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BIOCHEMICAL PROPERTIES OF ANTI-INFLAMMATORY DRUGS—V.

UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY SOME γ-RESORCYL AND OTHER DIHYDROXYBENZOL COMPOUNDS

M. W. WHITEHOUSE and P. D. G. DEAN

Department of Biochemistry, University of Oxford

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Abstract—Some new derivatives of 2, 6-dihydroxybenzoic (γ-resorcylic) acid were prepared and characterised.

Many lipophilic derivatives of γ-resorcylic acid and of 2, 4, 6-trihydroxybenzoic acid uncouple phosphorylation in liver mitochondria and inhibit ATP-linked biosynthetic reactions in a connective tissue (cartilage). Of over 40 hydroxybenzoyl compounds examined, 5-phenylazo-γ-resorcylic acid and γ-resorcylanilide were the most potent drugs *in vitro*. γ-Resorcylic acid itself is devoid of these *in vitro* drug activities but two of its potential metabolites, γ-resorcylaldehyde and *N*-γ-resorcylglycine (2, 6-dihydroxyhippuric acid), were potent uncouplers of oxidative phosphorylation. These findings are discussed in the light of controversial reports that γ-resorcylic acid is/is not an antirheumatic drug.

The uncoupling activity of γ-resorcyl derivatives was related to their pK_a's (< 8) and their considerable lipophilic character. This last property could in turn be related to intramolecular hydrogen bonding between the ortho phenolic groups and the carbonyl function.

γ-Resorcylic acid was excreted largely unchanged in the urine after its ingestion by two healthy subjects: no evidence was obtained that it was conjugated with glycine *in vivo*.

REID and his colleagues[1] suggested that the antirheumatic (anti-inflammatory) activity of salicylates was associated with a "chelate-ring" involving hydrogen bonding between the carboxylate ion and the *ortho* phenolic group. They predicted that γ-resorcylate (2, 6-dihydroxybenzoate) with two *ortho* phenolic groups would be a more effective drug than salicylate (2-hydroxybenzoate) and presented evidence that it was superior to salicylate for the treatment of rheumatic fever. These findings have been both confirmed[2] and disputed.[3] A similar claim that 2, 5-dihydroxybenzoate (gentisate) is an effective antirheumatic drug[4] has also been disputed.[5]

If it is true that anti-inflammatory drugs other than steroids and antimalarials act by uncoupling oxidative phosphorylation in peripheral tissues[6–10], then further doubt is cast upon these reports that γ-resorcylate and gentisate are effective antirheumatic agents. Neither of these compounds, unlike salicylate, uncouples oxidative phosphorylation in liver mitochondria[11, 12] or in connective tissues such as cartilage.[12, 13] By contrast 2, 3-dihydroxybenzoate (*o*-pyrocatechuate) is both an antirheumatic and anti-inflammatory drug[2, 6] and also uncouples oxidative phosphorylation.[11] Should it be proved that dihydroxybenzoates other than 2, 3-dihydroxybenzoate are active

antirheumatic drugs; then either this hypothesis, relating antirheumatic activity to uncoupling of oxidative phosphorylation, must be discarded or it must be supposed that gentisate and γ-resorcylate are metabolised *in vivo* to more lipophilic compounds since uncoupling potency is related to lipophilic character.[7, 12, 14] Some possible lipogenic transformations *in vivo* are loss of a phenolic group (dehydroxylation),[15] conjugation, or biological reduction to the corresponding aldehydes, which do uncouple oxidative phosphorylation (see below).

Certain substituted γ-resorcylic acids which would be more lipophilic than 2, 6-dihydroxybenzoic acid itself, by virtue of their substituent groups (halogens, benzyl, etc.), were recently reported to have anti-inflammatory activity in three different pharmacological assays.[16, 17] This report (i) presents: evidence that many lipophilic derivatives of γ-resorcylic acid, in contrast to the parent acid, are potent drugs *in vitro* in uncoupling oxidative phosphorylation, and (ii) offers an explanation to support the original claims that γ-resorcylate is an antirheumatic drug.

