Bioassay and Relative Cytotoxic Potency of Cyclophosphamide Metabolites Generated in Vitro and in Vivo

N. E. Sladek

Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455

SUMMARY

Cytotoxic potencies of cyclophosphamide, 4-ketocyclophosphamide, carboxyphosphamide, mechlorethamine (HN2), bis(2-chloroethyl)amine (nor-HN2), chlorambucil, acrolein, and cyclophosphamide metabolites generated in vitro and in vivo were determined via bioassay. Our bioassay procedure was to incubate the potential cytotoxic agent with Walker 256 ascites cells in vitro, inject these cells into host rats, record survival time, estimate the number of viable cells which must have been injected to account for the observed survival time, and calculate percentage cell kill from these estimates. A log-linear relationship between tumor cell kill and exposure time or drug concentration was observed. Cyclophosphamide and 4-ketocyclophosphamide were noncytotoxic, and incubation of the latter with a microsomal or 105,000 X g supernatant fraction did not activate it. Acrolein and carboxyphosphamide were only minimally cytotoxic. HN2, chlorambucil, and nor-HN2 all were cytotoxic; HN2 was most potent and nor-HN2 was least potent. The cytotoxic potency of cyclophosphamide metabolites generated in vitro and in vivo was expressed as the concentration of metabolite(s) in nor-HN2 or formaldehyde equivalents that was required to kill 90% of the tumor cells. For total cyclophosphamide metabolites obtained after the incubation of cyclophosphamide with hepatic microsomes or with a 9000 X g supernatant fraction and from blood or urine after cyclophosphamide injection, the concentrations of drug required to kill 90% of the tumor cells were 0.087, 0.42, 0.66, and 4.5 µM, respectively, when expressed in nor-HN2 equivalents, and were 0.035, 0.037, 0.041, and 0.17 µM, respectively, when expressed in formaldehyde equivalents. Cyclophosphamide, 4-ketocyclophosphamide, carboxyphosphamide, nor-HN2, and acrolein could not account for the cytotoxic activity of the cyclophosphamide metabolite generated in vitro by hepatic microsomal mixed-function oxidase action, since the latter was a more potent cytotoxic agent than any of these compounds, although it was less potent than HN2. Trapping of the microsome-generated metabolite with semicarbazide reduced its cytotoxic potency. These data support the contention that cyclophosphamide is activated (to a cytotoxic metabolite) primarily by mixed-function oxidase action of the hepatic endoplasmic reticulum which oxidizes it to aldophosphamide, and that aldophosphamide is inactivated (as a cytotoxic agent) by aldehyde oxidase (EC 1.2.3.1) and/or aldehyde dehydrogenase (EC 1.2.1.3), which oxidize(s) aldophosphamide to carboxyphosphamide. In addition, the data provide estimates of the potency of aldophosphamide as a cytotoxic agent relative to that of other alkylating agents.

INTRODUCTION

Cyclophosphamide (Chart 1, I) is a nitrogen mustard congener that has been used with some success in the treatment of both experimental and human neoplasias. However, the agent itself is virtually without cytotoxic activity and must be activated to exert its biological effect.

In a previous communication from this laboratory (28), 2-dimensional thin-layer chromatography provided data that demonstrated and/or were interpreted to mean (a) that incubation of cyclophosphamide with rat hepatic microsomes yielded a single NBP-reactive metabolite, aldophosphamide (Chart 1, III); (b) that aldophosphamide could be trapped as the semicarbazone derivative (Chart 1, VI) by the addition of semicarbazide to the incubation mixture; (c) that enzymatic action in hepatic cytosol converted aldophosphamide to carboxyphosphamide (Chart 1, IV); (d) that 1 hr after cyclophosphamide injection, carboxyphosphamide and aldophosphamide were present in approximately equal amounts in blood; and (e) that urine collected for 3 hr after cyclophosphamide injection contained one of the metabolites which oxidizes it to aldophosphamide, and that aldophosphamide is inactivated (as a cytotoxic agent) by aldehyde oxidase (EC 1.2.3.1) and/or aldehyde dehydrogenase (EC 1.2.1.3), which oxidize(s) aldophosphamide to carboxyphosphamide. In addition, the data provide estimates of the potency of aldophosphamide as a cytotoxic agent relative to that of other alkylating agents.

