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# DNA Damage in Intact Cells Induced by Bacterial Metabolites of Chloramphenicol

**Mike Isildar, Joaquin J. Jimenez, Grace K. Arimura, and Adel A. Yunis**

Departments of Medicine, Biochemistry and Molecular Biology, and Oncology, University of Miami School of Medicine, Miami

**Four chloramphenicol (CAP) metabolites known to be produced by intestinal bacteria were examined with respect to their capacity to induce DNA damage in intact cells. The induction of DNA single-strand breaks in Raji cells, activated human lymphocytes, and human marrow cells was assayed by the alkaline elution technique. One of the four compounds tested, dehydro-CAP, was capable of inducing DNA single-strand breaks in all three cell systems at concentrations of $10^{-4}$ M. This effect is comparable to that observed previously with nitroso-CAP, the nitroreduction intermediate of CAP. The nitroreduction of dehydro-CAP by human bone marrow cell homogenate was detected by the production of the corresponding amino derivative amounting to $5.6 \times 10^{-5}$ M from $2 \times 10^{-3}$ M substrate under aerobic conditions. In sharp contrast, nitroreduction of CAP by bone marrow could not be demonstrated. The genotoxicity of dehydro-CAP, its relative stability compared to the nitroso-CAP, and its nitroreducibility by bone marrow suggest that this bacterial metabolite of CAP may play a key role as a mediator of aplastic anemia in the predisposed host.**

**Key words: dehydrochloramphenicol, aplastic anemia**

## INTRODUCTION

The molecular mechanisms underlying aplastic anemia from chloramphenicol (CAP) are not clearly understood. On the basis of detailed comparative studies with CAP, thiamphenicol (TAP; in which the p-$NO_2$ group is replaced by a methylsulfonyl, $SO_2$-$CH_3$, moiety) and nitroso-CAP, a nitroreduction product of CAP, we have postulated that aplastic anemia is mediated by nitroreduction metabolites of CAP via DNA damage to stem cells [1–8]. Thus nitroso-CAP, a presumed nitroreduction intermediate of CAP, was found to be much more toxic in vitro than CAP. In micromolar concentrations nitroso-CAP suppresses CFU-GM growth irreversibly [5], inhibits DNA synthesis in bone marrow [5,6], causes complete degradation of isolated DNA in vitro [7], and induces DNA strand breaks in intact cells [8]. Whereas these observations underline the toxic nature of nitroreduction intermediates of CAP, they do not necessarily single out nitroso-CAP itself as the toxic intermediate. Indeed, because of its unstable nature, it is unlikely that nitroso-CAP can reach its target organ, the bone marrow. In recent studies on the reactions and disposition of nitroso-CAP in human blood and in rat liver, it was found that the compound was effectively eliminated within seconds in the blood, and no nitroso-CAP passed the liver when concentrations of less than 1 mM and 0.5 mM were used, respectively [9,10].

For a candidate CAP intermediate to mediate bone marrow damage it must either be produced in the marrow itself or must be transported in stable form to the bone marrow. In man, metabolic transformation of CAP may be effected in liver [11] or by microorganisms in the intestinal lumen [12]. Accordingly, it is possible that one or more bacterial metabolites of CAP could serve as mediators of bone marrow damage from this drug. In the present study we examined a series of compounds known to be produced by intestinal bacteria with respect to their capacity to induce DNA damage in intact cells. One of four compounds examined, dehydro-CAP (DHCAP), was

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Address reprint requests to Adel A. Yunis, M.D., Department of Medicine (R-38), University of Miami School of Medicine, P.O. Box 016960, Miami, FL 33101.

