatic activity is independent of the radiation dose given to the mitochondria before the sonic treatment. After sonic treatment, the activity of the control samples is as great as that of the samples receiving the high doses of radiation. Since the level of enzymatic activity attained after sonic vibration is the same, regardless of the radiation dose, the possibility

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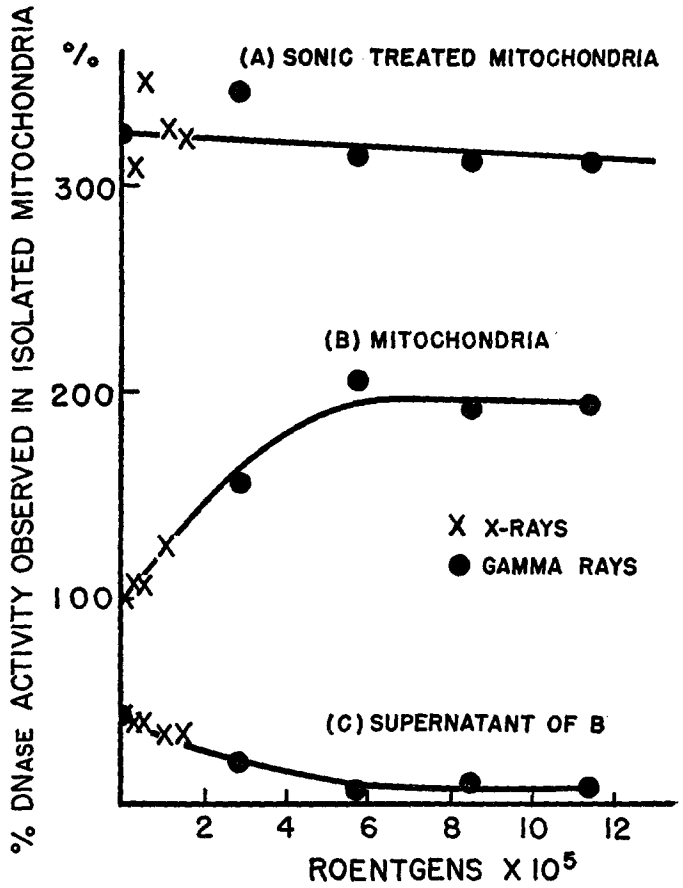
that radiation destroys activators or inhibitors of DNase II seems remote (Text-fig. 1). Although there is a decrease in the enzymatic activity of the supernatant fluid of the irradiated preparations, as can be seen from curve *C* of Text-fig. 1, this depression of enzymatic activity is small compared to



TEXT-FIG. 1. DNase II activity of irradiated and sonically treated mitochondrial fractions. The x-ray data are from a previous paper by one of the authors (1).

the elevation of activity in the mitochondria (curve *B*). It may be surmised, therefore, that the increased activity observed in these mitochondrial suspensions is due to changes in the state of the enzyme associated with particulate material.

B. Acid-Soluble Fraction in the Supernatant Fluids of Irradiated Mitochondria.—After the mitochondria were removed from irradiated and control samples by centrifugation, the resultant supernatant fluid was treated with

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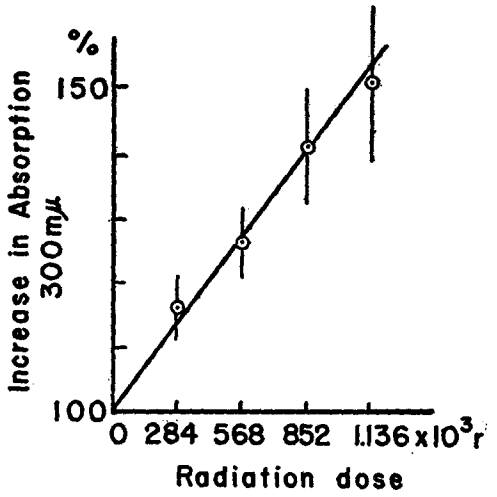
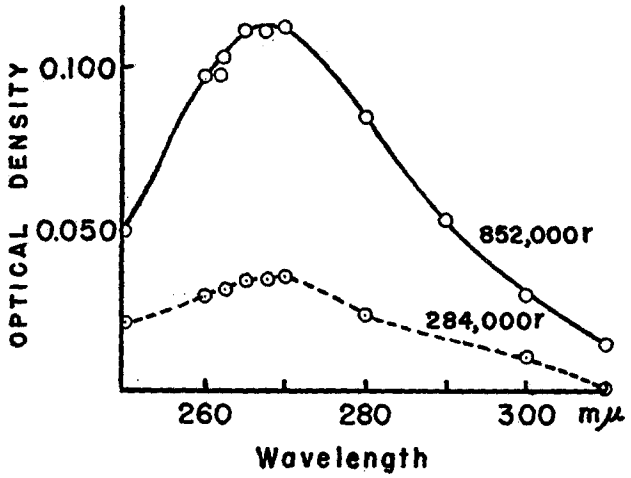
5 per cent trichloroacetic acid and centrifuged at 1,060 *g* for 10 minutes. The deproteinized fluid thereby obtained was examined at 300 mμ in the Beckman



