Biochemical Pharmacology, 1963, Vol. 12, pp. 37–46. Pergamon Press Ltd., Printed in Great Britain.

EFFECTS OF CHLORPROMAZINE ON BIOLOGICAL MEMBRANES—I.

CHLORPROMAZINE-INDUCED CHANGES IN LIVER MITOCHONDRIA

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(*Received* 10 *July* 1962; *accepted* 23 *August* 1962)

Abstract—The swelling of rat liver mitochondria suspended in isotonic sucrose at pH 7·4 was measured spectrophotometrically and by water and sucrose entry. Chlorpromazine at concentrations of 5×10^{-6} to 10^{-4} M inhibited such swelling to the same degree, as measured by all three methods. Higher concentrations also caused agglutination of the mitochondria suspended in either isotonic sucrose or KCl. In the KCl medium chlorpromazine at 5×10^{-6} to 10^{-4} M did not inhibit swelling whether measured spectrophotometrically or by water entry; the reason for this has not yet been elucidated. Chlorpromazine sulfoxide had no effect on mitochondria suspended in either isotonic sucrose or KCl.

THE present study was begun as the result of an analogy drawn between certain effects of dinitrophenol or thyroxine and chlorpromazine (CPZ) on liver mitochondria. It was reasoned that, since all three compounds inhibit oxidative phosphorylation, CPZ might also affect mitochondrial permeability as do the other two.[1] Inhibitory permeability effects of CPZ *in vitro* were indeed reported from this laboratory[2] in 1958 and again in 1961[3] and 1962.[4] Recently, Axelrod *et al.*[5] and Dengler and Titus[6] found that CPZ inhibited the *in vivo* uptake of labeled noradrenaline by various mammalian organs or the *in vitro* uptake by tissue slices, including those made from brain cortex. These findings were interpreted as resulting from inhibition of permeability of the cell membrane to the radioactive compound. The experiments described here indicate that CPZ (10^{-5} M) similarly inhibits the uptake of water and sucrose by rat liver mitochondria. Under the same conditions, chlorpromazine sulfoxide at concentrations up to 5×10^{-4} M produces little or no inhibition of mitochondrial swelling.

METHODS

Inorganic chemicals were of CP grade, Mallinckrodt Chemical Works, St. Louis, Mo. Chlorpromazine·HCl (CPZ·HCl) and chlorpromazine sulfoxide·HCl (CPZ-SO·HCl) were gifts of Smith, Kline & French Laboratories, Philadelphia, Pa. Sucrose and glycerol were of reagent grade, and from J. T. Baker Chemical Co., Phillipsburg, N. J. Triton X-100 was obtained from the Rohm and Haas Co., Philadelphia. Disodium ethylenediamine tetraacetate (sodium versenate or Na-EDTA) was obtained from the Fisher Scientific Co., Fairlawn, N. J. Tris (hydroxymethyl)-aminomethane was

* This work was supported by Grant B-2653 from the National Institute of Neurological Diseases and Blindness.

purchased from the Sigma Chemical Co., St. Louis, Mo. Tris or versenate buffers were adjusted to the proper pH with NaOH and made up with glass-distilled H_2O.

Rat liver mitochondria were prepared according to the method of Schneider and Hogeboom[7] except that the suspending medium was 0·25 M sucrose-0·002 M EDTA, pH 7·4. Aliquots of suspensions kept at 1 to 4° were then used for spectrophotometric experiments and to determine sucrose and water content in the following manner.

A stock solution of CPZ·HCl (5·0 mg/ml) was made with glass-distilled H_2O. A suitable amount was added to a Beckman cuvet to give the desired final concentrations. Next, a calculated amount of water and 0·32 M sucrose-0·02 M Tris buffer, pH 7·4, was added to make 2·94 ml. In some experiments, 0·16 M KCl-0·02 M Tris buffer, pH 7·4, was substituted for the sucrose-Tris buffer solution. The mitochondrial suspension was added (0·06 ml) to give a final volume of 3·0 ml. It was so regulated that the initial optical density (O.D.) of the reaction mixture lay between 0·400 and 0·600 at 520 mμ. Appropriate controls not containing the drug were simultaneously prepared. Readings were begun immediately after addition of mitochondria and mixing.

