Studies *in vitro* on the Effects of 1H,2H,4H(5H)-Octafluorocyclohexane and 1H,4H(2H)-Nonafluorocyclohexane on Enzymes and Organelles

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A comparison has been made of the effect of 1H,2H,4H(5H)-octafluorocyclohexane, which is highly toxic (LD₅₀ 17 mg./kg. in rats), and of 1H,4H(2H)-nonafluorocyclohexane, which is relatively non-toxic (LD₅₀ > 440 mg./kg. in rats), on the respiration of rat liver homogenates and mitochondria *in vitro*. 1H,2H,4H(5H)-Octafluorocyclohexane strongly inhibited the respiration of both homogenates and mitochondria, but neither compound had any significant effect on glycolysis or on glutamate dehydrogenase or NADH-cytochrome *c* reductase activity. 1H,2H,4H(5H)-Octafluorocyclohexane, however, caused a very marked inhibition of cytochrome oxidase activity, causing an almost complete lesion in this region of the respiratory chain. 1H,4H(2H)-Nonafluorocyclohexane was without effect in this respect. A marked decrease in turbidity of mitochondrial suspensions at 520 nm. was caused by addition of both compounds; the effect being greater with 1H,2H,4H(5H)-octafluorocyclohexane. ATP, Mg²⁺ and bovine serum albumin did not reverse these changes. Mitochondrial adenosine triphosphatase activity was increased twofold by the toxic compound, but only slightly by the non-toxic compound. Electron-microscopic examination of mitochondria treated with 1H,2H,4H(5H)-octafluorocyclohexane revealed gross morphological damage, whereas the effect of 1H,4H(2H)-nonafluorocyclohexane appeared to be merely to cause swelling. The results obtained account, to some extent at any rate, for the toxic effects of 1H,2H,4H(5H)-octafluorocyclohexane.

During investigations into the synthesis of fluorinated cyclohexanes carried out in the Department of Chemistry, University of Birmingham, workers observed that some compounds caused undesirable effects such as slight giddiness. Subsequent study of the toxicities of polyfluorocyclohexanes showed a wide range of values for LD₅₀ in rats, ranging from 17 mg./kg. for OFCH* to 440 mg./kg. for NFCH. Since the compounds as a group are chemically inert, the only reported property being dehydrofluorination on treatment with strong alkali (Godsell, Stacey & Tatlow, 1958; Nield, Stephens & Tatlow, 1959), it was of interest to study the mechanism of their toxic action. The two compounds chosen for investigation were OFCH, which is very toxic (LD₅₀ 17 mg./kg.), and NFCH, which is relatively non-toxic (LD₅₀ > 440 mg./kg.). Studies of the metabolism *in vivo* of OFCH were not possible, but it was found that in the rabbit and rat NFCH was excreted in expired

air largely unchanged, only about 2% apparently undergoing dehydrofluorination (H. G. Bray & J. A. F. Wickramasinghe, unpublished work).

The present paper reports the effects *in vitro* of OFCH and NFCH on the metabolism of rat liver and their effect on the morphology of rat liver mitochondria.

MATERIALS AND METHODS

Animals. Adult (4–5 months) Wistar-strain rats weighing 200–250 g. were used. They were starved overnight before use. Most of the rats used in these experiments were female, but a few male rats were also used to exclude the possible effect of sex difference on the results.

Materials. ATP, ADP, AMP, NAD⁺, NADH, NADP⁺, cytochrome *c*, FDP, rotenone, antimycin A and all the crystalline enzyme preparations were bought from Sigma Chemical Co. (St Louis, Mo., U.S.A.). The other chemicals were analytical or reagent grade and purchased from British Drug Houses Ltd., Poole, Dorset. Glass-distilled water was used for preparation of all the solutions. NFCH and OFCH were synthesized in the Department of Chemistry, University of Birmingham, and generously supplied to us. These compounds are water-insoluble and so they were used as a solution in propylene glycol, which was found to have no effect on the enzyme systems studied.