EXPERIMENTAL

A. Chemical

Compounds were either obtained from Aldrich Chemical Co., Milwaukee, U.S.A.; British Drug Houses Ltd., Poole, Dorset; Mr. D. J. Drain (Smith and Nephew Research Ltd.) and Dr. H. J. Rylance (Edinburgh Pharmaceutical Industries Ltd.); or were synthesised by standard procedures (as given in Beilstein's Handbuch) and recrystallised to give m.p.s within at least 5° of literature values. 2, 6-dihydroxybenzaldehyde, m.p. 155–6°, was kindly donated by Dr. J. R. Merchant (Institute of Science, Bombay). Other compounds were prepared and characterised as follows: 5-Phenylazoresorcylic acids and 4-phenylazoresorcinol (Beilstein and refs. 18, 19) were distinguished by paper ionophoresis at pH 7. 1, 3-Dihydroxynaphthoic acid was synthesised from phenylacetic acid and diethylmalonate.[20]

2,6-*Dihydroxybenzanilide* was prepared from 2,6-dihydroxybenzoyl chloride.[21] It gave a blue coloration with ferric chloride in ethanol, had λ_{max} (ethanol) at 277 mμ and 328 mμ (shoulder) and m.p. 198°. ($C_{13}H_{11}O_3N$ requires C, 68·0%; H, 4·79%; N, 6·12%; found C, 67·56%; H, 4·75%; N, 6·42%).

2,4-*Dihydroxybenzanilide* was prepared from 2,4-dihydroxybenzoic acid.[22] It gave a red-violet coloration with ferric chloride in ethanol, had λ_{max} (ethanol) at 268 mμ and m.p. 176° (Ref. 22 gives 139°) ($C_{13}H_{11}O_3N$ requires N, 6·12%, found N, 6·11%).

2,6-*Dihydroxyhippuric acid* (N-Dihydroxybenzoyl-glycine, γ-resorcylglycine) γ-Resorcylic acid (1·4 g), ethyl aminoacetate (1·03 g) and dicyclohexylcarbodiimide (2·06 g) were dissolved in 50 ml of chloroform-methylene dichloride (1·1 v/v) and the solution stirred at room temperature for 4 hr. Acetic acid (0·5 ml) was added, the mixture filtered and the filtrate was washed successively with dilute bicarbonate, acid and water. After drying ($MgSO_4$) the solvents were removed by evaporation. The residual ester was hydrolysed with 0·6 g KOH and 20 ml methanol with stirring at room temperature. After 2 hr the solution was acidified with dilute H_2SO_4 and methanol removed at 25°. The product was extracted into ethyl acetate (3 × 50 ml), from which it was extracted in turn with 2 N potassium bicarbonate (3 × 75 ml), and finally extracted into ethyl acetate again on acidifying the combined bicarbonate extracts. After washing with acid and water, and drying over sodium sulphate, the ethyl acetate

was removed and the product recrystallised twice from aqueous ethanol, m.p. 142–5° ($C_9N_9O_5N$ requires N, 6·64%; found N, 6·50%).

The product gave a blue coloration with ferric chloride, green fluorescence in the u.v. and reddish-orange coloration with the Altmann reagent (*p*-dimethylaminobenzaldehyde in acetic anhydride) for hippuric acids. It was immobile on paper ionophoresis at pH 2·7 (monochloracetate buffer) but mobile at pH 7 (phosphate buffer). [γ-Resorcylic acid is mobile at both pH's].

2-*Acetoxy*-6-*hydroxybenzoic acid* (6-*Acetoxysalicylic acid, monoacetylresorcylic acid*). 2, 6-Di-hydroxybenzoic acid monohydrate (from water m.p. 165° (d) 2·4 g) was dissolved in acetic anhydride (3·5 ml) and cooled. Concentrated perchloric (2 drops) was added and the solution was warmed to 50°. This temperature was maintained for 10 min. The solution was cooled and cold water (100 ml) added, then extracted with ether (2 × 200 ml) and the ethereal extracts combined, washed with water and the solvents evaporated *in vacuo* at 90° for 10 min. The residual gum was crystallised from petroleum ether (b.p. 60°–80°) and ether. The diacetate crystallised first as plates m.p. 113–5°, λ_{max} 276 mμ (EtOH) and showed no colour with ferric chloride (aq.). The monoacetate was recovered as needles from the mother liquors m.p. 126–7° λ_{max} 308 mμ ε, 3900 (EtOH), and gave a purple colour with ferric chloride (aq.); (Found: C, 54·7%; H, 4·32%. $C_9H_8O_5$ requires: C, 55·0%; H, 4·1%). [2, 6-Dihydroxybenzoic acid gives a blue coloration with ferric ions.]

