Cyclophosphamide and Urinary Bladder Toxicity*

FREDERICK S. PHILIPS, STEPHEN S. STERNBERG, ALICE P. CRONIN, AND PEDRO M. VIDAL

(Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York, New York)

SUMMARY

Single doses of cyclophosphamide can regularly induce bladder damage in rats and dogs. Changes develop rapidly and, within 24 hours, consist of ulceration of the mucosal epithelium, hemorrhage and edema in all bladder tissues, and necrosis in smooth muscle and small arteries. The epithelium and submucosa of the renal pelvis are similarly affected. Renal tubular necrosis is seen in a minority of the affected animals.

During the first few hours after injection high concentrations of reactive material are excreted in the urine. Most of the alkylating activity is due neither to cyclophosphamide nor to bis(2-chloroethyl)amine. When reactive urine is introduced by catheter into dog bladders, it induces the typical changes seen after intravenous injection of cyclophosphamide. Bladder damage can be prevented by promoting an active diuresis during the first few hours after the injection of the agent.

These observations show that bladder damage is probably a local response to contact by toxic bladder urine. They suggest means for preventing bladder complications in patients. They also provide evidence for the in vivo transformation of cyclophosphamide into circulating cytotoxic moieties.

Bladder damage is an untoward effect of cyclophosphamide (I, Chart 1) which has been seen in dogs (28) and in patients (2, 5, 14). It appears more frequently in patients given large doses at widely spaced intervals of 2 or more weeks than in those receiving daily treatment with repeated, small doses. Since the regimen of intermittent, high doses is the more efficacious mode of treatment of experimental tumors (3, 4, 15, 16, 22), it would be unfortunate if its clinical trial were discouraged because of the bladder complication.

It occurred to us that the lesion might be prevented by simple measures which were suggested by previous findings of other workers. Of particular importance was the evidence indicating that in rats treated with I a circulating, cytotoxic intermediate is formed in extratumoral tissues. Brock has shown that rat tumors, which are effectively destroyed by treatment with I in vivo, are actually resistant to high concentrations of the agent in vitro. Under the same in vitro conditions the tumor cells are, nevertheless, highly susceptible to IV (Chart 1) and to other polyfunctional, alkylating agents (3, 4). Presumably such cells are unable to transform I into the reactive bis(2-chloroethyl)amine, IV, by hydrolysis of the N-P bond (bond 'a,' Chart 1). Brock has proposed that in vivo I is initially converted into the noncyclic phosphamide, II (Chart 1), and that this intermediate is responsible for the chemotherapeutic effects of cyclophosphamide. His speculation is supported by the in vitro cytotoxicity of an N,N'-dimethyl derivative of II and also by the lack of in vitro activity of a phosphoric acid ester derivative of III (4).

Foley, Friedman, and Drolet (7) recently obtained direct evidence for extratumoral transformation by finding a cytotoxic factor in the serum of rats shortly after the intraperitoneal injection of I. The toxic serum inhibited mammalian cell cultures against which I is inactive. Cytotoxic activity was also generated when I was incubated in homogenates of mouse liver but not when it was incubated in homogenates of mouse tumors, even though these were chemotherapeutically susceptible in vivo. The same investigators have also shown that III is cytotoxic both in vitro and in

* Aided by CCNSC Contract No. SA-48-p4-2445 from the National Cancer Institute, USPHS.

Received for publication July 10, 1961.
in vivo and have suggested that it may be the primary product of in vivo hydrolysis (9, 10).

A cytotoxic factor, which appears promptly in the blood and circulates for an appreciable time after administration of I, might be expected to undergo renal excretion. Such a substance, potent enough to damage mammalian cells in vitro, could directly disturb the bladder mucosa especially if heavily concentrated by the usual renal mechanisms. It is the purpose of the present report to show (a) that the urine of rats and dogs does in fact contain high concentrations of alkylating substances most of which are neither I nor IV, (b) that the contaminated urine is responsible for the bladder lesion induced by cyclophosphamide, and (c) that the lesion can be prevented with simple, diuretic measures which presumably protect by promoting the excretion of a dilute urine and by increasing the frequency of micturition.

MATERIALS AND METHODS

The animals used were male CFN rats, 190-350 gm., and adult, mongrel dogs of both sexes. They were maintained as previously described with the exception that the dogs were fed a 1:1 mixture of water and Purina Dog Chow (19).

The agents injected were cyclophosphamide\(^1\) (I) and bis(β-chloroethyl)amine hydrochloride, hereafter designated as nor-HN\(_2\)-IICl.\(^2\) They were dissolved in 0.85 per cent NaCl or distilled water immediately before administration; rats received different doses in the constant volume of 1 ml/100 gm.

Tissues were prepared for histologic study, and blood samples were obtained and cells enumerated as in earlier work (19, 20). The nucleated cells in the bone marrow of the rat femur were counted as follows: the bone was removed from animals which had been anesthetized with ether and killed by exsanguination. The two extremities were removed by sawing transversely across the femur through the distal end of the lesser trochanter and through the proximal end of the patellar surface. (A Handee Grinder with a 1" radial saw was used to avoid crushing and fragmenting the shaft.) One end of the shaft was attached by rubber tubing to a glass syringe containing 5 ml. of 1 per cent acetic acid. The contents of the syringe were ejected through the shaft into a 10-ml volumetric flask and brought to mark with the dilution fluid. To disperse the marrow cells the flask contents were passed repeatedly by syringe through a 20-gauge hypodermic needle. The suspension was diluted 1:10 in a white blood cell pipette, and the nucleated cells were counted by hemocytometer. The count in each corner box (1 mm. X 1 mm.) X 10\(^6\) was taken to be equal to the sum of nucleated cells in the shaft.

