ionic species), maximum at 264 mμ, ϵ_{max} 12020; minimum at 232 mμ, ϵ_{min} 1470; at pH 6.50 (neutral species), maxima at 219 and 272 mμ, ϵ_{max} 7720 and 14890, respectively; minima at 206 and 242 mμ, ϵ_{min} 5940 and 510, respectively; at pH 12 (anionic species) maximum at 265 mμ, ϵ_{max} 8370, shoulder at *ca.* 225 mμ, minimum at 243 mμ, ϵ_{min} 2,280.

Anal. Calcd. for $C_4H_4FN_3O$: C, 37.22; H, 3.12; N, 32.55; F, 14.72. Found: C, 37.35; H, 3.15; N, 32.28; F, 14.70.

4,6-Diamino-2-pyrimidone (XVI) from XV.—XV (100 mg.) was heated with alcoholic ammonia in a sealed tube for 6 hr. at 140°. After cooling, the solid was filtered and recrystallized from hot water, 60 mg. (62%), no m.p. <300°. The product demonstrated the same ultraviolet absorption spectra and the same R_f by paper chromatography in several systems when compared with an authentic sample[18] of XVI.

2,4-Dimethoxy-6-fluoropyrimidine.—2,4,6-Trifluoropyrimidine (6.7 g., 0.05 mole), dissolved in a mixture of 50 ml. each of anhydrous methanol and dry benzene, was cooled to 15°, and a solution of sodium (2.3 g., 0.10 mole) in 50 ml. of anhydrous methanol was added, keeping the temperature below 20°. After standing several hours, the suspension was filtered through a Celite Pad and the filtrate evaporated *in vacuo* at *ca.* 30°. The residue was taken up in ether and washed with water; the ether solution was dried over sodium sulfate and evaporated *in vacuo*. The oily residue was purified by dissolving in dioxane, decolorizing the solution with Norit, and reprecipitating by addition of water. The product, 5.8 g., m.p. 51–53°, exhibited ultraviolet absorption maxima in water at 212 and 246 mμ. An aliquot sublimed at 100° (20 mm.) yielded prisms, m.p. 54–55° (lit.[14] m.p. 54–56°, absorption maximum at 245.5 mμ).

Anal. Calcd. for $C_6H_7FN_2O_2$: F, 12.01. Found: F, 12.23.

5-Chlorocytosine.—Cytosine (1 g., 0.009 mole) was dissolved in 20 ml. of warm glacial acetic acid. To the yellow solution was added 1.3 g. (0.01 mole) of N-chlorosuccinimide (NCS) and the temperature held at 105° for 1.5 hr. The NCS dissolved within 5 min.; gradually thereafter, precipitation of product began. After cooling, the suspension was filtered and the precipitate washed with water. The crude product was suspended in water and brought to pH 11 with ammonium hydroxide. The resulting solution was treated with glacial acetic acid and the precipitated product recrystallized from water, 0.9 g. (69%), browns at 285°, m.p. 291–292° dec. Ultraviolet absorption properties: in 0.1 *N* HCl (cationic species), maxima at 217 and 293 mμ, ϵ_{max} 11,340 and 8250, respectively; minimum at 248 mμ, ϵ_{min} 760; at pH 7.6 (neutral species), maxima at 216 and 282 mμ, ϵ_{max} 12,460 and 4940, respectively; minima at 212 and 257 mμ, ϵ_{min} 12,340 and 2850, respectively; at pH 12–14 (anionic species), maximum at 296 mμ, ϵ_{max} 6850; minimum at 256 mμ, ϵ_{min} 920.

Anal. Calcd. for $C_4H_4ClN_3O$: C, 33.01; H, 2.77; N, 28.87; Cl, 24.36. Found: C, 33.15; H, 2.84; N, 28.60; Cl, 24.05.