* Abbreviations: OFCH, 1H,2H,4H(5H)-octafluorocyclohexane; NFCH, 1H,4H(2H)-nonafluorocyclohexane; TMPD, NNN'-tetramethyl-*p*-phenylenediamine; ATPase, adenosine triphosphatase.

Preparation of liver homogenates. The rats were killed by a blow on the head and bled through the nose. The livers were immediately removed and placed in ice-cold 0.25M-sucrose solution. All subsequent operations were carried out at 0–4°. Tissue homogenates (10% wet wt./vol.) were prepared in 0.25M-sucrose solution in a Potter–Elvehjem glass homogenizer fitted with a Teflon pestle.

Isolation of liver mitochondria. These were prepared according to the method of Johnson & Lardy (1967), which is a slightly modified version of the method of Schneider (1948). A 10% liver homogenate prepared as above was centrifuged at 600g for 10 min. The supernatant fraction was decanted and saved. The pellets containing whole cells, nuclei, tissue fragments and some mitochondria were washed and resuspended in the requisite volume of ice-cold 0.25M-sucrose. The resultant resuspended material was again centrifuged at 600g for 10 min. The supernatant fractions were combined with that obtained before and the final pellets were discarded. According to Johnson & Lardy (1967) this washing contributes not only to the final yield of mitochondria but also to their integrity, apparently by permitting the recovery of some of the larger mitochondria. The combined supernatants were centrifuged at 15000g for 5 min. The supernatant from this centrifugation was discarded along with any lightly packed pink material. The pellets were resuspended as the nuclear pellet was and centrifuged at 15000g for 5 min. This procedure of resuspension and centrifugation was repeated twice to ensure minimum microsomal contamination and the final pellet was suspended in 0.3M-sucrose to give a protein concentration of 10–12 mg. of protein/ml. and used for all the experiments recorded here. All centrifugations were carried out in a refrigerated MSE automatic Superspeed 50 centrifuge.

Measurement of uptake of oxygen. This was done manometrically in a Warburg apparatus (Braun model V85). Uptake of O₂ in whole liver homogenates was measured at 37°, and that of mitochondrial fractions at 25°.

For uptake of O₂ of whole homogenates the flasks contained the following materials, in a final volume of 3.0 ml.: 150 μmoles of phosphate buffer, pH 7.6, 50 μmoles of glucose, 15 μmoles of ATP (neutralized), 15 μmoles of MgCl₂ and 1 ml. of 10% homogenate. To investigate the effect of NFCH and OFCH, these were added in requisite concentration as given in Table 1. A control reaction mixture with only propylene glycol was also included in the experiment; 0.2 ml. of 30% KOH was added in the central well of all Warburg flasks.

In the determination of uptake of O₂ by mitochondrial fractions, the Warburg flasks contained, in a final volume of 3.0 ml.: 90 μmoles of potassium phosphate buffer, pH 7.4, 30 μmoles of sodium malonate, 15 μmoles of 2-oxoglutarate, 3 μmoles of EDTA, 3 μmoles of ADP, 3 μmoles of AMP, 60 μmoles of glucose, 15 μmoles of MgCl₂, 0.015 μmole of cytochrome *c*, 80–100 units of hexokinase and 0.5 ml. of mitochondrial suspension (3–4 mg. of protein/ml.). All the components of the reaction mixture were adjusted to pH 7.4 with KOH or NaOH. Hexokinase and MgCl₂ were added last, just before the addition of mitochondrial suspension, which was kept at 0°. NFCH or OFCH was added (5 μmoles/ml.) to note their effects and suitable controls with propylene glycol were simultaneously carried out. The central well of all Warburg flasks contained 0.2 ml. of 30% KOH and thermal equilibration was carried out for 10 min. as a routine procedure.

Pretreatment of mitochondria with NFCH and OFCH. This was done at room temperature (25°). Equal volumes of isolated mitochondrial preparation in 0.30M-sucrose were incubated for 15 min. with NFCH or OFCH (10 μmoles/ml.). One sample was left at 25° without any further additions, whereas another sample was incubated for the same time with only propylene glycol (0.1 ml.). Where necessary the pretreated mitochondria were centrifuged down and separated from the supernatant.