As a test of accuracy the marrow count was compared in the right and left femoral shafts of each of a series of ten control male rats (251-283 gm.). The average (± 1 s.d.) of the means of the ten pairs was 144 ± 40 X 10\(^6\) cells per shaft (range: 53-176 X 10\(^6\)). Though there was more than a threefold range in the mean marrow counts, the left and right shafts of each animal agreed closely—the average deviation of the ten pairs from their respective mean values was 3.8 per cent (range, 0.6-9.1 per cent).

URINE COLLECTION

Urine was obtained from rats and female dogs deprived of food overnight but given free access to water. The rats were intubated intragastrically with water, 5 ml/100 gm, 30-60 minutes before and again 60-90 minutes after intraperitoneal injections of cyclophosphamide (or of 0.85 per cent NaCl). Some rats also received, 30 minutes prior to cyclophosphamide, subcutaneous doses of 2 gm/kg of a 20 per cent solution (w/v) of mannitol (NF, Mallinckrodt). Immediately after the injections the animals were placed in pairs in metabolism cages with drinking water available. Urine was collected at hourly intervals from receivers packed in ice and diluted to 100 ml. with water. The female dogs were also given water intra-
gastrically, 25 or 50 ml/kg. One-half hour later
they were anesthetized with pentobarbital sodi-
um, 30 mg/kg given intravenously; their bladders
were catheterized, drained, and rinsed with iso-
tonic saline, and a continuous intravenous infusion
of 5 per cent dextrose was begun at 3–4 ml/min.
Urine was collected hourly in iced receivers; at
the end of each period the bladder was rinsed with
10 ml of isotonic saline, and the rinse was added
to the collection. At the end of the first hourly
collection, which was used as a control, cyclo-
phosphamide was injected intravenously.

All collections were either stored in the frozen
state or chilled with ice when being used for
analysis. No significant changes in reactivity were
seen in samples that were analyzed on different
days during periods as long as 1 week after collect-

**NBP-reactivity**

The colorimetric determination of alkylating
activity in urine was based on the quaternization
of the reagent, γ-(4-nitrobenzyl)-pyridine (NBP),
into a derivative which is blue when alkaline.
The procedure and reagents employed were those pre-
viously described for the analysis of watersoluble ethylenimines (6) with the addition, when
needed, of a preliminary hydrolysis with acid.

**Before hydrolysis.—** One ml. of an appropriate
dilution of urine (or of nor-HN₂·HCl, 40 µg/ml),
2 ml. of water, 1 ml. of 0.05 m phthalate buffer,
and 1 ml. of reagent (NBP, Aromil Chemical or
Matheson, Coleman and Bell) were pipetted in
turn into a pyrex tube calibrated at 10 ml. A
series of such tubes, including the standard of
nor-HN₂·HCl and a reagent blank, were im-
mersed together in boiling water. Exactly 15
minutes later they were chilled in ice. Each tube
in turn was removed from the ice and allowed to
warm at room temperature for exactly 2 minutes.
In rapid succession 4 ml. of acetone, 1 ml of
molar K₂CO₃, and water to mark were added and
mixed. At exactly 3.5 minutes after removal from
the ice absorbance was measured in a Klett-
Summerson colorimeter with a green filter (No.
54). The rigid schedule described was adopted
because of the rapid fading of the blue derivative.
The reagent blank, 30–40 Klett units of absorb-
ance, was deducted from the readings of standard
and unknowns. NBP-reactive substances in the
urine samples were then calculated by direct pro-
centage with the absorbance of the standard as
equivalent concentrations of nor-HN₂·HCl.

**After hydrolysis.—** One ml. of dilute urine (or of
a standard of nor-HN₂·HCl, 40 µg/ml, or of
cyclophosphamide, 60 µg/ml), and 1 ml of 1.00 M
HCl were added to Pyrex test tubes. A series of
such tubes, including a reagent blank and the
standard, were immersed together in boiling water.
Exactly 10 minutes later they were cooled in ice
and then were neutralized by addition of 1 ml of
1.00 M NaOH. The remaining steps were those de-
scribed above, beginning at the addition of buffer
and NBP-reagent. (It is important that the HCl
and NaOH be exactly equivalent; the NBP-
reaction is poor if the pH of the system departs
appreciably from the buffer range.)

**Notes.—** Absorbance varied directly with the
concentration of nor-HN₂·HCl (4–40 µg/ml) and
of hydrolyzed cyclophosphamide (10–100 µg/ml).
Absorbances obtained with standard 40 µg/ml
solutions of nor-HN₂·HCl before and after hy-
drolysis ranged between 185 and 203 and between
138 and 175 Klett units, respectively. There was,
thus, a loss of NBP-reactivity by hydrolysis
averaging about 14 per cent (7–25 per cent, seven
determinations). Conversely, the NBP-reactivity
of cyclophosphamide was increased by hydrolysis.
With concentrations between 60 and 100 µg/ml
the NBP-reactivity before hydrolysis was found
to average 6.7 per cent of that obtained after
hydrolysis (4.2–9.1 per cent, sixteen determina-
tions). The reactivity of hydrolyzed solutions of
cyclophosphamide (60–100 µg/ml) averaged 99
per cent of that of molar equivalent amounts of
nor-HN₂·HCl (89–110 per cent, seven determina-
tions). To test for possible interference by
urinary constituents nor-HN₂·HCl was added in
a final concentration of 38 µg/ml to four 1-hour
urine collections, each from a different pair of rats
that had been hydrated and given injections of
isotonic saline as described above. The recovery
was 120–132 per cent before hydrolysis and 107–
110 per cent after hydrolysis; there is no obvious
explanation for the increased NBP-reactivity.
Cyclophosphamide was also added to the same
collections in the final concentration of 70 µg/ml.
The recovery was 8.0–8.4 per cent before hydro-
dysis and 100–104 per cent after hydrolysis.

