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SHORT COMMUNICATIONS

Concerning the differences in uncoupling activity of isomeric dinitrophenols

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2,4-DINITROPHENOL is well known as a potent uncoupling agent.[1] Two of its isomers, 2,6- and 3,5-dinitrophenols, are also potent inhibitors of mitochondrial phosphorylation.[2, 3] Parker[4] suggested that there may be a correlation between the acidity of a phenol and its uncoupling potencies but this correlation breaks down when all the dinitrophenols are considered together. For example, 3,5-dinitrophenol is the least acidic of the six isomeric dinitrophenols but is also the most potent of the six isomers as an uncoupler of oxidative phosphorylation. We therefore sought some further explanation than just differences in p*Ka*[4] or lipid solubility[2, 3] to account for the differences in uncoupling activity of the individual dinitrophenols. Our experiments have shown that there are some further distinctions between the isomers with regard to (i) their uptake by yeast cells at neutral pH and (ii) their binding to the amino groups of bovine plasma albumin. These latter differences in biophysical properties may also be correlated with the different potencies of the individual dinitrophenols, perhaps with more significance than previous correlations between uncoupling activity and merely physical properties (e.g. p*K*, lipid solubility).

EXPERIMENTAL

2,3-, 3,4- and 3,5-dinitrophenols were prepared by procedures given in Beilstein's Handbuch. [Ring-^{14}C]-2,4-Dinitrophenol was obtained from Volk Chemical Co., Chicago. [Carboxyl-^{14}C] salicylic acid was obtained from the Radiochemical Centre, Amersham. Rat liver mitochondria were isolated and their phosphorylation quotient (P/O ratio) with succinate as substrate determined as described.[5] Binding of 0·5 mM dinitrophenol to 0·1 mM albumin in 0·1 M sodium phosphate pH 7·4 was determined colorimetrically using 2,4,6-trinitrobenzaldehyde (0·1 mM) and reading the optical density at 525 mμ after 30 min at 20°. (Blanks contained appropriate dinitrophenol and albumin but not aldehyde.)

Fresh bakers' yeast (D.C.L.) was washed in 0·15 M sodium chloride at 0°, centrifuged and resuspended by stirring in 0·1 M sodium phosphate, pH 6·8 to give a suspension of 1 g yeast/ml fluid. Three ml of this suspension was diluted with 4 ml 0·1 M phosphate, pH 6·8 containing a dinitrophenol (final concn. = 0·5 mM) and incubated for 30 min at 0°. The bulk of the yeast was removed by centrifugation and the supernatant deproteinized by the Somogyi procedure (with $ZnCl_2$ and NaOH added separately). The dinitrophenol in the aqueous phase was determined spectrophotometrically at the appropriate wavelengths recorded in Table 1.

For radioactivity measurements, centrifuged yeast cells or rat liver mitochrondrial pellets were heated with 4 ml 5% (w/v) sodium deoxycholate and 2 ml 2 N NaOH in a boiling water bath for 30 min. Aliquots of this lysate and the original aqueous supernatant were then taken for liquid scintillation counting (and appropriate corrections applied for quenching).

RESULTS

The relative potencies of the six dinitrophenols in uncoupling phosphorylation in rat liver mitochondria were found to be (in declining order): 3,5 > 2,4 > 2,6 $\simeq$ 3,4 > 2,3 $\simeq$ 2,5 (Table 1). This order of activity does not agree exactly with another comparison of four dinitrophenols where it was reported[3] that the 2,5-isomer is more potent than the 2,6-isomer, though we confirm that the 2,5-isomer is less potent than the 2,4-isomer.[3, 7]

Table 1 also compares the relative partition characteristics of the six isomeric dinitrophenols when dissolved in 0·1 M sodium phosphate, pH 6·8 and then (*a*) extracted with an equal vol. of organic solvent, or (*b*) incubated with washed bakers' yeast for 30 min at 0°. The 2,3- and 2,5-dinitrophenols, which are the weaker acids and weaker uncoupling agents, were less readily taken up by the yeast

than the 2,4- and 2,6-dinitrophenols, which are the stronger acids and more potent uncoupling agents. No innate difference between the former and latter pairs of compounds, as regards lipophilic character, is evident from just comparing their relative partition characteristics when distributed between three organic solvents and neutral salt solutions.

TABLE 1. SOME PHYSICAL AND BIOPHYSICAL PROPERTIES OF THE DINITROPHENOLS

Dinitrophenol	p*Ka**	λ† (mμ)	Partition coefficients with Yeast at 0° (30 min incubation)	*n*-Octanol	*n*-Butanol	Tri *n*-butyl phosphate	Uncoupling‡ E.D. 40–60 (μM)	% Inhibition§ TNB–albumen reaction
2,3-	5·0	396	0·3	1·3	0·6	2·0	100	42
2,4-	4·1	388	1·2	0·03	1·1	2·3	30	65
2,5-	5·2	398	0	1·1	1·6	11	100	35
2,6-	3·7	388	0·6	0·2	4·5	0·3	40	49
3,4-	5·4	400	0·5	3·4	0·7	20	40	38
3,5-	6·7	374	6·7	3·8	47	14	20	52
Salicylic acid	3·0	306		0·1	0·3	0·5	600	18

* Data from refs. 8–10.

