Salicylanilides: A New Group of Active Uncouplers of Oxidative Phosphorylation

Abstract. *Investigations using housefly mitochondria revealed several salicylanilide derivatives as the most effective uncoupling agents of oxidative phosphorylation reported so far. Even greater inhibition of the associated P_i–adenosine triphosphate exchange was shown with these compounds in both housefly and rat-liver mitochondria, and close similarity was observed between the two types of organelles.*

Uncouplers and inhibitors of oxidative phosphorylation have a wide range of biocidal activities. Dinitro-*o*-cresol has been extensively used as an insecticide and herbicide, and pentachlorophenol and the trialkyltin derivatives have insecticidal, molluscicidal, herbicidal, and fungicidal properties (*1*). More recently, salicylanilides have been shown to possess fungicidal, bacteriostatic, cestocidal, and molluscicidal properties, and 5-chloro-2′-chloro-4′-nitrosalicylanilide has become an agent for schistosomiasis control (*2*). Whitehouse has shown that 0.1 m*M* salicylanilide and 0.02 m*M* *N*-salicyloyl anthranilate are uncouplers of oxidative phosphorylation in rat-liver mitochondria (*3*).

We now report a new series of salicylanilide derivatives that effect 50-percent inhibition of the P_i–ATP (adenosine triphosphate) exchange reaction in housefly and rat-liver mitochondria at concentrations of $10^{-9}M$, and appear to be the most effective uncouplers of oxidative phosphorylation yet recorded.

Mitochondria were obtained by homogenization of housefly thoraces or of rat liver in 0.25*M* sucrose plus 1m*M* EDTA and, after preliminary centrifugation at 600*g* to remove the nuclei and cellular debris, were centrifuged at 3000*g* (8000*g* for rat-liver mitochondria) at 0° to 4°C. Portion of the washed mitochondrial suspension was added to an incubation mixture consisting of 1-m*M* ATP, 1 m*M* KH_2PO_4, 15 m*M* KCl, 0.01 m*M* $MgCl_2$ and 2.5 m*M* EDTA in 50 m*M* tris-HCl buffer at *p*H 7.4. After 5 minutes for equilibration, the salicylanilide derivatives in dimethylformamide, at concentrations not exceeding 1 percent by volume, and approximately 500,000 counts of $H_3{}^{32}PO_4$ were added, and the mixture was allowed to incubate for 30 minutes at 25°C. Samples were then removed, and the P_i–ATP exchange was analyzed by the modified Berenblum-Chain extraction procedure of Walters and Cooper (*4*).

Oxygen uptake was measured manometrically for 60 minutes at 25°C, with each flask containing 1 m*M* adenosine diphosphate, 30 m*M* KH_2PO_4, 15 m*M* KCl, 2 m*M* EDTA, 5 m*M* $MgCl_2$, 15 m*M* pyruvate as substrate, 30 m*M* glucose, 200 units of hexokinase in 50 m*M* tris-HCl buffer at *p*H 7.4, and mitochondria from ten housefly thoraces. The reaction was stopped with trichloroacetic acid, and inorganic phosphate was determined by the method of Fiske and Subbarow (*5*).

Adenosine triphosphatase activity was determined by incubation of mitochondria from 20 housefly thoraces for 30 minutes at 25°C in flasks containing 10 m*M* ATP and 50 m*M* tris-HCl buffer at *p*H 7.4 in a total volume of 2.0 ml. Inorganic phosphate was determined by the method of Fiske and Subbarow and corrected for nonenzymic hydrolysis.

For both the P_i–ATP exchange reaction and the oxidative phosphorylation experiments, the values for percentage inhibition were plotted versus the negative logarithm of the inhibitor concentration, and the pI_{50} values were determined by inspection of the straight-line portions of the curves. Each curve was determined from a minimum of five concentrations of inhibitor, with each experiment performed twice. These values are presented (Table 1) with the pI_{50} values obtained in our laboratories of other uncouplers and inhibitors of oxidative phosphorylation.

The salicylanilide derivatives III–XIII (Table 1) were powerful inhibitors of the P_i–ATP exchange and clearly affected oxidative phosphorylation at lower concentrations than did the nitrophenols XVI and XVII (Table 1), substituted trifluoromethylbenzimidizide XIV (Table 1) (*6*), carbonyl cyanide *m*-chlorophenylhydrazone XV (Table 1) (*7*), oligomycin XIX (Table 1) (*8*), and the trialkyltins XVIII (Table 1) (*9*).

