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UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY SOME SALICYLAMIDE DERIVATIVES

JEAN E. LEADER and M. W. WHITEHOUSE

Department of Biochemistry, University of Oxford

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Abstract—N-(2,6-dihydroxybenzoyl)-2- and 4-aminobenzoic acids, β- and γ-resorcylimides were prepared, characterized and tested for uncoupling activity.

O-acyl (and N-acyl) derivatives of salicylamide, of γ-resorcylamide and of picric acid uncoupled oxidative phosphorylation and were hydrolysed to the parent phenol by rat liver mitochondrial preparations: salicylamide and picric acid, added as such, had no effect on mitochondrial phosphorylation. N-salicyl-anthranilic acid and salicylimide were unique in their powerful uncoupling activity: the corresponding 2,4- and 2,6-dihydroxybenzoyl (β- and γ-resorcyl) derivatives were much less active in this respect.

These findings are discussed with respect to physico-chemical determinants of uncoupling activity, the concept of drug latentiation and the anti-inflammatory activity of 4-oxo-benz-1 : 3-oxazines (cyclic derivatives of salicylamide).

THERE is general agreement that salts of salicylic acid uncouple oxidative phosphorylation but salicylamide does not.[1–3] This distinction is important for understanding why salicylamide is of little value as an antirheumatic drug, although it is a useful analgesic.[3, 4]

It was noted previously that certain N-substituted and other acidic derivatives of salicylamide (e.g. salicylanilide and γ-recorcylamide) uncoupled oxidative phosphorylation both in a connective tissue (cartilage) and in isolated rat liver mitochondria.[3, 5] This report extends these observations and indicates that several acidic derivatives of salicylamide, notably salicylimides, exhibit uncoupling activity and might therefore be of value as potential non-analgesic drugs such as antirheumatic agents, anticoagulants or biocides—the pharmacological activity of which is probably, in part at least, dependent upon acidic properties and/or ability to uncouple oxidative phosphorylation.

EXPERIMENTAL

Methods

Oxidative phosphorylation in rat liver mitochondria, respiring on succinate in the presence of these drugs, was determined as described.[6] Compounds with marked uncoupling activity were also tested directly for their action upon the yeast hexokinase[7] used to trap the ATP (newly synthesized by the mitochondria).

Enzymic hydrolysis (deacylation) of drugs was determined as follows. Drugs (acylated salicylamides), usually 0·5 mM, were incubated for 30 min at 37° with 1 ml of the buffer–substrate–cofactor medium (used to determine P:O ratios), and either 2 ml mitochondrial suspension or 2 ml of the mannitol-sucrose medium[6] (in which mitochondria were normally suspended). Protein was precipitated with an

equal volume of ethanol and removed by centrifuging and the ethanolic supernatant examined by paper ionophoresis or chromatography on 3 mM paper in benzene-acetic acid-water (125 : 72 : 3, v/v).

pK_a's were determined either by spectrophotometric titration[5] or by measuring the pH when a solution of the drug in water or 50% (v/v) ethanol was half-neutralized with 40 mM sodium hydroxide (in water or 50% ethanol).

Partition coefficients were determined spectrophotometrically,[6] with the aqueous phase at pH 7.

Chemicals

Carsalam was donated by Dr. B. K. Martin (Aspro-Nicholas, Slough) and A.350 by Prof. E. Arrigoni-Martelli (Instituto Chemioterapico Italiano, S. Grato di Lodi, Milano, Italy). Other compounds were obtained from commercial supply houses and checked for purity, synthesized by standard procedures (as given in Beilstein's Handbuch etc.) or prepared and characterized as follows. Analyses were by Weiler and Strauss, Oxford [R_f values for paper chromatography in benzene-acetic acid-water, 125 : 72 : 3 (v/v) unless specified otherwise].

2,6-*dihydroxybenzoyl chloride* was prepared by adding 1·15 ml thionyl chloride to 2·5 g dried γ-resorcylic acid = 2,6-dihydroxybenzoic acid (Aldrich Chemical Co. Milwaukee, Wis, recrystallized from water, m.p. 167°) in 75 ml sodium-dried benzene. The mixture was refluxed for 5 hr then used immediately. When aqueous ammonia (sp. gr. 0·88) was added to a portion of this mixture, a substance m.p. 218–220° (d) was obtained identical with a commercial sample (Aldrich) of 2,6-dihydroxybenzamide m.p. 220–222°.

