0026-895X/86/050478-06$02.00/0

MOLECULAR PHARMACOLOGY, 29:478–483

Effect of *N*-Hydroxyparacetamol on DNA, RNA, and Protein Synthesis and Chromatin Structure

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Received August 16, 1985; Accepted February 14, 1986

SUMMARY

N-Hydroxyparacetamol treatment of lymphoblastoid cells gave rise to a dose-dependent decrease in DNA, RNA, and protein synthesis. Inhibition of DNA synthesis was less marked in medium at pH 6.5 than at pH 9.0. *N*-Hydroxyparacetamol appeared to inhibit DNA synthesis at least in part through alterations to chromatin structure. This compound produced a dose-dependent and time-dependent loss in the superhelix density of DNA as determined by nucleoid sedimentation analysis. Alkaline elution data as well as sucrose gradient analysis revealed that this decrease in sedimentation did not arise through single strand breakage to DNA. The structrual alterations to chromatin caused by *N*-hydroxyparacetamol appeared to have been repaired after 6 hr. However, sedimentation of "repaired" nucleoids in the presence of ethidium bromide was markedly different from sedimentation of untreated nucleoids. These results suggested that some *N*-hydroxyparacetamol remained associated with nucleoprotein, thus interfering with the binding of ethidium. Furthermore, both RNA and protein synthesis were markedly inhibited by *N*-hydroxyparacetamol, demonstrating a major effect on cell function. The widespread effects of *N*-hydroxyparacetamol could be accounted for by changes to chromatin structure or by a more general effect on cellular metabolism. Either of these effects could account for the dramatic cytotoxicity of this compound. A concentration of 2.5 mM reduced cell viability by 96% after 3 days.

Paracetamol is a commonly used analgesic available without prescription. Therapeutic doses are believed to be extremely safe (1–3), but overdoses cause centrilobular hepatic necrosis in humans (4, 5) and experimental animals (6–8). Renal necrosis in Fisher 344 rats can be induced by paracetamol (9), and more recent evidence shows that it may also cause renal necrosis in humans (10). Damage to both of these organs occurs after a metabolite of paracetamol covalently binds to cellular macromolecules when glutathione levels are depleted. A number of monocyclic *N*-acetylarylamines, such as phenacetin (11, 12), acetanilide (13), and *p*-chloroacetanilide (14), are capable of being *N*-hydroxylated by cytochrome P-450 mixed function oxidases. In the light of these findings it was originally proposed that metabolic activation of paracetamol to a toxic arylating metabolite occurred via *N*-hydroxylation to *N*-hydroxyparacetamol (15, 16). Detection in the urine of *meta*-substituted sulfhydryl metabolites of paracetamol (17–21) led to the proposal that *N*-acetyl-*p*-benzoquinone imine was the ultimate toxic species arising from *N*-hydroxyparacetamol by dehydration (22, 23). However, a number of reports questioned the production of *N*-hydroxyparacetamol from paracetamol (24–26). Although *N*-acetyl-*p*-benzoquinone imine was the ultimate reactive metabolite of paracetamol, it was believed to arise via a different pathway. Results indicate that *N*-hydroxyparacetamol is a metabolite of *N*-hydroxyphenacetin rather than paracetamol (27). Therefore, it might also be expected that *N*-acetyl-*p*-benzoquinone imine can be derived from phenacetin.

N-Hydroxyparacetamol causes liver and renal damage in intraveneously injected rats and mice (28). In addition, *N*-hydroxyparacetamol is highly toxic to bacteria although it is not mutagenic in the *Salmonella*/Ames test (29). The present study was undertaken to investigate the possible effects of *N*-hydroxyparacetamol on DNA structure and function in cultured human cells. The effects of this compound on DNA are relevant to nephropathy and renal pelvic tumors associated with excessive usage of phenacetin-containing compound analgesics.

Methods

Cell culture. Epstein-Barr virus-transformed lymphoblastoid cells obtained from the Queensland Institute of Medical Research (Herston, Queensland) were used in this study. The cells had a doubling time of approximately 24 hr and were diploid when this study commenced. To ensure that the cells were growing in log phase at the commencement

[1] S. Djordjevic, N. Hayward, and M. Lavin, unpublished observations.

ABBREVIATIONS: RPMI 1640, Roswell Park Memorial Institute Medium 1640; FCS, fetal calf serum; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetate; TNE buffer, 10 mM Tris, pH 6.8, containing 150 mM NaCl and 2 mM EDTA.

