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Bisphenols—Uncouplers of phosphorylating respiration*

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HEXACHLOROPHENE [2,2′-methylenebis-(3,4,6-trichlorophenol)] (Fig. 1) dichlorophene [2,2′-methylenebis (4-chlorophenol)], bithionol [2,2′-thiobis (4,6-dichlorophenol)] and other chlorinated bisphenolic compounds are used extensively in consumer products and in agriculture as antibacterial and antifungal agents. Although these chemicals are known to be quite toxic to animals[1,2]† and recent studies have shown[3] that chronic exposure of rats to hexachlorophene results in damage to the nervous system, there is little information on the mechanisms for their toxicity.

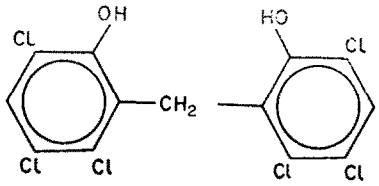

FIG. 1. Hexachlorophene [2,2′-methylenebis-(3,4,6-trichlorophenol)].

Hexachlorophene has been shown to inhibit the cytochrome oxidase, lactate dehydrogenase and succinoxidase systems in rats.[4] We have recently confirmed the inhibition of electron transport enzymes *in vitro* and in addition have shown that hexachlorophene is a potent uncoupler of oxidative phosphorylation.[5] In earlier studies, Thorsell[6] had reported that low concentrations of hexachlorophene stimulated microsomal adenosinetriphosphatase (ATPase) activity in the liver fluke while higher concentrations were slightly inhibitory.

Various attempts have been made to relate uncoupling efficacy to the physicochemical properties of uncouplers. In particular, based upon studies of isomeric series of compounds, it has been suggested that lipid solubility[7] and ionization state[8] are important. In this report we present evidence that a series of bisphenols including hexachlorophene readily uncouple oxidative phosphorylation and stimulate ATPase activity in intact mitochondria and that uncoupling efficacy correlates with the pK_a.

The isolation of rat liver mitochondria and the manometric determination of oxygen and phosphorus uptake have been described previously.[5] In the present studies, however, oxidative phosphorylation was estimated at 30° and the uncoupling reagents‡ were added in 0·1 M glycylglycine buffer (pH 7·4) containing 5% ethanol. The same buffer–alcohol mixture minus the uncoupler was added to the control flasks. Percentage inhibition of phosphorus uptake, measured at four different uncoupler concentrations, was plotted against the negative logarithm of the inhibitor concentration[9] and the results were then expressed as the concentration producing 50 per cent inhibition of phosphorus uptake. ATPase activity was determined at 30° by the method of Weinbach and Garbus.[10]

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† H. S. Nakaue, F. N. Dost and R. H. Buhler, submitted for publication.

‡ The chlorinated bisphenols: 2,2′-methylenebis (3,4-dichlorophenol); 2,2′-methylenebis (3,5-dichlorophenol); 2,2′-methylenebis (4,6-dichlorophenol); hexachlorophene [2,2′-methylenebis (3,4,6-trichlorophenol)]; 2,2′-dihydroxy-3,3′,5,6,6′-pentachlorodiphenylmethane; 2,4-dihydroxy-2,3,3′,5,5′,6-hexachlorodiphenylmethane; and 2,2′-oxybis (tetrachlorophenol) were kindly donated by the Givaudan Corp., Clifton, N.J. The fish eradicant, squaxin [bis(2-hydroxynaphthyl) methane], was a gift of Dr. C. MacPhee, University of Idaho, Moscow, Idaho. The dichlorophene [2,2′-methylenebis (4-chlorophenol)] and bis (2-hydroxyphenyl) methane were purchased from the Aldrich Chemical Corp., Milwaukee, Wis.; bithionol [2,2′-thiobis (4,6-dichlorophenol)] was purchased from Pfaltz & Bauer Company, Flushing, N.Y.; 2,4-dinitrophenol was purchased from Sigma Chemical Company, St. Louis, Mo.; and pentachlorophenol was purchased from Eastman Organic Chemicals, Rochester, N.Y. All other chemicals and solvents were obtained either from Sigma Chemical Company, St. Louis, Mo. or the Mallinckrodt Chemical Works, St. Louis, Mo.

