acid, trigallic acid, catechol, phloroglucinol, and glucose.

Tannin has been shown to be a rather potent antibacterial substance; its possible influence upon the various surveys for the presence of antibacterial agents in higher plants should be investigated during the surveys.

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Colorimetric Method for Determination of Uracil Mustard and Related Alkylating Agents

By H. G. PETERING and G. J. VAN GIESSEN

A colorimetric method for uracil mustard has been developed which is equally useful for other aromatic and aliphatic nitrogen mustard antitumor drugs. This method has been used to determine these drugs in plasma pools and for the study of their kinetics of hydrolysis and reactivity with nucleophilic reagents.

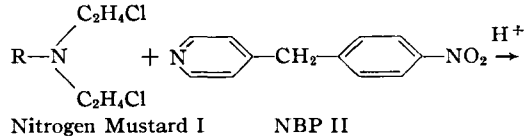
SEVERAL COLORIMETRIC methods for the determination of specific nitrogen mustards have been described by Klatt, Griffin, and Stahlin (1), Friedman and Boger (2) and Ausman, Crevar, Hagedorn, Bardos, and Ambrus (3). These methods all are based on the method for alkylating agents first reported by Epstein, Rosenthal, and Ess (4) which involves reaction of these agents with 4-(*p*-nitrobenzyl) pyridine. A photofluorometric method for ethylenimino and chloroethylamino groups has also been devised by Mellett and Woods (6). This latter method has the advantage of extreme sensitivity and thus may be useful for blood-level studies; it has the disadvantage of possible large losses because of involved and laborious manipulations.

The colorimetric methods are simpler than the fluorometric method and are useful in kinetic studies and in following the levels of these drugs when they are used in surgical perfusion studies. However, when we tried them with uracil mustard, 5-bis(2-chloroethyl) aminouracil, a number of difficulties were encountered. Since the method of Mellett and Woods was not found suitable for our purposes, we decided to attempt a modification of the colorimetric method of Klatt, Griffin, and Stahlin (1) so that a variety of nitrogen mustards could be determined.

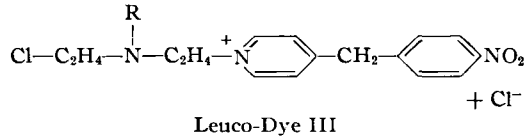
This method which is equally useful with aliphatic, aromatic, and heterocyclic nitrogen mustards and which can be conveniently used to determine these antitumor agents in perfusion blood pools, and on tissue extracts, is presented here.

EXPERIMENTAL

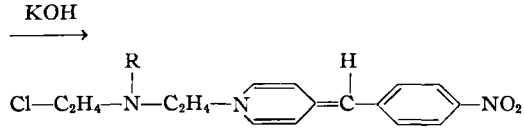
The reaction of alkylating agents with 4-(*p*-nitrobenzyl) pyridine (NBP) (II), which produces the chromophore, is carried out in our method under conditions similar to those originally described by Epstein, *et al.* (4). In the reaction formulated in Eq. 1, the solubility of the reaction product III is dependent on whether R is alkyl, aryl, or heterocyclic. Similarly the color formed from the chromophore IV when alkali is added will depend on the nature of R. In addition, Epstein, *et al.* (4), showed that the type of alkali used is of importance in the intensity of color formation. All of these matters were carefully considered in formulating the method outlined below.



Nitrogen Mustard I NBP II



Leuco-Dye III



Chromophore IV

The method worked out for uracil mustard (*Ia*) was found to give good results for dopan (*Ib*), chlorambucil (*Ic*), tris-chloroethylamine hydrochloride (*Id*), and HN-2 (Merck Mustargen) (*Ie*). The structures of these compounds, all of which are used as antitumor agents, are given in Table I.

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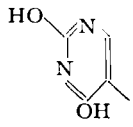
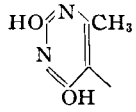
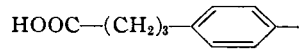
TABLE I.—STRUCTURE OF NITROGEN MUSTARDS INVESTIGATED WITH GENERAL FORMULA R—N $(CH_2—CH_2Cl)_2$

	Trade Name	Chemical Name	R Group
I*a*	Uracil mustard	5-Bis-(2-chloroethyl)-aminouracil	
I*b*	Dopan	5-Bis-(2-chloroethyl)-amino-6-methyluracil	
I*c*	Chlorambucil	4′-Bis-(2-chloroethyl)-amino-3-phenylbutyric acid	
I*d*	HN-3	Tris-(2-chloroethyl)-amine - 1·HCl	$Cl—CH_2—CH_2—$
I*e*	HN-2	Bis-(2 chloroethyl)-methylamine · HCl	$CH_3—$

Apparatus.—A Beckman B spectrophotometer was used throughout this work, but any other suitable instrument can be used. The color developed in this method was most accurately determined at 600 mμ.

