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IMPORTANCE OF SULFHYDRYL GROUPS FOR THE UNCOUPLING ACTIVITY OF NON-STEROIDAL ACIDIC ANTI-INFLAMMATORY DRUGS AND VALINOMYCIN IN OXIDATIVE PHOSPHORYLATION

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Abstract—In media at pH 7·4, the uncoupling activity on oxidative phosphorylation with sodium succinate (5 mM) as substrate was studied in liver mitochondria for many anti-inflammatory drugs (e.g., ibuprofen, 0·25 mM; flufenamic acid, 0·20 mM; pyrazinobutazone, 0·25 mM; indomethacin, 0·20 mM; and methiazinic acid, 1 mM) Mersalyl (0·035 mM) doesn't inhibit the respiration induced by 2,4-dinitrophenol, but inhibits respiration induced by all these drugs. This inhibition was not reversed by 2,4-dinitrophenol (20 μM), Janus Green B (50 μM) or any other uncoupling agents in media with or without phosphate. This is the proof that this action is independent of the ability of Mersalyl to prevent phosphate penetration into mitochondria. Meanwhile, cysteine (0·15 mM) was able to reverse the inhibition of Mersalyl in media with as well as without phosphate and restored the uncoupling activity of the anti-inflammatory drugs. This competition between cysteine and Mersalyl is "dose-dependent". A similar action of Mersalyl and cysteine was also observed on the uncoupling activity of valinomycin (0·2 μg). These results suggest that acidic anti-inflammatory drugs and valinomycin exert their uncoupling activity by the way of fixation on membrane sites rich in sulfhydryl groups. These sites might be similar for the two kinds of drugs. There is a slight inhibition of the uncoupling activity of valinomycin and acidic anti-inflammatory drugs observed with nupercaine (0·6 mM).

THE UNCOUPLING activity of non-steroidal anti-inflammatory drugs (NSAI) on mitochondrial oxidative phosphorylation has been known for a long time. In 1958, Adams and Cobb[1] suggested that the anti-inflammatory activity of NSAI may be related to their capacity to inhibit ATP biosynthesis of mitochondria selectively without inhibiting mitochondrial respiration. This concept has been extensively criticized. Even Adams and Cobb themselves showed that 2,4-dinitrophenol (DNP), a potent uncoupling agent,[1] has no anti-inflammatory effects and that amidopyrine, a well known NSAI,[1] fails to uncouple oxidative phosphorylation. Some of these objections were in turn criticized and rejected by Whitehouse.[2] In certain animal models, DNP does exhibit some anti-inflammatory properties,[3,4] but this drug is normally very rapidly metabolized in the liver.[5,6] On the other hand, rubazonic acid,[7] one of the metabolites of amidopyrine, is a very potent uncoupling agent. In an important review, Whitehouse[2] listed all the NSAI compounds that have an uncoupling activity: salicylates,[8] with the exception of γ-resorcylic acid and gentisic acid that are now claimed to have poor clinical value;[9] some hydroxyaniline homologues of paracetamol;[2] some derivatives of

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griseofulvin;[2] anthranilates such as flufenamic acid and mefenamic acid;[10] pyrazolones like phenylbutazone;[11] cinchophene derivatives;[2,11] mepacrine;[12] aryl alkanoic acids like ibufenac;[13] indoleacetic acids like indomethacin;[13,14] 18 β-glycyrrhetinic acid (exonolone);[11] gold salts[15] and some other heavy metal salts (essentially, bismuth,[15] selenium[15] and antimony).[2]

Many suggestions for the sites of uncoupling activity have been made. Fluharty and Sanadi[16] have proposed the involvement of vicinal dithiols and Heytler[17] has suggested the presence of a thiol group adjacent to a primary amino group.

Mersalyl is known to act on mitochondrial phosphorylation by inhibiting the penetration of inorganic phosphate into the mitochondrion,[18–20] but it is also a thiol reagent.

Valinomycin is a potent uncoupling antibiotic that exerts its activity by increasing the mitochondrial permeability to alkali cations.[21,22] This drug acts as a neutral carrier for cations with a decreasing activity following the sequence $H^+ > Rb^+ > K^+ > Cs^+ > Na^+ > Li^+$.[23] The present communication demonstrates the importance of sulfhydryl groups within the mitochondrial membrane for the uncoupling activity of NSAI (and valinomycin).