**CHCl₃-solubility.—** Since cyclophosphamide
and nor-HN₂ were readily extracted by CHCl₃,
analyses before and after shaking with the solvent
provided an estimate of the maximum possible
contribution of these substances to the total NBP-
reactivity of urine samples. For this purpose 1 ml
of urine, 4 ml of 0.10 molar NaHCO₃, and 25 ml
of CHCl₃ were pipetted into a 60-ml glass-
stoppered bottle and shaken mechanically for 30
minutes. One ml of the aqueous layer was mixed
with 1 ml of 0.08 M HCl (or 2 ml of 0.04 M). One
ml. of the neutralized mixture was then hydrolyzed and analyzed for NBP-reactivity as described above. The reactivity after extraction is expressed below in per cent of that found in unextracted urine.

In tests with solutions of cyclophosphamide (0.6–1 mg/ml) only 5 per cent of the NBP-reactivity remained in the aqueous layer after CHCl₃ extraction (3–7 per cent, five determinations). This was not due to decomposition of cyclophosphamide in the alkaline solution, since NBP-reactivity remained unchanged when the CHCl₃ was omitted during the shaking procedure. A solution of nor-HN₂·HCl (0.6 mg/ml) was also found after extraction to have only 4 per cent of its initial reactivity. Most of the nor-HN₂ was recovered by shaking 10 ml. of the CHCl₃ layer for 10 minutes with 5 ml. of 0.1 M HCl. The acid extract contained 71 per cent of the expected NBP-reactivity.

CYCLOPHOSPHAMIDE: 
SINGLE INTRAPERITONEAL DOES

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Mortality</th>
<th>Day of Death</th>
<th>LD₅₀ (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>333</td>
<td>8/9</td>
<td></td>
<td>182</td>
</tr>
<tr>
<td>222</td>
<td>5/9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>4/9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0/9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Male Rats, 190–330 gm

CHART 2.—Fate of rats given injections of cyclophosphamide.

TABLE 1

<table>
<thead>
<tr>
<th>Day after injection†</th>
<th>Weight Loss (per cent)</th>
<th>Marrow Count (10⁶ cells)</th>
<th>Leukocyte count</th>
<th>Number with</th>
<th>Wet weight of bladder (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Neutrophils (10⁶/cu mm)</td>
<td>Lymphocytes (10⁶/cu mm)</td>
<td>Bladder lesions</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td>141 ± 53</td>
<td>2.1 ± 0.7</td>
<td>8.3 ± 2.3</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1, 2, 6</td>
<td>35, 58, 53</td>
<td>2.8 ± 1.4</td>
<td>0.5 ± 3.0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.7, 2</td>
<td>20, 10, 28</td>
<td>0.5, 0.7, 0.6</td>
<td>0.1, 0.3, 0.1</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>12, 10, 7</td>
<td>10, 4, 8</td>
<td>0.2, 0.0</td>
<td>0.0</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>6, 12, 5</td>
<td>2, 3, 8</td>
<td>0.3, 0.0</td>
<td>0.0</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>25, 16, 9</td>
<td>10, 28, 54</td>
<td>0.2, 0.2, 0.2</td>
<td>0.0</td>
<td>2</td>
</tr>
<tr>
<td>12–15</td>
<td>19, 16, 15</td>
<td>87, 104, 115</td>
<td>1.5, 2.4, 2.4</td>
<td>0.4, 1.8, 1.4</td>
<td>3</td>
</tr>
</tbody>
</table>

* 244–322 gm.
† The treated animals were sacrificed in groups of 3, selected at random, on each of the days listed. The data for each group are presented in the rows; the data for individual animals are given in the same order in each column. One control animal was also sacrificed with each experimental group: the pooled results are given as averages ± standard deviations.
week, when the count of nucleated cells in the bone marrow of the femoral shaft was lowest. Recovery of hematopoiesis was evident at 8 days in the femoral counts and at 12–13 days in the blood.

Each of the eighteen rats of Table 1 had damaged bladders. By gross observation the disturbance was as marked at 1 and 2 days as at later times. The mucosa of the 1-day bladders contained numerous hemorrhages. In one there was a raised hematoma-like mass on the serosal surface continuous through the wall, with a hemorrhagic area in the mucosa. The bladders were thickened and spongy, and their wet weight was significantly increased (see Table 1). Edema was prominent in perivesical tissues, especially so in the contiguous ventral prostate. The ureters and ureteropelvic junctions of two of the 1-day animals were distended with fluid.

All the gross disturbances persisted through the 1st week, as shown in Table 1. In two of the 6-day rats blood clots and bloody fluid distended the bladders, ureters, and renal pelvices. The kidneys of the pair were enlarged and edematous, with parenchyma narrowed by enlarged, hollow, hydronephrotic pelvic cavities. By 8 days the ureters were less distended than before, and by 12–13 they were normal in size. Perivesical edema had also subsided at this time. The bladders were still enlarged and thickened at 12–13 days but showed at most only a few, faint petechial hemorrhages.

Microscopic study revealed extensive ulceration of the mucosa in the 1-day bladders. In ulcerated areas the epithelium was necrotic, and a bloody exudate extruded into the lumen containing cellular debris, fibrin, and inflammatory cells. Where the mucosa was intact, the epithelium showed various focal changes such as thinning, atypia, and karyorrhexis. Ulceration remained prominent through the 6th day; but repair and regeneration were already evident at 4 days, when mitotic activity was seen in the epithelium outside of zones of ulceration, and fibroblasts, histiocytes, and polymorphonuclear leukocytes were found in the submucosa (Figs. 1–4). By 8 days the epithelium of two bladders was intact, and in the third the ulcers were smaller than in the earlier specimens. At this time the epithelium was thicker than normal, and cellular atypia was prominent; mitoses were still abundant (Fig. 3). By 12–13 days the epithelium was intact and normal, but histiocytes laden with hemosiderin were found scattered throughout the submucosa.