Estimation of mitochondrial pellet H_2O

Direct estimation of mitochondrial pellet water was made by minor modifications of the gravimetric method of Price *et al.*[8] When the 3-ml reaction mixture was prepared for spectrophotometric assay, two tared 50-ml polyethylene test tubes were filled with 30 ml of the same reaction mixture at room temperature (25 to 27°). One tube was immediately centrifuged at 4° in an International centrifuge, model HR-1. The speed was brought to 25,000 × *g* (maximal) in 3 min and the centrifuge was then shut off. Total centrifugation time was 9 to 10 min. The clear supernatant was decanted at room temperature. This zero-time tube was then drained and dried according to Price *et al.*[8] and finally weighed on a semimicro analytical balance. If it had to stand for a time, it was covered with parafilm tightly stretched over the top to prevent water evaporation. After an appropriate interval, usually 10 or 20 min, the second tube was treated like the first. The difference between wet weights of the mitochondrial pellets from the first and second tubes represents the gain in weight of mitochondrial pellet water. The tubes with their contents were dried *in vacuo* in a desiccator over P_2O_5 for 24 h at 37°. The tubes were then weighed to give the dry weights of the mitochondrial pellets. Water-content values were obtained by subtracting pellet dry weight from pellet wet weight. The percentage of mitochondrial water was calculated from the formula:

$$\frac{\text{weight of mitochondrial pellet } H_2O}{\text{weight of wet mitochondrial pellet}} \times 100$$

The gain in per cent mitochondrial pellet H_2O at the end of the 10- or 20-min experimental period was calculated by subtracting the per cent H_2O determined at zero min from that at 10 or 20 min.

Mitochondrial nitrogen

The micro-Kjeldahl method of Heller *et al.*[9] was used with minor modifications. For this purpose the wet or dry mitochondrial pellets were made up to a known volume with glass-distilled H_2O and homogenized briefly with a hand Ten-Broek homogenizer until the suspension was smooth; aliquots giving about 0·5 mg of nitrogen were then analyzed.

Isolated mitochondria made in sucrose alone and in sucrose-EDTA did not differ significantly in total nitrogen content, showing that no measurable adsorption of the added EDTA occurred. Similar analyses of mitochondrial preparations with and without Tris buffer showed that Tris also was not measureably adsorbed.

Mitochondrial protein-N

Aqueous trichloracetic acid solution (100 g/100 ml solution) and water were added to the wet or dry mitochondrial pellet to make the trichloracetic acid concentration in the final suspension 5%. The mixture was homogenized, allowed to stand for 10 min, and then centrifuged at 20,000 × *g* for 10 min. The precipitate was washed once with 5% trichloracetic acid (w/v), centrifuged, and the nitrogen of the precipitate determined as above.[9]

Mitochondrial pellet sucrose

Dried pellets from 30·0-ml aliquots of mitochondrial suspension were homogenized with 3-ml portions of glass-distilled H_2O and made up to 5·0 ml. Then 5 ml of 4 N HCl was added and, after mixing, the suspension was hydrolyzed at 100° for 30 min, cooled, adjusted to pH 5·6 with NaOH, and made up to 30 ml with glass-distilled H_2O; 5·0-ml aliquots were removed to determine the glucose in the pellet by the method of Somogyi.[10] The glucose content was appropriately converted to sucrose content by the factor 342/180.

Drug uptake by mitochondrial suspensions

Complete ultraviolet spectra for chlorpromazine, trifluoperazine, and chlorpromazine sulfoxide indicated that the first two could be measured by their absorption peak at 255 mμ and the last at 275 mμ. To supernatants from mitochondrial suspensions (0·06 ml mitochondrial preparation + 2·94 ml sucrose-Tris or KCl-Tris) which had been allowed to stand for 10 or 20 min at 25° before centrifugation at 0 to 4° at 15,000 × *g* for 10 min, enough drug was added to make the final concentration 1×10^{-5} M. Optical density values obtained on the Beckman DU spectrophotometer at the appropriate absorption peak then represented the 100% value of the drug added. The same amount of mitochondria added to similar suspension media containing 1×10^{-5} M drug were treated similarly and the O.D. density readings of the clear supernatants recorded. The per cent absorption was then calculated from these two readings.