MATERIAL AND METHODS

Preparation of mitochondria. Liver from Wistar albino rats (fasted for 24 hr before the experiment, killed by cervical disruption and then bled) of about 200 g was chilled immediately after removal by immersion in sucrose, 0·25 M, with EDTA, 2 mM. The liver was cut with scissors into pieces of approximately 0·5 cm in thickness and weighed. After four thorough washes with 0·25 M sucrose + 2 mM EDTA, the pieces of liver were divided in two equal parts and transferred into two Potter–Elvehjem homogenizers with Teflon pestles containing 30 ml sucrose, 0·25 M, + 2 mM EDTA. The tissue was homogenized for 1 min with a rotating speed of about 750 rev/min and the temperature was maintained around 0° by working in the cold room with an ice container. The homogenate was filtered through gauze and then centrifuged for 5 min at 900 *g* (2700 rev/min) in the rotor SS-34 of the Sorvall RC2-B refrigerated centrifuge. After the first centrifugation, the supernatant was centrifuged again at 4500 *g* (6250 rev/min) for 10 min in the same rotor. The mitochondrial pellet (after carefully discarding the fluffy layer) was gently resuspended by hand homogenization with a loosely fitting Teflon pestle (clearance 0·4 mm) in 10 ml of cold 0·25 M sucrose + 2 mM EDTA and centrifuged for 10 min in the same rotor at 12,500 *g* (10,100 rev/min). The final mitochondrial pellet was rinsed with cold 0·25 M sucrose + 2 mM EDTA and then resuspended in a volume of 2 ml of the same solution to give a stock suspension of mitochondria containing about 45 mg protein/ml. Mitochondrial protein was estimated by the Folin method.[24] All the solutions used for mitochondrial preparation were at pH 7·4.

Pharmacological studies. Substrate oxidation rates, ADP/O ratios and uncoupling activities were determined polarographically[25] by means of a Clark electrode (Yellow Springs Instrument Company, Yellow Springs, Ohio) fitted to a plexiglasss chamber of 2·0 ml capacity. The Clark electrode was connected to a Gilson Medico Electronics Oxygraph. The complete system usually contains about 4·5 mg of mitochondrial protein added to 2 ml of incubation medium containing: 20 mM KCl, 5 mM $MgCl_2$, 10 mM potassium phosphate buffer (pH 7·4), 225 mM sucrose and 20 mM Tris HCl

buffer (pH 7·4). NSAI and inhibitors were dissolved in ethanol and were added as small volumes (± 10 μl) of stock solutions. This amount of ethanol has no significant effect *per se* on the enzyme activities assayed. Modifications of this medium are indicated in the Results section. The respiratory control ratio (RCR) was defined as the ratio of the respiration rate in the presence of added ADP to that observed after the removal of the added ADP by phosphorylation reactions.[26] Respiratory ratios (R.R.) were defined as the ratios of respirations rates in the presence of inhibiting or uncoupling agents to that observed in state 4. The respiratory states of the mitochondria were defined according to Chance and Williams.[26] The initial or starting value (100%) for the O_2 uptake curves represents 200–240 μM O_2. Experiment, as listed in the tables, means "one specific mitochondrial preparation". All the incubations and polarographic traces were repeated at least three times in each experiment to allow the evaluation of standard deviations.

Reagents. Antimycin, ADP, valinomycin and atractylate were products from Cal-Biochem (Los Angeles, Calif.); sodium Mersalyl and oligomycin were obtained from Sigma Chemical Co. (St. Louis, Mo.). Tributyl–tin–acetate was obtained from the Tin Research Institute (Greenford, Middlesex, Great Britain); tetra-methyl-*p*-phenylene diamine was obtained from Fluka (Buchs, Switzerland) and dichlorocyclohexyl carbodiimide was a generous gift from Dr. D. D. Tyler (Royal Veterinary College, London, Great Britain). NSAI drugs were obtained from the following drug companies: Geigy; Parke, Davis & Co.; Merck, Sharp & Dohme; Upsa (Agens, France); Specia (Société Parisienne d'Expansion Chimique, Paris, France); Seresci (Brussels, Belgium); Boots (Nottingham, Great Britain). Janus Green B (5-chloro-3 diethylamino-7-*p*-dimethyl-aminophenyl-azo-5-phenylphenazine) was obtained from Aldrich Chemical Co. (Milwaukee, Wis.). All the other chemicals were of the purest grade commercially available.