Edema and hemorrhage were also striking disturbances in the 1- to 4-day bladders. They were diffuse and uniform throughout the submucosa and focal in all other bladder tissues. They were still present in the 6- and 8-day specimens, and, even as late as 12–13 days, two of the three bladders, though free of hemorrhage, had focal areas of submucosal and subserosal edema.

Renal lesions were also seen. In two of the 1-day rats the mucosa of the renal pelvis showed hemorrhage, necrosis, and ulceration (Fig. 5). Between 2 and 6 days four other animals had pelvic changes consisting of cellular atypia in the epithelium and subepithelial hemorrhages. Foci of internal hydronephrosis (in nine rats) and of necrosis in convoluted tubules (in seven rats) were also found, mostly between 4 and 8 days. Five of the last nine rats of the series had necrosis of the tubules in the distal portion of the papilla (Fig. 6).

Microscopic study of the ventral prostates confirmed the presence of the edema which had been seen in the gross examinations. In some instances inflammatory cells and erythrocytes were present in the edema fluid. Since the epithelium, stroma, and vascular tissues of the prostate showed no other injury, it seemed likely that the edema had not originated locally but had drained from the contiguous tissues of the damaged bladders.

The rapidity of epithelial regeneration was quite remarkable when contrasted with the bone marrow arrest shown in Table 1. The bladder epithelium was well on its way to complete restitution at 6 days, when the bone marrow showed the maximum aplasia.

Although mice were not studied extensively, some preliminary findings may be useful to others. Male, Hauschka/ICR animals, 20–25 gm, were used. The LD₉₀ of single, intraperitoneal doses was found to be 550 mg/kg. Doses of 750 mg/kg killed within less than 24 hours: nine of ten animals given this dose and autopsied at 24 hours had grossly hemorrhagic bladders. Three of ten other mice were killed at 7 days after 385 mg/kg intraperitoneally. All three had gross and microscopic bladder lesions (Fig. 4). The remaining seven were killed at 8 days; four of these had grossly abnormal bladders which were not studied microscopically.
After the above study had shown that a high dose of cyclophosphamide (1.2 × LD₉₀, see Chart 2) uniformly induced severe bladder disturbances, it became of interest to determine the sensitivity of the organ to sublethal doses of the agent. Table 2 summarizes the results evaluated by gross inspection of animals which were killed by etherization at 2 days after single intraperitoneal injections. Bladder lesions were considered present if hemorrhages were seen in the mucosa. The data show that doses as low as 29 mg/kg (0.16 × LD₉₀) were toxic to the bladder: all six rats given this dose had bladder hemorrhages, and the wet weight of the organ was significantly greater than that of control rats. The absence of perivesical edema and hydroureter in the same six animals indicates that the bladder lesion was probably less severe than in rats receiving higher doses of cyclophosphamide.

The data in Table 2 also show that the injection of nor-HN₂ did not induce bladder damage. It should be noted that the doses of 42 and 100 mg/kg of nor-HN₂·HCl are, respectively, the molar equivalents of 66 and 148 mg/kg of cyclophosphamide. Such findings indicate that the bladder lesion caused by cyclophosphamide cannot be attributed to prerenal formation of nor-HN₂.

**NBP-reactivity in urine.**—Within the first 60 minutes after injection of cyclophosphamide the urine contained appreciable concentrations of NBP-reactive substance. This is shown graphically in Chart 3 in the hourly collections from a pair of animals given 66 mg/kg intraperitoneally. In this and another pair receiving the same dose the 4-hour cumulative excretion of NBP-reactivity was equal, respectively, to 69 and 70 per cent of the dose given (expressed in molar equivalents of nor-HN₂·HCl which was used as the analytical standard). Table 3 is a summary of all experiments in which the concentration of NBP-reactive materials in urine was determined during the first hours after cyclophosphamide. Table 4 gives the portions of the dose recovered in the urine in the same experiments.

There are several aspects of the data in Chart 3 and Tables 3 and 4 which have some bearing on the nature of the urinary NBP-reactive material. The total reactivity after hydrolysis was not more than twice that found in nonhydrolyzed urine. Had the NBP-reactive material been solely cyclophosphamide, the results obtained after hy-

### TABLE 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>No.</th>
<th>Number with</th>
<th>Wet weight of bladder† (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bladder lesions</td>
<td>Perivesical edema</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>222</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>148</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>6</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>66</td>
<td>6</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>6</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>nor-HN₂·HCl</td>
<td>100</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* 310-386 gm.
† Averages ± standard deviations.

**Chart 3.**—Excretion of NBP-reactivity in rat urine. The triangles are concentrations expressed as equivalent amounts of nor-HN₂·HCl. The circles are cumulative recovery in per cent of dose given.
drolysis would have exceeded by ca. fifteen-fold the pre-hydrolysis values (see "Methods"). The fact that hydrolysis increased the values also indicates that the reactive materials were not solely nor-HN2. Such considerations do not exclude the possibility that the reactivity was due to a mixture of cyclophosphamide and nor-HN2 in approximately equal concentrations. However, this is not supported by the low order of NBP-reactivity found in the urine of rats given the soluble in CHCl3, and for this reason can be attributed to reactive substances other than nor-HN2 and cyclophosphamide. It seemed likely that these substances were either II or III (see Chart 1) or mixtures thereof (see "Discussion")—a supposition consistent with the data in Table 5 showing that organic P was excreted in amounts that were approximately the molar equivalent of the NBP-reactivity.