N-(2,6-*dihydroxybenzoyl*)-*anthranilic acid.* 6·74 g of sodium anthranilate in 25 ml dimethylformanide was added to 150 ml 2,6-dihydroxybenzoyl chloride solution and the mixture refluxed for 4 hr. The mixture was cooled and extracted with 100 ml 2N sodium hydroxide after adding 50 ml ether. The aqueous layer was diluted with 100 ml water and acidified to pH 3 with conc. hydrochloric acid to deposit an oil which spontaneously crystallized within a few days. Recrystallization from water gave a cream coloured product m.p. 224–226° ($C_{14}H_{11}NO_5$ requires C 61·4%, H 4·1%, N 5·1%; found C 61·0%, H 4·6%, N 4·9%). This product gave a purple-green coloration with ethanolic ferric chloride and a pink colour with the Ehrlich reagent (*p.* dimethylaminobenzaldehyde) in acetic anhydride. It chromatographed on paper with R_f 0·69 (anthranilic acid R_f 0·81; 2,6-dihydroxybenzoic acid, R_f 0·40) and on paper ionophoresis, was mobile at pH 7·0 but immobile at pH 2·7. Further confirmation of its structure was obtained by comparison of its u.v. spectrum with those of other compounds of similar structure (Table 1).

N-(2,6-*dihydroxybenzoyl*)-*p.aminobenzoic acid.* Using sodium *p.*aminobenzoic and the acid chloride as described above, yielded a product which was recrystallized from aqueous acetone, m.p. 302–304°(d) ($C_{14}H_{11}NO_5$ requires C 61·4%, H 4·1%, N 5·1%; found C 60·2%, H 4·1%, N 5·1%) R_f 0·56 (*p.*aminobenzoic acid, R_f 0·68), giving the same colour reactions with ferric chloride and the Ehrlich reagent, as were given by dihydroxybenzoyl-anthranilic acid. Further confirmation of its structure was provided by its u.v. spectrum (Table 1).

O-*acetyl-salicylamide*,[8] m.p. 138–140° (from ethyl acetate) gave no coloration with ethanolic ferric chloride.

N-*acetyl-salicylamide*,[8] m.p. 142–144° (from benzene) gave a reddish-brown coloration with ethanolic ferric chloride.

O-*benzoyl-salicylamide* was prepared by adding 1·5 ml benzoyl chloride to 1·37 g salicylamide in pyridine solution, heating; at 60° for 2 min, cooling and adding water. The product, recrystallized from benzene-petroleum ether (b.p. 60–80°, m.p. 140–142°), gave no coloration with ethanolic ferric chloride.

TABLE 1. ULTRA VIOLET SPECTRA OF IONISED AND UNIONISED FORMS OF SOME SALICYLANILIDE DERIVATIVES

Compound	Unionised form* (at mμ)			Ionised form† (at mμ)		
Salicylanilide	242	267	304	239	273	340
N-Salicyl-anthranilic acid	237	261	324	242(s)	275	342
N-Salicyl-*p*-aminobenzoic acid	237	294		246	290	346
γ-Resorcylanilide		273		233	288	342(s)
N-(γ-Resorcyl)-anthranilic acid		273	312	236	289	345(s)
N-(γ-Resorcyl)-*p*-aminobenzoic acid	236(s)	298		246(s)	301	348(s)
γ-Resorcylic acid		251	321	247	309	
Anthranilic acid	236	273		242	314	
p-Aminobenzoic acid	237	297			270	

Principal absorption maxima or shoulders (S) between 230 and 390 mμ.

(γ-resorcyl = 2,6-dihydroxybenzoyl).
* examined at pH's below 2.
† examined at pH 10.

N-*benzoyl-salicylamide*. O-benzoyl-salicylamide (1 g) was boiled with 50 ml water for 1 min. The product recrystallized from ethanol, m.p. 210° absorbed light at 317 mμ (unsubstituted phenol) and gave a reddish-brown coloration with ethanolic ferric chloride.

N-*acetyl*-2,6-*dihydroxybenzamide* was prepared by adding 2 ml acetic anhydride and 15 g ice to 1·53 g γ-resorcylamide (2,6-dihydroxybenzamide) in 5 ml 3N sodium hydroxide, shaking vigorously for 5 min and collecting the solid product. This was recrystallized from water m.p. 174–176°, ($C_9H_9NO_4$ requires C 55·4%, H 4·7%, N 7·2%; found C 55·3%, H 4·9%, N 7·0%). The compound gave a purple-green colour with ferric chloride (γ-resorcylamide, R_f 0·52 gives a blue colour) had an R_f 0·71 and was mobile on electrophoresis at pH 7 (unsubstituted resorcinol groups).