TEXT-FIG. 2. Optical absorption of deproteinized supernatant fluid from irradiated mitochondrial fractions.



TEXT-FIG. 3. Spectral absorption curve of deproteinized supernatant fluid from irradiated mitochondrial fractions.

spectrophotometer. As is evident in Text-fig. 2, the optical density is directly proportional to the radiation dose. In order to obtain further information as to the nature of the absorbing material, the spectral absorption curve was extended into the ultraviolet range. The data in Text-fig. 3 show that the maximal absorption of the material occurs between 260 and 270 mμ. On the

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basis of the ultraviolet absorption pattern, it is assumed that the substances released from the mitochondria during irradiation are nucleotides or similar compounds.

C. Irradiation of Whole Liver Homogenate.—Whole liver homogenate was irradiated by the same method used for the mitochondrial fraction. No alteration of DNase II activity was found.

D. Appearance of the Mitochondria in the Electron Microscope.—A comparison of the electron microscopic appearance of non-irradiated and irradiated mitochondria (Figs. 1 and 2 respectively) shows the existence of radiation-induced structural damage consisting of rupture of mitochondrial membranes and fragmentation of the cristae mitochondriales. There are also a swelling of the mitochondria and an apparent loss of matrix in the irradiated preparations. The fragmented membranes and cristae, however, still show the characteristic double structure (Fig. 2, arrows).

DISCUSSION

The experiments presented above confirm earlier findings (1) that ionizing radiation causes an increase in mitochondrial DNase II activity in isolated fractions.

On the basis of the present experiments, a hypothesis as to the mechanism underlying the increase in DNase II activity during irradiation can be formulated. The phenomenon can be explained most readily on the basis of the demonstrated structural damage. Investigation of normal and irradiated mitochondria with the electron microscope showed that exposure to γ-rays results in rupture of the mitochondrial membranes as well as in a disruption of the internal mitochondrial structure. If one assumes that the DNase II molecules are associated with certain mitochondrial structures (the cristae mitochondriales or the external mitochondrial membrane) and/or with the mitochondrial matrix, then the increased enzymatic activity after exposure to x- or γ-rays can be explained on the basis of fragmentation and release of the structural elements or matrix, thus making the enzyme more readily available to the substrate. Since the membranes of the damaged mitochondria seem to retain their own structure when the organelle is fragmented, and since the enzyme does not go into solution, it may be postulated that the enzyme is normally firmly associated with the intact structures. It may even form a part of the structure of the organelle, although this has not been proven by the present experiments.

The above hypothesis is supported by the data on postirradiation sonic treatment. These data show that the total effect of irradiation plus sonic treatment is the same regardless of the radiation dose. Therefore, the mechanism by which each technique manifests its effect must be the same. If this were not true, one would expect the effects to be additive and the samples receiving high radiation doses to reach higher enzymatic activities after sonic

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treatment than the samples receiving little or no radiation. It is known that sonic vibration causes breakage of particulates in suspension and the present study has shown disruption of the mitochondrial structure by radiation, indicating that the common mechanism for increasing enzymatic activity is the production of structural damage.

This hypothesis is supported further by experiments which show that other methods of disrupting mitochondria also result in an increase in DNase II activity. The present study has shown an increase in DNase II activity after sonic treatment and irradiation and Okada (7) has shown a similar increase in activity in mitochondria broken by osmotic shock on being put into distilled water, and by homogenization in a Waring blendor. Thus there is strong evidence for a causal relationship between the disruption of the mitochondrial structure and the alteration in enzymatic activity.