5-Chloroisocytosine.—Isocytosine was chlorinated by a procedure identical to that used for 5-chlorocytosine. Yield of recrystallized product was 43%, m.p. 306–307° dec. Ultraviolet absorption properties: in 0.1 *N* HCl (cationic species), maxima at 223 and 272 mμ, ϵ_{max} 8370 and 6260, respectively; at pH 5.61 (neutral species), maximum at 299 mμ, ϵ_{max} 4270, shoulder at 270–280 mμ; minimum at 253 mμ, ϵ_{min} 1870; at pH 11 (anionic species), maxima at 232 and 286 mμ, ϵ_{max} 6910 and 5730, respectively; minima at 222 and 256 mμ, ϵ_{min} 6320 and 1480, respectively.

Anal. Calcd. for $C_4H_4ClN_3O$: C, 33.01; H, 2.77; N, 28.87; Cl, 24.36. Found: C, 33.00; H, 2.84; N, 29.09; Cl, 24.62.

Spectrophotometric Studies.—Ultraviolet absorption data were determined with a Cary recording spectrophotometer, Model 15, using buffers and techniques previously described.[22] The apparent pK_a values are accurate to ±0.05 pH unit and were determined spectrophotometrically by methods previously employed.[22,37]

Paper Chromatography.—Chromatographic analyses were performed by the ascending method using Schleicher and Schuell 597 paper in the following systems: (a) butanol–water (86:14); (b) ethanol–water (85:15); (c) 2-propanol–water (70:30). The compounds were visualized on the paper chromatograms under ultraviolet light.

Key to Charts.—The structures of all "hydroxypyrimidines," *e.g.*, V, X, XII, etc., are drawn in the carbonyl (lactam) form. It is understood that such representation need not necessarily reflect the true tautomeric state.

Acknowledgment.—The authors wish to thank Drs. E. Kober and H. Schroeder of the Olin Mathieson Chemical Corp., New Haven, Conn., for providing experimental details on their synthesis of trifluoropyrimidine prior to publication, and Dr. George Bosworth Brown for helpful discussions and continued interest.

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Synthesis of Possible Cancer Chemotherapeutic Compounds Based on Enzyme Approach. V. Tetrazolium Nitrogen Mustards[1]

K. C. TSOU AND HELEN C. F. SU

Central Research Laboratories, The Borden Chemical Company, Philadelphia, Pennsylvania, Harrison Department of Surgical Research and the Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania

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Several tetrazolium salts (I) and formazans (II) that bear a nitrogen mustard group have been synthesized. The introduction of a tetrazolium ring into an aromatic nitrogen mustard drastically reduces its toxicity. The tetrazolium nitrogen mustards can be reduced to the more toxic formazan nitrogen mustards. For certain types of cancer with dehydrogenase activity these tetrazolium salts are of interest as possible chemotherapeutic agents. Furthermore, if *in vivo* reduction did take place beyond the formazan stage, a potent nitrogen mustard, N,N-bis-(2-chloroethyl)-*p*-phenylenediamine, lethal to tumor cells, would be liberated. The synthesis, preliminary toxicity, and antitumor activity in animal screening of these new nitrogen mustards and related tetrazolium salts are discussed.

The synthesis of possible cancer chemotherapeutic compounds based on differences in the distribution of enzymes between normal and cancer cells is one of the promising areas of cancer chemotherapy. Previously, we reported the synthesis of several series of alkylating compounds[2–4] which had been designed to take advantage of the low esterase[5,6] or high phosphoramidase[7] content of tumor cells. The present paper reports our initial attempt to design and synthesize new chemo-

(1) (a) This work was supported by research Grant CY-2530 from the National Cancer Institute, National Institutes of Health, Bethesda, Maryland; (b) address inquiries to this address.

(2) (a) K. C. Tsou, H. C. F. Su, C. Segebarth, and U. Mirarchi, *J. Org. Chem.*, **26**, 4987 (1961); (b) H. C. F. Su, C. Segebarth, and K. C. Tsou, *ibid.*, **26**, 4990, 4993 (1961).

