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INHIBITION OF RESPIRATION BY PHENACETIN IN ISOLATED TUBULES AND MITOCHONDRIA OF RAT KIDNEY

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Abstract—Phenacetin, an analgesic drug thought to exert nephrotoxic effects *in vivo*, was found to inhibit respiration in isolated rat kidney tubules metabolizing endogenous substrate or exogenous glutamine, glucose or lactate. With isolated rat kidney mitochondria the oxidation of glutamate or succinate was strongly inhibited by phenacetin; in each case State 3 respiration and State 3u (uncoupled) respiration were affected to the same extent, indicating that phenacetin exerted its influence directly on the respiratory chain. The effects of phenacetin on the oxidation of NADH and succinate by submitochondrial particles in the presence of various electron acceptors suggested that at least two oxidoreduction reactions of the respiratory chain were susceptible to inhibition by phenacetin. One of these reactions was that catalysed by succinate dehydrogenase, while the other probably lay between reduced NADH dehydrogenase and coenzyme Q. The possibility that impairment to the oxygen-metabolising capacity of the kidney cell might contribute to the perceived cytotoxicity of phenacetin is discussed.

There exists a considerable body of evidence, both clinical and experimental, to suggest that the analgesic drug phenacetin (4-ethoxy acetanilide), either alone or in combination with acetyl salicylate, can cause degenerative kidney disease in man and other mammals (for review see [1]). The lesions, which appear to be caused by anoxia [2], occur primarily in, but are not always confined to, the medulla. It is in this region that *N*-acetyl *p*-aminophenol and *p*-phenetidine, two important metabolic derivatives of phenacetin, are strongly concentrated under experimental conditions [3–5], though whether the parent compound is itself concentrated there is disputed [5, 6]. Possible mechanisms whereby phenacetin and its metabolites might restrict the supply of oxygen to the affected areas include local methaemoglobinaemia [2, 5, 7], and ischaemia due either to a lowering of erythrocyte deformability [2, 5] or to the precipitation of the drug and its products in the loop of Henle and surrounding tissue [8].

In the studies reported here it was observed that phenacetin, at high aqueous concentration, strongly inhibited respiration in intact kidney cells and in isolated kidney mitochondria, the inhibition being due to effects exerted directly on the mitochondrial respiratory chain. It is suggested that any such impairment to the oxygen-metabolizing capacity of kidney cells *in vivo* could itself lead to tissue damage and also aggravate injury caused by restriction to the oxygen supply.

MATERIALS AND METHODS

Chemicals. Hyaluronidase, collagenase, L-lactic acid, NADH, *N,N,N′,N′*-tetramethyl *p*-phenylenediamine, 2,6-dichlorophenol indophenol, menadione and rotenone were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; L-ascorbic acid, sodium succinate, L-glutamic acid and D-glucose were from BDH Ltd., Poole, Dorset, U.K.; L-glutamine was from E. Merck AG Darmstadt, Germany; 2,4-dinitrophenol was from Hopkin & Williams Ltd., Chadwell Heath, Essex, U.K.; phenazine methosulphate was from Calbiochem, San Diego, CA, U.S.A. All other reagents were A.R. grade.

Preparation and incubation of kidney tubules. Tubules, isolated from the kidneys of adult albino rats (Wistar strain) as described previously [9], were suspended in a phosphate-buffered medium [10] at approx. 5 mg tubule protein/ml. To 2 ml aliquots of this suspension were added substrates and 0.03 ml of warm ethanol containing 0–20 μmoles phenacetin. Incubations were carried out at 37° in Warburg respirometers (Braun, Model V 166) and the consumption of oxygen was followed manometrically [11].

Preparation and incubation of kidney mitochondria. A recently described procedure was used for the isolation of mitochondria from rat kidney cortex [12]. Up to 3 mg of mitochondrial protein was incubated at 30° in 2.5 ml of reaction medium in the chamber of a Rank oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.). The reaction medium was that detailed by Bustamante *et al.* [12] except for the omission of bovine serum albumin and the inclusion of 1.2% (v/v) ethanol, and in some cases, 8 mM phenacetin. Substrates, ADP and 2,4-dinitrophenol were added as shown.

Oxidoreductase activities of submitochondrial particles. Submitochondrial particles (SMP) were prepared by exposing a suspension of kidney mitochondria to ultrasound for 2 min in a Branson Sonifier model B-12 and then removing unbroken mitochondria and dense particles by centrifugation at 25,000 *g* for 10 min. The supernatant, which contained the SMP,

was tested for various oxidoreductase activities in the presence and absence of phenacetin.

