MODE OF EFFECT OF OSTRUTHIN, A PHENOLIC COUMARIN ON RESPIRATION AND OXIDATIVE PHOSPHORYLATION OF RAT LIVER MITOCHONDRIA*

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Ostruthin (6-geranyl-7-hydroxycoumarin) in concentrations of 5—30 nmol/mg protein behaves as an uncoupler. It eliminates the respiratory control in liver mitochondria, decreases the ratio of ADP/O uptake, and stimulates ATPase activity. It also acts as an inhibitor of the exchange reaction between ^{32}Pi and ATP. Higher concentrations induce the swelling of mitochondria, bring about changes in the permeability of the membrane, and significantly alter the ultrastructure of mitochondria. A considerable quantity of mitochondrial proteins and a part of the phosphorus compounds are released into solution during this process. The investigation of the distribution of typical enzymes of membranes and matrix (monoamine oxidase, cytochrome oxidase, succinate dehydrogenase, and malate dehydrogenase) has shown that the mitochondrial components released come from matrix. Ostruthin concentrations higher than 50 nmol/mg protein cause fragmentation of membranes observable in ultrathin slices in the electron microscope. The fragmentation of both the inner and the outer mitochondrial membrane was demonstrated by the method of freeze etching.

In earlier studies of the effect of natural coumarins on bacteria[1], yeast[2], and oxidation reactions in sarcosomal fragments[3] the high inhibitory potency of 6-geranyl-7-hydroxycoumarin (ostruthin) has been demonstrated. The effect of ostruthin on yeast manifested itself by an increased ratio of glucose catabolized anaerobically to glucose catabolized aerobically and by a decrease of esterification of inorganic phosphate[4]. The fact that alcoholic fermentation remains unaltered in the presence of oxygen shows that ostruthin interferes with processes involved in energy transformation and conservation. In this study we made an effort to determine whether ostruthin may be classified as an uncoupler of the phenolic type[5,6]. A part of the results has been presented in preliminary form[7].

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EXPERIMENTAL

Liver mitochondria were prepared from male rats by the method of Schneider and Hogeboom modified by Nelson[8]. They were washed twice with 30 ml of 0·25M sucrose and suspended in a mixture of 0·25M sucrose and 0·2 mM-EDTA adjusted to pH 7·4 by Tris-HCl buffer. The submitochondrial particles were prepared by 15 min sonication of a subcooled rat liver mitochondria suspension.[9]

The rate of oxygen uptake was measured amperometrically at 25°C by a Clark electrode. The standard reaction mixture contained 188 mM sucrose, 40 mM-Tris-HCl buffer, pH 7·4, 0·15 mM-EDTA, 2·5 mM-$MgSO_4$, 1·5 mM potassium phosphate, and the mitochondria (1—2 mg of proteins); the total volume was 2 ml. The substrates were added in a volume of 10 µl, their final concentration was 6 mM. Other compounds (ostruthin, methyl ostruthin, geraniol) were dissolved in ethanol and added in a volume of 5—10 µl.

The ATPase activity was measured in terms of inorganic phosphate released in a standard reaction mixture lacking potassium phosphate with 1·3 mg of mitochondrial protein. The reaction was triggered by the addition of 3·6 nmol of ATP and of varying ostruthin concentrations. The incubation was allowed to proceed 10 min at 30°C. The total volume of the reaction mixture was 1·8 ml.

The $^{32}P_i$-ATP *exchange* reaction was carried out in 1 ml of a medium containing 1·6 mg of mitochondrial protein and 100 000 cpm of $^{32}P_i$. The composition of the reaction mixture and the extraction procedure were those described by Nielsen and Lehninger[10].

The swelling of mitochondria was measured in terms of absorbance decrease at 546 nm. The measurements were made in Unicam SP 1800 or Cary 118 recording spectrophotometer in a cell thermostated at 25°C. The reaction medium contained 150 mM ammonium phosphate or 0·25M sucrose, 5 mM-Tris HCl (pH 7·4), 0·2 mM-EDTA, and mitochondria in quantities sufficient to keep the absorbance at 546 nm between 0·5 and 0·8. The total volume was 3 ml. When an energy source was present (100 mM ammonium or potassium succinate), 0·72 µg of antimycin A and 0·45 µg of rotenone was added to the reaction medium.