Enzyme assay procedures. All spectrophotometric assays were done at 25° in a Unicam SP.500 spectrophotometer with a 1 cm. light-path.

Glutamate dehydrogenase (EC 1.4.1.2). This was assayed according to the method of Schmidt (1963). Since the equilibrium lies in favour of amino acid formation, glutamate dehydrogenase was assayed by following the reverse reaction. The assay mixture contained, in a final volume of 3.0 ml.: 100 μmoles of tris-HCl buffer, pH 8.0, 0.15 μmole of NADH, 7.8 μmoles of EDTA, 4.5 μmoles of ammonium acetate, 24 μmoles of 2-oxoglutarate and 0.03 ml. of suitably diluted mitochondrial preparation. The reaction was started by the addition of 2-oxoglutarate. The oxidation of NADH to NAD⁺ was followed spectrophotometrically at 340 nm. Antimycin (1 μg./ml.) was added as a precautionary measure to eliminate possible reoxidation of the reduced nucleotide via the electron-transfer chain. The enzyme was also assayed in the presence of cholate (0.5 mg./mg. of mitochondrial protein). The detergent was added immediately before the enzyme was assayed. Glutamate dehydrogenase was assayed after preincubation of mitochondria with NFCH or OFCH. It was assayed both in the mitochondrial suspension and also in the resulting supernatant, after spinning down the preincubated mitochondria.

NADH-cytochrome *c* reductase (EC 1.6.2.1). The method of Mackler & Green (1956) was used to assay this enzyme. The reduction of cytochrome *c* was followed spectrophotometrically at 550 nm. The assay mixture contained, in a final volume of 3.0 ml.: 120 μmoles of potassium phosphate buffer, pH 7.6, 0.3 μmole of NADH, 0.15 μmole of cytochrome *c*, 1 μmole of KCN and a suitable amount of mitochondrial suspension to give optimum activity. The enzyme was added last to start the reaction. Where mentioned, antimycin was used at a concentration of 1 μg./ml. and rotenone was used at a concentration of 3 μg./ml. of assay mixture. The enzyme was assayed after pretreatment of the mitochondria with NFCH or OFCH. Suitable controls were simultaneously carried out.

Cytochrome oxidase (EC 1.9.3.1). Mitochondrial cytochrome oxidase activity was assayed manometrically at 30° according to the method of Griffiths & Wharton (1961), which is essentially the same as described by Slater (1949). The Warburg flasks contained, in a final volume of 3.0 ml.: 60 μmoles of potassium phosphate buffer, pH 7.3, 60 μmoles of ascorbate (freshly neutralized to pH 6.6), 3 μmoles of EDTA, 0.3 μmole of cytochrome *c* and 0.6 ml. of suitably diluted mitochondrial suspension (5–6 mg. of protein/flask). Where indicated, 10 μmoles of OFCH or NFCH were added to the assay medium. Ascorbate was initially placed in the side arm and tipped in after 6 min. thermal equilibration. Readings were commenced 1 min. after mixing. Cytochrome oxidase activity was also assayed in mitochondria pretreated with NFCH or OFCH.

Assay of TMPD-ascorbate oxidation system. Jacobs (1960) has reported that P:O ratios approaching unity for