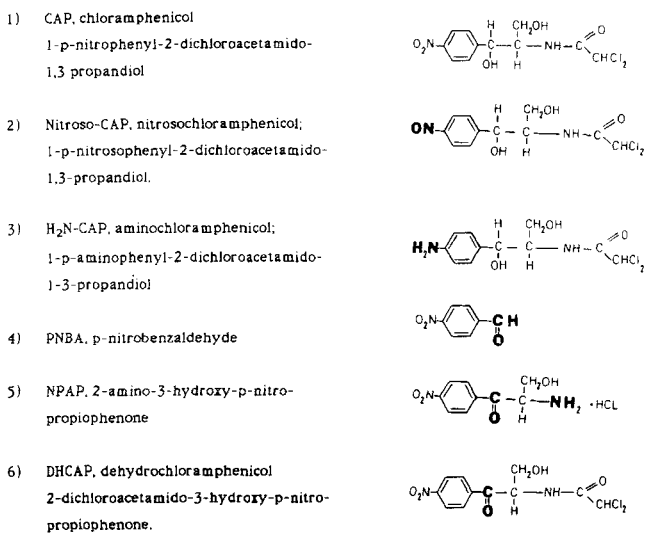


**Fig. 1. Chloramphenicol and its analogues.**

capable of inducing DNA single-strands breaks in Raji cells, activated human lymphocytes, and human bone marrow cells at concentrations comparable to those of nitroso-CAP. Evidence is presented that genotoxicity from DHCAP is related to its nitroreduction by the target cell.

## MATERIALS AND METHODS

### Drugs

The analogues of CAP known to be produced by bacteria [12] are of three types (see Fig. 1): (1) Aminochloramphenicol ($H_2N$-CAP) is a final nitroreduction product of CAP. (2) p-nitrobenzaldehyde (PNBA) and p-nitrophenyl-2-amino-3-hydroxy-propanone·HCl (NPAP) are products of CAP via oxidation at C-1-hydroxyl and hydrolysis. (3) 2-dichloroacetamido-3-hydroxypropio-p-nitrophenone (DHCAP) possesses a structure very similar to that of CAP, except the C-1-hydroxyl group of CAP is oxidized to a ketone moiety. PNBA was obtained from Aldrich, Milwaukee, WI. All the other drugs, CAP, nitroso-CAP, NPAP, DHCAP, and $H_2N$-CAP, were synthesized and kindly provided to us by Dr. Davide Della Bella, Zambon, S.p.A., Milan, Italy.

### Reagents

Tetrapropyl ammonium hydroxide and NADPH were from Sigma, St. Louis, MO; Sarkosyl NL-30 was from Ciba-Geigy Corp., Greenboro, NC; N-(1-naphthyl) ethylenediamine dihydrochloride was from Eastman Kodak Co., Rochester, NY; sodium nitrite and ammonium sulfamate were from Mallinckrodt Chemicals, St. Louis, MO; [2-$^{14}$C] thymidine (55 mCi/mmol), [methyl-$^3$H] thymidine (82 Ci/mmol) and Aquasol were obtained from New England Nuclear, Boston, MA; polyvinyl chloride filters (25 mm, 2-$\mu$m pore size) was from Millipore Corp., Bedford, MA.

### Cell Preparation and Labelling

**PHA-stimulated lymphocytes.** Blood was collected from normal human subjects in phenol-free heparin (10 U/ml). After gravity sedimentation, the white-blood-cell-rich protein was harvested and the cells were washed once in Dulbecco's modified Eagle's medium with 10% fetal calf serum (DME-FCS) and resuspended in medium (2–3 × $10^6$ cells/ml); $^{14}$C-thymidine (0.5 $\mu$Ci/ml) was added and incubation was continued for an additional 24 hr. Cells were then washed once with medium and further incubated in fresh medium containing $10^{-5}$ M unlabelled thymidine for 4 hours in order to chase the labelled thymidine into high-molecular-weight DNA.

**Bone marrow cells.** Bone marrow was obtained from normal human subjects, after informed consent, by needle aspiration from the posterior iliac crest. It was collected in phenol-free heparin and fractionated on Histopaque 1077 (Sigma Diagnostics, St. Louis, MO) to eliminate most of the erythrocytes and mature granulocytes before use. Bone marrow cells were suspended in DME-FCS (2–3 × $10^6$ cells/ml) containing 0.5 $\mu$Ci/ml $^{14}$C-thymidine and incubated for 24 hr at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Chasing with cold thymidine was then done as for lymphocytes.

