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EFFECT OF *N*-HYDROXYPARACETAMOL ON CELL CYCLE PROGRESSION

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Abstract—*N*-hydroxyparacetamol treatment of rat kidney cells gave rise to a dose-dependent decrease in DNA synthesis. A concentration of 1.0 mM *N*-hydroxyparacetamol at pH 7.2 decreased the level of DNA synthesis to 13.0 ± 2.3% of the control value after 1 hr incubation. This compound also caused a perturbation of cell cycle progression. A concentration of 0.44 mM *N*-hydroxyparacetamol induced G1/S and S phase blocks. These delays became evident at approximately 12 hr after treatment and persisted until about 15 hr when cells started to recover. It seems unlikely that *N*-hydroxyparacetamol inhibits DNA synthesis and perturbs cycle progression through alterations to DNA structure as such, since this compound failed to alter the migration pattern of naked plasmid DNA.

Paracetamol is an antipyretic analgesic which when consumed in excessive amounts produces a characteristic, and sometimes fatal, centrilobular hepatic necrosis as well as acute renal failure [1–4]. Paracetamol is converted to a chemically reactive arylating agent by a cytochrome P450/NADPH dependent reaction and covalently binds to cellular macromolecules producing pathological changes. A correlation between loss of renal glutathione, covalent binding of paracetamol metabolites to renal tissue, and paracetamol related renal cortical necrosis has been established [5, 6]. This correlation suggests that conversion of paracetamol to an electrophilic metabolite by cytochrome P450 is a critical step in the pathogenesis of acute paracetamol nephrotoxicity.

The main route of metabolism and excretion of paracetamol involves conjugation with sulphate and glucuronide with only a small portion of the drug being transformed into reactive intermediates. A number of monocyclic *N*-acetyl arylamines are capable of being *N*-hydroxylated by cytochrome P450 mixed function oxidases [7–9]. Thus, initially it was proposed that metabolic activation of paracetamol to a toxic arylating metabolite occurred via *N*-hydroxylation to *N*-hydroxyparacetamol [10, 11]. Detection in the urine of meta-substituted sulphydryl metabolites of paracetamol [12–16] led to the proposal that *N*-hydroxyparacetamol rapidly dehydrates to the ultimate toxic species *N*-acetyl-*p*-benzoquinone imine (NAPQI) [17, 18]. However, several reports questioned the production of *N*-hydroxyparacetamol from paracetamol although there seems to be agreement that NAPQI is the ultimate toxic metabolite of paracetamol [19–21]. HPLC and gas chromatography–mass spectrometry revealed that hamster liver microsomes form *N*-hydroxyparacetamol from *N*-hydroxyphenacetin and not from paracetamol [22]. Thus NAPQI may also be one of the ultimate toxic intermediates in phenacetin metabolism.

N-hydroxyparacetamol has been reported to inhibit DNA, RNA and protein synthesis, alter the sedimentation of nucleoids and decrease cell viability in lymphoblastoid cells [23]. The main objective of the study reported here is to gain a greater understanding of the possible interaction of *N*-hydroxyparacetamol, or its spontaneous dehydration product NAPQI, with naked DNA and also to determine the effects of this compound on cell cycle progression.

MATERIALS AND METHODS

Cell culture. A normal rat kidney fibroblast cell line (NRK-49F) obtained from ATCC, Rockville, Maryland, was used in this study. The cells had a doubling time of approximately 24 hr and were diploid when this study commenced. In order to ensure that the cells were growing in log phase at the commencement of experiments they were always subcultured 30 hr prior to use. Cells were grown as monolayer cultures in RPMI-1640 medium (GIBCO) supplemented with 5% foetal calf serum (FCS), penicillin (100 I.U./ml) and streptomycin (60 μg/ml) in a humidified atmosphere of 5% CO_2 in air, at 37°.

