Biochemical Pharmacology, Vol. 28, pp. 3057–3062

0006-2952/79/1015-3057 $02.00/0

INHIBITION OF RESPIRATION AND GLUCONEOGENESIS BY PARACETAMOL IN RAT KIDNEY PREPARATIONS

KAY E. PORTER and ANTHONY G. DAWSON
Department of Biochemistry, University of Sydney, N.S.W. 2006, Australia

(*Received* 31 *March* 1979; *accepted* 9 *May* 1979)

Abstract—Paracetamol, at concentrations up to 10 mM, caused a reversible, concentration-dependent inhibition of respiration in isolated rat-kidney tubules metabolizing glucose, glutamine, lactate or glutamate. It also strongly inhibited the synthesis of glucose from glutamine or lactate and brought about a significant fall in the cell ATP level. Paracetamol lowered both coupled and uncoupled respiration in isolated kidney mitochondria oxidizing glutamate, but had no effect on respiration supported by succinate. Experiments with submitochondrial particles revealed that the drug did not influence the activity of NADH dehydrogenase but slowed the rate at which electrons were transferred from reduced NADH dehydrogenase to cytochrome *b*. The implications of these findings for paracetamol cytoxicity are discussed.

Paracetamol (4-hydroxyacetanilide), at normal therapeutic dose levels, is considered to be a safe and effective analgesic drug. However, at high doses, both in experimental animals and in man, the drug is acutely hepatotoxic and nephrotoxic [1–11]. Potter *et al.* [10] proposed that liver damage is due to the microsomal degradation of paracetamol, giving rise to an excess of aryl radicals which bind and inactivate intracellular proteins. A similar model has been used to explain kidney damage [11]. While this hypothesis is widely accepted and is well supported experimentally, other studies indicate that paracetamol, and some related compounds such as phenacetin and *p*-aminophenol, exert direct inhibitory effects on certain metabolic processes *in vitro* [12, 13]. It has been suggested that these effects might also contribute to the cell injury that occurs *in vivo*.

In an earlier study [12] we reported that phenacetin, an analgesic drug closely related to paracetamol, strongly inhibited respiration in isolated rat-kidney cells and kidney mitochondria. An investigation into the effects of paracetamol on kidney metabolism was therefore undertaken and the results are presented here. It was found that paracetamol, like phenacetin, slowed mitochondrial respiration by inhibiting electron transport between NADH dehydrogenase and cytochrome *b*. Respiratory inhibition was also seen in intact cells and was accompanied there by a fall in the cell ATP and a diminished rate of gluconeogenesis. The relevance of these observations to the recorded cytoxic actions of paracetamol *in vivo* is discussed.

MATERIALS AND METHODS

Chemicals. Hyaluronidase, collagenase, hexokinase, glucose 6-phosphate dehydrogenase, L-lactic acid, NADH, ADP, ATP, bovine serum albumin and 2,6-dichlorophenol indophenol were obtained from Sigma Chemical Co., St.Louis, MO, U.S.A.; sodium succinate, L-glutamic acid, D-glucose and ethylenediaminetetra-acetate (EDTA) were from BDH Ltd., Poole, Dorset, U.K.; paracetamol (*p*-hydroxyacetanilide) and L-glutamine were 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 cells. Fragmented kidney tubules, isolated from adult albino rats (Wistar strain) as described previously [14], were suspended in phosphate-buffered medium [15]. Incubation systems were prepared by adding cell suspension (5–10 mg protein) to buffered medium containing substrate, 2% (v/v) ethanol and 0–10 mM paracetamol, bringing the total volume to 2.0 ml. Incubations were carried out at 37°C in Warburg respirometers and the consumption of oxygen was followed manometrically [16]. Incubations were terminated by the addition of 0.2 ml 3N $HClO_4$ and the precipitated protein was removed by centrifugation. The deproteinized supernatants were neutralized with 3 N KOH and, after removal of the $KClO_4$ precipitate, the neutralized solutions were used for glucose and ATP determinations.

