of thyroid extract given by mouth the serum cholesterol level gradually reverts to normal values in every experiment. The ratio of free to esterified cholesterol stays within normal limits during the whole experiment and seems not to be appreciably changed by chronic ANTU poisoning.

Discussion. The experiments demonstrate that chronic poisoning with ANTU produces a reversible rise in serum cholesterol in thyroidectomized dogs treated with adequate doses of thyroid hormone. From our experiments it cannot be determined whether ANTU influences the metabolism of cholesterol or the distribution of cholesterol between blood and tissues. We can assume therefore, that ANTU produces a direct effect on serum cholesterol quite independent from an indirect effect due to the antithyroid properties of the drug.

The large dose of thyroid hormone used (640 mg daily) without inducing signs or symptoms of hyperthyroidism reflects the tolerance of dogs to large doses of thyroid hormone. The dog, in proportion to its body weight, has apparently a much greater capacity than does man to inactivate exogenous thyroid hormone. This inactivation seems to take place in the extrathyroid tissues. Therefore both normal and thyroidectomized dogs can tolerate large doses of thyroid hormone without damage.[7]

Summary. Chronic poisoning with alpha naphthylthiourea (ANTU) produces a reversible rise in serum cholesterol in thyroidectomized dogs maintained on a dose of thyroid hormone adequate to prevent thyroid deficiency. This indicates that the effect of ANTU on serum cholesterol is at least partly independent from its property as an antithyroid drug.

We wish to thank Mrs. Aurora Bradford for her assistance in the cholesterol determinations, and Miss Frieda Faiman for statistical analysis of the data.

[7] Danowski, T. S., Man, E. B., and Winkler, A. W., *Endocrinology*, 1946, **38**, 230.

16890

On the Chemical Sterilization of Blood and Blood Plasma.*

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The present study was undertaken in a search for a suitable means of chemically sterilizing human plasma and whole blood without so altering the blood components as to make them unsuitable for intravenous injection. The high incidence of homologous serum jaundice (4.5 to 7.2%) resulting from the injection of pooled plasma has become a serious problem in the use of pooled lyophylized plasma. At the present time only those methods involving the irradiation of plasma with ultraviolet light,[1] x-ray, or high speed electrons[2] have offered promise of a solution to this problem. Of these methods, only electron bombardment has appeared to offer any promise in the sterilization of whole blood. All of these irradiation technics require the use of highly specialized equipment which precludes the possibility of treating plasma or blood except at specially equipped plants.

The specifications for a chemical sterilizing agent are dictated by the future intra-

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† The authors are indebted to Dr. Elizabeth M. Yagle for the immunological data in this report.

[1] Wolf, A. M., Mason, J., Fitzpatrick, J., Schwartz, O., and Levinson, O., *J.A.M.A.*, 1947, **136**, 476.

[2] Trump, J. G., personal communication.

venous use of the plasma or whole blood. (1) It must not alter the plasma proteins in such a way as to make them toxic or antigenic. (2) The agent used must be nontoxic or must readily be converted to a nontoxic substance on standing or on suitable neutralization with a second nontoxic substance; (3) It should cause minimal or no changes in the immunological components of the blood; (4) It should not cause hemolysis nor increased fragility of red blood cells. In the present investigation, a member of the nitrogen mustard group, methyl-bis (beta-chloroethyl) amine hydrochloride has been chosen for study for a number of reasons. (a) This compound has been extensively studied as a chemotherapeutic agent against the leukemias and is available in purified form;[3] (b) Its cytotoxic action is believed to be due to its effect on nucleoproteins toward which it has been shown to react competitively to a marked degree;[4-7] (c) In buffered aqueous solution the compound readily hydrolyzes to form relatively nontoxic endproducts; (d) The mechanism of action of this substance on biological systems closely parallels that of ionizing radiations.