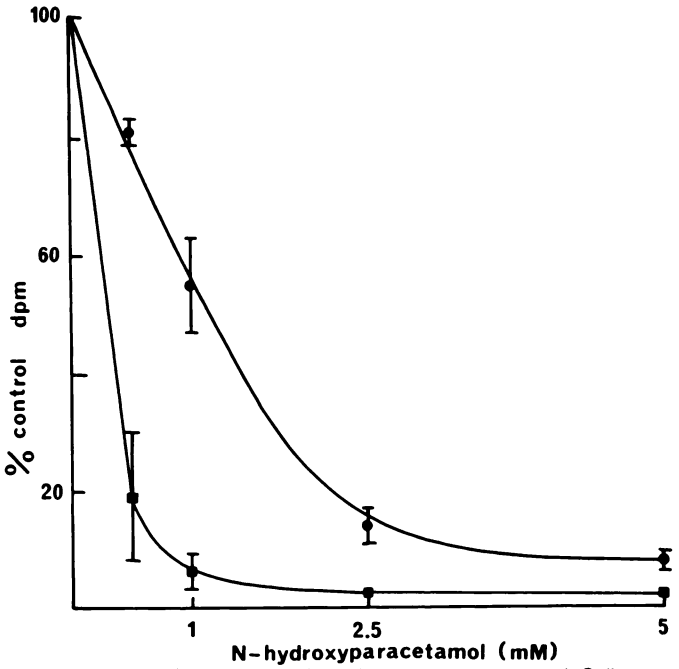

Fig. 1. Inhibition of DNA synthesis by *N*-hydroxyparacetamol. Cells were treated with various concentrations of *N*-hydroxyparacetamol for 60 min in medium at pH 6.5 (●) or at pH 9.0 (■). DNA synthesis was subsequently determined by incorporation of [^{3}H]thymidine over a 90-min period. *Error bars* represent SE of two to five separate experiments.

of experiments they were always subcultured 1 day prior to use. Cells were grown as suspension cultures in RPMI 1640 (GIBCO) supplemented with 10% FCS, penicillin (100 IU/ml), and streptomycin (60 μg/ml) in a humidified atmosphere of 5% CO_2 in air, at 37°.

Inhibition of DNA, RNA, and protein synthesis. A 5-ml stock of 5 mM *N*-hydroxyparacetamol was prepared by dissolving 4.2 mg of *N*-hydroxyparacetamol in RPMI 1640 without bicarbonate, buffered with 21 mM Hepes, adjusted to pH 6.5. This solution was further diluted with the medium to give final concentrations of *N*-hydroxyparacetamol in the range 0–5 mM. Solutions of *N*-hydroxyparacetamol prepared in RPMI 1640 without bicarbonate, buffered with 13 mM sodium tetraborate, adjusted to pH 9.0, were also prepared. Cells (10^6) prelabeled for 18 hr with [^{14}C]thymidine (0.01 μCi/ml; 60 mCi/mmol) were pelleted at 200 × *g*, resuspended in 1-ml volumes of the various concentrations of *N*-hydroxyparacetamol, and then transferred to tissue culture plates. Incubations were carried out for 60 min at 37° in air. After incubation, cells were harvested and washed once in RPMI 1640 before being resuspended in 1.2 ml of RPMI 1640 containing 10% FCS. Samples were then reincubated at 37° for 30 min in a humidified atmosphere of 5% CO_2 in air. Eight 100-μl aliquots of each cell suspension were removed and dispensed into wells of a microtiter plate. For DNA synthesis 100 μl of [^{3}H]thymidine (5 μCi/ml, 25 Ci/mmol, Amersham) was added to each microwell. [^{3}H]Uridine (1 μCi/ml, 30 Ci/mmol) was used to measure RNA synthesis and protein synthesis was determined by incorporation of [^{3}H]methionine (10 μCi/ml, 74 Ci/mmol). Incubations were carried out for 60 min in air supplemented with 5% CO_2 at 37°, and were terminated by placing the microtiter plates in a −70° Revco freezer. Samples were subsequently thawed at room temperature, and acid-precipitable DNA was collected onto GF/A glass fiber filters (Whatman) using a multicell harvesting device. The GF/A strips were then washed with water followed by ice-cold 5% trichloroacetic acid before being washed in absolute ethanol. When dried, they were counted in a Beckman LS-250 scintillation counter using toluene scintillation fluid. Counts per min were converted to disintegrations per min with the aid of a computer program. The ratio of incorporated ^{3}H-label to ^{14}C-prelabel in acid-precipitable material in treated samples was expressed as the percentage of that incorporated into untreated samples incubated under the same pH conditions as cells exposed to *N*-hydroxyparacetamol.

