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THE EFFECT OF CERTAIN PHENOTHIAZINES ON THE STRUCTURE AND METABOLIC ACTIVITY OF SARCOSOMES OF GUINEA PIG HEART*

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Abstract—The metabolic effects of phenothiazine derivatives have been attributed to the inhibition of several of the most fundamental properties of the mitochondria. Enzymes not linked to the mitochondrial activity were also reported as being inhibited by phenothiazine compounds. However, most of the effects on isolated mitochondria should be considered a consequence of their action on the mitochondria itself. The effects of several phenothiazine compounds on the normal properties of mitochondria were then studied by polarographic determinations; their effect on the fine structure of these subcellular particles was studied by turbidimetry and by electronmicroscopy.

REPORTS of the possible effect of phenothiazine (PTZ) compounds on the respiratory properties of cells include the following. Allemby and Collier[1, 2] showed that PTZ compounds depressed oxygen uptake by animal tissues and homogenates and inhibited brain hexokinase and succinoxidase of rat liver mitochondria. The relationships between the chemical structure of chlorpromazine and its inhibitory effect on enzymes such as cytochrome oxidase and ATPase were studied by Bernsehn *et al.*;[3] Dawkins *et al.*[4] explained the effect of phenothiazines on the central nervous system on the basis of their effect on cytochrome oxidase and $NADH_2$-cytochrome c oxido-reductase systems. Also, the inhibitory effect of phenothiazines on the respiratory mechanism of cells was attributed to their chelating properties.[3, 5, 6] It has been admitted that PTZ compounds compete with the metals from the metaloproteins, which could be an explanation for their inhibitory effect. Bacila and Medina,[7] on the other hand, demonstrated that the inhibitory effect displayed by PTZ on the oxidative phosphorylation is common to all PTZ derivatives. They have also shown that PTZ is able to inhibit oxidative phosphorylation and to prevent the stimulatory effect of 2,4-dinitrophenol on the respiration of isolated sarcosomes of guinea pig heart. In certain conditions, when oxidative phosphorylation and the stimulating effect of 2,4-dinitrophenol are inhibited, sarcosomes are still able to respire in the presence of succinate.

Because inhibition of several activities of the respiratory chain has been attributed to PTZ, this work was undertaken in order to study the possible effect of such compounds on the structure and the integrity of isolated sarcosomes.

MATERIAL AND METHODS

Guinea pig heart sarcosomes were obtained by the method proposed by Medina and

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Bacila.[8] This method follows the general procedure of Cleland and Slater[9] with the modification for brain mitochondria proposed by Voss *et al.*[10] in which 0·21 M mannitol is used instead of sucrose. Guinea pig hearts were minced, washed, and then homogenized in a loose-fitting Potter-Elvehjem homogenizer. The washing and extraction medium contained mannitol 0·21 M, sucrose 0·075 M, Tris 0·01 M, and EDTA 0·2 mM (pH 7·4). The homogenate was centrifuged for 5 min at 1,500 *g*, the sediment discarded, and the supernatant centrifuged 15 min at 10,000 *g*. The sedimented sarcosomes were washed twice. To prepare the final suspension of sarcosomes, 1 ml of the extraction medium for each heart used was added to the sediment. The average protein content of this suspension was equal to 1·4 mg protein/ml.

Respiration and oxidative phosphorylation were assayed polarographically by an oxygen electrode described by Voss *et al.*[11] This oxygen electrode is built with a platinum electrode of the stationary type combined with a magnetic stirrer; a KCl bridge links the platinum electrode with a calomel reference electrode. The magnetic stirrer provides a rapid mixture of the components of the system, which are in a reaction chamber within a glass vessel through which water circulates, thus maintaining a constant temperature.

Oxidative phosphorylation was measured according to the polarographic technique of Chance and Williams,[12] the P/O ratios being measured as ADP/O ratios. Respiratory control coefficients (RC) were calculated as the ratio between the rate of respiration in the presence of substrate and ADP (active state of respiration) and the rate of respiration after the inorganic phosphate acceptor was consumed.