**Raji cells.** A rapidly growing human lymphoblastoid cell line (Raji cells, CCL-86, ATCC, Rockville, MD) with a doubling time of 22 hr was maintained in DME-FCS. Exponentially growing Raji cells (5 × $10^5$ cells/ml) were incubated for 22 hr in medium containing 0.5 $\mu$Ci/ml of $^{14}$C-thymidine, followed by chasing with cold thymidine as described above.

**C51 cells.** A rapidly growing rat leukemic cell line [13] with a doubling time of 10 hr was maintained in DME-FCS medium. Exponentially growing C51 cells (3 × $10^5$ cells/ml) were incubated 20 hr in medium containing 1 $\mu$Ci/ml of $^3$H-thymidine. Excess $^3$H-thymidine was removed, cells were washed, and $^3$H label was chased with cold thymidine for 2 hr; $^3$H-thymidine-labelled C51 cells were used as an integral standard in alkaline elution assays for the determination of DNA single-strand breaks (vide infra).

After DNA labelling was completed, cells were incubated with drug under aerobic conditions for 3 hr at 37°C. The drug concentrations used and the time exposure (3 hr) were such that cell viability as determined by trypan blue exclusion exceeded 90% and was comparable to controls.

### Alkaline Elution Technique for DNA Single-Strand Breaks

Following drug treatment, the cells were washed and alkaline elution was performed by using the method of

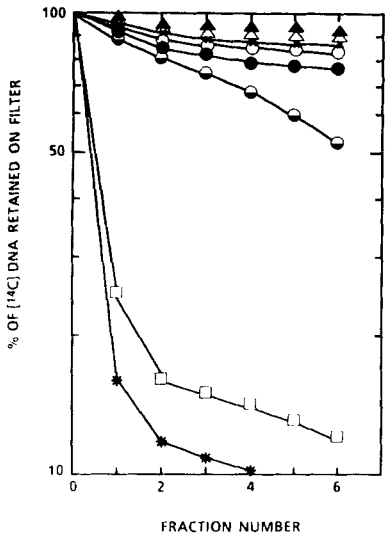


**Fig. 2. Alkaline elution profiles of DNA of Raji cells after exposure to drug for 3 hr. × control (DMSO = 0.5%), ○ CAP, 8 × $10^{-4}$ M; ● NPAP, $10^{-4}$ M; ◒ NPAP, 2 × $10^{-4}$ M; △ $H_2N$-CAP, 2 × $10^{-4}$ M; ▲ PNBA, 2 × $10^{-4}$ M; □ DHCAP, $10^{-4}$ M; ✳ Nitroso-CAP, $10^{-4}$ M.**

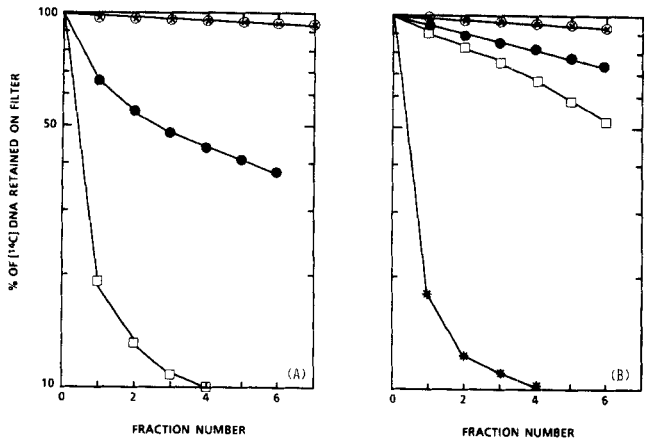


**Fig. 3. Alkaline elution profiles of Raji cells after treatment with increasing concentration of DHCAP and NPAP. A: × control (DMSO, 0.5%), ○ DHCAP, 2 × $10^{-5}$ M; ● DHCAP, 5 × $10^{-5}$ M; □ DHCAP, $10^{-4}$ M. B: × control (DMSO, 0.5%); ○ NPAP, 5 × $10^{-5}$ M; ● NPAP, $10^{-4}$ M; □ NPAP, 2 × $10^{-4}$ M; ✳ nitroso-CAP, $10^{-4}$ M.**