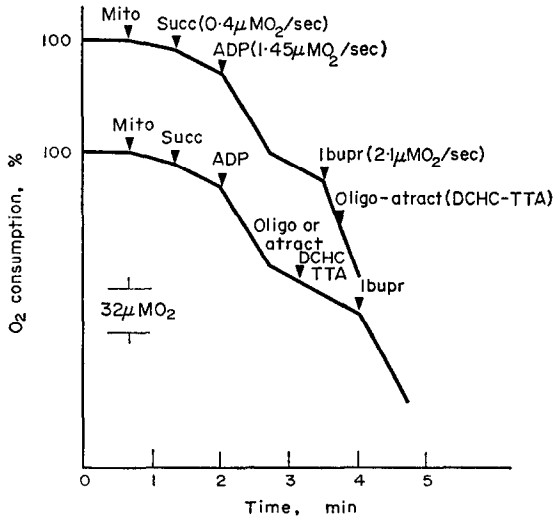

FIG. 1. Uncoupling effect of ibuprofen. Mito = mitochondria, 4·5 mg protein; Succ = succinate of sodium, 5 mM; Ibupr = ibuprofen, 0·25 mM. ADP = adenosine diphosphate, 125 μM; Oligo = oligomycin, 4 μg; Atract = atractylate, 50 μM; DCHC = dicyclohexylcarbodiimide, 20 μM; TTA = tributyl–tin–acetate, 1·6 μM.

RESULTS

The control of mitochondrial respiration by adenosine diphosphate (ADP, 125 μM) is indicated in the polarographic trace of Fig. 1 in the presence of succinate (5 mM) as substrate.

TABLE 1. ADP/O RATIOS AND RESPIRATORY CONTROL RATIOS (RCR) WITH SUCCINATE AND α-KETOGLUTARATE*

Substrates	Concn (mM)	ADP/O	RCR
Succinate	5	1·95 ± 0·35	3·60 ± 0·52 (5)
α-Ketoglutarate	5	3·10 ± 0·42	5·80 ± 0·94 (5)

* Results expressed as mean values ± standard deviation.
Number of experiments from which mean values were calculated are given in parentheses.

The ADP/O ratios and the RCR are indicated in Table 1 and are in good correlation with the type of substrate oxidized.[25]

The addition of NSAI after the exhaustion of this ADP effect (state 4) stimulates the O_2 consumption in the same way as the classical uncoupling agent DNP, 20 μM, (R.R. = 6·8 ± 1·2, mean values from five experiments).

The different types of NSAI used and the R.R. obtained are shown in Table 2.

The same stimulation was observed with valinomycin (0·2 μg), which promotes essentially the uptake of K^+ by mitochondria (R.R. = 8·10 ± 1·26, mean values from five experiments).

TABLE 2. RESPIRATORY RATIOS (R.R.) WITH VARIOUS TYPES OF NON-STEROIDAL ACIDIC ANTI-INFLAMMATORY DRUGS*

Chemical types	Drugs	Concn (mM)	R.R. with succinate (5 mM)	R.R. with α-ketoglutarate (5 mM)
Two arylalkanoic derivatives	Isobutylphenylpropionic acid	0·25	4 ± 1·22 (5)	4·1 ± 0·97 (3)
	Indomethacin	0·2	3·2 ± 0·54 (5)	3·6 ± 0·88 (3)
Three anthranilic derivatives = fenamates	Flufenamic acid	0·2	4·3 ± 1·31 (5)	4·2 ± 1·27 (3)
	Mefenamic acid	0·5	3·5 ± 0·94 (5)	3·7 ± 1·01 (3)
	Niflumic acid	0·2	5·1 ± 0·75 (5)	5·3 ± 0·80 (3)
Two pyrazolone derivatives	Phenylbutazone	0·25	4·2 ± 0·58 (5)	4·2 ± 0·74 (3)
	Pyrazinobutazone†	0·25	4·3 ± 1·12 (5)	4·5 ± 0·94 (3)
One phenothiazine derivative	Methiazinic acid	1	2·8 ± 0·60 (5)	2·9 ± 0·34 (3)

* Results expressed as mean values ± standard deviation.
Number of experiments from which mean values were calculated are given in parentheses.
† Piperazine salt of phenylbutazone.

As predicted by their known activities,[27,28] oligomycin (4 μg) dicyclohexylcarbodiimide (20 μM) and tributyl-tin-acetate (1·6 μM) did not inhibit the uncoupling effect of the NSAI,[29,30] just as is the case for DNP and valinomycin (Fig. 1). This is clearly indicated by the R.R of Table 3.