1 This research was supported by USPHS Grant 15477. Part of this material appeared in abstract form (24). This is Paper 5 in the series on "Cyclophosphamide Metabolism."

2 Research Career Development Awardee of the National Cancer Institute, USPHS (1-KO4-CA70383-01).

Received December 29, 1972; accepted February 19, 1973.
Bioassay of Cyclophosphamide Metabolites

Chart 1. Proposed route of cyclophosphamide metabolism.

MATERIALS AND METHODS

Animals and Dosage Schedules. Male Holtzman rats (230 to 270 g; The Holtzman Co., Madison, Wis.), pretreated with phenobarbital sodium (Merck & Co., Inc., Rahway, N. J.), were used as the source of blood, urine, or hepatic mixed-function oxidase. Phenobarbital, 40 mg/kg, was injected i.p. in a volume of about 0.5 ml of 0.9% NaCl solution every 24 hr for 5 days before sacrifice or injection of cyclophosphamide.

The antineoplastic activities of cyclophosphamide metabolites, other alkylating agents, and acrolein were quantitated via bioassay in which male Holtzman rats (90 to 100 g) were used. In all experiments, rats were fed a standard chow diet ad libitum.

Tissue Preparation, Hepatic 9000 X g Supernatant and Microsomal Cyclophosphamide Metabolism, and Preparation of Metabolites for Bioassay. All rats were pretreated with phenobarbital and sacrificed between 8 and 9 a.m. Hepatic

5 Cyclophosphamide was generously supplied by Dr. W. A. Zygmunt, Mead Johnson Research Center, Evansville, Ind. Carboxyphosphamide and 4-ketocyclophosphamide were generously supplied by Dr. R. F. Struck, Southern Research Institute, Birmingham, Ala.
9000 X g supernatant and microsomal fractions were obtained as described previously (25).

The incubation mixture and conditions were as described previously (25), except that (a) the concentration of cyclophosphamide was 0.69 mM; (b) a 9000 X g supernatant fraction or microsomal fraction obtained from 50 mg wet liver was used per incubation flask; (c) the mixture was incubated for 15 (microsomal fraction) or 30 min (9000 X g supernatant fraction); (d) semicarbazide hydrochloride, 37.5 μmoles/reaction flask, was used in some of the experiments; and (e) nicotinamide, 4.0 mM, and DPN, 2.0 mM, were added when the 9000 X g supernatant fraction was used. After the incubation, we stopped the reaction by placing the incubation mixture into an ice-water bath. Aliquots of the incubation mixtures were deproteinized and assayed for alkylating activity and aldehyde content as previously described (25, 28). Aliquots of the original incubated reaction mixture were then removed and diluted with the appropriate unreacted incubation mixture to the desired concentration of nor-HN2 equivalents for bioassay. The unreacted incubation mixture, which consisted of the complete reaction mixture kept at 4° for the appropriate length of time, served as a control as well as a diluent, since preliminary experiments revealed that metabolites possessing alkylating activity (and/or which were aldehydes) were not formed under these conditions.

Preparation of Cyclophosphamide Metabolites Obtained from Rat Blood for Bioassay. Phenobarbital-pretreated rats were anesthetized with ether, a dorsal incision was made, and the blood supply to both kidneys was occluded by ligation. One hr later, 2.6 ml of 0.9% NaCl solution (control rats) or 100 mg of cyclophosphamide dissolved in 2.6 ml of 0.9% NaCl solution were injected i.p. One hr after injection, the abdominal cavities were opened under ether anesthesia, and 1-ml urine samples were collected. After dilution with diluted (1 to 10) urine from control rats to attain the desired concentration of nor-HN2 equivalents for bioassay. Aliquots of the urine samples were assayed for alkylating activity and aldehyde content as previously described (25, 28). Aliquots of the original urine samples from rats that received cyclophosphamide were then diluted 1/100 with water, centrifuged to remove any solids, and further diluted with diluted (1 to 100) urine from control rats to attain the desired concentration of nor-HN2 equivalents for bioassay. Diluted (1 to 100) urine from control rats also served as a control in the bioassay experiments.