The determination of the protein content of the sediment and supernatant of the mitochondrial suspension exposed to ostruthin was carried out by the modified biuret method[11]. The phosphorus content was determined after conversion to inorganic phosphate by the method of Chen and coworkers[12].

The enzyme activities of the sediment and supernatant of the mitochondrial suspension were determined from the change in absorbance in Cary 118 recording spectrophotometer at room temperature. The activity of monoamine oxidase was measured at 250 nm in a medium composed of: 1·65 ml of 0·1M phosphate buffer, pH 7·6, 0·1 ml of 96% ethanol, 0·2 ml of 0·1M benzylamine, and 10—50 µl of sample. The activity of cytochrome oxidase was measured at 550 nm in a medium containing 1·65 ml of 0·1M phosphate buffer, pH 7·4, 0·3 ml of 0·1M ferrocytochrome c, and 10—50 µl of sample. In experiments with detergents 0·1 ml of 12% Tween 40 or 50 µl of 4% sodium deoxycholate in phosphate buffer were added. The activity of succinate dehydrogenase was measured at 600 nm using phenazine methosulfate and dichlorophenolindophenol[13]. The activity of malate dehydrogenase was measured at 340 nm in a mixture containing 1·6 ml of 0·12M glycine buffer, pH 10, 0·3 ml of 1M-L-malate, 50—80 µl of sample, and 50—80 µl of 0·0275M-NAD^+.

Electron microscopy of ultrathin slices. The samples bound for fixation were withdrawn directly from the spectrophotometer cells (0·1 ml of the medium) after measurement of the swelling. Fixation was effected in a ten-fold volume of 1% glutaraldehyde, 30 min at room temperature.

The washed sample was exposed to 1% osmic acid for 1 h. The mitochondria were dehydrated in acetone and the samples were embedded in Durcupan. In several instances the objects were suspended in 1% agar after the fixation. Ultrathin slices (500—600 Å) were made in a Reichert ultramicrotome. The slices were contrasted by lead(II) — citrate according to Reynolds[14]. The freeze-etching replicas were made in the apparatus manufactured by Balzers. The mitochondria were fixed prior to the freezing 30 min in 1% glutaraldehyde and transferred to 20% glycerol for 30 min. The ultrathin slices and the replicas were scanned in Tesla BS 242 and 413 electron microscopes.

RESULTS

Ostruthin concentrations considerably lower than those inhibiting the activity of succinate oxidase of mitochondrial fragments stimulate the ATPase activity and respiration in state 4 (Table I). The effect of ostruthin depends on its ratio to the mitochondrial protein. The RC-values (4·16 and 5·3 with succinate and β-hydroxybutyrate as substrates, respectively) markedly decrease in the presence of even 5 nmol of ostruthin per mg of protein and drop to the value of 1 at 20 – 25 nmol of ostruthin per mg of protein. The ratio of ADP/O uptake decreased with the succinate from a value of 1·67 to 0·27 in the presence of 17 nmol of ostruthin per mg of protein.

In the concentration range of 10 – 30 nmol/mg of mitochondrial protein ostruthin behaves as a typical uncoupler. It also inhibits the $^{32}P_i$-ATP exchange reaction (50% inhibition at 22 nmol/mg protein). It may be assumed that the uncoupling effect of ostruthin is the result of its phenolic character. The uncoupling effect disappears after methylation of the phenolic hydroxyl and can be prevented by bovine serum albumin[15]. This protein was demonstrated in earlier studies to be an efficient reactivator of succinate dehydrogenase (of sarcosomal fragments) inhibited by ostruthin[3]. Fig. 1 shows the effect of various ostruthin concentrations on the oxidation of succinate by liver mitochondria in state 4. It can be seen that low albumin con-

TABLE I

Action of Ostruthin on Oxidative Phosphorylation in Rat Liver Mitochondria

The conditions of the measurement are described under Experimental.