Inhibition of DNA synthesis. Freshly divided NRK-49F cells were plated out at 3.0×10^5 cells per plate and were allowed to grow for approximately 30 hr in RPMI-1640 medium supplemented with 5% foetal calf serum. The medium was then removed and the plates washed twice in colourless Hanks (a balanced salt solution) to remove serum proteins. Two millilitres of RPMI-1640 buffered with 21 mM Hepes, pH 7.2, was added to the cells so that the volume of medium just covered the bottom of the plate. *N*-hydroxyparacetamol was added to these plates to give final concentrations of 0, 0.25, 0.4, 0.5, 0.75 and 1.0 mM. Cells were then incubated under

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5% CO_2 in air at 37° for 1 hr. After incubation, the plates were washed three times with colourless Hanks before 1.9 ml of RPMI-1640 + 5% FCS was added. One hundred microlitres of [^{3}H]-thymidine (15 μCi/ml, 25 Ci/mmol, Amersham) was also added to each plate and incubation was carried out for 90 min at 37°. Acid-precipitable DNA was collected onto Whatman GF/A glass fibre discs and incorporation of radioactivity determined by scintillation counting.

Effect of N-*hydroxyparacetamol on DNA structure*. In these experiments the effects of *N*-hydroxyparacetamol and its decomposition products on plasmid DNA were studied. *E. coli* strain JM101 transformed with the plasmid pBR322 was grown overnight at 37° in 500 ml of tryptone-yeast medium containing ampicillin (50 μg/ml). Spectinomycin (100 μg/ml) was then added and incubation carried out for 12 hr to amplify plasmid at 37° according to the method of Mukhopadhyay *et al.* [24]. Extraction of plasmid DNA was essentially by the alkaline lysis method followed by CsCl–ethidium bromide equilibrium centrifugation according to the method of Maniatis *et al.* [25].

Concentrations ranging from 0 to 5 mM were used to examine the effects of *N*-hydroxyparacetamol on plasmid DNA structure. Ten microlitres of plasmid DNA (0.7 μg) was mixed with 10 μl of various concentrations of *N*-hydroxyparacetamol in 10 mM Tris (pH 8) containing 1 mM EDTA. Incubations were carried out at 37° for 1 hour and reactions were "stopped" by addition of 4 μl of 6× gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol 40% (w/v) sucrose in H_2O). All plasmid samples were run in 0.8% agarose gels, pH 8 buffer (0.04 M Tris–acetate, 1 mM Na_2 EDTA). Gels were run in a horizontal electrophoresis apparatus immersed in buffer containing 5 μg/ml ethidium bromide and subsequently photographed under u.v. The negatives of these photographs were scanned by densitometry to determine the relative amounts of the various forms of plasmid DNA.

Cell cycle effects of N-*hydroxyparacetamol*. The effects of *N*-hydroxyparacetamol on cell cycle progression were studied by the stathmokinetic procedure of Dosik *et al.* [25]. Cells at 3.0×10^5/ml were plated out as described in the previous section. After washing, 4 ml of 21 mM Hepes-RPMI-1640 was added to each of 20 tissue culture plates. *N*-hydroxyparacetamol was added to half the plates to give a final concentration of 0.44 mM, the other 10 plates remained untreated. Incubation was carried out at 37° for 1 hr followed by two washings with colourless Hanks. Cells were then incubated in the presence of colcemid (0.05 μg/ml). Samples were harvested at 0, 4, 6, 8, 10, 12, 13, 15, 17 and 20 hr after treatment to follow the progression of cells through the cycle. After centrifugation, cells were resuspended in 200 μl of PBS, fixed by adding 200 μl of methanol, and stored for up to 24 hr prior to analysis on a Fluorescent Activated Cell Sorter (FACS IV Becton Dickinson). One hundred microlitres of a fluorescent DNA stain (250 mg/ml propidium iodide (Sigma), 1 mg/ml Ribonuclease (Boehringer, Mannheim) and 1.0% triton-X100) was added to 400 μl of fixed cells on ice for approximately 30 min prior to analysis.

