Biochemical Pharmacology, Vol. 24, pp. 2149–2152. Pergamon Press, 1975. Printed in Great Britain.

FURTHER STUDIES CONCERNING THE EFFECTS OF 2-ARYL-1,3-INDANDIONES ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

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(*Received* 5 *March* 1975; *accepted* 9 *May* 1975)

Abstract—The uncoupling activities of a series of substituted 2-aryl-1,3-indandiones are highly dependent on the rate at which energy is conserved. The latter process was varied by using different substrates or by adding different amounts of amytal. The uncoupling activities determined with ascorbate as the substrate did not differ fundamentally from those obtained with glutamate and both are mainly governed by lipophilicity and electronic effects.

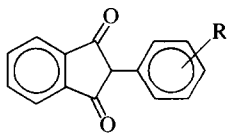
INTRODUCTION

2-Aryl-1,3-indandiones are uncouplers of oxidative phosphorylation in isolated rat liver mitochondria[1, 2] and this property was studied in our previous paper using glutamate as the substrate[2]. It was noted that the compounds fell into two categories, namely compounds without *ortho* substituents which have properties reminiscent to 2,4-dinitrophenol (DNP) and *ortho*-substituted derivatives which behave differently. The latter type of compounds inhibit respiration before maximal uncoupling has been reached (see Table 1) and as inhibition of respiration has been reported to enhance the potency of uncouplers[3] we studied the uncoupling activities of these compounds in more detail by using different substrates. The activities obtained were compared with the values from our previous work using multiple regression analysis.

MATERIALS AND METHODS

Special chemicals. Most of the 2-aryl-1,3-indandiones were synthesized in our laboratories as described previously[4]. The halogen substituted compounds were kindly supplied by Dr J. A. Durden Jr., Union Carbide Corporation, South Charleston, U.S.A. ATP, hexokinase (type III) and rotenone were purchased from Sigma Chemical Co. Tetramethyl-*p*-phenylenediamine (TMPD) was obtained from British Drug Houses and amytal from

Table 1. Physicochemical parameters and uncoupling activities of 2-aryl-1,3-indandiones

R		π*	σ†	E_s^0‡	resp.$_{50}$§ (%)	$-\log C_{50}$‖ (M^{-1})	$-\log C'_{50}$¶ (M^{-1})
H (PID)		0·00	0·00	2·48	100	3·74	4·14
2-methyl		0·56	0·29	1·24	74	3·46	3·65
2-ethyl		1·02	0·41	1·17	70	3·65	3·75
2-isopropyl		1·53	0·56	0·77	90	3·92	4·04
2-*t*-butyl		1·98	0·69	−0·30	78	3·66	3·85
2,6-dimethyl		1·12	0·58	0·00	40	3·45	3·50
2-methyl-6-ethyl		1·58	0·70	−0·07	50	3·65	3·71
2,6-diethyl		2·04	0·82	−0·14	62	3·77	3·89
2-methyl-6-isopropyl		2·09	0·85	−0·47	57	3·93	3·93
2,6-diisopropyl		3·06	1·12	−0·94	100	4·11	4·37
2-trifluoromethyl		0·88	1·61	0·08	40	3·08	3·22
2-fluoro		0·14	0·93	2·02	86	3·25	3·48
2-chloro		0·71	1·28	1·51	66	3·28	3·38
2-bromo		0·86	1·35	1·32	47	3·31	3·45
2-methyl-6-chloro		1·27	1·57	0·27	22	3·32	3·33
2,6-dichloro		1·42	2·56	0·54	30	3·11	3·04

* From ref. 8; † from ref. 9; ‡ from ref. 10.

§ Respiration rate using glutamate as the substrate at 50% uncoupling as % of control (from ref. 2).

‖ C_{50}: uncoupling activity determined with glutamate as the substrate (from ref. 2).

¶ C'_{50}: uncoupling activity determined with ascorbate + TMPD + rotenone as the substrate.

ACF Chemiefarma N.V., Maarssen, The Netherlands. All other chemicals were analytical grade preparations from the usual commercial sources.