Experimental. I. Virucidal and Bactericidal Action of HN2. In the virus experiments a New Jersey strain of vesicular stomatitis virus was used.‡ The mice used were 3- to 4-week-old Swiss strain. After several mouse passages in our laboratory, the LD_{50} of the virus was found to be 0.03 ml intracerebrally of a $10^{-6.8}$ dilution. A 10% mouse brain suspension was prepared in rabbit serum, distributed to ampules, and frozen and stored in liquid oxygen for subsequent experiments. The inoculum was always 0.03 ml intracerebrally in mice and the titer of

TABLE I. Virucidal Action of HN2 in Human Serum, Plasma, and Whole Blood.

	Description % virus suspension	Time interval, days	LD/50 control	(D) Dosage mg/l vs. Mortality (M)						Final pH
				D	M	D	M	D	M	
1.	1% in 95% serum		$10^{-5.2}$	500	3/8*					8.4
	same plus 2% 0.15 M $NaHCO_3$	5		500	3/8					8.5
	same plus 5% 0.15 M $NaHCO_3$	5		500	7/8					8.6
						200				
2.	1% in 95% serum	5	$10^{-5.2}$	200	6/8	200†	8/8	400	6/8	500	3/8	7.9 to 8.4
				600	0/8	800	0/8	1000	0/8			
3.	5% in 95% serum	2	—	400	5/5	500	5/5	600	3/5	800	3/5	
		5	—	400	5/5	500	5/5	600	3/5	800	3/5	8.0 to 8.2
4.	2% in 100% serum	3	$10^{-5.7}$	500	8/20	550	1/20	600	0/20	700	0/20	7.7 to 8.2
5.	1% in 98% citrated plasma‡	3	$10^{-6.8}$	400	0/10	500	0/10	550	0/10	600	0/10	7.0 to 7.03
6.	1% in 98% citrated plasma‡	3	$10^{-6.5}$	250	1/4	300	0/5	350	0/4	400	0/5	6.6 to 7.05
7.	1% in 90% citrated blood‡	3	$10^{-5.8}$	400	1/5§	500	0/5	550	0/5	600	0/5	6.67 to 6.73
8.	1% in 90% citrated blood‡	3	$10^{-6.8}$	250	3/5	300	2/5	350	0/4	400	0/4	6.68 to 6.85

* Denominator denotes number of mice tested, numerator denotes number of deaths.
† Second 200 mg/l added after 24 hr.
‡ In citrated blood and plasma experiments, enough $CaCl_2$ was added to remove the citrate, in this case 0.01 cc of 5 M $CaCl_2$ per cc of blood and plasma. Also enough heparin to prevent clotting once citrate was removed. The addition was made just before inoculation of animals.
§ Original bottle had to be diluted 1:10 because of excess of $CaCl_2$ which was toxic to the mice.

[3] Gilman, A., and Philips, F. S., *Science*, 1946, **103**, 409.

[4] Tenbroeck, C., and Herriott, R. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 271.

[5] Rose, H. M., and Gellhorn, A., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 83.

[6] Fruton, J. S., Stein, W. H., Stahlman, M. A., and Golumbic, C., *J. Org. Chem.*, 1946, **11**, 571.

[7] Gjessing, E. C., and Chanutin, A., *Cancer Research*, 1946, **6**, 593.

‡ Received from Dr. Carl E. Duffy.

the control sample of virus was never less than 10^{-5}, even after standing for 5 days at 4°C. In each experiment the stock virus was added to the medium desired to make a concentration of 1 to 5% brain, the HN2 added in saline immediately after dissolving, and the mixtures and controls allowed to stand for 3 to 5 days in the icebox to allow the HN2 to react with the virus and the excess HN2 to hydrolyze. The material was then tested by inoculation in mice and any questionable deaths decided by passage of the mouse brain.

The results of studies on the virucidal action of HN2 in the presence of serum, ACD citrated plasma, and whole blood are presented in Table I. A consistent effect of pH has been observed, with more favorable results at pH values of 6.7 to 7.2 than at higher pH values. Destruction of the stomatitis virus is complete in ACD citrated plasma at 300 mg/l and in ACD citrated whole blood at 500 mg/l, while sterilization in serum required from 250 to more than 800 mg/l depending upon the pH and amount of serum added.

