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BIOCHEMICAL PROPERTIES OF ANTI-INFLAMMATORY DRUGS—III.

UNCOUPLING OF OXIDATIVE PHOSPHORYLATION IN A CONNECTIVE TISSUE (CARTILAGE) AND LIVER MITOCHONDRIA BY SALICYLATE ANALOGUES: RELATIONSHIP OF STRUCTURE TO ACTIVITY

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Abstract—At low drug levels, sodium salicylate and other drugs which uncouple oxidative phosphorylation in liver and muscle mitochondria selectively inhibit (i) the incorporation of inorganic phosphate into organic phosphates and (ii) the incorporation of inorganic sulphate into polysaccharide sulphates by bovine cartilage *in vitro*, without inhibiting the oxidation of glucose or octanoate by this tissue. Processes (i) and (ii) are also inhibited by respiratory inhibitors (nitrogen atmosphere, cyanide). It is concluded that salicylate will uncouple oxidative phosphorylation in connective tissues, as well as in muscle, kidney, liver etc.

The relationship of chemical structure to activity in uncoupling phosphorylation in cartilage and in rat liver mitochondria, was studied for 80 salicylate analogues. The partial structures given in figure 1 were essential for uncoupling activity: within this structural requirement, potency was governed by lipophilic character.

The following compounds were notably more active than salicylate *in vitro*: *o*.hydroxynaphthoates, phenylsalicylates, 2-mercaptobenzoate and lipophilic N-substituted derivatives of PAS, salicylamide and anthranilic acid. N-Salicyloylanthranilate, 3,5-diiodosalicylate and 5-phenylazosalicylate were the most active drugs *in vitro* uncovered in this survey. Nitrosalicylates were much less active than the corresponding nitrophenols.

SALTS of salicylic acid and certain other *ortho* hydroxybenzoyl derivatives exhibit anti-inflammatory properties and are employed extensively for the treatment of rheumatic diseases. At least part of the response to these drugs must involve their action upon the connective or supportive tissues of the body. In previous studies[1–3] of salicylate action *in vitro*, circumstantial evidence was obtained that salts of salicylic acid at concentrations of 2mM or greater, uncoupled oxidative phosphorylation in cartilage, cornea and heart valves. Several research workers have described the uncoupling of oxidative phosphorylation in isolated liver or muscle mitochondria by 1–5mM salicylate[4–10] and compared its action with that of some other phenols, notably 2,4-dinitrophenol, which also uncouple oxidative phosphorylation.[4, 7, 8, 10]

This report provides further evidence that salicylates do indeed uncouple phosphorylation from cellular oxidation in cartilage tissue. The activity of many salicylate analogues as 'uncoupling agents' influencing cartilage metabolism has been compared with their potency in uncoupling oxidative phosphorylation in liver mitochondria. Several analogues of salicylic acid, which were not studied by previous

workers, are included in this survey. Some of these findings were presented to the recent Symposium on Salicylates.[11]

EXPERIMENTAL

Materials

N-cyclohexyl-salicylamide was prepared by heating methyl salicylate with 2 volumes of cyclohexylamine under an air condenser for 2 hr at 160°. After removal of excess amine with dilute hydrochloric acid, the product was recrystallised from benzene–*n*-hexane as white feathery needles, m.p. 84° (Ref. 12 gives 85–86°).

Several compounds were kindly donated by Dr. B. K. Martin (Aspro-Nicholas Ltd., Slough), Mr. D. J. Drain (Smith and Nephew Research Ltd., Hunsdon), Dr. S. S Adams, (Boots Pure Drug Co. Ltd., Nottingham). Dr. G. W. Cheeseman (Queen Elizabeth College, London), Dr. Idris Jones (Chemical Research Laboratory, D.S.I.R., Teddington) and Dr. J. L. Gorringe (Parke, Davis and Co., Hounslow). Other salicylate derivatives were synthesised by standard procedures or obtained from commercial supply houses. These compounds were checked for authentic m.p. and where necessary, were further purified by distillation, sublimation or recrystallisation.

Tracheas from 2-year-old cattle were obtained immediately after slaughter and brought to the laboratory packed in ice. Tracheal cartilage was dissected from the accompanying muscle, fat and loose connective tissue and mechanically sliced to a thickness of 0·5 mm (±0·05 mm) with a modified McIlwain tissue chopper. These cartilage slices were kept in chilled 0·9% (w/v) sodium chloride until disposed amongst the incubation flasks.

Biochemical procedures

Oxidative phosphorylation in rat liver mitochondria was measured by standard procedures[9] at 30° with succinate as substrate. Isolated mitochondria were re-suspended in a medium containing 0·21 M mannitol and 0·07 M sucrose. 2 ml of this suspension were added last to a medium containing 0·5 ml 0·1 M sodium phosphate, pH 6·8, 0·1 ml 0·3 M sodium succinate, pH 6·8, 0·1 ml 0·25 M potassium chloride, 0·1 ml 0·025 M adenosine triphosphate (ATP)*, 0·1 ml 0·3 M glucose, 0·05 ml 0·3 M potassium fluoride, 0·05 ml 0·3 M magnesium chloride and 0·1 ml distilled water or aqueous drug solution contained in the main compartment of a Warburg cup: 10μg (approx. 300 Kunitz-McDonald units) of crystalline yeast kexokinase, (Boehringer and Soehne, Mannheim) contained in 0·2 ml 1% (w/v) aqeous glucose was added to the side arm of each Warburg vessel. Oxygen uptake was measured manometrically for a period not exceeding 25 min. Hydrolysis of ATP by liver mitochondria was, measured at 20° in Tris hydrochloride buffer, pH 7·4.[13]

Cartilage slices equivalent to approx. 50 mg dry weight were incubated with sodium sulphate-^{35}S ($^{35}S_i$) for 3–5 hr at 37° with shaking in a modified Krebs-Ringer phosphate medium.[1] Radioactivity incorporated into the cartilage mucopolysaccharide sulphates (PS) was measured after papain digestion of the washed and dried incubated slices and fractionation of the digests with Rivanol.[2]

* Abbreviations used throughout the text are ATP = adenosine-5′-triphosphate; S_i = inorganic sulphate; PS = polysaccharide sulphates; P_i = inorganic phosphate; P_{org} = organic phosphates Rivanol (Ethodin) = 6,9-diamino-2-ethoxyacridinium lactate.

