In previous studies we have found that a single exposure of dog kidneys to a relatively high dosage of irradiation resulted in a significant suppression of the erythropoietic and excretory functions of the kidney (9). Alkylating chemotherapeutic agents are frequently described as radiomimetic agents (5), because many of their biological effects are similar to those following ionizing radiation. The alkylating drugs are similar to radiation in that they are mutagenic, carcinogenic, induce chromosome abnormalities, and produce growth inhibition (12). It has been suggested that the mutagenic action of alkylating agents in biological systems is due to their capacity to alkylate with DNA at the 7-nitrogen of guanine moieties (13). To determine whether the alkylating agents have an inhibitory effect, such as that seen after irradiation of the kidney, erythropoietin production by the kidney, dog kidneys, isolated in situ, were perfused bilaterally with blood alone or blood containing alkylating drugs. Plasma erythropoietin titers were measured in the dogs after kidney perfusion and following an injection of cobalt. The response is compared with that of control nonperfused dogs.

MATERIALS AND METHODS

Male and female mongrel dogs weighing between 8 and 15 kg. were used throughout these studies. All surgical procedures were carried out while the dogs were anesthetized with 30 mg pentobarbital/kg. Microhematocrits were determined on blood samples on each dog with heparinized capillary tubes. Blood urea nitrogen was measured by the method of Gentzkow (11). Erythropoietin assay was carried out in the fasted rat according to the procedure of Fried et al. (10). Male rats of the Sprague-Dawley strain were used, five in each erythropoietin assay. Young, healthy rats, weighing 165–195 gm. each, were fasted for 32 hours prior to the first injection of substance being assayed, and maintained in the fasted state throughout the duration of the bio-assay, water being permitted ad libitum. Two ml. of plasma or the test substances were injected daily for 2 days. On the 3d day a tracer dose of 1 μc. Fe59-labeled citrate was given intravenously, and standards were prepared for later counting. Sixteen hours later 1 ml. of blood was obtained by cardiac aspiration, and the Fe59 incorporation into red cells was calculated according to the following formula:

\[
Fe^{59} \text{ uptake} = \frac{\text{net counts per ml. blood} \times 0.05 \text{ body weight}}{\text{net counts injected}}
\]

In these experiments the blood volume was assumed to be 5 per cent of the body weight.

A dosage of 250 μmoles cobaltous chloride/kg was injected subcutaneously as the erythropoietic stimulus in each dog. Approximately 60 ml. blood was removed from the femoral artery for erythropoietin assay prior to and at several time intervals following the cobalt injection. Plasma samples were assayed for cobalt by a modification of the technic of Almond (2). Each sample contained less

The kidneys were removed for microscopic studies after the 24-hour blood sample was withdrawn.

Chart 1 is a schematic diagram of kidney perfusion system No. 1. The renal arteries and veins, abdominal aorta, and vena cava were exposed through an anterior abdominal incision. The ovarian or spermatic vein, posterior lumbar arteries, and veins near the points on the abdominal aorta or vena cava where the renal arteries and veins are given off, were ligated. Chokers were passed around the vena cava approximately 1–2 cm. above and below the point where the renal veins are given off. Chokers were also placed around the right and left adrenolumbar veins. Curved 20-gauge needles with dulled tips were used to cannulate the renal arteries. The needle cannulae were attached to polyethylene tubing which was adapted to 3/4-inch Argyle tubing. The perfusion system was filled with 700- to 800-ml. donor dog blood containing 30 mg. heparin sodium as the anti-coagulant and 60 mg. papavine hydrochloride to keep the kidney blood vessels dilated during the perfusion. A Davol heart-lung perfusion pump with single-ended ventricle and 20 ml. Actuator1 was used to circulate the blood through the perfusion system. An Abbott Pulmo-Pak bubble oxygenator with a Saran anti-foam column was used to oxygenate the blood in the perfusion system. The blood was warmed in a bottom drain bottle reservoir and maintained at 37°C. in a constant-temperature water bath. As shown in Chart 2, the blood was circulated through both renal arteries to the kidney, and the venous effluent from the kidneys was returned through the renal veins to the vena cava, where it was picked up by a polyethylene catheter which had been previously passed up the left iliac vein. The catheter was held in place by means of a choker, and a second choker higher up on the vena cava prevented the passage of blood into the peripheral circulation. The drugs were added to the perfusion system through a side-arm from the arterial side of the perfusion system at a point just after the blood leaves the pump. The blood containing the drug was perfused through the kidneys for 15 minutes, after which time the kidney was perfused with 150 ml. of fresh arterial donor dog blood to remove any residual drug from the blood contained in the kidneys. The arterial cannulae and venous catheter were immediately removed to re-establish venous return from the hind limbs and complete circulation to the kidneys. The dogs were given

1 Obtained from International Medical Instruments Corp., Stoneham, Mass.
Sup. Vena Cava

Right Kidney

Left Kidney

Inf. Vena Cava

Supplied through the courtesy of Burroughs Wellcome & Co., Tuckahoe, N. Y.

