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PREVENTION OF PARACETAMOL-INDUCED LIVER INJURY BY FRUCTOSE

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Abstract—Hepatic cell injury was studied in an *in vitro* system using rat liver slices incubated in two stages. During the first 2 hr slices were exposed to 10 mM paracetamol, this was absent during the subsequent 4 hr of incubation. Cell damage was quantified at the end by measuring leakage of lactic dehydrogenase, increase in water content and potassium loss. Treatment of slices with 20 mM fructose in the second period of incubation prevented paracetamol-induced damage. The effect of fructose was not modified by the continued presence of paracetamol in the second incubation period. The inhibition of glycolysis either with 1 mM NaF or 10 μM iodoacetate blocked the effect of fructose. The protective effect afforded by fructose was not duplicated by the addition of lactate. All these findings strongly suggest an increase in intracellular ATP levels as the most probable explanation for the protective effect of fructose, and point to fructose as a potentially useful therapeutic tool for protection of the liver late in paracetamol intoxication.

A number of model systems have been used to investigate paracetamol toxicity. These include whole animals, especially rats, mice and hamsters [1–3], rat liver slices [4], isolated hepatocytes of various species of suspension [5–7], hepatocytes attached to tissue culture dishes and sub-cellular fractions such as mitochondria and microsomes [8, 9].

These studies have led to a successful understanding of the initial phases of toxicity, namely paracetamol metabolism, generation of reactive metabolites by cytochrome P450 linked enzymes, especially the P450IA2 and IIE1 isoenzymes, metabolite binding to glutathione (GSH), exhaustion of GSH and release of reactive metabolite to bind to macromolecules or cause oxidative damage, when large doses of paracetamol are taken [1–10].

The studies have also led to strategies of early prevention of paracetamol-induced liver injury, first in the model systems, where giving -SH donors such as *N*-acetyl cysteine and methionine proved successful. Clinical trials of treatment of patients who had taken large overdoses have confirmed the usefulness of methionine and acetyl cysteine [11].

However, the later steps of cell injury, subsequent to exhaustion of GSH supplies and release of reactive metabolite into the cell, have been far more difficult to understand or treat. Three major threads of argument about the nature of injury have emerged. These concern covalent binding of metabolite, presumably *n*-acetyl-*p*-benzoquinoneimine (NAPQI), to essential macromolecules, oxidation of macromolecules especially cation pumping ATPases by the action of quinone metabolites in an oxygen recycling step leading to formation of oxygen radicals, and inhibition of mitochondrial function possibly by paracetamol itself. It may well be that several points of cell function are injured (e.g. mitochondrial electron flow, calcium pumping by endoplasmic reticulum ATPase, oxidation of protein SH groups). Failure of intracellular calcium ion regulation is often proposed as a late common step leading to cell breakdown [12–14]. The ability of intracellular EDTA, some antioxidants and reducing agents like dithiothreitol to block late stages of cell breakdown would seem to support this view that there are many reversible steps late on in the path to cell breakdown after paracetamol attack [6, 15]. However, none of these agents acting late on in paracetamol injury have proved effective *in vivo*. In order to prevent breakdown of the cell after attack by paracetamol and its metabolites we may have to protect several sites of injury.

Liver slices are close to the original tissue in being sensitive to paracetamol if taken from rats pretreated with phenobarbitone and in showing a slow development of cell injury over an incubation time of about 6 hr [4, 16]. The concentration of paracetamol in the peripheral blood of poisoned patients, or rats, can reach around 5 mM at its peak, and the 10 mM concentration used in the present experiments is probably not far off the concentration found in the portal blood, when large amounts of paracetamol are present in the gastrointestinal tract.

Recently, it has been shown that fructose can protect completely against hypoxic damage in the liver [17]. The mechanism of protection afforded by fructose has been related to its ability to provide glycolytic ATP. Since one of the steps in cell death in paracetamol damage may be failure of mitochondrial function; we have studied the effect of fructose as a glycolytic substrate in preventing cell death. We have applied fructose in the second phase of injury, in liver slices, at the stage after paracetamol exposure and metabolism.