Cartilage slices were also incubated for at least 3 hr with sodium orthophosphate-^{32}P ($^{32}P_i$) in a Krebs–Ringer medium constituted with 0·15 M Tris hydrochloride, pH 7·4 in place of the conventional buffers. Washed and dried cartilage slices were digested overnight with papain in 0·5 ml of 0·04 M potassium phosphate buffer, pH 6·4 at 45° to release non-protein constituents. Inorganic phosphate (and $^{32}P_i$) was precipitated at 4° by adding 0·2 ml of a mixture, pH 9·2 containing 5·5% (w/v) $MgCl_2.6H_2O$ and 10% (w/v) NH_4Cl dissolved in 1·5 M ammonium hydroxide. ^{32}P remaining in the supernatants after centrifugation was assayed either by drying 100-μl aliquots on sand-blasted aluminium discs and counting with an end-window Geiger-Muller tube or by adding 50-μl aliquots to 5 ml 'Diotol'[2] for liquid scintillation counting.

The quantity of cartilage slices taken for each incubation was determined either by (i) direct weighing of the dried slices after incubation, or (ii) turbidometric determination of the polysaccharide sulphates liberated by papain digestion, with Rivanol,[1] or (iii) colorimetric determination of the polyuronides (chondroitin sulphates) after papain digestion of the cartilage slices, by the following procedure. 50-μl Aliquots of the papain digests were heated for 40 min in a boiling water bath with 2·5 ml of a reagent composed of 0·13% (w/v) recrystallised orcinol and 0·004% $CuCl_2.2H_2O$ dissolved in 7N hydrochloric acid. The green colour which developed was extracted into 5 ml isoamyl alcohol. The alcohol layer was clarified by centrifugation and its optical density measured at 660 mμ after standing for 24 hr.

All measurements of ^{32}P and ^{35}S incorporation into cartilage slices in parallel incubations, were then corrected for variation in the quantity of cartilage between the different incubation flasks.

Drug action in vitro

Drugs were added to incubation flasks from stock solutions prepared in water, 20% (v/v) aqueous dimethylformanide (DMF) or 100% DMF. Stock solutions in 100% DMF were discarded after 2 days (kept at 4°). Aqueous drug solutions were first neutralised with the theoretical equivalent of sodium bicarbonate. Equivalent quantities of sodium bicarbonate were added directly to the incubation medium before the addition of acidic drugs dissolved in 100% DMF, if the final drug concentration exceeded 0·2 mM. Where appropriate DMF alone was added to the drug-free control incubations at concentrations not exceeding 1·5% (v/v) (normally 0·75% for mitochondrial incubations and 0·5% for cartilage incubations).

Mitochondria or cartilage slices were added last of all to each incubation flask after the pH of their contents had been checked.

Drug action on cartilage slices was assessed by comparing the radioactivity of the $^{32}P_{org}$ and PS-^{35}S fractions from drug-incubated slices with the radioactivity of the same fractions from slices incubated without drugs. Values quoted in Tables 1–3 represent the arithmetic mean of duplicate radioactivity assays (agreeing within 8%) for $^{32}P_{org}$ or PS-^{35}S from one of a pair of simultaneous incubations containing the same level of a given drug, where the values for $^{32}P_{org}$ or PS-^{35}S from each of these duplicate incubations agreed within 10%. All values in the Tables (1–6) from experiments with drugs are expressed as the percentage of the corresponding mean values of $^{32}P_{org}$, PS-^{35}S or the phosphorylation quotient (P/O ratio) from at least two drug-free controls.

RESULTS

Evidence for oxidative phosphorylation in cartilage

Cartilage slices incubated with sodium orthophosphate-^{32}P ($^{32}P_i$) incorporated radioactivity into a fraction which was not precipitated by magnesium and ammonium ions at an alkaline pH in the presence of excess (non-radioactive) inorganic phosphate. After 4 hr incubation at 37° with $^{32}P_i$, approximately 12–20% of the radioactivity released from the washed cartilage slices by papain digestion was not precipitated with the P_i and rerepresents organic phosphates-^{32}P ($^{32}P_{org}$). Much of the radioactivity of this organic fraction was precipitated with organic bases (Proflavine, Rivanol, cationic detergents) at pH 7 and by trichloracetic acid at 4°. The proportion of the $^{32}P_{org}$ fraction which was acid-soluble varied in different experiments between approximately 40 and 80% of the total $^{32}P_{org}$.

TABLE 1. EFFECT OF RESPIRATORY INHIBITORS AND UNCOUPLING AGENTS ON THE INCORPORATION OF (a) $^{32}P_i$ INTO ORGANIC PHOSPHATES (P_{org}) AND (b) $^{35}S_i$ INTO POLYSACCHORIDE SULPHATES (PS) BY TRACHEAL CARTILAGE SLICES.

Radioactivities of the P_{org} and PS fractions expressed as percentage of values in the controls (no additions).

Addition	Conc. (mM)	$32_{P_{org}}$ (%)	PS-^{35}S (%)
None	—	100	100
Nitrogen atmosphere	—	26	34
Iodoacetate	4	0	0
Cyanide	0·1	80	82
Glucose	10	155	140
Salicylate	4	45	22
4-Hydroxybenzoate	4	105	95
2,4-Dinitrophenol	0·05	65	53
2,5-Dinitrophenol	0·05	94	83
Phenylbutazone	0·5	35	34
Glycyrrhetate	0·05	20	15

^{32}P incorporation into the P_{org} fraction was proportional to the length of the incubation period up to 5 hr. ^{32}P incubation was much reduced when the slices were incubated at 15° or in an atmosphere considerably enriched with nitrogen (Table 1). Inhibitors of aerobic cellular oxidation (cyanide, fluoroacetate, iodoacetate, malonate) also depressed this incorporation of ^{32}P into the P_{org} fraction.