Nucleoid sedimentation analysis. The effect of *N*-hydroxyparacetamol on DNA structure was investigated using a variation of the method of Cook and Brazell (30). Cells were treated with *N*-hydroxyparacetamol at pH 6.5 over the range 0–5 mM as described above. After incubation, cells were washed once in PBS and resuspended in PBS at 2–4 × 10^6/ml. One hundred-μl aliquots of cell suspensions were added to 300 μl of nucleoid lysis solution (2 M NaCl, 0.01 M EDTA, 0.5% Triton X-100, pH 8) and layered on top of 10–30% neutral sucrose gradients. Lysis was allowed to proceed for 10 min before the gradients were centrifuged at 37,000 rpm for 15–30 min, at 12° in a Beckman L2-65B ultracentrifuge, using an SW41 rotor. The position of the nucleoids was determined by measuring absorbance at 254 nm after pumping the gradients through an LKB Uvicord S absorbance monitor. Triplicate or duplicate sets of both untreated and treated cells were employed in each centrifugation run. Sedimentation of nucleoids from treated cells

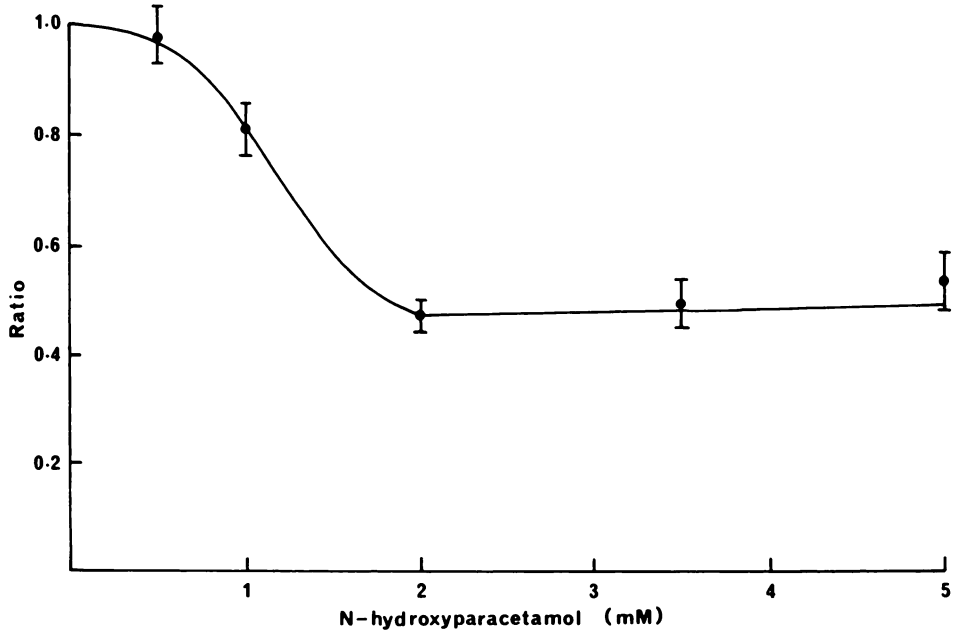

Fig. 2. Effect of *N*-hydroxyparacetamol concentration on sedimentation of nucleoids. Incubation conditions were as described in Methods. The ratio refers to the distance sedimented by treated nucleoids divided by that for untreated nucleoids. *Error bars* represent SE of 3 to 12 experiments.

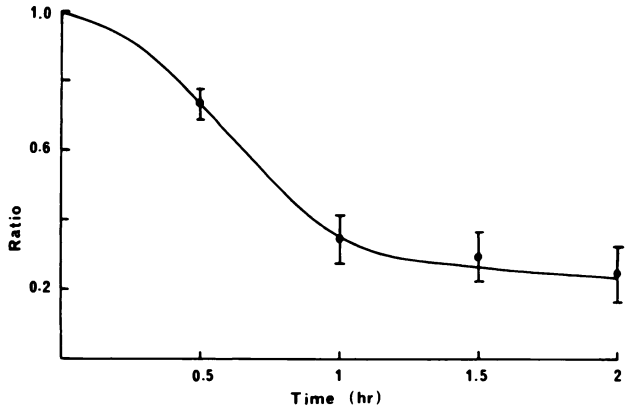

Fig. 3. Effect of incubation times with 2 mM *N*-hydroxyparacetamol on sedimentation of nucleoids. Incubation conditions were as described in the text. The ratio is as described in the legend to Fig. 2. *Error bars* represent SE of four or five experiments.