3, 5-*Dibromo*-2, 4, 6-*trihydroxybenzoic acid*. 2, 4, 6-trihydroxybenzoic acid (1·7 gm.) was dissolved in acetic acid (30 ml.) and treated with 5 ml bromine in acetic acid (1:4 v/v). The product precipitated almost instantaneously and was crystallised from warm water as fine needles m.p. 191–2° (d) ($C_7H_4Br_2O_5$ requires C, 25·6%; H, 1·22%; Found C, 25·4%; H, 1·30%).

B. Biochemical

Drug action on oxidative phosphorylation was studied using rat liver mitochondria respiring on succinate as the substrate.[12, 23] Hexokinase activity in the presence of added drugs was measured at pH 8 to 9.[24] The effect of γ-resorcylate derivatives on tracheal cartilage metabolism was studied by measuring the incorporation of ^{32}P from inorganic phosphate ($^{32}P_i$) into organic phosphates and of ^{35}S from inorganic sulphate ($^{35}S_i$) into mucopolysaccharide sulphates, as described previously.[12]

Urine (from 2 human subjects) was collected in several individual batches for 7 days after ingestion of a single 2 g dose of twice recrystallised 2, 6-dihydroxybenzoic acid (m.p. 166°) and stored at 4° (with a few drops of added chloroform) until processed.

C. Physical

Acid dissociation constants were determined approximately, by titration of aqueous solutions in 0·1 M Tris (hydroxymethyl)aminomethane or 0·1 M disodium hydrogen phosphate, containing the compound under investigation (1–4 mg/100 ml) and 1–4% (v/v) ethanol, with either N hydrochloric or citric acids. The extinction of the the solution measured at the appropriate absorption maximum in the range 310–390 mμ was plotted graphically against the pH, determined with a pH-meter (Electronic Instruments Ltd., Richmond, Surrey); the approximate pK_a (± 0·1 pH unit) being obtained either from the point of inflection, or as the mid-point of a fairly symmetrical titration curve.

RESULTS

In order to test Reid's original hypothesis, some less acidic and more lipophilic derivatives of γ-resorcylic and phoroglucinolcarboxylic (2, 4, 6-trihydroxybenzoic) acids were compared with their corresponding salicyl (2-hydroxybenzoyl) and 2, 4-dihydroxybenzoyl (β-resorcyl) analogues for their ability (i) to uncouple oxidative phosphorylation in rat liver mitochondria respiring on succinate; and (ii) to depress energy-linked metabolism in bovine tracehal cartilage slices *in vitro*, as measured by the effects of these compounds on the incorporation of inorganic phosphate ^{32}P into the organic phosphates and upon the ATP-dependent incorporation of inorganic sulphate-^{35}S into the cartilage mucopolysaccharide sulphates.

Uncoupling of oxidative phosphorylation by some dihydroxybenzoyl compounds

Table 1 shows that (a) only salicylic acid of the parent acids uncoupled oxidative phosphorylation; and (b) amongst at least two types of derivatives which are less hydrophilic than the parent acids, i.e. esters, dibromo acids, (Category 1) the β-resorcyl compounds (and the related 2, 4, 6-trihydroxybenzoyl derivatives) are actually more potent than the γ-resorcyl isomers; but (c) amongst at least five other classes of more lipophilic derivatives (Category 2), the γ-resorcyl derivatives are much more potent uncouplers of oxidative phosphorylation than the β-resorcyl isomers. The 2, 4, 6-trihydroxybenzoyl derivative of both categories exhibited uncoupling activities intermediate between those of the β- and γ-resorcyl derivatives.

TABLE 1. EFFECT OF SOME *o*-HYDROXYBENZOYL DERIVATIVES ON PHOSPHORYLATION COUPLED TO SUCCINATE OXIDATION IN RAT LIVER MITOCHONDRIA

Class of compound	P/O ratios as percentage of controls (incubated without drugs)				
	Conc $\times 10^{-4}$ M	2-hydroxy derivative (salicyl)	2, 6-dihydroxy derivative (γ-resorcyl)	2, 4-dihydroxy derivative (β-resorcyl)	2, 4, 6-trihydroxy derivative
Benzoic acids	25	0	97	100	100
Methyl benzoates	25	80	70	15	48
3, 5-Dibromobenzoic acids	3	0	100	32	90
Benzamides	25	96	0	92	65
Benzanilides	0·25	83	0	93	
Acetophenones	5	92	2	88	67
Benzaldehydes	5	80	5	62	15
5-Phenylazobenzoic acids	0·1	68	0	100	

P/O ratios (Drug-free controls) were within the range 1·4–1·8.