Kohn and co-workers [14,15], which is modified to include an internal standard [16] or irradiated $^3$H-thymidine labelled C51 cells (750 rads from $^{60}$Co gamma radiation source). The elution protocol is briefly described as follows: At the end of the drug treatment, 1 × $10^6$ drug-treated $^{14}$C cells were mixed with 1 × $10^6$ irradiated internal standard $^3$H-labelled C51 cells and loaded onto polyvinyl chloride filters; the cell suspension was gravity filtered. Then the cells on the filters were washed with 5 ml ice-cold PBS and lysed at room temperature with 5 ml of 0.2% Sarkosyl NL-30, 2M NaCl, 0.04 M $Na_2$ EDTA, pH 10, for at least 15 min. Lysis solution was drained by gravity and the filters were washed with 3 ml of 0.02 M $Na_2$ EDTA, pH 10. The DNA on the filters was eluted with 0.02 M $H_4$ EDTA solution at pH 12.2 which was adjusted with tetrapropyl ammonium hydroxide. About ten fractions, 3–4 ml each, were collected at 90-min intervals from each filter at a constant flow rate, and 1-ml aliquots of these radioactive fractions and the filters were counted in a scintillation counter. Early eluted radioactivity represents short single strands of DNA. Intact DNA and long strands are retained on the filters. Results of experiments with and without internal standards showed qualitatively very similar elution profiles. Therefore, our data are presented here as present of $^{14}$C-DNA retained on the filters vs. the fraction numbers.

### Nitroreduction

Reduction of nitro moieties of CAP and DHCAP by homogenized Raji cells and normal human bone marrow cells were investigated under aerobic conditions, and the quantity of nitroreduction products was determined as $H_2N$-CAP and $H_2N$-DH CAP, respectively, by Bratton-Marshall colorimetric assay as described previously [17,18].

## RESULTS

### Drug-Induced DNA Damage in Raji Cells

The DNA alkaline elution profile of Raji cells after treatment with drugs listed in Figure 1 is shown in Figure 2. In each case nitroso-CAP was used as a reference since it was shown previously to cause DNA single-strand breaks [8]. CAP (8 × $10^{-4}$ M), $H_2N$-CAP (2 × $10^{-4}$ M), and PNBA (2 × $10^{-4}$ M) had no significant effect on the rate of elution. In contrast, DHCAP at only $10^{-4}$ M caused a marked increase in the rate of elution comparable to that caused by nitroso-CAP at similar concentration. NPAP caused only a slight increase in the elution rate at $10^{-4}$ M, but the effect was more evident at 2 × $10^{-4}$ M. A dose-response pattern for DHCAP and NPAP in Raji cells is shown in Figure 3. Both drugs caused a dose-dependent increase in the DNA elution rate; however, DHCAP was considerably more potent exhibiting a significant effect at 5 × $10^{-5}$ M.

### Pattern of DNA Elution in Drug-Treated Lymphocytes

As observed in Raji cells, next to nitroso-CAP DHCAP was the most effective drug in increasing the rate of DNA elution (Fig. 3). However, the extent of the DHCAP effect in lymphocytes (Fig. 4) is significantly less than that noted in Raji cells; the reason for this difference is not apparent. Nitroso-CAP, on the other hand, was equally effective in lymphocytes and Raji cells. It can also be noted that the DNA elution rate of

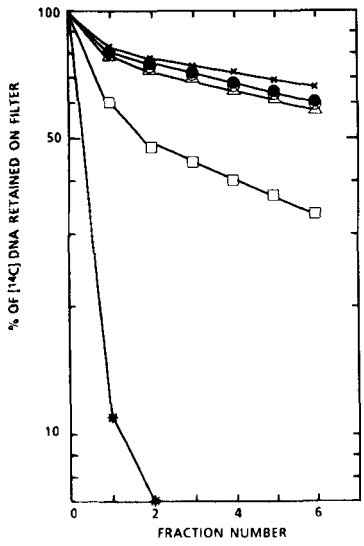


**Fig. 4. Alkaline elution profiles of DNA of PHA-stimulated normal human lymphocytes after exposure to drugs for 3 hr. × control (DMSO, 0.15%), ○ CAP, 8 × $10^{-4}$ M; ● NPAP, $10^{-4}$ M; □ DHCAP, $10^{-4}$ M; ✳ nitroso-CAP, $10^{-4}$ M.**