Both 2,4-dinitrophenol (50 μM) and sodium salicylate (4mM) which uncouple oxidation phosphorylation in liver, brain and muscle mitochondria, inhibited ^{32}P metabolism in the cartilage slices. At the same concentrations their respective isomers, 2,5-dinitrophenol and 4-hydroxybenzoate had very little effect upon ^{32}P incorporation. These latter phenols are virtually inactive in uncoupling oxidative phosphorylation in liver mitochondria. At these concentrations, neither 2,4-dinitrophenol or salicylate inhibits the oxidation of glucose-^{14}C, acetate-^{14}C or octanoate-^{14}C by cartilage slices.[2]

Non-phenolic drugs known to uncouple oxidation phosphorylation in liver mitochondria such as phenylbutazone[14] and salts of glycyrrhetic acid also inhibited ^{32}P incorporation into the P_{org} fraction of cartilage.

All the reagents which inhibited $^{32}P_i$ incorporation, also inhibited the incorporation of $^{35}S_i$ into the cartilage polysaccharide sulphates (Table 1) which is an endergonic process requiring ATP.[16]

These findings indicate that drugs which uncouple oxidation phosphorylation (i.e. ATP synthesis) in liver and heart muscle preparations, can also selectively inhibit phosphate metabolism in a connective tissue without impairing the cellular oxidative processes. These results do not exclude the possibility that $^{32}P_i$ is also metabolised by anaerobic pathways,[17] which might be expected to be insensitive to uncoupling agents.

Inhibition of cartilage phosphorylation and uncoupling of oxidative phosphorylation in liver slices by salicylate analogues

Tables 2 and 3 show the relative activities of some hydroxybenzoyl derivatives compared with sodium salicylate, as measured by their effect on (i) ^{32}P incorporation into the organic phosphate fraction and ^{35}S incorporation into the polysaccharide sulphates by cartilage slices *in vitro*, and (ii) the phosphorylation quotient (P/O) ratio of isolated rat liver mitochondria respiring in the presence of succinate. The P/O ratio

TABLE 2. EFFECT OF SOME SALICYLATE ANALOGUES ON THE METABOLISM OF CARTILAGE SLICES AND LIVER MITOCHONDRIA.

All values expressed as percentage of $^{32}P_{org}$, PS-^{35}S, P/O ratio or ATP-ase activity of controls without drugs.

Compound	Conc. (mM)	Cartilage		Liver mitochondria	
		$32P_{org}$ (%)	PS-^{35}S (%)	P/O* (%)	ATP-ase (%)
None	—	100	100	100*	100
Salicylate	2·5	60	65	0	1210
	0·5	90	90	60	710
3-Hydroxynaphth-2-oate	0·5	7	12	0	1260
6-Hydroxynaphth-2-oate	0·5	98	100	92	97
3-Phenylsalicylate	0·5	12	15	0	695
0-Acetyl-Salicylate	2·5	93	81	75	560
0-Acetyl-3-Phenylsalicylate	0·5	84	82	20	710
Salicylamide	2·5	96	100	96	113
Salicylanilide	0·1	42	45	40	1280
Gentisate	2·5	97	98	100	124
Gentisaldehyde	0·4	65	45	56	510

* P/O ratio (succinate) was in the range 1·35–1·70 for the drug-free controls.

without added drugs was not closely reproducible from one mitochondrial preparation to another but varied within the range 1·2–1·7. The degree of uncoupling by a given concentration of sodium salicylate was remarkably constant between different mitochondrial preparations, even though the P/O ratios in the absence of salicylate varied so widely. Experiments with six different preparations of mitochondria to measure the uncoupling by 0·5 mM salicylate gave P/O ratios with salicylate which were 61%, 46%, 60%, 65%, 68% and 60% respectively of the P/O ratios of the controls (with 0·5 mM sodium benzoate or without any additions).

Table 2 also records the relative activity of the (latent) mitochondrial ATP-ase

TABLE 3. EFFECT OF SOME FURTHER SALICYLATE ANALOGUES ON THE METABOLISM OF CARTILAGE AND ON OXIDATIVE PHOSPHORYLATION IN LIVER MITOCHONDRIA.

$^{32}P_{org}$, PS-^{35}S and P/O ratio are expressed as percentage of these same values in controls incubated without drugs.

Compound	Cartilage			Liver	
	Conc. (mM)	$^{32}P_{org}$ (%)	PS-^{35}S (%)	Conc. (mM)	P/O (%)
Salicylate	3·5	60	47	0·5	65
2,3-Dihydroxybenzoate	3·5	81	70	2·5	74
2-Hydroxyphenylacetate	3·5	92	86	3	49
Melilotate	3·5	108	100	3	70
Hexahydrosalicylate	3·5	97	105	3	97
3-Methylsalicylate	3·5	40	18	0·5	39
4-Methylsalicylate	3·5	46	27	0·5	52
5-Methylsalicylate	3·5	34	25	0·5	47
2-Mercaptobenzoate	3·5		37	0·1	38
Salicylaldehyde	0·5	46	32	0·5	80
2-Acetoxybenzaldehyde	0·5	44	25	0·5	76
2,4-Dihydroxybenzaldehyde	0·5	30	36	0·5	68
2,4-Dimethoxybenzaldehyde	1·0	92	100	0·5	94

when fresh mitochondrial preparations were incubated with added ATP in the presence of salicylate or some of its analogues. Each of these four independent measurements of cartilage and liver mitochondrial metabolism, as influenced by some of these salicylate analogues, revealed the same order of relative activity amongst these diverse compounds as (i) an inhibitor of P_i and S_i incorporation and (ii) an activator of mitochondrial ATP-ase. Thus it appears that the salicylate ion and each of these potent salicylate analogues uncouples oxidative phosphorylation in cartilage (and thereby inhibits ATP-dependent processes such as polysaccharide sulphate biosynthesis) in a similar manner to their action upon coupled oxidation phosphorylation within liver mitochondria.

Oxidative phosphorylation in liver mitochondria was generally more sensitive to these compounds, being inhibited by lower concentrations of salicylate or its analogues, than the phosphorylation process(es) in cartilage tissue. The principal exceptions to this generalisation were *o*-hydroxyarylaldehydes which, relative to the parent *o*-hydroxyacid, were more potent inhibitors of cartilage metabolism than of oxidative phosphorylation in liver mitochondria (also see below).

Relationship of uncoupling activity to chemical structure amongst salicylate analogues

N.B. Throughout this discussion and in Tables 2–6, "salicylate" refers to the ionised form of salicylic acid or its derivatives and not to esters of salicylic acid unless specified.