NADH oxidase and succinoxidase activities were determined by using the oxygen electrode as described above.

NADH oxidase was also assayed spectrophotometrically, as were NADH: ferricyanide oxidoreductase and NADH: menadione oxidoreductase activities. Previously described assay methods [13] were adapted for split-beam spectrophotometry using a Varian Techtron u.v.-VIS Spectrophotometer (model 635). Assays were conducted at 30° in 2.5 ml systems by following the rate of change in absorbance at 340 nm of a system containing submitochondrial particle suspension relative to that of a system containing an equivalent volume of the suspending medium. The reaction medium comprised 50 mM Tris–sulphate buffer (pH 8), 0.3 mM NADH, 1.2% (v/v) ethanol, and when required, 8 mM phenacetin. The electron acceptors were oxygen, 1 mM potassium ferricyanide or 0.2 mM menadione, the last being introduced in methanol to give a final methanol concentration of 2% (v/v). 1 mM KCN was included except when O_2 was the acceptor.

Assays based on methods previously described [14] were used in determining oxidoreductase activities in which succinate acted as the electron donor. The reaction mixture comprised 50 mM sodium phosphate buffer (pH 7.8), 24 mM succinate, 1 mM KCN, 1.2% (v/v) ethanol and, when required, 8 mM phenacetin. The electron acceptor was either 1.2 mM potassium ferricyanide or 0.36 mM phenazine methosulphate (PMS) together with 0.06 mM 2,6-dichlorophenol indophenol (DCPIP). The general procedure was similar to that described above for NADH oxidation except that the reduction of ferricyanide or DCPIP was followed, at wavelengths of 420 nm and 600 nm respectively.

Protein measurements. Protein was determined by a biuret method [15], with bovine serum albumin as standard.

Statistical analysis. All results are presented as the mean ± the standard error of the mean. Significance of difference was estimated by the Student's *t*-test.

RESULTS

Respiration of kidney tubules. Figure 1 shows that the consumption of oxygen by tubules provided with glutamine proceeded at a constant rate for at least 2 hr at 37°, as did respiration when either lactate or glucose was substituted for glutamate (data not shown). Addition of 5 mM or 10 mM phenacetin led to falls of 34 per cent ($P < 0.01$) and 50 per cent ($P < 0.001$) respectively in the rate of glutamine-supported respiration. The inhibition by phenacetin was exerted immediately and remained constant throughout the incubation.

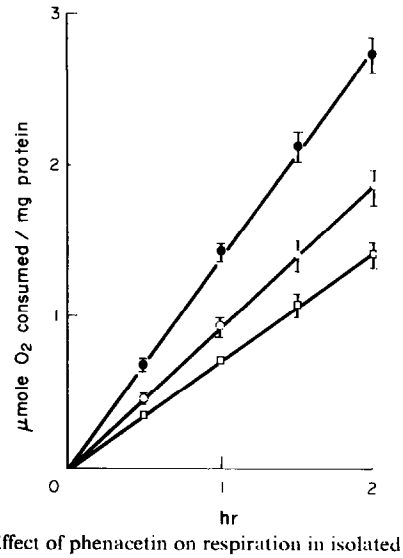


Fig. 1. Effect of phenacetin on respiration in isolated kidney tubules metabolizing glutamine. Tubules (approx. 10 mg protein) were incubated at 37° in 2 ml of the buffered medium containing 10 mM L-glutamine and 1% (v/v) ethanol. (●) control, (○) + 5 mM phenacetin, (□) + 10 mM phenacetin. The points represent mean values ± SEM for 3 separate experiments.

Table 1 contains results showing that phenacetin also inhibited oxygen consumption by tubules not supplied with exogenous substrate and by tubules provided with glucose or lactate. The degree of inhibition varied slightly according to the substrate but was statistically significant ($P < 0.01$) in all cases.

Respiration of intact mitochondria and submitochondrial particles. In Table 2 it is shown that phenacetin strongly inhibited the oxidation of glutamate by intact mitochondria when either ADP or the uncoupler 2,4-dinitrophenol was present (State 3 and State 3u respiration respectively) but had no effect on the slow and variable respiration that occurred in the absence of ADP and dinitrophenol (State 4 respiration).