Preparation of Cyclophosphamide Metabolites Obtained from Rat Urine for Bioassay. Phenobarbital-pretreated rats were given i.p. injections of 2.6 ml of 0.9% NaCl solution (control rats) or 100 mg of cyclophosphamide dissolved in 2.6 ml of 0.9% NaCl solution. The rats were placed in restraining cages, and 1-ml urine samples were collected. After dilution with water and centrifugation to remove any solids present, aliquots of the urine samples were assayed for alkylating activity and aldehyde content, as previously described (25, 28). Aliquots of the original urine samples from rats that received cyclophosphamide were then diluted 1/100 with water, centrifuged to remove any solids, and further diluted with diluted (1 to 100) urine from control rats to attain the desired concentration of nor-HN2 equivalents for bioassay. Diluted (1 to 100) urine from control rats also served as a control in the bioassay experiments.
Bioassay of Cyclophosphamide Metabolites

Potency as a function of exposure time or drug concentration (actual, or in nor-HN2 or formaldehyde equivalents) was then calculated and expressed in terms of an LC_{90}.

We determined the concentration-log response curves, LC_{90}'s, and the precision of their respective estimates by submitting the response values to logarithmic transformation and subsequent linear regression analysis, as described by Goldstein (17). All calculations were performed by means of a Wang 600 computer with a program that provided values of LC_{90}'s and 95% confidence intervals of their estimates.

RESULTS

Initial experiments revealed a log-linear relationship between the number of Walker 256 ascites cells injected and survival time (Chart 2). The tumor cell population multiplied with an apparent doubling time of 10.7 hr. The data predict that the survival time for a host rat that received 1 viable tumor cell (injected i.p.) will be 346 hr postinjection. Credence to this prediction is provided by the observation that, among several hundred experimental animals, all rats (with 6 exceptions) died in less than 346 hr or lived for at least 1000 hr, at which time the experiments were terminated. The 6 rats which were exceptions died in less than 450 hr. The data also estimate that when the tumor cell population reaches 6 billion cells, the host animal will die. In subsequent experiments, survival times were recorded, and the number of viable cells injected was estimated from the curve presented in Chart 2. From these estimates, percentage tumor cell kill was calculated and the cytotoxic potency of cyclophosphamide metabolites and other agents was quantitated.

The cytotoxic potencies of several alkylating agents, as determined via the bioassay described, are shown in Table 1 and Chart 3. In all cases, a log-linear relationship between tumor cell kill and drug concentration was observed (Chart 3). HN2 was by far the most potent agent tested. In agreement with reports from several laboratories, where other experimental tumor systems were used to estimate cytotoxic potency (22, 23, 30, 31), carboxyphosphamide was only minimally cytotoxic, and 4-ketocyclophosphamide and cyclophosphamide were noncytotoxic. Incubation of 4-ketocyclophosphamide—which has been identified as a minor urinary metabolite of cyclophosphamide (4, 5, 19, 30) and which can be produced by incubation of liver slices with cyclophosphamide (22)—with a microsomal fraction or a 105,000 X g supernatant fraction did not result in the activation of 4-ketocyclophosphamide, i.e., conversion to a cytotoxic metabolite (data not presented). This indicates that 4-keto-cyclophosphamide is not an intermediate metabolite in the formation of the cytotoxic metabolite of cyclophosphamide, in agreement with the work of other laboratories (22, 30).

The relative extinction coefficients of these drugs in the NBP assay were determined, and their cytotoxic potencies in nor-HN2 equivalents were calculated (Table 1). These calculations were for the purpose of comparing cytotoxic potencies of these agents with those of cyclophosphamide metabolites.