TABLE 1. UNCOUPLING EFFECT AND PARTITION COEFFICIENTS OF CHLORINATED BISPHENOLS AND RELATED COMPOUNDS

No.	Compound	Concentration causing 50% inhibition of phosphorus uptake* (μM)	Concentration producing 50% increase in ATPase activity† (μM)	Hexane/buffer partition coefficient
	Methylenebisphenol			
1	2,2′-Dihydroxy-3,3′,5,6,6′-pentachlorodiphenylmethane	0·25	0·10†	120
2	2,2′-Methylenebis (3,4,6-trichlorophenol); hexachlorophene	0·31	0·19‡	16·1
3	2,2′-Methylenebis (3,5-dichlorophenol)	0·39	0·088‡	5·7
4	2,2′-Methylenebis (4,6-dichlorophenol)	0·57	0·35	21·6
5	2′,4-Dihydroxy-2,3,3′,5,5′,6-hexachlorodiphenylmethane	1·1	§	74·6
6	2,2′-Methylenebis (3,4-dichlorophenol)	2·2	0·55	5·0
7	2,2′-Methylenebis (4-chlorophenol); dichlorophene	7·9	2·7	5·0
8	Bis (2-hydroxyphenyl) methane	16	§	0·4
	Thiobisphenol			
9	2,2′-Thiobis (4,6-dichlorophenol); bithionol	0·90	0·38‡	32·0
	Oxybisphenol			
10	2,2′-Oxybis (tetrachlorophenol)	2·5	0·80	8·4
	Methylenebisnaphthol			
11	Bis (2-hydroxynaphthyl) methane	500	175	3·8
	Other phenols			
12	Pentachlorophenol	3·0	0·90‡	7·7
13	2,4-Dinitrophenol	16·7	4·8	0

* Control phosphorus and oxygen uptakes averaged 4·50 μmoles/mg of protein and 1·73 μatoms O/mg of protein respectively. Respiration rates were unaffected at uncoupling concentrations of each of the compounds, except bis(2-hydroxyphenyl)methane. For the latter compound at a concentration affecting 50 per cent inhibition of phosphorus uptake, 30 per cent inhibition of oxygen uptake was observed.

† Concentration producing 50 per cent of maximum 2,4-dinitrophenol effect.[9]

‡ Maximum stimulation observed exceeded that found with 2,4-dinitrophenol by 33–78 per cent.

§ Fifty per cent stimulation was not achieved at concentrations of 1·5 μM [2′,4-hydroxy-2,3,3′,5,5′,6-hexachlorodiphenylmethane] and 37·5 μM [bis (2-hydroxyphenyl)methane].

The effect of each compound on ATPase activity was also measured at four different concentrations and the level of uncoupler causing 50 per cent of the maximal effect obtained with 2,4-dinitrophenol then was determined.[9] Reaction mixtures contained 1·5–2·1 mg/ml of mitochondrial protein for measurement of oxidative phosphorylation and 1·6 mg/ml of mitochondrial protein for determination of ATPase activity.

Dissociation constants were determined as described by Fogg *et al.*,[11] except that distilled water was used in place of 0·1 M sodium perchlorate. Partition coefficients were determined spectrophotometrically. Ten μl ethanol containing 0·065 μmole of a given bisphenol, 5 ml of 0·1 M phosphate buffer (pH 6·75) and 5 ml hexane was measured into a culture tube. The stoppered tubes were shaken in a Burrell wrist-action shaker for 50 min at room temperature. The absorption spectra of the bisphenols in the hexane phase were measured on a model 11 Cary recording spectrophotometer and the partition coefficients were then calculated on a basis of the previously determined extinction coefficients for the individual bisphenols.

The chlorinated bisphenols strongly inhibit phosphorylation in rat liver mitochondria with little or no effect on oxygen uptake (Table 1). These compounds, in general, uncoupled oxidative phosphorylation at considerably lower concentrations than did 2,4-dinitrophenol or pentachlorophenol. Uncoupling activity for the chlorinated bisphenols tended to increase with each successive addition of chlorine to the aromatic ring. Hexachlorophene, with six symmetrically located chlorine atoms was one of most effective uncoupling agents, reducing mitochondrial phosphorylation 50 per cent at a concentration of 0·31 μM. Only one other compound, the asymmetric dihydroxy-3,3′,5,6,6′-pentachlorodiphenylmethane, was more active, yielding a 50 per cent reduction in oxidative phosphorylation at 0·25 μM. Factors other than the number of chlorine atoms were also important in determining activity, since 2,2′-methylenebis (3,5-dichlorophenol) was a more effective uncoupler than 2,2′-methylenebis (4,6-dichlorophenol) and both of these tetrachlorophenes were, in turn, considerably more active than the isomeric 2,2′-methylenebis (3,4-dichlorophenol).

