at fault because it has been thoroughly evaluated and found to give the same results as the assay employing the labeled nucleoside as substrate [5]. An alteration in the cells and consequent alteration in the kinase cannot be ruled out. However, this is also unlikely because the K_m for adenosine has remained the same over the 16 years that we have worked with the HEp-2 enzyme. The presence of impurities in TCN could have affected the results but, even if present, impurities are unlikely to account for a difference of two orders of magnitude unless they themselves were substrates or good inhibitors. The purity of the non-radioactive sample used earlier could not be re-examined because none of it remains; however, the [^{14}C]TCN had a high degree of radiopurity (97% as determined by paper chromatography). We therefore think that our reported results for TCN were in error for reasons that cannot now be explained. It is likely that the kinetic constants found for the phosphorylation of TCN by the L1210 and HEp-2 adenosine kinases are representative of those of adenosine kinases from mammalian cells, for among the many substrates that have been studied no large differences were found with enzymes from HEp-2 cells [5], L1210 cells [6] and rabbit liver [8, 9].

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Covalent binding and the mechanism of paracetamol toxicity

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Paracetamol is a popular, over-the-counter, analgesic drug which is safe and well tolerated at therapeutic doses, but causes chiefly centrilobular hepatic necrosis when taken in overdose [1, 2]. The specific mechanism for the toxicity of paracetamol is a matter of controversy. Covalent binding of paracetamol metabolites to essential cellular macromolecules has been most extensively studied, particularly by Mitchell and colleagues [2, 3], and the received concept stemming from the work of these authors is that generalized covalent binding is the trigger that leads to cell necrosis. This view has received wide support [4–6] but some evidence contrary to the covalent binding theory has also been presented [7, 8]. Also we have recently reported that covalent binding of paracetamol metabolites to cell protein is not sufficient to account for cell injury as a consequence of paracetamol toxicity [9]. In this communication, we expand on our findings on the role of the covalent binding mechanism, and report a simple technique capable of separating and investigating cells which have been either damaged or undamaged after paracetamol treatment under exactly the same incubation conditions in the same flask. These studies provide evidence against the 'general covalent binding' theory and reinforce our previous observation that, on its own, general covalent binding of paracetamol is not sufficient cause for the cell necrosis observed in paracetamol-induced injury.

Materials and methods

Isolated rat hepatocytes were prepared and used as previously described [9]. [^{14}C]Paracetamol used for the covalent binding studies was purchased from Amersham International (Amersham, U.K.), and purified by a high-performance liquid chromatography (HPLC) technique [10] before use. Percoll was purchased from Pharmacia (Great Britain) Ltd. (Hounslow, U.K.). Ten times concentrated Hank's balanced salt solution (HBSS) was obtained from Gibco Bio-Cult (Uxbridge, U.K.). Damaged and undamaged cells were separated using Percoll which was prepared and used as follows.

Percoll solution was mixed with 10 × HBSS in the ratio of 9:1 (v/v) to give an isotonic stock solution of density 1.13 g/ml. This was further diluted with normal strength HBSS in the ratio of 42.5:57.5 (v/v) to give a working solution of density 1.06 g/ml.

Aliquots (15 ml) of the working Percoll solution, in universal containers, were used as 'columns' for separation of the damaged and undamaged cells in a given cell suspension. Usually 3–4 ml of the cell suspension was loaded on top of a Percoll solution column, taking care to avoid mixing the cell suspension into the Percoll, and then centrifuged at 40 *g* for 10 min in an angle rotor. At the end of the centrifugal run, mixing was again avoided and the cells were recovered. The damaged dead cells which remained at the top of the Percoll column were recovered by sucking off with a plastic bulb pipette, and the undamaged, apparently live cells which had migrated through the Percoll solution and pelleted were recovered after decanting the Percoll solution. Cell viability of both the damaged and the

Table 1. Covalent binding of paracetamol in damaged and undamaged cells

Cell sample	nmole [^{14}C]paracetamol bound/mg protein at time (hr) of incubation 0.5	1.0	2.0	4.0
Total cells	1.10 ± 0.40	2.43 ± 0.77	2.95 ± 0.29	5.20 ± 1.55
Damaged cells	2.44 ± 1.34	3.47 ± 1.87	5.76 ± 3.54	8.79 ± 3.71
Undamaged cells (pre-culture)	0.56 ± 0.18	1.44 ± 0.50	2.10 ± 0.16	4.90 ± 3.68
Attached cells, 22–24 hr later (post-culture)	0.85 ± 0.11	1.04 ± 0.45	1.39 ± 0.61	3.20 ± 0.46

Isolated rat hepatocytes were incubated in the presence of [^{14}C]paracetamol, in shaking flasks, for the times indicated. At the end of each incubation period a small fraction of the total cell suspension was collected and analysed for covalent binding. The remaining cell suspension was centrifuged through Percoll solution (density 1.06 g/ml), and the cells separated into either the damaged or the undamaged cell fractions. The undamaged cells were washed with sterile William's Medium E containing 2.5% foetal calf serum, and a small fraction of the cleaned cells was taken for analysis of covalent binding. The remaining washed cells were suspended in William's medium E + 2.5% FCS, and incubated in tissue culture for 22–24 hr. At the end of the culture period the live cells attached to culture plates were scraped off and analysed for covalently-bound paracetamol. The damaged cells obtained after Percoll separation were not cleaned and were analysed directly for covalent binding. Results are mean ± S.D. for three separate experiments.

undamaged cell fractions was confirmed by trypan blue exclusion, and shown to be less than 5% (six determinations) for the damaged cells and greater than 95% (six determinations) for the undamaged cells.

Results and discussion

The results are set out in Table 1 and show that the extent and time course of covalent binding of paracetamol to hepatocytes *in vitro* are similar to those observed *in vivo* by Mitchell and colleagues [2]. Cell injury is associated with a greater degree of bound paracetamol. When the paracetamol-killed cells at time 1 hr are compared with the surviving cells at 4 hr, it can be seen that the 4 hr survivors contain more paracetamol than the 1 hr killed cells, and that even after 22 hr culture, cells contain as much bound paracetamol as the cells killed by paracetamol at 1 and 2 hr. These results suggest that covalent binding of paracetamol to cellular macromolecules in general is not sufficient to account for cell necrosis in paracetamol injury.

To test the hypothesis that the undamaged cells really were intact and not only at a stage soon after which they would also become damaged if incubated for longer periods, these were washed with sterile tissue culture medium and incubated for 22–24 hr in tissue culture. Analysis of covalent binding in the cells attached to the tissue culture plates and recovered at the end of the culture period indicated that the cells not only retained the covalently-bound paracetamol, but that this was retained at levels similar to those present at the beginning of the culture period (see Table 1).

To date, major support for the covalent binding mechanism has been provided chiefly by studies in which the extent of covalent binding has been correlated with the extent of tissue necrosis. These studies have provided only a measure of the 'end-point' of the cell necrotic process and do not really give an indication of the initiation point of this process. From these studies, covalent binding is merely a measure of extent of exposure to reactive metabolites. The experimental procedures described in this communication have overcome part of this problem and can be used in investigating the role of covalent binding of paracetamol in both damaged and undamaged cells. The data obtained from these studies provide evidence that suggests that general covalent binding of paracetamol to cellular macromolecules is not on its own the main cause of paracetamol-induced cell death. It is likely that binding and inactivation of specific sites are the real initiators of the process leading, through many steps, to eventual cell death.

The cell separation technique described offers a different approach in the study of drug-induced cell injury and has potential for the investigation of the specific events leading to cell death.

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