MATERIALS AND METHODS

Animals. Male Wistar rats (OLAC, Bicester,

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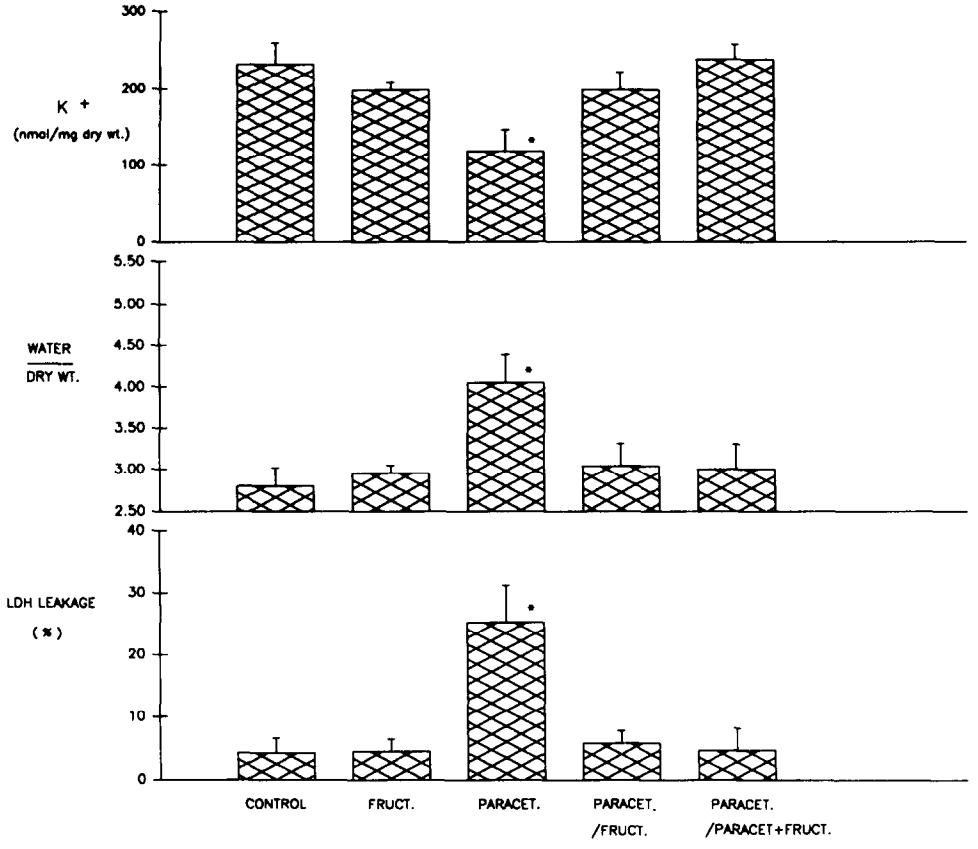

Fig. 1. Effect of 20 mM fructose added in the second stage of incubation (2–6 hr) in the presence and absence of 10 mM paracetamol, in slices previously exposed to 10 mM paracetamol for the first 2 hr of incubation (0–2 hr). Slice K^+ content, slice water content and LDH leakage from slice into the external medium were determined as described in Materials and Methods. LDH leakage is expressed as the percentage of enzyme activity found in the medium in comparison with the amount originally present in the slice. K^+ content is expressed as nmol/mg of dry weight slice and water as mg water/mg of dry wt of slice. Results are means ± SD of at least five experiments performed with slices obtained from different rats and using the mean of the duplicate slices taken in each experiment as a single measurement. * $P < 0.05$ as compared with control group.

