STUDIES ON THE MECHANISM OF ACTION OF THE NITROGEN AND SULFUR MUSTARDS IN VIVO *

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The biologic actions of the sulfur and nitrogen mustards have been reviewed recently,[1] and detailed descriptions of the toxicologic and pathologic effects of these compounds have been submitted for publication.[2,3] At LD_{50} doses, via any route of administration, these compounds produce a characteristic toxicologic *systemic* effect consisting of anorexia, weight loss, diarrhea, and leukopenia, terminating in death 3 to 5 days after injection. Clinical and pathologic examinations show that lymphopenia appears within 6 to 12 hours after injection and is associated with lymphocytic destruction and involution of the lymphatic tissue, thymus, and spleen. This is followed by progressive decrease in granulocytes to the low level of 200 to 300 cells per cmm. within 3 days after injection, associated with aplasia of the bone marrow; and diarrhea beginning 2 days after injection, associated with demonstrable desquamative degenerative changes in the intestinal mucosa.[3]

This report consists of a series of experiments designed to elucidate the mechanism whereby these effects are produced.

THE "ALARM REACTION" AND ITS RELATION TO THE LYMPHOCYTOTOXIC ACTION OF NITROGEN MUSTARD

The systemic intoxication produced by the mustard compounds in rats resembles in many respects a pattern of organic changes that has been termed by Selye [4,5] the "alarm reaction."† This pattern is alleged to follow the subjection of normal rats to a wide variety of unrelated, sublethal injurious procedures, *e.g.*, cold, traumatic injury, excessive muscular exercise, spinal shock, acute infection, and injections of formaldehyde, morphine, atropine, and adrenalin.[5] According to Selye's description, this reaction consists of *"a rapid decrease* in the *size of the thymus, spleen, lymph glands* and liver; disappearance of fat tissue; edema formation, especially in the thymus and loose retroperi-

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† "An alarm reaction develops after the administration of any drug administered in sublethal doses, unless the specific pharmacological actions of the drug exert such a violent selective action on vital centers (heart, respiratory center, etc.) that death ensues as a result of this selective action before any marked general damage occurs." Selye.[5]

toneal connective tissue; accumulation of pleural and peritoneal transudate; *loss of muscular tone;* fall of body temperature; *formation of acute erosions in the digestive tract,* particularly in the stomach, *small intestine* and appendix; loss of cortical lipoids and chromaffin substance from the adrenals; and sometimes hyperemia of the skin, exophthalmos, increased lachrymation and salivation. In very severe cases, focal necrosis of the liver and dense clouding of the crystalline lens may be observed." (The italics are ours and indicate features which are common in animals intoxicated with the mustard compounds.) Marked leukocytosis with lymphopenia usually occurs within 48 hours with the "alarm reaction," but leukopenia develops if the treatment is severe.[6]

Microscopically, the lymphoid tissue in the "alarm reaction" presents a picture of massive lymphocytic necrosis and fragmentation with contraction of the organ and increase in reticular elements and frequent hemorrhages.[7] The adrenal cortex becomes hyperplastic with a loss of lipoid granules, while the medulla loses its chromaffin granules and vacuoles appear in the periphery of its cells; if the "alarm reaction" is severe the cells undergo necrosis. Other changes occur in the pancreas, the gastric and intestinal mucosa, and the liver.

Selye postulated that the "alarm reaction" is due to a common substance released in the body by all varieties of noxious stimuli. In normal rats this substance induces adrenal hypertrophy and hypersecretion, which in turn causes involution of the lymphatic tissue, for in the absence of the adrenal glands the "alarm reaction" does not induce lymphatic atrophy.[5] Selye stated that "So far, the only substances with which we have been able to cause thymus involution in the adrenalectomized rat are cortical extract and estrone."

It is to be assumed under Selye's hypothesis that the mustard compounds, because of their severe and prolonged intoxicating effect, must invoke some degree of the "alarm reaction." It is necessary to demonstrate, however, that the specific pathologic effects attributable to the mustard compounds are not indirectly due to the "alarm reaction." In the case of the lymphatic tissue, this was shown in adrenalectomized rats as follows.

THE EFFECT OF METHYL-BIS (β-CHLOROETHYL) AMINE HYDROCHLORIDE (HN_2·HCl)* IN ADRENALECTOMIZED RATS

Before a definitive experiment could be performed, the toxicity of HN_2·HCl in adrenalectomized rats was determined. Such rats, ade-

* HN_2 is the official designation of this nitrogen mustard compound; HN_2·HCl is the hydrochloride salt.

quately maintained on salt, are unduly sensitive to HN_2 intoxication both in regard to dosage and to the acceleration of intoxication; 10 mg. per kg. of the HCl salt given subcutaneously are fatal in 3 to 6 hours after injection, whereas most normal rats survive for 72 hours; 3 mg. per kg. cause death in 48 to 60 hours, instead of 78 to 100 hours for the controls; and 1 mg. per kg. ($\frac{1}{2}$ LD_{50}) is fatal in 3 to 4 days whereas controls survive such a dose without difficulty. A dose of 3 mg. per kg. was used in the experiment reported.