Schneider and Hogeboom (4) have shown an increase in DNase activity of isolated mitochondria after sonic treatment. They, however, report that almost two-thirds of the enzyme becomes soluble after sonic treatment. The present study has shown essentially no solubilization of the enzyme after irradiation. This discrepancy may be explained either by differences in the methods used to isolate the mitochondria, or the methods of enzymatic assay, or by differences in the effects of sonic treatment and irradiation. Schneider and Hogeboom isolated and treated their mitochondria in 0.25 M sucrose. It has been shown by Witter *et al.* (8) that such mitochondria do not resemble mitochondria *in situ* as closely as do mitochondria isolated in the medium used in the present study. The assay methods are such that Schneider and Hogeboom, by adding magnesium sulfate to their reaction mixture, measured a combination of both DNase I and II, while in the present study the use of versene insured the measurement of only DNase II.

This leads to two possibilities for the association between the enzyme and the mitochondrion. It has become apparent that the DNase II molecules in the isolated mitochondrion are masked from the substrate in some way, and that methods which disrupt the mitochondrial structure also increase the availability of the enzyme and thus its activity. What is not clear is whether the enzyme molecules are associated with the matrix or with the structural elements of the mitochondrion. On the basis of the experiments presented here, the present authors believe that the enzyme is bound in some way to the structural elements, and that this association can be disrupted by various procedures.

It is of interest to note that structural damage has been reported for mitochondria irradiated *in vivo* in thyroid tissue by McQuade *et al.* (9) and that Ryser *et al.* (10) have reported increased fragility for irradiated, isolated mitochondria.

It is believed by the present authors that other radiation-induced changes in intracellular enzymatic activities, such as those reported by van Bekkum

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(11) and by Maxwell and Ashwell (12) for spleen and by Aronson *et al.* (13) for yeast, as well as those reported by Ryser *et al.* (10) and Fritz-Niggli (14) for isolated mitochondria, may have a close relationship with changes in the structure of the subcellular elements.

Recent work by de Duve *et al.* (15) has suggested that DNase, as well as other hydrolytic enzymes, is localized in the lysosomes, a new particulate fraction, intermediate in size between mitochondria and microsomes. No attempt was made in the present study to isolate these particulates separately. It has been suggested in a paper by Novikoff *et al.* (16) that the lysosomes may be the dense bodies found in parenchymatous cells of liver, and Bennett (17) has suggested that these dense bodies may be phagocytosed inclusions found in liver parenchymal and endothelial cells, in mesothelial cells, and in macrophages. The morphology of these phagocytosed bodies in endothelial cells of liver has been described by Parks and Peachey (18) and by Parks *et al.* (19) who show them to be bounded by a single membrane, similar in appearance to the cell membrane. Since both the phagocytosed inclusions and the mitochondria presumably depend on membranes for structural integrity, it is probable that the doses of radiation which have been shown in the present study to disrupt virtually all the mitochondria would also disrupt the bodies identified with the lysosomes, if any of these bodies were present in the preparations. Examination of sections from several preparations in the present study, however, revealed the presence of few, if any, particulates resembling those identified by Novikoff *et al.* (16) with the lysosomes.

The data reported by de Duve on mitochondrial and lysosomal fractions isolated simultaneously (15) indicate that a considerable portion of the total DNase activity of the homogenate is isolated with the mitochondria. It certainly has not been proven, at present, that DNase is exclusively a lysosome enzyme, and therefore the present work proceeded on the assumption that at least a part of the total enzyme is found in the mitochondria. If, however, it should be shown that DNase is localized in the lysosomes, the present experiments will have to be extended.

The present study has shown that an alteration of enzymatic activity could not be observed when liver homogenates suspended in 0.44 M sucrose were irradiated. The failure to demonstrate the effects of irradiation in homogenates is probably due to the presence of protective substances. The addition of cysteine to mitochondrial suspensions increased the DNase II activity even in the absence of irradiation. It is impossible therefore to determine whether or not the protective substances in homogenates consist of cysteine or other —SH group–containing substances.

SUMMARY

1. Exposure of isolated liver mitochondria to high doses of gamma rays from a Co^{60} source causes the level of DNase II activity to increase. Treatment of the mitochondria with sonic vibration causes a further elevation of the activity to a level which is independent of the prior radiation dose.

2. Such increased mitochondrial DNase II activity appears to be due to

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the "structural damage" of the subcellular particulates caused by the ionizing radiation. Other methods of disrupting the mitochondrial structure also cause increased DNase II activity. A causal relationship between the structural alteration and the increased enzymatic activity is postulated.

3. The DNase II activity appears to be closely associated with the structural elements of the mitochondria and remains associated with the fragments after irradiation.

4. Upon irradiation, the mitochondrial suspension releases ultraviolet-absorbing materials which are probably nucleotide in nature.