Table 2 shows that some γ-resorcyl derivatives are more potent than the corresponding salicyl or β-resorcyl compounds in affecting cartilage metabolism and almost certainly uncouple oxidative phosphorylation in this connective tissue.[12] The usnic acids which are naturally occurring γ-resorcyl derivatives known to uncouple oxidative phosphorylation[25] (see Table 3) also inhibited cartilage metabolism.

Some potential metabolites of γ-resorcylic acid, which are each less acidic than the parent acid (which has pK_a, 1·3), were examined for ability to uncouple oxidative phosphorylation. Both the monomethyl and dimethyl ethers (6-methoxysalicylic and

2, 6-dimethoxybenzoic acid) were inactive but N-(γ-resorcyl) glycine (2, 6-dihydroxyhippuric acid) was found to be a potent uncoupling agent (Table 3). The corresponding metabolite of salicylic acid, salicyluric acid (N-salicylglycine) was inactive. By this *in vitro* assay, γ-resorcylglycine is about 10 times as potent as salicylic acid and is apparently even more potent than γ-resorcylamide. In contrast to 6-methoxysalicylic

TABLE 2. EFFECT OF SOME *o*-HYDROXYBENZOYL DERIVATIVES ON CARTILAGE METABOLISM

Compound	Conc. $\times 10^{-4}$ M	$^{32}P_{org.}$ (%)	PS-^{35}S (%)
Salicylanilide	0·5	54	77
γ-Resorcylanilide	0·2	16	41
Salicylamide	25	96	100
β-Resorcylamide	10	93	83
γ-Resorcylamide	5	53	56
2-Hydroxyacetophenone	10	72	86
2, 4-Dihydroxyacetophenone	10	80	73
2, 6-Dihydroxyacetophenone	5	30	47
D-Usnic acid	0·1	39	24

Radioactivity of organic phosphate (P_{org}) and polysaccharide sulphate (PS) fractions expressed as percentage of these same values in controls incubated without drugs.

TABLE 3. EFFECT OF SOME FURTHER *o*-HYDROXYBENZOYL DERIVATIVES AND SOME TRIONES ON OXIDATIVE PHOSPHORYLATION

Compound	Conc. $\times 10^{-4}$ M	P/O (%)	Compound	Conc. $\times 10^{-4}$ M	P/O (%)
Salicylic acid	10	10	p. Orsellinic acid	25	44
6-Acetoxysalicylic acid	50	25	3, 5-Dibromo-orsellinic acid	7·5	57
2, 6-Diacetoxybenzoic acid	50	98	[3, 5-Dibromo-γ-resorcylic acid	7·5	48]
6-Methoxysalicylic acid	45	100	1, 3-Dihydroxy-2-naphthoic acid	5	70
Salicyluric acid	25	92	Ethyl dihydroxynaphthoate	5	90
N-γ-Resorcylglycine	0·5	58	[1-Hydroxy-2-naphthoic acid	1	25]
γ-Resorcylamide	1·0	55	Methyl 2, 3-dihydroxybenzoate	25	87
3, 5-Dihydroxybenzamide	25	97	Methyl gentisate	25	85
2, 6-Dimethoxyacetophenone	5	91	Gentisaldehyde	5	58
2, 5-Dihydroxyacetophenone	6	71	Indan-1, 3-trione	5	35
Phloretin	5	69		2·5	82
Phlorizin	15	71	2-Acetyl-indan-1, 3-trione	5	5
D-Usnic acid	0·1	39	2-Benzoyl-indan-1, 3-trione	2·5	38
L-Usnic acid	0·1	31			

P/O ratios expressed as % of that found in controls incubated without drugs.

acid, 6-acetoxysalicylic acid did uncouple oxidative phosphorylation, but was less potent in this respect than salicylic acid (Table 3).