Methods

Fifty-six adult female rats, weighing from 150 to 210 gm., were offered 1 per cent saline solution in tap water beginning 24 hours before operation and during the course of the experiment. Four groups were employed.

Group I. (*8 Sham-operated Control Rats.*) The rats were placed on limited rations so that they suffered a slight progressive weight loss. It is difficult to prepare a control group whose food intake and weight loss will parallel that seen after HN_2 since changes in fluid balance and gastric distention with food complicate the picture in the intoxicated rat. It was felt, however, that animals on decreased food intake for a short period would furnish a more adequate control than normal well fed rats.

Group II. (*10 Adrenalectomized Rats.*) These were fed *ad libitum.*

Group III. (*18 Sham-operated Rats Receiving 3 mg. per kg. of HN_2·HCl Subcutaneously 24 Hours after Operation.*) These animals were fed *ad libitum.*

Group IV. (*20 Adrenalectomized Rats Receiving 3 mg. per kg. of HN_2·HCl Subcutaneously 24 Hours after Operation.*) These animals were fed *ad libitum.*

Operations were performed under ether anesthesia. Twenty-four hours after operation 3 mg. per kg. of HN_2·HCl in 0.8 cc. of saline solution were given to each animal in the appropriate groups. This represents an LD_{99} dose in normal animals, the LD_{50} being 1.9 mg. per kg. The rats were weighed daily. Sacrifices were performed by exsanguination from the abdominal aorta under ether anesthesia. Blood counts and smears were taken from the freely flowing aortic blood at autopsy. Organs were removed, blotted to free them of blood, and weighed to the nearest milligram on a Roller-Smith torsion balance. The lungs were weighed in tared beakers and then dried for 18 hours at 88 to 90° C. The data on the weight of the organs are insufficient to justify corrections for body weight or statistical analysis; only the averages and the extreme range of weights for each organ are given.

TABLE I

Effects of 3 mg. per kg. of HN2·HCl Methyl-bis (β-chloroethyl) Amine Hydrochloride on the Normal and Adrenalectomized Rat; Body and Organ Weights at Time of Sacrifice

	Controls		Injected with 3 mg. per kg. of HN2·HCl				
			Group III Sham-operated			Group IV Adrenalectomized	
			Time of sacrifice after injection, in hours				
	Group I Sham-operated, diet restricted	Group II Adrenalectomized	24	48	72	24	48
No. of rats	8	10	5	4	8	4	5
Pre-treatment wgt. (gm.)	193 (182–200)	182 (170–195)	184 (170–200)	184 (172–200)	195 (185–208)	184 (165–200)	190 (175–200)
Aver. wgt. at sacrifice (gm.)	178 (169–192)	181 (168–208)	172 (164–184)	163 (150–178)	162 (150–185)	175 (159–197)	171 (142–192)
Aver. wgt. of liver (gm.)	5.90 (5.2–6.3)	6.45 (5.4–8.4)	7.35 (7.1–7.5)	6.30 (5.2–7.5)	5.70 (5.2–6.4)	5.50 (5.0–5.8)	6.25 (4.9–7.9)
Aver. wgt. of spleen (mg.)	865 (660–1,260)	1,140 (719–1,948)	541 (375–804)	446 (337–536)	233 (168–285)	590 (431–664)	452 (357–566)
Aver. wgt. of adrenals (mg.)	50 (42–55)		61 (48–79)	58 (51–72)	79 (62–94)		
Aver. wgt. of thymus (mg.)	155 (110–206)	280 (172–392)	102 (73–139)	79 (61–100)	39 (33–61)	94 (51–113)	94 (65–126)
Aver. wgt. of cervical lymph node (mg.)	73 (45–100)	79 (54–117)	55 (53–56)	48 (34–70)	23 (15–30)	71 (54–90)	59 (31–81)
Aver. wgt. of heart (mg.)	584 (517–660)	575 (512–648)	584 (520–726)	525 (451–587)	585 (577–634)	543 (483–626)	463 (464–634)
Aver. wet wgt. of lungs (gm.)	1.44 (1.25–1.71)	1.45 (1.23–1.79)	1.53 (1.07–2.39)	1.09 (0.91–1.22)	1.17 (1.01–1.34)	1.45 (1.40–1.48)	1.40 (1.32–1.51)
Aver. dry wgt. of lungs (mg.)	306 (276–351)	294 (259–346)	343 (252–504)	284 (223–331)	314 (276–341)	302 (295–308)	305 (262–362)
Aver. per cent of water in lungs	78.7 (77.9–79.3)	79.7 (79.0–80.7)	77.2 (75.2–78.9)	73.8 (70.4–76.3)	73.3 (72.7–73.9)	79.1 (77.5–80.0)	78.3 (76.1–79.4)
Aver. total white cell count	12,260	13,000	3,240	450	300	1,600	1,070

The results are given in Table I and the several observations of interest are discussed under the appropriate group.

Results

Group I. The sham-operated control group on a restricted diet lost about 8 per cent of their body weight, representing a moderate degree of inanition. Necropsy findings were not remarkable. Organ weights are tabulated in Table I.