the cytochrome oxidase step can be obtained by using TMPD to reduce cytochrome *c*. In this system, TMPD acts as the artificial electron carrier between ascorbate and the cytochrome *c* region of the respiratory chain. The system was assayed manometrically, according to the method of Sanadi & Jacobs (1967). The Warburg flasks contained, in a final volume of 3.0 ml.: 60 μ moles of tris-HCl buffer, pH 7.5, 30 μ moles of potassium phosphate buffer, pH 7.5, 30 μ moles of ascorbate (freshly neutralized), 20 μ moles of $MgCl_2$, 3 μ moles of TMPD, 5 μ moles of rotenone and 0.6 ml. of mitochondrial suspension (5–6 mg. of protein/flask). Where indicated, 10 μ moles of NFCH or OFCH were added per flask and suitable controls were carried out as usual. The side arms of the Warburg flasks contained 15 μ moles of ATP, pH 7.5, 100 μ moles of glucose and 0.05 ml. of hexokinase (10 mg./ml.). As we were not measuring phosphorylation, the TMPD-ascorbate oxidation system was equally effective when 10 μ moles of ADP were added to the reaction mixture, instead of using ATP, glucose and hexokinase for continuous ADP regeneration. The central wells of all Warburg flasks contained 0.2 ml. of 30% KOH. After thermal equilibration for 6–7 min. the side-arm contents were tipped into the main compartments of the flasks and uptake of O_2 was measured as usual. This assay was also carried out with pretreated mitochondria.

Adenosine triphosphatase (EC 3.6.1.3). This enzyme was also assayed after pretreatment of the mitochondria with NFCH or OFCH. As usual, suitable controls were simultaneously carried out. The assay procedure to determine mitochondrial ATPase was principally based on the method of Lardy & Wellman (1953) and Potter, Siekevitz & Simonson (1953). However, the final concentrations of different compounds in the reaction medium were modified according to Myers & Slater (1957) to produce optimum activity at pH 7.4. The reaction mixture contained, in a final volume of 1.0 ml.: 50 μ moles of tris-HCl buffer, pH 7.4, 75 μ moles of KCl, 50 μ moles of sucrose, 2 μ moles of ATP, 1 μ mol of $MgCl_2$ and mitochondrial suspension (0.1–0.2 mg. of protein). 2,4-Dinitrophenol (0.1 mM) was added to all assay systems.

Mitochondrial ATPase activity was measured immediately after pretreatment with NFCH or OFCH. For measurement of ATPase the mitochondria were incubated in the reaction mixture for 15 min. at 25°. The reaction was stopped by trichloroacetic acid and the precipitated protein was removed by centrifugation. Inorganic phosphate was determined in the supernatant by the method of Fiske & Subbarow as modified by Sumner (1944).

Measurement of mitochondrial swelling. This was measured according to Hunter & Smith (1967) by following the decrease in turbidity at 520 nm. Suspensions of isolated mitochondria were diluted in 0.3 M-sucrose to give an extinction reading between 0.6 and 0.7. In a final volume of 3.0 ml., 10 μ moles of NFCH or OFCH were added, quickly mixed by stirring with a glass rod and the decrease in E_{520} was followed. Owing to the insoluble character of these compounds, 10 μ moles/3 ml. was the highest concentration that could be used in experiments involving spectrophotometric measurements. The effects of lower concentrations were also investigated. Suitable controls, i.e. with and without propylene glycol, were simultaneously carried out.

Reversal of mitochondrial swelling. Mitochondrial swelling was induced by addition of NFCH or OFCH as above. ATP (5 μ moles/ml.), $MgCl_2$ (5 μ moles/ml.) and bovine serum

albumin (2.7 mg./ml.) were added to this medium and any reversal of swelling, which would be indicated by an increase in E_{520} , was followed spectrophotometrically.

Determination of protein. This was estimated spectrophotometrically, according to Lowry, Rosebrough, Farr & Randall (1951), caseinogen being used as the standard.

Electron microscopy. Mitochondria were fixed in 2% glutaraldehyde in 0.1 M-phosphate buffer, pH 7.4, for 2 hr. Samples were post-fixed in 1% osmium tetroxide, pH 7.4, dried and embedded in Araldite. Thin sections were post-stained with uranyl acetate (25% in methanol) and examined with an A.E.I. EM 6 electron microscope.