The bactericidal effects of HN2 have been studied on several organisms. In the absence of plasma, *B. coli* is killed by dilutions of HN2 as low as 100 mg/l. In inactivated citrated plasma at pH 7.1, the sterilizing dosage of HN2 is 450 mg/l after storage of the treated material 5 days in the icebox. Under the same conditions hemolytic streptococcus was killed at 450 mg/l, *Staphylococcus aureus* (strain 209 P) at 800 mg/l and *Pseudomonas aeruginosa* at 350 mg/l. Each organism was treated with HN2 at increments of 50 mg/l of HN2 up to 500, and increments of 100 mg/l up to 1200. 0.1 ml aliquots were used in each instance for subculturing and the treated tubes and controls were made up to contain initially about 1 million organisms per ml. A study of the time course of destruction of *B. coli* showed that the organisms were progressively destroyed over a 3 day period during which the active vesicant is present. At the end of this time the organisms will resume growth at 37° in the treated samples if not entirely killed.

II. Toxicity of HN2 treated Blood and Plasma. The toxicity of freshly dissolved HN2 has been extensively investigated and is of the order of about 3 mg/kg to various animals by the intravenous route. If allowed to stand in unbuffered solution there is an accumulation of the ethylenimonium ions which under certain circumstances may increase the toxicity beyond the original value due to the greater neurotoxic action of these intermediates.[9]

In bicarbonate buffer at pH 8.0 or in biological systems containing adequate buffering power to permit the degradation of the ethylenimonium ion at the end of 3 days at room temperature the chloroethyl and ethylenimonium compounds have disappeared and the toxicity decreased to the order of one thousandth its original value.[8] In the present experiments it has been found that appreciable toxicity may remain at 3 days in preparation stored in the refrigerator at 4-10°C, but after 5 days mixtures of blood, plasma or serum containing up to 1000 mg/l of HN2 are entirely innocuous to mice. Dosages as high as 5% of the body weight intravenously or 15% of the body weight intraperitoneally of citrated plasma containing 600 mg/l of HN2 hydrochloride are tolerated by mice without evidence of unfavorable reactions if the citrate effect is avoided with calcium chloride. The marked slowing of the decomposition by low pH values should, however, be emphasized.

Further evidence of the harmless nature of the end products of hydrolysis of HN2 in blood and plasma and of the probable lack of serious antigenic responses have been obtained in experiments on dogs and man. HN2 treated citrated plasma was administered to a dog weighing 18½ lb at 3 to 6 day periods as follows: 6 doses of 100 ml of plasma treated with 100 mg/l; twice with 100 ml of plasma containing 250 mg/l; once with 50 ml containing 2000 mg/l; twice with 100 ml containing 600 mg/l. Another dog initially weighed 23½ lbs and was intravenously ad-

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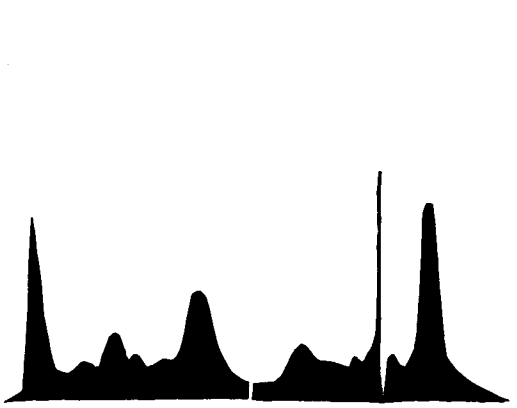
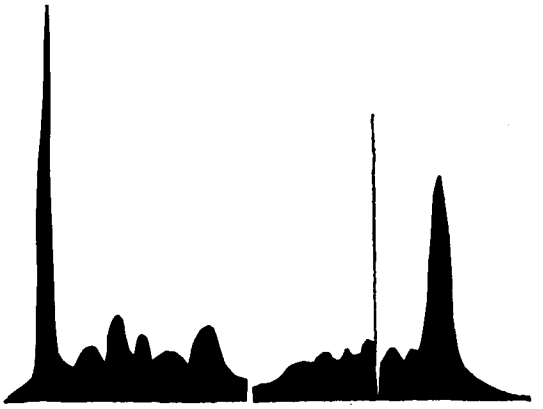
[9] Golumbic, C., and Bergmann, M., *J. Org. Chem.*, 1946, **11**, 536.