Preparation and incubation of kidney mitochondria. Mitochondria were isolated from rat kidney cortex by the method of Bustamante *et al.* [17] and were incubated at 30°C in a Rank oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.) as described in an earlier report [12]. The ethanol concentration in the reaction medium was 2% (v/v) and up to 10 mM paracetamol was also included. Substrates, ADP and 2,4-dinitrophenol were added as indicated in the text.

Preparation of submitochondrial particles. The method was based on that of Dionisi *et al.* [18]. Mitochondria, isolated as above, were suspended in 2 mM EDTA, pH 9, at a concentration of about 20 mg protein/ml. The suspension was exposed to ultrasound for 2 min in a Branson Sonifier operating at 20 kHz and a power output of approx 7 W. Unbroken mitochondria and dense particles were removed by centrifugation at 25,000 *g* for 10 min and the submitochondrial particles (SMP) were sedimented from the supernatant by centrifugation at 144,000 *g* for 45 min. The SMP were finally suspended in 0.25 M sucrose at roughly 10 mg protein/ml.

Assay of oxidoreductase activities in submitochondrial particles. NADH oxidase activity (i.e. NADH:O_2 oxidoreductase) was determined by measuring either the rate of oxygen consumption or the rate of disappearance of NADH when SMP were incubated with NADH. For oxygen consumption measurements the SMP were incubated at 30°C in the oxygen electrode. The reaction medium was that used for mitochondria, containing 2% (v/v) ethanol and up to 10 mM paracetamol. The reaction was initiated by addition of 0.5 mM NADH.

Spectrophotometric assays of NADH oxidase and NADH:ferricyanide oxidoreductase were carried out at 30°C in a Varian Superscan 3 UV-VIS spectrophotometer operating in split-beam mode as described previously [12]. SMP were suspended in a reaction medium comprising 50 mM Tris–sulphate (pH 8.0), 2% (v/v) ethanol, 0–10 mM paracetamol and 0.3 mM NADH. The rate of disappearance of NADH was followed at 340 nm with either oxygen or 1 mM potassium ferricyanide as the final electron acceptor. One millimolar KCN was also added when ferricyanide was used.

Succinate dehydrogenase activity was determined spectrophotometrically [12] by following the reduction of 2,6-dichlorophenol indophenol at 600 nm when SMP were incubated at 30°C in a medium comprising 50 mM sodium phosphate buffer (pH 7.8), 24 mM succinate, 1 mM KCN, 0.36 mM phenazine methosulphate, 0.06 mM 2,6-dichlorophenol indophenol, 2% (v/v) ethanol and 0–10 mM paracetamol.

Reduction of cytochromes by NADH. SMP (up to 4 mg protein) were suspended at 25°C in 2.5 ml Tris–sulphate buffer (pH 8.0), containing 2% (v/v) ethanol and 0–6 mM paracetamol. Using an Aminco DW-2 UV-VIS spectrophotometer operating in dual wavelength mode the increases in absorbance due to the reduction of cytochromes *b*, $c_1 + c$ and $a + a_3$ were monitored after the addition of 0.8 mM NADH. Recordings were continued until the solution was fully depleted of oxygen as evidenced by a further abrupt rise in the reduction level of the cytochromes. The wavelength pairs employed were 562–575 nm (cytochrome *b*), 550–540 nm (cytochrome $c_1 + c$) and 605–630 nm (cytochrome $a + a_3$).

Glucose and ATP determinations. Glucose present in neutralized samples of the deproteinized cell incubation systems was estimated by following the reduction of $NADP^+$ at 340 nm in systems containing ATP, hexokinase and glucose 6-phosphate dehydrogenase [19]. This method was used in preference to the more common glucose oxidase–peroxidase procedure of Krebs *et al.* [20] because of the observed interference of paracetamol with the latter method (see also [21]).

ATP was measured by an enzymatic method [22] modified slightly so that the assay medium contained 22 mM triethanolamine buffer (pH 7.5), 11 mM glucose, 1.7 mM $MgCl_2$, 0.7 mM EDTA and 0.03 mM $NADP^+$.

Protein measurements. Protein was determined by the method of Lowry *et al.* [23] and the linear transform equation of Coakley and James [24] was applied, Bovine serum albumin was used as the standard.