Chart 3. Cytotoxicity of cyclophosphamide metabolites and several other alkylating agents to Walker 256 carcinosarcoma ascites cells: concentration response. Points, mean ± S.E. of 4 rats; *, HN2; •, cyclophosphamide metabolites (concentration expressed in nor-HN2 equivalents) generated in the absence of semicarbazide hydrochloride by a hepatic microsomal preparation; •, cyclophosphamide metabolites (concentration expressed in nor-HN2 equivalents) generated in the presence of semicarbazide hydrochloride by a hepatic microsomal preparation; ○, chlorambucil; ◦, nor-HN2; □, carboxyphosphamide; x, 4-ketocyclophosphamide; ○, cyclophosphamide. All experimental details are as described in “Materials and Methods.”
generated in vitro and in vivo, since the latter can be quantitated in terms of nor-HN2 equivalents but could not be quantitated absolutely.

We generated cyclophosphamide metabolites by incubating cyclophosphamide with hepatic microsomes and appropriate cofactors. Tumor cells were then exposed for various time periods to a fixed concentration of metabolites, either 0.25 or 0.5 M in nor-HN2 equivalents. Results of these experiments show a log-linear relationship between tumor cell kill and exposure time; show that tumor cell kill is a function of drug concentration, as well as of time of exposure to the drug; and predict that, on the average and at the highest concentration of drug used in these experiments, less than 1 viable cell will remain if exposure time is increased to more than 72 min whereas, at the lower concentration of drug, a much longer exposure time is required to achieve the same result (Chart 4).

The bioassay procedure used in these experiments makes unavoidable the injection of some unmetabolized cyclophosphamide. Conceivably, cyclophosphamide itself or cyclophosphamide metabolites formed in vivo (i.e., after injection) could account for some of the cytotoxic effects observed in these experiments. Microsomal incubation mixtures contained 0.69 M cyclophosphamide. Routinely, 0.1 ml of incubation mixture containing 0.069 mmole of cyclophosphamide was added to 5 ml of tumor cells, 1 million cells per ml. After incubation, 0.5 ml of tumor cell suspension, containing a maximum of 0.0069 mmole cyclophosphamide, was injected into approximately 100-g rats, i.e., a maximum dose of 0.069 mmole cyclophosphamide per kg. In addition, a cytotoxic effect due to other components of the incubation media itself, e.g., microsomal protein, might be anticipated. However, in these experiments, no tumor cell kill was observed following the exposure of 5 ml of tumor cell suspension to 0.1 ml of unreacted incubation mixture (containing 0.069 mmole of cyclophosphamide) for 1 hr at 4°. This suggested that, for the metabolite to exert its cytotoxic effect, metabolic activity of some sort is required while the cells are in contact with the agent (e.g., transport of the metabolite into the cell) and/or that chemical reactivity of the cytotoxic agent is abolished at this temperature (data not presented).

Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>Range of drug concentrations tested (µM)</th>
<th>Relative extinction coefficients</th>
<th>Actual</th>
<th>In nor-HN2 equivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN2</td>
<td>0.005–0.04</td>
<td>0.86</td>
<td>0.008</td>
<td>0.007</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>0.31–2.48</td>
<td>0.56</td>
<td>0.52</td>
<td>0.29</td>
</tr>
<tr>
<td>nor-HN2</td>
<td>0.2–1.6</td>
<td>1.00</td>
<td>0.83</td>
<td>0.83</td>
</tr>
<tr>
<td>Carboxyphosphamide</td>
<td>2.0–16.0</td>
<td>0.85</td>
<td>6.3</td>
<td>5.4</td>
</tr>
<tr>
<td>4-Ketocyclophosphamide</td>
<td>10.0–1000.0</td>
<td>0.002</td>
<td>&gt;1000.0</td>
<td>&gt;2.0</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>1.0–100.0</td>
<td>0.02</td>
<td>&gt;100.0</td>
<td>&gt;2.0</td>
</tr>
</tbody>
</table>

a In the NBP assay (relative to that obtained for nor-HN2, which is arbitrarily assigned a value of 1.00).
b Each value represents an estimate of the LC₉₀ and, in parentheses, the 95% confidence interval of that estimate.