Antidiuretic effects.—It was noted during the

### TABLE 3
CONCENTRATION OF NBP-REACTIVE SUBSTANCE* IN RAT URINE AFTER SINGLE INTRAPERITONEAL DOSES

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (mg/kg)</th>
<th>Time (hr.)</th>
<th>Before hydrolysis (mg/ml)</th>
<th>After hydrolysis (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide</td>
<td>222</td>
<td>0-1</td>
<td>2.5, 1.4</td>
<td>1.6, 1.7, 1.5, 1.2</td>
</tr>
<tr>
<td></td>
<td>66</td>
<td>1-2</td>
<td>2.8, 1.3</td>
<td>0.4, 0.4, 0.3, 0.3</td>
</tr>
<tr>
<td>nor-HN2·HCl</td>
<td>142</td>
<td>0-1</td>
<td>0.8, 0.8, 0.7, 0.6</td>
<td>0.4, 0.4, 0.3, 0.3</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>1-2</td>
<td>0.3, 0.3</td>
<td>0.7, 0.7</td>
</tr>
<tr>
<td>Saline†</td>
<td>0-1</td>
<td></td>
<td>0.1, 0.1</td>
<td>0.5, 0.4</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td></td>
<td>0.1, 0.1</td>
<td>0.5, 0.4</td>
</tr>
</tbody>
</table>

* Calculated as nor-HN2·HCl
† In animals receiving mannitol subcutaneously.

molar equivalent doses of nor-HN2·HCl (see Tables 3 and 4).

Table 5 also shows that most of the urinary NBP-reactive material was not due to cyclophosphamide or nor-HN2. In each experiment of Table 5 the total dose (90–130 mg/kg) was divided equally between two animals which were caged together. NBP-reactivity in the urine collected during the first 2 hours was determined after hydrolysis, with cyclophosphamide used as the analytical standard. The recovery ranged between 32 and 53 per cent of the dose given. More than 60 per cent of the NBP-reactivity was

The data in Tables 4 and 5 suggest that the urinary recovery of NBP-reactive materials during the first 2 hours after injection varied inversely with the dose of cyclophosphamide. This needs further study, since it could conceivably have bearing on the relation between chemotherapeutic efficacy and schedule of dosage (see "Introduction").

### TABLE 4
NBP-REACTIVE SUBSTANCE* RECOVERED IN RAT URINE AFTER SINGLE INTRAPERITONEAL DOSES

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (mg/kg)</th>
<th>Before hydrolysis (per cent)</th>
<th>After hydrolysis (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide</td>
<td>222</td>
<td>15, 17</td>
<td>55, 50, 56, 57</td>
</tr>
<tr>
<td></td>
<td>66</td>
<td>39, 31, 28, 34</td>
<td>38, 41</td>
</tr>
<tr>
<td>nor-HN2·HCl</td>
<td>142</td>
<td>3, 3</td>
<td>3, 2</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>2, 3</td>
<td>2, 3</td>
</tr>
</tbody>
</table>

* Calculated as nor-HN2·HCl.
† In animals receiving mannitol subcutaneously.
above studies that the volume of urine from rats given cyclophosphamide was significantly less during the first few hours than that from saline-injected controls. Since antidiuresis would tend to increase the concentration of reactive materials in urine, it seemed worth while to explore this effect in greater detail. Pairs of animals were hydrated with 5 ml/100 gm of water by intragastric intubation and were given immediately intraperitoneal injections of either isotonic saline or 66 or 222 mg/kg of cyclophosphamide. Urine volumes were measured at 30-minute intervals thereafter. The results are depicted in Chart 4. Whereas the performance of the controls was the expected response to the water-load, excretion was significantly delayed in the animals given 66 mg/kg and markedly inhibited in those receiving 222 mg/kg.

### TABLE 5

**Excretion of Organic Phosphate and NBP-Reactivity in Rat Urine after Single Intraperitoneal Doses of Cyclophosphamide**

Urine collected for 2 hours after injection.

<table>
<thead>
<tr>
<th>Dose (µMoles)</th>
<th>P₀ (µMoles)</th>
<th>NBP-reactivity*</th>
<th>Ratio: P₀/NBP-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (µMole)</td>
<td>% CHCl-insoluble (per cent)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>195</td>
<td>78</td>
<td>71%</td>
<td>1.1</td>
</tr>
<tr>
<td>195</td>
<td>93</td>
<td>73%</td>
<td>1.3</td>
</tr>
<tr>
<td>195</td>
<td>68</td>
<td>68%</td>
<td>1.1</td>
</tr>
<tr>
<td>179</td>
<td>64</td>
<td>64%</td>
<td>0.9</td>
</tr>
<tr>
<td>179</td>
<td>92</td>
<td>92%</td>
<td>1.0</td>
</tr>
<tr>
<td>179</td>
<td>92</td>
<td>92%</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* Calculated as cyclophosphamide after hydrolysis.

The same experiment was repeated with one addition: all animals received subcutaneously at 0 time 2 gm/kg of mannitol. As shown in Chart 4 the osmotic diuretic blocked the antidiuretic effect of 66 mg/kg and substantially reduced that of the higher dose.

**Prevention of bladder damage.**—As shown in Table 6, water diuresis effectively reduced the severity of bladder damage caused by 66 mg/kg of cyclophosphamide; almost complete protection was achieved by the addition of the osmotic diuretic, mannitol. The one failure in the latter group had only a few, scattered petechial hemorrhages in the bladder mucosa, and the weight of the organ was only 83 mg.

As might have been expected the diuretic treatment was limited in effectiveness. Neither of the procedures was successful against the high dose of 222 mg/kg. Presumably the antidiuretic effect of this dose coupled with the relatively short duration of diuresis following water and mannitol (see Chart 4) could explain the failure.