TABLE 3. EFFECT OF SOME CLASSICAL INHIBITORS OF MITOCHONDRIAL RESPIRATION ON ISOBUTYLPHENYLPROPIONIC ACID STIMULATION OF O_2 CONSUMPTION*

Inhibitors	R.R.
1 Succinate 5 mM, no inhibitors	4·0 ± 1·22 (5)
2 Tetramethyl-*p*-phenylene diamine 0·1 mM + ascorbate 5 mM, no inhibitors	5·2 ± 0·82 (3)
3 Antimycin 2 μg + 1	0·25 ± 0·04 (3)
4 Antimycin 2 μg + 2	5·2 ± 0·82 (3)
5 KCN 0·15 mM + 1	0·11 ± 0·02 (3)
6 Oligomycin 2 μg + 1	4·0 ± 1·22 (4)
7 Tributyl-tin-acetate 1·6 μM + 1	4·0 ± 1·22 (2)
8 Dicyclohexylcarbodiimide 20 μM + 1	4·0 ± 1·22 (2)
9 Atractylate 50 μM + 1	4·0 ± 1·22 (2)

* Results expressed as mean values ± standard deviation.
Number of experiments from which mean values were calculated are given in parentheses.

The same thing is observed for atractylate (50 μM), an inhibitor of the ADP penetration into mitochondria,[31,32] as shown in Fig. 1 and Table 3.

The acceleration of oxygen consumption by uncoupling effects of NSAI is prevented by antimycin (2 μg), except when the succinate or α-ketoglutarate substrates are replaced by an artificial electron donor system composed of ascorbate (5 mM) and

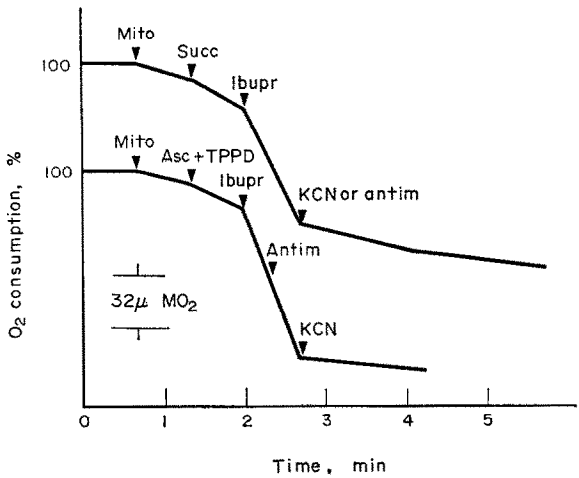

FIG. 2. Inhibition by antimycin and potassium cyanide. Mito = mitochondria, 4·5 mg protein; Succ = succinate of sodium, 5 mM; Ibupr = ibuprofen, 0·25 mM; Asc = ascorbate, 5 mM; TPPD = tetramethyl-*p*-phenylene diamine, 0·1 mM; Antim = antimycin, 2 μg; KCN = potassium cyanide, 15 mM.

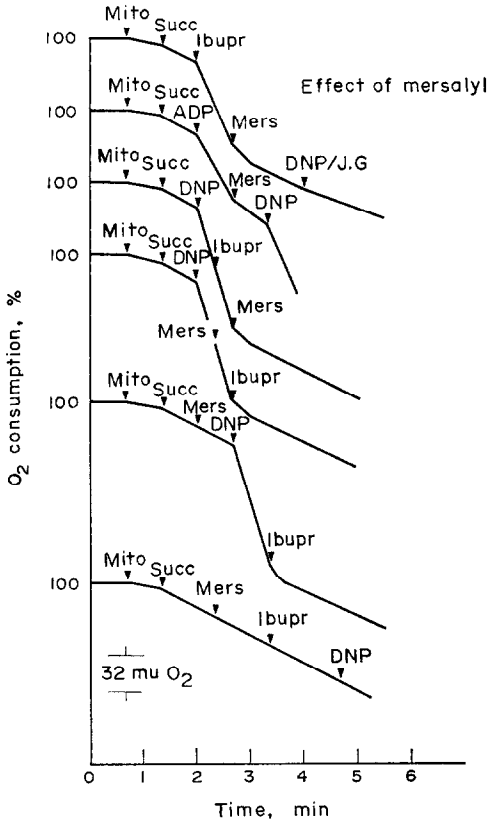

FIG. 3. Inhibition by Mersalyl in a medium with phosphate. Mito = mitochondria, 4·5 mg protein; Succ = succinate of sodium, 5 mM; ADP = adenosine diphosphate, 125 μg; Ibupr = ibuprofen, 0·25 mM; Mers = Mersalyl, 35 μM; DNP = 2,4-dinitrophenol, 20 μM; J.G. = Janus Green B, 20 μM.