Group II. Two of the 10 adrenalectomized rats were sacrificed at 1 day, 2 at 2 days, and 6 at 8 days after operation; the organ weights of these animals, which were not appreciably different, are averaged together in Table I. The rats seemed well during the experiment. On 1 per cent saline drinking water, adrenalectomized animals gained 10 to 15 gm. in weight immediately after operation because of retention of fluid, which they then lost gradually. At sacrifice the thymuses and spleens were larger than those of Group II (controls, diet restricted). The lungs showed a slight increase in water. The other organs were not significantly altered, and no adrenal tissue was found. Leukocyte counts were normal, and the blood smears, while suggesting an increase in lymphocytes, were too few for valid interpretation.

Group III. Three mg. per kg. of $HN_2 \cdot HCl$ given subcutaneously produced severe effects in normal rats. At 24 hours the rats had lost a little weight and seemed moderately depressed, at 48 hours slight diarrhea appeared, and between 48 and 72 hours marked symptomatic changes developed. The rats appeared cold, were hunched-up, and huddled together. An almost continuous watery diarrhea, which contained considerable mucus, completely soaked the anal region and lower abdomen, and weight loss was marked. Neurologic changes were not noted, but the rats became more depressed and inactive. Those not sacrificed died between 80 and 102 hours.

Five rats were sacrificed at 24 hours, 4 at 48 hours, and 8 at 72 hours. The organ weights were averaged separately for each of these groups. At 24 hours the stomach contained a moderate amount of food, and in the small intestine small amounts of air and mucus were found. One animal had a small hemorrhagic patch in the ileum, and the mesenteric nodes were reddened. The thymus, spleen, and lymph nodes were decreased in size, and the adrenals appeared to be larger than is normal. The lungs were of normal weight but the fluid content appeared to be slightly reduced. The fall in white blood cell count was already marked and, although the lymphocytes were most severely depressed, the granulocytes had begun to fall also. At 48 hours the stomach was moderately filled with food, and the fluid in the small intestine appeared increased. The lymph nodes were hemorrhagic and those in the ileoce-

cal region were markedly so. The spleen and thymus were about half of the control weights and the lymph nodes were reduced in size. The suggestive increase in adrenal weight was still present. A remarkable finding was the sharp fall in the wet weight of the lungs, with retention of normal dry weight, indicating approximately 30 per cent decrease in the water content of the lungs. The average total leukocyte count had fallen to 450. At 72 hours, the stomach was distended with considerable food and fluid, and the intestines and cecum contained excess amounts of fluid, often including mucus. The colon was usually empty, but contained almost pure mucus in one animal. Small petechial hemorrhages often were found in the mesentery and hyperemic areas were present in the duodenum and lower ileum, the latter in one rat containing several deep erosions of the mucosa. Peyer's patches were hemorrhagic and the regional lymph nodes were small and red. The spleen, thymus, and lymph nodes were reduced to about 25 to 35 per cent of their control size. The increase in adrenal weight was unquestionable, and the livers were decreased in weight. The water content of the lungs remained decreased, being about 25 per cent of that of control group II.

Group IV. Rats receiving 3 mg. per kg. of $HN_2 \cdot HCl$ 24 hours after adrenalectomy showed a rapid acceleration in the development of toxic symptoms. Two animals died within 7 hours. At 24 hours the remaining rats appeared very weak, depressed, and had mild watery diarrhea; at 48 hours they were either dead or moribund; those remaining were huddled together, cold, moved with difficulty, and all died within 60 hours after injection. Diarrhea was moderate and weight loss was not marked. Four rats were sacrificed at 24, and 5 rats at 48 hours. At 24 hours the stomach was filled largely with fluid, the duodenum was slightly congested, and the small intestine greatly distended with clear fluid. The entire animal seemed excessively moist, and there was free peritoneal and thoracic fluid. The spleen, thymus, and lymph nodes were reduced in size, as markedly as those seen in the control animals treated with $HN_2 \cdot HCl$ (group III). The lymph nodes seemed slightly hemorrhagic. The water content of the lungs was slightly greater than is normal. The average total leukocyte count was 1,600. At 48 hours the rats were moribund when sacrificed. Their stomachs and small intestines were greatly distended with fluid. The walls of the duodenum were reddened and frequently hemorrhagic. The mesentery had a few small petechial hemorrhages and the fat had a peculiar white, granular appearance. The decrease in weight of the thymus and spleen was not much greater than that at 24 hours, and the weights of these organs did not differ significantly from those in group III. The water content of the lungs was only slightly decreased.

The average leukocyte count was 1,070 with 80 per cent lymphocytes; this total count was suggestively higher than that seen in group III.

Microscopic Observations

In the adrenalectomized rats injected with 3 mg. per kg. of HN_2·HCl, some intact lymphocytes could be found in the thymus, and generally the lymphatic tissue, although containing a diminished number of lymphocytes, appeared to be less severely affected than that of normal rats given the same dose. In another experiment, adrenalectomized rats, receiving 10 mg. per kg. of HN_2·HCl and sacrificed 3 to 6 hours after injection when they appeared moribund, showed severe lymphocytic fragmentation and chromatin débris in the thymus and lymph nodes, but again these effects seemed somewhat less than in the controls treated with the same dose. It should be noted that although 1 mg. per kg. of HN_2·HCl given subcutaneously, a sublethal dose in normal rats, is fatal to adrenalectomized rats, the histologic evidence of injury to the lymphoid tissue, bone marrow, and gastrointestinal tract is no more marked in the adrenalectomized rats than in the controls.