Respiration and oxidative phosphorylation. Rat liver mitochondria were isolated according to the technique described by Myers and Slater[5] and protein content was determined by the method of Lowry *et al.*[6].

Respiration and oxidative phosphorylation were measured as described previously[2]. The medium (3 ml) contained 32 mM sucrose, 15 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 50 mM Tris–HCl, 16·7 mM potassium phosphate, 0·9 mM ATP, 16·7 mM glucose, 7 units hexokinase and about 5 mg of mitochondrial protein. The final pH was 7·5. Drugs were added as an ethanolic solution of 0·1 ml.

The substrates used were: glutamate (8 mM), succinate (8 mM) + rotenone (1 μg/ml) or ascorbate (8 mM) + TMPD (0·2 mM) + rotenone (1 μg/ml).

The respiratory rate was varied by adding different amounts of amytal.

Correlations. The relationship between uncoupling activity and physicochemical parameters (see Table 1) was studied using multiple regression. The following statistical parameters will be mentioned: r is the correlation coefficient, s is the standard deviation, F is the overall statistical significance of the equation and the Student's t-test values of the regression coefficients are placed between brackets.

RESULTS

Figure 1 shows that inhibition of respiration lowers the P/O ratio considerably in the presence of uncouplers. This is in accordance with the results of Tsou and Van Dam[3]. In order to establish whether the uncoupling activities are substrate-dependent, some experiments were carried out with three different substrates, namely glutamate, succinate + rotenone and ascorbate + TMPD + rotenone in concentrations as described under Methods. The results are presented in Figs. 2 and 3. Respiration was not inhibited by DNP and 2-phenyl-1,3-indandione (PID) in the concentrations used but the *ortho*-substituted compounds did produce an inhibitory effect on glutamate and succinate oxidation, whereas oxidation of ascorbate remained unaffected (see Fig. 3).

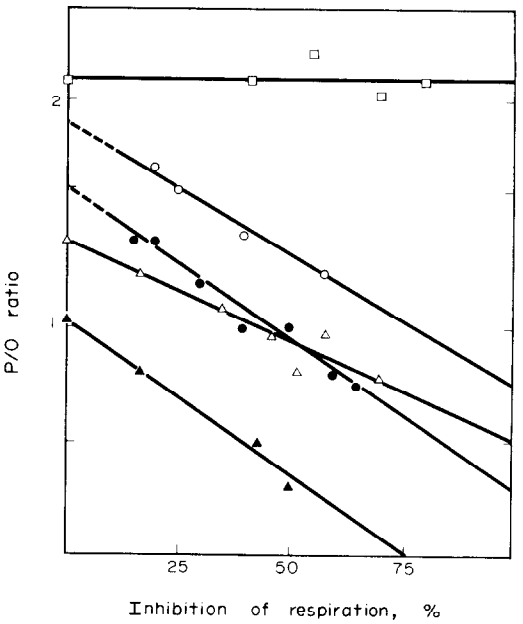


Fig. 1. Effects of respiration rate on uncoupling activity. The respiration rate was varied by changing amytal concentrations. PID: 0·12 mM (△) and 0·20 mM (▲); 2-(2-methylphenyl)-1,3-indandione: 0·13 mM (○) and 0·25 mM (●); control (□).

Because of this finding, the uncoupling activities of a series of *ortho*-substituted derivatives were determined using ascorbate + TMPD + rotenone as the substrate. It was found that, under this condition, none of the compounds inhibited respiration before 100 per cent uncoupling had been reached, which is in accordance with the results of Wilson and Merz[7].

The activity of an uncoupler has been characterized by the concentration at which the P/O ratio is reduced to 50 per cent (C_{50}) and these values are listed in Table 1.