Statistical analyses. Most results are expressed as the mean ± S.E.M. for several separate experiments.

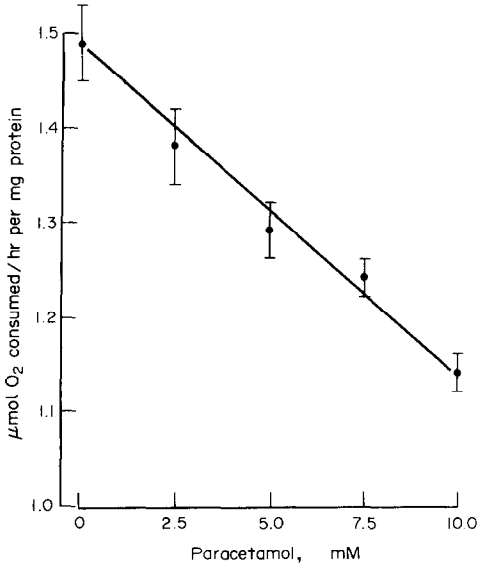

Fig. 1. Influence of paracetamol on the uptake of oxygen by kidney tubules metabolizing glutamine. Tubules (5–8 mg protein) were incubated for 1 hr at 37° in 2 ml of the buffered medium containing 5 mM L-glutamine, 2% (v/v) ethanol and paracetamol as indicated. Each point represents the mean ± S.E.M. for 4 experiments.

Significance of difference was estimated by the Student's *t*-test.

RESULTS

Respiration, gluconeogenesis and ATP levels in intact kidney cells. The results in Fig. 1 indicate that in cells provided with glutamine as an oxidizable substrate the consumption of oxygen was inhibited by paracetamol in a concentration-dependent fashion. The degree of inhibition exerted by each concentration of paracetamol was constant throughout the 60 min incubation period, as portrayed in Fig. 2. It was found that the

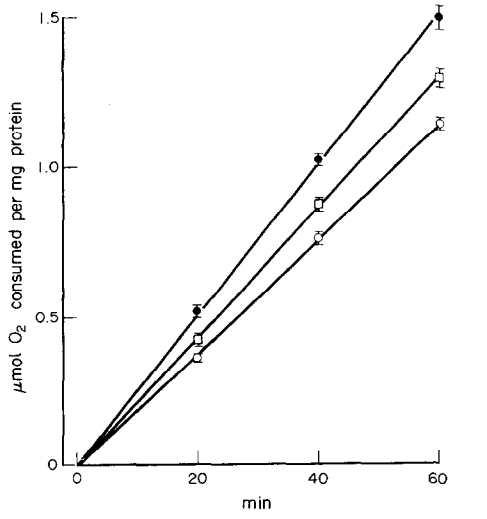

Fig. 2. Effect of paracetamol on the respiration of kidney tubules metabolizing glutamine. Experimental conditions as for Fig. 1. Each point represents the mean ± S.E.M. for 4 experiments. (●) Control; (□) +5 mM paracetamol; (○) +10 mM paracetamol.

Table 1. Effect of paracetamol on the uptake of oxygen by kidney tubules supplied with various substrates.

Substrate added	Oxygen uptake (μmoles/hr per mg protein) Control	+5 mM PC	+10 mM PC
None	0.89 ± 0.03	0.69 ± 0.03†	0.59 ± 0.03†
2 mM D-Glucose	1.18 ± 0.04	0.99 ± 0.03*	0.89 ± 0.02†
5 mM L-Lactate	1.61 ± 0.02	1.37 ± 0.05†	1.22 ± 0.04†
5 mM L-Glutamate	1.35 ± 0.02	1.18 ± 0.03†	1.03 ± 0.03†

Tubules (5–7 mg protein) were incubated for 1 hr at 37° in 2 ml of the buffered medium containing 2% (v/v) ethanol with substrate and paracetamol (PC) at the concentrations indicated. Results are expressed as the mean ± S.E.M. for 3 experiments.