These experiments demonstrate that methyl-*bis* (β-chloroethyl) amine hydrochloride can induce lymphocytic destruction and involution of the thymus, spleen, and lymph nodes in the absence of the adrenal gland. Quantitatively, however, the lymphocytotoxic effect seems to be somewhat less severe in adrenalectomized rats than in normal ones injected with the same dose. The involution of the lymphatic tissue, therefore, results from a direct action of the nitrogen mustard, or is mediated by a different mechanism than that occurring in the "alarm reaction." Since, by definition,[5] the "alarm reaction" is involved in nitrogen mustard intoxication, it is possible that this mechanism may contribute to a slight extent to the lymphatic involution seen in rats intoxicated with HN_2·HCl. LeBlond and Segal [8] have been able to induce involution of the thymus in adrenalectomized rats by x-rays, although these effects were quantitatively less severe than those seen in normal rats. The nitrogen mustards seem to produce a somewhat similar effect.

DEMONSTRATION OF THE DIRECT ACTION OF HN_2·HCL INJECTED INTRAVENOUSLY, ON THE INTESTINAL TRACT *

It is shown in the following experiment that the intestinal injury induced by HN_2·HCl is due to a *direct* and rapidly completed action

* It had previously been shown in this laboratory that exclusion of the biliary secretion in rats did not prevent HN_2 from inducing characteristic intestinal injury. Since one could have anticipated this result from the experiment cited above, the biliary exclusion experiment is not described in detail.

of this compound on the intestinal tract. This demonstration was accomplished by temporarily occluding the circulation to the intestinal tract during varying, short periods of time after the intravenous injection of the compound, and then observing the nature of the injury produced.

Rats were anesthetized with ether, the abdomen opened, and most of the small intestine exteriorized and placed on cotton soaked with saline solution. Rubber-sheathed hemostats were placed about 5 cm. distal to the pylorus and 1 cm. proximal to the ileocecal valve, and a third hemostat was placed across the root of the mesentery. By this ring of hemostats, circulation to the major portion of the small intestine was occluded for 15 minutes. Control rats subjected to this procedure suffered no ill effects, and gave no evidence of significant histologic damage to the intestinal mucosa.

Following the application of the clamps, 2 mg. per kg. of $HN_2 \cdot HCl$ (1.6 LD_{50}) was injected into the vena cava, and 15 minutes later the clamps were removed. The rats were sacrificed at 60 and 72 hours. Grossly and microscopically the small portion of the duodenum and ileum outside the occluding clamps showed the characteristic injury due to the nitrogen mustards,[8] whereas the remainder of the small intestine, which had been surrounded by the clamps, was normal. The toxicity of $HN_2 \cdot HCl$, however, was not appreciably altered by this procedure.

This experiment clearly demonstrated that $HN_2 \cdot HCl$ had a rapidly completed, and presumably direct, action on the mucosa of the small intestine.

DEMONSTRATION OF THE DIRECT ACTION OF $HN_2 \cdot HCL$ INJECTED INTRAVENOUSLY, ON THE BONE MARROW OF THE RAT AND RABBIT

It is shown in the following experiments that $HN_2 \cdot HCl$ has a direct and rapidly completed action on the bone marrow. This demonstration was accomplished by temporarily occluding the circulation to the hind legs during and for varying short periods after the intravenous injection of $HN_2 \cdot HCl$.

Rats were anesthetized with ether, the abdomen opened, and an arterial clamp placed on the abdominal aorta and inferior vena cava just below the renal vessels. Two mg. per kg. of $HN_2 \cdot HCl$ (1.6 LD_{50}) were then injected into the vena cava just above the clamp, and the clamp removed after intervals varying from 5 to 15 minutes. Operated controls carried the clamp for 15 minutes without ill effects and with no changes in the bone marrow. The rats were sacrificed 72 and 96 hours after injection.

At the time of sacrifice, the control rats injected with $HN_2 \cdot HCl$ had

extreme leukopenia, the leukocyte count being in the range of 50 to 250 per cmm. with cells usually too few to make a differential count significant. Microscopic sections showed the femoral, vertebral, sternal, and humeral marrow to be aplastic. The rats subjected to temporary vascular occlusion had leukocyte counts averaging 1700 per cmm. at 72 hours and 4,000 per cmm. at 96 hours after injection, with the differential count consisting predominantly of granulocytes. Histologic study (Figs. 1 and 2) showed aplastic sternal and humeral marrow, whereas the femoral marrow was extremely cellular and especially rich in myeloid elements. The marrow in the lumbar vertebrae below the level of the occluded aorta was very cellular, whereas that from the upper thoracic vertebrae was aplastic.