Effects of NFCH and OFCH in vitro on mitochondrial structure. Mitochondria were prepared as described and finally suspended in 0.3 M-sucrose, pH 7.4. Equal volumes of this mitochondrial suspension were pretreated with NFCH and OFCH. Suitable controls were simultaneously set up. After incubation at room temperature the mitochondria were quickly centrifuged and collected as small pellets. They were then fixed for 2 hr. in 2% glutaraldehyde, pH 7.4. The glutaraldehyde was then drained off and fixed mitochondrial pellets were washed in 0.1 M-phosphate, pH 7.4, and kept overnight in cold buffer and later prepared for electron micrographs.

Effects of NFCH and OFCH in vivo on mitochondrial structure. These were studied by preparing electron micrographs of mitochondria isolated from the livers of rats dosed with 7 mg. of either OFCH or NFCH. The former compound proved fatal in 20–25 min. and the rat given NFCH was killed at the end of the same period after dosage. The livers were removed immediately and mitochondria prepared for electron microscopy as described above.

RESULTS

Effect of NFCH and OFCH on respiration of whole homogenates and mitochondria. Table 1 shows the effect of NFCH and OFCH on the uptake of oxygen by whole liver homogenate. Propylene glycol, the solvent in which these compounds were dissolved,

Table 1. *Effects of NFCH and OFCH on rat liver respiration*

Experimental details are given in the text.

Assay system	Initial rate*	Inhibition† (%)
1 Whole homogenate respiration		
(a) Without additions	40	—
(b) With propylene glycol (0.3 ml.)	39	—
(c) With NFCH (10 μ moles/ml.)	38	3
(d) With OFCH (10 μ moles/ml.)	5	87
2 Mitochondrial respiration		
(a) Without additions	13	—
(b) With propylene glycol (0.2 ml.)	11	—
(c) With NFCH (5 μ moles/ml.)	10	9
(d) With OFCH (5 μ moles/ml.)	0	100

* Uptake of O_2 (μ l./10 min.).

† Calculated in terms of values from controls containing propylene glycol.

did not affect the rate of respiration. It can be seen from this table that $10\mu\text{moles}$ of OFCH/ml. markedly inhibited uptake of oxygen by liver homogenate, whereas NFCH at this concentration did not affect the respiration appreciably. Owing to the insoluble nature of these compounds, higher concentrations could not be used in experiments *in vitro*. Table 1 also shows the effect of $5\mu\text{moles}$ of NFCH or OFCH/ml. on mitochondrial respiration. OFCH completely inhibited the mitochondrial uptake of oxygen whereas the less toxic NFCH had only a very slight effect on mitochondrial respiration. The effects of further lower concentrations of OFCH ($0.5\text{--}1.0\mu\text{mole/ml.}$) were also studied and even the lowest concentration inhibited mitochondrial uptake of oxygen to an extent of 30–40%. NFCH did not affect mitochondrial respiration at these lower concentrations. Mitochondrial respiration seemed to be very sensitive to the inhibitory effect of OFCH. Propylene glycol had a slight inhibitory effect on mitochondrial respiration. Oxidation of succinate was similarly affected by OFCH, but NFCH had no significant effect.

Effect of NFCH and OFCH on mitochondrial swelling. The markedly different effects of these compounds on mitochondrial respiration focused our attention on this subcellular fraction and it was thought worth while to study their effect on mitochondrial morphology. When NFCH or OFCH was added to suitably diluted mitochondrial suspensions, a very marked decrease (60–70%) in E_{520} was noted. From Fig. 1 it can be seen that there was a very rapid and sharp decrease in E_{520} with OFCH ($5\mu\text{moles/ml.}$) within 10–15 min. and this was much smaller with NFCH, being only 30–40% of that produced by the more toxic OFCH. Propylene glycol did not affect E_{520} under similar conditions. When the protein content of the supernatants (separated after induction of mitochondrial swelling with OFCH and NFCH) was determined, it showed a marked increase with OFCH and also to a lesser degree with NFCH. As mentioned above, there was an approximately fourfold increase (from 0.24 to 0.98 mg./ml.) in the supernatant separated from OFCH-treated mitochondria, which represented about 40–50% of the total mitochondrial protein added originally, whereas the increase in protein content after treatment with NFCH was appreciably less (from 0.24 to 0.60 mg./ml.). Protein content of the sample treated with propylene glycol alone did not show an increase; in fact the value was consistently found to be slightly less than in the normal control. This increase was not due to contamination of a lysosomal protein, as was shown by the absence of a lysosomal marker enzyme, e.g. acid phosphatase, from the supernatants.