(i) Effects of increased hydrophilic character:

2,3-Dihydroxybenzoate is the only isomer of the four monohydroxysalicylates which has any effect upon cartilage metabolism.[1] It is also the only isomer which uncouples oxidative phosphorylation in liver mitochondria.[4] In each respect it is much less active than salicylate. This point was re-examined in view of the fact that com-

mercial preparations of dihydroxybenzoates often contain some salicylate.[18] It was found that chromatographically pure 2,3-dihydroxybenzoate had uncoupling activity only approximately 20% that of salicylate (Table 3).

The following sodium salts each uncoupled oxidative phosphorylation in liver mitochondria and inhibited cartilage phosphorylation by no more than 15% at 3 mM: salicylurate (salicyloylglycine), 6-methoxysalicylate, 5-sulphosalicylate, 4- and 5-aminosalicylates, 2- and 4-hydroxyisophthalates and *o*-hydroxynicotinate (2-hydroxypyridine-3-carboxylate). Thus an increase in the molecular charge or water solubility effectively abolished drug activity (see also section (iii) below).

(ii) Effect of increased lipophilic character:

Nuclear-substituted salicylates which are less water-soluble than salicylate were more active drugs *in vitro* than salicylate itself. The following evidence suggests that this greater drug activity was associated primarily with a general increase in lipophilic character rather than a more specific activation of either the phenol or carboxylate groups.

(a) Isomeric methylsalicylates (Table 3), phenylsalicylates and *o*-hydroxynaphthoates (Tables 2 and 4) were approximately equipotent; the positions of the substituents were relatively unimportant in determining the drug activity (compared with that of salicylate).

(b) Drug activity increased with increasing hydrophobic character of the nuclear substituents i.e. methyl < phenyl < phenylazo and bromo < iodo, and not according to the relative magnitudes of the inductive effect of each substituent.

(c) *o*-Hydroxynaphthoates ($C_{11}H_7O_3^-$) were less potent than phenylsalicylates ($C_{13}H_9O_3^-$) suggesting that the proportion of carbon to oxygen in the molecule, rather than (conjugated) aromatic character, governed drug potency in this instance.

(d) Abolition of the hydrophilic carboxylate ion in several instances enhanced uncoupling activity, e.g. *o*-hydroxyarylaldehydes, salicylanilide (Table 2). It was found that 2,4- and 2,5-dihydroxybenzaldehydes uncouple oxidative phosphorylation at 0·5 mM, whereas their parent acids (β-resorcylic, gentisic) are inactive at 2·5 mM.[1, 4]

Salicylaldehyde, *o*-vanillin (2-hydroxy-3-methoxybenzaldehyde) and *o*-hydroxynaphthaldehydes were each more potent as inhibitors of cartilage metabolism than salicylate, *o*-vanillate and the *o*-hydroxynaphthoates respectively but none of these aldehydes was appreciably more active than the parent acid in uncoupling oxidative phosphorylation in liver mitochondria. This discrepancy in the relative drug activities between aldehyde and acid according to the tissue substrate, could not be wholly attributed to mitochondrial oxidation of aldehydes to the (less active) acids: a considerable proportion (at least 50%) of the added aldehydes could be recovered from incubations with respiring mitochondria (for 30 min at 30°). (These recovered aldehydes were identified by their u.v. absorption and ionophoretic mobility at pH 7 and 9 on paper, and quantitated spectrophotometrically).

(iii) Lipophilic/hydrophilic character as a determinant of drug activity:

Table 4 shows two examples where highly active (lipophilic) salicylate anlogues were rendered much less active by introduction of a hydrophilic group into the molecule viz. an amino group into 3,5-diiodosalicylate and an extra (non *ortho*) phenolic group into 3-hydroxy-2-naphthoate. Conversely, decreasing the hydrophilic character of 4-aminosalicylate (inactive at 2 mM[4]) by N-acylation, N-alkylation or N-arylation

TABLE 4. RELATIVE ACTIVITY OF FURTHER SALICYLATE ANALOGUES AS UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

P/O expressed as percentage of P/O ratio in controls (without drugs).

Compound	Conc. ($\times 10^{-4}$M)	P/O (%)	Compound	Conc. ($\times 10^{-4}$M)	P/O (%)
Salicylate	10	25	1-Hydroxy-2-naphthoate	1	37
	5	60	1-Acetoxy-2-naphthoate	2	35
			3-Hydroxy-2-naphthoate	1	42
3,5-Dibromosalicylate	2	4	3,5-Dihydroxy-2-naphthoate	2·5	60
	0·25	84	2-Hydroxynaphthalene-1-acetate	1	84
(3,5-Dibromo-4-hydroxybenzoate	2	78)	3-Hydroxy-2-naphthoylethanolamide	2	80
5-Iodosalicylate	0·25	68	3-Hydroxy-2-naphthoylhydrazide	2	88
			Embonate (Pamoate)	1	72
3,5-Diiodosalicylate	0·25	10	1-Hydroxy-2-naphthaldehyde	0·5	56
4-Aminosalicylate	10	100	3-Hydroxy-2-naphthaldehyde	0·5	55
3,5 Diiodo-4-aminosalicylate	0·25		3-Phenylsalicylate	1	22
		50	2-Acetoxy-3-phenylbenzoate	2	28
4-N-Phenylaminosalicylate	1	78	5-Phenylsalicylate	1	10
4-(2.4-Dinitrophenylamino) salicylate	1	8	5-Phenylazosalicylate	0·5	0
(4-(2.4-Dinitrophenylamino) benzoate)	2·5	91)		0·1	50
0-*n*. Valerylsalicylate	25	54	5-(Diphenylmethyl) salicylate	1	0
0-Benzoylcalicylate	25	80	2-Hydroxyacetophenone	30	27
2.4-Dihydroxybenzoate	25	100		10	89
Methyl 2.4-dihydroxybenzoate*	25	23	2,2′-Dihydroxybenzophenone	7	61
Methyl salicylate*	25	80	2-Acetonaphth-1-ol	1	87
Phenyl salicylate*	3	60	2-Hydroxybenzonitrile	10	84
Benzyl salicylate*	2	87			

* esters

induced uncoupling activity—the degree of which was largely governed by the lipophilic nature of the N-substituent. Similarly, esterifying an inactive hydroxysalicylate e.g. 2,4-dihydroxybenzoic acid, conferred uncoupling activity (Table 4).