Rabbits were anesthetized with ether and, after suitable preparation of the skin, the aorta and vena cava were exposed through a left lateral abdominal incision. A rubber-sheathed hemostat was clamped on the two vessels during and for times varying for 2 to 15 minutes after the injection of 2 to 3 mg. per kg. of HN_2·HCl (around the LD_{50}) into an ear vein.* Several complications may result from this procedure. If the clamp is left on too long (15 minutes or longer, usually), a gradual paralysis of the hind legs may occur, presumably due to injury in the spinal cord, since the legs do not become edematous, cold, or gangrenous. A dose of 3 mg. per kg. of HN_2·HCl in the clamped animal frequently produces various neurologic manifestations such as tremors, ataxia, and convulsions, which in some instances seemed to lead to death within a period seen only when two to three times this dose is given to the normal rabbit. This would suggest, incidentally, that the lower part of the body accounts for a considerable amount of the chemical when it is injected into the intact animal. Because of these effects, a dose of 2 mg. per kg. with a clamping period of 2 to 5 minutes is recommended for a relatively uncomplicated preparation.

This experiment was repeated many times with uniform results in protecting the bone marrow of the lower part of the body.† The results obtained on 5 representative rabbits are detailed below, and the blood counts are shown in Text-Figure 1.

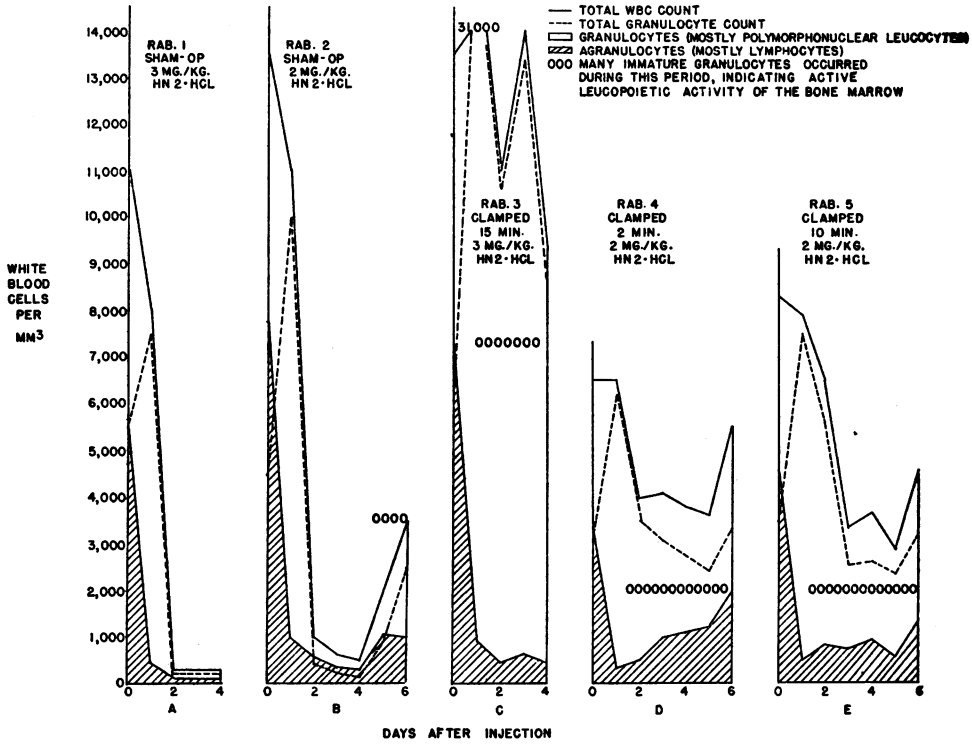
Rabbit 1. (*A Sham-operated Control.*) Rabbit 1 was given 3 mg. per kg. of HN_2·HCl intravenously. Diarrhea appeared on the second day, weakness was progressive, and death occurred 92 hours after injection. The daily leukocyte and differential counts (Text-Fig. 1-A)

* It is possible to produce occlusion of the abdominal aorta and vena cava by inflating a blood pressure cuff around the rabbit's abdomen. The use of this simplified technic has not been thoroughly investigated as yet.

† Preliminary experiments have shown that the reticulocyte count in the clamped rabbit injected with HN_2·HCl is severely depressed, although active regeneration of granulocytes is in progress. In view of the lymphocytotoxic action of the nitrogen mustards, this observation merits more detailed investigation.

were typical for the dose received. Typical pathologic findings were noted at autopsy, and there was no evidence of inflammation at the operative site.

Rabbit 2. (*Clamp Applied on Vena Cava for 5 Minutes Only, 2 mg. per kg. Injected.*) Rabbit 2 showed weight loss, diarrhea on the third day, and beginning recovery in weight 7 days after injection. The



Text-Figure 1. Effects of the direct action of nitrogen mustard on the bone marrow of the rabbit. A and B illustrate leukotoxic action of nitrogen mustard at two levels of dosage. In C, D, and E the protective effect of clamping the aorta and vena cava for varying periods during which similar dosages of nitrogen mustard were injected is illustrated.

leukocyte and differential counts are shown in Text-Figure 1-B and are in accord with our previous observations for this dosage. Nine days after injection the rabbit appeared almost completely recovered.