O,O′-*diacetyl*-2,6-*dihydroxybenzamide* was prepared by adding one drop of 72% (w/w) perchloric acid to a mixture of 1 g γ-resorcylamide and 1·5 ml acetic anhydride and warming at 60° for 5 min. The mixture was cooled, the product collected and washed with water. It was recrystallized from benzene, m.p. 160–162 ($C_{11}H_{11}NO_5$ requires CH_3CO 36·3%, found 35·0%). The product, R_f 0·80 gave no coloration with ethanolic ferric chloride and the u.v. spectrum (in methanol) also indicated no unsubstituted phenolic groups (peak at 208 mμ only).

O,O′,N-*triacetyl*-2,6-*dihydroxybenzamide* was prepared by heating 1·53 g γ-resorcylamide with 5·7 ml acetic anhydride and 0·28 g anhydrous sodium acetate under reflux for 1 hr. Water (20 ml) was added to the cooled mixture and the pink solid which separated was recrystallized from benzene, m.p. 124–126° ($C_{13}H_{13}NO_6$ requires CH_3CO 46·3%, found 45·5%). It give no coloration with ferric chloride.

O,O'-*dibenzoyl*-2,6-*dihydroxybenzamide* was prepared by warming 1·5 ml benzoyl chloride and 1·53 g γ-resorcylamide in pyridine solution at 60° for 2 min. The product which separated on adding water, was recrystallized from aqueous ethanol, m.p. 162–164°, R_f 0·91 ($C_{21}H_{15}NO_5$ requires C 69·8%, H 4·2%, N 3·9%; found C 69·1%, H 4·2%, N 3·7%). This compound gave no coloration with ethanolic ferric chloride and the u.v. spectrum (in methanol) showed no phenolic absorption at about 310 mμ (peaks only at 212 (shoulder) and 240 mμ).

O,N'-*dibenzoyl*-2,4-*dihydroxybenzamide* was prepared from β-resorcylamide by the method described above. The product crystallized from aqueous alcohol, m.p. 200–201°, ($C_{21}H_{15}NO_5$ requires C 69·8%, H 4·2%, N 3·9%; found C 69·9%, H 4·5%, N 3·6%), had R_f 0·87 (β-resorcylamide, R_f 0·33) and gave a reddish brown colour with ethanolic ferric chloride. Confirmation that only one (the *p*-) phenolic group was substituted was obtained from the u.v. spectrum (peaks at 218 (shoulder), 260 and 307 mμ).

Salicylimide (2,2'-*dihydroxydibenzamide*) was prepared by heating 7·5 g salicylamide with 7·75 g phosphorous pentoxide until the mixture turned orange. Water was then added and the mixture filtered. The product was washed with ether to extract unchanged salicylamide and recrystallized from ethanol, m.p. 208°, R_f 0·58 (salicylamide, R_f 0·76). It gave a reddish brown colour with ferric chloride.

2,2',6,6'*tetrahydroxydibenzamide* (γ-resorcylimide) was prepared by heating 4·6 g γ-resorcylamide with 15 g polyphosphoric acid for 24 hr at 100°. The resulting reddish-yellow viscous liquid was diluted with 40 ml hot water and filtered. The product was recrystallized from aqueous ethanol, m.p. 284–288°, R_f 0·82 ($C_{14}H_{11}NO_6$ requires C 58·2%, H 3·8%, N 4·9% found C 60·9%, H 3·7%, N 5·0%). It gave a brown coloration with ethanolic ferric chloride.

2,2',4,4'-*tetrahydroxydibenzamide* (β-resorcylimide) was prepared from β-resorcylamide by the procedure described above. It was recrystallized from water m.p. 308–310° after losing water of crystallization at 190° ($C_{14}H_{11}NO_6.2H_2O$ requires C 51·7%, H 4·6%, N 4·3%; found C 51·9%, H 4·6%, N 4·6%), and had a low R_f 0·05 (β-resorcylamide R_f 0·33) suggesting firmly-bound water. It gave a reddish coloration with ethanolic ferric chloride.

Hexafluorodiacetamide b.p. 744, 134–136° was prepared by refluxing trifluoroacetamide with trifluoroacetic anhydride.[9]

O-*acetyl-picric acid* was prepared by warming 1 g picric acid with 1·12 ml acetic anhydride in the presence of 1 drop of 72% perchloric acid at 60° for 15 min. The product was precipitated by the addition of 20 ml water and recrystallized as an almost colourless compound from ether m.p. 76°.