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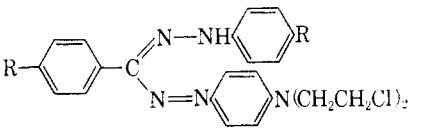
Table I

Formazans

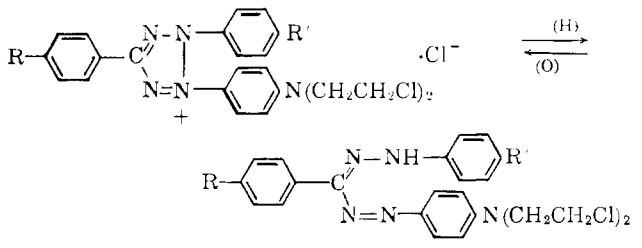


No.	R	R′	Empirical formula	Color of crystals	M.p., °C.	Yield, %	Analyses[a] Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found	Chlorine Calcd.	Chlorine Found
IIA	H	H	$C_{23}H_{23}Cl_2N_5 \cdot HCl$	Dark reddish	200–201	34	57.93	57.78	5.07	4.97	14.69	14.86	22.30	22.21
IIB	H	NO_2	$C_{23}H_{22}Cl_2N_6O_2$	Dark reddish with green luster	184–186	60	56.93	56.61	4.56	4.43	17.32	16.99	14.61	14.93
IIC	NO_2	NO_2	$C_{23}H_{21}Cl_2N_7O_4$	Dark violet	231.5–233	32	52.08	52.23	3.99	4.17	18.49	18.12	11.39	13.50

[a] Analyses were done by Dr. S. M. Nagy, Microchemical Laboratory, Belmont, Massachusetts.

therapeutic agents that may be useful in certain types of cancer with different dehydrogenase activity.

It is well known that the reduction of colorless tetrazolium salts to colored formazans is useful for the demonstration of dehydrogenase activity.[8] It is also well known that introduction of an electron-withdrawing group on the benzene ring of an aromatic nitrogen mustard reduces the basicity of the amine, and consequently reduces the rate of formation of ethyleniminmonium ion. Therefore, tetrazolium salts bearing a nitrogen mustard group (I) have a low rate of hydrolysis or ethyleniminmonium ion formation and lower toxicity because the tetrazolium ring can exert a strong electron-withdrawing influence on the aromatic amine. However, when



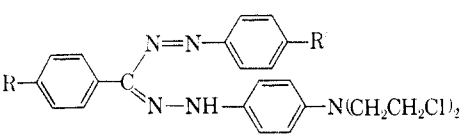
IA, R = R′ = H
IB, R = H; R′ = NO_2
IC, R = R′ = NO_2

IIA, R = R′ = H
IIB, R = H; R′ = NO_2
IIC, R = R′ = NO_2

affected by high succinic dehydrogenase activity, such as known in uterine[9] and other types of cancer[10] they could be reduced to the formazans (II) in which the electromeric effect of the 3-N would impart an increase in electron density on the nitrogen atom of the mustard, and thereby increase the basicity of the amine and the toxicity of the nitrogen mustard. The correlation of toxicity of nitrogen mustard and the basicity of the amine has been substantiated by Ross.[11] Further encouragement to this rationale was provided by an early report of Siegert, *et al.*,[12] in which they demonstrated that ascites tumor cells could actually reduce the tetrazolium salt beyond the formazan state. Thus, if similar *in vivo* reactions were to take place in our designed compounds, an even more potent nitrogen mustard, N,N-bis(2-chloroethyl)-*p*-phenylenediamine (III), lethal to tumor cells, would be liberated.

After exploring numerous approaches to the synthesis of these compounds, the successful route was that starting from the hydrochloride of N,N-bis(2-chloroethyl)-*p*-phenylenediamine (III), which could be prepared readily by the method of Everett and Ross.[13] This compound is a potent vesicant and must be handled with extreme care. Diazotization of III to its diazonium salt (IV) and coupling to the corresponding phenylhydrazone yield the formazans (II). The introduction of nitro groups in the formazan has been shown[14] to improve the substantivity of formazan and the ease of reduction of the tetrazolium salts in tissue.[15,16] The purification of the dinitro substituted formazan was quite difficult as the starting dinitrophenylhydrazone formed a weak complex with the formazan. The physical properties and analyses of the formazans are summarized in Table I.