Turbidimetric determinations were carried out at 600 mμ in a Beckman DU spectrophotometer. To the two cuvets used for the spectrophotometric readings, 2·9 ml of the reaction media containing 0·25 M mannitol, 0·01 M Tris, 0·2 mM EDTA, 0·01 M phosphate buffer (pH 7·4), and 0·01 M KCl, final pH 7·4, were added and followed by the addition of 0·1 ml of a suspension of sarcosomes containing 1·4 mg protein/ml. The first cuvet was used as a control to show the changes in turbidity that the suspension of sarcosomes could undergo at room temperature. To the second cuvet, different amounts of PTZ were added and the changes followed by optical density.

Two aliquots of each mitochondrial suspension containing 1 μmole of promethazine were taken after 1-min and 60-min incubation, respectively, and fixed for electron microscopy in a buffered solution of osmic acid at 1%, according to the method of Palade.[13] The mitochondrial suspension (0·15 ml) added with promethazine was transferred to a plastic centrifuge tube containing 1·5 ml of a 1% osmic acid solution buffered with Versene-acetate buffer (pH 7·4). This suspension was kept at room temperature and then centrifuged at 10,000 *g*. After 30 min of the fixation time, including centrifugation, the sediment was washed several times in 70% ethanol and embedded in methacrylate for the final slicing. A control suspension of mitochondria was also examined by electron microscopy. The electron micrographs were taken in a Siemens electron microscope from the Instituto Butantan, São Paulo.

The following PTZ compounds were used in the present work. Thiodiphenylamine base (phenothiazine); promethazine: 10-(2-dimethylaminopropyl)phenothiazine; chlorpromazine: 2-chloro-10-(3-dimethylaminopropyl)phenothiazine; levomepromazine: 2-methoxy-10-(2-methyl-3-dimethylaminopropyl)phenothiazine; perphenazine: 1-(2-hydroxyethyl)-4-[3-(2-chloro-10-phenothiazinyl)propyl]piperazine; pro-

chlorperazine: 2-chloro-10-[3-(1-methyl-4-piperazenyl)propyl]phenothiazine; alimemazine: neutral tartarate of 10-(3-dimethylamino-2-methylpropyl)phenothiazine.

RESULTS

Effect on respiration and oxidative phosphorylation of heart sarcosomes

Phenothiazine compounds showed a very marked effect on the normal properties of heart sarcosomes. This was shown by measuring respiration and oxidative phosphorylation of isolated guinea pig heart sarcosomes (Table 1).

TABLE 1. THE EFFECT OF PTZ COMPOUNDS ON THE NORMAL PROPERTIES OF ISOLATED SARCOSOMES OF GUINEA PIG HEART

System: 1·9 ml of aerobic medium containing 0·33 M sucrose, 0·02 M phosphate buffer (pH 7·4,) 0·01 M potassium chloride, and 0·1 ml sarcosome suspension (1·4 mg protein/ml). The first experiment was a control with no PTZ added. Sarcosome was always preincubated with the corresponding PTZ compound in the following experiments. In every instance substrate respiration rate was determined and was followed by the addition of ADP. The respiration rate was stimulated ("active respiration") and after the inorganic phosphate acceptor was consumed the respiration rate leveled off to the substrate respiration level. The respiratory control coefficient (RC) was measured as the ratio between the respiration rate during ADP phosphorylation and the rate of respiration after the ADP was consumed. Per cent inhibition of RC was then taken as a measurement of the PTZ effect on mitochondria, the inhibition values obtained considering the control as 100 per cent.