(iv) Requirement for an *ortho* phenolic group:

Sodium salts of other *ortho* monosubstituted benzoic acids with dissociation constants close to that of salicylic acid, pK 3·0 (e.g. 2-chlorobenzoic, pK 2·9; 2-nitrobenzoic, pK 2·2) did not uncouple oxidative phosphorylation at 2·5 mM.

Hexahydrosalicylate (*cis* 2-hydroxycyclohexane-carboxylate) with an alcoholic group in place of the phenolic group, was also inactive. 2-Methoxybenzoate (3 mM) and 2-methoxybenzanilide (0·2 mM) had no effect upon oxidative phosphorylation in liver or cartilage, in contrast to salicylate and salicylanilide.

The uncoupling action of salicylate and its analogues is evidently not a property of the phenolic group alone. 4-Hydroxybenzoate (Table 1), 4-hydroxybenzaldehyde, 4-hydroxybenzanilide (Table 5) and 6-hydroxy-2-naphthoate (Table 2) were inactive at those concentrations at which their respective *ortho*phenolic isomers completely abolished oxidative phosphorylation.

Acetylation of the phenolic group in salicylate, salicylaldehyde, *o*-hydroxynaphthoates and phenylsalicylates in every case afforded somewhat less active drugs *in vitro* than the parent phenols. It was found that not more than 20% of the acetylsalicylate added to cartilage or liver mitochondrial incubations (4 hr at 37° and 30 min at 30° respectively) was hydrolysed to salicylate, (which was determined spectrophotometrically at 300 mμ after extraction at pH 1 into ether). The degree of hydrolysis was usually higher when these other phenol acetates were incubated under the same conditions. Thus it would seem that most, if not all, of the uncoupling activity observed with these acetylated phenols is actually due to the phenol itself formed by spontaneous or enzymic hydrolysis during the experiments. O-Benzoysalicylate, O-propionylsalicylate and O-*n*-valerylsalicylate also uncoupled oxidative phosphorylation in liver mitochondria (Table 4) but all these phenolic esters of salicylic acid were less active than salicylate.

An *ortho* phenolic group is not absolutely essential for uncoupling activity amongst *o*-substituted benzoates: 2-mercaptobenzoate[8] (Table 3) and lipophilic N-substituted anthranilates[15] (see Table 5) are also potent uncoupling agents.

(v) Requirement for the *ortho* carbonyl group:

2-Substituted phenols without an *ortho* carboxyl or carbonyl group were either very much less active than salicylate or completely inactive in uncoupling oxidative phosphorylation when tested at 3 mM: this is twice the concentration of salicylate required to abolish oxidative phosphorylation in liver mitochondria.

Uncoupling potency diminished rapidly in the series: salicylate, 2-hydroxyphenylacetate, 2-hydroxy-β-phenylpropionate (melilotate, Table 3). Likewise 2-hydroxy-1-naphthaleneacetate is very much less active than the *o*-hydroxynaphthoates. *o*-Hydroxyarylacetates are very much weaker complexers of ferric ions than salicylate and the *o*-hydroxynaphthoates. Saligenin (2-hydroxybenzyl alcohol), 2-hydroxybenzenesulphonamide, 2-hydroxyphenylarsonic acid and 1-hydroxynaphthalene-2-sulphonate had no uncoupling action at 2·5 mM, even though the two latter compounds, like salicylate, firmly bind ferric ions.

Ortho-nitrophenol, which is isosteric with the salicylate ion but only a weak complexer of ferric ions, is also very much less effective than salicylate in

TABLE 5. UNCOUPLING ACTIVITY OF SOME SALICYLAMIDE DERIVATIVES.

Tested on rat liver mitochondria with succinate as substrate P/O expressed as percentage of P/O ratio in controls (without drugs)

Compound	Conc. ($\times 10^{-4}$M)	P/O (%)	Compound	Conc. ($\times 10^{-4}$M)	P/O (%)
Salicylhydrazide	25	100	N-Methylsalicylanilide	1·5	96
Salicylhydroxamate	25	78	N-Phenyl Salicylanilide	1·5	86
N-Salicoylpiperidine	25	100	3-Hydroxy-2-naphthoylanilide	1·5	63
N-Benzyl Salicylamide	1·0	80	3-Hydroxy-2-naphthoyl-p.phenetidide	1·5	0
N-Cyclohexylsalicylamide	1·5	64	N-Salicyloyl-4-amino benzoate	1·5	100
	5	0			
N-Benzoylsalicylamide	1·5	41	N-Salicyloyl anthranilate	0·2	56
Salicylanilide	1·5	0	N-Benzoyl anthranilate	2·5	0
	0·6	60		0·5	87
2-Acetoxybenzanilide	0·6	62	(N-Benzoyl-4-aminobenzoate	2·5	94)
2-Methoxybenzanilide	1·5	100	(Octylphenol	1·5	85)
4-Hydroxybenzanilide	1·5	100			

uncoupling oxidative phosphorylation (Table 6),[4] in contrast to *p*-nitrophenol.

Salicylaldoxime, which complexes transition metal ions but has no carbonyl group was quite inactive at concentrations at which salicylaldehyde abolishes oxidative phosphorylation. Thus the presence of the carbonyl group, rather than ability to complex metal ions *per se*, seems to determine uncoupling activity.

A carboxylate ion was not essential for uncoupling activity. Water soluble salicylate esters (Table 4) and certain N-substituted salicylamides (see below and Table 5) also uncoupled oxidative phosphorylation and inhibited the incorporation of $^{32}P_i$ and $^{35}S_i$ by cartilage. 2-Hydroxyphenyl alkyl ketones showed no significant uncoupling activity at concentrations at which they were water soluble (0·5–1·5 mM). 2·2′-Dihydroxybenzophenone though able to uncouple oxidative phosphorylation (Table 4), was very much less active than phenylsalicylates (salts) and phenyl salicylate (ester). 2-Hydroxybenzonitrile did not significantly incouple oxidative phosphorylation.