Effect on enzymes. When a standard mitochondrial enzyme like glutamate dehydrogenase was assayed,

OFCH or NFCH did not have any inhibitory effect, whether added directly to the assay medium or in mitochondria pretreated with these compounds. It was in fact observed that glutamate dehydrogenase activity was stimulated in pretreated mitochondria, NFCH increasing the initial rate by 20% and OFCH by 40%. This stimulation of the enzyme could be due to mitochondrial swelling or damage induced by these compounds. When, however, the assay was performed in the presence of cholate, there was no stimulation of the enzyme activity and the compounds did not have any adverse effects either. It has been mentioned that the protein content of the supernatants separated from pretreated mitochondria showed a marked increase and it was thought worth while to assay glutamate dehydrogenase in these supernatants. Pretreated mitochondria were separated by centrifugation and when glutamate dehydrogenase was assayed separately, both in the pretreated mitochondria (resuspended in 0.3M-sucrose) and in the supernatant, the whole activity could be recovered from the mitochondrial fractions and the supernatants did not have any detectable activity. This showed that even after mitochondrial swelling or damage induced by these compounds (as evidenced by electron micrographs and turbidity measurements) an intramitochondrial enzyme like glutamate dehydrogenase was still completely retained in the mitochondria.

It is well known that any agent that induces mitochondrial swelling or damage consequently

Fig. 1. Effects of OFCH and NFCH on mitochondrial swelling. (1) Without any additions; (2) with propylene glycol; (3) with $10\mu\text{moles}$ of NFCH/3ml.; (4) with $10\mu\text{moles}$ of OFCH/3ml. Experimental details are given in the text.

stimulates mitochondrial ATPase. When mitochondrial ATPase was assayed after pretreatment of mitochondria with OFCH there was a twofold increase in ATPase activity (Table 2). NFCH-treated mitochondria also showed some increase in ATPase activity, but this was much less than the increase in OFCH-treated mitochondria. Propylene glycol did not have any stimulatory effect on mitochondrial ATPase; on the contrary the value was found to be consistently slightly lower than the normal value.

To study the effects of OFCH and NFCH on the electron-transfer chain, NADH-cytochrome *c* reductase activity was assayed. The initial rate of the enzyme reaction was decreased by about 50% in the presence of OFCH (directly added to the assay medium) or in OFCH-treated mitochondria. In contrast, the less toxic NFCH did not affect the activity of this enzyme. The inhibition due to OFCH was only partial and the net decrease in E_{550} was the same as the normal value and it seemed that only the rate has been slowed, without any decrease in the total amount of product formed. It was significant that specific inhibitors like rotenone or antimycin inhibited this enzyme in all the differently treated mitochondria, showing the presence of an undamaged antimycin-sensitive segment of the respiratory chain.

When the respiratory chain cytochrome *b* → cytochrome *c* (antimycin-sensitive) was found to be undamaged in the presence of OFCH, it was thought that the lesion might be at a point beyond cytochrome *c* of the respiratory chain. To check this possibility mitochondrial cytochrome oxidase activity was measured in the presence of NFCH and OFCH. It can be seen from Fig. 2 that OFCH caused a very marked inhibition of cytochrome oxidase activity and that there was hardly any uptake of oxygen after 30 min. In contrast, NFCH or propylene glycol did not affect this enzyme. The results plotted in Fig. 2 show the effect of directly added OFCH or NFCH: there was no significant difference in the nature or degree of inhibition produced in the mitochondria pretreated with OFCH. Propylene glycol or NFCH did not have any effect even after pretreatment. This clearly indicated that OFCH caused a major lesion in the cytochrome oxidase region of the respiratory chain, which could account for the previously observed inhibitory effects of OFCH on mitochondrial respiration (Table 1).