Exposure of tumor cells to cyclophosphamide metabolites, generated in vitro (0.5 µM in nor-HN2 equivalents), for 1 hr at 4° did not produce any measurable tumor cell kill. This suggested that, for the metabolite to exert its cytotoxic effect, metabolic activity of some sort is required while the cells are in contact with the agent (e.g., transport of the metabolite into the cell) and/or that chemical reactivity of the cytotoxic agent is abolished at this temperature (data not presented).

Data describing the cytotoxic potency of cyclophosphamide metabolites generated in vitro and in vivo are presented in Charts 3, 5, and 6 and in Table 2. As before, and in all cases, tumor cell kill as a function of drug concentration, whether expressed in nor-HN2 or formaldehyde equivalents, was log-linear (Charts 3, 5, and 6).

Cyclophosphamide metabolites generated in vitro by microsomal enzyme action were highly potent as cytotoxic agents, with an LC₉₀ in nor-HN2 equivalents of 0.087 µM, and the data predict that in this system and with an exposure time of 1 hr, a concentration of 0.6 µM is required to reduce the tumor cell population to less than 1 (Charts 3 and 5; Table 2). A comparison of the LC₉₀'s (expressed in nor-HN2 equivalents) obtained for carboxyphosphamide, 4-ketocyclophosphamide, or cyclophosphamide (Table 1) with that obtained for the metabolite(s) found after microsomal activation, also expressed in nor-HN2 equivalents (Table 2), leads one to the conclusion that the former cannot account for the cytotoxic activity found in the latter. Only 1 detectable NBP-reactive metabolite, presumably aldophosphamide, is generated under the incubation conditions used in these experiments (28). Thus, most or all of the cytotoxic activity found after microsomal activation appears to be due to aldophosphamide.

If such is indeed the case, aldophosphamide appears to be a much more potent cytotoxic agent than is chlorambucil or nor-HN2, although not as potent as HN2 (Tables 1 and 2).

A bioassay of cyclophosphamide metabolites generated in vitro by a microsomal preparation in the presence of semicarbazide yielded an LC₉₀ of 0.28 µM expressed in nor-HN2 equivalents, a marked decrease in cytotoxic potency (Table 2; Chart 3). Evidence has previously been obtained and...
interpreted to mean that incubation of cyclophosphamide with a microsomal preparation in the presence of semicarbazide, a known aldehyde trap, results in enzymatic oxidation of cyclophosphamide to aldophosphamide, followed by chemical reaction of semicarbazide and most of the aldophosphamide formed to yield the corresponding semicarbazone derivative, i.e., aldophosphamide semicarbazone (28). Our experiments lend support to this interpretation and to the contention previously made (28) that the predominant cytotoxic cyclophosphamide metabolite is an aldehyde, presumably aldophosphamide.

While the extinction coefficients of aldophosphamide and aldophosphamide semicarbazone in the NBP assay are not known, previous experiments (28) suggest they are very similar; thus, the differences in cytotoxic potency observed are probably real in the sense that they are not due to differences in actual concentrations of the 2 agents, differences which could exist as a result of expressing concentrations in nor-HN2 equivalents. Under the conditions of the experiment, semicarbazide converts most, but not all, of the aldophosphamide formed to the corresponding semicarbazone derivative (28). Thus, the cytotoxic effect of metabolites generated in the presence of semicarbazide is due in part to free aldophosphamide and in part to aldophosphamide semicarbazone, the exact proportion being unknown. For this reason, the LC90 obtained for this preparation represents a numerical value lower than that which would be obtained if all of the aldophosphamide formed was converted to the semicarbazone derivative.