Reagents.—Five per cent of 4-(*p*-nitrobenzyl) pyridine (NBP) obtained from Aromil Chemical Co., Baltimore, Md., was dissolved in acetone and used as the chromophore-producing reagent. Potassium hydroxide (1*N*) in 90% ethanol was used as the base for color development.

Nitrogen Mustard Solutions.—Uracil mustard, dopan, chlorambucil, and HN-3 were synthesized in the Upjohn Laboratories; HN-2 was obtained as Mustargen from Merck and Co. Stock solutions of the first four compounds were prepared by dissolving 10 mg. of each drug in 5 ml. of dimethylacetamide (DMA) and diluting these solutions to 100 ml. with absolute ethanol or saline. (The drugs are more stable if the dilution is made in absolute ethanol.) A stock solution of Mustargen, which is a mixture of 10 mg. of HN-2 and 90 mg. of sodium chloride, was prepared by dissolving 100 mg. in 10 ml. of water and then diluting 1 ml. of this solution with 0.5 ml. of DMA and enough absolute ethanol or saline to bring the volume to 10 ml. Thus each stock solution contained the same weight concentration of drug and the same dilution of DMA. Aliquots of these stock solutions were used to obtain the standard curves and to explore the recovery of uracil mustard from plasma.

Buffer Solutions.—Phthalate buffer of pH 4.0 was prepared according to Clark (5), and the acetate buffer of pH 4.6 according to the directions of Friedman and Boger (2).

PROCEDURES

Standardization Curves.—An aliquot of stock solution of I*a*, I*b*, I*c*, I*d*, or I*e* in the range of 10–70 mcg. was placed in a 12-ml. centrifuge tube. To this was added 1.0 ml. of pH 4.0 phthalate buffer, 1.0 ml. of NBP reagent, 1.0 ml. of 0.9% saline, and enough absolute ethanol to bring the volume to 4.0 ml. This mixture with a boiling chip was then heated for 20 minutes in a water bath at 80°. A blank without the mustard but with 1.0 ml. of absolute ethanol was treated in the same way.

After the heating period, during which the condensation was completed, the tubes were plunged into an ice bath and after being cooled were individually treated for maximum color formation and subsequent spectrophotometric analysis at 600 mμ. The contents of each tube were carefully transferred to a 5-ml. volumetric flask, 0.1 ml. of 1.0 *N*

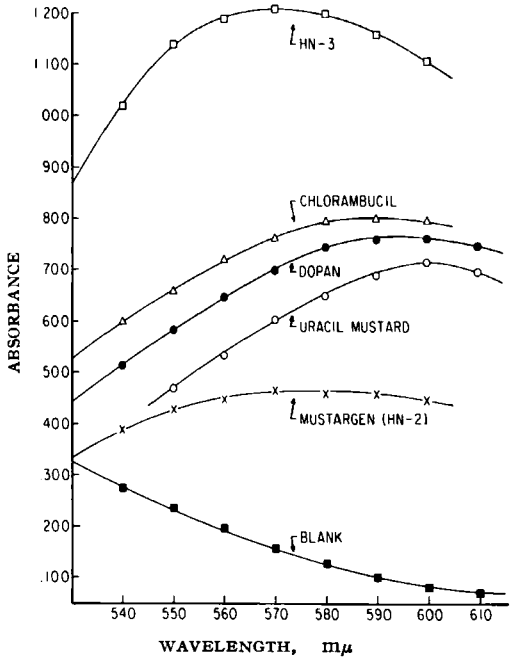


Fig. 1.—Ultraviolet absorption curves of chromophores produced by reaction of NBP reagent with several nitrogen mustards.