Relationship of uncoupling activity to chemical structure amongst salicylamide derivatives (*Table* 5)

Salicylamide itself does not uncouple oxidative phosphorylation[4] (Table 2) but several of its less water soluble derivatives were found to be quite active uncoupling agents. Although at least one derivative which is a primary amide, 3,5-dibromosalicylamide, was very active in this respect (at 0·5 mM), at this concentration other dibromophenols (2,4-dibromo-*o*-cresol, 3,5-dibromosaligenin) also uncoupled oxidative phosphorylation indicating that the dibromophenolic portion of the bromoamide, rather than the *o*-hydroxyamide moiety, conferred drug activity. Other lipophilic primary amide drivatives of salicylamide such as 5-phenylazosalicylamide and 5-diphenylmethylsalicylamide were so poorly water-soluble that they could not be tested adequately but in saturated solutions, they appeared to be inactive.

On the other hand, salicylimides and other N-substituted salicylamides exhibited a range of uncoupling activity which could be correlated with their relative lipophilic character *provided* there was a free hydrogen on the amide nitrogen, i.e. secondary amides. Hydrophilic secondary amides and all tertiary amides examined were virtually devoid of uncoupling activity. e.g. salicylurate, salicylhydroxamate, salicylhydrazide, salicylmorpholide, N-salicyloylpyrrolidine, N-salicyloylpiperidine (all tested at 2·5 mM). 3-Hydroxy-2-naphthoyl-hydrazide and -ethanolamide were each less active than the parent acid (Table 4). When tested at twice the concentration at which salicylanilide completely uncouples oxidative phosphorylation, the N-methyl and N-phenyl derivatives of salicylanilide had no effect on phosphate uptake: neither of these compounds complexed ferric ions. It must be concluded that the CONH-group is essential for uncoupling activity amongst salicylamide derivatives.

4-aminosalicylanilide, N-cyclohexylsalicylamide and N-benzylsalicylamide were each less active than salicylanilide. 2-Methoxybenzanilide, positional (phenolic) isomers of salicylanilide (2-benzamidophenol, 4-hydroxybenzanilide), and the most hydrophobic phenols of similar mol. wt. to salicylanilide e.g. *n*-octylphenol, nonylphenol, were all virtually inactive. N-phenyl-benzohydroxamate, which is another structural isomer of salicylanilide and like salicylanilide strongly complexes ferric ions, had no uncoupling activity: this finding confirms the earlier conclusions that ability to bind metal ions is of less importance than specific structural requirements. *o*-Acetylsalicylanilide proved to be as active as salicylanilide itself in uncoupling

phosphorylation in liver mitochondria. Spectrophotometric measurements of the salicylanilide formed an incubating this *o*-acetate with mitochondria in parallel incubations showed it to be at least 40% hydrolysed during this incubation period.

The two esters, benzyl and phenyl salicylate were each less active in uncoupling oxidative phosphorylation than their isoelectronic analogues, N-benzyl-salicylamide and salicylanilide (Table 5).

Exceptional observations

The following observations could not be simply reconciled with the structural considerations or the importance of lipophilic/hydrophilic character discussed hitherto.

(a) At concentrations at which it was soluble in the aqueous buffer media, 3-hydroxy-2-naphthoylanilide was less active in uncoupling oxidative phosphorylation than salicylanilide. Substitution in the anilide moiety e.g. *m*-nitro, *p*-ethoxy groups did however enhance the uncoupling activity (as it does in the case of salicylanilide) in agreement with the increased lipophilic character.

(b) Embonate (pamoate; 1,1'-methylene-bis-3-hydroxy-2-naphthoate), which might be considered a 'molecular dimer' of an *o*-hydroxynaphthoate, was rather less active than the *o*-hydroxynaphthoates on a mole-for-mole basis at concentrations at which it was wholly soluble in the aqueous buffer medium used in these experiments.

(c) Likewise aurin-tricarboxylate which might be regarded as a 'molecular trimer' of salicylate was not as active as salicylate on a mole for mole basis. The related (tri)phenol, aurin (*p*-rosolic acid) was more potent than salicylate (sic), completely abolishing oxidative phosphorylation at 0·5 mM, whereas phenol has no uncoupling activity at 5 mM.

(d) The uncoupling activity of 3-and 5-mononitrosalicylates and of 3,5-dinitrosalicylate was much less than that of the corresponding nitrophenols (Table 6) indicating that the activity of the nitrosalicylates was predominantly due to the non-salicylate part of the molecule. The presence of the carboxyl group diminished or abolished the

TABLE 6. COMPARISON OF THE UNCOUPLING ACTIVITIES OF (a) NITROSALICYLATES AND THEIR CORRESPONDING NITROPHENOLS AND (b) SOME HALOSALICYLATES AND RELATED HALOPHENOLS.

Tested with rat liver mitochondria oxidising succinate P/O expressed as percentage of P/O ratio in controls (without drugs).

Salicylate	Conc. (mM)	P/O (%)	Phenol	Conc. (mM)	P/O (%)
3-Nitro	2	68	2-Nitro	2	34
5-Nitro	0·2	54	4-Nitro	0·2	1
3,5-Dinitro	0·25	95	2,4-Dinitro	0·1	0
5-Iodo	0·1	0	4-Iodo	0·2	88
3,5-Dibromo	0·1	26	2,4-Dibromo-6-methyl	0·1	89

drug activity of the nitrophenol, presumably by increasing the hydrophilic character of the molecule. Picric acid which is isosteric with 3,5-dinitrosalicylate is not an uncoupling agent at 0·2 mM and like dinitrosalicylate is very much more water

soluble (and is a stronger acid) than 2,4-dinitrophenol. These considerations did not appear to apply to halogen-substituted salicylates which were each more active than the related halophenols (Table 6).

(e) The uncoupling activity of 2,4-dihydroxybenzaldehyde (β-resorcylaldehyde) appeared to be greater than that of salicylaldehyde. Likewise with the esters, methyl 2,4-dihydroxybenzoate was more potent than methyl salicylate in uncoupling oxidative phosphorylation in liver mitochondria. Unless these two salicyl compounds are more rapidly metabolised (reduced?) to inactive compounds (i.e. not salicylate) within respiring mitochondria, than are the corresponding β-resorcyl compounds (for which we have been unable to obtain evidence so far), these particular observations cannot be simply reconciled with the many other findings that the more water-soluble the compound; the lower its uncoupling activity.