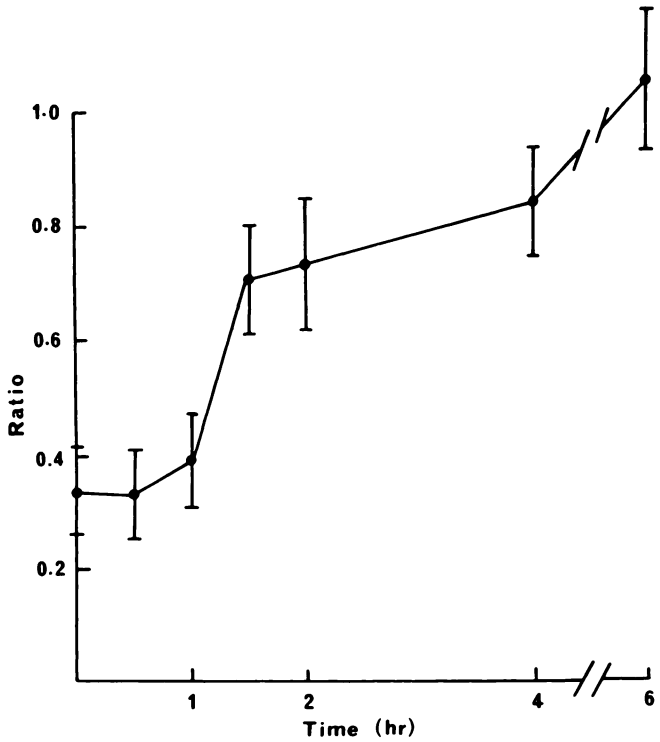

Fig. 4. Repair of structural alterations to DNA induced by 2 mM *N*-hydroxyparacetamol. Cells treated with *N*-hydroxyparacetamol for 60 min were washed and transferred to fresh medium. Nucleoid sedimentation was subsequently carried out at various times. The ratio is as described in legend to Fig. 2. *Error bars* represent SE of three to seven experiments.

was expressed as a ratio of the distance sedimented by untreated nucleoids.

The ability of lymphoblastoid cells to repair damage to chromatin induced by *N*-hydroxyparacetamol was studied. Restoration of superhelix density of DNA determined by nucleoid sedimentation was taken as evidence for repair. After incubation with 2 mM *N*-hydroxyparacetamol at pH 6.5, as described above, cells were harvested and washed twice in RPMI 1640 before being resuspended in RPMI 1640 plus 10% FCS at 3–5 × 10^5/ml. Cells were then incubated for various times at 37° in air containing 5% CO_2. Subsequently, the cells were harvested, washed once in PBS, and resuspended in PBS at 2–4 × 10^5/ml prior to sedimentation analysis. Nucleoid sedimentation was also conducted in sucrose gradients containing ethidium bromide in the range 0–10 μg/ml, after cells treated with 2 mM *N*-hydroxyparacetamol (pH 6.5) had undergone either 0-hr or 6-hr repair incubation, as described above. Sedimentation in the presence of ethidium bromide was used to determine whether the increase in sedimentation after 6 hr was due to restoration of the normal degree of supercoiling of the DNA.

Alkaline sucrose gradient analysis. Cells were labeled with [^{3}H] thymidine (1 μCi/ml, 25 Ci/mmol, Amersham) for 24 hr and transferred to fresh unlabeled medium for 2 hr, to deplete labeled precursor pools. Cells were subsequently treated with 5 mM *N*-hydroxyparacetamol (pH 6.5) as described for inhibition of DNA synthesis experiments. After incubation, cells were washed once in saline/EDTA and resuspended in saline/EDTA at 10^6/ml. Sedimentation was carried out in 5–24% isokinetic alkaline sucrose gradients as previously described (31).

The molecular weight of DNA was determined with the aid of a computer program.