Phenothiazine compound (pre-incubation)	Respiration rate with α-ketogluta-rate, 10 mM	Respiration rate during ADP (active respiration)	Controlled respiration rate after ADP	Respiratory control co-efficient (RC)	Per cent inhibition of the RC
No addition	0·30	0·92	0·30	3·0	
Levomepromazine ($1·8 \times 10^{-4}$ M)	0·29	0·68	0·35	1·94	35·3
Chlorpromazine ($1·9 \times 10^{-4}$ M)	0·21	0·47	0·26	1·8	40·0
Perphenazine ($4·9 \times 10^{-4}$ M)	0·19	0·29	0·19	1·5	50·0
Promethazine (5×10^{-4} M)	0·29	0·86	0·47	1·8	40·0
Alimemazine ($8·0 \times 10^{-4}$ M)	0·20	0·50	0·27	1·8	40·0
Prochlorperazine ($6·5 \times 10^{-5}$ M)	0·29	0·53	0·25	2·1	30·0
Phenothiazine ($2·0 \times 10^{-5}$ M)	0·25	0·66	0·32	2·0	33·3

It can be seen that all PTZ compounds inhibit the normal properties of sarcosome. Thus, such inhibition seems to be a general property of the thiodiphenylamine itself rather than an effect of any other chemical group found in its derivatives.

Effect on the physical properties of isolated heart sarcosomes

The physical effect caused by the addition of PTZ to a suspension of isolated heart sarcosome was analysed by turbidimetry at 600 mμ in a Beckman DU spectrophotometer. Immediately after the addition of the PTZ derivative to the suspension of mitochondria there was a sudden increase in optical density, followed by a continuous decrease. A control containing only sarcosomes and no PTZ was used. The readings

were carried out against a blank of reaction medium. Figure 1 shows the results obtained with chlorpromazine and alimemazine. Table 2 gives numerical values of results obtained by the addition of different concentrations of PTZ.

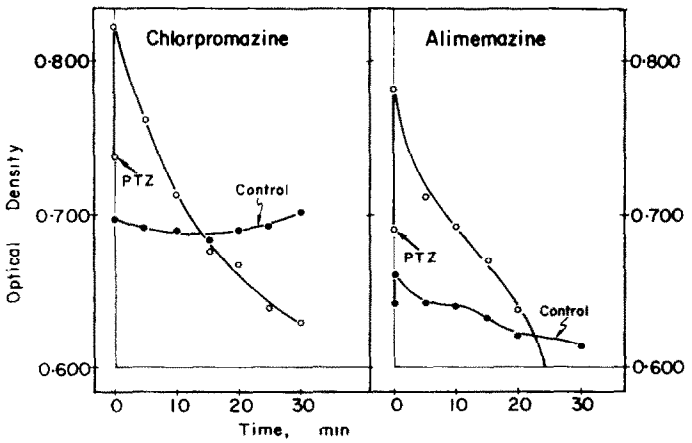

FIG. 1. Effect of PTZ on the suspension of isolated heart sarcosomes. System: 2·9 ml of a solution containing 0·33 M sucrose, $1 \cdot 10^{-3}$ M Tris, $1 \cdot 10^{-4}$ M EDTA (final pH 7·4), 0·1 ml of the sarcosome suspension containing 1·4 mg protein/ml. Arrows indicate the addition of 0·5 μmoles of PTZ to the suspension of sarcosomes. Readings were done in spectrophotometer at 600 mμ. Values express optical densities taken against a blank containing only the suspension medium.

TABLE 2. EFFECT OF CONCENTRATION OF PHENOTHIAZINES ON THE STRUCTURE OF ISOLATED HEART SARCOSOMES

System: 2·9 ml of medium containing 0·33 M sucrose, $1 \cdot 10^{-3}$ M Tris, $1 \cdot 10^{-4}$ M Versene (final pH 7·4), 0·1 ml sarcosome suspension (1·4 mg protein/ml). Reading done in Beckman DU spectro-photometer at 600 mμ. Values express change in optical density 30 min after the addition of the drug.

	Chlorpromazine	Levomepromazine	Alimemazine	Promethazine
μmoles in 3 ml	Change in optical density			
0·1	0·045	0·028	0·030	0·015
0·2	0·050	0·110	0·070	0·050
0·3	0·132	0·145	0·135	0·055
0·4	0·175	0·150	0·230	0·080
0·5	0·200	0·170	0·170	0·060

Effect on the structure of isolated sarcosomes

The spectroscopic results shown in the previous experiments were checked by electron micrographs of isolated sarcosomes to which promethazine was added. Normal sarcosomes are shown in Fig. 2; Figs. 3 and 4 show the suspension of sarcosomes 60 sec and 60 min after the addition of promethazine. It can be seen that phenothiazine has a definite effect on the structure of isolated sarcosomes.