Ostruthin nmol/mg protein	Oxygen uptake µgatom O/min g protein				ATPase activity nmol P_i/min. g protein
	succinate		β-hydroxybutyrate		
	state 3	state 4	state 3	state 4	
—	71	17	43	8	28
24	95	92	46	39	55

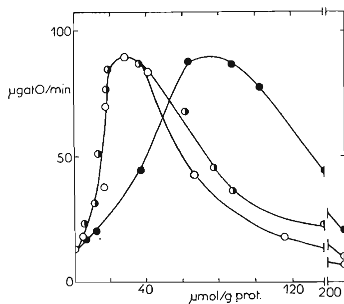

FIG. 1

Uncoupling Effect of Ostruthin on Rat Liver Mitochondria

The conditions of the measurement and the reaction medium are described under Experimental. Ostruthin as given, the values of oxygen uptake are based on g of protein. Quantity of albumin in reaction mixture (nmol): ○ 0; ◑ 10; ● 40.

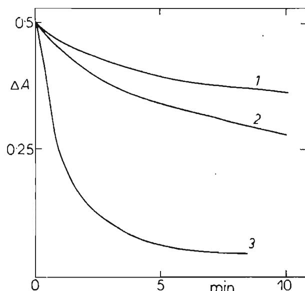

FIG. 2

Time Profile of Swelling of Mitochondria Induced by Ostruthin

The conditions of the measurement as described. Ostruthin concentration (μmol/g protein): *1* 31; *2* 47; *3* 62.

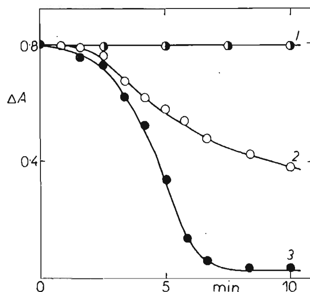

FIG. 3

Swelling of Mitochondria Induced by Ostruthin *3*, Methylostruthin *2*, and Geraniol *1*

The conditions of the measurement as described. The concentrations of the compounds were gradually raised by 25 nmol in 50-s intervals.

centrations do not affect the stimulation of respiration brought about by ostruthin. When the ratio of ostruthin to albumin was approximately equimolar, albumin decreased the inhibitory effect of ostruthin on oxidases. When the quantity of albumin is double the quantity of ostruthin, the stimulation of respiration requires higher ostruthin concentrations. Even in this case, however, a maximal "uncoupling effect" can be achieved. By contrast, the original inhibitory effect of higher ostruthin concentrations on succinate oxidase cannot be achieved in the presence of albumin. A perfectly analogous behavior was observed with liver mitochondria oxidizing β-hydroxybutyrate.

Ostruthin concentrations which caused the uncoupling of oxidative phosphorylation (from 10 nmol/mg protein up) stimulated the phosphate-induced swelling of mitochondria in an ammonium succinate medium. This stimulation, however, cannot be ascribed to the effect of ostruthin on succinate or phosphate transport into the mitochondria since a similar effect showed ostruthin also in the presence of an impermeable cation. The swelling is independent of energy supply and occurs also in the presence of antimycin A and rotenone during inhibited respiration. Such an effect is being ascribed to a so-called passive swelling usually suppressed by the opposite osmotic pressure, *i.e.* in a sucrose solution. Ostruthin brings about the swelling of mitochondria even in 0·25M sucrose. Its time profile as a function of ostruthin concentration is shown in Fig. 2.

