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UNCOUPLING OF OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA WITH DESASPIDIN AND RELATED PHLOROBUTYROPHENONE DERIVATIVES

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Abstract—The effect of desaspidin, ortho-desaspidin, flavaspidic acid, nor-flavaspidic acid and desaspidinol on respiration and oxidative phosphorylation in rat liver mitochondria was studied.

All these substances uncouple oxidative phosphorylation. Desaspidin and ortho-desaspidin show maximum activity in the concentration 2–4 $\times$ 10^{-6} M, the other substances in concentrations of 1·6–6·4 $\times$ 10^{-5} M. Respiration is inhibited more or less distinctly by these concentrations, especially when glutamate or succinate are used as substrate. When pyruvate and malate are employed as the substrate, this effect is less pronounced.

In a medium free of terminal phosphate acceptor respiration is stimulated by desaspidin and by flavaspidic acid to values corresponding to those obtained with phosphate acceptor or with 2:4-dinitrophenol.

The connection between chemical structure and uncoupling effect as well as the possible connection between the former and the biological effects of the substances tested are discussed briefly.

INTRODUCTION

THE anthelmintic action of Dryopteris fern extract is exerted via its toxic effect on contractile protoplasm,[1] but it is biologically active in many other ways also. It possesses, for example, haemolytic[2] and bactericidal properties *in vitro*.[3,4] Flavaspidic acid glucaminate has been shown to have an inhibitory effect on the Ca^{2+} activated adenosine-triphosphatase in rat heart muscle homogenate *in vitro*.[5]

Several pure substances with anthelmintic effect have been isolated from the crude extract. All these substances are closely related chemically and can be characterized as phlorobutyrophenone derivatives.[6] The LD_{50} for mice has recently been determined for a number of these substances.[7] Desaspidin has a strong anthelmintic effect against human *Diphyllobothrium* infection.[8]

Experiments conducted for the purpose of studying the mode of action of desaspidin showed that it stimulates anaerobic glycolysis in fish tapeworm (*Diphyllobothrium latum*), and to some extent also its respiration, while it is a powerful stimulant of the respiration of human amniotic cells in tissue cultures.[9] These observations prompted the present study of the effect of desaspidin on oxidative phosphorylation. It will be

shown in the present work that desaspidin, ortho-desaspidin, flavaspidic acid, norflavaspidic acid and desaspidinol (see Fig. 1) uncouple oxidative phosphorylation in rat liver mitochondria.

MATERIAL AND METHODS

Mitochrondria of rat liver were obtained by differential centrifugation of rat liver homogenate in 0·25 M sucrose. Young white rats (Wistar) were killed by decapitation, the liver was extirpated and placed immediately in ice cold sucrose, weighed and cut with scissors and homogenized. A glass homogenizer with a Teflon pestle was used. To remove cell fragments and nuclei the homogenate was centrifuged at 600 **g** for 10 min in an International refrigerated centrifuge at 0 °C with rotor 296. The mitochondria were then centrifuged at 5000 **g** for 10 min and resuspended twice. The procedure as a whole was carried out in a refrigerated room at 0 °C. Mitochrondria corresponding to 0·3 g of initial, wet liver weight, suspended in 0·3 ml of 0·25 M sucrose, were used for each individual experiment.

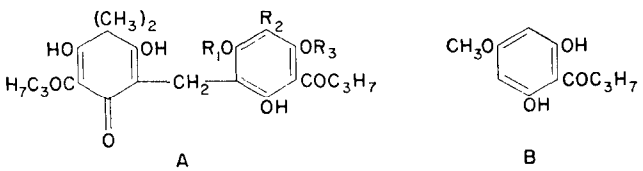

FIG. 1. The formulae for the substances tested. (A) Nor-flavspidic acid: $R_1 = R_2 = R_3 = H$. Flavaspidic acid: $R_2 = CH_3$; $R_1 = R_3 = H$. Desaspidin: $R_1 = CH_3$; $R_2 = R_3 = H$. Ortho-desaspidin: $R_3 = CH_3$; $R_1 = R_2 = H$. (B) Desaspidinol (phlorobutyrophenone-4-methyl ether).

Respiration was determined according to Warburg's direct method [10] in *c.* 15 ml Warburg flasks with a side arm. The medium contained per flask 50 μmoles of potassium phosphate buffer, pH 7·5 100 μmoles of KCl, 16 μmoles of $MgCl_2$, 2 μmoles of adenosine triphosphate (ATP), 75 μmoles of sucrose, 60 μmoles of glucose and an excess of hexokinase ("Sigma", yeast, grade III), and substrate as specified in the respective tables. The total volume of liquid in the flasks was 2 ml plus 0·2 ml of 2N KOH in the centre well.