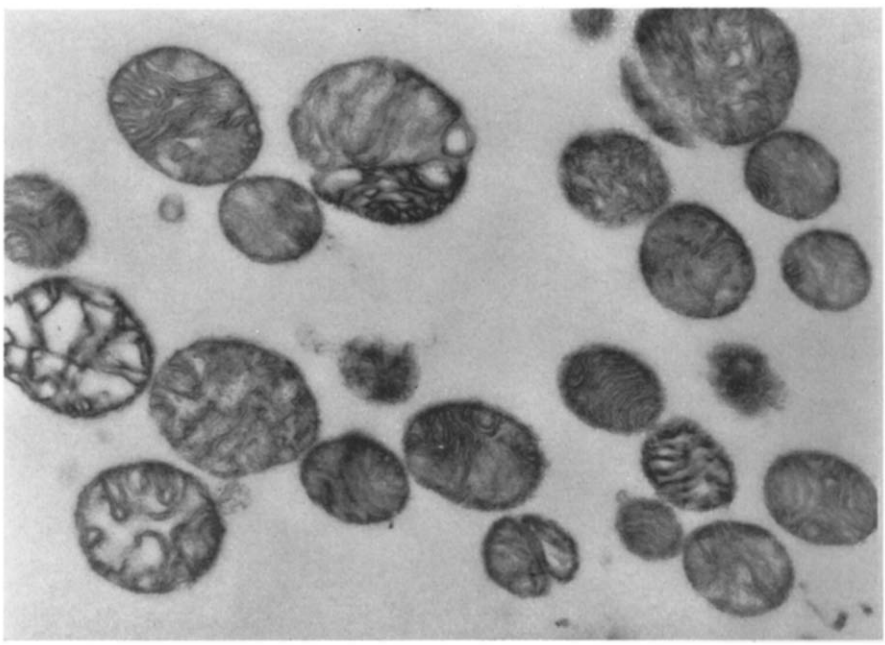

FIG. 2. Electron micrograph of isolated guinea pig heart sarcosomes. Sarcosomes suspended in a medium containing 0·33 M sucrose, $1 \cdot 10^{-3}$ M Tris, and $1 \cdot 10^{-4}$ M Versene, final pH 7·4. Fixation (30 min) in 1% osmic acid, and embedding in methacrylate (× 7, 100 from the original and × 24, 800 total amplification). The isolated guinea pig heart sarcosomes show integrity and continuity of the mitochondrial cristae in parallel with the larger axis. The changes that can be observed are either artificial or due to the aging of the mitochondria, which can explain the appearance of a few empty sites or of the possible swelling. The interrelations of the internal and external membranes and the mitochondrial cristae are so intimate that they do not allow a perfect differentiation between the two osmophilic layers and a less osmophilic intermembrane space.

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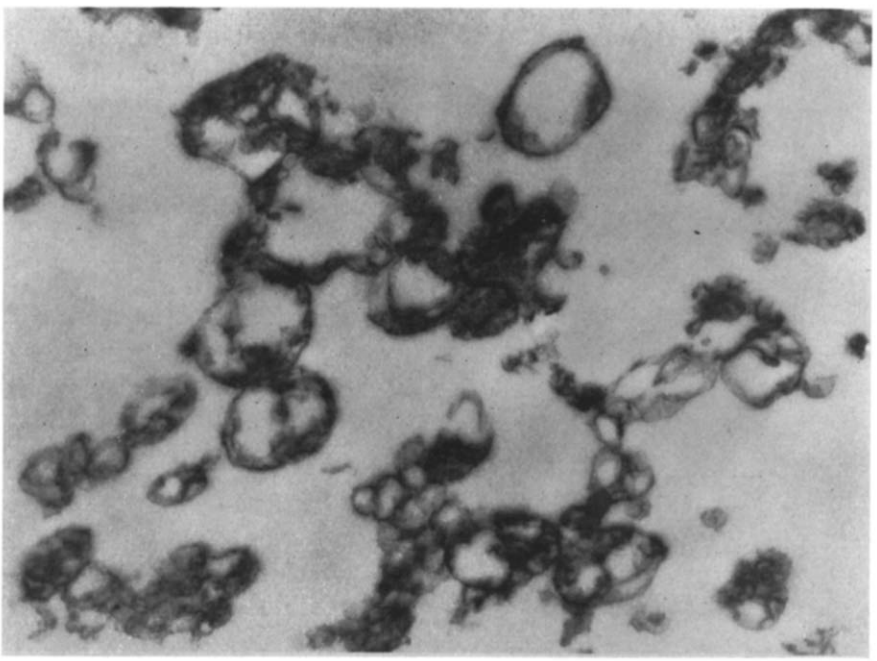