Alkaline elution. The presence of single strand breaks in DNA and/or protein-DNA association was determined according to the method of Kohn (32). Cells prelabeled with [^{14}C]thymidine (0.1 μCi/ml) were treated with 5 mM *N*-hydroxyparacetamol as described above. Treated cells were mixed with an equal aliquot of untreated cells prelabeled with [^{3}H]thymidine (1 μCi/ml). After washing in 10 TNE buffer, cells were gently deposited onto Millipore BS-2 filters, lysed with Triton solution (0.69% Triton X-100, 2.6 M NaCl, 130 mM Tris, pH 8, and 2.7 mM EDTA) and treated either with or without proteinase K (1 mg/ml) in 0.2% Sarkosyl in TNE, pH 10, for 60 min and then washed with 5 mM EDTA. DNA was eluted with tetramethylammonium hydroxide/EDTA, pH 12.2, and collected at 90-min intervals for 13.5 hr.

Cell viability. Cells (approximately 2 × 10^6), previously pelleted at 200 × *g*, were resuspended in 2-ml volumes of either 1 mM or 2.5 mM *N*-hydroxyparacetamol (pH 6.5) and incubated for 60 min at 37° in air. After incubation, cells were harvested and washed twice in RPMI 1640 before being resuspended at approximately 2–3 × 10^5/ml in medium containing 10% FCS. Cells were subsequently maintained at 37° in a humidified atmosphere of air supplemented with 5% CO_2.

At various times up to 3 days, the cell suspensions were mixed thoroughly and 0.5-ml aliquots were removed for viability determinations. Trypan blue (0.1 ml, 0.4% w/v in PBS) was added to each sample and the number of viable cells, i.e., those that excluded the dye, was counted. Viabilities of all treated cell samples were expressed as a percentage of the change in viability of untreated cells with time.

Results

A dose-dependent decrease in DNA synthesis occurs after treatment of cells with *N*-hydroxyparacetamol at pH 6.5 (Fig. 1). A relatively rapid decline in synthesis occurs at doses up to 2.5 mM *N*-hydroxyparacetamol, with a levelling off between 2.5 mM and 5 mM. The effect of *N*-hydroxyparacetamol on DNA synthesis at pH 9.0 was also determined. At this pH, *N*-hydroxyparacetamol rapidly dehydrates in solution to *N*-acetyl-*p*-benzoquinone imine (33). Fig. 1 shows that DNA synthesis is inhibited by *N*-hydroxyparacetamol to a much greater extent under alkaline conditions. Concentrations up to 5 mM *N*-hydroxyparacetamol did not reduce cell viability below that for untreated cells after the 60-min incubation period.

Nucleoid sedimentation analysis reveals that DNA structure is dramatically altered by *N*-hydroxyparacetamol. A dose-dependent decrease in sedimentation of nucleoids is seen with doses of *N*-hydroxyparacetamol up to 2 mM, above which the sedimentation rate remains approximately the same to 5 mM (Fig. 2). A time-dependent decrease in nucleoid sedimentation occurs when cells are incubated with 2 mM *N*-hydroxyparacetamol. Fig. 3 shows that the sedimentation rate continues to decrease for up to 2 hr incubation at this concentration, being reduced to 24 ± 8% of the value for untreated cells.

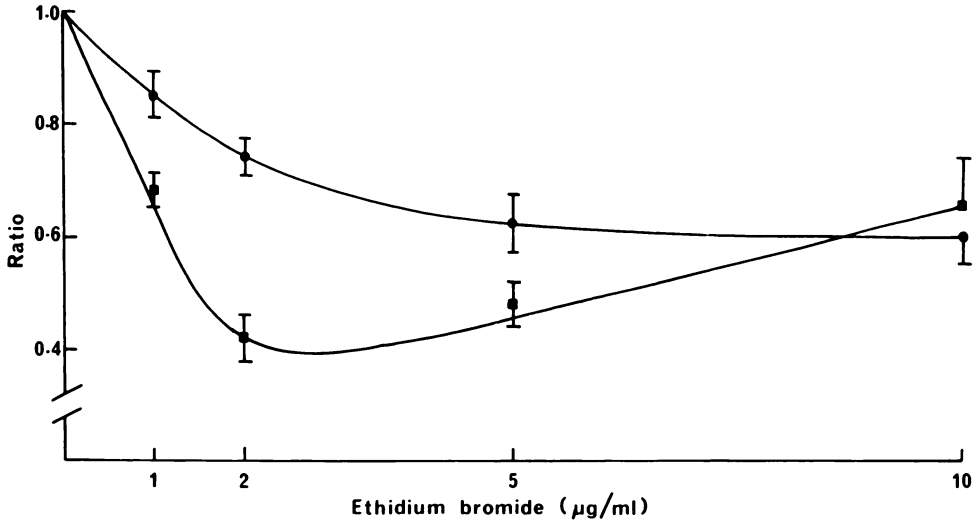

Fig. 5. Effect of ethidium bromide concentration on nucleoid sedimentation from untreated cells (■) and cells treated with 2 mM *N*-hydroxyparacetamol that have been allowed to repair for 6 hr (●). The ratio is as described in the legend to Fig. 2. *Error bars* represent SE of seven or eight experiments.