TABLE 4. IRREVERSIBILITY OF MERSALYL EFFECTS BY CLASSICAL UNCOUPLERS*

Drugs	R.R.
1 Isobutylphenylpropionic acid 0·25 mM	4 ± 1·22 (5)
2 Mersalyl 35 μM + 1	1 ± 0·23 (3)
3 DNP 20 μM	6·8 ± 1·2 (5)
4 DNP 20 μM + 1	6·8 ± 1·2 (3)
5 Mersalyl 35 μM + 3	6·8 ± 1·2 (3)
6 Mersalyl 35 μM + 3 + 1	1·1 ± 0·26 (2)
7 Mersalyl 35 μM + 1 + Janus Green B 50 μM	0·9 ± 0·22 (2)

* Results expressed as mean values ± standard deviation.
Number of experiments from which mean values were calculated are given in parentheses.

tetramethyl-*p*-phenylene diamine (0·1 mM), as shown in Fig. 2. Respiration in the presence of these latter substrates was blocked by potassium cyanide, 15 mM (Table 3).

In a medium with phosphate (10 mM), the uncoupling effect of the NSAI is inhibited by sodium Mersalyl (35 μM). This inhibition is observed regardless of whether Mersalyl is added to the incubation medium before or after the NSAI. Mersalyl by itself (at such a low concentration) has no effect on the uncoupling activity of DNP (20 μM); but when the uncoupling activity of the NSAI was blocked by adding Mersalyl this blocking effect was not reversed by DNP and even not by Janus Green B, 20 and 50 μM (Fig. 3 and Table 4).

In a medium without phosphates, when ADP (125 μM) failed to stimulate mitochondrial respiration the same relations were observed between NSAI and Mersalyl. This blocking activity is apparently not related to the ability of Mersalyl to block the penetration of phosphate anions into the mitochondrion, as shown by Tyler[18,20] for the inhibition by this organic mercurial of uncoupling activity induced by calcium (Fig. 4).

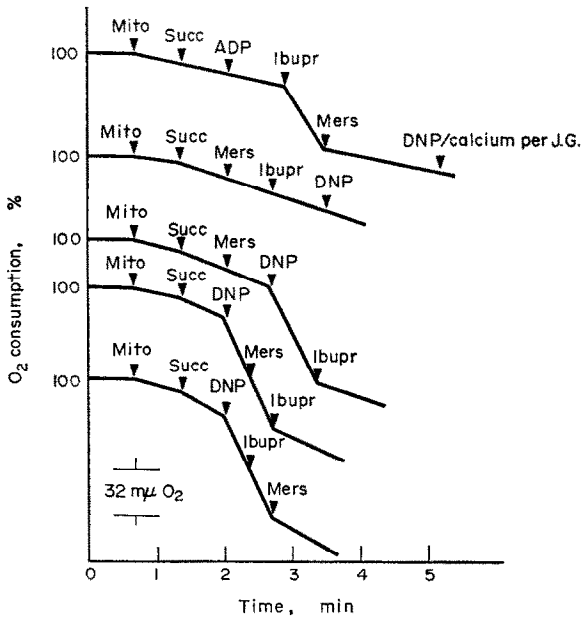

FIG. 4. Inhibition by Mersalyl in a medium without phosphate. Mito = mitochondria, 4·5 mg protein; Succ = succinate of sodium, 5 mM; ADP = adenosine diphosphate, 125 μg; Ibupr = ibuprofen, 0·25 mM; Mers = Mersalyl, 35 μM; DNP = 2,4-dinitrophenol, 20 μM; J.G. = Janus Green B, 20 μM.

In Table 5 the inhibition effect of Mersalyl in a medium with phosphate, in the presence of 5 mM succinate as respiratory substrate, is clearly shown on the R.R. induced by NSAI. Very similar results have been found when the same experiments have been performed in a phosphate-free medium.

The effect of Mersalyl (for the same concentration, 35 μM) on the uncoupling activity induced by the antibiotic valinomycin (0·2 μg) in a medium with and without phosphate is identical to its effect on blocking the uncoupling activity of NSAI (Fig. 5 Table 5).