Tables 1 and 3 also show that at least four acidi cγ-resorcylates other than γ-resorcylic acid itself, can uncouple oxidative phosphorylation: they are *p*-orsellinic (4-methyl-2, 6-dihydroxybenzoic) acid; the 3, 5-dibromo and 5-phenylazo derivatives of

γ-resorcylic acid and 1, 3-dihydroxy-2-naphthoic acid. The latter compound was however much less active than any of the three *o*-monohydroxynaphthoic acids[12] and was actually a weaker uncoupling agent than 1, 3-dihydroxynaphthalene[23] (which completely abolishes oxidative phosphorylation at 1 mM). Orsellinic acid, dibromo- and phenylazo-γ-resorcylic acids were however more potent uncoupling agents than the corresponding resorcinols.[23] These 4 particular γ-resorcylic acids all migrated much more rapidly than salicylic acid on ionophoresis at pH 2·7 (monochloracetate buffer) and like γ-resorcylic acid itself, are evidently more acidic than salicylic acid (pK_a 3·0). [Ionophoresis also indicated that the samples tested were not contaminated with their decarboxylation products, the corresponding resorcinols.]

The uncoupling activity of methyl 2, 4-dihydroxybenzoate was exceptional.* None of the other three isomeric methyl hydroxysalicylates (Tables 1 and 3) significantly uncoupled oxidative phosphorylation; not even the 2, 3-dihydroxybenzoate, although 2, 3-dihydroxybenzoic acid is the only monohydroxysalicylic acid which uncouples oxidative phosphorylation.[11, 12] This property of the 2, 4-dihydroxy ester was all the more exceptional since, in at least two other instances the 2, 4-dihydroxybenzoyl derivatives were no more active than the related 2, 5-dihydroxybenzoyl compounds (acetophenones and aldehydes, Tables 1 and 3); but the 2, 5-dihydroxy ester (methyl gentisate) was inactive.

2, 6-dimethoxyacetophenone and 3, 5-dihydroxybenzamide (Table 3) were both inactive demonstrating the requirement for the ortho phenolic groups for uncoupling activity. Phloretin was more potent than its glycoside, phloridzin, in uncoupling oxidative phosphorylation.

None of the drugs which uncoupled oxidative phosphorylation inhibited the hexokinase preparations, used to trap the newly synthesized ATP as glucose-6-phosphate.

Uncoupling of oxidative phosphorylation by β-triones

Two α-carbonyl substituted β-diketones, 2-acetyl and 2-benzoyl-indan-1, 3-dione, were examined for uncoupling activity since they might be considered chemical analogues of γ-resorcyl compounds existing in the tautomeric form (i.e. as triketones or diketo-enols). These two particular triones were each more potent than indan-1, 3-dione[23] in uncoupling oxidative phosphorylation (Table 3) but the benzoyl compound was much less active than 2-phenyl-indan-1, 3-trione[23].

Metabolism of γ-resorcylic acid

Each of the authors being in excellent health, ingested recrystallised γ-resorcylic acid. Urine samples collected within 1 hr gave the blue coloration with ferric chloride which is characteristic for the acid or its glycine conjugate. Urine samples collected for 24 hr before ingestion of the acid and for 48 hr after its ingestion, were extracted successively at an alkaline pH with ethyl acetate and *n*-butanol and again after acidification, with each of these solvents. Ferric (chloride)-positive material was found only in post-ingestion urine extracts and identified by paper ionophoresis at pH 7 and pH 2·7 as unchanged γ-resorcylic acid (by its mobility at pH 2·7) and confirmed

* The 2, 4-dihydroxy ester was prepared from the parent acid by three different procedures (methyl iodide on the silver salt, diazomethane or methanol and the acid): each preparation had the correct analysis and gave m.p. of 79° (monohydrate) and 118° (anhydrous) and uncoupled oxidative phosphorylation at 2mM by more than 50%.

by paper chromatography in isopropanol-ammonia-water (8:1:1 v/v) and benzene-acetic acid-water (125:72:3 v/v). γ-Resorcylic acid (m.p. 167° after crystallisation from water) was recovered from ethyl acetate extracts of acidified urine collected 3 hr after ingestion and previously extracted at pH 10 with ethyl acetate. γ-Resorcylglycine was not detected as a significant metabolite.

Salicyluric acid was detected in the urine after ingesting salicylic acid and in very small quantities after ingesting γ-resorcylic acid (which contained only traces of salicylic acid).