RESULTS

In Fig. 1 the effect of chlorpromazine on liver mitochondria suspended in sucrose-Tris buffer can be seen. A decrease in optical density is generally attributed to mitochondrial swelling when the organelles are suspended in isotonic sucrose.[11] The drug significantly inhibited, at the 95% confidence level or better,[12] the fall in optical density at concentrations of 10^{-4} to 5×10^{-6} M. Occasionally, inhibition was recorded in the presence of 10^{-6} M CPZ.

An opposite effect of CPZ—enhancement of the fall in O.D.—was seen at a concentration of 3 to 5×10^{-4} M. Although this might seem to indicate increased swelling, it was eventually proved to be caused by two other factors. First, just as high concentrations of CPZ cause hemolysis of human erythrocytes,[4, 13] they also disrupt mitochondria. The data in Table 1 show that a 34% drop in mitochondrial nitrogen

occurred when the organelles were suspended in 0·32 M sucrose at a CPZ concentration of 5×10^{-4} M. This effect accounts for part of the increased drop in O.D. seen with 5×10^{-4} M CPZ. It was also observed that the mitochondria began to settle out of the suspension at this concentration. Microscopic examination revealed a marked agglutination of mitochondria (Fig. 2).

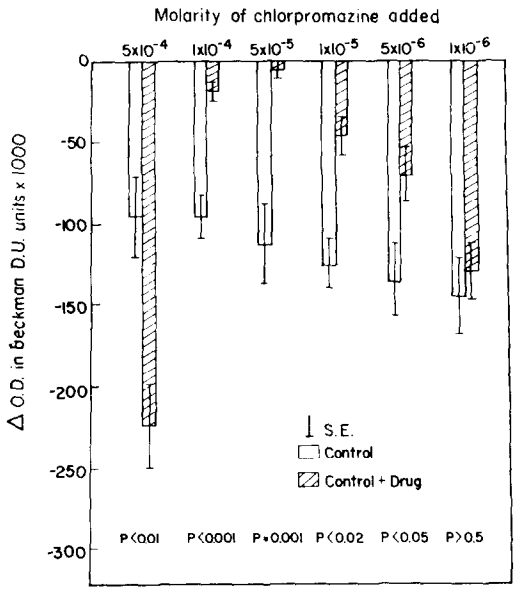

FIG. 1. Effect of exposure to chlorpromazine on the swelling of 0·06 ml of rat liver mitochondrial preparation suspended in 2·94 ml of 0·32 M sucrose + 0·02 M Tris buffer, pH 7·4. Exposure time of the mitochondria to the medium was 10 min; temperature 25°. Each concentration of drug and the control were tested on at least 12 different mitochondrial preparations. P values were determined by Student's *t* test for small samples. O.D. readings taken at 520 mμ.

Figure 2A is a photograph of the mitochondrial suspension in the absence of drug. Fig. 2B, 2C, and 2D show the degree of agglutination in the presence of 5×10^{-5} M, 10^{-4} M, and 5×10^{-4} M CPZ, respectively, as seen in dark-field photographs at 20 × magnification. The white or light gray masses represent mitochondria. At CPZ concentrations of less than 5×10^{-5} M, photographs were indistinguishable from that of Fig. 2A, the control. Fig. 2E is an oil-immersion, light-field photomicrograph at a magnification of 1000× of the suspension shown in Fig. 2D. Individual mitochondria can be seen in each clump. Such marked agglutination accounts for much of the decrease in O.D. A similar agglutination, although not so extensive, is seen when rat brain mitochondria are exposed to this CPZ concentration in isotonic sucrose.