Since OFCH was found to be a potent inhibitor of cytochrome oxidase, it was thought that assay of TMPD-ascorbate oxidase would provide further supporting evidence regarding the lesion in this region of the respiratory chain. It can be seen from Fig. 3 that OFCH did inhibit uptake of oxygen via TMPD-ascorbate oxidation and the degree of

inhibition was similar to that of cytochrome oxidase. Results given in Figs. 2 and 3 clearly indicate an almost complete lesion of the respiratory chain in the cytochrome oxidase region. NFCH did not have any inhibitory effect and, as can be seen from Fig. 3, the rate of uptake of oxygen was slightly stimulated in the presence of NFCH.

It would be worth while to add here that neither of these compounds had any significant inhibitory effect on 'glycolysis'. This was measured both manometrically, in terms of production of carbon dioxide, and spectrophotometrically, by assaying individual glycolytic enzymes, e.g. hexokinase (EC 2.7.1.1), glucose phosphate isomerase (EC 5.3.1.9), triose phosphate isomerase (EC 5.3.1.1),

Table 2. *Effect of OFCH and NFCH on mitochondrial adenosine triphosphatase activity*

Reaction medium and conditions of assay are given in the text.

Assay system	ATPase activity (μ moles of P_i /mg. of protein/hr.)
Without additions	12.3
+ Propylene glycol	10.2
+ NFCH + propylene glycol	13.6
+ OFCH + propylene glycol	21.0

Fig. 2. Effects of OFCH and NFCH on cytochrome oxidase. (1) Without any additions; (2) with propylene glycol; (3) with 5 μ moles of NFCH/ml.; (4) with 5 μ moles of OFCH/ml. Experimental details are given in the text.

glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12), lactate dehydrogenase (EC 1.1.1.27) etc.

Effects of NFCH and OFCH in vitro on mitochondrial structure. Plate 1 shows the effect of NFCH and OFCH on mitochondrial structure. It can be seen that propylene glycol did not have any significant effect on mitochondrial size or conformation. Electron micrographs clearly show the swelling and damaging effects of OFCH, where the cristae and inner matrix are not visibly differentiated from the boundary membranes and appear as hollow vesicles. NFCH-treated mitochondria show a considerable degree of swelling; the cristae have unfolded and disappeared but still retain the gross morphology of control mitochondria suspended in 0.3M-sucrose, as evidenced by the granular appearance of the matrix. These electron micrographs clearly demonstrate the drastic morphological changes induced by OFCH, whereas the effect of NFCH seems to be much less severe.

Effects of NFCH and OFCH in vivo on mitochondrial structure. Effects *in vivo* were naturally not expected to be as drastic as results *in vitro*. It is understandable that in experiments *in vivo*, where the compounds were given by stomach tube, the possibility of penetration of these compounds within such a short time (20–25 min.) is less. Visible morphological changes would therefore be expected to be less than under conditions *in vitro*. But even then, as can be seen from Plate 2, in the mitochondria isolated from an OFCH-fed animal the outer membranes seem to have swollen and separated from the inner membranes and show a definite tendency to rupture, whereas the inner membranes seem to be more densely packed and contracted. The mitochondria isolated from an NFCH-fed animal did not show such changes and resembled a normal mitochondrial preparation isolated in 0.25M-sucrose. It is noteworthy that there were some changes *in vivo*, even when the OFCH-fed animal had died within a short period of 20–25 min.

DISCUSSION

Results obtained *in vitro* so far clearly indicate some significant effects of OFCH, which seem to have a direct relationship to its toxic effects *in vivo*.

The present experiments provide results that may be classified as (1) enzymological and (2) morphological.