Table 1. Effect of phenacetin on respiration in isolated rat kidney tubules

Substrate	μmoles Oxygen consumed/hr per mg protein		
	control	+5 mM phenacetin	+10 mM phenacetin
None	0.80 ± 0.02	0.57 ± 0.01*	0.49 ± 0.02*
10 mM D-glucose	1.20 ± 0.04	0.98 ± 0.02*	0.77 ± 0.06*
10 mM L-lactate	1.48 ± 0.01	1.20 ± 0.01*	0.99 ± 0.04*
10 mM L-glutamine	1.42 ± 0.06	0.92 ± 0.07*	0.70 ± 0.04*

Tubules (approx. 10 mg protein) were incubated for 1 hr at 37° in 2 ml of buffered medium containing substrates and phenacetin as indicated. The results are given as the means ± S.E.M. for 3 separate experiments.

*Values with and without phenacetin are significantly different $P < 0.01$.

Table 2. Effect of phenacetin on mitochondrial respiration

Preparation, substrate and no. experiments	Additions	nmoles Oxygen atoms consumed/min per mg protein		% Change due to phenacetin
		control	+8 mM phenacetin	
Intact mitochondria				
8 mM L-glutamate (3)	none	15 ± 5	15 ± 7	+10 ± 10
	ADP	138 ± 25	65 ± 11*	−53 ± 2
	DNP	168 ± 19	92 ± 14*	−46 ± 3
8 mM succinate (3)	none	70 ± 14	66 ± 9	− 2 ± 9
	ADP	293 ± 34	189 ± 14*	−35 ± 3
	DNP	352 ± 36	248 ± 21*	−29 ± 2
10 mM L-ascorbate + ‡1 mM TMPD (3)	none	617 ± 94	703 ± 98	+15 ± 11
	ADP	877 ± 94	888 ± 98	+ 1 ± 1
	DNP	940 ± 115	924 ± 23	− 2 ± 1
Submitochondrial particles				
1 mM NADH (6)	none	283 ± 10	85 ± 5†	−70 ± 2
8 mM succinate (3)	none	68 ± 3	48 ± 2†	−30 ± 1
10 mM L-ascorbate + 1 mM TMPD (1)	none	500	569	+14

Intact mitochondria or submitochondria particles were incubated at 30° in 2.5 ml of the reaction medium containing substrates and phenacetin as indicated. Respiration was determined with substrate only and after addition of either 1 μmole ADP or 0.04 mM 2,4-dinitrophenol (DNP). Results are given as mean values ± S.E.M. for the number of experiments indicated in parentheses in the first column.

*Values with and without phenacetin are significantly different, $P < 0.05$.

†$P < 0.01$.

‡TMPD, *N,N,N′,N′*-tetramethyl *p*-phenylenediamine.

The same general effects were observed when succinate served as the substrate, State 3 and 3u respiration being inhibited by phenacetin while State 4 respiration was not consistently affected. In contrast, with ascorbate plus tetramethyl phenylenediamine (TMPD) as electron donor, phenacetin had no significant or reproducible effect on respiration under any of the conditions used. It was concluded that, because phenacetin inhibited State 3 and State 3u respiration to the same extent, it must have acted directly on the respiration chain rather than indirectly through the coupling mechanism. Furthermore, the inhibition must have been exerted prior to cytochrome *c*, the point at which electrons from TMPD enter the chain.

These findings were confirmed by studies on submitochondrial particles, the results of which are presented in Table 2 and Fig. 2. Phenacetin inhibited the reduction of oxygen by NADH via the respiratory chain in a concentration-dependent fashion (Fig. 2). As indicated in Table 2, 8 mM phenacetin inhibited NADH oxidation by 70 per cent and succinate oxidation by 30 per cent but had no effect on the rate of oxidation of ascorbate plus TMPD.