The results of the present study and from our previous paper were analyzed by multiple regression using the Hansch approach[8]. The substituent constants π, σ and E_s^0 are also listed in Table 1 and the following regression equations were obtained:

$$-\log C_{50} = 3{\cdot}074 + 0{\cdot}500\pi - 0{\cdot}293\sigma + 0{\cdot}173E_s^0$$
$$(8{\cdot}775) \quad (-8{\cdot}434) \quad (3{\cdot}481) \quad (1)$$

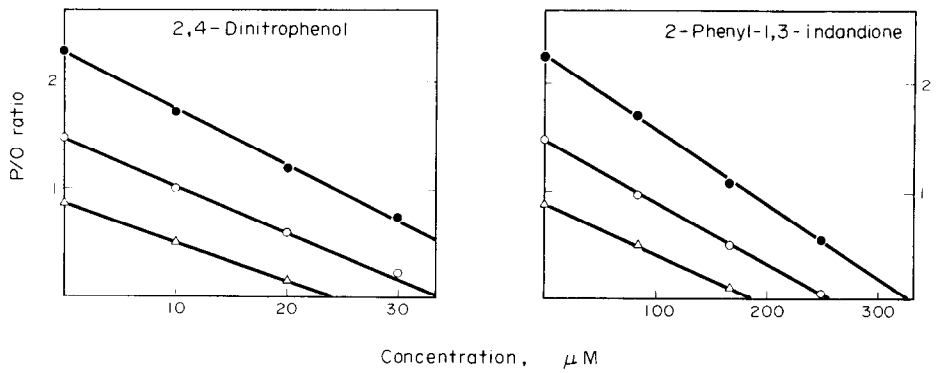


Fig. 2. Uncoupling of oxidative phosphorylation by DNP and PID. The substrates used are glutamate (●), succinate + rotenone (○) and ascorbate + TMPD + rotenone (△).

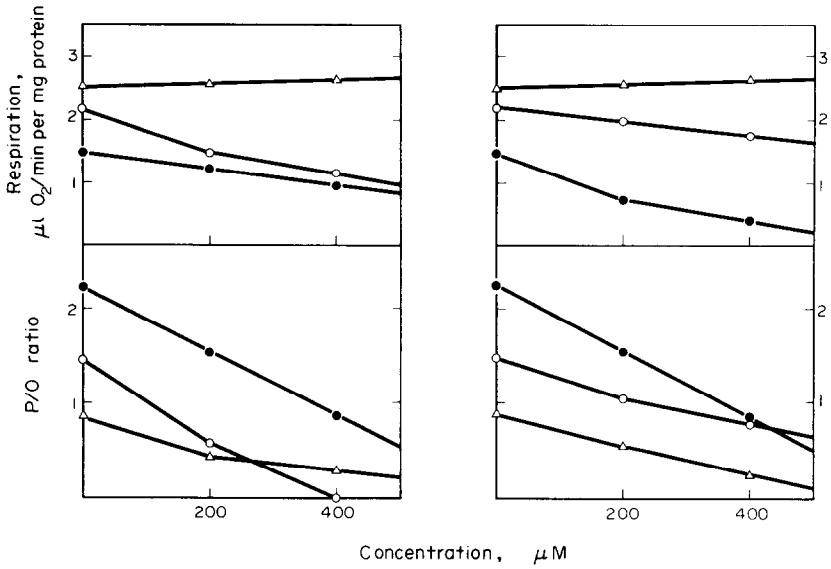


Fig. 3. Uncoupling of oxidative phosphorylation by 2-(2-methylphenyl)-1,3-indandione (*left*) and 2-(2,6-dimethylphenyl)-1,3-indandione (*right*). The substrates used are glutamate (●), succinate + rotenone (○) and ascorbate + TMPD + rotenone (△).

$$n = 15 \quad r = 0{\cdot}977 \quad s = 0{\cdot}076 \quad F = 75{\cdot}37$$

$$-\log C'_{50} = 3{\cdot}152 + 0{\cdot}554\pi - 0{\cdot}359\sigma + 0{\cdot}227E_s^0 \quad (2)$$
$$(7{\cdot}816) \quad (-8{\cdot}303) \quad (3{\cdot}663)$$

$$n = 15 \quad r = 0{\cdot}970 \quad s = 0{\cdot}096 \quad F = 58{\cdot}77.$$

There exists an intercorrelation between $-\log C_{50}$ and $-\log C'_{50}$ values with $r = 0{\cdot}953$. PID was omitted from the calculations as its presence deteriorated the correlation considerably. The latter demonstrates clearly the difference in activity between *ortho* and non-*ortho* compounds.