Chlorinated bisphenols having an oxygen or sulfur bridge were also strong uncouplers but were less active than the corresponding methylenebisphenols. Thus, replacement of the methylene bridge in 2,2′-methylenebis (4,6-dichlorophenol) with a thio bridge to form 2,2′-thiobis (4,6-dichlorophenol) diminished the effectiveness by nearly half.

In general, there appeared to be a good correlation between the inhibitory effects on oxidative phosphorylation and the antibacterial[12] and antifungal[13] activities of the chlorinated bisphenols that were tested.

The chlorinated bisphenols also stimulated mitochondrial ATPase activity, yielding a structure–activity relationship somewhat analogous to that found for uncoupling activity (Table 1). Some of the differences between 50 per cent uncoupling and ATPase stimulating concentrations may result from the use of different mitochondrial protein levels[5] in the two analyses. We are at a loss to explain the low ATPase activity associated with compounds such as 2,4-dihydroxy-2,3,3′,5,5′,6-hexachlorodiphenylmethane.

Although we observed a tendency for the more lipophilic compounds to be the most effective uncouplers, the correlation between uncoupling activity and partition coefficient was relatively poor (Table 1). For example, while 2,2′-methylenebis (4-chlorophenol) has about the same lipid solubility as 2,2′-methylenebis (3,5-dichlorophenol), the latter compound was about 20 times more effective in uncoupling oxidative phosphorylation.

Uncoupling activity of the bisphenols appears to be more closely associated with the acidity of the compound (Fig. 2). There was a linear relationship between uncoupling activity and the pK_a of bis (2-hydroxyphenyl) methane, its chlorinated derivatives and thio analog, 2,2′-thiobis (4,6-dichlorophenol). As the acidity of the uncoupler increased, the concentration of chlorinated bisphenol necessary to affect oxidative phosphorylation decreased. There was also a progressive decline in pK_a of the chlorinated bisphenols as the number of chlorine substituents increased, explaining the previously noted direct correlation between the number of chlorine substituents and uncoupling activity. Only in the case of the oxygen-linked 2,2′-oxybis (tetrachlorophenol) was the activity of the chlorinated bisphenols less than would be anticipated on the basis of its acidity.

Pentachlorophenol, 2,4-dinitrophenol and bis (2-hydroxynaphthyl)methane failed to superimpose on the pK_a–activity plot constructed for the chlorinated bisphenols (Fig. 2). Nevertheless, Parker[8] has demonstrated that chlorophenol and nitrophenol uncouplers, including pentachlorophenol and 2,4-dinitrophenol, do exhibit a linear correlation between pK_a and the logarithm of the 50 per cent uncoupling concentration. In the case of these compounds, however, the plot, while parallel, occurs to the left of that for the chlorinated bisphenols. The shifted correlation curve results from the fact that the simple phenols are intrinsically less active against mitochondrial systems than are the chlorinated bisphenols. A series of similar acidity-uncoupling activity plots could presumably be constructed for each structurally similar class of weak acid uncouplers. In fact, correlations between acidity and the concentration affecting mitochondrial systems have already been shown for several other groups of uncouplers.[8,14–17]