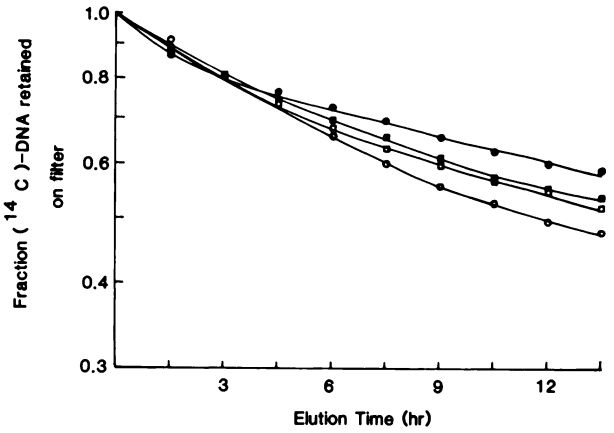

Fig. 6. Investigation of *N*-hydroxyparacetamol-induced formation of single strand breaks in DNA and protein-DNA associations using alkaline elution. Cells were treated at 37° with 5 mM *N*-hydroxyparacetamol at pH 9.0 for 60 min as described under Methods. ■, untreated cells, no proteinase K treatment; ●, untreated cells with proteinase K treatment; □, treated cells without proteinase K; ○, treated cells with proteinase K treatment. *Points* represent the mean of three experiments.

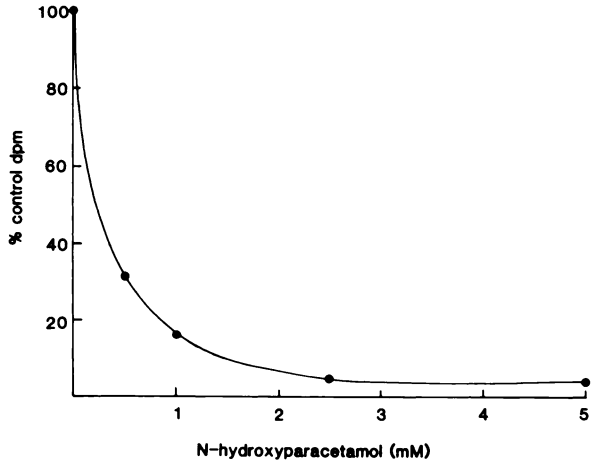

Fig. 7. Inhibition of RNA synthesis by *N*-hydroxyparacetamol. Cells were treated with various concentrations of *N*-hydroxyparacetamol for 60 min at 37°, pH 9.0. RNA synthesis was subsequently determined by incorporation of [^{3}H]uridine over a 60-min period. *Points* represent the mean of two separate experiments.

The ability of lymphoblastoid cells to repair the structural alterations induced by *N*-hydroxyparacetamol was investigated using the technique of nucleoid sedimentation analysis (Fig. 4). There is little or no change in the sedimentation rate of nucleoids from 2 mM *N*-hydroxyparacetamol-treated cells that were allowed to carry out repair for 30 min. However, upon further incubation, a time-dependent increase in sedimentation is observed, reaching a value approximately the same as that for untreated cells after 6 hr.

Sedimentation in the presence of ethidium bromide was used to determine whether nucleoid DNA from cells that had undergone 6 hr of repair after treatment with 2 mM *N*-hydroxyparacetamol, had been restored to a normal supercoiled form. Fig. 5 reveals that nucleoids from these cells sediment quite differently from those of untreated cells in the presence of ethidium. Nucleoids from treated cells do not follow the biphasic response that is characteristic of those from untreated cells. In contrast, they show a relatively slow reduction in sedimentation up to 5 μg/ml of ethidium (62 ± 5% of control), which fails to change to a significant degree as the ethidium concentration increases to 10 μg/ml (60 ± 5%).