Supplied by the Lederle Laboratories, Pearl River, N. Y.

**RESULTS**

The procedure to be followed in perfusing blood containing the alkylating drugs through the kidney according to the technic described as perfusion system No. 1 (Chart 1) involved occlusion of the renal arteries and veins for 1 hour. Therefore, it seemed important to determine the influence of renal occlusion alone on the erythropoietic response to an injection of cobalt. Chart 3 demonstrates the plasma levels of erythropoietin at 12, 18, and 24 hours after an injection of cobalt in intact dogs or dogs exposed to a 1-hour renal occlusion. A significant increase in erythropoietin levels was seen in intact dogs at 12 and 18 hours following the injection of cobalt. However, the erythropoietin titers had returned to normal values after 24 hours. The dogs that had been exposed, 18 hours previously, to a 1-hour renal occlusion showed significant increases in plasma erythropoietin levels at 12, 18, and 24 hours after the cobalt stimulus. These findings indicate that bilateral renal artery and vein occlusion for 1 hour in the dog does not interfere with the erythropoietic response to cobalt. The 18- and 24-hour plasma levels of erythropoietin were actually significantly greater in the dogs exposed to renal occlusion than those of intact dogs.

Chart 4 depicts the plasma levels of erythropoietin at 12, 18, and 24 hours after an injection of cobalt in dogs treated, according to the technic outlined in perfusion system No. 1 (Chart 1), with saline vehicle, chlorambucil, or thioTEPA. As indicated previously the saline vehicle or alkylating drugs remained in the kidneys for 1 hour while the renal arteries and veins were clamped. As indicated by the control curve (Chart 4) there was a significant elevation in plasma erythropoietin titers at 12, 18, and 24 hours following cobalt in dogs exposed to renal artery and vein occlusion alone. The plasma levels of erythropoietin (Chart 4) were significantly less at 12, 18, and 24 hours after cobalt in dogs whose kidneys had been previously occluded.
perfused with either chlorambucil or thioTEPA. Perfusion of the kidneys with thioTEPA resulted in a more marked suppression of the erythropoietic response to cobalt, as indicated by significantly less erythropoietin at 12 and 18 hours after cobalt in the thioTEPA group, than perfusion with chlorambucil. Therefore, it may be concluded that treatment of the dog kidney with alkylating agents significantly suppresses the erythropoietic function of the kidneys.

The renal occlusion experiments described in Charts 3 and 4 resulted in a moderate amount of histological damage to the kidney. Studies of the microscopic sections of kidneys removed from dogs 42 hours after the 1-hour renal occlusion revealed a moderate amount of ischemic degeneration. There was evidence of early degeneration of the epithelium lining the proximal and distal tubules as indicated by vacuolization of the cytoplasm. A slight amount of congestion of the glomerular tuft with red blood cells was also seen. Most kidneys showed the presence of renal casts and hemolysis within the intralobular arterioles. These ischemic changes were more marked when renal occlusion was combined with treatment with chlorambucil. The ischemic changes noted in the kidneys treated with thioTEPA were not as marked as those receiving chlorambucil. The renal tubular and glomerular changes were only slightly greater in the animals treated with thioTEPA than those from animals exposed to renal occlusion alone.

Because of the extensive ischemic degenerative changes noted in the kidneys exposed to renal occlusion alone or in combination with the alkylating drugs, it was decided to use a perfusion system whereby the blood could be oxygenated by circulating it through the kidney with the use of a heart perfusion pump and artificial lung. Chart 5 demonstrates the results obtained from bilateral perfusion of dog kidneys for 15 minutes with the use of perfusion system 2 (Chart 2) with blood alone or blood containing chlorambucil or thioTEPA. Eighteen hours following kidney perfusion, and after the dog had completely re-

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**Chart 3.** Influence of renal artery and vein occlusion in the dog on the erythropoietic response to a cobalt injection. The per cent change in Fe⁵⁹ incorporation in red cells is expressed as the per cent of the mean 0 time Fe⁵⁹ incorporation values, which is indicated as the 100 per cent value. Each point represents the mean value for ten dogs exposed to renal occlusion and six intact control dogs. The 18- and 24-hour points in the renal artery occlusion group were found to be significantly different at the 5 per cent level from those of the intact controls.