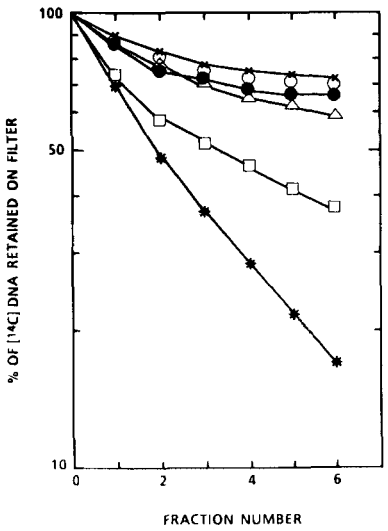


**Fig. 6. Alkaline solution profiles of DNA of normal human bone marrow cells after exposure to drugs for 3 hr. × control (DMSO, 0.5%), ○ CAP, 8 × $10^{-4}$ M; ● NPAP, $10^{-4}$ M; □ DHCAP, $10^{-4}$ M; ✳ nitroso-CAP, $10^{-4}$ M.**

control lymphocytes was relatively higher than that of control Raji cells. A dose-response pattern for DHCAP and NPAP in lymphocytes is shown in Figure 5. A detectable increase in the rate of DNA elution can be observed at 2 × $10^{-5}$ M DHCAP, increasing further with higher concentrations. Only a slight effect was noted from NPAP at 2 × $10^{-4}$ M.

### Effect of Bone Marrow

Figure 6 shows the alkaline elution profiles of DNA of bone marrow cells which have been treated with the

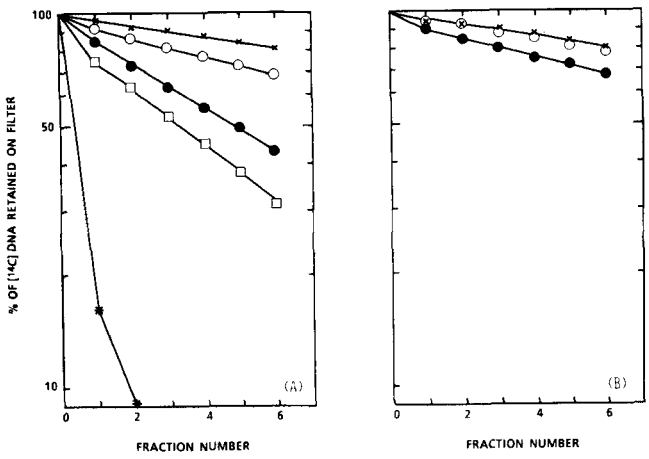


**Fig. 5. Alkaline elution profiles of DNA of PHA-stimulated normal human lymphocytes after treatment with increasing concentrations of DHCAP and NPAP. A: × control (DMSO, 0.5%); ○ DHCAP, 2 × $10^{-5}$ M; ● DHCAP, 5 × $10^{-5}$ M; □ DHCAP, $10^{-4}$ M; ✳ nitroso-CAP, $10^{-4}$ M. B: × control (DMSO, 0.5%); ○ NPAP, $10^{-4}$ M; ● NPAP, 2 × $10^{-4}$ M.**

drugs. As in lymphocytes, only DHCAP and nitroso-CAP caused a clear-cut increase in the elution rate at the concentration of $10^{-4}$ M.

### Nitroreduction of DHCAP by Raji Cells and Bone Marrow Cells

Table I shows that the p-$NO_2$ moiety of DHCAP was readily reduced by homogenates of Raji cells and normal human bone marrow cells under aerobic conditions. Thus, the concentration of reduction product, $H_2N$-DHCAP derivative, formed in the reaction mixture in the 4-hr period reached 2 × $10^{-5}$ M at 2 × $10^{-3}$ M DHCAP when $10^8$ Raji cells/ml were used. A similar reduction was observed for human bone marrow cells, and the amount of reduction product formed was cell-concentration dependent: 5 × $10^{-5}$ M at 5 × $10^7$ cells/ml and 5.6 × $10^{-5}$ M at 2 × $10^8$ cells/ml. In contrast, no detectable $H_2N$-CAP was formed from CAP itself.