### STUDIES IN DOGS

**Bladder lesion.**—Thirteen female and twelve male dogs were deprived of food overnight (but given water to drink freely) before being given injections intravenously of cyclophosphamide. Two received 100 mg/kg; one died at 20 hours and was autopsied immediately; the other was killed with intravenous pentobarbital at 3 days. Thirteen dogs received 50 mg/kg: eleven of this group were killed at 24 hours; the other two, at 3 days. Two animals received 25 mg/kg and were killed at 24 hours. The remaining eight dogs were hydrated with 50 ml/kg of water by stomach tube 60 minutes before receiving 50 mg/kg of cyclophosphamide; each of these eight was paired with one
of the above nonhydrated animals given the same dose. All eight were killed 24 hours later, along with their paired, nonhydrated controls.

Sixteen of the seventeen nonhydrated dogs had bladder damage; the one exception had received the lowest dose of cyclophosphamide, 25 mg/kg. In most animals the gross changes were severe. The bladders were thick and edematous, and large areas of hemorrhage were scattered throughout the mucosa and submucosa (Figs. 7 and 8). In three, hemorrhages were also present on the serosal surface; in one of these petechial hemorrhages were also found on the serosa of the uterus where it was in contact with the bladder. In six

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Treatment*</th>
<th>No.</th>
<th>Bladder lesions</th>
<th>Perivesical edema</th>
<th>Hydro-ureter</th>
<th>Wet weight of bladder (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>222</td>
<td>None</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>14</td>
<td>145 ± 19†</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>4</td>
<td>141 ± 37†</td>
</tr>
<tr>
<td></td>
<td>Water + mannitol</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>109 ± 28†</td>
</tr>
<tr>
<td>66</td>
<td>None</td>
<td>23</td>
<td>23</td>
<td>11</td>
<td>4</td>
<td>113 ± 19†</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>11</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>88 ± 30†</td>
</tr>
<tr>
<td></td>
<td>Water + mannitol</td>
<td>11</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>68 ± 9†</td>
</tr>
<tr>
<td>Control‡</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>67 ± 8</td>
</tr>
</tbody>
</table>

* The experimental rats were starved overnight with free access to water; their weight was then 190–205 gm. All were given injections of cyclophosphamide at 0 min. Those treated with water alone received 5 ml/100 gm by intragastric intubation at −30 to −60 min; a second dose of water was given 60 min. after the 222 mg/kg dose of cyclophosphamide. Those treated with water and mannitol received, respectively, at −30 min. 5 ml/100 gm by intubation and 2 gm/kg subcutaneously (1 ml/100 gm of a 20% solution).
† P < 0.01 for difference from controls ("t" test).
‡ The control animals of Table 2.

bladders yellow, green, or black plaques or membranes adhered to the surface of the mucosa. Edema of perivesical fat was seen in three. Three of the sixteen bladders (all from animals given 50 mg/kg) were less severely affected: edema was moderate, and the hemorrhages were small or petechial.

Microscopic study of the thirteen severely damaged bladders revealed diffuse and marked hemorrhage and edema in the submucosa; in some of the thirteen these changes were also present in the mucosal epithelium (Fig. 9), the smooth muscle, and the serosa. The mucosa was diffusely ulcerated in all (Fig. 10). Complex exudates of fibrin, erythrocytes, inflammatory cells, desquamated epithelium, and protein protruded into the

were as a group conspicuously different from those of nonhydrated dogs. By gross and microscopic inspection four were wholly normal, and three had a few petechial hemorrhages in the submucosa. Only one resembled bladders seen in nonhydrated animals; microscopically this was similar to the less severely damaged examples described above.

Renal lesion.—Damaged kidneys were found in eleven of the 25 animals described above without obvious relation to sex or the prior administration of water. In nine the lesion consisted of an occasional focus of necrosis in convoluted tubules in the outer medulla (Fig. 14). In two, however, there was severe, diffuse necrosis of convoluted tubules, mainly distal, in both cortex and medulla and of most collecting tubules. Protein casts were
prominent (Figs. 15 and 16). Where the necrosis was incomplete, the tubular epithelium showed hyaline droplet degeneration. In spite of the severity of the disturbance the more proximal portions of proximal convoluted tubules remained undamaged. (Both of the severe renal disturbances occurred in dogs given 50 mg/kg of cyclophosphamide; one of the pair had been hydrated before injection).

**NBP-reactivity in urine.**—The renal excretion of NBP-reactive substance was determined in two female dogs for the first 6 hours after 50 mg/kg of cyclophosphamide. The recovery was equivalent to 56 per cent of the dose in the dog described in Table 7 and to 46 per cent in the second animal. In Table 7 the NBP-reactivity before hydrolysis averaged 67 per cent of the values obtained after hydrolysis, and more than half of the reactivity was CHCl₃-insoluble. In the second dog the same calculations averaged, respectively, 81 per cent (range: 68–90 per cent) and 60 per cent (range: 47–73 per cent). As noted above in rats such results show that much of the reactive substance was not unchanged cyclophosphamide. This also followed from consideration of the rate of excretion of NBP-reactivity. Had it been due solely to cyclophosphamide, it would have been maximal in the earliest urine collections. Actually, however, excretion was greater in both dogs during the 2d and 3d hours than during the 1st hour (see Table 7)—a finding indicating the formation in vivo of a metabolite of cyclophosphamide. The fact that the excretion of P₃, kept pace with that of NBP-reactivity was also consistent with this interpretation. In Table 7 the P₃:NBP-reactivity averaged 0.99 and in the second dog, 1.08 (range: 0.95–1.26).