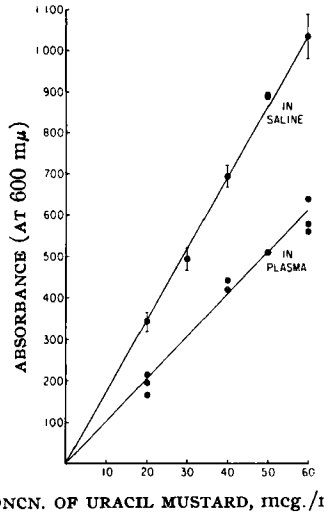


Fig. 2.—Beer's law plot of uracil mustard in saline compared with similar amounts extracted from plasma. These data indicate the extent of decomposition or binding of uracil mustard which can be expected in determining the concentration of this drug in blood.

KOH was added, and the volume brought to 5.0 ml. with ethanol. Each flask was thoroughly mixed, and placed in a cell for spectral analysis. The absorbance was observed to decrease at the rate of 1% per minute after the addition of the alkali, making it important to read the color at a designated time interval after mixing the reagents. All of our data for standard curves were obtained by making the readings within 2–3 minutes after the addition of alkali. Under these conditions a plot of absorbance *versus* concentration of drug gave a linear relationship (Beer's law) up to 70 mcg. in all instances.

Recovery of Uracil Mustard from Blood Plasma.—The following procedure was used to determine the recovery of uracil mustard (I*a*) from blood plasma. To heparinized rat plasma, uracil mustard was added from a DMA-saline stock solution in amounts so that 1.0 ml. of plasma contained 20–60 mcg. of I*a*. One milliliter of such plasma was immediately extracted with 2.0 ml. of acetone, making certain that thorough mixing and extraction occurred. The precipitated proteins were removed by centrifugation and the extract saved. The precipitate was extracted twice more with 1.0 ml. of acetone. The combined extract was then cooled in an ice bath and again centrifuged to remove residual protein. To this extract, in a 40-ml. centrifuge tube, was added 1.0 ml. of NBP reagent, 1.0 ml. of pH 4.0 phthalate buffer, and several milliliters of ethanol to control foaming.

This mixture was then heated at 60° for 5 minutes and at 80° for 20 minutes, during which the desired condensation occurred and acetone, which can react in the final color development, is removed. The resultant reaction product was then treated for color formation as described above. The chromophore produced from the drug extracted from plasma fades more rapidly than it did in the case of the standard curve investigation, probably because of the presence of unidentified soluble reactants. The rate of decrease of absorbance is about 5% per minute which makes it imperative that the readings be made quickly after the addition of alkali.

RESULTS

One of the important problems which must be resolved in the colorimetric determination of nitrogen mustards by the use of NBP reagent is the selection of the wavelength (or band) for maximum sensitivity of color determination. This varies enough for different nitrogen mustards that it accounts for some of the differences in the methods now in use. Our data in Fig. 1 indicate that the selection of 600 mμ, the maximum for the uracil mustard color, is satisfactory for the other compounds tested, although each has a somewhat different maximum. However, if 562 mμ had been selected as indicated in the work of Klatt, *et al.* (1), and 540 as indicated by Friedman and Boger (2), the results with uracil mustard (I*a*), dopan (I*b*), and chlorambucil (I*c*) would have been lower and thus decreased the sensitivity of the test.

Since excellent linear plots of optical density *versus* concentration (Fig. 2) were obtained in every case, the optical density constants for 10 mcg. per ml. are compared in Table II. These data indicate the applicability of the method to a wide variety of nitrogen mustards with comparable sensitivity in all cases except that of HN-2, which was obtained as Mustargen (Merck). Since Mustargen is a mixture of HN-2 and sodium chloride, no real basis for purity or lack of decomposition was available and it is likely that the lower results reflect less HN-2 available in the sample. Nevertheless, the method gave excellent agreement with Beer's law; thus the color with HN-2 by this method is consistent and determinable.

In all reports on the reaction of NBP with nitrogen mustards, the pH of the reaction mixture has been cited as critical; indeed it is important to have an acidic medium present as the reaction kinetics have shown. Epstein, *et al.* (4), indicated a phthalate buffer at pH 4.0 was the best for the original study. Klatt, *et al.* (1), stated that the pH should be between 4.5 and 5.0 and attempted to achieve this by adding acetic acid to the mixture before heating. Friedman and Boger (2) found acetate buffer at pH 4.6 to be better than phthalate buffer at the same