Hippuric acid was copiously excreted in the urine 48 hr before and 48 hr after ingesting the γ-resorcylate, on ingesting a test sample of sodium benzoate. Ingestion of sodium salicylate was followed by the excretion of some salicyluric acid, indicating that glycine conjugation was not apparently impaired.

Acidity of some salicyl and resorcyl derivatives

Rough determinations of the pK_a's in water at room temperature of several of these compounds showed that both γ- and β-resorcyl derivatives were each more acidic than the corresponding salicyl compounds (Table 4), in agreement with the greater acidity of many resorcinols compared with that of the corresponding phenols.[23] In some instances the γ-resorcyl compounds were more acidic than the β-resorcyl isomers (benzamides and benzanilides); the reverse was true for other compounds (acetophenones, benzoate esters). Other isomers of these dihydroxybenzoyl compounds appeared to be less acidic than the β- or γ-resorcyl derivatives: e.g. 3, 5-dihydroxybenzamide, pK 8·8; methyl 2, 3-dihydroxybenzoate, pK 9·0; 2, 5-(3, 6)-dihydroxyacetophenone, pK 9·6.

TABLE 4. ACID DISSOCIATION CONSTANTS (AS pK_a's ± 0·1 pH UNIT) OF SOME SALICYL, β- AND γ-RESORCYL DERIVATIVES

Compounds	pK_a's of		
	2-Hydroxy (Salicyl)	2, 4-Dihydroxy (β-resorcyl)	2, 6-Dihydroxy (γ-resorcyl)
Benzaldehydes	[8·8], 8·6	7·0	7·0
Benzamides	[8·9], 8·7	7·5	7·1
Benzanilides	7·5	6·8	6·3
Acetophenones	[10·8]	7·1	8·0
Methyl benzoates†	[10·2]	7·9	8·7

* Values in square brackets taken from Ågren (Ref. 35). Other values measured spectrophotometerically in aqueous ethanol (1 to 4% v/v) solution at 20°.

† esters.

DISCUSSION

Physicochemical properties of o-hydroxybenzoic acids

γ-Resorcylic acid (pK_a, 1·3) is a stronger acid than salicylic acid (pK_a, 2·98) but even if hydroxybenzoates penetrated biological membranes solely in the form of undissociated molecules, this could not explain why salicylic acid and 2, 3-dihydroxybenzoic acid (pK_a, 2·86) are pharmacologically active[6, 12] but gentisic acid (pK_a, 2·97) is inactive. Yeast cells apparently exclude gentisate and 2, 4-dihydroxybenzoate ions at pH 4·5 but will take up salicylate, 2, 3- and 2, 6-dihydroxybenzoate

ions.[26] Being more water soluble, dihydroxybenzoates would be expected to partition less extensively than salicylic acid into immiscible lipid phases. Spectrophotometric measurements of various hydroxybenzoates in aqueous salt solutions, pH 6·8 before and after partial extraction into non-hydroxylic solvents (such as chloroform, diethyl ether and pentan-2-one) have indicated no significant differences between the partioning of 2, 3- and 2, 6-dihydroxybenzoate ions. However, γ-resorcylate (2, 6-dihydroxybenzoate) partitions even more readily then salicylate (in turn more readily than 2, 3-dihydroxybenzoate) into water-immiscible hydroxylic solvents such as lower aliphatic alcohols, cyclohexanol and benzyl alcohol. These physicochemical considerations give little guidance as to why salicylate and 2, 3-dihydroxybenzoate should be effective drugs *in vitro* and *in vivo* and why 2, 6-dihydroxybenzoate is inactive *in vitro*.

Although it appears that optimal uncoupling activity is found with compounds with pK's greater than 3,[23] it is evident from the uncoupling activities of 1, 3-dihydroxynaphthoate and of some substituted γ-resorcylic acids that a pK 2 is itself no absolute bar to uncoupling activity, provided the molecule is sufficiently lipophilic.

Concerning claims for the therapeutic-activity of γ-resorcylic acid

Our observations have shown that many derivatives of γ-resorcylic acid with pK's considerably higher than that of the parent acid, are moderately potent drugs *in vitro*. Of special interest is the fact that one potential metabolite of γ-resorcylic acid, γ-resorcyl-glycine, is actually much more potent than salicylic acid *in vitro* and is at least as active in uncoupling oxidative phosphorylation as two of the most potent anti-inflammatory drugs in current use, indomethacin and phenylbutazone.[8] However, we could find no evidence that this glycine conjugate was formed *in vivo*, in a very limited study upon ourselves, after ingestion of single doses of the acid.