The oxidation of the formazan to the tetrazolium salt was accomplished in a dry tetrahydrofuran solution of the formazan by the use of isoamyl nitrite as the oxidizing agent. The physical properties are summarized in Table II. The absorption spectra of the formazans and tetrazolium salt are summarized in Table III. The tetrazolium salts are very water-soluble and were found to be reduced by liver or kidney succinic dehydrogenase preparations to the insoluble colored formazans, in the presence of sodium succinate. Even though only one formazan nitrogen mustard was synthesized in each case, it is theoretically possible to form two formazans from the reduction of a tetrazolium salt. The isomeric form (II′) would, however, be expected to be more toxic than II, and therefore its formation will strengthen the rationale in an *in vivo* study.



II′

It might be necessary to mention here that in conjunction with the synthesis of these compounds, the antitumor activity of several tetrazolium salts including 2,3,5-triphenyl-2H-tetrazolium chloride (TTC), 3-(*p*-

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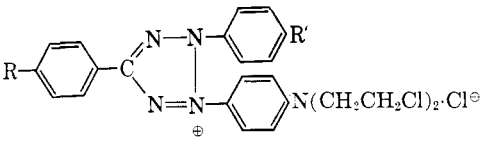
TABLE II

TETRAZOLIUM CHLORIDES



No.	R	R′	Empirical formula	Color of crystals	M.p., °C.	Yield, %	Analyses[a] Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found	Chlorine Calcd.	Chlorine Found
IA	H	H	$C_{23}H_{22}Cl_3N_5$	Yellow	117–120	75	58.16	57.94	4.67	4.92	14.76	14.27	22.41	22.07
IB	H	NO_2	$C_{23}H_{21}Cl_3N_6O_2 \cdot H_2O$	Orange	151–154	30	51.36	51.61	4.31	4.22	15.63	15.30	19.78	20.05
IC	NO_2	NO_2	$C_{23}H_{20}Cl_3N_7O_4$	Orange-red	175–177	37	48.88	48.76	3.57	3.86	17.37	17.01	18.84	18.55

[a] Analyses were done by Dr. S. M. Nagy, Microchemical Laboratory, Belmont, Massachusetts.

TABLE III

ABSORPTION SPECTRA OF FORMAZANS AND TETRAZOLIUM CHLORIDES[a]

Compound	Absorption maxima, mμ: λ_{max}	ϵ_{max}	λ_{max}	ϵ_{max}
IA	405	1,481	...	...
IB[b]	445	7,020	...	...
IC	455	6,026	...	...
IIA[c]	400	12,000	500	20,500
IIB	390	19,800	525	31,400
IIC	400	30,000	535	33,500

[a] Water solution in concentrations of IA, 2.6×10^{-4} *M*; IB, 1.44×10^{-4} *M*; IC, 1.21×10^{-4} *M*. Acetone solution in concentrations 10^{-5} *M* for IIA, IIB, IIC. [b] Monohydrate. [c] Hydrochloride.

even though at a slightly toxic dose, nitro-BT does show activity in Sarcoma-180 and Carcinoma-755 systems. While there is no *a priori* reason to correlate this difference in activity to nitro substitution, this finding strengthened our interest in the preparation of these nitro substituted tetrazolium nitrogen mustards in the present work. Further screening data are still needed to complete the assessment of antitumor activity of the tetrazolium nitrogen mustards synthesized in this work. Interestingly, at the same nontoxic dose, the formazan IIA does show higher activity than the tetrazolium chloride IA in the Walker 256 system (Table V). The mononitrosubstituted compounds have only been screened in the Dunning Leukemia system, and neither