N-*acetyl- and* N-*benzoyl-picramic acids*,[10] were yellow solids, m.p. 204–206° and 225–226° respectively.

RESULTS*

Properties of salicylamides

Table 2, which combines some previous data[3, 5] with more recent results, shows that N-salicyl-anthranilic acid is exceptional in its powerful uncoupling action (i.e. ability to lower the P : O ratio). γ-Resorcyl-anthranilic acid was less potent an uncoupling

* "γ-Resorcyl" is used hereafter as a synonym for the 2,6-dihydroxybenzoyl radical and "β-resorcyl" for the 2,4-dihydroxybenzoyl radical.

agent than N-benzoylanthranilic acid. This finding is in sharp contrast to the fact that many other γ-resorcyl derivatives, including the amide and anilide, are more potent uncoupling agents than the corresponding salicyl compounds.[5] The low partition coefficients for γ-resorcyl-anthranilic acid together with its weak (phenolic) acidity, relative to γ-resorcylanilide, suggest why the acid is in fact only a weak drug *in vitro*; being neither a sufficiently strong phenol nor sufficiently lipophilic to compare with the *o*-hydroxybenzanilides (in drug activity).

TABLE 2. UNCOUPLING ACTIVITY AND OTHER PROPERTIES OF SOME SALICYLANILIDE DERIVATIVES

Compound	Phenolic pK_a (1% EtOH)	Conc. (10^{-4}M)	P/O ratio (as % control)	Partition coef.*	
				n-octanol	$CHCl_3$
None	—		100		
N-Benzoyl-anthranilic acid		1·0	72	2·2	0·32
		2·5	0		
N-Benzoyl-*p*-aminobenzoic acid		2·5	94		
Salicylanilide	7·5	0·6	60		23
N-Salicyl-anthranilic acid		0·2	75	5·5	0·55
		0·5	28		
N-Salicyl-*p*-aminobenzoic acid		1·5	100		
γ-Resorcylanilide	6·3	0·1	35	27	28
N-(γ-Resorcyl)-anthranilic acid	7·8	1·0	100	2·1	0·16
		2·5	75		
		5·0	25		
N-(γ-Resorcyl)-*p*-aminobenzoic acid	6·6	5·0	90	2·5	

* Between organic solvent and aqueous salt solutions, pH 7·0

In all instances, the N-acyl-*p*-aminobenzoic compounds were inactive when tested at concentrations at which the corresponding N-acyl-anthranilic acids exhibited uncoupling activity.

Properties of acyl-salicylamides (salicylimides)

Table 3 indicates that N-acyl derivatives of salicylamide (e.g. N-acetyl, N-benzoyl and N-salicyl = salicylimide itself) are able to uncouple mitochondrial phosphorylation in contrast to salicylamide itself (which is inactive in this respect). Salicylimide was the most active compound in this series. The increase in activity could, at first sight, be correlated simply with both the increase acidity of these compounds (salicylamide, p*K* 8·7; N-benzoyl-derivative p*K* 6·1, salicylimide p*K* 6·25 (in 33% ethanol)) and with their greater lipophilic character, indicated by their relative partition coefficients.

Surprisingly, it was found that the non-acidic O-acylated salicylamides (but not O-methyl derivatives) were also able to uncouple oxidative phosphorylation, although less effectively than the corresponding N-acyl derivatives. Examination of the incubation medium revealed that salicylamide was formed when both O- and N-acyl-salicylamides were incubated with mitochondria. The salicylate ion was present following incubations with salicylimide. The very similar uncoupling activity of the mono-, di- and tri-acetyl derivatives of γ-resorcylamide (Table 3) lent support to the

idea that the real uncoupling agent was the same in each case, probably γ-resorcylamide. This unsubstituted amide was always found in the medium, following incubation of mitochondria with any of these acetylated γ-resorcylamides, but it was not formed when these acetyl compounds were incubated either without mitochondria in the salt medium at pH 6·8 or with mitochondria previously denatured by heating.