In order to obtain contaminated urine with maximal concentration of NBP-reactivity, three other anesthetized, female dogs were given 50 mg/kg but without prior hydration. Four successive 1-hour urine collections were obtained from each animal for use in an experiment to be described below. The rate of urine production by the three nonhydrated animals varied between 0.1 and 0.2 ml/min, whereas the NBP-reactivity after hydrolysis, expressed as cyclophosphamide-equivalents, ranged between 3.5 and 9.6 mg/ml (average: 6.6 mg/ml.) In the two previous dogs the flow rates during the first 4 hours had been 5–10 times greater, averaging 2.9 ml/min in the animal of Table 7 and 0.9 ml/min in the other. The mean NBP-reactivity in their urine collections had been correspondingly lower: 0.3 and 1.2 mg/ml, respect-

<table>
<thead>
<tr>
<th>TIME OF COLLECTION (HOURS)</th>
<th>NBP-reactivity</th>
<th>P₃ (μmoles)</th>
<th>RATIO P₃/NBP-REACTION AFTER HYDROLYSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before hydrolysis (μmoles)</td>
<td>After hydrolysis (μmoles)</td>
<td>CHCl₃-insoluble (per cent)</td>
</tr>
<tr>
<td>-1-0</td>
<td>0</td>
<td>0</td>
<td>42</td>
</tr>
<tr>
<td>0-1</td>
<td>91</td>
<td>170</td>
<td>51</td>
</tr>
<tr>
<td>1-2</td>
<td>194</td>
<td>294</td>
<td>74</td>
</tr>
<tr>
<td>2-3</td>
<td>217</td>
<td>201</td>
<td>51</td>
</tr>
<tr>
<td>3-4</td>
<td>56</td>
<td>94</td>
<td>57</td>
</tr>
<tr>
<td>4-5</td>
<td>56</td>
<td>94</td>
<td>78</td>
</tr>
<tr>
<td>5-6</td>
<td>59</td>
<td>57</td>
<td>78</td>
</tr>
</tbody>
</table>

* Calculated in terms of a hydrolyzed cyclophosphamide standard.
Toxicity of NBP-reactive urine.—The collections from the nonhydrated dogs described above were used for a direct test of the bladder toxicity of NBP-reactive urine. Each of the three animals served as a urine donor for an anesthetized female recipient. Immediately before the end of the 1st hour the recipient's bladder was catheterized, drained, and rinsed with isotonic saline. All but about 1 ml. of the donor's collection was then introduced into the recipient's bladder, where it was retained by clamping the catheter. At the end of each of the next 3 hours the recipient's bladder was drained and then given the next donor collection. After the 4th hour of exposure the recipient's bladder was drained and rinsed with three successive 10-ml. portions of isotonic saline. The donor's bladder had been washed similarly after removal of the 4-hour urine. Both animals were killed 1 day later.

To compare with the direct effect of contaminated urine, 10-ml. portions of a 5 mg/ml solution of cyclophosphamide were introduced at the beginning of each of 4 successive hours into the bladders of each of four anesthetized, female dogs. These bladders were drained and washed as in the above recipients, and the animals were killed 1 day later. The concentration of cyclophosphamide had been chosen to be within the range of NBP-reactivity after hydrolysis (see above). The 10-ml. volume also approximated the amounts of contaminated urine introduced into the recipient bladders (6-13 ml.). From the amounts of fluid drained from the catheters at the end of each hour of exposure, the two groups diluted the bladder contents with comparable amounts of urine: 4-10 ml. in the three recipients and 5-12 ml. in the cyclophosphamide-treated dogs.

The urine drained from two of the cyclophosphamide-treated bladders was analyzed. The NBP-reactivity after hydrolysis (in terms of cyclophosphamide-equivalents) ranged between 2.6 and 3.7 mg/ml in the eight hourly collections; the recovery averaged 54 mg/collection (50 mg. of cyclophosphamide had been instilled); reactivity before hydrolysis was 7-10 per cent of these values; and only 4-7 per cent of the total was CHCl3-insoluble. These results show that cyclophosphamide is relatively stable when in urine within the lumen of the bladder.

Minor changes were found in each of the cyclophosphamide-treated bladders. Gross inspection of the mucosa showed bluish-red discolorations in the neck region in two; diffuse, light pink congestion throughout the mucosa in two; and small areas of petechial hemorrhage in the neck in three and in the dome in one. The microscopic changes were also minor. There was neither ulceration nor edema. In the submucosa a few petechial hemorrhages were seen in two bladders and small foci of inflammatory cells in two. Congested vessels were found in the neck region. It is conceivable that these alterations were due as much to the trauma of catheterization as to the exposure to cyclophosphamide. None were the equal of those seen in animals given intravenous doses.

The three donor-recipient pairs were impressively different. The luminal surface of each of the six bladders was hemorrhagic, more marked in recipients than in donors. Microscopic study showed widespread ulcers with purulent bases in the mucosa of two recipients and one donor. Thinning of the epithelium was seen in the others. Widespread edema and hemorrhage were prominent in the submucosa and present as well in all other tissues in five of the bladders; in one donor these were only focal and submucosal. Three recipients and one donor had focal muscle necrosis; one recipient showed fibrinoid degeneration of arteries in the mucosa, submucosa, and serosa. All such changes were the equivalent of those found previously in the unanesthetized males and females given intravenous doses of cyclophosphamide.