Results with and without paracetamol are significantly different; * $P < 0.05$, † $P < 0.01$.

decrease in respiration was not confined to cells metabolizing glutamine but was also evident when no substrate was added and when glucose, lactate or glutamate was provided as substrate (Table 1). Furthermore, as shown in Fig. 3, the formation of glucose from either glutamine or lactate was strongly inhibited by paracetamol, again in a concentration-dependent manner. Coinciding with this decrease in gluconeogenesis was a fall in the cell ATP level. With glutamine as substrate the cell ATP level at the end of the incubation period was much lower in systems containing 10 mM paracetamol (2.40 ± 0.18 nmoles ATP/mg protein) than in systems lacking paracetamol (4.03 ± 0.43 nmoles/mg protein). Paracetamol thus lowered the ATP concentration by 40% ($P < 0.02$).

In other experiments it was observed that the effect of paracetamol on respiration was reversible. Cells preincubated with 10 mM paracetamol for 30 min and then washed with fresh, paracetamol-free medium respired at the same rate as cells which were both preincubated and washed in paracetamol-free medium. Respiration in the treated and untreated cells was equally susceptible to inhibition by paracetamol during the experimental incubation period.

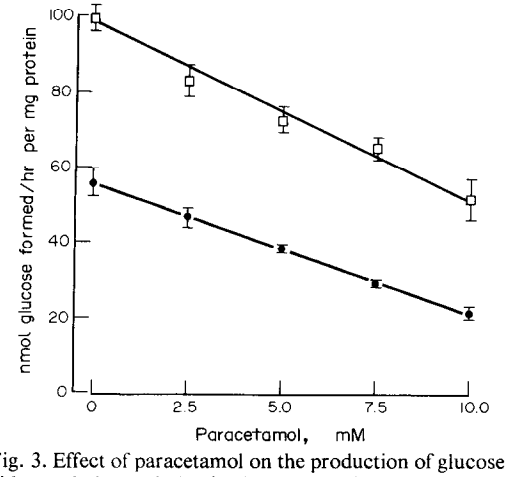

Fig. 3. Effect of paracetamol on the production of glucose by kidney tubules. Tubules (5–8 mg protein) were incubated for 1 hr at 37° in 2 ml of the buffered medium containing 2% (v/v) ethanol with paracetamol and substrate as indicated. Each point represents the mean + S.E.M. for 3 experiments. (□) 5 mM L-lactate; (●) 5 mM L-glutamine.

Because the inhibition exerted by paracetamol was independent of the nature of the exogenous substrate it seemed likely that the drug exerted its effect on processes common to the oxidative metabolism of all of them, most probably the oxidation of intramitochondrial tricarboxylate cycle intermediates. Accordingly experiments with mitochondrial preparations were undertaken.

Respiration in intact kidney mitochondria. The data in Table 2 show that glutamate-dependent respiration in mitochondria was strongly inhibited by paracetamol. State 3 respiration (ADP present) was decreased by 53 per cent in the presence of 10 mM paracetamol while state 3u respiration (uncoupled by 2,4-dinitrophenol) fell by 51 per cent. There was also a small but consistent inhibition of respiration in the absence of ADP and dinitrophenol (state 4). In marked contrast, paracetamol had no detectable effect on succinate-dependent respiration under similar experimental conditions.

These results suggested that paracetamol interfered directly with the flow of electrons along the respiratory chain and that the susceptible step was not normally traversed by electrons originating from succinate.

Respiratory and oxidoreductase activities of submitochondrial particles. The oxidation of NADH by SMP using oxygen as the ultimate electron acceptor was inhibited over 60 per cent by 10 mM paracetamol. This was observed both in experiments where oxygen consumption was monitored (data not shown) and in experiments where NADH oxidation was followed spectrophotometrically (Table 3). However, when ferricyanide was introduced as the electron acceptor and the terminal respiratory chain was blocked by cyanide, paracetamol did not affect the rate of oxidation of NADH. This showed that the drug inhibited the reoxidation of reduced NADH dehydrogenase by the respiratory chain but did not influence the activity of NADH dehydrogenase *per se*. Similarly, paracetamol did not alter the rate at which electrons were transferred from succinate to dichlorophenol indophenol via phenazine methosulphate, a process dependent on the activity of succinate dehydrogenase.