Incubation of cyclophosphamide with a rat hepatic 9000 X g supernatant fraction was previously shown to yield 2 NBP-reactive metabolites, with the proportion of the 2 metabolites, believed to be aldophosphamide and carboxyphosphamide, depending on the length of the incubation; the longer the incubation, the greater the carboxyphosph-

Table 2

<table>
<thead>
<tr>
<th>Source of cyclophosphamide metabolites</th>
<th>Range of drug concentrations tested (µM)</th>
<th>LC90a (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In nor-HN2 equivalents</td>
<td>In formaldehyde equivalents</td>
</tr>
<tr>
<td>Microsomal incubation mixture (incubated in the absence of semicarbazide)</td>
<td>0.025–0.50</td>
<td>0.010–0.080</td>
</tr>
<tr>
<td>Microsomal incubation mixture (incubated in the presence of semicarbazide)</td>
<td>0.13–1.15</td>
<td>b</td>
</tr>
<tr>
<td>9000 X g supernatant incubation mixture</td>
<td>0.25–2.00</td>
<td>0.022–0.176</td>
</tr>
<tr>
<td>Blood</td>
<td>0.28–2.24</td>
<td>0.017–0.136</td>
</tr>
<tr>
<td>Urine</td>
<td>1.86–14.88</td>
<td>0.07–0.56</td>
</tr>
</tbody>
</table>

a Each value represents an estimate of the LC90 and, in parentheses, the 95% confidence interval of that estimate.

b Not determined.
N. E. Sladek

Chart 5. Cytotoxicity (in nor-HN2 equivalents) of cyclophosphamide metabolites, generated in vitro and in vivo, to Walker 256 carcinosarcoma ascites cells. Concentration response. Quantitated are the relative cytotoxic effects of cyclophosphamide metabolites obtained from urine (○), blood (●), 9000 x g supernatant incubation mixture (♦), and microsomal incubation mixture (♦). Concentrations of cyclophosphamide metabolites were quantitated by the NBP assay and are expressed in terms of nor-HN2 equivalents. All other experimental details are as described in "Materials and Methods." Points, mean ± S.E. of 4 rats.

phamide/aldophosphamide ratio (28). A probable explanation is that microsomal mixed-function oxidase activity results in the oxidation of cyclophosphamide to aldophosphamide and that aldophosphamide is then further oxidized to carboxyphosphamide by nonmicrosomal enzymes, possibly aldehyde oxidase and/or DPN-linked aldehyde dehydrogenase (20, 22, 28–31); the latter enzymes remain functional in vitro for a longer time than does microsomal mixed-function oxidase (data not presented).

Carboxyphosphamide and aldophosphamide appear to have similar extinction coefficients in the NBP assay, but when cytotoxic potency is expressed in nor-HN2 equivalents, aldophosphamide appears to be much more potent than carboxyphosphamide in the bioassay used in these experiments (Tables 1 and 2). Thus, if cytotoxic potency is expressed in terms of nor-HN2 equivalents and if all of the experiments thus far discussed have been properly interpreted, the cytotoxic potency of metabolites generated by incubation of cyclophosphamide with a 9000 x g supernatant fraction should be less than the cytotoxic potency of a metabolite preparation generated by incubation of cyclophosphamide with a microsomal fraction. This turned out to be the case. The LC50 (in nor-HN2 equivalents) of cyclophosphamide metabolites generated by a 9000 x g supernatant fraction was 0.42 μM, compared with 0.087 μM when cyclophosphamide metabolites were generated by a microsomal fraction (Table 2; Chart 5).

One hr after cyclophosphamide injection, carboxyphosphamide and aldophosphamide are present in approximately equal amounts in blood, whereas urine collected for 3 hr after cyclophosphamide injection contains large amounts of carboxyphosphamide and relatively small amounts of aldophosphamide and other NBP-reactive metabolites (28). In accordance with the previous discussion then, cytotoxic potency expressed in nor-HN2 equivalents should be greatest for metabolites generated in vitro by microsomal enzyme action, intermediate for metabolites obtained from blood, and lowest for metabolites obtained from urine; this proved to be true, since the LC50 values in nor-HN2 equivalents for these preparations were 0.087, 0.66, and 4.5 μM, respectively (Table 2 and Chart 5). The LC50 of 4.5 μM obtained for cyclophosphamide metabolites present in urine is very close to that obtained for carboxyphosphamide (5.4 μM) when both are expressed in nor-HN2 equivalents. This observation supports the contention that little aldophosphamide is present in urine, and carboxyphosphamide makes up the bulk of urinary cyclophosphamide metabolites (4, 5, 28, 30, 31).