DISCUSSION

The four compounds shown in Chart 1 are probable contributors to the alkylating activity of urine following administration of cyclophosphamide. (A fifth compound arising from rupture of bond 'c' in compound II or bond 'b' in III is unlikely, since it would spontaneously degrade into IV [8]). Although some cyclophosphamide, I, and small amounts of nor-HN2. IV, may be involved, the present studies favor the proposal that II or III or mixtures of both constitute most of the reactive materials. (It is also conceivable that derivatives of II and III with bond 'a' intact are present in reactive urine, since both compounds contain groups known to be susceptible to metabolic transformation: the aliphatic amino of II, the alcohol of III, and the phosphoric acid hydroxyl and phosphamide-N of both [24].) This proposal is consistent with the following evidence: the inextractability by CHCl3 of most of the alkylating activity, the large fraction of the activity that is available for reaction without prior hydrolysis, the paced excretion of molar equivalent amounts of organic phosphate and of reactive materials, the delay in excretion of maximal activity in keeping with the build-up in vivo of a metabolite, and the relatively low recovery of urinary activity after injection of nor-HN2. HCl.
Isolation of the major reactive component from contaminated urine may clarify the nature of the cytotoxic intermediate which has been proposed by Brock (3, 4) and Foley et al. (7, 9, 10) to be involved in the in vivo actions of cyclophosphamide. Pending this it is reasonable to suppose that the same urinary component is also responsible for the bladder damage caused by reactive urine.

The present work has shown that the injury to the bladder mucosa is probably a local response to contact by toxic bladder urine rather than a selective cytotoxic effect of blood-borne substances. There is one observation of other investigators which may affect this conclusion. Phosphamidase activity has been selectively localized by histochemical means in the mucosal epithelium of the rat bladder (18). Since phosphamide hydrolysis is essential for the ultimate transformation of cyclophosphamide or of its noncyclic intermediates into nor-HN₂, the presence of a phosphamidase in the epithelium could account for its susceptibility. Contiguous tissues could be affected by diffusion of the cytotoxic end-product. It follows that activation of hematogenous cyclophosphamide could be an alternative or additional mechanism of bladder damage. This, however, would be discordant with the relatively low incidence of renal tubular necrosis in cyclophosphamide-treated animals; for the proximal and distal convoluted tubular epithelium of the rat is the most active site of histochemical phosphamidase activity among all body tissues—far in excess of that detected in bladder epithelium (18). Renal epithelium is also endowed with highly active acid and alkaline phosphomonoesterases which may be involved in cyclophosphamide activation; the same enzymes are by contrast negligible in bladder epithelium (18).

Those kidney lesions which have been found in the present work are, as in the case of bladder damage, probably due to the local effects of contaminated urine. Indeed, the relative insusceptibility of the kidney does not support the concept that the selective cytotoxic effects of cyclophosphamide are due to selective activation by and consequent destruction of cells rich in the requisite hydrolytic enzymes.

As shown above bladder damage was easily prevented in rats and dogs by promoting a brisk diuresis during the first few hours after administration of cyclophosphamide. This was accomplished by hydration in both species and by the additional use of an osmotic diuretic in rats. It would seem likely that similar regimens could be employed in patients to reduce the incidence of bladder complications. It is also likely that the risk of bladder damage would be higher in dehydrated individuals than in those with a normal flow of urine. Cyclophosphamide probably should not be administered when urine flow is inadequate.

The renal excretion of reactive materials needs further study. This could be helpful in the clinical management of cyclophosphamide therapy. It may also be revealing in regard to the mechanism responsible for the greater efficacy of intermittent, high-dose regimens in the treatment of experimental tumors (see "Introduction"). If cyclophosphamide and its cytotoxic intermediates are excreted at different rates or have different renal thresholds and if the same substances vary in the relation of efficacy to toxicity, renal mechanisms could be important factors in dose-response relationships.

**ADDENDUM**

The authors have recently learned of two other studies which are pertinent to the present work. Kallenbach and Schattenfroh have described a...
Fig. 7.—The mucosal surface of the bladder from a dog, 24 hours after it had been hydrated and injected with 50 mg/kg. This bladder showed neither gross nor microscopic changes.

Fig. 8.—Severe hemorrhage and edema in the bladder mucosa of a dog 24 hours after receiving 50 mg/kg without prior hydration. This animal had been paired with the dog of Figure 7. Both bladders were photographed together on the same film employing an infra-red filter.

Fig. 9.—Inter- and intracellular edema and hemorrhage in bladder epithelium of a dog 3 days after 100 mg/kg.

Fig. 10.—Bladder ulcer in a dog 24 hours after 50 mg/kg. Note hemorrhagic exudate extending into lumen; intact mucosa is at left.

Fig. 11.—Nuclear fragmentation in epithelium and submucosa in bladder of a dog 24 hours after 50 mg/kg.

Fig. 12.—Muscle necrosis in the bladder of a dog 24 hours after 50 mg/kg.
Fig. 13.—Severe muscle damage in the bladder of a dog 3 days after 50 mg/kg. Note abscess formation.

Fig. 14.—Single-cell necrosis in convoluted tubular epithelium 24 hours after 50 mg/kg.

Figs. 15 and 16.—Severe necrotizing nephrosis in the dog of Figure 13. In Figure 15 note both the completely necrotic tubules and the tubules containing protein casts. Some intact tubules may be seen at lower right and upper left. Figure 16 is a higher magnification showing both tubular necrosis and tubules with flattened epithelium which are distended with casts. Some of the epithelial cells are irregular and hyperchromatic.
toxic nephrosis in rats treated chronically with relatively low, daily doses of cyclophosphamide (Langenheck Arch. Klin. Chir., 296:517–27, 1961). Arnold and Klose have proposed a new scheme of hydrolysis of cyclophosphamide different from that shown in Chart 1 (Arzneimittel-forsch., 11:159–63, 1961). They suggest that the reactive intermediate formed in vivo is the bis(β-chloroethyl)amine salt of cyclo-O,N-propylene esteramide phosphoric acid. It is, however, unlikely that such a salt remains undissociated in the circulation and that both the anionic and cationic moieties are excreted by the kidney at identical rates. For this reason the salt is probably not the primary agent responsible for the reactivity of urine and for bladder toxicity.

REFERENCES

Cyclophosphamide and Urinary Bladder Toxicity
Frederick S. Philips, Stephen S. Sternberg, Alice P. Cronin, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/21/11/1577

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.