TABLE IV

ANTITUMOR ACTIVITY OF SOME TETRAZOLIUM SALTS[a]

Compound	Tumor	Dose, mg./kg.	Survivors	Wt. change, *T/C*[b]	Tumor wt., or survival days, *T/C*	Per cent *T/C*
TTC	Sa-180	2.0	...	...	...	78
	Ca-755	1.4	...	...	...	111
	L-1210	1.6	...	...	...	100
INT	Sa-180	40.0	5/6	−1.3/−0.6	496/825	60
		25.0	6/6	−1.3/−0.1	497/455	110
	Ca-755	32.0	8/10	0.2/2.4	1411/1855	76
	L-1210	32.0	5/6	−1.2/0.9	7.6/8.3	91
NT	Sa-180	12.5	6/6	−0.4/−0.8	889/855	100
	Ca-755	11.3	4/10	−1.1/2.4	656/1855	35 (toxic)
		5.5	10/10	2.3/2.5	1082/1545	70
	L-1210	11.3	6/6	−1.0/0.9	7.5/8.3	90
Nitro-BT	Sa-180	6.3	3/6	−1.7/−0.8	247/770	33 (toxic)
		3.0	3/6	−1.9/−0.6	380/825	46 (toxic)
		1.5	5/6	−1.2/−0.8	757/855	89
	Ca-755	1.2	9/10	1.3/2.4	912/1855	49
		1.2	9/10	0.1/2.5	1156/1545	74
	L-1210	1.2	6/6	−1.1/2.6	7.8/9.4	85
BT	Sa-180	20.0	6/6	−1.3/−1.3	761/672	110
	Ca-755	9.0	10/10	2.0/2.0	1622/1545	104
	L-1210	9.0	6/6	1.9/2.0	10.2/9.3	109

[a] Screening data obtained through the generous cooperation of CCNSC. Acknowledgment is due to Drs. H. Bond, R. B. Ross, and J. Leiter for these data. [b] *T/C* = test/control.

iodophenyl)-2-(*p*-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT), 3,3′-(4,4′-biphenylene)-bis(2,5-diphenyl)-2H-tetrazolium chloride (NT), 3,3′-(3,3′-dimethoxy-4,4′-biphenylene) - bis(2,5-diphenyl)-2H-tetrazolium chloride (BT), and 3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-bis[2-(*p*-nitrophenyl)-5-phenyl]-2H-tetrazolium chloride(nitro-BT) were submitted by us to the Cancer Chemotherapy National Service Center (CCNSC) and were examined in a preliminary manner. The results are summarized in Table IV. It was found that activity nor toxicity was noticed with 1.0–100.0 mg./kg. doses when administered subcutaneously in oil.

Preliminary toxicity data of these compounds were determined by Dr. S. P. Kramer of the Department of Surgery of Sinai Hospital, Baltimore, Maryland. In general, the formazans are found to be more toxic than the tetrazolium salts, even though as nitrogen mustards, they are both relatively low in toxicity. For example, at a 40 mg./kg. (or 77 μmoles/kg.) dose of IB, no toxic symptoms were observed, and animals were

found to be healthy when sacrificed. The low toxicity of these tetrazolium salts became even more evident in the CCNSC primary antitumor screening. Preliminary findings to date indicate that the LD_{50} of these tetrazolium salts is at least 50–100 mg./kg., which makes them a class of low toxicity nitrogen mustards. The dinitro compounds were found to be only slightly more toxic than the mononitro compounds.