We also examined whether the uncoupling effect is related to the swelling of mitochondria and tested the effect of methylated ostruthin and geraniol from this viewpoint. Fig. 3 shows the swelling of mitochondria in a sucrose medium, induced by successive additions of ostruthin, its methyl ether, and by geraniol alone. The methylation of the phenolic hydroxyl of ostruthin slightly weakens yet not eliminates its effect on mitochondrial swelling. Comparable concentrations of geraniol itself are without effect on the mitochondria. Likewise, 7-hydroxycoumarin has no effect on the mitochondria. The swelling can be brought about also in the absence of the free phenolic hydroxyl. The swelling depends on the presence of the hydrophobic geranyl chain and a more polar substituent on the coumarin ring. The structure requirements are the same as those necessary for the inhibition of respiration. The induction of the swelling caused by ostruthin and methylostruthin indicated that both compounds altered the permeability of the membrane. Unenergized swelling takes place also in the sucrose medium in the presence of an impermeable ion (potassium ion). This shows that the changes in membrane permeability are nonspecific and independent of the proton transport[16]. The impairment of the permeable barrier is such that it leads to the release of mitochondrial components. As shown in Table II a considerable quantity of proteins is released into the solution from mitochondria during their interaction with ostruthin. The quantity of "solubilized" proteins increases proportionally to ostruthin concentrations. The values given in Table II show that more than 60% of mitochondrial proteins are released into solution

if the ratio is 75 nmol of ostruthin per mg of protein. Together with the proteins a part of the phosphorus compounds are also released into solution by the effect of ostruthin (Table III). Their quantity is markedly lower than the quantity of the

TABLE II

Release of Mitochondrial Proteins by Ostruthin

The mitochondrial preparation (2 mg of proteins) was incubated with ostruthin 15 min in 1 ml of 0·25M sucrose at 25°C. Ostruthin was added as ethanolic solution; the concentration of ethanol was 1—2·5%. The mixture after incubation was centrifuged 10 min at 20 000*g* and both the supernatant and the sediment were analyzed for proteins.

Ostruthin nmol/mg protein	Protein, mg		Found %
	sediment	supernatant	
0[a]	1·70	0·14	92
27	1·35	0·59	97
54	1·00	0·93	96
75	0·70	1·12	91

[a] Contains 2·5% ethanol.

TABLE III

Effect of Ostruthin on the Release of Phosphorus Compounds from Rat Liver Mitochondria and Submitochondrial Particles

Five mg of mitochondrial proteins or 3·7 mg of particle proteins in 1 ml of 0·25M sucrose containing 2 mM EDTA was incubated with ostruthin 15 min at room temperature; subsequently the mixture was centrifuged 10 min at 20 000*g* (mitochondria) or 30 min at 144 000*g* (particles). The sediments and supernatants were analyzed for phosphorus content (*cf.* Experimental).

Preparation	Ostruthin nmol/mg protein	Protein[a], μg P/mg		Sum
		sediment	supernatant	
Mitochondria	0	12·17	0·50	12·67
	36	10·62	1·96	12·58
	89	9·60	2·98	12·58
Particles	0	4·50	1·59	6·09
	108	4·35	1·31	5·66

[a] The values represent both organic and inorganic phosphate. The quantity of the latter found after deproteinization of the mitochondria without mineralization[12] was 0·66 μg P/mg protein.

proteins. Even at considerably higher ostruthin concentrations (89 nmol/mg) roughly one quarter of total phosphorus only can be released from the mitochondria. The results clearly show that the action of ostruthin results in an impairment of the mitochondrial structure permitting the passive transport of ions and the passage of proteins and phosphorus compounds through the mitochondrial membrane.

The nature of the phosphorus compounds released can be judged by the results of an experiment (Table III) in which mitochondrial fragments were exposed to the action of ostruthin. In this case the phosphorus compounds were not released into the supernatant by ostruthin; therefore these compounds are obviously not phospholipids of the mitochondrial membranes. To cast light on the origin of the proteins liberated we examined the activity of several enzymes which are typical of the individual mitochondrial parts. To investigate the outer membrane we chose monoamine oxidase, the inner membrane was judged by the activity of cytochrome oxidase and succinate dehydrogenase; a typical enzyme of the mitochondrial matrix is malate dehydrogenase. The activities of these enzymes in the sediment and supernatant after the action of ostruthin on the mitochondrial suspension are summarized in Fig. 4.