**Chart 4.** Influence of chlorambucil and thioTEPA on kidney erythropoietin production in dogs. The per cent change in Fe⁵⁹ incorporation in red cells is expressed as the per cent of the mean 0 time Fe⁵⁹ incorporation values, which is indicated as the 100 per cent value. The group exposed to renal occlusion alone contained ten dogs, the group treated with chlorambucil contained seven dogs, and the group perfused with thioTEPA contained six dogs.

**Chart 5.** Influence of perfusing dog kidneys with blood alone or blood containing chlorambucil or thioTEPA on plasma erythropoietin. Erythropoietin titers are expressed as per cent Fe⁵⁹ incorporation in red cells. The enclosed numbers at the bottom of each bar represents the total number of rats in that group. Each group is the mean value obtained from plasma from five dogs. The line at the top of each bar indicates standard error of the mean, and the asterisk means significantly different at the 5 per cent level from the 0-time sample.
covered from the anesthesia, the dogs were given injections of cobalt and sacrificed 12 hours later. At the end of 12 hours blood was removed for erythropoietin assay to compare with the 0 time sample. The kidneys were removed for histological studies, and blood was withdrawn for microhematocrits and BUN determinations. The set of bars (Chart 5) demonstrates the mean Fe$^{59}$ incorporation values in rats given injections of saline or plasma obtained from intact dogs before and 12 hours after the cobalt stimulus. As indicated from the Fe$^{59}$ values from plasma removed from intact nonperfused dogs, cobalt produces a significant increase (108 per cent) in plasma erythropoietin in dogs 12 hours after the cobalt stimulus. The second set of bars also demonstrates that cobalt produced a significant (118 per cent) increase in erythropoietin titers at 12 hours in dogs whose kidneys have been previously perfused for 15 minutes with blood alone. Perfusion of the dog kidneys with blood containing chlorambucil resulted in significantly less erythropoietin in blood 12 hours after the cobalt stimulus than that of dogs whose kidneys were perfused with blood alone. The 12-hour titers in the chlorambucil group were significantly greater than the 12-hour titers in the cobalt group. The baseline plasma erythropoietin levels in dogs whose kidneys were perfused with the alkylating drugs were essentially normal, and no significant histological change was observed. An occasional degenerative change would develop in the kidneys if the alkylating drugs are often ad-

Table 1 shows the body weight, hematocrit, BUN, and plasma erythropoietin levels in the dogs 10 hours following kidney perfusion (system No. 2) with blood or blood containing chlorambucil or thioTEPA. The baseline plasma erythropoietin levels were significantly higher in the controls than in either the chlorambucil or thioTEPA groups. The BUN values for the plasma in the control and drug perfusions were greater than the average value for intact control dogs (16.9 ± 1.1 mg. per cent) studied in our laboratory. The increase in BUN values in all groups may have been related to a mild suppression of the excretory function of the kidney. The decrease in plasma erythropoietin levels in dogs whose kidneys were perfused with the alkylating drugs may have been associated with their inhibitory effects on kidney erythropoietin production, which is necessary to maintain normal erythropoiesis.

**DISCUSSION**

In the present studies it has been demonstrated that perfusion of the kidneys, isolated in situ, with blood containing chlorambucil or thioTEPA inhibits the rise in plasma erythropoietin levels which is usually seen following an injection of cobalt. In addition, perfusion of the kidneys with these drugs resulted in a significant decrease in baseline plasma erythropoietin levels. Therefore, it seems clear that the alkylating agents antagonize the response of the kidney to a potent erythropoietic stimulus such as cobalt and also inhibit the baseline erythropoietin production which is necessary to maintain normal erythropoiesis. The mechanism by which alkylating agents interfere with erythropoietin production by the kidney may be related to the changes produced by these agents in such vital macromolecules of the cell as DNA or RNA. The effects of the alkylating drugs are relatively nonselective, and all functions of the renal cells that are directly related to DNA, RNA, or protein metabolism are probably vulnerable. It is difficult to understand how these agents can suppress the erythropoietic function of the kidney within such a short period of time if their effect on DNA is the only factor responsible for this inhibitory action. The organic mercurial diuretics, which affect predominantly the renal tubules, have also been found to interfere with erythropoietin production (8), but whether the target molecules acted upon by these two substances are the same is not known.