RESULTS

A previous report from this laboratory has demonstrated that *N*-hydroxyparacetamol or a derivative causes a marked inhibition of DNA synthesis in human lymphoblastoid cells [23]. Experiments in the present study confirm this inhibition in cells (NRK-49F) derived from a target organ for this compound. A rapid decline in DNA synthesis occurred in the concentration range from 0.25 to 0.75 mM with a levelling off at concentrations greater than 0.8 mM (Fig. 1). A concentration of 1.0 mM *N*-hydroxyparacetamol decreased the level of DNA synthesis to 13.0 ± 2.3% of the control value.

N-hydroxyparacetamol has been reported to cause a dose-dependent and time-dependent loss in the superhelix density of DNA in mammalian cells as determined by nucleoid sedimentation analysis [23]. Plasmid pBR322 was used to study the effects of *N*-hydroxyparacetamol on DNA structure. The plasmid was exposed to a range of concentrations of *N*-hydroxyparacetamol. Figure 2 shows the migration pattern of *N*-hydroxyparacetamol treated plasmid DNA. Concentrations up to 5.0 mM appear to have no effect on naked supercoiled DNA since no changes in the relative amounts of relaxed circular and supercoiled DNA were observed as the dose of *N*-hydroxyparacetamol was increased.

Since *N*-hydroxyparacetamol inhibited DNA synthesis, it seemed conceivable that this compound might interfere with the progression of cells through the cell cycle. An obvious point of interference would be expected to be in S phase but it is possible that *N*-hydroxyparacetamol could also interfere with the passage of cells between G1 and S phase as well as S phase and G2. The stathmokinetic method of Dosik [26] was employed to study the effects of *N*-hydroxyparacetamol on cell cycle progression. This method prevents cells re-entering the G1 phase due to a block in mitosis by colcemid and therefore allows a means of quantitating the number of cells in any stage of the cycle with time after treatment. Figure 3 shows a series of histograms of cell number versus DNA content for untreated cell cultures. With

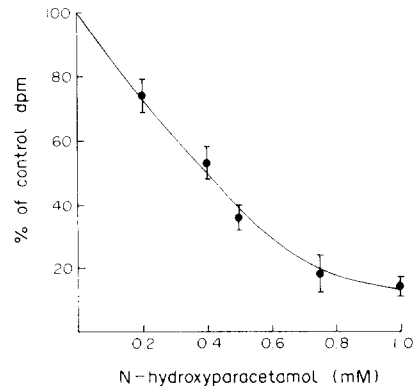

Fig. 1. Effect of *N*-hydroxyparacetamol concentration on DNA synthesis in NRK-49F cells. Cells were treated for 1 hr with *N*-hydroxyparacetamol as described in Methods and synthesis was subsequently determined by incorporation of [^{3}H]-thymidine over a 90 min period.