Since compounds like thyroxine cause swelling of mitochondria suspended in KCl as well as in sucrose solutions, the effect of CPZ was now tested on mitochondria suspended in 0·16 M KCl–0·02 M Tris buffer, pH 7·4. At high concentrations (1 to 5×10^{-4} M) agglutination was marked, and an increase in O.D. fall was noted in Fig. 3. At lower concentrations, however, the CPZ had no effect on the endogenous O.D. fall, contrary to the findings for mitochondria suspended in sucrose media. The

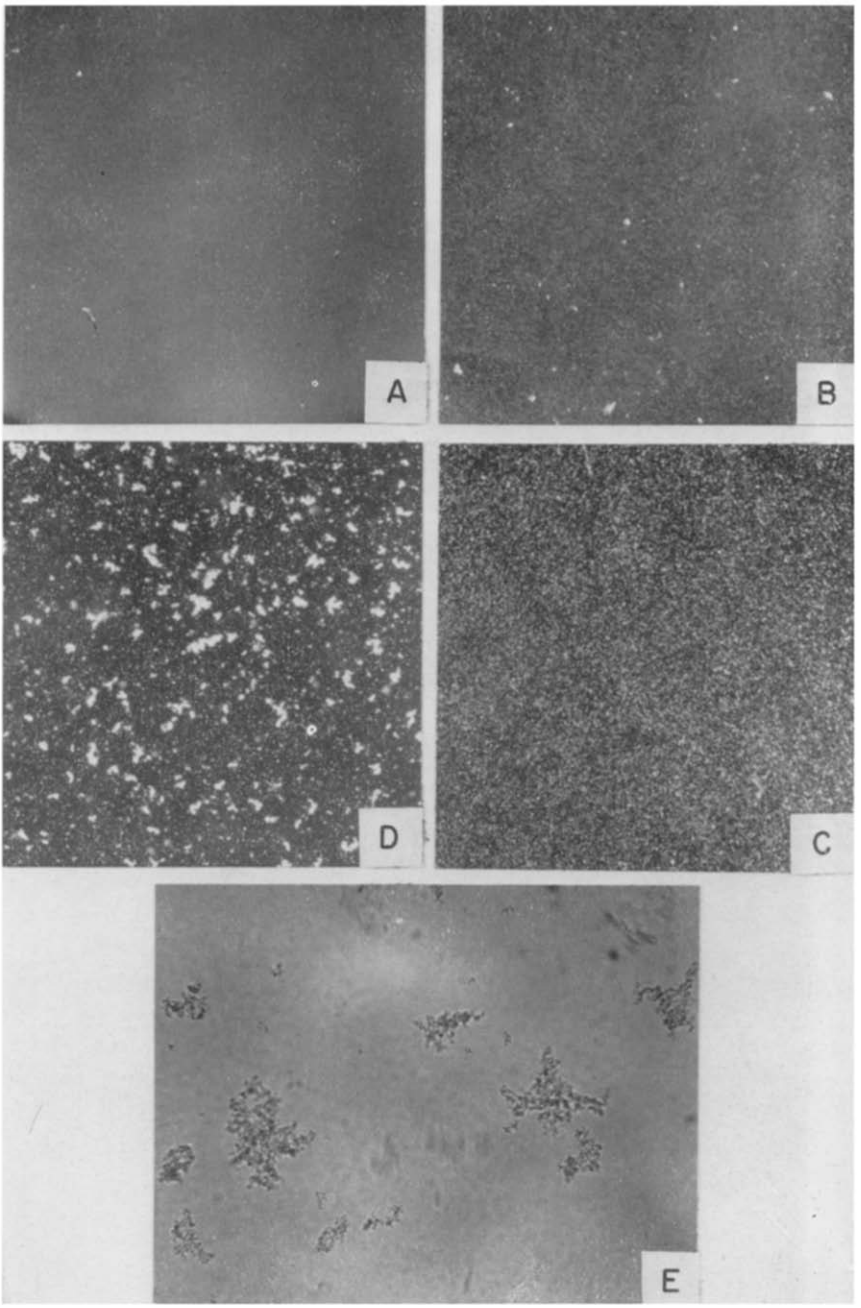

FIG. 2. Agglutinating effect of chlorpromazine on a suspension of 0·06 ml of a rat liver mitochondrial preparation suspended in 2·94 ml of 0·32 M sucrose + 0·02 M Tris buffer, pH 7·4. Exposure time of mitochondria to drug was 10 min; temperature 25°. Fig. 2A-2D, dark-field photomicrographs at 20× magnification. A, control. B, 5×10^{-5} M chlorpromazine present. C, 10^{-4} M chlorpromazine present. D, 3 to 5×10^{-4} M chlorpromazine present. E, the same preparation as in D at 1000× magnification in a light field photograph.

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reason CPZ did not inhibit the O.D. fall in this case is not yet clear. Certainly the adsorption of CPZ at 10^{-5} M concentration is almost as great in the mitochondria suspended in KCl as in sucrose. Table 2 indicates that the extent of adsorption is 58% for the former and 70% for the latter in the zero-minute specimens. There is a slight increase to 64·5 and 81·5% after 10-min incubation.