TABLE II.—ABSORBANCE OF CHROMOPHORES DEVELOPED WITH DIFFERENT NITROGEN MUSTARDS

	Mol. Wt.	Eq. Wt.	Absorbance at 600 mμ: Per 10 mcg. of Drug ± S.E.	Absorbance at 600 mμ: Per 0.1 μm. of Drug
Uracil mustard	252	126	0.173 ± 0.007	0.437
Dopan	266	133	0.185 ± 0.007	0.492
Chlorambucil	304	152	0.199 ± 0.006	0.605
HN-3 HCl	241	80	0.282 ± 0.014	0.679
HN-2 HCl (Mustargen)	192.5	96	0.117 ± 0.003	0.223

pH. We have compared the color developed with Ia and Id when acetate buffer of pH 4.6 and phthalate buffer of pH 4.0 are used. Our results show that the original work of Epstein, *et al.* (4), is valid, and that a phthalate buffer of pH 4.0 gives considerably higher values. We therefore held to the original buffer selection.

Potassium hydroxide (1.0 *N*) in 90% ethanol was used for final color development instead of either K_2CO_3 solutions or triethylamine as suggested by Epstein, *et al.* (4), and Klatt, *et al.* (1). Thereby we have avoided the problems of solubility of K_2CO_3 when acetone or ethanol are present and the critical nature of the water concentration as well as the difficulties which are inherent in safely handling triethylamine and in keeping it stabilized. This modification contributed greatly to the suitability of the method for both aqueous-soluble and aqueous-insoluble nitrogen mustard derivatives.

In other studies we found that DMA is not only a useful solvent for making up stock solutions of uracil mustard and other alkylating agents, but also that its presence at 2–5% in a saline solution of these drugs increase their stability. From our data DMA at levels up to 10% in the solutions of any of the mustards investigated does not affect the chromophore development or the subsequent color formation. Therefore, nitrogen mustard solutions containing DMA may be prepared for kinetic or biological studies without fear of altering the analytical data when this method is used.

The addition of uracil mustard in DMA-saline or ethanol-saline solutions to plasma and the immediate analysis of this mixture according to the method described above gave consistent values of about 60% recovery of the added drug. These data which are shown in Fig. 2 compared with the standard curve in the absence of plasma indicate the rapid inactivation of the drug or its complexing with plasma proteins. When trichloracetic acid precipitation of plasma protein and extraction of uracil mustard were attempted, no color development at all was obtained. Similarly when proteins were precipitated by boiling for 3 minutes in the presence of hydrochloric acid according to the method of Friedman and Boger (2) no evidence of uracil mustard in the extract was obtained. When, however, the method of Klatt, *et al.* (1), was used and the plasma was added directly to the reaction mixture, color development of intensity similar to that found by our method was obtained, although the consistency of the results was not satisfactory. Thus it is evident that uracil mustard is affected by the methods for removal of plasma protein.

DISCUSSION

Of the colorimetric methods mentioned earlier for the determination of nitrogen mustard drugs useful in cancer chemotherapy, only that of Klatt, Griffin, and Stahlin (1) has been shown to be suitable for both aromatic and aliphatic compounds. This method, however, has several shortcomings which the present method avoids. Since the pH of the reaction medium for the production of the chromophore is critical, we have chosen to use a definite buffer—phthalate buffer at 4.0—rather than adjusting the acidity with glacial acetic acid as Klatt, *et al.*, did. Furthermore, we have eliminated the use of triethylamine as the alkali for color development to avoid handling a highly toxic reagent which is unstable and requires frequent purification and have substituted instead ethanolic potassium hydroxide which is more readily available and easier to handle.

We have avoided the use of methods for extracting the drugs from plasma which will destroy the drug as Klatt, *et al.*, also attempted to do. We, however, prefer the extraction of the drug from plasma with acetone in which it is stable, although we have used the method of Klatt, *et al.*, with some success. We have found that uracil mustard is unstable when trichloracetic acid is used for extraction purposes or when the plasma is heated to boiling in the presence of hydrochloric acid as Friedman and Boger (2) recommended.

Only the method of Mellett and Woods (6) offers advantages over that described here in that it is more sensitive because of the use of a fluorometric method. Our experience with this method indicated to us that in its present form it was not suitable for routine use by laboratory technicians. In addition, we feel that the results with uracil mustard by this method as reported recently by Mellett and Woods (6) indicate very great losses during the laborious manipulations which involve the use of strong hydrochloric acid, known to cause destruction of uracil mustard. Nevertheless, if a more sensitive method is required than that described here, fluorometric methods of analysis should be investigated and developed.

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