The findings in the present study may be of toxicological significance because the alkylating agents are often ad-

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**TABLE 1**

**INFLUENCE OF PERFUSION OF DOG KIDNEYS WITH BLOOD ALONE OR BLOOD CONTAINING CHLORAMBUCIL ON THIOTEPA ON HEMATOCRIT AND BUN**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (kg.)</th>
<th>Hematocrit (per cent)</th>
<th>BUN (mg.%)</th>
<th>Plasma erythropoietin (% Fe$^{59}$ incorp.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12.6 ± 1.06‡</td>
<td>42.0 ± 3.4</td>
<td>61.4 ± 1.7</td>
<td>10.3 ± 1.8</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>13.6 ± .70</td>
<td>45.3 ± .88</td>
<td>56.0 ± 10.9</td>
<td>6.0 ± .7§</td>
</tr>
<tr>
<td>thioTEPA</td>
<td>13.5 ± 1.4</td>
<td>42.8 ± 1.7</td>
<td>31.0 ± 5.8§</td>
<td>5.0 ± .29§</td>
</tr>
</tbody>
</table>

* Blood sample removed for determinations 18 hours after kidney perfusion.
† Five dogs in each group.
‡ Standard error of the mean.
§ Means significantly different from the controls at the 5% level.
administered alone or in combination in the treatment of neoplastic diseases (14). The hematopoietic depression seen after the administration of these agents (3, 15) may be related to a suppression of erythropoietin production in the kidney by these compounds, as well as a direct cytotoxic effect on the bone marrow. It is interesting to note that Ehrlich's initial observation on papillary necrosis with alkylating agents (6) was studied for many years, note that Ehrlich's initial observation on papillary necrosis with alkylating agents (6) was studied for many years, but it was considerably later before it was observed that bone marrow depression (3) accompanied the renal toxicity. We know now that the clinically useful alkylating agents do not produce an observable histological change in the kidney in the usual clinical dosages (15). The slight fall in erythrocyte and hemoglobin values which usually occurs within a few days after systemic treatment with moderate dosages of alkylating agents (7) is probably a direct effect of these substances on bone marrow. However, a corresponding reduction in kidney erythropoietin production may be responsible for the maintenance of the anemia.

The finding that thioTEPA was more effective than chlorambucil in suppressing erythropoietin production by the kidney may be related to the permeability of the renal cells to these agents or to their relative affinities to a reactive center on a receptor site necessary for erythropoietin production. It seems possible that thioTEPA may gain entrance to the renal cells which produce erythropoietin with greater ease than chlorambucil, because of the differences in their ionizations (1), and in addition may have a greater affinity for guanine nucleosides (13).

The finding that plasma erythropoietin titers in dogs given injections of cobalt and exposed to renal occlusion eventually achieve a higher level and remain elevated for a longer period of time than those of intact dogs is evidence that cobalt is a more potent erythropoietic stimulus when given on a background of renal hypoxia. Erythropoietin titers in plasma from dogs exposed to kidney perfusion with blood alone were also higher than those of intact dogs and could have also been the result of slight renal hypoxia during cannulation of the renal arteries. Although the plasma erythropoietin levels in the renal ischemic dogs were not significantly different from those of intact dogs prior to the cobalt injection, the greater elevation in plasma erythropoietin following renal occlusion may represent erythropoietin produced by cobalt and renal hypoxia combined. It may be possible to produce an elevation in plasma erythropoietin with renal hypoxia alone with the use of prolonged partial renal occlusion and a more sensitive assay method. The higher erythropoietin titers in dogs exposed to renal occlusion may also be related to a change in the excretory function of the kidney. The ischemic damage to the kidney may have resulted in a decrease in the rate of renal excretion of both cobalt and erythropoietin. However, none of the plasma samples analyzed at the various time intervals following the cobalt injection contained a sufficient amount of cobalt to stimulate radioactive iron incorporation in red cells of the assay animal. Therefore, the enhanced erythropoietic activity of the plasma from the renal occlusion dogs was not due to the presence of cobalt.

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