FIG. 3. Electron micrograph of isolated guinea pig heart sarcosomes 60 seconds after the addition of promethazine. To a suspension of sarcosomes 1·0 μmole of promethazine was added. Fixation (30 min) in 1% osmic acid was followed 60 sec after the addition of promethazine; embedded in methacrylate (increase $\times 7$, 100 from the original and $\times 24$, 800 total). There is a complete modification of the structure and morphology of the sarcosomes which are shrunk and agglutinated. There is a disappearance of the normal structure and a change in the mitochondrial cristae followed by a greater adherence of both osmophilic membranes from the α-cytomembrane, which allows a greater visualization of the shape of the sarcosome. There are several intramitochondrial clear spaces in contrast to the greater density of the rest of the membranes and the mitochondrial cristae which are agglutinated. In the upper left side of the figure part of an α-cytomembrane can be seen, showing the two membranes and osmophilic points separated by a clearer space with a clearer and less osmophilic space. The mitochondrial cristae have completely lost the normal aspect they showed before the addition of promethazine.

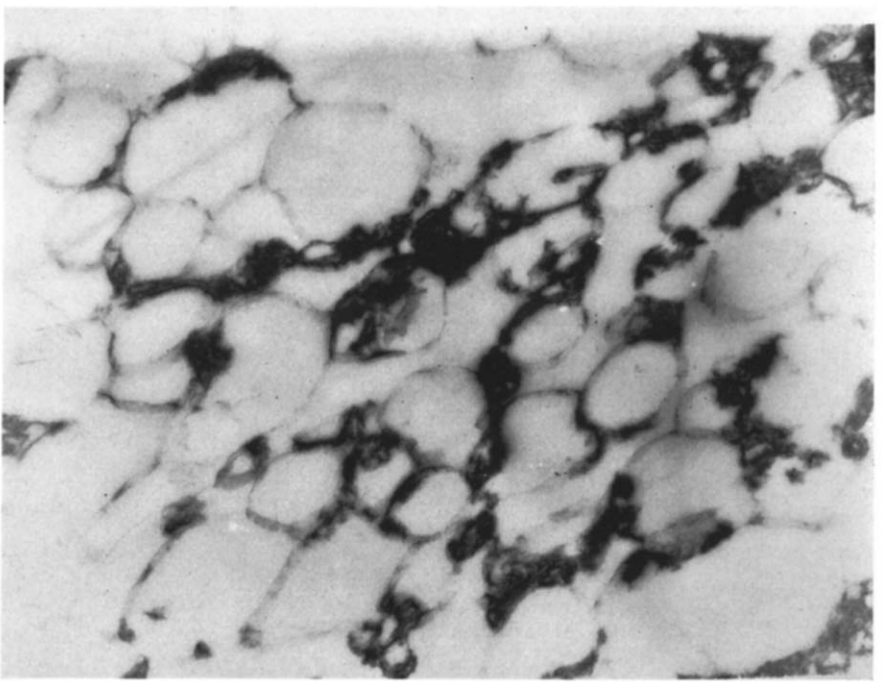

FIG. 4. Electron micrograph of isolated sarcosomes 60 minutes after the addition of promethazine. Electron micrograph from the same suspension of sarcosomes from Fig. 2 taken 60 min after the addition of promethazine. Marked alterations took place in the structure of the sarcosomes. Water entry into the sarcosomes was followed by an increase in the size of the particle ($\times$ 7, 100 from the original. Total amplification $\times$ 24, 800). The aspects shown in Fig. 3 were followed by a general swelling of all sarcosomes, which became vacuolated, showing a distention of the α-cytomembrane with the two osmophilic layers well delineated and generally keeping their integrity. The swollen and less osmophilic appearance of the intermembrane space is evident. The rest of the mitochondria cristae were pressed toward one side of the intermitochondrial space against the α-cytomembrane.