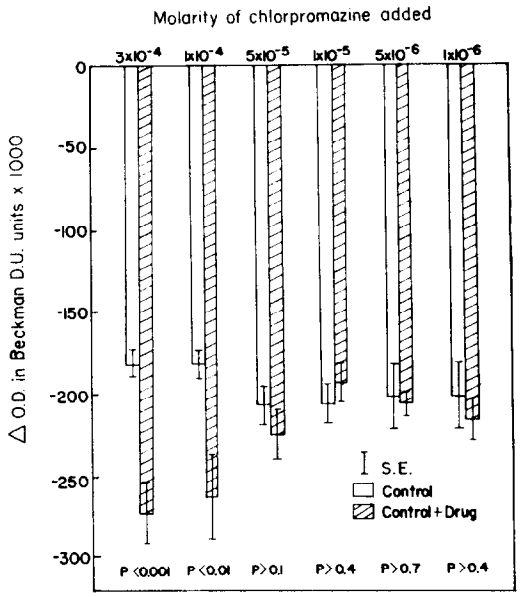

FIG. 3. Effect of chlorpromazine on the swelling of 0·06 ml of rat liver mitochondrial preparation suspended in 2·94 ml of 0·16 M KCl + 0·02 M Tris buffer. Conditions as in Fig. 1.

TABLE 3. EFFECT OF EXPOSURE TO CHLORPROMAZINE ON SUCROSE ENTRY, OPTICAL DENSITY, AND WATER CONTENT OF RAT LIVER MITOCHONDRIA SUSPENDED IN ISOTONIC SUCROSE

	Mitochondrial suspension without chlorpromazine		Mitochondrial suspension with 10^{-5} M chlorpromazine	Inhibition due to drug (%)	No. of animals
Optical density change (Beckman DU units × 1000)	−167 ± 22*	$P < 0.01$	−64 ± 17	64	7
Change in % mitochondrial pellet H_2O	6·5 ± 0·8	$P < 0.01$	1·8 ± 1·5	72	7
Mitochondrial sucrose entry mg/mg mitochondrial N	4·0 ± 0·6	$P < 0.01$	1·4 ± 0·6	66	7

* Standard error of the mean. P values were determined from the *t* test for small samples, and a level of 0·05 or less was considered significant.

All three parameters were measured on each of seven Wistar rats. Mitochondria from 1·0 g liver made up to 1 ml according to Schneider and Hogeboom;[7] 0·06 ml mitochondrial suspension added to 0·32 M sucrose–0·02 M Tris buffer, pH 7·4, containing 10^{-5} M chlorpromazine·HCl to make 3·0 ml total volume and spectrophotometric readings taken at 520 mμ for 10 min on the Beckman DU spectrophotometer using standard Beckman cuvets with a 1-cm light path.