U.K.) weighing 120–200 g were fed stock pellets (SDS, Witham, U.K.) and given Na phenobarbitone solution (1 mg/mL) as the sole source of drinking water for at least 5 days [18]. Vitamin E (5 mg α-tocopherol acetate in two drops of olive oil) was given by mouth 12–24 hr before the rats were killed. Rats were killed by exsanguination under fentanyl citrate (0.105 mg/kg, i.m.) and diazepam (2.5 mg/kg, i.p.) anaesthesia (Janssen, Wantage, U.K.). The liver was rapidly removed and slices of 0.3 mm thickness or less were cut by hand on a Stadie-Riggs stage with a long razor blade (A. H. Thomas Co., Philadelphia, PA, U.S.A.). Slices weighing 80–120 mg were put into 25-mL Erlenmeyer flasks containing 5 mL of Ringer solution with the following composition: 125 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 1 mM NaH_2PO_4, 1 mM $CaCl_2$, 10 mM glucose, 15 mM Hepes buffer, pH 7.4 at 37° as previously described [4]. The slices were put into the Ringer solution at room temperature and the experiment started by placing the flasks into an incubator bath at 37° under oxygen with shaking (90 strokes/min). After 2 hr the slices were taken out of the first flask and reincubated in the fresh Ringer solution for a further 4 hr.

Chemicals. Paracetamol, fructose, ethionine, iodoacetate and other chemicals were purchased from the Sigma Chemical Co. (Poole, U.K.) or BDH Ltd (Poole, U.K.) and were of analytical grade.

Measurement of enzyme leakage, K^+ and water content. Injury was assessed by measuring leakage of lactate dehydrogenase from the slice into the medium, and loss of potassium and increase in water content of the slices, as previously described [4, 16]. Lactate dehydrogenase (LDH) released into the Ringer solution was expressed as a percentage of the amount of enzyme originally present in the flask, based on the original slice weight and LDH assays of homogenates of slices sampled before incubation [16, 19].

For measurement of water and potassium content slices were removed from the incubation medium, blotted and weighed. They were dried at 90°