Alkaline elution and alkaline sucrose gradient analysis were carried out to determine whether the marked reduction in the nucleoid sedimentation after treatment of cells with *N*-hydroxyparacetamol was due to the production of single strand breaks and/or alkali-labile sites in the DNA. No significant reduction in molecular weight of DNA from cells treated with 5 mM *N*-hydroxyparacetamol was observed by gradient analysis. Furthermore, *N*-hydroxyparacetamol-treated DNA was not eluted from filters by alkali more rapidly than untreated DNA either with or without proteinase K treatment (Fig. 6).

In view of the marked inhibition of DNA synthesis, apparent structural alterations to chromatin, and no evidence for appearance of breaks in DNA, the effects of *N*-hydroxyparacetamol on protein and RNA synthesis were also studied. The results in Fig. 7 show that RNA synthesis is inhibited by *N*-hydroxyparacetamol in a dose-dependent manner. Protein synthesis is also inhibited to a similar extent by *N*-hydroxyparacetamol, being reduced to 26% of that for untreated cells at a concentration of 0.5 mM.

The effect of *N*-hydroxyparacetamol on cell viability was determined. Fig. 8 depicts cell viabilities up to 3 days post-

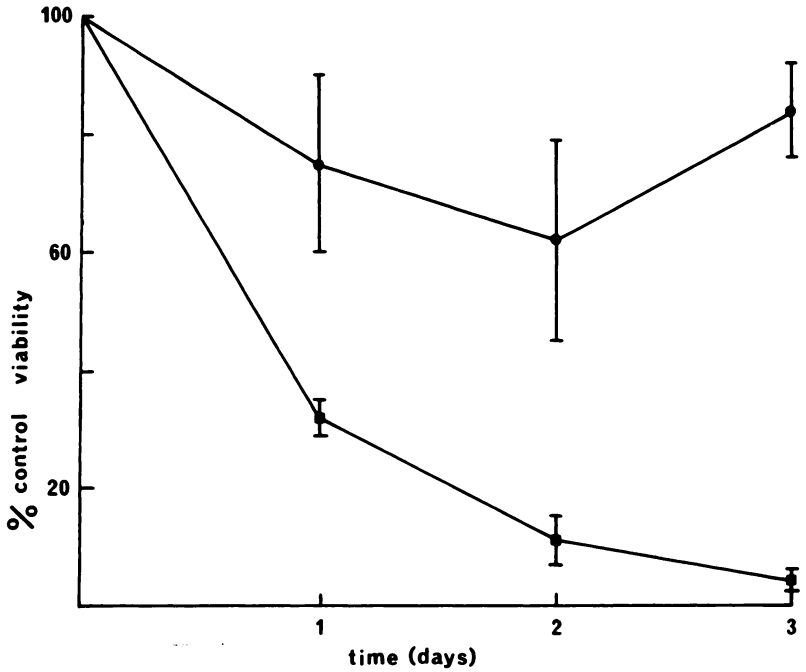

Fig. 8. Effect of 1 mM (●) and 2.5 mM (■) *N*-hydroxyparacetamol on cell viability. Incubation conditions were as described in Methods. Viabilities were determined by trypan blue exclusion. *Error bars* represent SE of three experiments.

treatment with either 1 mM or 2.5 mM *N*-hydroxyparacetamol. A rapid decline in viability is observed after teatment of cells with a concentration of 2.5 mM, decreasing to a level of only 4% of the control value after 3 days. Cell death also occurs after treatment with 1 mM *N*-hydroxyparacetamol, but at a considerably slower rate than after treatment with the higher concentration. Three days after treatment with 1 mM *N*-hydroxyparacetamol, a small increase in cell survival is observed compared to the value obtained 2 days after treatment, indicating recovery from damage. Colony formation in agar supports the high level of cytoxicity observed using trypan blue exclusion. At a dose of 1 mM *N*-hydroxyparacetamol, colony survival was reduced to 4% of the untreated value at pH 6.5 and to less than 1% at pH 9.0.