Reduction of cytochromes by NADH. The above results point towards paracetamol inhibiting the flow of electrons from reduced NADH dehydrogenase through a region of the respiratory chain not normally accessible to electrons derived from succinate. This suggests

Table 2. Effect of paracetamol on the uptake of oxygen by kidney mitochondria

Substrate (No. expts.)	Additions	Oxygen uptake (nmoles oxygen atoms/min per mg protein) Control	+2 mM PC	+4 mM PC	+6 mM PC	+8 mM PC	+10 mM PC
L-Glutamate (3)	None	31 ± 1	30 ± 3	26 ± 2	28 ± 2	20 ± 2	20 ± 1*
	ADP	153 ± 6	146 ± 4	122 ± 2*	104 ± 6†	87 ± 5†	72 ± 7†
	DNP	191 ± 1	180 ± 4	157 ± 7*	132 ± 6†	111 ± 5†	93 ± 11†
Succinate (5)	None	81 ± 3					77 ± 4
	ADP	232 ± 6					216 ± 9
	DNP	323 ± 5					331 ± 13

Mitochondria (1.4–1.6 mg protein) were incubated at 30° in 2.5 ml of the buffered incubation medium containing 8 mM substrate, 2% (v/v) ethanol, with paracetamol (PC) as indicated. Respiration was determined before and after the addition of either 0.2 mM ADP or 0.04 mM 2,4-dinitrophenol (DNP). Results are expressed as the mean ± S.E.M. for the number of experiments shown in brackets in column 1.

Results with and without paracetamol are significantly different; * $P < 0.01$, † $P < 0.001$.

the region between NADH dehydrogenase and coenzyme Q. In an attempt to add support to this idea, by demonstrating unequivocally that the partial block in electron transport occurred prior to the acceptance of electrons by the cytochrome components of the chain, experiments were performed in which the reduction of cytochromes in SMP oxidizing NADH in the presence of oxygen was followed spectrophotometrically. The results of these experiments are presented in Fig. 4. It is clear that paracetamol not only increased the duration of the aerobic steady-state (i.e. prior to the advent of anoxia), consistent with its role as an inhibitor of electron transport, but also brought about a fall in the aerobic steady-state reduction levels of all the cytochromes. This latter effect was most pronounced with cytochrome *b* (Fig. 4a), the aerobic reduction level of which fell from 57.9 ± 2.4 per cent (3 experiments) under control conditions to only 29.1 ± 3.8 per cent (3 experiments) in the presence of 6 mM paracetamol ($P < 0.002$). Neither this decrease nor the smaller ones seen with cytochrome $c_1 + c$ and $a + a_3$ was due to any change in the spectral characteristics of the cytochromes in the presence of the drug. This was shown by experiments in which the cytochromes were chemically reduced by dithionite in the presence or absence of paracetamol. It is also interesting to note that under anoxic conditions NADH, while capable of reducing virtually all of the chemically-reducible cytochrome $c_1 + c$ and $a + a_3$, was able to reduce less than one-third of the chemically-reducible cytochrome *b*, indicating that only a minor portion of the cytochrome *b* in SMP lies on the direct route of electron flow between NADH and oxygen. Furthermore, the proportion of the chemically-reducible cytochrome *b* capable of being reduced by NADH appeared to be lowered significantly in the presence of paracetamol. No explanation for this effect can be offered at this time.

DISCUSSION

Most clinical and experimental studies into the cytotoxicity of paracetamol have been concerned with the damage to the liver that ensues from the administration of high doses of the drug, though there are a few reports to suggest that damage to the kidney and other organs also occurs, particularly after prolonged use [1–5]. Acute hepatocellular injury appears to depend on the metabolism of the drug [6] and is associated with a fall in the intracellular level of glutathione and with an increase in the degree of covalent binding of paracetamol metabolites to cell macromolecules [7–9]. These observations led to the proposal [10] that paracetamol is metabolized by the hepatic microsomal drug-metabolizing system to potentially injurious molecules which are normally rendered inactive by becoming conjugated with glutathione. However, once the amount of these