Desaspidin and other tested substances prepared at the research laboratories of Medica Ltd. were employed. A small amount of the substance (6–10 mg) was dissolved rapidly in a few drops of 0·1 N KOH and diluted to the desired concentration with phosphate buffer. The solution obtained was used immediately.

Immediately after the mitochrondria, glucose and hexokinase were added to the medium from the side arm and the flasks were placed in the Warburg apparatus for 5 min thermoequilibration. The oxygen uptake was then determined for 15–20 min, depending on the activity of the preparation, at a temperature of 30 °C. The reaction was stopped with 1 ml of 10 per cent trichloroacetic acid and the flasks were placed on ice. The oxygen uptake for the experimental period as a whole, inclusive of the thermoequilibration, was obtained through extrapolation. The phosphate uptake was

determined by the isotope distribution method.[11] The ^{32}P used was a phosphate in carrier-free solution, obtained from the Radiochemical Centre, Amersham, England.

RESULTS

All the substances tested inhibited oxidative phosphorylation in rat liver mitochondria, although the concentrations required for the purpose varied as can be seen from the series of experiments in Table 1. Desaspidin and ortho-desaspidin were

TABLE 1. THE EFFECT OF DESASPIDIN AND SOME RELATED COMPOUNDS ON THE RESPIRATION AND PHOSPHORYLATION OF RAT LIVER MITOCHONDRIA

Each Warburg flask contained 50 μmoles of potassium phosphate buffer, pH 7·5, 100 μmoles of KCl, 16 μmoles of $MgCl_2$, 75 μmoles of sucrose, 20 μmoles of L-glutamate, 2 μmoles of ATP. Immediately after the mitochondria, 60 μmoles of glucose + hexokinase was added from the side arm. Final volume, 2·0 ml; 0·2 ml 2 N KOH in the centre well. The temperature was 30 °C. The duration of the experiment was 15–20 min. The figures in brackets indicate the value in per cent of the controls.

Exp. no.	Substance tested	μatoms O	μmoles P	P/O
1	—	11·3 (100)	30·2 (100)	2·7
	5×10^{-7} M desaspidin	11·5 (102)	27·4 (91)	2·4
	1×10^{-6} M desaspidin	11·2 (99)	10·8 (66)	1·8
	2×10^{-6} M desaspidin	9·4 (83)	6·7 (22)	0·7
	4×10^{-6} M desaspidin	7·4 (66)	3·1 (10)	0·4
2	—	11·5 (100)	30·0 (100)	2·6
	5×10^{-7} M *o*-desaspidin	11·4 (99)	29·8 (99)	2·6
	1×10^{-6} M *o*-desaspidin	11·5 (100)	24·8 (83)	2·1
	2×10^{-6} M *o*-desaspidin	10·9 (95)	14·5 (48)	1·3
	4×10^{-6} M *o*-desaspidin	6·1 (53)	1·7 (6)	0·3
3	—	11·3 (100)	29·5 (100)	2·6
	8×10^{-6} M flavaspidic acid	11·0 (97)	24·9 (84)	2·3
	$1{\cdot}6 \times 10^{-5}$ M flavaspidic acid	9·6 (85)	18·5 (63)	1·9
	$3{\cdot}2 \times 10^{-5}$ M flavaspidic acid	5·6 (50)	4·6 (16)	0·8
	$6{\cdot}4 \times 10^{-5}$ M flavaspidic acid	1·8 (16)	1·3 (4)	0·7
4	—	11·9 (100)	29·2 (100)	2·5
	4×10^{-6} M nor-flavaspidic acid	10·8 (91)	25·1 (86)	2·3
	8×10^{-6} M nor-flavaspidic acid	11·2 (94)	22·5 (77)	2·0
	$1{\cdot}6 \times 10^{-5}$ M nor-flavaspidic acid	9·9 (83)	12·4 (42)	1·3
	$3{\cdot}2 \times 10^{-5}$ M nor-flavaspidic acid	6·1 (51)	2·1 (7)	0·3
	$6{\cdot}4 \times 10^{-5}$ M nor-flavaspidic acid	3·6 (30)	1·0 (3)	0·3
5	—	11·2 (100)	29·8 (100)	2·7
	4×10^{-6} M desaspidinol	10·3 (92)	26·8 (90)	2·6
	8×10^{-6} M desaspidinol	10·3 (92)	25·4 (85)	2·5
	$1{\cdot}6 \times 10^{-5}$ M desaspidinol	9·0 (80)	21·4 (72)	2·4
	$3{\cdot}2 \times 10^{-5}$ M desaspidinol	7·7 (69)	11·9 (40)	1·5
	$6{\cdot}4 \times 10^{-5}$ M desaspidinol	5·3 (47)	1·2 (4)	0·2