The P_i–ATP exchange reaction was more susceptible to the action of salicylanilides than was the overall phosphorylation sequence. These results are partially explained by the findings of Löw *et al.* (*10*) who reported that the P_i–ATP exchange was mainly associated with the first phosphorylating site of the respiratory chain, and was more labile

Table 1. Effects of salicylanilides and other compounds on mitochondrial oxidative phosphorylation and the associated P_i–ATP exchange. Mean specific activities of uninhibited P_i–ATP exchange reactions: housefly and rat-liver mitochondria, 1.93 and 0.66 μmoles of $^{32}P_i$ incorporated per milligram of protein per 30 minutes at 25°C, respectively. pI_{50}, negative logarithm of the molar concentration producing 50-percent inhibition.

Compound	P_i–ATP exchange (pI_{50})		Oxidative phosphorylation (pI_{50})*, uptake	
	Housefly	Rat liver	Oxygen	Phosphate
I. Salicylanilide	4.97	4.88	4.33	4.44
II. 3-OH,2-naphthylenecarboxanilide	6.44	6.35	5.72	5.78
III. 5-Cl,2′-Cl,4′-NO_2-salicylanilide	7.59	7.69	6.48	6.64
IV. 5-Cl,3-phenyl,2′,5′-diCl-salicylanilide	7.90	7.84	6.52	6.67
V. 5-Cl,3-phenyl,3′,4′-diCl-salicylanilide	8.03	7.81	6.44	6.66
VI. 5-Cl,3-(*p*-Cl-phenyl), 4′-Cl-salicylanilide	8.03	7.60	6.48	6.60
VII. 5-Cl,3-phenyl,2′,4′,5′-triCl-salicylanilide	8.42	8.19	7.10	7.26
VIII. 5-Cl,3-phenyl,3′-Cl,4′-NO_2-salicylanilide	8.56	8.21	6.94	7.20
IX. 5-Cl,3-(*p*-Cl-phenyl),2′-Cl,5′-NO_2-salicylanilide	8.75	8.58	7.23	7.42
X. 5-Cl,3-(*p*-Cl-phenyl),2′,4′,5′-triCl-salicylanilide	8.76	8.76	7.48	7.62
XI. 5-Cl,3-(*p*-Cl-phenyl),2′-Cl,4′-CN-salicylanilide	8.85	8.66	7.88	7.97
XII. 5-Cl,3-(*p*-Cl-phenyl), 2′-Cl,5′-CF_3-salicylanilide	9.08	8.70	7.42	7.58
XIII. 5-Cl,3-*t*-butyl,2′-Cl,4′-NO_2-salicylanilide	9.14	8.77	8.27	8.34
XIV. 4,5,6,7-TetraCl,2-CF_3-benzimidizide	7.06		5.87	6.24
XV. Carbonyl cyanide *m*-Cl-phenylhydrazone	7.30		6.48	6.88
XVI. 2,4-Dinitrophenol	4.72		3.93	4.13
XVII. 4,6-Dinitro-*o*-cresol	5.50		4.59	4.88
XVIII. Tributyltin chloride	7.04		6.14	6.07
XIX. Oligomycin *a* + *b*	7.32		6.62	6.59

* Housefly mitochondria only; mean control P:O value, 2.4.

Table 2. Effects of salicylanilides and dinitrophenol on housefly mitochondrial adenosine triphosphatase (ATPase). Each value is the average result from two experiments. Mitochondrial protein, 2.2 mg per flask.

Compound (molar conc.)	Oligomycin (2 μ per flask)	ATPase activity (P_i, μmole/30 min)
Control	−	3.83
	+	0.41
Dinitrophenol (10^{-4})	−	17.50
	+	0.69
IX (10^{-7})	−	17.25
	+	0.72
X (10^{-7})	−	17.25
	+	0.69
XI (10^{-7})	−	17.80
	+	0.75
XII (10^{-7})	−	17.80
	+	0.75
XIII (10^{-8})	+	12.05
	−	0.69
XIII (10^{-7})	−	18.34
	+	0.75