DISCUSSION

The property of chlorpromazine to inhibit respiratory processes (Allemby and Collier,[1, 2] Bernsehn *et al.*,[3] and Dawkins *et al.*[4]) is shown in the present paper to be a general feature of PTZ compounds. Bacila and Medina[7] showed that these compounds also prevent the stimulation of respiration by 2,4-dinitrophenol.

PTZ compounds (Table 1) have a definite effect on the normal properties of sarcosomes by lowering the respiratory control coefficient of the sarcosome suspension. Respiratory control coefficients are calculated as the ratio between the respiration rate during ADP or active state of respiration and the controlled respiration rate after ADP, the respiration rates being calculated as μmoles O_2/sec per l. According to the polarographic method of Chance and Williams[12] the addition of a limiting amount of the inorganic phosphate acceptor to a sarcosome suspension is followed by the stimulation of the respiration. The respiration rate levels off to the controlled state of respiration after ADP as soon as the inorganic phosphate acceptor is consumed. The respiratory control coefficient is, in this way, an experimental parameter established polarographically, which proves to be very useful for the study of the coupling state of the mitochondrial preparation as well as for the analysis of the effect of different agents such as those that uncouple or inhibit oxidative phosphorylation. It can be seen by the analysis of the data in Table 1 that the addition of PTZ compounds to a suspension of heart sarcosomes seems to cause an inhibition of the active state of respiration; that is, the respiration rate following the addition of ADP. Bacila and Medina[7] have already shown that the preincubation of guinea pig heart sarcosomes with concentrations of perphenazine ranging from $6{\cdot}2 \times 10^{-5}$ M to $6{\cdot}2 \times 10^{-4}$ M prevents oxygen uptake during the active state of respiration, the depressing effect being proportional to the concentration of the drug. From these considerations it seems that PTZ compounds can hardly be considered as uncouplers of oxidative phosphorylation with the same meaning given to 2,4-dinitrophenol[14] which, when added to a suspension of sarcosomes, gives lower respiratory control coefficient values either because the rate of respiration never levels off or, when it does, it never goes back to the substrate respiration levels. PTZ compounds, on the other hand, never stimulate respiration but give lower respiratory control coefficients because they depress the respiratory rates during the active state of respiration. A similar fact seems to occur with thyroxine, whose uncoupling properties are described by Martius and Hess[15] and Lardy and Feldott.[16] Ernstern and Lindberg[17] suggest that it acts on the mitochondrial structure, the same not being true for 2,4-dinitrophenol; Tapley *et al.*[18] think that this hormone acts by labilizing the mitochondrial structure. Lee and Williams,[19] on the other hand, showed that thyroxine binds in a labile manner to the mitochondria; Chappell and Greville[20] studied the swelling and shrinking of the mitochondria in relation to the action of thyroxine. Bronk[21] found that it inhibits oxidative phosphorylation by combination with a particular protein of the mitochondria. Medina and Bacila[22] found an effect with thyroxine similar to that now shown for PTZ compounds: that active state of respiration of guinea pig heart sarcosomes is also prevented by the hormone. It is obviously difficult to extrapolate and to correlate PTZ to what is known for thyroxine. However, the visible effects of PTZ compounds and of thyroxine on the respiration and oxidative phosphorylation of isolated heart sarcosomes as they were demonstrated polarographically are very

similar but are undoubtedly different from those of drugs that act like 2,4-dinitrophenol.