5. The possibility of localization of DNase activity in the lysosome fraction of de Duve (15) is discussed. It is felt that DNase II is at least in part a mitochondrial enzyme and that probably the conclusions drawn here would be applicable to any DNase II present in the lysosomes as well.

6. Irradiation of whole liver homogenate causes no increased DNase II activity. The experiments do not provide any information on the presence or action of protective substances in the homogenate.

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EXPLANATION OF PLATES

PLATE 65

FIG. 1. Rat liver mitochondria isolated in 0.44 M sucrose. The mitochondria are relatively well preserved with the double structure of the mitochondrial membrane (*MM*) and the cristae mitochondriales (*CM*) shown where the plane of the section is favorable. Most of the mitochondria are intact. Extramitochondrial debris is probably contamination from remnants of the endoplasmic reticulum. × 52,000.

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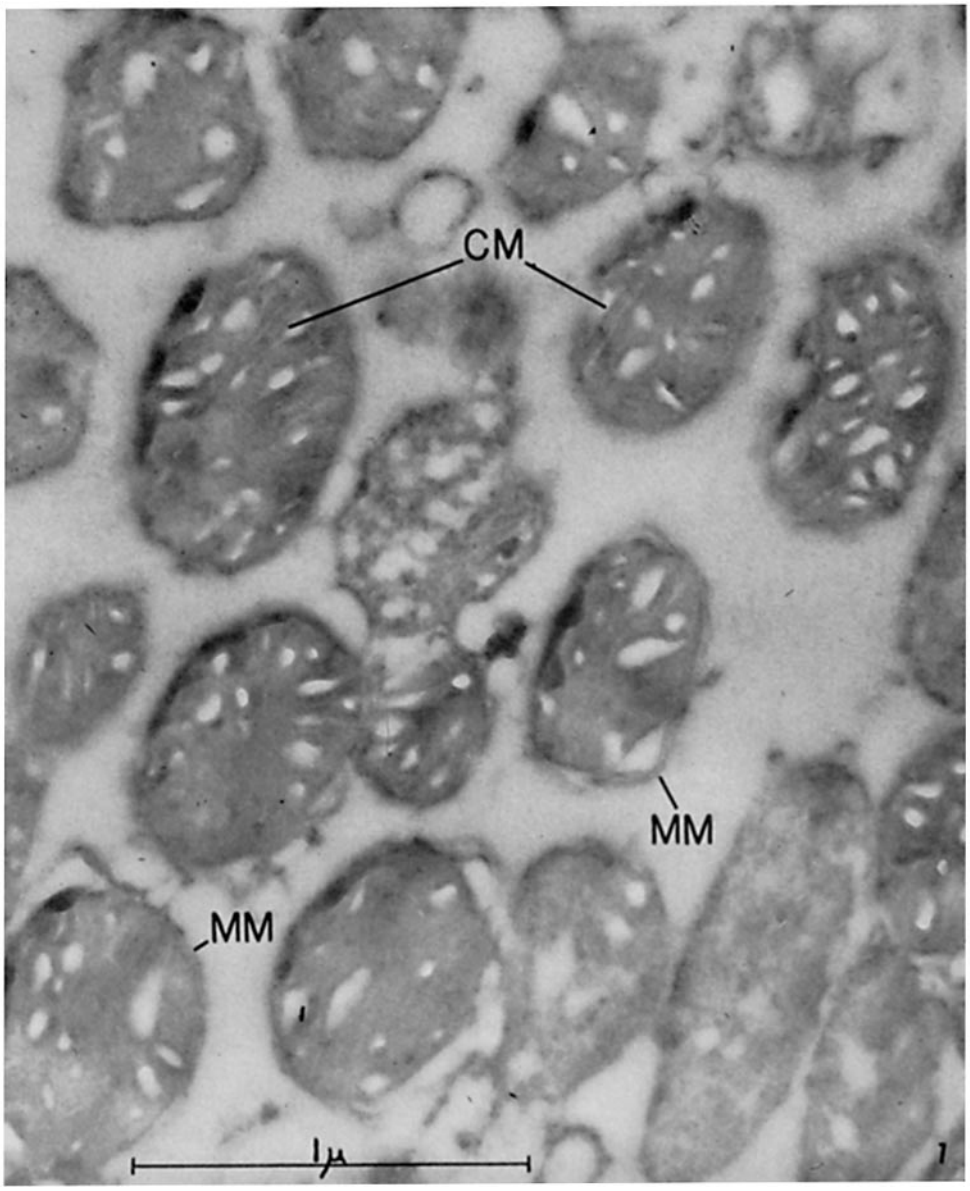

(Okada and Peachey: Desoxyribonuclease II activity)

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PLATE 66

FIG. 2. Rat liver mitochondria isolated in 0.44 M sucrose and irradiated with 10^6 roentgens of gamma rays. The mitochondria appear swollen, many are broken, and there is considerable fragmentation of the cristae mitochondriales in those in which the outer membrane is still intact. Double structure is still seen in many places (arrows). × 44,000.