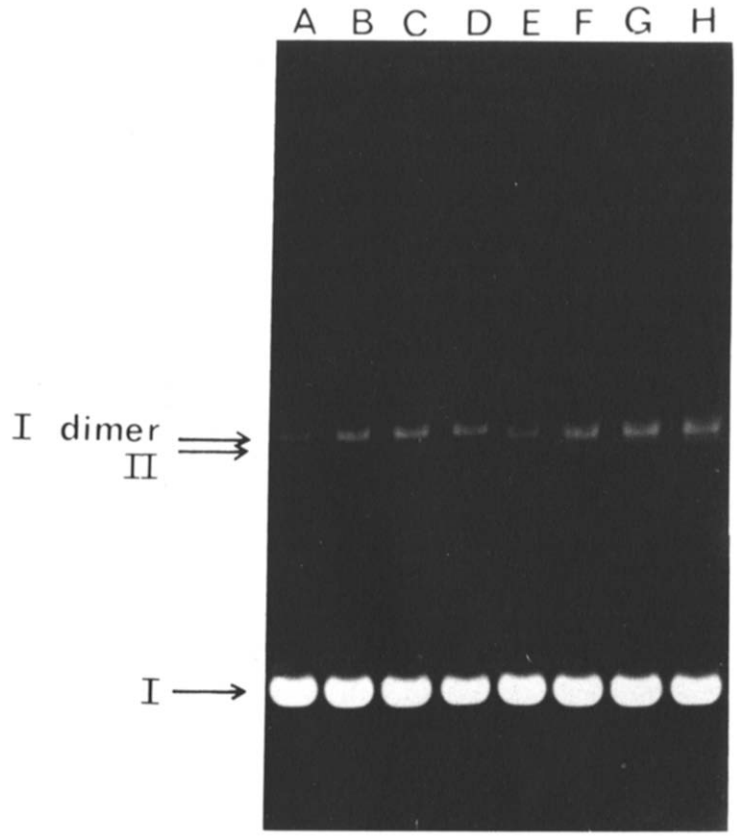

Fig. 2. Effect of increasing concentration of *N*-hydroxyparacetamol on naked plasmid DNA. pBR322 (0.7 μg) was incubated with *N*-hydroxyparacetamol at 1 mM (lanes C,D), 3 mM (lanes E,F), 5 mM (lanes G,H), or untreated (lanes A,B). I = supercoiled plasmid DNA; II = relaxed circular DNA.

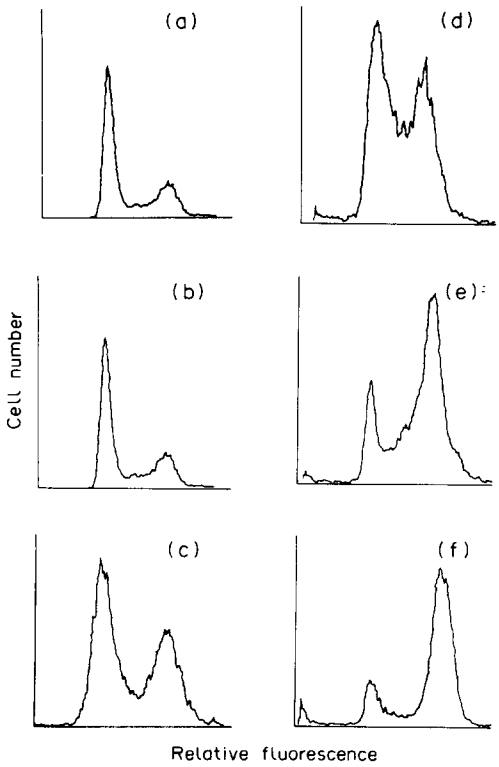

Fig. 3. Flow cytometry of untreated NRK-49F cells using the stathmokinetic procedure. In the presence of colcemid cells gradually accumulate in G2 + M phases: a = 0; b = 4; c = 10; d = 12; e = 15; f = 20 hr.

increasing time in the presence of colcemid, cells accumulated in G2 and M phases, finally giving proportions of 10.0% (G1), 28.0% (S) and 62.0% (G2 + M). The effect of *N*-hydroxyparacetamol on the rate of movement of these cells through the cell cycle is depicted in Fig. 4. The block seen in Fig. 3 due to the colcemid did not become apparent until the 15 hr time point in treated cells. Cells were seen to accumulate at the G1/S region 12 hr after treatment with a block also appearing in S phase from 12 to 15 hr after incubation with *N*-hydroxyparacetamol.

Data from a number of different experiments have been used to quantify the effect of *N*-hydroxyparacetamol on the rate of progression of cells through the different phases (Fig. 5). These results compare the rate of movement of treated and untreated cells. A marked effect due to *N*-hydroxyparacetamol treatment is obvious at all stages after approximately 12 hr. Untreated cells show a rapid decline in G1 phase cell numbers with time, reaching approximately 5% after 20 hr. On the other hand the rate of loss of cells from G1 is considerably