NADH dehydrogenase and succinate dehydrogenase activities of submitochondrial particles. Spectrophotometric assay of NADH oxidase confirmed that phenacetin strongly inhibited the oxidation of NADH via the respiratory chain with oxygen as the terminal electron acceptor. This is shown in Table 3. However, when the respiratory chain was blocked by cyanide and either ferricyanide or menadione was used as electron acceptor, phenacetin had no inhibitory effect. Because the oxidation of NADH by these two acceptors was unaffected by 5 μM rotenone, in contrast to the greater than 90 per cent inhibition that occurred when oxygen was the acceptor, it was

Table 3. Effect of phenacetin on the oxidation of NADH and succinate by submitochondrial particles using various electron acceptors

Substrate	Acceptor	No. experiments	nmoles Substrate oxidized/min per mg protein		% Change due to phenacetin
			control	+8 mM phenacetin	
0.3 mM NADH	oxygen	3	130 ± 15	39 ± 4†	−70 ± 1
0.3 mM NADH	1 mM ferricyanide	4	2200 ± 110	2360 ± 120	+ 7 ± 1
0.3 mM NADH	0.2 mM menadione	3	14 ± 2	15 ± 2*	+ 9 ± 1
24 mM succinate	1.2 mM ferricyanide	3	67 ± 9	39 ± 3*	−40 ± 4
24 mM succinate	0.36 mM PMS *plus*‡ 0.06 mM DCPIP	3	143 ± 12	73 ± 6†	−49 ± 2

Assays were conducted at 30° in 2.5 ml systems by following the rate of change in the absorbance of a system containing submitochondrial particle suspension relative to that of a system containing an equivalent volume of the suspending medium. The wavelengths used were 340 nm for NADH, 420 nm for ferricyanide and 600 nm for DCPIP. Reaction mixtures were as described in the Methods section and includes 1 mM KCN when acceptors other than oxygen were used. Phenacetin was present as indicated. The results are given as mean values ± S.E.M.

*Values with and without phenacetin are significantly different $P < 0.05$.

†$P < 0.01$.

‡PMS, phenazine methosulphate; DCPIP, 2,6-dichlorophenol indophenol.

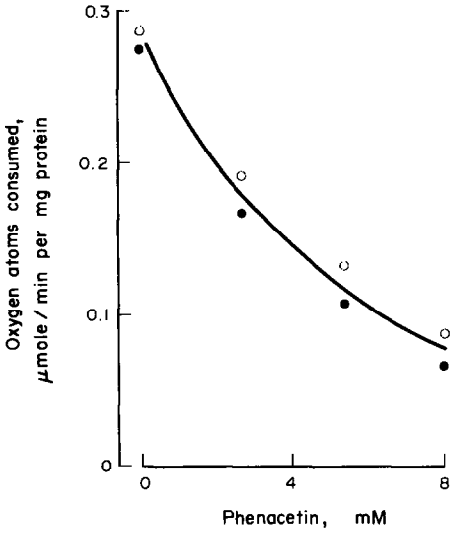
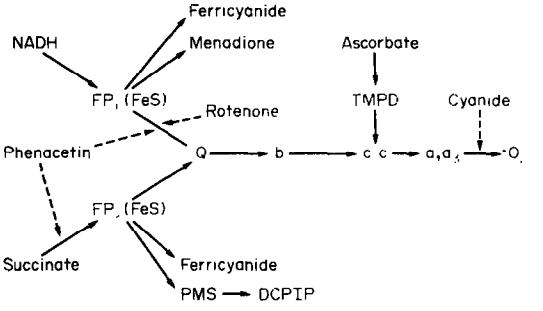


Fig. 2. Effect of phenacetin on respiration in submitochondrial particles oxidizing NADH. Particles were incubated at 30° in 2.5 ml of reaction medium containing 1 mM NADH, 1.2% (v/v) ethanol and phenacetin as shown. The values obtained in two separate experiments are presented.



Scheme 1. Electron transport in mitochondrial preparations and the possible sites of inhibition by phenacetin. Abbreviations and symbols used are as follows: FP_1, NADH dehydrogenase; FP_2, succinate dehydrogenase; FeS, nonhaem iron-sulphur proteins; Q, coenzyme Q (ubiquinone); b, c_1, c, a, a_3 cytochromes; PMS, phenazine methosulphate; DCPIP, 2,6-dichlorophenol indophenol; TMPD, *N,N,N',N'*-tetramethyl *p*-phenylenediamine. The full line arrows show the direction of electron flow and the dashed arrows indicate inhibition.

concluded that both received electrons directly from the reduced flavin moiety of NADH dehydrogenase. It appeared, therefore, that phenacetin did not affect the rate at which NADH dehydrogenase was reduced by NADH but strongly inhibited the rate at which the enzyme was reoxidized by the respiratory chain.