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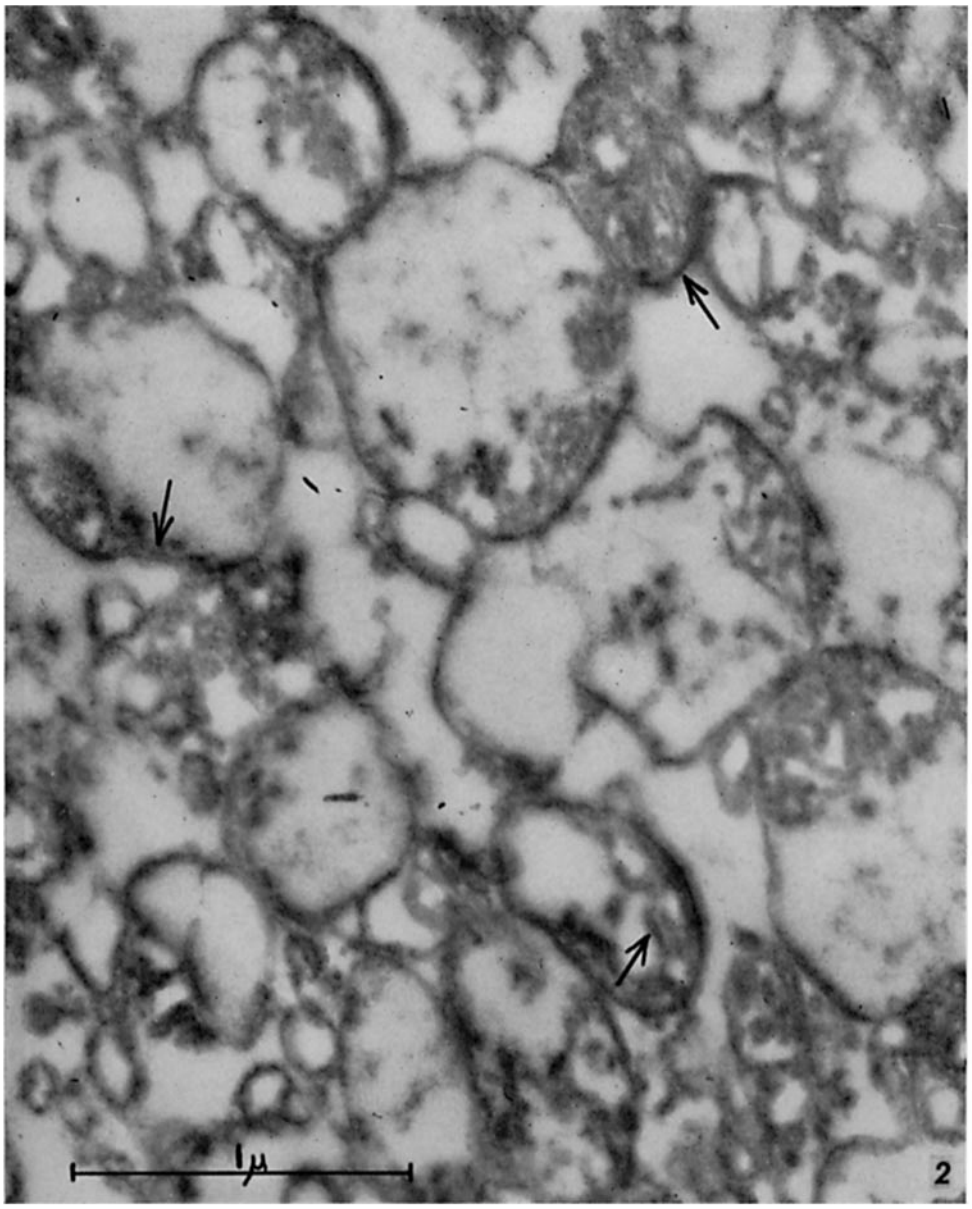

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