Table 3 indicates that, when CPZ inhibits the O.D. fall of liver mitochondria suspended in isotonic sucrose at a concentration of 10^{-5} M it also inhibits to the same extent the entrance of sucrose and water into the mitochondrial pellet (64·0 to 72·0%) as measured by the methods outlined. Spectrophotometric data as well as both H_2O and sucrose entry were determined for each mitochondrial preparation in this study and are therefore directly comparable.

TABLE 4. EFFECT OF A 10-MIN EXPOSURE TO CHLORPROMAZINE SULFOXIDE ON OPTICAL DENSITY AND WATER CONTENT OF RAT LIVER MITOCHONDRIA SUSPENDED IN ISOTONIC SUCROSE

	Mitochondrial suspension without chlorpromazine sulfoxide		Mitochondrial suspension with 10^{-5} M chlorpromazine sulfoxide	No. of animals
Optical density change (Beckman DU units × 1000)	−126 ± 23*	P > 0·5	−147 ± 29	4
Change in % mitochondrial pellet H_2O	2·5 ± 1·9	P > 0·3	5·1 ± 2·3	4

* Standard error of the mean. P values calculated from *t* tests for small samples, and levels of 0·05 or less were considered significant. The same four Wistar rats were used to measure both optical density and mitochondrial pellet-water changes. Liver mitochondrial suspensions were prepared and tested as in Table 3.

Table 4 lists the results of experiments similar to those in Table 2 except that CPZ-sulfoxide at 10^{-5} M was present instead of CPZ. The O.D. drop noted at this concentration is not appreciably altered by the drug, nor is there any inhibition of the concomitant gain in mitochondrial pellet water. There is, instead, a tendency for mitochondrial water content to increase rather than fall in the presence of 10^{-5} M CPZ-SO.

TABLE 5. EFFECT OF EXPOSURE TO CHLORPROMAZINE ON OPTICAL DENSITY AND WATER CONTENT OF RAT LIVER MITOCHONDRIA SUSPENDED IN ISOTONIC KCL

	Mitochondrial suspension with chlorpromazine			Mitochondrial suspension with 10^{-5} M chlorpromazine	
Exposure:	10 min			10 min	
Optical density change (Beckman DU units × 1000)	(7) −141 ± 30*		P > 0·05	(7) −193 ± 5	
Exposure:	10 min	20 min		10 min	20 min
Change in % mitochondrial pellet H_2O	(7) 2·2 ± 1·2	(2) 6·6		(7) 3·4 ± 1·5	(2) 7·4

* Standard error of the mean. P values calculated from *t* tests for small samples, and levels of 0·05 or less were considered significant. Figures in parentheses represent the number of experiments performed. Liver mitochondria were prepared as in Table 3 but suspended in 0·160 M KCl–0·02 M Tris buffer, pH 7·4, for testing.

Table 5 shows that the failure of CPZ at 10^{-5} M to inhibit the O.D. drop of the mitochondrial suspension in 0·16 M KCl correlates with the failure to affect the concomitant entry of water. In fact, there is again a tendency to further swelling, rather than inhibition of swelling after a 10- or 20-min exposure of the mitochondria to KCl-Tris and 10^{-5} M CPZ.

DISCUSSSION

Because CPZ at 10^{-5} M is effective in inhibiting swelling of mitochondria without producing significant agglutination, this concentration was chosen to test for a possible correlation between spectrophotometric and mitochondrial water and sucrose changes. It was indeed found that the inhibition of swelling caused by the drug was accompanied by a decrease in the entry of H_2O into the mitochondrial pellet (Table 3), the decrease being 64 to 72% by both methods.

Similar experiments are now being carried out by the more direct water-entry measurements to determine whether CPZ is inhibitory at agglutinating concentrations or when other complicating factors enter the picture, such as the addition of a swelling agent with the drug.

For some time it has been known that when mitochondria are suspended in sucrose media, some sucrose enters the mitochondria.[11] Analysis of sucrose entry shows that 10^{-5} M CPZ inhibits the permeability of the mitochondria to both water and sucrose to the same degree that it inhibits mitochondrial particle swelling.