The activity of monoamine oxidase in the sediment is not decreased by the action of ostruthin. The activity of the enzyme on the whole is low and could not be detected at all in the supernatant. The cytochrome oxidase activity of the sediment was de-

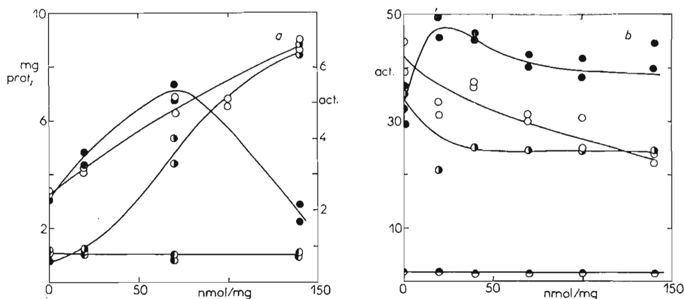

FIG. 4

Release of Enzyme Activities from Mitochondria by Ostruthin

Conditions as described in the legend to Table III. *a* Proteins of supernatant ○, malate dehydrogenase activity (in tens of nanocat) of supernatant ◑ and sediment ●; monoamine oxidase (ncat) in sediment ◐. *b* Succinate dehydrogenase (μcat) in sediment ◑ and supernatant ⊖; cytochrome oxidase (ncat) in sediment without Tween ○ and with 0·6% of Tween 40 ●.

creased after the action of ostruthin and the activity of the enzyme in the supernatant was not proved beyond doubt. The decrease of this activity in the sediment cannot unequivocally be ascribed to the liberation of this enzyme from the inner membrane since it could also be the result of the inhibitory action of ostruthin on the enzyme. Neither did a clear-cut response furnish measurements carried out in the presence of surface-active compounds known to decrease the effect of ostruthin[17]. Also in these experiments the activity of the enzyme in the sediment was lower after the action of ostruthin. Similar results were obtained when the activity of succinate dehydrogenase was determined. The effect of ostruthin on mitochondria decreased the activity of the enzyme in the sediment, no increase of its activity in the supernatant, however, was shown. The cumulation of proteins in the supernatant cannot be ascribed to the decomposition either of the outer or the inner membrane. If the ratio is 70 nmol of ostruthin per mg of protein, more than 60% of proteins are released from the sediment; the slight decrease of activities of structurally bound enzymes does not correspond to this value.

The measurement of the activity of malate dehydrogenase afforded entirely unambiguous results. The increase of the enzyme activity in the supernatant is directly proportional to the increase of ostruthin concentration. This provides evidence of the release of proteins from the mitochondrial matrix; the activity increase parallels the increase in the quantity of the proteins liberated. It is surprising that the activity of malate dehydrogenase in the sediment first increases and decreases only at higher ostruthin concentrations, in accordance with the increase of this activity in the supernatant. This discrepancy cannot be explained by assuming oxidation of the arising NADH since the activity of malate dehydrogenase was not affected by potassium cyanide and antimycin A. We checked therefore whether all malate dehydrogenase activity is liberated when the sediment is suspended in a hypotonic medium. The suspension of mitochondria in 0·25M sucrose shows a low activity only (720 ncat/g protein) which increases 2–4-times (to 1750 ncat/g) after incubation in 0·01M Tris-HCl buffer at pH 7·4. A 15-min sonication of the hypotonic suspension led to an additional 4–5-fold activity increase (to 7130–8570 ncat/g).

The changes in mitochondria structure brought about by ostruthin were investigated by electron microscopy. Figs 5*a*–*e** show ultrathin slices of rat liver mitochondria. The control mitochondria (Fig. 5*a**) are of relatively uniform shape, the space between the outer and the inner membrane is larger in some cases as a result of the fixation procedure. The volume of the matrix is reduced, as evidenced also by the relatively strong electron density. The degree of contamination with microsomes or swollen mitochondria is low.