roughly equally active, and at their maximal effect in the concentration $2–4 \times 10^{-6}$ M. For the other substances the concentrations required were considerably higher, $1{\cdot}6–6{\cdot}4 \times 10^{-5}$ M. Parallel with the phosphorylation-inhibiting effect there was a more or less distinct inhibition of respiration, but it was clearly weaker than the effect on phosphate uptake. This is indicative of uncoupling of oxidative phosphorylation.

lower than those necessary for the complete inhibition of phosphorylation in the above experiments. Similar effects have been reported with DNP and with some other known uncouplers.[13] In higher concentrations, on the other hand, the respiration-inhibiting effect emerged.

TABLE 3. STIMULATION OF RESPIRATION WITH DESASPIDIN AND FLAVASPIDIC ACID IN MEDIUM WITHOUT TERMINAL PHOSPHATE ACCEPTOR

The medium was as in Table 1 but without glucose and hexokinase unless specially stated. The figures in brackets indicate the value in per cent of the control experiment with glucose and hexokinase.

Additions	μatoms O
60 μmoles glucose + hexokinase	7·4 (100)
none	1·2 (16)
1×10^{-6} M desaspidin	2·6 (35)
2×10^{-6} M desaspidin	7·8 (105)
4×10^{-6} M desaspidin	5·8 (78)
$1{\cdot}6 \times 10^{-5}$ M flavaspidic acid	3·2 (43)
$3{\cdot}2 \times 10^{-5}$ M flavaspidic acid	8·6 (116)
$6{\cdot}4 \times 10^{-5}$ M flavaspidic acid	2·7 (36)
5×10^{-5} M DNP	7·6 (103)
1×10^{-4} M DNP	7·6 (103)

DISCUSSION

There is at present little evidence as to the mode of action of these uncoupling substances. However, a certain connection can be discerned between the uncoupling effect and the chemical structure of the substance. Proceeding from nor-flavaspidic acid, the introduction of a methoxy group into the compound involves an appreciable increase in activity. Whether this group is introduced in the *ortho-* or *para*-position in relation to the butyryl group (*o*-desaspidin or desaspidin, respectively), does not seem to have any greater effect on the activity. The introduction of a methyl group (flavaspidic acid) does not affect the activity to any greater extent, but appears to increase the respiration-inhibiting effect to some degree. That the double-ring structure is not necessary for the uncoupling effect appears from the fact that desaspidinol is nearly as active as nor-flavaspidic acid. On the other hand, desaspidinol has a much weaker effect than the corresponding double-ring compound, i.e. desaspidin.

It seems interesting that the substances are at the same time strong anthelmintics and uncouplers of oxidative phosphorylation. Atebrin, which is commonly used as an anthelmintic against tapeworms, is also known to be an uncoupler of oxidative phosphorylation. At present it is impossible to conclude whether this similarity in biological action of two anthelmintics with entirely different chemical structure is more than a coincidence. It should, on the other hand, be observed that the phloro-butyrophenone derivatives tested, unlike atebrin[14] do not in maximally uncoupling concentrations completely inhibit glutamate oxidation and that some of them seem to be active in lower concentrations than most uncouplers reported so far. As pointed out in another connection,[9] it is improbable that the anthelmintic effect of these substances is confined only to disturbances in the aerobic processes of the worm. It is still too early to express opinions concerning the possible connection between the various

biological effects of the compounds in question and their uncoupling effect on oxidative phosphorylation. It might be pointed out, however, that the concentrations needed for uncoupling agree in the main with those demonstrated to be biologically active in various systems *in vitro*.

Known facts indicate that the active substances present in Dryopteris fern extract produce disturbances in the energy-generating processes of cells. These disturbances have not yet been fully explained but it is possible that a part of the manifold effects of the substances in question on various living organisms can be traced to their effects on energy metabolism. Further studies are required on this point.

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