LC50's for cyclophosphamide metabolites generated in vitro and in vivo were also expressed in formaldehyde equivalents (Table 2; Chart 6). The rationale for expressing LC50's in formaldehyde rather than nor-HN2 equivalents was that if aldophosphamide is the sole or major source of cytotoxic action, LC50's obtained with these preparations and expressed in formaldehyde equivalents should be approximately the same, because essentially noncytotoxic metabolites (such as carboxyphosphamide and perhaps other NBP-reactive, nonaldehydic metabolites, which appear to be responsible for the wide variance in LC50's obtained when these values are expressed in nor-HN2 equivalents) would not give a positive

Chart 6. Cytotoxicity (in formaldehyde equivalents) of cyclophosphamide metabolites, generated in vitro and in vivo, to Walker 256 carcinosarcoma ascites cells. Concentration response. Quantitated are the relative cytotoxic effects of cyclophosphamide metabolites obtained from urine (○—○), blood (●—●), 9000 x g supernatant incubation mixture (♦—♦), and microsomal incubation mixture (♦—♦). Concentrations of aldehyde(s) were quantitated and are expressed in terms of formaldehyde equivalents. All other experimental details are as described in "Materials and Methods." Points, mean ± S.E. of 4 rats.
aldehyde test. This prediction was approximately validated, in that the LC₉₀'s in formaldehyde equivalents were, respectively, 0.035, 0.037, and 0.041 μM for cyclophosphamide metabolites obtained (a) after incubation of cyclophosphamide with hepatic microsomal fraction, (b) after incubation of cyclophosphamide with 9000 X g supernatant fraction, and (c) from blood after cyclophosphamide injection (Table 2). The LC₉₀ (0.17 μM) expressed in formaldehyde equivalents, observed with cyclophosphamide metabolites obtained from urine after cyclophosphamide injection, was different from the 3 values already mentioned. A tenable explanation is that the high dose of cyclophosphamide injected into rats in these experiments caused some tissue damage which resulted in the excretion of aldehydes normally not excreted and thus not accounted for in the blank. These aldehydes would be included in the total aldehyde content (since the aldehyde assay used in these experiments is nonspecific) and would effectively serve to dilute the small amount of aldophosphamide present, so that cytotoxicity per unit formaldehyde equivalent would be less than that expected if aldophosphamide was the sole source of aldehydic functional groups as well as of cytotoxic action. A similar argument could serve as an explanation for the much smaller discrepancy observed between the LC₉₀ when cyclophosphamide metabolites are obtained from blood and that observed when they are generated in vitro; in the latter case, an appropriate blank value can be obtained.

Alarcon and Meienhofer (1) demonstrated that microsomal oxidation of cyclophosphamide generates acrolein and suggested that this cytotoxic aldehyde may account for some or all of the antitumor activity of cyclophosphamide. In our experiments, the LC₉₀ for acrolein was 8.75 μM (data not presented). The extinction coefficient for acrolein in the aldehyde assay used in these experiments is only 0.06 that of formaldehyde; the LC₉₀ for acrolein in formaldehyde equivalents then, is 0.525 μM, a value considerably greater than the LC₉₀'s in formaldehyde equivalents obtained for cyclophosphamide metabolites generated in vitro or in vivo. Thus, acrolein cannot account for the cytotoxic activity of cyclophosphamide metabolites and, at most, could be responsible for only a very small portion of the total cytotoxic activity due to cyclophosphamide metabolites.