## DISCUSSION

Our previous studies on CAP and TAP have served to reinforce the hypothesis that the p-$NO_2$ group of CAP may be the structural feature underlying aplastic anemia from this drug [1–3]. Subsequent investigation strongly suggested that the p-$NO_2$ group exerts its effect through metabolic reduction and the formation of toxic intermediates [4,5]. One such intermediate, nitroso-CAP, was shown to be much more toxic than CAP in vitro [5], and in contrast to CAP, it inhibits DNA synthesis at low concentration [5] and causes damage to isolated DNA in vitro [7] as well as to DNA in intact cells [8]. Both animal and human liver tissue can reduce the p-$NO_2$ group of CAP to the amino form, presumably producing

the toxic intermediates [11]. Intestinal bacteria are also known to produce $H_2N$-CAP from CAP [12]. Whereas it appears from these observations that nitroso-CAP is produced in humans and is cytotoxic, it is unlikely that it can serve as the mediator of marrow damage since it is extremely unstable and will likely be eliminated before reaching its target [9,10]. The possibility that the p-$NO_2$ group can undergo reduction in the target organ has been considered, but no reduction of CAP could be demonstrated in human marrow [19].

In addition to $H_2N$-CAP, other metabolic products of the drug are produced by intestinal bacteria [12] and possibly in the liver. It was therefore reasoned that one or more bacterial metabolites of CAP may serve as mediators of bone marrow damage.

The bacterial metabolite may itself be toxic and stable enough to reach the marrow. Alternatively, the metabolite may, in contrast to CAP, serve as a better substrate for nitroreduction by bone marrow and therefore generate the toxic intermediate in situ. Both of these possibilities are supported by the data described herein. Among the metabolites examined, DHCAP was, next to nitroso CAP, the most potent in causing DNA single-strand breaks in all three systems. The relatively smaller effect observed in bone marrow (as compared for Raji cells, for example) could probably be explained by a smaller fraction of cells in DNA synthesis in marrow. NPAP also induced DNA single-strand breaks in a concentration-dependent manner but was much less effective. None of the others showed an effect, and CAP itself was without effect at concentrations eightfold higher. The ability of DHCAP to produce DNA damage is consistent with its inhibitory effect on DNA synthesis reported previously [20].

Although the alkaline elution technique is among one of the best methods that are used in studying DNA damage induced by genotoxic agents, this technique provides end-point responses. Therefore, from our present results, the exact molecular mechanism for the initiation of DNA strand breakage cannot be ascertained. This is true, especially in our systems where the cells were exposed to the drugs at 37°C for a duration of 3 hr. Contrary to radiation investigations in which studies are carried out at 0°C and the exposure is virtually instantaneous [16,21], our systems do not permit us to distinguish easily between strand breaks formed from direct-hit of the DNA by the reactive intermediates and those caused by enzymatic repair of the damaged DNA. Perhaps, this distinction can be achieved by using cell systems which are deficient in excision repair and under conditions in which excision DNA repair is inhibited [21]. Whatever the mechanism, it is clear that DHCAP causes detectable DNA damage in intact cells at concentrations equivalent to or lower than those considered therapeutic for CAP ($5 \times 10^{-5}$–$1 \times 10^{-4}$ M). Perhaps more important is that at these concentrations DHCAP, in contrast to nitroso-CAP, is relatively stable when incubated with fresh human blood or liver tissue in vitro. Thus at 30 min 35 and 65% of DHCAP can be recovered from blood and liver, respectively, as determined by HPLC analysis [22]. Accordingly, any DHCAP formed elsewhere should find its way to the bone marrow before inactivation.