Ascending Descending

FIG. 1a.

Electrophoretic pattern of citrated plasma treated with 250 mg/l of HN2. (Barbital buffer, pH 8.6, r/2 0.01.)



Ascending Descending

FIG. 1-b.

Electrophoretic pattern of control plasma from same sample.

ministered HN2 treated citrated whole blood as follows: 6 times with 100 ml of blood treated with 100 mg/1; once with 100 ml of blood containing 250 mg/1. Both animals remained in excellent condition throughout and following the treatment period. Their final weights following the last injection were 21 and 25¼ lbs respectively.

One normal individual has received a total of 5 doses of HN2 treated (600 mg/1) citrated type O plasma at approximately 2 week intervals as follows: 0.5 ml intradermally, 10 ml subcutaneously, 20 ml intravenously, 250 ml intravenously, and 250 ml intravenously. A second individual who has become locally sensitized to the unreacted beta-chloroethyl vesicant by repeated contact has received several intradermal, subcutaneous and intravenous injections of the same material without any evidence of sensitivity to the reaction products of the HN2 and plasma. No detectable reactions of any type have followed any of these tests. They clearly demonstrate the harmless nature of the final mixtures (10 days after mixing) and suggest that antigenic reactions are not likely to occur.

III. Biochemical Studies. Equally important with the virucidal activity and the nontoxic nature of the blood or plasma-HN2 reaction product are the alterations which occur in the plasma proteins and the red blood cells. A series of studies was, therefore, undertaken to ascertain the degree of alteration of some well known plasma proteins and of red blood cell properties and constituents.

A. Plasma Constituents: The extent to which added HN2 appears in the protein free filtrate has been roughly estimated by measurement of the amount of nitrogen in the protein free filtrate before and after the addition of various amounts of HN2 hydrochloride.

Most of the added HN2 appears in the protein free filtrate fifteen minutes after its addition while at 2 days and 5 days less than half of the added nitrogen is still present. The possibility of a part of the HN2 reacting with both NPN constituents and protein via the 2 available ethylenimonium groups should not be disregarded. Urea nitrogen determinations showed that no urea as measured by the urease method had disappeared.

The effect of HN2 on the A/G ratio has been determined. No measurable effect of 1000 mg/1 could be demonstrated 10 days later, while a marked difference was evident between samples stored at room temperature and those which were refrigerated. Additional evidence of the minor nature of the changes is seen in the electrophoretic pattern data prepared through the courtesy of Dr. Charles Janeway and Dr. John Newell, Massachusetts Blood Center, presented in Fig. 1. Visually the pattern exhibited a slight broadening of the albumin peak and increase of the ϵ-globulin peak.

The plasma fibrinogen concentration has

TABLE II.
Effect of HN2 on Prothrombin in Acid Citrated Whole Blood at pH 6.7-6.8 Three Days After Treatment.

HN2, mg/l	Prothrombin, % fresh control	HN2, mg/l	Prothrombin, % fresh control
Control	82	650	17
Control with HCl eq. to 1000 mg/l	74	700	15
450	38	750	12
500	16	800	10
550	22	900	9
600	16	1000	6

TABLE III.
Effect of HN2 on Plasma Prothrombin of Citrated Plasma and Whole Blood Treated with 600 mg/l of HN2.

Hours	Prothrombin, % of normal control		
	Plasma	Whole blood*	Whole blood†
1	54	80	—
1½	60	66	66
3	46	46	50
20	42	46	46
27	37	46	46
44	37	42	46
51	37	40	40
68	34	44	48
77	34	52	52
92	34	44	52

* Plasma separated after one-half hour.
† Separated just before each prothrombin determination.

been measured before and after treatment with HN2. No decrease is produced by concentrations up to 1000 mg/l. Clot formation progressively fails with the addition of higher concentrations (complete inhibition at about 1%) even in the presence of large excesses of added thrombin.