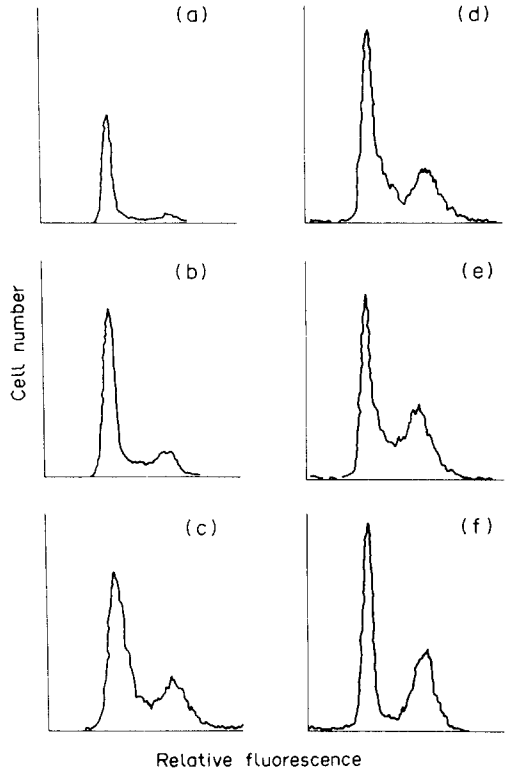

Fig. 4. Accumulation of 0.44 mM N-hydroxyparacetamol treated NRK-49F cells in G2+M phases with time after treatment. As in Fig. 3 colcemid was added after treatment to block the progression of cells in mitosis: a = 0; b = 4; c = 10; d = 12; e = 15; f = 20 hr.

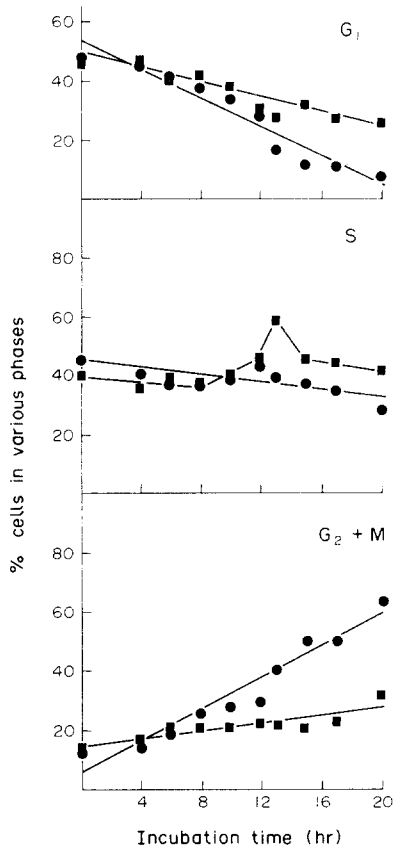

Fig. 5. Rate of progression of untreated (●) and *N*-hydroxyparacetamol treated (■) cells through the cell cycle. Pooled data from a number of different experiments similar to those described in Figs. 3 and 4 were used to determine individual points.

retarded after treatment with *N*-hydroxyparacetamol; approximately 30% still remaining in G1 after 20 hr. Again in S phase, little effect was observed up to 12 hr, at which time treated cells began to accumulate in S phase while untreated cells continued to move into G2 + M. The delay of treated cells in G1 and S phases was reflected by a reduced rate of entry into G2 + M.

DISCUSSION

A rapid decrease in DNA synthesis was observed after treatment of rat kidney fibroblasts with *N*-hydroxyparacetamol at pH 7.2 at 37°. This result is in keeping with a marked inhibition of DNA, RNA and protein synthesis previously observed in human lymphoblastoid cells exposed to *N*-hydroxyparacetamol [23].

NAPQI has been implicated as a toxic intermediate of *N*-hydroxyparacetamol metabolism. Evidence for this came from *in vivo* studies with rats and mice where high amounts of mercapturic acid and cysteine conjugates of paracetamol were formed after injection with *N*-hydroxyparacetamol. These conjugates are expected from the reaction of NAPQI with glutathione. Moreover, *p*-aminophenol has been demonstrated to inhibit DNA synthesis, alter chromatin structure, and decrease cell viability more effectively under conditions that are known to enhance its autoxidation to *p*-benzoquinone imine, a structural analogue of NAPQI ([28] and unpublished observations from this laboratory). During the complex decomposition of *N*-hydroxyparacetamol a number of highly reactive products are formed including transient nitrone intermediates [21]. Although some of these metabolites would be expected to be toxic to cells, it is unlikely that they contribute significantly to the observed effects on macromolecular synthesis, cell viability and cell cycle progression, as their formation would be low in the presence of cellular reducing agents [21, 29].