Rabbit 3. (*Clamp Applied on Aorta and Vena Cava for 15 Minutes, 3 mg. per kg. Injected.*) The day following injection, rabbit 3 showed generalized neurologic symptoms. The animal was weak and incoordinate in its movements, and these symptoms were present until death. Three days after injection a very severe diarrhea appeared, and the rabbit became progressively weaker, dying 104 hours after injection. The leukocyte counts were unprecedented, as shown in

Text-Figure 1-C. The lymphocyte count fell precipitously and recovery did not occur. Granulocytes were greatly increased in number, and within 24 hours there was an increase in banded cells, polymorphonuclear leukocytes containing basophilic granulations, macropolycytes, and occasional myelocytes, this picture persisting until death. Ninety-six hours after injection the leukocyte count was 9,000 and the smear showed occasional myelocytes and normoblasts, and the differential count showed 11 per cent banded and 83 per cent segmented polynuclear leukocytes, 3 per cent basophils, and 3 per cent lymphocytes. At autopsy no gross inflammation was found; the incision appeared to be healing, and the intestines, as was expected, were distended with fluid. Smears of the bone marrow showed an aplastic humeral marrow and a hyperplastic femoral marrow.

Rabbits 4 and 5. (Clamp Applied for 2 and 10 Minutes, Respectively, and 2 mg. per kg. Injected.) Rabbits 4 and 5 developed the usual picture of HN_2 intoxication, except for the fall in granulocytes. Banded polynuclear leukocytes and pseudo-eosinophilic granulocytes with basophilic granulations were present 24 hours after injection and persisted during the period of observation. One animal survived, and the other died with purulent peritonitis 12 days after injection. The total leukocyte and differential counts are shown in Text-Figure 1, D and E.

Comment

These studies demonstrate that the action of HN_2·HCl on the cells of the bone marrow is a rapidly completed one, probably accomplished within less than 2 minutes after injection. This strongly suggests, therefore, that this compound has a direct action on the hematopoietic cells. It further shows that systemic intoxication does not interfere with the process of active granulopoiesis. Also, the data make clear that leukopenia, *per se,* is not essential in the lethal effects of the tested compound.

The availability of a technic whereby the major portion of the hematopoietic tissue of the body may be temporarily destroyed, while preserving a small area of actively regenerating bone marrow, should be of considerable use in other hematologic investigations.

DEMONSTRATION OF THE DIRECT ACTION OF TRIS (β-CHLOROETHYL) AMINE HYDROCHLORIDE (HN_3·HCL),* INJECTED INTRAVENOUSLY, ON THE INTESTINAL TRACT

Clamping experiments on the intestinal tract, similar to those described above with HN_2·HCl, were carried out with HN_3·HCl in rats and rabbits. HN_3 produced effects similar to those reported for HN_2,

* HN_3·HCl is the official designation of the hydrochloride salt of this compound.

indicating that the former also has a rapidly completed action on the intestinal tract. It is probable that this will be true also in the case of the bone marrow.

DEMONSTRATION OF THE DIRECT ACTION OF BIS (β-CHLOROETHYL) SULFIDE (H),* INJECTED INTRAVENOUSLY, ON THE INTESTINAL TRACT AND BONE MARROW

H is poorly soluble and unstable in water. Water, therefore, was not a suitable vehicle for intravenous injection, and solvents, such as propylene glycol and thiodiglycol, were used. This factor seems to have complicated a simple interpretation of the results obtained by the clamping technic.

Methods and Results

Rats. It was first shown that by occluding the circulation to the small intestine during and for 5 minutes after the intravenous (inferior vena cava) injection of 2.5 mg. per kg. of neat H, the clamped portion of the gut was protected from injury. Similarly, clamping of the abdominal aorta for 5 minutes during and for 5 minutes after the intravenous injection of 1 mg. per kg. of H in propylene glycol protected the bone marrow distal to the clamp, whereas the bone marrow proximal to the clamp was destroyed. These results were in accord with those obtained with the nitrogen mustards.

Needham, Cohen, and Barrett [9] reported, however, that they had been unable to obtain protection of the distal bone marrow in the rat against a dose of 2.0 mg. per kg. of H in thiodiglycol, injected intravenously, by applying a clamp to the abdominal aorta for 60 minutes. In order to resolve this discrepancy, 8 groups of 6 to 12 rats each were treated as shown in Table II. The results are summarized briefly therein.

This experiment confirmed both our previous experiment and the results obtained by Needham and co-workers.[9] The protective effect of vascular occlusion was found only when a dose of 1 mg. per kg. of mustard in propylene glycol was given. With a higher dose of mustard, or with thiodiglycol as a solvent, the protective effect of temporary vascular occlusion was not demonstrated. The explanation of the failure to obtain consistent protection of the distal bone marrow in these experiments is not apparent.