DISCUSSION

Ortho-substituted 2-aryl-1,3-indandiones inhibit the oxidation of glutamate and succinate by rat liver mitochondria, and the extent of inhibition is dependent on the substrate concerned (see Fig. 3: the oxidation of succinate is inhibited more obviously by 2-(2-methylphenyl)-1,3-indandione than by 2-(2,6-dimethylphenyl)-1,3-indandione, whereas the reverse is true for glutamate oxidation). This would imply that these compounds affect the different components of the respiratory-chain in unlike manner, as was postulated by Wilson and Merz for other types of uncouplers[7]. It is, however, also quite possible that the competitive inhibition of substrate accumulation is the underlying mechanism. DNP has been reported to inhibit the accumulation of β-hydroxybutyrate in a more pronounced way than the accumulation of succinate, whereas the opposite was found for dicoumarol[11] and similar differences would occur in the case of arylindandiones. This possibility can only be confirmed by determining simultaneously the extra- and intra-mitochondrial concentrations of uncoupler and substrate.

It was concluded in our previous paper that any change in substrate concentration entails a small alteration in C_{50} value (Fig. 2 in ref. 2). However, when C_{50} values are determined by using different substrates the variation in these values becomes much more obvious: for PID the C_{50} values with glutamate, succinate and ascorbate are 17, 13 and 10×10^{-5} M, respectively and for DNP 21, 17 and 13×10^{-6} M (see Figs. 2 and 3). This is in accordance with the theory that the extent of uncoupling is dependent on the rate at which energy is conserved, as postulated by Tsou and Van Dam[3]. They found that uncouplers tended to be more potent in the case of inhibited respiration. Figure 1 shows that this is also the case with a few 2-aryl-1,3-indandiones. The ratio of respiration rates when the three substrates mentioned above are used is 1 : 1·4 : 1·7 and thus the ratio of energy conservation is 3 : 2·8 : 1·7. The same order was found for the C_{50} values determined with these three substrates. A similar situation was seen with the *ortho*-substituted compounds (Fig. 3). In this latter case, inhibition of respiration is highly dependent on the substrate used, and the strongest inhibition is always accompanied by the largest uncoupling activity. It would seem, therefore, that the extent of uncoupling is governed by the rate at which energy is conserved and also by the uncoupler concentration. Since this rate and the accumulation of uncouplers in mitochondria are effected by substrates in unlike manner (succinate is a more potent inhibitor of DNP accumulation than glutamate[12]), such experiments can never be used to determine uncoupling at each coupling site as indicated by Katyare *et al.*[13].

There exists a remarkable agreement between the two series of uncoupling activities in Table 1. Generally speaking, the $-\log C'_{50}$ values are somewhat higher than the $-\log C_{50}$'s and the differences are more pronounced if there is less inhibition of glutamate oxidation (cf. 2-(2,6-diisopropylphenyl)-1,3-indandione and 2-(2-methyl-6-chlorophenyl)-1,3-indandione). This is in accordance with the theories discussed above. Inhibition of glutamate oxidation causes an increase in $-\log C_{50}$ value so that the difference between $-\log C_{50}$ and $-\log C'_{50}$

values diminishes. There is also an agreement between equations 1 and 2, indicating that the different coupling sites are essentially the same. In both cases, π and σ are the most important parameters, whereas steric effects are of little consequence.

The difference between compounds with *ortho* substituents and compounds without these substituents, reported in our previous paper[2] might have been due to inhibitory effects of the *ortho* substituted derivatives on respiration rate but the present results show that this difference also exists if oxidation rate is unaffected (experiments using ascorbate as the substrate) as in both regression calculations PID appeared to be an outlier. In another study it was concluded that a difference in lipophilicity between the two groups of compounds is probably responsible for this phenomenon[14].

The above considerations demonstrate clearly that within the series of 2-aryl-1,3-indandiones, the *ortho*-substituted derivatives occupy a peculiar position.

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