A different picture emerged when succinate served as electron donor (Table 3). The succinate dehydrogenase-catalyzed transfer of electrons either to ferricyanide or to dichlorophenol indophenol via phenazine methosulphate was inhibited by phenacetin. The degree of inhibition was similar to that of succinate oxidation in intact mitochondria (cf. Table 2), suggesting that phenacetin inhibits succinate-supported mitochondrial respiration through an action on succinate dehydrogenase activity.

DISCUSSION

Very few investigations into the specific effects of phenacetin on cell metabolism, and particularly on mitochondrial activities, have so far been reported. Raab *et al.* [16, 17] found that chronic administration of the drug to rats led to decreases in the activities of some mitochondrial enzymes in liver and kidney. It was suggested that the decreases were caused either by an altered rate of enzyme synthesis or by structural changes in the inner mitochondrial membrane; no direct influence of phenacetin on mitochondrial enzyme activities, including that of succinate dehydrogenase, was detected in *in vitro* assays. In contrast, the present work shows clearly that, in kidney mitochondria *in vitro*, phenacetin inhibits at least two electron transfer reactions of the respiratory chain. One of these is catalysed by succinate dehydrogenase and, judging from the greater sensitivity of phenacetin of NADH oxidation as compared with succinate oxidation, the other probably lies between reduced NADH dehydrogenase and coenzyme Q. These events are illustrated in Scheme 1 which depicts a simplified version of the respiratory chain, the points at which electron acceptors receive electrons, and the likely sites of inhibition by rotenone and phenacetin. It might be predicted that the susceptibility of the two main branches of the respiratory chain to inhibition by phenacetin could have adverse consequences for the whole oxidative energy metabolism of a kidney cell exposed to the drug. This prediction appears to be borne out by the observation that phenacetin strongly inhibited respiration in intact kidney cells using endogenous substrate or exogenous glucose, glutamine or lactate, all of which are considered to be important respiratory fuels *in vivo* [18] and are utilized rapidly *in vitro* [19].

These findings could, therefore, form the basis of another mechanism by which phenacetin might exert a cytotoxic effect. Previous studies on phenacetin nephrotoxicity, or more accurately analgesic nephrotoxicity, have suggested that the cause of cell damage is anoxia. Three main explanations have been offered for the fall in the oxygen supply to the injured tissue: they are,

1. products of phenacetin metabolism cause local methaemoglobinaemia and thus bring about a fall in the oxygen carrying capacity of the blood [2, 5, 7],
2. phenacetin and related substances oxidatively damage the erythrocyte membrane causing a loss of deformability and so restricting their passage through the capillaries [1, 2, 5],
3. analgesic drugs, including phenacetin, and their metabolite are so concentrated in the kidney that they precipitate and create a physical barrier to the flow of blood [8, 20]. In this regard it has been suggested that phenacetin itself might be only mildly cytotoxic but its ability to enhance the effectiveness of antidiuretic hormone could lead to increases in the intrarenal concentrations of its more toxic metabolites [21].

The present work can shed no light on the value of any of the above explanations but suggests that phenacetin might bring about anoxic damage not only by restricting the supply of oxygen to the cells but also by impairing their ability to metabolize oxygen.

The relevance of the present study to the *in vivo* situation may be questioned on the grounds of whether

or not the phenacetin concentrations used in the study could be attained in the *in vivo* kidney. This is difficult to answer with any degree of certainty because there is dispute as to whether phenacetin is concentrated by the kidney [5, 6]. However, a number of reports indicate that the intrarenal concentrations of some analgesic drugs and their metabolites, including *N*-acetyl *p*-aminophenol the major metabolic derivative of phenacetin, might rise close to saturation levels, especially during antidiuresis (for references see [21]). This suggests that concentrations approximating those used in this study could be reached *in vivo*. Also in doubt is the matter of whether, *in vivo*, it is phenacetin itself or its metabolic derivatives which are nephrotoxic. In unpublished work we have evidence that *N*-acetyl *p*-aminophenol also inhibits respiration in kidney tubules. Moreover, it is not inconceivable that other metabolites of phenacetin might be more inhibitory to respiration than is phenacetin. This possibility is at present engaging our attention.

In conclusion it is proposed that the cytotoxicity of phenacetin might be in part related to its ability to inhibit certain reactions of the mitochondrial respiratory chain. This effect, particularly if accompanied by a decrease in the supply of oxygen to the tissue, might help explain anoxic renal damage associated with phenacetin intake in mammals.

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