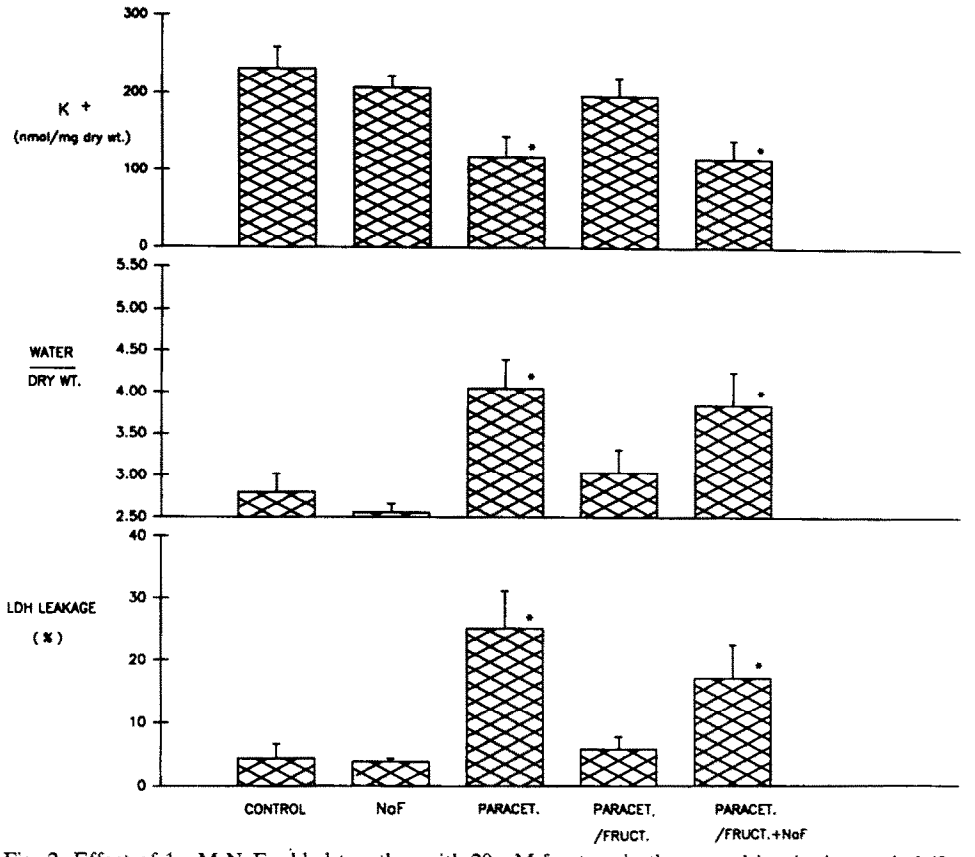

Fig. 2. Effect of 1 mM NaF added together with 20 mM fructose in the second incubation period (2–6 hr) in slices previously exposed to paracetamol for 2 hr (0–2 hr). Slice K^+ content, slice water content and LDH leakage from slice into the external medium were determined as described in Materials and Methods. Results are means of at least five experiments performed in duplicate with slices obtained from different rats. * $P < 0.05$ as compared with control groups.

overnight and weighed again. Water content was expressed as milligram water per milligram dry weight of liver slice. Potassium content was measured in an HCl extract of the dried piece of the liver, by atomic absorption, as previously described, and expressed as nmoles K^+ per milligram dry weight of liver slice [4].

Statistical analysis. Duplicate flasks were set up for each incubation condition in each experiment on the liver slices from one rat. The mean value for the duplicate slices was used as one data point for the analysis of results from at least five separate experiments. Significance of the differences were determined using analysis of variance (ANOVA) with a $P < 0.05$ being taken as indicating significant differences. Application of a *t*-test gave the same results of significance where indicated in the text.

RESULTS

As described before, slices incubated with 10 mM paracetamol for 2 hr showed little sign of injury, but when moved to a second incubation medium without paracetamol, developed a progressive and extensive leak of soluble enzyme content (lactic dehydrogenase or isocitrate dehydrogenase) from 2 hr in the second incubation onwards. This was accompanied by loss of potassium and increase in water content of the slices, all of which were readily measurable after 4 hr in the second incubation medium. In contrast, slices incubated without exposure to paracetamol showed a minimal leakage of enzyme and maintained a high potassium content [4]. The use of fentanyl in anaesthesia, instead of ether, did not alter the response of the liver slices to paracetamol.

Figure 1 shows the effect of fructose added in the incubation system after the 2 hr paracetamol exposure. Fructose (20 mM) on its own caused only minor effects in control slices (fall in K^+ and increase in water), but when added to those previously exposed to paracetamol it was able to protect the slices against the toxic effects of the drug. The LDH leakage, loss of K^+ and increase in water content were almost completely prevented as compared with the untreated slices, and slices incubated with paracetamol alone ($P < 0.05$). The effect afforded by fructose was also maintained in the continued presence of 10 mM paracetamol in the second incubation. The addition of fructose together with paracetamol during the first stage of incubation (0–2 hr) did not modify the subsequent toxic effect of paracetamol on the slices (data not shown).

Figure 2 shows the effect of 1 mM NaF added together with fructose in the second period of