Table 3. Effect of paracetamol on the oxidation of NADH and succinate by submitochondrial particles

Substrate	Electron acceptor	Substrate oxidized (μmoles/min per mg protein) Control	+4 mM PC	+10 mM PC
0.3 mM NADH	Oxygen	0.47 ± 0.04	0.30 ± 0.01*	0.16 ± 0.01†
0.3 mM NADH	Ferricyanide	2.88 ± 0.10	3.13 ± 0.13	3.28 ± 0.15
24 mM Succinate	PMS + DCPIP	0.13 ± 0.02		0.11 ± 0.01

Assays were conducted at 30° by following the rate of change of absorbance in a system containing submitochondrial particles relative to that in a system lacking them. The wavelengths used were 340 nm for NADH and 600 nm for DCPIP. Reaction mixtures were as described in Materials and Methods and paracetamol (PC) was present as indicated. Results are expressed as the mean ± S.E.M. for 3 experiments.

Results with and without paracetamol are significantly different; * $P < 0.01$, † $P < 0.001$. PMS, phenazine methosulphate; DCPIP, 2,6-dichlorophenol indophenol.

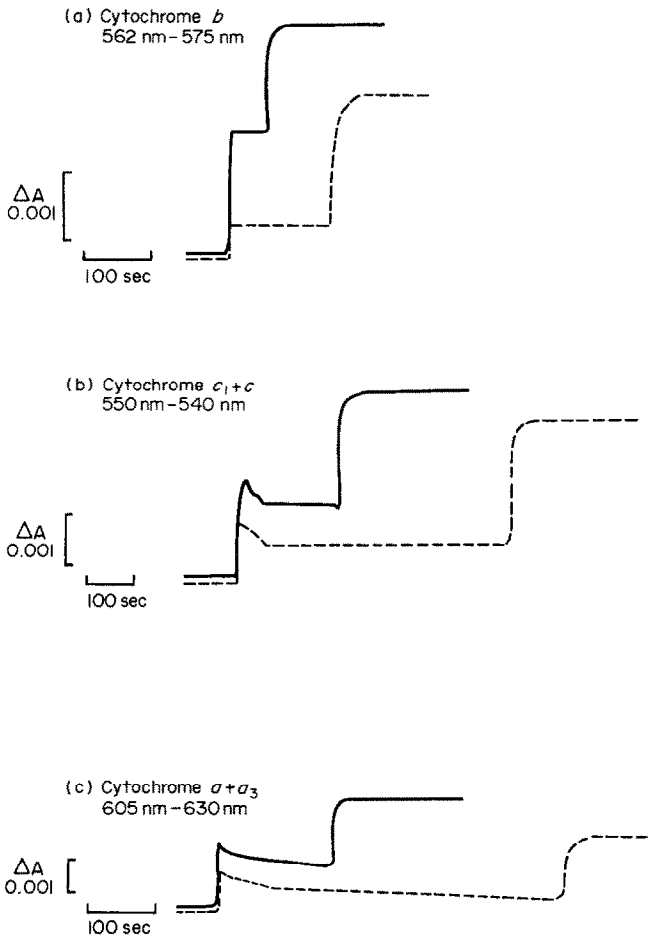

Fig. 4. Effect of paracetamol on the reduction levels of the cytochromes in submitochondrial particles oxidizing NADH. Submitochondrial particles [(a) 2.7 mg protein; (b) and (c) 0.9 mg protein] were suspended at 25°C in 2.5 ml Tris–sulphate buffer (pH 8), containing 2% (v/v) ethanol, with or without paracetamol. The increase in absorbance after the addition of 0.8 mM NADH was followed at the wavelength pairs indicated in the figure. (——) Control; (– – – –) +6 mM paracetamol.

toxic metabolites exceeds the detoxifying capacity of the glutathione, cellular damage occurs due to their binding to and inactivating macromolecules, especially proteins, within the cell. A similar sequence of events has been proposed to occur in the kidney [11]. Though the toxic metabolites have not yet been identified, it is likely that they are aryl radicals, possibly formed by way of *N*-hydroxyparacetamol [10, 25, 26].