If the mitochondria are incubated 15 min in methanol or ethanol (25 μl/ml of suspension), the aspect of the ultrathin slices of the mitochondria remains practically

* See insert facing p. 656.

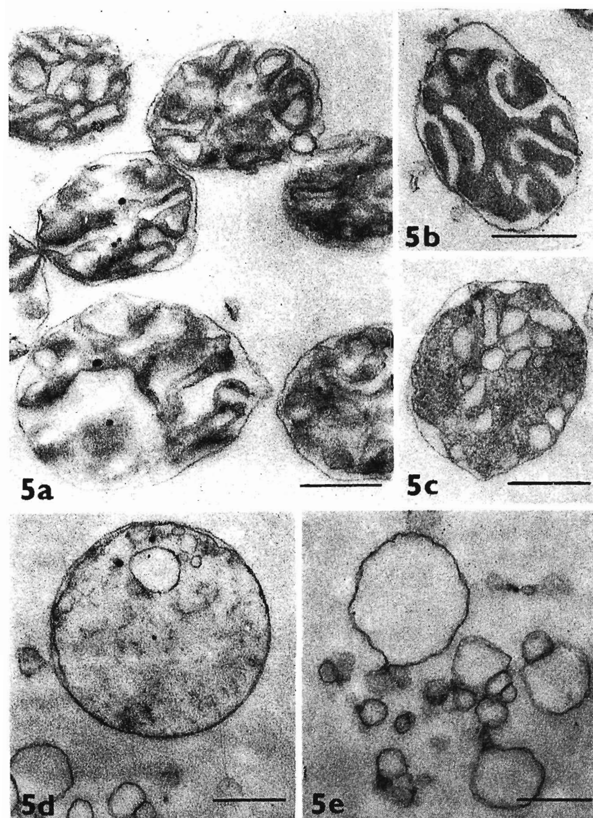

FIG. 5

Ultrathin Slices of Rat Liver Mitochondria

a Control; *b* after incubation with methanol (25 μl/ml); *c*, *d*, *e* incubation with ostruthin (30, 60, 80 nmol/mg protein). The abscissa on the photograph represents 0·5 μm.

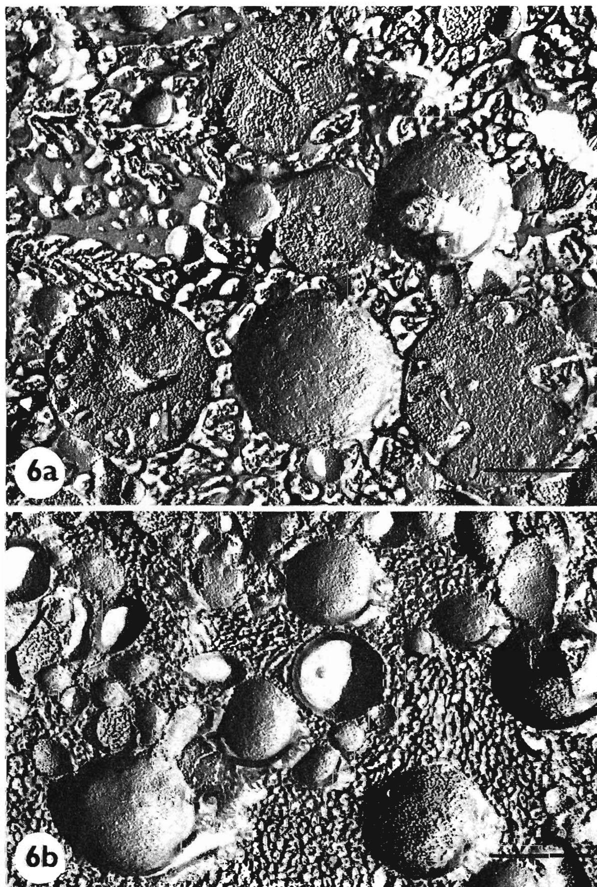

FIG. 6

Freeze Fracture Replicas of Isolated Rat Liver Mitochondria

a Controls; *b* after incubation with 50 nmol of ostruthin. The abscissa on the photograph represents 0·5 μm.

unaltered (Fig. 5*b**). Neither are observable any changes in structure after incubation with 3·5 and 16 nmol of ostruthin/mg of mitochondrial protein. However, uncoupling takes place at these concentrations (Fig. 1).