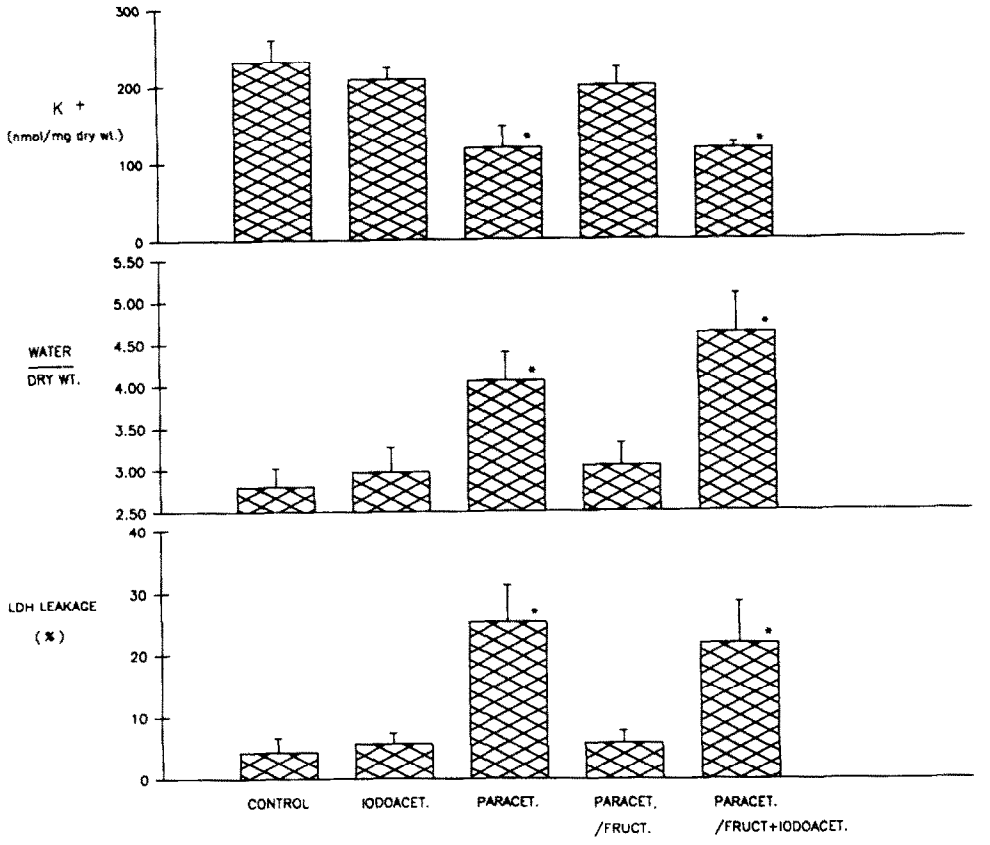

Fig. 3. Effect of 10 μM iodoacetate added together with 20 mM fructose in the second incubation period (2–6 hr) in slices previously exposed to 10 mM paracetamol for 2 hr (0–2 hr). Slice K^+, water content and LDH leakage from slice into the external medium were determined as described in Materials and Methods. Results are means of at least five experiments performed in duplicate with slices obtained from different rats. *$P < 0.05$ as compared with control groups.

Table 1. Effect of ethionine on the protection afforded by fructose against paracetamol toxicity in liver slices

Incubation time 0–2 hr	Incubation time 2–6 hr	LDH leakage (%)	K^+ content (nmol/mg dry wt)	Water/dry wt (mg/mg)
Ringer	Ringer	4.3 ± 2.3	231 ± 28	2.8 ± 0.2
Ringer	Ethionine	3.9 ± 0.1	234 ± 6.4	2.9 ± 0.1
Paracetamol	Ringer	25.2 ± 5.9*	118 ± 28*	4.1 ± 0.2*
Paracetamol	Fructose	6.3 ± 2.2	201 ± 23	3.0 ± 0.3
Paracetamol	Ethionine	30.6 ± 4.6*†	82 ± 12*†	4.6 ± 0.4*†
Paracetamol	Ethionine + fructose	9.3 ± 4.4	194 ± 24	3.3 ± 0.4

Each value represents the mean ± SD of at least five separate experiments performed in duplicate assays.

The final concentrations of fructose and ethionine were 20 and 5 mM, respectively, while the paracetamol concentration was 10 mM.

Cell injury was assessed as LDH leakage from slices into the medium at 6 hr expressed as a percentage of the original content of LDH in the slice.

K^+ content and water content of slices were measured as described in Materials and Methods.

* Means statistically different from control values (Ringer), $P < 0.05$.

† Means statistically different from paracetamol treated group, $P < 0.05$.