This theory of cytotoxicity is plausible and well supported by the available experimental evidence, notwithstanding the fact that instances have been reported in which the correlation between tissue damage and the extent of covalent binding, or between tissue damage and glutathione depletion, is uncertain [25–27]. In the investigations reported here no attempt has been made to shed light on this mode of action of paracetamol, but the results obtained suggest that other mechanisms might also play a part in the cytotoxic actions of the drug. The paracetamol concentrations used in this work are of the same order as those which occur in the serum after severe paracetamol overdose [8, 28, 29] and which have been used in other experimental toxicity studies [1, 7–9, 27, 30]. Furthermore, since paracetamol can be concentrated by the *in vivo* kidney [31, 32], its concentration there could be even higher than in the serum. Hence the respiratory inhibition observed in isolated kidney cells, which was shown to be due to the partial blockage of mitochondrial electron transport between NADH dehydrogenase and cytochrome *b*, might also easily occur *in vivo*. A likely consequence of such inhibition would be a fall in the overall metabolic performance of the tissue, leading in the longer term to visible cell damage. In particular it could be predicted that various energy-dependent biosynthetic and osmotic processes would suffer most since respiration is the major means whereby energy is made available to them. This idea was substantiated by the data showing that in isolated kidney cells supplied with either glutamine or lactate, two important renal fuels [33], paracetamol caused a marked decrease in the cell ATP level with an associated fall in the rate of gluconeogenesis. Direct inhibition of renal and hepatic gluconeogenesis by paracetamol *in vitro* was also observed by Tange *et al.* [13] although the gluconeogenic capacity of liver

and kidney cells isolated from rats that had earlier received an intravenous injection of paracetamol appeared to be unimpaired. However, this might be explained in terms of the reversibility of paracetamol effects observed in the present study. Cases have been reported in which paracetamol overdose led to hypoglycaemia in human patients [2, 34], and although the validity of the data on plasma glucose concentrations has been questioned on methodological grounds [21], the results appear to be well founded. Record *et al.* [34] also found that in patients suffering from acute paracetamol intoxication the plasma concentrations of lactate and ketone bodies were markedly elevated. It could be surmised that the high levels of these substances reflected a decrease in the rate at which they were extracted from the blood and metabolized by tissues which normally use them as oxidizable or, in the case of lactate, gluconeogenic substrates.

Finally, it should be stressed that the actions of paracetamol on mitochondrial respiratory processes are unlikely to be confined to the kidney since these processes are common to all aerobic tissues. Hence, even though different tissues might not prove to be equally susceptible to the actions of paracetamol, respiratory inhibition should not be overlooked as a possible contributory factor in assessing the causes of tissue damage caused by paracetamol intoxication. Even if this action is insufficient on its own to account for the observed injury, it could be expected to aggravate damage brought about through other mechanisms. Furthermore, a possible role for other metabolites of paracetamol, notably *p*-aminophenol, should not be excluded from consideration [35–37].