**DISCUSSION**

Cyclophosphamide was originally synthesized as the “transport” form of a drug which would be activated by enzymes within target (tumor) cells (2). That is, cyclophosphamide itself was designed to be inactive as an antineoplastic agent but was to have the potential of being converted to an antineoplastic agent by certain cellular enzymes, namely, phosphatases and phosphoramidases. Selective toxicity was to be achieved because the concentrations of these enzymes was believed to be much higher in tumor cells, compared to normal cells (18). Subsequent experimentation has revealed that cyclophosphamide itself is indeed without antineoplastic activity but that selective or even nonspecific activation in neoplastic cells does not occur (3, 6–8, 16, 22, 25, 31). Rather, activation was shown to be catalyzed by mixed-function oxidase action, the majority of which activity is located in hepatic endoplasmic reticulum (7–9, 11–13, 15, 16, 20, 21, 25–28).

In accordance with the observations of Hill et al. (20), the evidence presented herein and in a previous communication (28) suggests that the principal cytotoxic metabolite of cyclophosphamide is an aldehydic compound possessing alkylating activity. More specifically, evidence now available (while not conclusive) supports the hypothesis that aldophosphamide, which is an aldehydic compound possessing alkylating activity, is the cytotoxic metabolite of cyclophosphamide (20, 28, 29). Aldophosphamide, or at any rate, the cyclophosphamide metabolite generated by hepatic microsomal mixed-function oxidase action, is highly cytotoxic in the bioassay used to evaluate this action, e.g., on a relative basis it was much more cytotoxic than cyclophosphamide, 4-ketocyclophosphamide, carboxyphosphamide, nor-HN2, or chlorambucil, although it was not as cytotoxic as HN2. These data and other evidence strongly support the belief that the scheme presented in Chart 1 is descriptive of the major pathway of cyclophosphamide metabolism. Thus, it would appear that activation of cyclophosphamide (to a cytotoxic metabolite) occurs when it is oxidized to aldophosphamide by a mixed-function oxidase of the hepatic endoplasmic reticulum (7–9, 11–13, 15, 16, 20, 21, 25–28) and that aldophosphamide is inactivated (as a cytotoxic agent) by aldehyde oxidase and/or DPN-linked aldehyde dehydrogenase, which oxidize(s) aldophosphamide to carboxyphosphamide (4, 5, 20, 22, 28–31). DPN-linked aldehyde dehydrogenase activity has been detected in the cytosol and mitochondria of many tissues; highest activity was found in liver cells (14). Oxidation in vivo of aldophosphamide to a relatively noncytotoxic metabolite, carboxyphosphamide, appears to proceed rather rapidly, as evidenced by the observation that urine, even when collected at time points soon after cyclophosphamide administration, contains little aldophosphamide but a lot of carboxyphosphamide (28).

The possibility exists that the primary metabolite of cyclophosphamide activation, presumed to be aldophosphamide, is inactive or minimally active as a cytotoxic agent and that it is further converted, not only to carboxyphosphamide, but also to another metabolite that is highly cytotoxic. This conversion could be the result of enzymatic action or spontaneous degradation. No experimental evidence for or against such a possibility has yet been reported.

It is apparent that selective toxicity does not occur as a result of the initial activation process. However, selective toxicity could be achieved by selective inactivation. Thus, it may well be that cyclophosphamide (aldophosphamide)-sensitive normal and neoplastic cells possess little aldehyde oxidase and/or DPN-linked aldehyde dehydrogenase activity, the consequence of which is that they would be unable to inactivate aldophosphamide by converting it to carboxyphosphamide. Cyclophosphamide (aldophosphamide)-insensitive normal and neoplastic cells would possess relatively high aldehyde oxidase and/or DPN-linked aldehyde dehydrogenase activity, so that they would be able to inactivate aldophosphamide by converting it to carboxyphosphamide. Experi-
ACKNOWLEDGMENTS

The author gratefully acknowledges the technical assistance of Barbara Carpenter.

REFERENCES

Bioassay and Relative Cytotoxic Potency of Cyclophosphamide Metabolites Generated *in Vitro* and *in Vivo*

N. E. Sladek

*Cancer Res* 1973;33:1150-1158.

Updated version  Access the most recent version of this article at:  
http://cancerres.aacrjournals.org/content/33/6/1150

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.