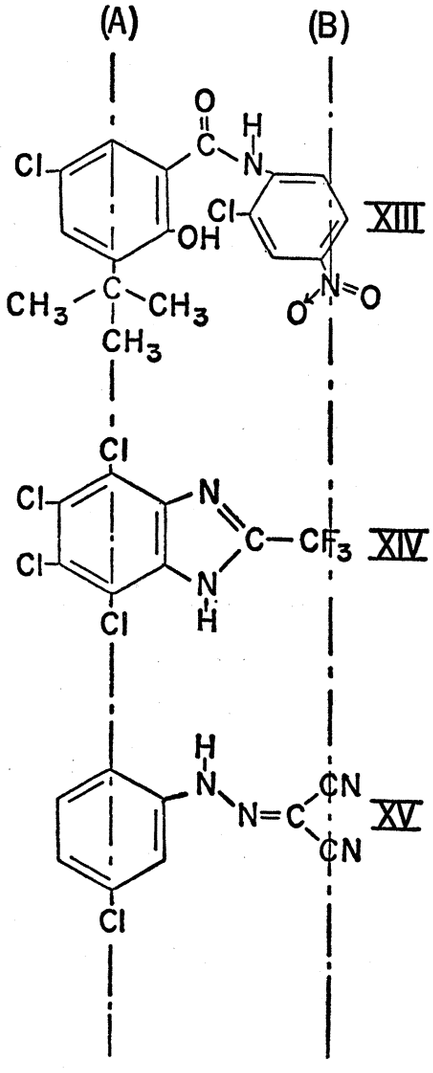

Fig. 1. Structural similarities among various uncouplers of oxidative phosphorylation. (A) Represents the plane of symmetry through the halogenated aromatic rings and (B) is the corresponding plane of symmetry through the electron withdrawing groups.

in many respects than the overall phosphorylating sequence.

Although the salicylanilides inhibit pyruvate oxidation in housefly mitochondria, they are categorized as uncoupling agents on the ground that adenosine triphosphatase, stimulated by the salicylanilides, is inhibited by oligomycin. Table 2 compares the salicylanilides with 2,4-dinitrophenol in effect on adenosine triphosphatase activity of housefly mitochondria in the presence and absence of oligomycin. In this capacity the salicylanilides are shown to be approximately 1,000 to 10,000 times more effective than dinitrophenol, which finding agrees favorably with the relative effectiveness of these compounds on the P_i–ATP exchange and oxidative phosphorylation.

Although the mode of action of these various uncouplers of oxidative phosphorylation is still obscure, they appear to possess certain common structural features. Inspection of molecular models reveals the presence of strong electron-withdrawing centers, such as NO_2, CN, or CF_3, located within a certain spatial distance from a halogenated aryl ring (Fig. 1). These common features and the greatly enhanced effect on oxidative phosphorylation and the related P_i–ATP exchange, resulting from the attachment of an additional bulky group such as naphthyl (II), biphenylyl (IV–XII), or *t*-butyl (XIII), suggest that inhibition results from preferential adsorption at an active site on the enzyme surface.

The close correspondence, in inhibition of the P_i–ATP exchange by the salicylanilide derivatives, between housefly and rat-liver mitochondria demonstrates the essential similarity of oxidative phosphorylation in vertebrates and invertebrates. These relatively simple and extremely active salicylanilides should prove to be useful tools for further investigation of this fundamental process.

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Nucleotide Sequence of KB Cell 5S RNA

Abstract. *The nucleotide sequence of 5S RNA derived from KB carcinoma cell ribosomes has been determined. The molecule has a length of either 120 or 121 nucleotides with uridine at its 3′-terminus and guanylic acid at its 5′-terminus. If, in addition to Watson-Crick base-pairing, one accepts occasional base-pairing of guanylic acid to uridylic acid, long sequences of complementary nucleotides can be identified within the molecule. Two regions of the molecule contain sequences complementary to four or five bases in the pentanucleotide sequence guanylic acid, ribothymidylic acid, pseudouridylic acid, cytidylic acid, guanylic acid, which is common to most transfer RNA molecules. This is the first time the sequence of an animal-cell RNA has been determined.*

In many cell species, the ribosomes contain a low-molecular-weight ribonucleic acid (*5S* RNA) whose function is unknown (*1*, *2*). The introduction by Sanger *et al.* of an improved procedure for the separation and identification of P^{32}-labeled oligonucleotides by two-dimensional paper electrophoresis (*3*) has facilitated the study of the primary structure of *5S* RNA and other low-molecular-weight homogeneous RNA species. Enzymatic digests of *5S* RNA yield only a small number of oligonucleotides that are specific for the cell species of its origin and are present in quantities, which suggests that *5S* RNA has a single nucleotide sequence, or a limited number of closely related sequences (*4*–*6*).

We used this oligonucleotide frac-