TABLE 3. UNCOUPLING ACTIVITY AND OTHER PROPERTIES OF SOME SALICYLAMIDE AND SALICYLIMIDE DERIVATIVES

Compound	Conc. $\times 10^{-4}$M	P/O ratio (as % control)	Partition coef.* *n*-octanol	Partition coef.* $CHCl_3$
None	—	100		
Salicylamide (SAm)	25	100	7·8	3·1
O-Acetyl-SAm	10	80		
	25	0		
N-Acetyl-SAm	10	50		
	25	0		
O-Benzoyl-SAm	1	85		
	2·5	0		
N-Benzoyl-SAm	1	55	24	16
Salicylimide	1	0	83	7·3
γ-Resorcylamide (GRAm)	10	100	4·0	0·18
	25	8		
N-Acetyl-GRAm	2·5	100		
	5	7		
O,O′-Diacetyl-GRAm	2·5	100	0·24	2·6
	5	0		
O,O′,N,-Triacetyl-GRAm	2·5	92		
	5	0		
O,O′-Dibenzoyl-GRAm	1	95	4·2	18
	5	0		
γ-Resorcylimide	5	100	14	
β-Resorcylamide (BRAm)	25	95	0·7	0·04
O,N-Dibenzoyl-BRAm	2·5	97		56
	5	0		
β-Resorcylimide	5	95	7·3	

* Between organic solvents and aqueous salt solutions, pH 7·0

Spectrophotometric measurements showed that the degree of hydrolysis was approximately 50% after 30 min incubation of these acetates (0·5 mM) with rat liver mitochondria at 37°. Mitochondrial hydrolysis of these acyl (acetyl and benzoyl) derivatives was barely affected by inhibiting respiration with 1 mM cyanide, 1 mM deoxycholate or 50 mM malonate.

Both β and γ-resorcylimides were each devoid of significant uncoupling activity at concentrations at which they were freely soluble.

Properties of other compounds

A few other acidic imides, not related to salicylic acid, were also tested for uncoupling activity. They included phthalimide, its 4-nitro and 3,5-dihydroxy derivatives, and hexafluorodiacetamide (p*K* 3·4) : none displayed activity at mM concentrations (1 to 5 mM).

Picryl acetate uncoupled oxidative phosphorylation (Table 4) and also partly inhibited respiration. Picric acid had no effect on mitochondrial phosphorylation or respiration at the same concentration. Other labile phenol acetates (of *p*-nitrophenol, salicylamides) did not affect respiration at uncoupling concentrations, suggesting that the effect of picryl acetate on respiration was due to liberation of picric acid within the mitochondrion. Picryl acetate was hydrolysed at least three times as rapidly in the presence of mitochondria at pH 7 as in the absence of mitochondria.

N-acetylation of picramic acid (2-amino-4,6-dinitrophenol) considerably diminished its uncoupling activity (Table 4): N-benzoylation enhanced its uncoupling activity but the product was still less potent than 2,4-dinitrophenol as a drug *in vitro*.

TABLE 4. UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY SOME DERIVATIVES OF 2,4-DINITROPHENOL

Compound	Conc. ($\times 10^{-4}$M)	P/O ratio (% controls)
None	—	100
2,4-Dinitrophenol	0·5	0
Picric acid	2·5	100
Picryl acetate	2·5	50
Picramic acid	0·5	75
	1·25	25
N-Acetyl-picramic acid	2·0	100
	5·0	65
	10·0	0
N-Benzoyl-picramic acid	0·50	20
	1·25	0

Two cyclic derivatives of salicylamide (4-oxo-benz-1 : 3-oxazines, OBO), carsalam (2-oxo-OBO) and A.350 [I.C.I. 350; 2(β-chloroethyl)-2,4-dihydro-6-amino-OBO], did not uncouple phosphorylation at 2 mM.

Further observations. Apart from picryl acetate, none of the other potent uncoupling compounds mentioned in the three previous sections and Tables inhibited respiration coupled to succinate oxidation by more than 45 per cent or the yeast hexokinase reaction by more than 10 per cent.

In a few experiments, it was checked that phosphate uptake paralleled oxygen consumption throughout the normal duration of an incubation (15 min), both in the absence and presence of drugs (at levels uncoupling phosphorylation by not more than 60 per cent). It was therefore assumed in all further experiments that (any) phosphate uptake was proportional to oxygen uptake.

DISCUSSION

This attempt to enhance the uncoupling activity of each of the N-substituted anthranilyl[3] and γ-resorcyl[5] pharmacophores, by incorporating both in one molecule, was unsuccessful because the hybrid product (γ-resorcyl-anthranilic acid) was not sufficiently lipophilic to retain potent uncoupling activity. However, this product is approximately twice as potent as salicylic acid in suppressing mitochondrial phosphorylation. Likewise the attempt to improve upon salicylimide as an uncoupling agent, with the

resorcylimides, evidently failed for the same reason. A mixed imide, e.g. γ-resorcyl-salicylamide might perhaps be just sufficiently lipophilic to exhibit useful uncoupling activity.