Of all the substances studied to date, only prothrombin time (Quick's method) is markedly affected by the sterilizing dosage of HN2. Table II shows the effect of HN2 on apparent prothrombin activity and Table III shows the time course of the inactivation of prothrombin in plasma, whole blood, and whole blood separated one hour after addition of HN2. Whether the apparent inactivation of prothrombin is due to the effect of HN2 on prothrombin or accelerator globulin is being investigated.

Other substances studied to date included alkaline phosphatase, complement and immune bodies. None of these substances were markedly affected at 500 mg/l of HN2.

B. Effect of HN2 on Red Blood Cells. The possible use of chemical sterilizing agents in whole blood is predicated upon the ability of the chemical used to destroy the contaminating organisms without a concomitant destruction of the red blood cells or without so altering them as to render them short lived when introduced into the body. At the present time the *in vivo* survival of the red cells has not been adequately investigated but such studies have already been arranged.

Numerous samples of citrated whole blood have been treated with HN2 during the various studies carried out to date. Somewhat different results have been obtained on the 3 species studied. Human blood in ACD citrate-glucose solution have uniformly exhibited good storage qualities over periods up to 3 months as compared with the control bloods. In no case with dosages of HN2 hydrochloride of up to 800 mg/l have the treated specimens shown any evidence of deterioration greater than that of the same blood control. We have, in fact, sometimes observed that the treated bloods failed to exhibit hemolysis until several days after the untreated controls showed definite hemolysis. At dos-

TABLE IV.
Effect of HN2 on the Rate of Hemolysis of Red Blood Cells.

Dosage of NH2, mg/l	$NaHCO_3$	pH	Hemoglobin determined as oxyhemoglobin, g/100 ml of plasma	Hemoglobin determined as cyanmethemoglobin, g/100 ml of plasma
Control	No	6.70	1.28	1.30
450	"	6.63	1.46	1.50
600	"	6.69	1.40	1.38
800	"	6.67	1.39	1.40
1000	"	6.62	1.32	1.37
450	Yes	6.71	1.18	1.18
600	"	6.70	1.13	1.15
800	"	6.78	1.10	1.12
1000	"	6.73	1.14	1.17

(The ACD citrated blood was treated one hour after collection with the amount of HN2 indicated, with or without the calculated amount of $NaHCO_3$ to neutralize all 3 chlorides, stored without disturbing for 3 months, thoroughly mixed, centrifuged, and the hemoglobin determined in the supernatant plasma.)

ages of 450-600 mg per liter, careful studies have shown that the onset and rate of hemolysis of refrigerated whole blood is approximately equal in treated and untreated blood, if pH is adequately controlled. Observations on citrated human blood and rabbit blood have indicated that these are not affected by the sterilizing dosages of HN2. Dog blood, however, will not tolerate dosages of HN2 in excess of 250 mg/l. At 600 mg/l, the onset of hemolysis is rapid (within 24 hours) and hemolysis is almost complete within a week. This is in marked contrast to the behavior of human blood which remains in apparent good condition at the end of one week after treatment with as much as 1500 mg/l of HN2.

The results of application of various doses of HN2 upon red blood cell fragility has been investigated. Very little change was observed in fragility at sterilizing doses of HN2, but the cells became increasingly susceptible to hypotonic salt solutions as the dosage approached 1000 mg/l.

Discussion. It has been demonstrated that HN2 is capable of exerting a bactericidal and virucidal action in whole blood, blood plasma and blood serum without causing major alterations in the properties of either the plasma components or the red blood cells. These observations are supported by the findings of Tenbroeck and Herriott[3] and Rose and Gellhorn[4] on the virucidal action of this compound on 5 other viruses in the absence of blood. Because of the present inability to transmit to animals the agent of virus hepatitis, it has not been possible to study this virus until careful studies had been made upon the characteristics of treated plasma. It is felt that the present evidence is sufficiently strong to warrant the direct study of this virus as soon as a more extensive group of recipients have received the treated plasma.