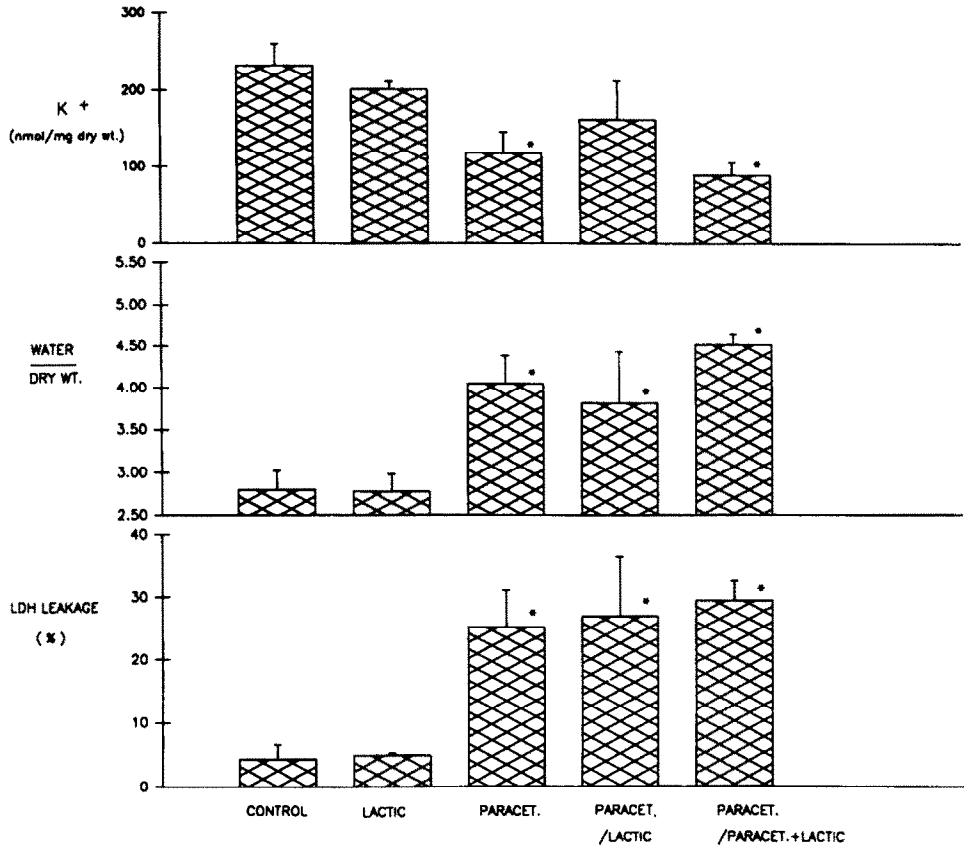

Fig. 4. Effect of 2 mM lactic acid added in the presence and absence of 10 mM paracetamol to slices previously incubated with 10 mM paracetamol for 2 hr (0–2 hr). Slice K^+ content, slice water content and LDH leakage from slice into the external medium were determined as described in Materials and Methods. Results are means of at least five experiments performed in duplicate with slices obtained from different rats. * $P < 0.05$ as compared with control groups.

incubation (2–6 hr). NaF on its own did not produce any effect in control slices. However, when added in the second stage with fructose it blocked the protection afforded by fructose. Values of LDH leakage, K^+ loss and increase in water content under these conditions were similar to those obtained in slices incubated with paracetamol only. Similar effects were obtained when 20 μM iodoacetate was used instead of NaF as inhibitor of the glycolysis. That is, iodoacetate also prevented the protective effect of fructose (see Fig. 3). In order to investigate whether the effect of fructose was due to the production of lactic acid through the glycolytic pathway, experiments where 2 mM lactic acid was included in the medium were performed and these results are shown in Fig. 4. Lactate (2 mM) did not protect the slices against the toxic effects of paracetamol.

Table 1 shows the effect of 5 mM ethionine on the second phase of incubation using liver slices previously incubated with and without paracetamol. Ethionine at this concentration did not produce any effect on the markers of cell damage in control slices, but when added to slices previously exposed to paracetamol for 2 hr, a small but significant increase in toxic effects was produced. Fructose but not lactate, was able to counteract the cell injury induced by the combination of paracetamol and ethionine.