Discussion

A rapid decrease in the rate of DNA synthesis is observed after treatment of lymphoblastoid cells with *N*-hydroxyparacetamol at pH 6.5 (Fig. 1). At this pH and 20°, *N*-hydroxyparacetamol is relatively stable (26, 28, 33), although the rate of decomposition is appreciably faster at higher temperatures and concentrations (26, 34). Therefore, in the present study (pH 6.5, 37°, and 2–5 mM), *N*-hydroxyparacetamol and small amounts of its reaction products would be present during the 60-min incubation. Decomposition of *N*-hydroxyparacetamol is highly complex. The initial step is dehydration to form *N*-acetyl-*p*-benzoquinone imine which further reacts to yield a variety of products (26, 34–36). Thus, the inhibition of DNA synthesis observed at pH 6.5 may be attributed either to the reaction of *N*-hydroxyparacetamol or one of its products with DNA. As the pH is increased to 9.0, the stability of *N*-hydroxyparacetamol is reduced to a minimum (34).Treatment of cells with *N*-hydroxyparacetamol in medium at pH 9.0 results in a more rapid decline in the rate of DNA synthesis than is observed when cells are incubated with this compound at pH 6.5 (Fig. 1). Furthermore, a marked inhibition of both RNA and protein synthesis was also observed after treatment with this compound at pH 9.0. This finding is consistent with the proposition that *N*-acetyl-*p*-benzoquinone imine is one product of *N*-hydroxyparacetamol that causes the inhibition of macromolecular synthesis. Additional evidence in support of *N*-acetyl-*p*-benzoquinone imine as a reactive intermediate of *N*-hydroxyparacetamol responsible for these effects comes from previous studies using *p*-aminophenol (37). The effects of *p*-aminophenol on DNA structure were found to be enhanced under conditions that were known to increase the formation of *p*-benzoquinone imine, a structural analog of *N*-acetyl-*p*-benzoquinone imine.

Alkaline sucrose gradient analysis and alkaline elution data reveal that no single strand breaks and/or alkali-labile sites are introduced into the DNA by doses of *N*-hydroxyparacetamol that cause a marked reduction in nucleoid sedimentation. Thus, it would appear that the observed decrease in nucleoid sedimentation is not due to the introduction of breaks into the DNA. Instead, *N*-hydroxyparacetamol, or a reaction product, may react with the DNA or nucleoproteins in a manner that could lead to the loss of supercoiling of the DNA. Data from this laboratory using a plasmid (PBR322) failed to show evidence of binding of *N*-hydroxyparacetamol or its reaction products to naked DNA.

Repair of *N*-hydroxyparacetamol-induced alterations to chromatin structure, determined by sedimentation of nucleoids, was essentially complete in 6 hr. Treated cells showed a loss of supercoiling in the presence of ethidium; however, no obvious minimum in sedimentation was observed. In addition, the sedimentation rate of nucleoids from *N*-hydroxyparacetamol-treated cells failed to increase at higher concentrations of ethidium. This indicates that, when treated "repaired" nucleoids are in their most relaxed state, further addition of ethidium does not cause a winding up of the DNA in the opposite (positive) sense. This is further supported by the observation that nucleoids fully relaxed by treatment with 2 mM *N*-hydroxyparacetamol (zero time point; Fig. 4) do not respond to ethidium (0–10 μg/ml), either by relaxing further or

by winding up in the opposite sense (data not shown). These observations can be explained if *N*-hydroxyparacetamol or a derivative were to bind to nucleoprotein and alter the pattern of protein-nucleic acid association. Alkaline elution data using proteinase K do not support the presence of protein-associated DNA strand breaks but do not rule out DNA-protein crosslinks. Furthermore, alkaline elution and alkaline sucrose gradient analysis provide no evidence for breaks induced directly in DNA by *N*-hydroxyparacetamol. Although it is evident that *N*-hydroxyparacetamol or a derivative inhibits DNA, RNA, and protein synthesis and alters the sedimentation pattern of nucleoids, it is not clear how this compound induces such dramatic structural changes. A more general effect in metabolism could explain inhibition of synthesis of these macromolecules and, indeed, the cytotoxicity of this compound. A recent report provides evidence that *N*-acetyl-*p*-benzoquinone imine exerts its cytotoxic effects by disruption of Ca^{2+} homeostasis secondary to the depletion of soluble and protein-bound thiols (38).

It is also possible that other decomposition products of *N*-hydroxyparacetamol such as *p*-nitrosophenol, *N*-acetoxyparacetamol, *p*-benzoquinone, or transient nitrone intermediates may be responsible for some of the observed effects on DNA, RNA, protein, and cell viability. However, these products are unlikely to have had a significant effect in this study since their formation would be low in the presence of cellular reducing agents (26, 35).

Acknowledgments

We wish to thank the Queensland Cancer Fund, the Australian Kidney Foundation, and the National Health and Medical Research Council for their support of this project. We are also grateful to Mark Davidson for preparation of medium.

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