It would seem that such attempts to chemically "cross-breed" proved uncoupling agents to develop new hybrid uncoupling drugs are generally not very successful: the failures, as illustrated above or by the nitrosalicylates,[3] outnumber the successes (such as N-salicyl-anthranilic acid and 3-hydroxycinchophene[3]). This has important theoretical implications concerning the mechanism of action of uncoupling agents of this type and confirms the impression that they do not act simply by sequestering essential metal ions,[3, 11] but rather, that they bind specifically to a protein receptor site within the mitochondrial lipid phase (probably the ε-amino group of a lysine residue[12, 13]) to exclude an essential intermediate (anion) from participating in the mitochondrial phosphorylation sequence.

Another important principle re-emerges from these observations that certain O-acylated phenols, which ought not to uncouple oxidative phosphorylation in the light of present theory,[3, 14, 15] yet do so although O-alkyl salicylamides do not.[3, 16] It is evident that such acyl derivatives are further examples of latent drugs[17] being hydrolysed to the active phenol *in situ*, but gaining access to their site of their action only because in their latent (masked) form they acquire the requisite degree of lipophilic character (not a property of salicylamide itself). Although many phenol esters are labile in aqueous media, in these experiments the hydrolysis of the salicylamide esters (and acyl salicylamides) was effected enzymically by the liver mitochondria preparations used to study phosphorylation. Abolishing the continuous production of hydrogen or hydroxyl ions (associated with electron transport) by respiratory inhibitors or detergents, did not affect mitochondria-dependent phenolic ester hydrolysis.

The superior uncoupling activity of salicylimide (compared with that shown by N-acetyl- and N-benzoyl-salicylamides) cannot be simply ascribed to its greater acidity but must be due, in part, to the fact that salicylimide hydrolyses within the mitochondrion to give two products—each of which is able to uncouple phosphorylation i.e. the salicylate ion and salicylamide. *In vivo*, salicylimide is not notably more active than sodium salicylate as an anti-inflammatory drug (Dr. B. K. Martin, personal communication), suggesting that it hydrolyses too rapidly *in vivo* (and not only within mitochondria?) to be significantly superior to the salicylate anion. Other N-acylamides would give only salicylamide and an acid devoid of uncoupling activity. These observations suggest that salicylamide possesses some (feeble) intrinsic uncoupling activity, although it is too hydrophilic to concentrate sufficiently within the mitochondrial lipid phase to normally exhibit this property. Other phenols with p*K*'s similar to that of salicylamide (p*K* 8·7) but with more lipophilic character, display weak uncoupling activity.[5, 11] Salicylamide itself exhibits feeble anti-inflammatory (anti-oedemic) activity in rats[18] and in view of its very rapid excretion (at least in man),[19] some of this pharmacological activity might be attributed to the amide itself, rather than to salicylic acid formed by hydrolysis *in vivo* (many anti-inflammatory drugs uncouple oxidative phosphorylation[4, 14]).

One corollary to these findings is that other phenols with sufficiently low p*K*'s, but little lipophilic character and normally devoid of uncoupling activity, should nevertheless be able to uncouple oxidative phosphorylation if presented to respiring mitochondria in a suitably labile and lipophilic form. This was dramatically demon-

strated with picryl acetate which uncoupled phosphorylation and was rapidly hydrolysed in mitochondrial incubations. Picric acid (2,4,6-trinitrophenol) itself is so highly ionised in aqueous solution (pK_a 0·3)[20] that it barely exhibits uncoupling activity.[3] The picryl acetate also inhibited respiration to some degree, probably due to some picric acid being bound within the mitochondrial lipid. These combined effects upon both mitochondrial phosphorylation and respiration indicate successful translocation of picric acid from the external aqueous phase to the drug-sensitive intramitochondrial sites, following acetylation and masking of the highly ionised phenolic group.

Another implication is that anti-inflammatory drugs such as aspirin or β-chloroethyl)-2,3-dihydro-4-oxo-6-aminobenz-1 : 3-oxazine (A. 350),[21] a cyclic derivative of salicylamide, which fail to uncouple oxidative phosphorylation in short term experiments *in vitro*, may nevertheless be latent uncoupling drugs *in vivo*. At least one 4-oxobenz-1 : 3 oxazine (Valmorin) has been shown to be metabolized *in vivo* to salicylic acid (via salicylamide?).[22]

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