The data in the present paper show, however, that the PTZ compounds have a definite and striking effect on the structure of mitochondria. Since PTZ compounds inhibit the metabolic activity of isolated mitochondria, experiments were carried out in order to see whether they would also induce structural changes in the mitochondria itself. It was shown (Fig. 1) that the addition of 0·5 μmoles of PTZ to a suspension of sarcosomes caused a sudden increase followed by a continuous decrease in optical density. Table 2 shows similar data in which increasing amounts of PTZ give a change in optical density proportional to the concentration of the drug. Quantitatively, the effect of promethazine was different from the other phenothiazine compounds used, always giving smaller changes in optical density. It was shown by electron microscopy that PTZ added to a suspension of sarcosomes agglutinates them, causing a shrinking of the sarcosomes along with a few morphological alterations such as disruption of the fine internal structure (Fig. 2). After 60 min of the addition of promethazine (Fig. 4) marked alterations took place in the structure of the sarcosomes. In spite of the results obtained by electron microscopy, it is still difficult to establish whether the structural changes induced by these compounds are primarily responsible for the effect of PTZ on the metabolic behavior of the mitochondria. No direct support is found for such a hypothesis in addition to the data presented here. Aghajanian,[23] for instance, studying the effect of chlorpromazine on the metabolic levels of brain mitochondrial respiration, correlated the insensitivity of the drug action in some of the metabolic levels of brain mitochondria to a high rate of electron transport rather than to high or low steady-state reduction of the electron carriers, while during slow transport the mitochondria is much more sensitive to the drug action. However, due to the fact that in these experiments Mg^{2+} was added to the reaction medium, it is a little difficult to follow his conclusions, considering that Voss *et al.*[10] demonstrated that Mg^{2+} is an uncoupler of oxidative phosphorylation for brain mitochondria.

The effect of chlorpromazine on biological membranes was studied *in vitro* by Guth and Spirtes[24, 25] and by Freeman and Spirtes.[26] The inhibition of permeability caused by chlorpromazine was also studied by Axelrod *et al.*[27] and by Dengler and Titus.[28] They found that chlorpromazine inhibited the uptake of labeled noradrenaline by mammalian tissues *in vivo* and similarly, *in vitro*, by tissue slices. Spirtes and Guth[29] found that 10^{-5} M chlorpromazine inhibited the uptake of water and sucrose by rat liver mitochondria, whereas a more concentrated solution of chlorpromazine sulfoxide (10^{-4} M), showed a very weak effect on the mitochondrial swelling. On the other hand, they reported that chlorpromazine added to a suspension of liver mitochondria in suitable concentrations shows an agglutinating effect on these subcellular particles. By measuring the water uptake as well as the sucrose entry, they were able to conclude that $1{\cdot}10^{-5}$ M chlorpromazine inhibited the permeability of the mitochondria to both water and sucrose to the same degree that it inhibited the swelling of mitochondria. They also concluded that chlorpromazine affects the passive transport of water and ions across biological membranes.

The sudden increase in optical density that follows the addition of PTZ to the suspension of mitochondria can be explained by the agglutination that occurs; the electron microscopy shows shrinking of the mitochondria upon the addition of PTZ. Furthermore, if the agglutination is not extensive, this effect combined with the shrink-

ing of the mitochondria offers an explanation for the increase in optical density. It is true that light-scattering has to be considered when optical density measurements are discussed in relation to the effects shown. Figure 2 shows that the isolated sarcosomes are spherical in shape so that Mier's calculation spheres for light-scattering can be applied.[30] The decrease in optical density can be explained in terms of the water (Fig. 4) in the mitochondria. This has the effect of increasing the size of the particles and thus increasing forward scatter to such a rate that it lowers optical density.

A possible interaction of PTZ with proteins has not yet been studied so that no data are available on this subject. However, recent and preliminary investigations at this laboratory (Medina and Bacila, unpublished data) indicate that PTZ compounds added to a suspension of actomyosin increase the optical density of the suspension, which could be an indication of the physical action of these compounds on the structure of the contractile protein. Actomyosin G, on the other hand, was unable to produce the F form in the presence of PTZ.

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