DISCUSSION

In previous experiments using rat liver slices we demonstrated that the addition of exogenous sulphur-containing amino acids increases the cellular GSH concentration thus providing protection in the first stage of paracetamol metabolism. However this increase in GSH did not afford protection in the second stage of cell run-down [4]. More recently, we demonstrated the ability of the electron acceptor 2,6-dichlorophenol indophenol (DCPIP) and also ethanol to protect against paracetamol in the second phase of incubation after paracetamol metabolism was ended [16]. These latter results showed that an alternative mode of cell protection could be to change the intracellular redox balance. It also seems possible that the mitochondria are a target for paracetamol or its metabolits.* If there was a major lesion in the mitochondria leading to a fall in ATP/ADP ratio, then repair of other lesions in the cell could be impaired and measures to increase ADP phosphorylation might protect the cells. The

* Nazareth WMA, Sethi JK and McLean AEM, manuscript in preparation.

experiments reported here were designed to provide glycolytic substrates to increase energy supplies to the damaged cells.

Our present results demonstrate that paracetamol injury in liver slices can be prevented by the addition of fructose to the medium after the end of paracetamol metabolism.

In order to simulate the conditions usually found in patients intoxicated with the drug, we also performed experiments which included paracetamol in the second phase of incubation along with fructose. These findings demonstrate the effectiveness of fructose in protecting against cell death even in the continued presence of paracetamol.

Two different inhibitors of the glycolytic pathway were used: NaF and iodoacetate which block fructose metabolism at different levels. It is well known that NaF produces an accumulation of 3-phosphoglycerate and 2-phosphoglycerate while iodoacetate causes accumulation of fructose 1,6-diphosphate [20]. Both inhibitors have in common their ability to reduce the formation of glycolytic ATP, however with iodoacetate NADH generation during glycolysis is also blocked. In our experiments the effects of iodoacetate and NaF were similar, they counteracted the protection afforded by fructose to the same extent.

Even though these inhibitors have numerous sites of action, the results suggest that ATP generation derived from fructose metabolism is probably more important than NADH generation in blocking the damage induced by paracetamol in slices.

Fructose as a good glycolytic substrate leads to rapid production of lactate [21, 22] but lactate added to the medium did not protect the slices. However, it is still possible that lactate penetrates cells in slices less well than fructose does.

The most likely explanation for the protection given by fructose is an increase in the intracellular concentration of ATP. Indeed, our experiments performed with ethionine suggest that the fall in the total ATP levels normally caused by ethionine [23] can potentiate the damage by paracetamol and that fructose could reverse the process, presumably by a compensatory change in ATP/ADP ratio. A decline in ATP levels can be a critical event in the development of cell damage [24]. Low ATP levels may lead to loss of activity of energy requiring calcium pumps and intracellular calcium may rise. That increase in intracellular calcium may secondarily activate phospholipases [25, 26] which cause breakdown of membrane phospholipids and may also be important for other critical cellular functions.

It was previously shown that fructose is particularly efficient in preventing hypoxic cell damage and its protective effect was related to its ability to provide glycolytic ATP [27]. Although anaerobic ATP production may only provide approximately 20% of that required by aerobic tissue, ATP produced glycolytically may be used primarily for membrane functions and Ca^{2+}-uptake by endoplasmic reticulum facilitating the survival of the cells [28–30]. It has been demonstrated that other carbohydrates such as xylitol and mannitol do not have the same ability to increase ATP levels as does fructose. Glucose fails to produce much glycolytic ATP because it is a poor substrate for hepatic glycokinase due to its high K_m [31].

Fructose tends to delay glucose release and counteract glycogen depletion induced by paracetamol as already demonstrated [32, 33]. Large doses of fructose given in circumstances of high ATP levels can depress ATP and inorganic phosphate levels in the liver during the rapid synthesis of fructose-1-phosphate [34, 35]. All these effects taken together make fructose a potential tool for investigation of the late toxic effects of paracetamol.

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