Table V

Comparison of Antitumor Activity of Tetrazolium Nitrogen Mustard IA and the Corresponding Formazan Nitrogen Mustard IIA in Walker-256 System

Compound	Dose, mg./kg.	Survivors	Wt. change, $T-C$	Tumor wt., T/C	Per cent T/C
IA	50.	6/6	33/41	6.6/5.9	111
	25.	6/6	39/41	4.7/5.9	79
	12.5	6/6	43/41	6.9/5.9	116
	6.25	6/6	44/41	6.1/5.9	103
IIA	50.	6/6	39/38	5.0/9.4	53
	25.	6/6	42/38	5.1/9.4	54
	12.5	6/6	41/38	6.5/9.4	69
	6.25	6/6	52/38	14.8/9.4	157

In reference to the rationale just presented, we did submit compound III for toxicity and antitumor screening. It was found to be almost 10 times more toxic than the tetrazolium salt IA. Furthermore, III was also found to be active in the Dunning Leukemia system and curative at 8 mg./kg. (Table VI). It was, however, only slightly active in the Ca-755 system (T/C = 63% at 0.05 mg./kg.).

In spite of the difficulty in synthesis, the low toxicity of these new tetrazolium nitrogen mustards and the increase in activity of the formazans and N,N-bis(2-chloroethyl)-*p*-phenylenediamine (III) validate our initial design of this class of compounds for possible cancer chemotherapy. Further studies of their behavior in enzymatic reduction by normal and neoplastic cells are in progress.

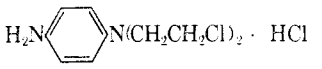
Experimental

Preparation of the Formazans.—N,N-bis(2-chloroethyl)-*p*-phenylenediamine hydrochloride (13.05 g., 0.048 mole) was suspended in 50 ml. of water and 60 ml. of concentrated hydrochloric acid at 0°. Diazotization was carried out with a sodium nitrite solution (4 g., 0.058 mole, in 20 ml. of water) in 30 min. Excess nitrite was destroyed by the addition of 0.3 g. of urea. The diazotized solution was then added dropwise to a pyridine solution (35 ml.) of benzaldehyde phenylhydrazone (8.4 g., 0.048 mole) at 0°, followed by 100 ml. of triethylamine. After stirring at room temperature overnight, the dark solution was diluted with an equal volume of ice-water. The dark reddish precipitate was collected and washed thoroughly with water and methanol. Recrystallization from acetone–methanol yielded the pure formazan as glistening red needles. The other two formazans were prepared by similar procedure, using the corresponding *p*-nitrophenylhydrazones. The physical constants and analyses are summarized in Table I.

Preparation of the Tetrazolium Chlorides.—A dry tetrahydrofuran solution (20 ml.) of the formazan (0.5 g.) was saturated with dry gaseous hydrogen chloride at 5°. Isoamyl nitrite (1 ml.) was added. The mixture was stirred at 5–10° for 2 hr., then at room temperature overnight. The orange-brown solution was concentrated *in vacuo* to its smallest volume. The residue was recrystallized from tetrahydrofuran–ether to obtain the tetrazolium salts as fine yellow or orange crystals. The physical constants and analyses are summarized in Table II.

Table VI

Antitumor Activity of N,N-Bis(2-chloroethyl)-*p*-phenylenediamine Hydrochloride (III)[a] on Dunning Leukemia System



Daily dose, mg./kg.	Survivors	Wt. change, T/C[b] (g.)	MST[c] (days), T/C	% Increase in life span
Administered s.c. in oil qd × 12				
10.0	1/2	−19/15	46.0/13.3	246
8.0	4/4	−22/15	∞/13.3	∞
6.0	4/6	−13/15	29.2/13.3	119
4.0	3/6	−3/14	28.3/12.8	121
3.0	6/6	−2/12	23.0/12.5	84
2.5	6/6	10/12	23.5/12.5	88
2.0	6/6	8/14	18.8/12.8	47
1.0	6/6	15/14	14.7/12.8	15
Administered i.p. in saline qd × 12				
1.5	0/6	−/19	7.0/13.6	Toxic
1.0	3/4	−10/19	16.3/12.6	29
1.0	4/4	−7/13	15.8/13.4	18
0.75	6/6	−10/19	15.7/12.6	25
0.50	1/1	9/13	13.0/13.4	−3

[a] Screening data obtained through the generous cooperation of CCNSC. [b] T/C = test/control. [c] MST = mean survival time.