Rabbits. Seven rabbits were subjected to the clamping procedure, and then injected intravenously with 4.0 mg. per kg. of H in propylene glycol (1.5 LD_{50}). In 2 rabbits the mesenteric arterial supply to a 15 cm. segment of the ileum was occluded during and for 15 minutes

* H is the official designation of this compound.

after the injection, and one rabbit was sacrificed at 72 hours and the other at 96 hours. These animals both showed extremely severe injury to the spleen, thymus, and bone marrow with terminal leukocyte counts of 900 and 350 per cmm., respectively. The portion of the intestinal tract subjected to the vascular occlusion was practically completely protected, whereas the remaining portion of the small intestine showed severe damage.

TABLE II

Protection Afforded the Femoral Bone Marrow of the Rat, by the Temporary Occlusion of Its Circulation, Against the Effects of the Intravenous Injection of H in Doses of 1 and 2 mg. per kg. in Propylene Glycol and Thiodiglycol

Group no.	No. of rats in group	Solvent used for injection	Intravenous dose of H	Duration of clamping	Effects on the bone marrow
			mg./kg.	*minutes*	
A	6	None	None	5	None
B	6	Propylene glycol	None	5	None
C	12	Propylene glycol	1	5	Severe leukopenia, but the femoral marrow was less severely injured than the sternal
D	6	Propylene glycol	2	5	Femoral and sternal marrow seem to be equally severely affected
E	6	None	None	20	None
F	6	Thiodiglycol	None	20	None
G	6	Thiodiglycol	1	20	Femoral and sternal marrow seem to be equally severely affected
H	6	Thiodiglycol	2	20	Femoral and sternal marrow seem to be equally severely affected

Five rabbits had clamps applied to both the mesenteric artery and abdominal aorta during and for 15 minutes after the injection of H. One animal died soon after injection, and an autopsy was not performed in another dying 75 hours after treatment. Three of the 4 rabbits did not develop leukopenia, and at 72 and 96 hours, at the time of sacrifice, their leukocyte counts were 2250, 2850 and 4100 per cmm.; the remaining animal had a count of 600 per cmm. Histologic examination was performed on 3 rabbits. Again the temporarily clamped gut was protected, and the spleen and thymus in these animals were severely damaged. The rabbits all had extremely severe injury to the sternal, humeral, and upper vertebral bone marrow, whereas 2 rabbits showed complete and one rabbit (the one with a white count of 600 per cmm.) showed partial protection of the femoral bone marrow.

These experiments show, in general, what is much more clearly shown in the case of the nitrogen mustards, namely, that occluding the circulation to a given tissue before and for 15 minutes after the intravenous injection of mustard will serve to protect it from the action of the agent.

DEMONSTRATION THAT THE BACTERIOSTATIC EFFECT OF RABBIT SERUM IS ALTERED BY THE INTRAVENOUS INJECTION OF HN_2 *

The demonstration that $HN_2 \cdot HCl$ has a rapidly completed action and disappears from the blood shortly after injection makes it necessary to find a secondary mechanism which is more directly responsible for the development of systemic intoxication. A study of alterations in the circulating blood of intoxicated animals was undertaken, and the results have been reported elsewhere in this series.[10] Among various approaches, the measurement of bacterial growth in serum as a possible method of demonstrating the appearance of toxic substances in the serum was considered. A simple experiment was performed, and this is briefly reported because of the unexpected results obtained.

Methods and Results

The growth of type A hemolytic streptococcus (C203) was determined in normal rabbit serum and in serum taken at various intervals after the intravenous injection of 3 mg. per kg. of $HN_2 \cdot HCl$. Blood was obtained by cardiac puncture, centrifuged, and the serum removed and refrigerated. One-tenth cc. of a 10^{-2} dilution of an 18-hour culture of washed streptococci was implanted in 0.9 cc. of each sample of serum, and colony counts were made at 0, 4, 8, and 24 hours after incubation.

The control serum of 4 rabbits, whether starved or fed, was bacteriocidal, bacteriostatic, or permitted slight growth of bacteria over the period of 8 hours' incubation. In these same rabbits, 6 samples of serum drawn within 6 hours after injection of $HN_2 \cdot HCl$ showed very little difference from the controls. Following this 6-hour period, and until the rabbits died, 3 to 5 days later, the serum became much more favorable for the growth of bacteria, and the rate of bacterial growth was strikingly accelerated.

Although the variability of bacterial growth in normal rabbit serum prevents any quantitative statement, enhanced bacterial growth of hemolytic streptococci occurred consistently in the serum of the intoxicated animals beginning 6 to 8 hours after the injection of an LD_{50}

* We wish to thank Dr. Colin MacLeod and Mrs. Edna Stone of the Department of Bacteriology, New York University College of Medicine, for assistance in carrying out this study.

dose of HN_2·HCl. This observation suggests that some alteration occurs in the blood beginning at 6 to 8 hours after injection which facilitates the growth of the test bacteria. It was not possible to pursue this observation further.

Summary and Conclusions

1. Methyl-*bis* (β-chloroethyl) amine hydrochloride (HN_2·HCl) was used as the type substance in this study. *Tris* (β-chloroethyl) amine hydrochloride (HN_3·HCl) and *bis* (β-chloroethyl) sulfide (H) were examined in more limited trials and, in the particulars tested, gave essentially the same results as did HN_2·HCl.