The lowest concentration of DHCAP which induces detectable DNA damage under the conditions described herein as determined by alkaline elution is $5 \times 10^{-5}$ M. In a previous study [20], DHCAP was found to inhibit CFU-GM growth by 72% at a concentration 100-fold

**TABLE I. Nitroreduction of DHCAP by Raji Cells and Normal Human Bone Marrow Cells***

| Tissue homogenate | Drugs | No. of cells in homogenate | Product concentration as amino derivative |
|---|---|---|---|
| Raji cells | Control, EtOH | $10^8$ cells/ml | — |
| | CAP | $10^8$ cells/ml | U[a] |
| | DHCAP | $10^8$ cells/ml | $2 \times 10^{-5}$ M |
| Bone marrow | Control, EtOH | $5 \times 10^7$ cells/ml | U |
| | DHCAP | $5 \times 10^7$ cells/ml | $5 \times 10^{-6}$ M |
| | Control, EtOH | $2 \times 10^8$ cells/ml | — |
| | CAP | $2 \times 10^7$ cells/ml | U |
| | DHCAP | $2 \times 10^7$ cells/ml | $5.6 \times 10^{-5}$ M |
| Boiled bone marrow | DHCAP | $2 \times 10^7$ cells/ml | U |

*The reaction mixtures contained 2 ml of tissue homogenate 40 μl drug solution in ethanol and 50 μl of NADPH solution. Results shown were obtained with drug and NADPH concentrations of $2 \times 10^{-3}$ M. The reaction mixtures were incubated with continuous shaking under aerobic conditions for 4 hr at 37°C. The lower limit of detectability of nitroreduction products was $10^{-6}$ M using the Bratton-Marshall assay.

[a]Undetectable.

lower ($5 \times 10^{-7}$ M). The apparent difference in the drug concentrations required to induce DNA damage ($5 \times 10^{-5}$–$10^{-4}$ M) vs. those required to inhibit CFU-GM growth ($5 \times 10^{-7}$–$10^{-6}$ M) may be related to differences in experimental conditions—for example, higher cell concentrations ($2–3 \times 10^6$ cell/ml) and shorter drug exposure (3 hr) for the DNA damage experiments. It should be noted here that currently nothing is known about the actual or potential serum concentrations of any of the CAP metabolites.

Our data also suggest that the ability of DHCAP to cause DNA damage is related to its nitroreduction by the target cell although they do not rule out a direct toxic effect from DHCAP itself, a question currently under investigation. Both Raji cells and normal human bone marrow cells were capable of reducing DHCAP but not CAP under aerobic conditions. That the induction of DNA damage is related to nitroreduction is also suggested by other investigations. For example, CAP, which is readily reduced by bacteria, is mutagenic in the Ames salmonella/microsome bioassay system [23], which is an indicator of DNA damage. CAP also induces DNA strand breaks in *E. coli* and *S. typhimurium* under aerobic conditions [23]. Furthermore, CAP produced damage in isolated DNA during its electrochemical reduction [24–26]. Olive and McCalla [27], Olive and Durand [28], and Olive [29,30] reported a correlation between DNA damage, cytotoxicity, and mutagenicity from nitrocompounds with their reduction potentials and/or electron affinity. It would therefore appear that DNA damage induced by DHCAP and other nitrocompounds is mediated by reactive nitroreduction intermediates. In our system the ready induction of DNA damage by DHCAP compared to CAP is believed to be due to ease of reducibility of the nitrogroup by the target cells because of the introduction of a conjugate side chain as a result of the presence of a carbonyl group adjacent to the benzene ring.

Nitroreduction is thought to be an important step in the activation of some procarcinogens such as 5-nitrofurans and 5-nitrothiophenes [31–35]. The mutagenic activity of metronidazole (Flagyl) has been attributed to its nitroreduction product [36]. Furthermore, it is known that enzymes which activate procarcinogens are present in human tissues such as liver and that the level of these enzymes can vary by as much as tenfold among individuals, possibly on a genetic basis [37]. Accordingly, the novel findings reported herein—that DHCAP known to be produced by intestinal bacteria and possibly in liver causes DNA damage and is reduced by bone marrow cells—open new avenues of investigations on the problem of CAP-induced aplastic anemia. The predisposed host could produce more toxic metabolites such as DHCAP, or his marrow may be capable of more extensive nitroreduction of these metabolites, thereby resulting in marrow stem cell damage ultimately leading to aplastic anemia and/or leukemia.

## ACKNOWLEDGMENTS

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