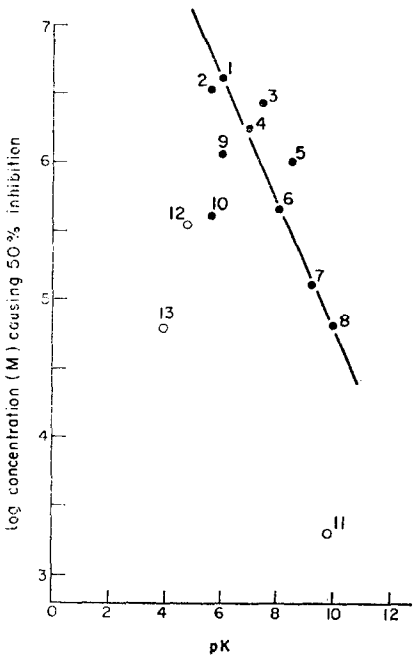

FIG. 2. Relationship between dissociation constant and concentration of phenol required to inhibit mitochondrial phosphorylation by 50 per cent. Bisphenol compounds: (1) dihydroxy-3,3′,5,6,6′-pentachlorodiphenylmethane; (2) hexachlorophene [2,2′-methylenebis (3,4,6-trichlorophenol)]; (3) 2,2′-methylenebis (3,5-dichlorophenol); (4) 2,2′-methylenebis (4,6-dichlorophenol); (5) 2′,4-dihydroxy-2,3,3′,5,5′,6-hexachlorodiphenylmethane; (6) 2,2′-methylenebis (3,4-dichlorophenol); (7) dichlorophene [2,2′-methylenebis (4-chlorophenol)]; (8) bis (2-hydroxyphenyl) methane; (9) bithionol [2,2′-thiobis (4,6-dichlorophenol)]; (10) 2,2′-oxybis (tetrachlorophenol). Other compounds: (11) squaxin [bis (2-hydroxynaphthyl) methane]; (12) pentachlorophenol; (13) 2,4-dinitrophenol.

Since the acidity-uncoupling activity plots for different sets of structurally related compounds do not superimpose, it is apparent that factors other than pK_a influence uncoupling potency. It is unlikely, however, that the relatively minor differences in lipid solubility of the uncoupling phenols and the bisphenols would alone be sufficient to account for the differences noted. Furthermore, the poor correlation observed between uncoupling efficacy and partition coefficient for the meythlene bridged bisphenols adds further support to the supposition that another factor (or factors) is also involved.

Weinbach and Garbus[18] have assembled indirect evidence that phenolic uncouplers interact with mitochondrial proteins leading to conformational changes of the phosphorylating enzymes and loss of the coupling function. Their hypothesis is compatible with the data correlating lipid solubility and pK_a of uncouplers and uncoupling potency, however, since they suggest that the uncoupler must first cross a lipoid boundary in the un-ionized state and then ionize in order to interact with charged groups of the protein.

A similar mechanism may account for the effect of the bisphenols on mitochondrial systems, since the amount of hexachlorophene bound to mitochondria is directly proportional to the degree of uncoupling[5] and the chlorinated bisphenols have also been shown to bind strongly to serum proteins[19] and to various polypeptides.[20] It is considered likely, therefore, that interaction of the bisphenol uncouplers with mitochondrial proteins is an essential part of the uncoupling process.

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Cytokinin activity of transfer RNA during chick embryogenesis

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A CLASS of N^6-substituted adenines, the cytokinins, promote proliferation and differentiation of plant tissues[1] and may have analogous effects on animal cells.[2] Nucleosides, corresponding to these bases, such as N^6-(Δ^2-isopentenyl)adenosine (2iPA), are constituents of bacterial, yeast, rat liver as well as plant *t*RNA (see ref. 1 for review). In *Escherichia coli*,[3] yeast[4] and *Lactobacillus acidophilus*,[5] cytokinin activity is present only in *t*RNA species that respond to codons containing *U* as the initial base. It is not known whether the promoting effect of the cytokinins on cellular growth is related to their presence in *t*RNA. This relationship might be reflected in a greater cytokinin content of *t*RNA derived from rapidly growing cells as has been shown in *E. coli*.[6] To investigate this possibility in animal cells, the cytokinin activity of *t*RNA was examined at stages of chick embryogenesis characterized by rapid growth and differentiation. The amount of cytokinin activity in total *t*RNA and the amount and chromatographic distribution of isoaccepting serine *t*RNA species corresponding to codons containing *U* as the initial base, were determined.

Transfer RNA was prepared from 4-, 8-, 12- and 19-day chick embryos by a procedure that included a DNase treatment as previously described.[7] Using $E^{0.1\%}_{260}$ (1 cm) for *t*RNA of 22, the yields were 71 (4-day), 71 (8-day), 45 (12-day) and 17 (19-day) mg *t*RNA per 100 g of tissue (wet wt.). The A_{260}/A_{280} ratios of the *t*RNA's ranged from 1·91 to 1·93. The *t*RNA was hydrolyzed and the hydrolysates were bioassayed for cytokinin activity by measuring their ability to promote growth of tobacco callus *in vitro* as described by Skoog *et al.*[8] Each *t*RNA preparation was assayed at several concentrations, with four replicate cultures for each concentration (Table 1). Activity of the preparation was expressed in kinetin equivalents (KE), i.e. micrograms of kinetin (N^6-furfuryladenine) required to give the identical growth response as determined by reference to a kinetin standard curve obtained from parallel tests. For comparison, values of cytokinin activity obtained from a sample each of yeast and *E. coli* *t*RNA assayed in the same manner are included.