2. The "alarm reaction" of Selye must be considered in interpreting the toxic effects of any drug. It is shown that only a minor and questionable rôle can be ascribed to this reaction in the production of the characteristic pattern of injury to the lymphatic and hematopoietic tissues and intestinal mucosa resulting from HN_2·HCl intoxication. Following the injection of HN_2·HCl in the rat, involution of the lymphatic tissue and lymphocytic destruction occur in the absence of the adrenal glands.

3. That the damage to the intestinal tract and bone marrow is a rapidly completed and presumably direct action of the agent, and is not related to the "alarm reaction," was shown by the following procedures:

a. By means of occluding the circulation to a portion of the small intestine during and for 5 to 15 minutes after the injection of a lethal dose of HN_2·HCl, that portion was protected from the damaging effect of the compound.

b. By means of occluding the circulation to the lower extremities by a clamp on the abdominal aorta and inferior vena cava during and for 2 to 15 minutes after the intravenous injection of HN_2·HCl, granulocytopenia in the peripheral blood was prevented, and at autopsy hyperplasia of the femoral marrow, with aplasia of the rest of the bone marrow in the body, was observed.

4. Beginning 6 hours after the injection of a lethal dose of HN_2·HCl in the rabbit, the growth of hemolytic streptococci in the serum is enhanced. This suggests that an alteration occurs in the blood making it more favorable for the growth of bacteria.

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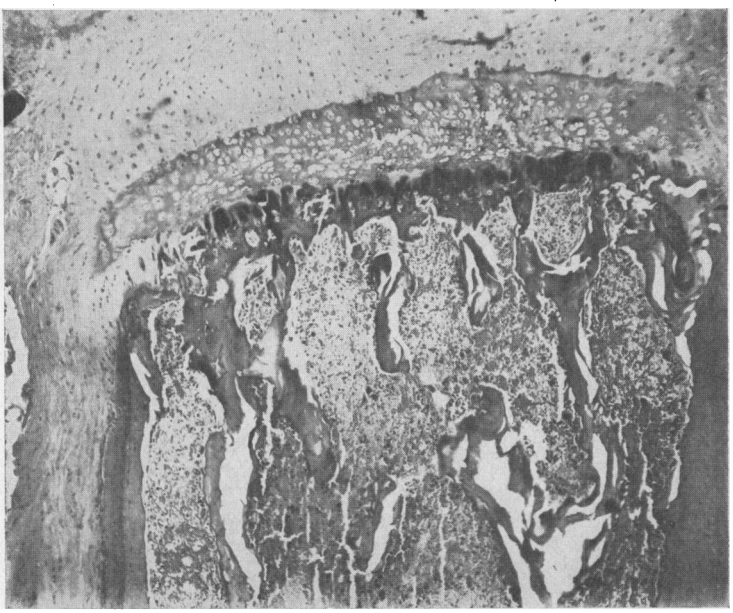
DESCRIPTION OF PLATE

PLATE 57

FIG. 1. Photomicrograph of a section of the sternum of a rat given 3.0 mg. per kg. of HN_2·HCl intravenously and sacrificed 70 hours afterward. The sinusoids are more prominent due to engorgement with red blood cells. The marrow is represented by fat spaces and cells and reduced numbers of stem cells imbedded in a matrix containing protein precipitate. Eosin and azure II stain.

FIG. 2. Femoral bone marrow of a rat protected from intoxication by HN_2·HCl by clamping the aorta for 10 minutes during and after the intravenous administration of 2 mg. per kg. Eosin and azure II stain.

1



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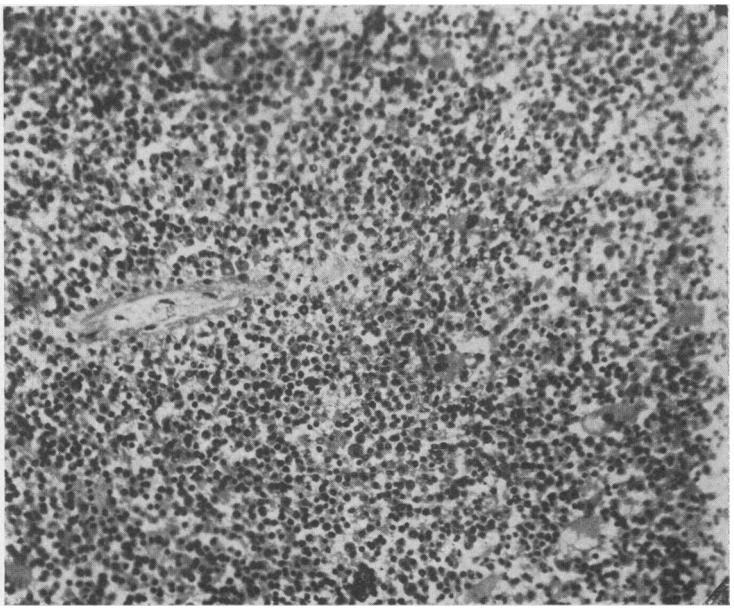

Karnofsky, Graef, and Smith Effects of Nitrogen and Sulfur Mustards