Glycine conjugation of salicylic acid proceeds rather less readily than hippurate formation from benzoic acid, in man.[27] No evidence was found that a glycine conjugate was excreted by dogs after feeding gentisic acid, an isomer of γ-resorcylic acid.[28] So it may well be that γ-resorcylic acid does not normally form a glycine conjugate because of steric hindrance, or some other factor (such as too low a pK), preventing its activation *in vivo* to form a coenzyme A derivative and subsequent conjugation with glycine. However, in Reid's original study,[1] it is possible that his clinical patients might have been "adapted" after any previous salicylate administration, and developed a more efficient glycine—conjugating mechanism than we ourselves possessed for dealing with the acid, following its ingestion in an acute dose. Another potential γ-resorcylate metabolite, the monomethoxy ether, does not appear to be formed readily by liver slices under conditions in which dihydroxy and trihydroxy-phenolic acids are *o*-methylated:[29] this hypothetical metabolite was tested in our studies and found not to be active. If γ-resorcylic acid were reduced *in vivo* to γ-resorcylaldehyde to any extent, this could represent another example of active drug biogenesis from an inactive precursor; the aldehyde being a very potent uncoupler of oxidative phosphorylation. At least one instance is known of the biological reduction of an aromatic acid (salicylic acid) occurring as an adaptive response to the substrate.[30]

These speculations would presumably also apply to 2, 4, 6-trihydroxybenzoic acid which has been claimed to be an effective antirheumatic drug:[2] which claim was not confirmed in at least one report.[31]

Uncoupling of oxidative phosphorylation by resorcyl derivatives

A number of natural products containing a 2, 6-dihydroxy-alkylphenone nucleus have been noted to uncouple oxidative phosphorylation, e.g. usnic acid,[25] phloridzin,[32] desaspidin.[33] These and the present observations indicate that the γ-resorcyl (i.e. 2, 6-dihydroxybenzoyl) group is certainly a pharmacophore for uncoupling activity and potential anti-inflammatory activity, although this fact escapes detection when γ-resorcylic acid itself is investigated. *In vitro* assays suggest that the γ-resorcyl pharmacophore is intrinsically more potent than the salicyl pharmacophore.

At least one γ-resorcyl derivative which is not a carboxylic acid exhibits anti-inflammatory activity: 2, 6-dihydroxyacetophenone (γ-resacetophenone) which was indicated as a potential anti-inflammatory drug in the present survey, was subsequently tested *in vivo* and found to have an activity approaching that of aspirin and exhibited up to 3 hr in the u.v. erythema test with guinea pigs (personal communication from Nicholas Research Institute Ltd., Slough). D- and L-usnic acids were virtually inactive in this assay, possibly because of poor distribution *in vivo* associated with their high degree of lipophilic character.[23]

The superior (uncoupling) activity of the γ-resorcyl pharmacophore (compared with that of the salicyl[12] or resorcinol[23] pharmacophores) can be related to the lower pK's of the γ-resorcyl derivatives, where this does not fall below 3. Other studies[23] have indicated optimal uncoupling activity is obtained with compounds having a p$K > 3$. This is further borne out by (i) the weaker uncoupling activity of dibromo-γ-resorcylic acid (p$K < 2{\cdot}5$) compared with dibromo-β-resorcylic acid (p$K > 2{\cdot}5$); (ii) the small gain in activity on inserting the 2-acetyl group into indan-1, 3-dione, when the pK falls sharply from 7·2 to 2·9;[34] and (iii) the activity of 2-nitroresorcinol[23] (pK 6·4) and inactivity of its isosteric and isoelectronic analogue, γ-resorcylic acid, in contrast to the fact that *o*-nitrophenol is a less potent uncoupler of oxidative phosphorylation than its isosteric (isoelectronic) analogue, salicylic acid.[12]

Table 4 shows that all the hydroxybenzoyl derivatives which were found to uncouple oxidative phosphorylation in this study, have pK_a's $\leqslant 8{\cdot}0$.