TABLE 5. EFFECTS OF MERSALYL AND CYSTEINE*

Drugs	Concn (mM)	R.R. with PO_4 and succinate (5 mM) 1	R.R. 1 + Mersalyl (35 μM) 2	R.R. 2 + Cysteine (0·15 mM) 3
Isobutylphenylpropionic acid	0·25	4 ± 1·22 (5)	1 ± 0·23 (6)	3·8 ± 1·07 (6)
Indomethacin	0·2	3·2 ± 0·54 (5)	1·1 ± 0·31 (3)	3·0 ± 0·67 (3)
Flufenamic acid	0·2	4·3 ± 1·31 (5)	0·9 ± 0·24 (4)	4·0 ± 0·59 (4)
Mefenamic acid	0·5	3·5 ± 0·94 (5)	0·95 ± 0·26 (3)	3·3 ± 0·34 (3)
Niflumic acid	0·2	5·1 ± 0·75 (5)	1·2 ± 0·32 (6)	5·2 ± 0·72 (6)
Phenylbutazone	0·25	4·2 ± 0·58 (5)	1·1 ± 0·24 (3)	4 ± 0·97 (3)
Pyrazinobutazone	0·25	4·3 ± 1·12 (5)	1·3 ± 0·25 (2)	3·9 ± 1·11 (2)
Methiazinic acid		2·8 ± 0·60 (5)	1 ± 0·32 (3)	2·2 ± 0·31 (3)
Valinomycin	0·2 μg	8·1 ± 1·26 (5)	1·3 ± 0·30 (5)	7·0 ± 1·24 (5)

* Results expressed as mean values ± standard deviation.
Number of experiments from which mean values were calculated are given in parentheses.

Although other uncoupling agents such as DNP are unable to reverse the inhibition induced by Mersalyl, adding cysteine to Mersalyl-poisoned mitochondria restored the uncoupling activity of the NSAI and valinomycin. This cysteine effect is detected with 0·05 mM, but it is necessary to add 0·15 mM cysteine to observe a complete restoration of the uncoupling activity of NSAI and valinomycin (Fig. 6) which appears from the results shown in Table 5. This effect of cysteine is also observed in a phosphate free medium, with very similar R.R.

The local anesthetic nupercaine (0·6 mM), which was described as an agent which might possibly prevent or reduce the K^+ flux induced by valinomycin,[33] was found to have a small but significant effect inhibiting the uncoupling activity induced by NSAI and valinomycin, as shown by the R.R. of Table 6. The effect of nupercaine is a little more evident with NSAI than with valinomycin (Fig. 7).

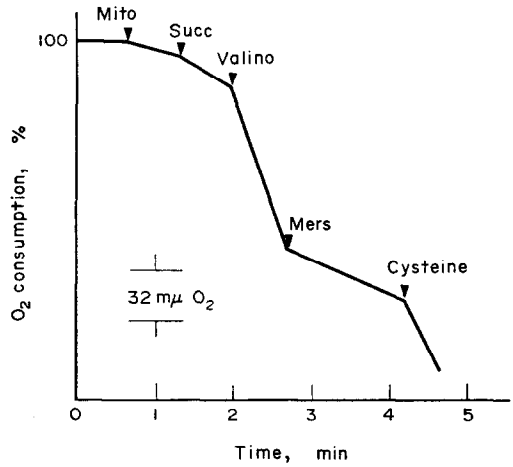

FIG. 5. Effect of valinomycin. Mito = mitochondria, 4·5 mg protein; Succ = succinate of sodium, 5 mM; Valino = valinomycin, 0·2 μg; Mers = Mersalyl, 35 μM; cysteine, 0·15 mM.

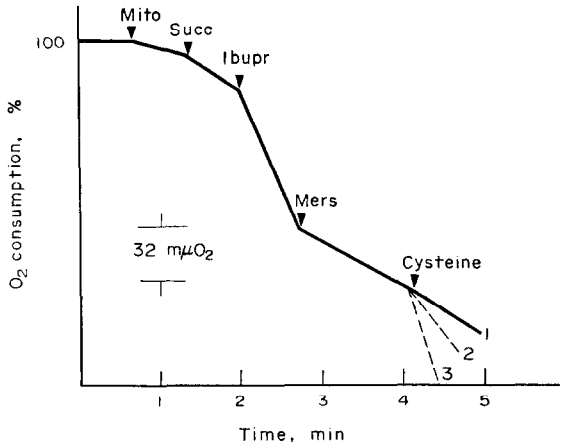

FIG. 6. Reversal of Mersalyl inhibition by cysteine. Mito = mitochondria, 4·5 mg protein; Succ = succinate of sodium, 5 mM; Ibupr = ibuprofen, 0·25 mM; Mers = Mersalyl, 35 μM; cysteine (1) = 0·05 mM; (2) = 0·10 mM; (3) = 0·15 mM.