The data presented in this paper strongly suggest that at least two variables are involved in the virucidal activity of HN2—the competition of the virus with plasma and whole blood components, and the effect of pH. Much of the effect of varying the percentage of plasma or serum on the virucidal activity of HN2 has now been traced to the buffering action of the blood. In contrast to the supposed advantage of a pH of about 8.0, it has been observed that virucidal action is greatly enhanced by decreasing the pH to 7.2 or below. It seems likely that the beneficial effect of a lower pH is due to a reduction in the rate at which HN2 decomposes and/or reacts with other competing substances.

On the basis of studies carried out to date, it seems likely that the required sterilizing dosage of HN2 will be no greater than 500 mg/l if the pH of the treated material is held at or below 7.2. ACD citrated blood pH values range from 6.7 to 7.0. Studies are now under way to establish the optimum pH for the virucidal and bactericidal action of HN2.

Summary. Methyl-bis (beta-chloroethyl) amine hydrochloride has been demonstrated to exert effective virucidal and bactericidal effects in the presence of either plasma, serum

or whole blood. The required virucidal dosage of HN2 is below 500 mg/l of blood or plasma if the pH is at or near 7.0. The end products of decomposition of the added HN2 are essentially nontoxic if the pH of the system is not too low. No evidence of antigenic or other toxic reactions has been produced in two dogs and two humans receiving repeated injections of treated plasma. Human red blood cells withstand the application of virucidal dosages of HN2 without hemolysis and with only a slight increase in fragility. Complement, immune bodies, phosphatase and fibrinogen are only slightly affected by sterilizing dosages of HN2. Prothrombin time is markedly prolonged by HN2.

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Timed Intravenous Infusion of Metrazol and Strychnine for Testing Anticonvulsant Drugs.*

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The validity of the use of metrazol for the evaluation of anticonvulsant drugs has been well established.[1,2] When given to animals, subconvulsant doses of metrazol will elicit electro-encephalographic patterns similar to those found in patients with petit mal.[3] The marked antagonism between metrazol and 3,5,5-trimethyloxazolidine-2,4-dione (Tridione) and the success of this drug in the treatment of petit mal epilepsy further substantiate the validity of the metrazol antagonism test as a method for screening anti-epileptic compounds.

The present metrazol test method employs the subcutaneous route of administration whereby an effective dose of the anticonvulsant is injected into a group of animals, followed, after time is allowed for absorption, by a subcutaneous dose (usually 90 mg/kg) of metrazol known to be convulsant in the non-protected animal. If the animals fail to have seizures, a second group is given a higher dose of metrazol. This procedure is repeated until a dose is attained which produces typical convulsions. In this way the degree of protection (or metrazol antagonism) furnished by a drug in question may be ascertained.

For several years we have used a timed intravenous infusion of metrazol in mice, and have found it to have some distinct advantages over the previous procedure. A similar procedure has been evolved for the measurement of strychnine antagonism by anticonvulsant drugs.

Method. The apparatus employed consists of a cone-shaped mouse holder made of plexiglass and attached to a metal base. The plexi-glass makes it possible to clearly observe the reactions of the animal within the holder. A slit, through which the tail of the mouse is pulled, extends the length of the cone at its top. Attached to the base is a small electric light connected in series through a transformer to a synchronous electrical timer. The synchronous timer is set so that the electric bulb lights every 10 seconds. The reflection of the light is transmitted throughout the transparent holder and thus is readily noted by the observer.

The mouse is pulled into the holder by drawing its tail through the slit in the cone, and the injection is made into a tail vein, using a 27 gauge needle. A 0.5% solution of metrazol or a 0.01% solution of strychnine sulfate is employed in all tests, and injected

* This study was subsidized in part by grants from the Bristol Laboratories and the Mallinckrodt Chemical Works.

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3 Goodman, L. S., Toman, J. E. P., and Swinyard, E. A., *Am. J. Med.*, 1946, **1**, 219.