γ-Resorcylamide and γ-resorcylanilide had the lowest pK's of the hydroxybenzamides and hydroxybenzanilides examined and, taken with other evidence of the critical nature of the pK for uncoupling activity,[23] this alone would explain why these two γ-resorcyl compounds are more potent drugs *in vitro* than the β-resorcyl- or salicylamides and anilides respectively. γ-Resacetophenone and γ-resorcylaldehyde did not have lower pK's than their β-resorcyl isomers: the superior drug activity of these resorcyl compounds must therefore be determined primarily by their greater lipophilic character. Table 5 records the partition coefficient for some hydroxybenzoyl derivatives between chloroform and aqueous salt solutions, pH 6·8 (of the same composition as was used for studies of drug action of oxidative phosphorylation). This last table indicates how much more readily the salicyl and γ-resorcyl derivatives can pass from a neutral aqueous medium into a non-aqueous (lipid) phase than can the isomeric 4-hydroxybenzoyl or β-resorcyl compounds. This is almost certainly due to the strong intramolecular hydrogen bonding between the carbonyl group and the *ortho* phenolic group in the former compounds (for which ample spectroscopic evidence is available). As a consequence, hydrophilic interactions between the phenolic groups and a hydroxylic solvent (involving intermolecular hydrogen bonds) are

minimal and the molecule acquires a degree of lipophilic character not shared with isomeric dihydroxybenzoyl compounds.

Therefore Reid's original hypothesis concerning the importance of such an interaction between neighbouring groups on the benzene nucleus in determining potential anti-inflammatory (anti-rheumatic) activity, might be considered vindicated (even though the original claim that γ-resorcylic acid is itself an anti-rheumatic drug,[1] may be disputed or refuted[3]).

The exceptional behaviour of methyl 2, 4-dihydroxybenzoate in uncoupling oxidative phosphorylation, which contrasts with the inactivity of methyl salicylate[12] and of other methyl dihydroxy benzoates, can also be explained by the much lower pK of this compound (Table 4) compared with the pK_a's of other hydroxybenzoate esters. Likewise β-resorcylaldehyde must owe its greater uncoupling activity than salicylaldehyde, to its considerably lower pK even though it is a far more polar molecule than salicylaldehyde (Table 5).

TABLE 5. PARTITION COEFFICIENTS OF SOME HYDROXYBENZOYL DERIVATIVES

Compounds	Partition coefficient ($CHCl_3/H_2O$) for 2-Hydroxy (salicyl)	4-Hydroxy	2, 4-Dihydroxy (β-resorcyl)	2, 6-Dihydroxy (γ-resorcyl)
Benzaldehydes	60	0·7	0·9	1·9
Acetophenones	60	1·2	3·0	11
Benzamides	3·1		0·04	0·18
Benzanilides	23	2·0	0·2	28

Compounds were partitioned between chloroform and an aqueous salt solution pH 6·8 and determined spectrophotometrically in the aqueous phase before and after extraction.

General conclusions

Carboxyl substituted derivatives of γ-resorcylic acid represent another class of supersalicylates (*cf.* Ref. 12); that is, derivatives of salicylic acid which are considerably more potent drugs than salicylic acid itself.

Compounds which are β- and γ-resorcyl derivatives might be considered as carrying two contiguous uncoupling pharmacophores, associated respectively with the *ortho* hydroxybenzoyl (salicyl) and *meta* dihydroxyphenyl (resorcinol) structures.[12, 23] The successful fusion of these two pharmacophores is however only achieved in the γ-resorcyl series: here the gain in intramolecular hydrogen bonding counteracts the usual increase in hydrophilic character (and loss of uncoupling activity[12]) associated with introducing another polar (e.g. phenolic)group into a salicylate derivative. The β-resorcyl derivatives which are generally inferior to the corresponding salicyl derivatives (with a few exceptions, e.g. aldehyde, esters), may be compared with the nitrosalicylic acids[12] as examples where fusion of two pharmacophores actually results in less active drugs (uncoupling agents) than the unsubstituted salicyl derivatives.

These present findings suggest that it would be worth carrying out anti-inflammatory assays upon γ-resorcylic acid derivatives in which the strongly acidic carboxyl group (pK_a 1·3) is substituted but the *ortho* carbonyl group is retained; if it is considered good strategy to attempt to develop supersalicylates as potential antirheumatic drugs. (We ourselves have reservations on this point.)

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