TABLE 6. EFFECTS OF NUPERCAINE*

Drugs	Concn	R.R.	
		Succinate (5 mM) alone	Succinate (5 mM) + nupercaine (0·6 mM)
Isobutylphenylpropionic acid	0·25 mM	4 ± 1·22 (5)	2·0 ± 0·29 (3)
Valinomycin	0·2 μg	8·10 ± 1·26 (5)	5·16 ± 0·32 (3)

* Results expressed as mean values ± standard deviation.
Number of experiments from which mean values were calculated are given in parentheses.

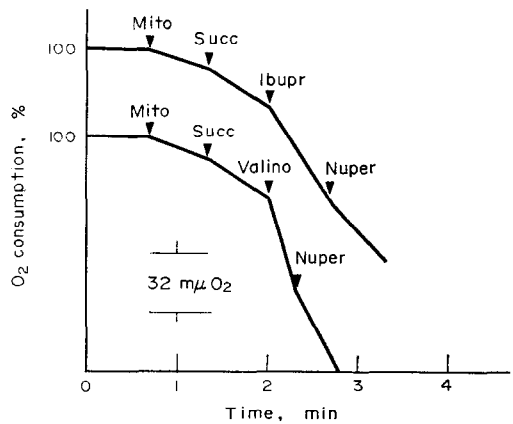

FIG. 7. Effect of nupercaine. Mito = mitochondria, 4·5 mg protein; Succ = succinate of sodium, 5 mM; Ibupr = ibuprofen, 0·25 mM; Valino = valinomycin, 0·2 μg; Nuper = nupercaine, 0·6 mM.

DISCUSSION

All the eight NSAI studied here stimulated the respiration of state 4 mitochondria, confirming their uncoupling activity demonstrated by other authors.[1,11,13,14] The fact that oligomycin and some of its analogues were unable to block this uncoupling suggests that, according to the chemical hypothesis of coupling activity, the reaction $X \sim I + P \rightleftharpoons X \sim P + I$ is not involved[34] (Fig. 8) in the mode of action of the NSAI.

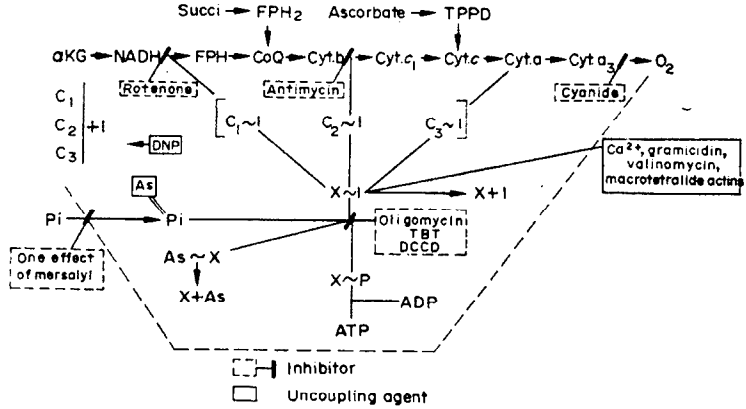

FIG. 8. Schematic representation of mitochondrial electron transport and oxidative phosphorylation as generally accepted for rat liver and kidney mitochondria. C_1-C_2-C_3 denote electron carriers at the coupling sites of the respiratory chain; I and X denote hypothetic energy-transfer carriers. The site and mode of action of the different inhibitors and uncouplers are indicated. αKG = α-ketoglutarate As = arsentate, CoQ = ubiquinone, Cyt = cytochrome, DCCD = dichlorocyclohexylcarbodiimide, DNP = 2,4-dinitrophenol, FPH_2 = succino and NADH dehydrogenases, NADH = nicotinamide adenine dinucleotides reduced, Pi = inorganic phosphate, Succi = succinate, tbt = tributyltin, TPPD = tetramethylparaphenylenediamine.