REFERENCES

1. E. M. Boyd and G. M. Bereczky, *Br. J. Pharmac.* **26**, 606 (1966).
2. D. G. D. Davidson and W. N. Eastham, *Br. Med. J.* **2**, 497 (1966).
3. D. M. Krikler, *Br. med. J.* **2**, 615 (1967).
4. D. R. Master, *Proc. R. Soc. Med.* **66**, 904 (1973).
5. I. T. Gilmore and E. Tourvas, *Br. med. J.* **1**, 753 (1977).
6. J. R. Mitchell, D. J. Jollow, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 185 (1973).
7. D. J. Jollow, J. R. Mitchell, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 195 (1973).
8. M. Davis, G. Ideo, N. G. Harrison and R. Williams, *Clin. Sci. molec. Med.* **49**, 495 (1975).
9. M. Davis, N. G. Harrison, G. Ideo, B. Portmann, D. Labadarios and R. Willimas, *Xenobiotica* **6**, 249 (1976).
10. W. Z. Potter, S. S. Thorgeirsson, D. J. Jollow and J. R. Mitchell, *Pharmacology* **12**, 129 (1974).
11. R. J. McMurtry, W. R. Snodgrass and J. R. Mitchell, *Toxic Appl. Pharmac.* **46**, 87 (1978).
12. C. J. Druery and A. G. Dawson, *Biochem. Pharmac.* **28**, 57 (1979).
13. J. D. Tange, B. D. Ross and J. G. G. Ledingham, *Clin. Sci. molec. Med.* **53**, 485 (1977).
14. A. G. Dawson, *Biochem. J.* **130**, 525 (1972).
15. A. G. Dawson, *Biochem. Pharmac.* **24**, 1407 (1975).
16. W. W. Umbreit, in *Manometric Techniques* (Eds W. W. Umbreit, R. H. Burgess and J. F. Stauffer), 3rd edn., p. 1. Burgess, Minneapolis (1957).
17. E. Bustamante, J. W. Soper and P. L. Pederson, *Analyt. Biochem.* **80**, 401 (1977).
18. O. Dionisi, T. Galeotti, T. Terranova and A. Azzi, *Biochim. biophys. Acta* **403**, 292 (1975).
19. H. U. Bergmeyer, E. Bernt, F. Schmidt and H. Stork, in *Methods of Enzymatic Analysis* (Ed H. U. Bergmeyer), 2nd edn p. 1196. Academic Press, New York (1974).
20. H. A. Krebs, D. A. H. Bennett, P. DeGasquet, T. Gascoyne and T. Yoshida, *Biochem. J.* **86**, 22 (1963).
21. I. Kaufmann-Raab, H. G. Jonen, E. Jahnchen, G. F. Kahl and U. Groth, *Clin. Chem.* **22**, 1729 (1976).
22. W. Lamprecht and I. Trautschold, in *Methods of Enzymatic Analysis* (Ed H. U. Bergmeyer), p. 543. Academic Press. New York (1963).
23. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
24. W. T. Coakley and C. J. James, *Analyt. Biochem.* **85**, 90 (1978).
25. I. C. Calder, K. Healey, A. C. Yong, C. A. Crowe, K. N. Ham and J. D. Tange, in *Biological Oxidation of Nitrogen* (Ed J. W. Gorrod), p. 308. Elsevier North Holland Biomedical Press, Amsterdam (1978).
26. K. Healey, I. C. Calder, A. C. Yong, C. A. Crowe, C. C. Funder, K. N. Ham and J. D. Tange, *Xenobiotica* **8**, 403 (1978).
27. D. Labadarios, M. Davis, B. Portmann and R. Williams, *Biochem. Pharmac.* **26**, 31 (1977).
28. L. F. Prescott, N. Wright, P. Roscoe and S. S. Brown, *Lancet* **1**, 519 (1971).
29. L. F. Prescott, R. W. Newton, C. P. Swainson, N. Wright, A. R. W. Forrest and H. Matthew, *Lancet* **1**, 588 (1974).
30. S. S. Thorgeirsson, H. A. Sasame, J. R. Mitchell, D. J. Jollow and W. Z. Potter, *Pharmacology* **14**, 205 (1976).
31. M. A. Barraclough and F. Nilam, *Experientia* **28**, 1065 (1972).
32. G. G. Duggin and G. H. Mudge, *J. Pharmac. exp. Ther.* **199**, 1 (1976).
33. R. F. Pitts, in *Physiology of the Kidney and Body Fluids*, 3rd edn p. 259. Year Book Medical Publishers, Chicago (1974).
34. C. O. Record, R. A. Chase, K. G. M. M. Alberti and R. Williams, *Clin. Sci. molec. Med.* **49**, 473 (1975).
35. I. C. Calder, C. C. Funder, C. R. Green, K. N. Ham and J. D. Tange, *Br. med. J.* **4**, 518 (1971).
36. C. A. Crowe, I. C. Calder, N. P. Madsen, C. C. Funder, C. R. Green, K. N. Ham and J. D. Tange, *Xenobiotica* **7**, 345 (1977).
37. C. A. Crowe, N. P. Madsen, J. D. Tange and I. C. Calder, *Biochem. Pharmac.* **26**, 2069 (1977).