Treatment of rat kidney fibroblast cultures with *N*-hydroxyparacetamol under conditions favouring its decomposition altered cell cycle progression. One hypothesis for control of cell division proposes that this is regulated at a particular point during the G1 period before initiation of DNA replication. Thus G1 control has been called the "restriction point" in mammalian cells and once cells pass this point they are committed to the mitotic cell cycle [30]. Exposure of rat kidney fibroblasts to 0.44 mM *N*-hydroxyparacetamol affected the rate of exit of cells from G1 at approximately 12 hr after treatment (Fig. 5). Presumably cells late in G1 at the time of treatment

are committed to enter S phase, while cells at an early stage of G1 (before the restriction point) are blocked temporarily due to N-hydroxyparacetamol treatment. This may allow cells time for DNA repair. Indeed evidence for repair of N-hydroxyparacetamol induced alterations to chromatin structure has been reported [23]. Again at approximately 12 hr after treatment, a block in S phase is evident. Cells in late S phase at the time of treatment enter G2 as witnessed by the gradual accumulation of cells in G2 from 1 to 10 hr after treatment. However, the rate of entry into G2 phase is slow compared to untreated controls. This delay is evident over the time period 12–17 hr after treatment and is due to the G1/S and S phase delays shown in Fig. 5. After 17 hr the rate of entry into G2 is beginning to increase indicating that the delays in G1/S and S phases are only temporary blocks.

It is possible that the G1/S and the S phase delays in drug treated cultures are due to a direct effect of N-hydroxyparacetamol (or a metabolite) on DNA metabolism. The studies reported here demonstrate that N-hydroxyparacetamol causes inhibition of DNA synthesis. However, Fig. 3 shows that the G1/S and S phase delayed cells can still progress to G2 phase, doubling their DNA content per cell prior to arrest in the premitotic compartment. This apparent anomaly can be explained by studies carried out by Hayward *et al.* [23] who showed that some of the structural alterations to chromatin are repaired within 6 hr after treatment of human cells with 2 mM N-hydroxyparacetamol. Furthermore, cell viability is restored to normal values within 3 days after treatment with 1 mM N-hydroxyparacetamol.

It would appear that N-hydroxyparacetamol inhibits DNA synthesis and alters cell cycle progression by interacting with cellular macromolecules other than DNA. Indeed there is evidence from this laboratory that N-hydroxyparacetamol also interferes with protein and RNA synthesis [23]. Concentrations ranging from 0 to 5 mM N-hydroxyparacetamol failed to alter the migration pattern of plasmid DNA after 1 hr incubation at 37° (Fig. 2). This conclusion is supported by Wirth *et al.* [31] who found that N-hydroxyparacetamol had no mutagenic effect on the *Salmonella* strain TA100 over the pH range 5–8. Furthermore the viability of the strain was not affected over this pH range in the presence of microsomal protein. However, in the absence of microsomal protein, N-hydroxyparacetamol was found to be very toxic indicating that the microsomal proteins may be protecting the bacteria from the toxic effects of N-hydroxyparacetamol by preferentially reacting with it or one of its decomposition products. Similar results were reported by Shudo *et al.* [32] with NAPQI. Dahlin and Nelson [33] using NAPQI showed that this compound is so reactive and cytotoxic that little of it reaches the liver when administered by intraperitoneal route, reaction occurred largely in the intraperitoneal space. More recent results from that laboratory suggest that paracetamol and NAPQI exert their cytotoxic effects by disrupting Ca^{2+} homeostasis after soluble and protein-bound thiols have been depleted [34]. The present study and recent results from our laboratory [23] demonstrate that N-hydroxyparacetamol also has marked effects on chromatin structure, DNA synthesis, cell cycle progression, and cell viability apparently without acting directly on DNA. Therefore it is possible that this compound or a derivative (NAPQI) reacts with one or more proteins involved in chromatin structure or that the changes observed at the level of DNA are due to Ca^{2+}-induced fragmentation of chromatin which has been reported previously [35].

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