Significant changes in the ultrastructure of mitochondria can be observed after the concentration of ostruthin has been raised above 20 nmol/mg protein (Fig. 5*c**). The electron density of the matrix decreases, the cristae are fragmented, and the intracristal space is reduced. In single cases parts of membranes separate from the mitochondria. The volume of the mitochondria increases. The matrix is almost dissolved in preparations with ostruthin concentration exceeding 50 nmol/mg protein. The inner membrane decomposes to little vesicles, the external membrane persists in the form of larger sacs (Fig. 5*d**). The final stage of ostruthin action on mitochondria (Fig. 5*e**) at concentrations around 70 nmol/mg protein represent clusters of smaller or larger sacs, most likely residues of the inner and outer membrane. This stage of ostruthin action on the structure of mitochondria is in good accordance with the results of the measurement of optical density and of the liberation of proteins and phosphorus compounds.

The freeze-etching replicas of control mitochondria retain their typical appearance (Fig. 6*a**). Convex surfaces reveal the fragmented EF surface of the outer membrane (the designation of the surface complies with the nomenclature introduced by Brandon and coworkers[18]); the layer situated below is thickly covered with granules and corresponds to the PF surface of inner membrane[19]. The concave surfaces are thickly covered with granules and correspond probably to the PF surface of the outer membrane. The fracture faces of the mitochondria have a granular appearance with insular cristae. The dynamics of changes of mitochondria structure resulting from the action of ostruthin is analogous to the changes observed with their ultrathin slices. The final stage in this case are also clusters of vesicles of different size (Fig. 6*b**). According to the fracture plane and granule density on the newly revealed surfaces we can distinguish between four types of broken membranes indicating the presence of both the outer and the inner membrane.

DISCUSSION

The results of experiments with liver mitochondria confirmed the postulate that ostruthin acts as a typical uncoupler. It complies with the characteristics of uncouplers of the phenol type. Like, *e.g.* 2,4-dinitrophenol and dicoumarol it stimulates respiration in controlled state 4 and the activity of ATPase. Its effect depends on its ratio to the mitochondrial phase. This is understandable since ostruthin is a lipophilic compound and its binding to mitochondria has a hydrophobic character, *i.e.* manifests itself by the partition effect. The effective concentration of ostruthin in this case is therefore its concentration in the lipid phase of the membrane. This holds

* See insert facing p. 656.

true even more generally for strongly lipidic phenolic uncouplers[20]; a similar observation has been made in experiments with the inhibition of succinate oxidase by coumarines[3]. A further increase of ostruthin concentration results in nonspecific inhibition of the oxidases.

Albumin, an efficient reactivator of coumarin-inhibited succinate oxidase, is far less efficient as an uncoupling eliminator. This finding is in perfect agreement with earlier data showing that ostruthin interacts with the mitochondrial membrane system in at least two different manners[21]. One type of interaction of higher affinity is characterized by a binding capacity of 40–50 nmol/mg (for fragments of heart mitochondria). If we take into account the fact that phospholipids are responsible for the substantial part of this interaction (nonspecific partition), the participation of proteins on this interaction (approximately 18 nmol/mg) can be correlated with the concentration required for maximal uncoupling. It can be assumed that this interaction interferes with the processes leading to ATP synthesis. The other type of interaction results in the inhibition of respiration, the lower affinity for ostruthin then leads to an easier elimination of this interaction by albumin.