† Wavelength for determining dinitrophenol concentration in the aqueous phase.

‡ Concentrations to reduce P/O ratio to 40–60 per cent of the value obtained in drug-free controls in three separate experiments.

§ With 0·1 mM albumen and aldehyde and 0·5 mM phenol in 0·1 M phosphate, pH 7·4.

The value of 1·2 obtained spectrophotometrically for the partition coefficient of [^{14}C]-2,4-dinitrophenol, between yeast and the salt solution, was lower than the value obtained (= 1·6) by measuring the distribution of radioactivity between the yeast and external aqueous phase. The disparity between these partition values for 2,4-dinitrophenol obtained from spectrophotometric and radioactivity measurements increased as the temperature of incubation was raised (up to 37°) but was reduced if cyanide (1 mM) was added to the incubation medium. As the radioactivity measurements always indicated a higher partition value, it is probable that even at 0° the yeast was metabolizing some of the 2,4-dinitrophenol to products which absorb more light at 388 mμ. Yeast was incubated at 0° with other dinitrophenols to minimize this "metabolic error" in spectrophotometrically determining the partition coefficient for other (non-radioactive) dinitrophenols.

Radioactivity measurements gave values of 4·4 ± 0·2 and 2·0 ± 0·1 for the partition coefficients of [^{14}C]-2,4-dinitrophenol and [^{14}C]-salicylate respectively, incubated with washed mitochondria (from female rate livers) and 0·1 M sodium phosphate, pH 6·8 for 30 min at either 0° or 37°, both with and without added cyanide. Comparison of these values with partition coefficients obtained with 3 organic solvents (Table 1) shows that these particular solvents are rather poor models for obtaining data on related lipophilic character, from which to predict the relative drug-partitioning between mitochondrial lipid and neutral aqueous salt solutions.

Trinitrobenzaldehyde reacts with bovine plasma albumin at pH 7·4 to give a red coloration (λ_{max} 425 and 525 mμ), the formation of which is inhibited by many acidic uncoupling agents.[6] Table 1 shows that the various dinitrophenols also inhibited colour formation but to different degrees. The actual degree of inhibition affords some measure of the competition between the particular uncoupling agent and the trinitrobenzaldehyde for the reactive amino groups of the albumin. Both the 2,4- and 3,5-dinitrophenols, despite the disparity in their p*K*'s, evidently bound the most strongly to these amino groups whereas the 2,5- and 3,4-isomers showed the least avidity for these groups. 2-Nitrophenol (p*Ka* 7·2) which is a very weak uncoupling agent[3, 7] did not inhibit the albumen-trinitrobenzaldehyde interaction under these conditions but 4-nitrophenol did (30 per cent quenching with 0·5 mM phenol). 4-Nitrophenol (p*Ka* 7·1) is a moderately potent uncoupler.[2–4]

2,4-Dinitrophenol binds strongly to plasma albumin[11] rendering it resistant to trypsin digestion[12] (indicating binding to a cationic group on the albumin) but bind less strongly to acetylated albumin.[13] It is also bound by a mitochondrial protein (probably at an amino group).[14]

In conclusion, the data indicate that the relative potency of certain phenolic drugs as uncouplers of oxidative phosphorylation may be determined primarily by their ability to cross a boundary membrane and to bind to a cationic site on a protein normally associated with energy-conservation, rather than merely by their relative acidity or lipid solubility. This is further exemplified by the hydroxybenzoic acids, of which salicylic acid most readily partitions into yeast[15] and is the most potent in uncoupling phosphorylation.[16]

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The inhibition of drug metabolism by antispermatogenic N,N′-bis(dichloroacetyl) diamines

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THAT one drug may alter the pharmacological potency of another is by now a well-established fact,[1–4] and the problem of the interaction of drugs has become a subject of widespread interest in recent years.[5, 6] Perhaps the best known of the inhibitors of drug metabolism, SKF 525-A (β-diethylaminoethyl-3-3-diphenylpropylacetate) has been shown to be firmly bound to subcellular membrane systems, and it has been proposed that this binding is related to the effects on microsomal metabolism by this compound.[7] In this communication we would like to present evidence for the inhibition of drug metabolism by two dichloroacetylamines, N,N′-bis(dichloroacetyl-1-8) octamethylenediamine (WIN 18,446), and N,N′-1,4-xylylene-bis(N-ethyldichloroacetamide) (WIN 13,099) which, like SKF 525-A, are firmly bound to subcellular membrane systems* and which have been shown to

* A. J. Merola, unpublished observation.