DISCUSSION

Oxidative phosphorylation in cartilage

If glucose is the major fuel for respiration and energy-yielding processes in cartilage tissue, a drug such as salicylate could only inhibit the incorporation of inorganic phosphate into organic phosphates, if this process were dependent upon glycolysis alone, by inhibiting one or more of the enzymes involved in the dissimilation of glucose to form lactate. For this there is no evidence. Measurements of the carbon dioxide-^{14}C production from glucose-^{14}C have shown that salicylate and other uncoupling agents do not impair glucose oxidation at drug levels which largely abolish phosphate uptake.[2] Oxidation of substrates other than glucose by cartilage such as acetoacetate or glutamate, could only generate organic phosphates from inorganic phosphate via a process of oxidative phosphorylation. The sensitivity of cartilage phosphorylation to the action of several uncoupling agents, including salicylate, at concentrations at which these drugs effectively inhibit oxidative phosphorylation in liver, kidney and muscle preparations is strong circumstantial evidence that these drugs uncouple the phosphorylation of adenosine-5-diphosphate linked to substrate oxidation and electron transport within the chondrocytes.

In these experiments with tissue slices, it is not possible to trap the newly-synthesised ATP-^{32}P formed from $^{32}P_i$. Much of the ATP-^{32}P must be lost again as the ATP is hydrolysed in driving endergonic processes in the tissue. Only a fraction of the ^{32}P would be transferred to such stable cell constituents as the nucleic acids—which might survive (a) the long incubation periods required, (b) proteolytic digestion of the tissue at an elevated temperature to dissolve the matrix and release intracellular constituents, and (c) the alkaline conditions for precipitating the excess inorganic phosphate—and are the subject of the final radioassay for ^{32}P incorporation. A far more sensitive and reliable index of ATP formation in the tissue slices, under the influence of a drug, is provided by measurements upon an ATP-dependent process—in this case, the incorporation of inorganic sulphate into the mucopolysaccharide sulphates which are abundant in cartilage tissue (over 20% dry weight). These polysaccharides are virtually insensitive to the somewhat drastic experimental conditions required to extract and fractionate them from the other cartilage constituents and are readily assayed for radioactivity.

That a drug is not specifically inhibiting polysaccharide synthesis as such but rather ATP biogenesis, must be inferred from observing whether or not the same drug

concentration produces quantitatively similar effects upon phosphate incorporation in the connective tissue and also inhibits oxidative phosphorylation in mitochondria isolated from other tissues e.g. liver. All drugs inhibiting oxidation or phosphorylation in a connective tissue would impair polysaccharide sulphation (at least after an interval), though drugs which inhibit polysaccharide synthesis and/or sulphation are not necessarily uncouplers of oxidative phosphorylation.

Evidence for the uncoupling of oxidative phosphorylation by salicylate analogues

The only certain evidence for uncoupling activity is afforded by direct or indirect measurements of drug action on each of the two processes of oxidation and phosphorylation. The stimulation of respiration in the whole animal, in animal tissue slices or in yeast which sometimes, but not always accompanies the uncoupling of oxidative phosphorylation, has been used formerly to screen several salicylate analogues for uncoupling activity. This procedure appears to be very unsatisfactory. Spurious results may be obtained if the drug antagonises an endogenous inhibitor of respiration, e.g. calcium ions, unrelated to normal respiratory control. In this present study it was found that many salicylate analogues were indeed potent uncoupling agents but did not stimulate mitochondrial respiration or the oxidation of glucose by cartilage slices. Even so, there is a measure of agreement between the results of these respiratory studies with whole animals and yeast, and the results reported here based solely on *in-vitro* assays for uncoupling action. Thus 4-methyl salicylate was the least active of the three *o*-hydroxycresotates (3-, 4- and 5-methylsalicylates) examined in rats:[19] it was also the least active of these three in the present *in-vitro* studies and was found to be inactive in yeast, whereas 3-methylsalicylate was active in the whole yeast assay.[20] 1-Hydroxy-2-naphthoate was more active than salicylate in yeast. However the yeast assay gave some results contrary to the present findings; salicylamide being found active and 3-phenylsalicylate inactive.[20] The whole rat assay showed 3-phenylsalicylate to be more potent *in vivo* than any of the three methylsalicylates.[19] This was confirmed in these present studies of drug action *in vitro*.

More confidence can be placed in the consistent parallelism between the relative drug potencies of at least forty salicylate anlogues, which emerged after testing each compound upon cartilage slices and liver mitochondria and making two (methodologically) independent biochemical measurements upon the metabolism of each tissue in the presence of these drugs.

The structure–action relationship

At least two parameters evidently govern the activity of a given salicylate derivative in uncoupling oxidative phosphorylation and influence ATP-dependent phenomena. The first is simply ability to partition from an aqueous phase into a lipid-rich phase, so facilitating transport though the cell wall and concentration within the mitochondrion itself—the ultimate site of drug action. The second is a specific structural requirement for which we will use the term 'pharmacophore'.[21]

The importance of lipophilic character in determining uncoupling activity has already been demonstrated for halophenols[22] and nitrophenols.[23] in simple isolated cellular and subcellular systems. These present studies have demonstrated that this is true also for another class of phenols *in vitro*. In the whole animal this may not be so evident. The degree of binding of salicylates to plasma proteins also increases with

increasing lipophilic character.[24] Thus an analogue which is more active than salicylate itself (a 'supersalicylate') in plasma-free test systems *in vitro* such as were used in this work e.g. an *o*-hydroxynaphthoate, if bound more firmly to plasma proteins *in vivo* than salicylate, might actually be less accessible to the intracellular receptor(s) than salicylate. It has been found that *o*-hydroxynaphthoates are in fact less active as anti-inflammatory drugs and as inhibitors of cartilage polysaccharide sulphation in intact rats than salicylate,[25] probably for this reason. This is disappointing because the only supersalicylates uncovered in this study, apart from salicylanthranilate, were all in fact much more lipophilic than salicylate and suggests that a search for more potent salicylate-like drugs based upon salicylate itself may be singularly unrewarding. The marginal nature of the drug activity of salicylate itself is evident by comparison with its amide or any of its monohydroxy derivatives. Any decrease in lipophilic character effectively abolishes drug activity.