The induction of the swelling falls behind the start of the uncoupling. This swelling can be induced to a certain degree also by methylostruthin which is lacking the uncoupling effect. 7-Hydroxycoumarin or geraniol are inefficient. It may be assumed that the action of ostruthin involves two effects. The first one dissipates the proton gradient on the mitochondrial membrane by electrogenic entry of protons into the mitochondria. This entry results in uncoupling and in the first stage of swelling which is lacking with methylostruthin. It has been shown with numerous uncouplers that this entry of protons may cause swelling by nonelectrogenic exchange of protons for Na^+- or NH_4^+-ions, yet not for K^+-ions[16]. The other effect manifests itself by deterioration of the permeable barrier of the inner membrane. A result of this is nonspecific entry of ions and sucrose into mitochondria, *i.e.* the second stage of the swelling. The effect is not directly related to the uncoupling effect and leads to more profound changes in mitochondrial structure at higher ostruthin concentrations. Fragmentation and later lysis of the cristae and the inner membrane take place. Proteins and phosphorus compounds are released into the mitochondrial medium.

There is no doubt that the proteins released come from matrix which passes into the medium after disintegration of the inner membrane. The activity of malate dehydrogenase, a typical matrix enzyme, increases in parallel with the increase of protein concentration. The increase in malate dehydrogenase activity of the sediment at low ostruthin concentrations is explained by the fact that this enzyme is not released by a mere decrease of the osmotic pressure of the medium. If we take the activity of malate dehydrogenase after sonication as 100% then the incubation in hypotonic medium will release (or make accessible to substrates) 20–25% of activity only. The original activity value without ostruthin (Fig. 4) should therefore be

4 – 5-times higher. We assume that low ostruthin concentrations make easier the release of malate dehydrogenase during hypotonic incubation and that this release is manifested by an activity increase.

The possibility that a part of the proteins are derived from the membranes can practically be eliminated. None of the activities of the enzyme markers in the supernatant increases. The fact that the activities of these enzymes slightly decrease in the sediment can be ascribed to their inhibition by ostruthin. The alternative that the absence of these activities in the supernatant is also caused by their inhibition is not likely. First, the concentration of ostruthin in the supernatant is far lower than in the sediment, *i.e.* in the lipid phase of the mitochondrial membranes; second, the activity of membrane-bound enzymes, such as succinate dehydrogenase, is considerably more sensitive to ostruthin than after their solubilization[22]. Very unlikely is also the possibility that the observed activity decrease in the sediment can be ascribed to the transition of enzymes into the supernatant in inactive form. The time profile of the decrease of activity of these enzymes does not substantiate any such assumption.

The phosphorus compounds released also originate in the matrix. The quantity of total phosphate found in the supernatant exceeds the values of intramitochondrial inorganic phosphate (0·66 μg of phosphorus/mg protein). This phosphate, however, is derived from organic phosphate compounds, such as nucleotides, contained in the matrix. It cannot be assumed that this phosphate originates in membrane phospholipids liberated by ostruthin since phosphate is not liberated from submitochondrial particles. The same results give also the experiments with sarcosome fragments. The earlier observed release of phospholipids from the complex with the structural protein does not occur with native membrane systems. Hence, the reconstituted system does not portray the behavior of the native membrane structure in this respect.

The ultrathin slices provide unequivocal evidence of ostruthin action on the structure of mitochondria. We did not demonstrate any morphological change of mitochondria at concentration levels causing uncoupling; this does not necessarily mean that there is no correlation between morphological change and uncoupling. Higher ostruthin concentrations lead, however, to considerable structural changes, from the disintegration and dissolution of the matrix up to the fragmentation of the inner and outer membrane. It is probable that the dynamics of the changes is not characteristic of ostruthin only but rather represents a general response of the mitochondria to their impairment[23]. It cannot be decided from the appearance of the membranes in the ultrathin slices whether they are fragments of the inner or the outer membrane. There exist, however, morphological criteria (density and placement of granules) according to which both types of fragments can be distinguished by the method of freeze-etching[24]. It can be concluded from our observations that the clusters of vesicles resulting from the action of ostruthin on mitochondria represent a mixture of fragments of both the inner and the outer membrane. It cannot be decided from our material whether some of the membranes is present more frequently or not.

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