It is important that CPZ membrane permeability is not affected by CPZ-sulfoxide, an extremely weak tranquilizer, over a wide concentration range. Nor did this drug cause mitochondrial agglutination, even at 5×10^{-4} M. Whether this lack of effect is due to the fact that CPZ-sulfoxide is not adsorbed by mitochondria, as claimed by Strecker,[15] is at present being investigated.

The reason for the ineffectiveness of CPZ in inhibiting swelling when added to mitochondria suspended in isotonic KCl-Tris buffer instead of sucrose solution is not yet clear. It is possible that the ionic medium, but not sucrose, causes some change in the organelle membranes. That the mitochondria swell in KCl as much or more than in sucrose is obvious from the spectrophotometric and water entry data in Table 5. Also, almost as much CPZ is bound by mitochondria suspended in KCl as in sucrose (Table 2) when the drug concentration is initially 10^{-5} M. Furthermore, CPZ at 1×10^{-4} and 5×10^{-4} M causes agglutination when added to mitochondria suspended in KCl, indicating that drug adsorption also occurs at these concentrations, and this effect is at least as strong in the ionic medium as in sucrose (Figs. 1 and 3). The actual extent of drug adsorption at these high concentrations has not yet been tested but, surely, agglutination indicates a drug effect on the mitochondrial surface.

During the course of the mitochondrial experiments in sucrose and KCl, it was noticed that the swelling of the organelles often increased as the fresh mitochondria were stored over a 2- or 3-h period. This was the case especially when they were suspended in sucrose (Fig. 1) and it necessitated the inclusion of a control with every two Beckman cuvets containing CPZ. When the mitochondria were fresh, they swelled more when suspended in KCl; at the end of 2 to 3 h the swelling rate was approximately the same in both the ionic and nonionic media.

The present experimental results, as well as some to be reported subsequently using aerobic and anaerobic techniques and Na^+ and K^+ determinations on mitochondrial

preparations and on human erythrocytes, suggest that CPZ affects the *passive* transport of water and ions across biological membranes.

The technique of measuring mitochondrial swelling by following, over a definite time period, changes in optical density of a mitochondrial suspension is not without its pitfalls.

Mitochondria in isotonic sucrose or KCl are roughly spherical in shape and possess a diameter in the range of 1 to 5 microns.[16] Suspensions of particles of this size scatter light to a considerable degree. Furthermore, this light scatter is not equal in all directions; a large percentage consists of forward scatter.[17] According to the equations of Mie, scatter for such particles varies directly with the index of refraction and particle size.[17] Scatter at angles greater than 2 to 3° to the side of incident rays tends to cause an apparent increase in optical density because of loss of light reaching the photoelectric cell. Forward scatter within 2 to 3° of the incident rays, on the other hand, will reach the photoelectric cell and tend to lower optical density. The resultant effect depends on the ratio of forward scatter to total scatter. The entrance of water into mitochondria results in an increase in particle size and a decrease in index of refraction. The increase in particle size may increase forward scatter sufficiently to cause a lower O.D. The decrease in index of refraction also decreases total scatter so that, again, a diminution of O.D. results. However, at the concentrations of mitochondria usually employed in spectrophotometric experiments (0·1 to 0·2 mg mitochondrial N per 3 ml suspension), secondary scatter accompanying a change in particle size becomes significant and, in addition, its effects are unpredictable; it may appreciably alter the amount of forward scatter and therefore the O.D.

The possible agglutination of mitochondria offers additional complications. Extensive agglutination brings about a lowering of optical density by decreasing the number of particles. It may therefore camouflage the optical effects of a change in individual particle size of the mitochondria (either swelling or shrinkage).

Because these considerations sometimes made the interpretations of spectrophotometric data difficult, another more direct method of measuring water uptake by mitochondria was sought. That of Price *et al.*[8] was chosen because of its simplicity. For the same reason sucrose entry data were also undertaken.

Acknowledgements—We are grateful for the invaluable and expert aid given us in carrying out these experiments by Mrs. Sophie Morgan and Mr. Jorge Fernandez.

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