These studies have indicated that the essential pharmacophore for uncoupling activity in a salicylate analogue (including salicylamides) is represented by the partial structure A (Fig. 1). The simplest expression of this is actually the salicylate ion. Other examples are to be found in non-salicylates such as the mono-enol form of phenylbutazone (1,2-diphenyl-4-*n*. butylpyrazolidine-3,5-dione)[14, 15] and dicoumarol,[26]

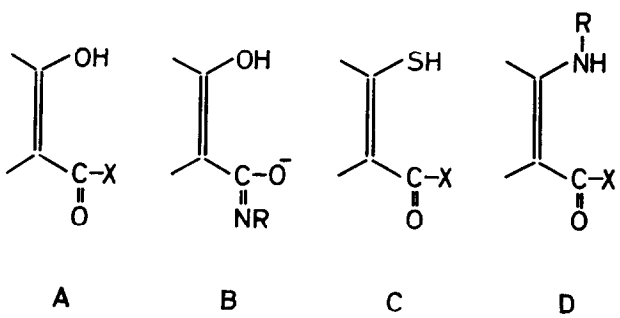

FIG. 1. Pharmacophores for uncoupling activity amongst salicylate analogues (2-substituted benzoyl derivatives).

which are both considerably more potent than salicylate in uncoupling oxidative phosphorylation. With salicylate analogues, the greatest uncoupling activity is found when X is potentially, if not in fact, a negatively charged group i.e. OH or NHR becoming O^- or NR^- on losing a proton. If X is NHR, this amide might actually ionise as the lactim tautomer within the lipid-rich phase of the mitochondrion, to give partial structure B with the negative charge on the oxygen, rather than as the ionised form represented by partial structure A with $X = NR^+$. It is clear that however this ionisation might occur, NHR is evidently much inferior to OH in conferring drug activity: R must be a hydrophobic group and the amide poorly water-soluble to match the salicylate ion in uncoupling potency. When X is an alkyl or an alkoxy group, uncoupling activity is very low. These considerations strongly suggest that some form of metal ion interaction, probably involving chelation within the mitochondrion, is associated with the uncoupling action of salicylate derivatives. It is clear that certain subtle stereochemical or physicochemical (solubility, pK?) requirements must be

satisfied. The inactivity of *o*-hydroxyarylsulphonates, *o*-hydroxyphenylarsonate, salicylaldoxime and arylhydroxamates demonstrates that, amongst salicylate analogues, ability to chelate metal ions does not of itself confer uncoupling activity.

Permissible variations in this pharmacophore are represented by partial structures C and D, where X is OH. These and other studies[8, 15] have shown that 2-mercaptobenzoate and N-substituted anthranilates are also potent uncoupling agents, in cartilage as well as in liver or heart muscle mitochondria.

N-salicyloylanthranilate which is about 25 times as active as salicylate *in vitro* appears to merit further study. On theoretical grounds it should be a superior drug to salicylanilide. Being less lipophilic than the anilide, it would probably attain a higher therapeutic concentration *in vivo* from a given dose—being less firmly bound by plasma proteins, less readily metabolised in the lipid-rich hepatic microsomes and less readily removed by the kidney. Anthranilic acid and its metabolites would probably be formed in the course of the metabolic inactivation of salicyloylanthranilate *in vivo*. These compounds are relatively non-toxic compared with the potential metabolites of salicylanilide such as phenylhydroxylamine, formed by hepatic hydroxylation of any aniline arising from hydrolysis *in vivo*. Salicyloylanthranilate is furthermore of interest as it represents the successful fusion of two moieties, each of which is a pharmacophore for uncoupling activity. In other instances where this might also be anticipated, the nitro salicylates, the uncoupling activities of the nitrophenol and salicylate portions of the molecule are not additive: the nitro group(s) activate the carboxyl group increasing its acidity (lowering the pK), thereby rendering the molecule much less lipophilic—in fact too hydrophilic for it to possess useful drug activity *in vitro*.

Relationship of uncoupling activity to therapeutic activity amongst salicylate analogues

If therapeutically active salicylate analogues are assessed by the following criteria,

(I) Ability to uncouple phosphorylation (and/or stimulate respiration)
(II) Inability to do so
(III) Clinical antirheumatic activity or anti-inflammatory activity in animals
(IV) Analgesic properties;

there is a remarkable overlap between compounds fulfilling criteria I and III on the one hand and those fulfilling II and IV on the other. Salicylate itself appears to be relatively unique in fulfilling I, III *and* IV.

For example, salicylamide,[27] many of its derivatives including salicyloylpiperidine,[28] and 2- or 4-hydroxyisophthalates[29] are useful analgesics but as shown here, they fail to uncouple oxidative phosphorylation. Moreover several derivatives of salicylamide in which the phenolic group is etherified have pronounced analgesic properties[30] but, as demonstrated in this study, an *ortho*phenolic group is essential for uncoupling activity. Hydroxyisophthalates and salicylamide have no significant anti-inflammatory activity in guinea pigs[31, 32] and clinical trials have shown that salicylamide and 4-hydroxyisophthalate are of no value as antirheumatic drugs.[33, 34] Many other analgesics, not chemically related to salicylate, also fail to uncouple oxidative phosphorylation[4, 35] and are devoid of anti-inflammatory activity in laboratory animals.[31, 32] Like salicylamides and the hydroxyisophthalates, they fulfil criteria II and IV but not I and III.

At the present, the correlation of uncoupling activity with antirheumatic potency (criteria I and III) is more limited amongst salicylate analogues. There is a paucity of clinical literature *establishing* antirheumatic activity in compounds other than salicylate salts and esters (which would be hydrolysed *in vivo*) and the *o*-hydroxycresotates (methylsalicylates), although there are several isolated reports claiming that a given salicylate derivative is clinically active. Unfortunately many of these claims lack published confirmation. Claims that gentisate (2,5-dihydroxybenzoate)[36] and γ-resorcylate (2,6-dihydroxybenoate)[37] are therapeutically active antirheumatic drugs have however been challenged in print.[38, 39] We can therefore set aside those objections based upon these now disputed claims, to the very useful hypothesis propounded by Adams and Cobb,[14] that ability to uncouple oxidative phosphorylation is related to anti-inflammatory and antirheumatic activity *in vivo*. [One such objection, often retailed, was that there were at least two (supposedly) active antirheumatic drugs i.e. gentisate, and γ-resorcylate, *unable* to uncouple oxidative phosphorylation.] Looking beyond salicylates congeners, we find many examples of a close correlation between uncoupling activity and antirheumatic or anti-inflammatory potency.[15, 35]

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