Enzymological effects of OFCH and NFCH. Experiments done so far show that the less-toxic compound, NFCH, had no significant effect on the different enzyme systems involved in respiration. In contrast, OFCH had a striking inhibitory effect on mitochondrial respiration and also on respiration in whole-tissue homogenates. These inhibitory

effects were produced within 10–15 min., which suggested a rapid penetration of the compound through cellular membrane(s). The inhibitory effect of OFCH on cytochrome oxidase is particularly

Fig. 3. Effects of OFCH and NFCH on TMPD-ascorbate oxidation system. (1) Without any additions; (2) with propylene glycol; (3) with 5 μ moles of NFCH/ml.; (4) with 5 μ moles of OFCH/ml. Experimental details are given in the text.

Fig. 4. Irreversible nature of mitochondrial swelling. (1) Without any additions; (2) with propylene glycol; (3) with 10 μ moles of NFCH/3ml.; (4) with 10 μ moles of OFCH/3ml. Experimental details are given in the text. BSA, Bovine serum albumin.

interesting and worth further attention. It is also noteworthy that the effect of OFCH seemed to be specifically on the cytochrome oxidase region of the respiratory chain, whereas the antimycin- or rotenone-sensitive segment of the respiratory chain remained virtually undamaged after pretreatment with OFCH. The nature of inhibition produced by OFCH seemed to be comparable with that of cyanide. It is likely that phosphorylation at sites I and II (i.e. antimycin-sensitive segment of the respiratory chain) may be unimpaired, since it has already been observed that the antimycin-sensitive part of the respiratory chain itself is not damaged by OFCH. Another noteworthy point which emerged during this investigation is that neither of these compounds affected 'glycolysis'.

Apart from the inhibitory effects of OFCH on mitochondrial respiration and the cytochrome oxidase region of the respiratory chain, the results obtained from studying ATPase and glutamate dehydrogenase are also interesting. It was quite puzzling to find that glutamate dehydrogenase activity was completely retained in the particulate fraction (after pretreatment with OFCH or NFCH) and that there was no detectable activity in the supernatant, although there was an appreciable increase in the protein content of the supernatant obtained after pretreatment. Other mitochondrial dehydrogenases also need to be studied from this point of view. The observed increase in the ATPase activity after pretreatment with OFCH and NFCH provides additional support to the morphological results reported in this paper.

Morphological effects of OFCH and NFCH. The morphological changes that were noted during the course of this investigation provide an interesting aspect of the problem and merit more detailed study. So far, morphological studies have been restricted to intact mitochondria and examination of further sub-fractions after pretreatment with these compounds would enable us to look into the degree and nature of changes induced in mitochondrial ultrastructure.

The marked decrease in E_{520} observed after addition of OFCH and NFCH indicated mitochondrial swelling or damage. This was subsequently found to be irreversible in that ATP, Mg^{2+} and bovine serum albumin did not reverse these changes (Fig. 4). This is unlike most of the different types of large-amplitude mitochondrial swelling reported so far. While discussing the morphological changes induced by these compounds, it is necessary to bear in mind that only NFCH seemed to cause a swelling of the mitochondria, whereas the changes induced by OFCH seemed to be quite different in nature (Plate 1). The mitochondrial swelling or decrease in E_{520} reported here is also different from the various other types of large-amplitude swelling reported so far, as the

medium used was simply 0.30M-sucrose, without any other added ions or energy source. All the various types of large-amplitude mitochondrial swelling reported so far indicate the role of mitochondrial thiols (Lehninger, 1962), which are associated with oxidative phosphorylation, electron transport and ATPase activity, processes intimately related with mitochondrial swelling and contraction (Fluharty & Sanadi, 1962; Chappell & Greville, 1963; Cooper, 1960). In contrast, mitochondrial swelling or changes induced by OFCH and NFCH are not energy-linked and are not blocked by sucrose or respiratory inhibitors as reported for other types of swelling by Lehninger (1962). Thus the morphological changes induced by NFCH and OFCH seem to be a more direct effect of these compounds on mitochondrial structure and probably have no relationship with their effects on respiration.

The electron-microscopic results at this stage do not permit us to say anything more than what the pictures show. It is difficult to say at this stage whether the hollow vesicles (Plate 1) produced as a result of treatment with OFCH have single- or double-membrane structures.

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