The inhibition of their activity by antimycin A or potassium cyanide is predicted by this hypothesis, if these drugs act at the level of $C_{1,2,3} \sim I$, as DNP is supposed to act ($C \sim I + \text{drug}_{\overrightarrow{\text{irreversible}}} + C + I + \text{drug}$), or at the level of $X \sim I$, as gramicidin or valinomycin are supposed to act.[34]

It is not possible from these observations to locate the exact site of action of the NSAI, but because most of them are lipid-soluble weak acids (p*K*a between 4 and 6), they might act in a manner similar to that of the better known uncoupling agents such as the halogenophenols and nitrophenols,[35] various derivatives of carbonyl-cyanide-phenylhydrazone,[36] and the substituted benzimidazoles.[37,38]

Mersalyl, a thiol inhibitor, has been described by Tyler[18,20] as an inhibitor of a phosphate transporter system into the inner membrane of the mitochondrion. This transporter system is probably altered by the formation of complexes between Mersalyl and the thiol groups of the inner membrane protein.

It seems that in the case of prevention of the accelerated respiration induced by NSAI, the phosphate transport system is not involved as far as the prevention by Mersalyl can be obtained in a phosphate-free as well as a phosphate-rich medium.

That thiol groups are of importance for the uncoupling activity of the NSAI is

suggested by the fact that cysteine is able to restore the uncoupling activity of the NSAI after the addition of Mersalyl. This activity of cysteine is concentration-dependent. The total restoration is observed when cysteine is equal to 6 equivalents of Mersalyl. This effect of cysteine in reversing the inhibition induced by Mersalyl is similar to those reported by Tyler,[18,20] except that in our experiments the phosphate transporter system seems of no importance.

The effect of Mersalyl on the uncoupling activity of NSAI is not overcome by DNP and not even by Janus Green B.[39] On the other hand, if DNP is present in the incubation medium, its uncoupling activity is not blocked by Mersalyl alone (at such a low concentration, 35 μM) or by an NSAI alone, but the presence of both these drugs in the medium, regardless of order of addition, inhibits the O_2 consumption.

These results differ from those described by Tyler[18,20] and also by Mockel and Dumont[40] who showed that DNP restored the O_2 consumption inhibited by Mersalyl when the uncoupling agent was calcium.

It is possible that the sites of action of DNP and NSAI on the mitochondria are closely related but not similar because, at the low concentration used in our experiments, Mersalyl inhibits only the NSAI activity, but when the NSAI occupy their sites and then are rendered inactive by Mersalyl, DNP can no longer uncouple because its sites might be now unavailable.

This effect of Mersalyl on DNP action or binding is also apparent when the sites are still presumably occupied by DNP, since the addition of NSAI and Mersalyl (but not each drug alone) inhibits the activity of DNP. A similar phenomenon was observed with valinomycin in place of NSAI.

The observed inhibition by Mersalyl with and without the presence of phosphate in the medium is also observed for the well known uncoupling agent,[41] valinomycin. The fact that here too the uncoupling activity is restored by cysteine suggests that thiol groups in the membrane are very important for this activity. These thiol groups may perhaps be located at the same site as those implicated in the NSAI uncoupling activity.

The uncoupling activity of valinomycin is related to its ability to induce an alkali flux (essentially K^+) through the mitochondrial membrane[21,22] in a manner similar to that of other antibiotics, like the macrotetralide actins,[22,42] or to the cyclic polyethers synthetized by Pedersen[43] that act as neutral carriers for cations (see review by Eisenman *et al.* [44]).

Nupercaine has been shown by Azzi and Scarpa[33] to inhibit specifically at low dose the K^+ transport into mitochondria, even in the presence of valinomycin. This is confirmed by the fact that we have found a slight inhibition by 0·6 mM nupercaine on the uncoupling activity induced by valinomycin.

The fact that we also found a slight (even more pronounced) inhibition with the same dose of nupercaine on the uncoupling activity induced by NSAI may be correlated with the existence of a common site and perhaps of a common mode of action (induced changes in the permeability of the mitochondrial membrane to the protons and the alkali cations) for valinomycin and NSAI.

These findings might finally suggest that, like valinomycin, the macrotetralid actins and the cyclic polyethers are neutral carriers, the NSAI could be *charged carriers* like some other molecules recently studied on artificial phospholipid bilayer membranes and that are also uncoupling agents[45–47] related to the salicylates.[48]

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