Aromatic Sulfonic Acids as Viral Inhibitors. Structure–Activity Study using Rhino, Adeno 3, Herpes Simplex, and Influenza Viruses

STIG ÅKERFELDT,*

Virology Department

GERTRUD WESTIN, AND TORSTEN JANSSON

Organic Chemistry Department, Research Department of the KABI Group, Stockholm 30, Sweden

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Forty-nine sulfonic acids, mainly derivatives of naphthalene and benzene, have been investigated as potential antiviral agents in various test systems using rhino virus 33342, adenovirus 3, herpes simplex virus, and several influenza strains. Some of the substances tested were active both *in vitro* and *in vivo*, especially certain dyes and derivatives of chromotropic acid. The structure–activity relationship is briefly discussed and certain conclusions are drawn as to desirable structures. Preliminary investigations of the mechanism of action of these compds showed that some substances are strongly absorbed to the virions and, consequently, might prevent the entry of virus into cells. Other compds seem to act on the cell membrane or inside the cells.

Certain dyes containing sulfonic acid groups are known to inhibit the effects of a number of viruses. For instance, Hurst, *et al.*,[1] demonstrated the protective properties of a number of azo dyes in mice infected by equine encephalomyelitis virus. Becht and Drzeniek[2] showed that both Congo red and Trypan red inhibited neuraminidase of Newcastle disease and fowl plaque viruses. Congo red was thought to inhibit release of virions from infected cells.

In this laboratory, Svehag and Geijer[3] have studied the effect of several dyes containing sulfonic acid groups on herpes simplex and rhino viruses in tissue culture systems. Of 11 dyes tested 8 gave good protection in the concn range of 10–50 μg/ml (see Table III). The purpose of the present investigation has been: (1) to investigate if smaller molecules, related to the dyes, also show antiviral activity; and (2) to determine the effect of structural variations on antiviral activity.

Experimental Section

The strains of viruses used were obtained from the National Bacteriological Laboratory, Stockholm, Sweden. Human amnion AV3 cells, human adult conjunctiva, and human embryonic lung cells were obtained from Flow Laboratories, Irwine, Scotland. Cells were grown in tissue culture glass tubes (12 × 100 mm), in Eagle's medium containing Earle's salt soln, and 5% of calf serum (totally 1,5 ml). Tubes with a homogeneous monolayer of cells were infected with virus suspension (0.15 ml), and the virus was allowed to absorb to cells for 1 hr at 33° for adeno 3 and rhino virus 33342, but 37° for herpes simplex.[4] The tubes were treated with the substances to be tested and dissolved in maintenance medium 24 hr and 0 hr before infection. The medium-containing substance was then changed every second day throughout the experiment. The viral infection dose was chosen so that *ca.* 100% cytopathogenic effect was obtained in the infected control tubes 5–6 days after infection. Usually 4 tubes were used for each concn of substance to be tested. Uninfected controls and a known antiviral agent were also included in each experiment. The amount of viral infection was determined by estimating the percentage of cells showing typical cytopathogenic changes. By plotting time *vs.* the log of percentage of cytopathogenic changes a straight line was usually obtained. The degree of protection was calcd from the equation: % protection = $100(VC - T)/VC$, where VC stands for per cent infected cells in virus control tubes and T, per cent infected cells in tubes containing test substance. VC was always close to 100.

For the animal experiments male 10- to 12-g mice (2–3 weeks old) of the NMRI strain were used. Intranasal infection (0.03 ml of virus suspension) of anesthetized (Et_2O_2) animals was applied. Usually a LD_{90} dose of virus was inocculated. Ten mice were used for each concn of substance to be tested.

Intracerebral injection of herpes simplex virus into mice was carried out by introducing the syringe about 3 mm above the eye to a depth of about 3 mm. The injected vol was 0.03 ml. The control animals tolerated one such injection of saline above each eye.

Synthesis of Sulfonic Acids.—Most of the compds required for this investigation were obtained from commercial sources or synthesized according to published procedures. The novel benzoyl and benzenesulfonyl derivatives of naphthalenesulfonic acids tested were easily obtained by conventional routes. The work-up procedure had, however, to be tailored to the individual compd.

The substances prepared were electrophoretically homogeneous (pH 1.9 acetate–formate buffer, potential gradient 30 V/cm, sepn time 2 hr), but often strongly retained H_2O and were difficult to obtain completely anhyd. For this reason the conventional elementary analyses were supplemented with Karl Fischer H_2O determinations (Table I). On the basis of these data the isolated compds usually contained 95–99% of organic substance, the remainder being mainly H_2O.

TABLE I

ANALYTICAL DATA FOR THE PREPARED COMPOUNDS[a]

No.	Yield, %	R_f[b]	Formula	Analyses[c]
13		1.1	$C_{17}H_{11}NNa_2O_9S_2$[d]	CNS[e]
15	53	1.3	$C_{17}H_{11}NNa_2O_7S_2$	CNS[f]
16	10	1.7	$C_{16}H_{11}K_2N_2O_8S_3$	CNS[g]
17	72	1.3	$C_{17}H_{11}NNa_2O_7S_2$	CNS[h]
18	10	1.5	$C_{16}H_{13}NO_8S_3$	CNS[i]
19		1.6	$C_{17}H_{11}NNa_2O_7S_2$	CNS[j]
20	77	1.6	$C_{16}H_{11}NNa_2O_8S_3$	CNS[k]

[a] The elementary analysis were carried out at the analytical Department of the chemical Institute, University of Lund, Sweden. [b] Electrophoretic migration rates compared to those of the corresponding unacylated products. [c] Corrected for H_2O content. [d] Na content 10.1%. [e] *Anal.* Calcd: C, 42.2; N, 2.90; S, 13.3. Found: C, 36.9; N, 2.82; S, 12.0. [f] *Anal.* Calcd: C, 45.2; N, 3.10, S, 14.2. Found: C, 44.9; N, 3.02; S, 13.6. [g] *Anal.* Calcd: N, 2.70; S, 18.5. Found: N, 2.51; S, 16.1. [h] *Anal.* Calcd: S, 14.2. Found: S, 13.8. [i] *Anal.* Calcd: C, 43.3; N, 3.16; S, 21.7. Found: C, 37.2; N, 2.71; S, 17.9. [j] *Anal.* Calcd: C, 45.2; N, 3.10; S, 14.2. Found: C, 44.6; N, 3.30; S, 13.8. [k] *Anal.* Calcd: C, 39.4; N, 2.87. Found: C, 40.8; N, 2.97.

(1) E. W. Hurst, P. Meloin, and J. M. Peters, *Brit. J. Pharmacol.*, **7**, 455 (1952).

(2) H. Becht and R. Drzeniek, *J. Gen. Virol.*, **2**, 261 (1968).

(3) S. E. Svehag and S. Geijer, unpublished results.

(4) S. Osterhout and I. Tamm, *J. Immunol.*, **83**, 442 (1959).

The commercially obtained sulfonic acids, used as starting materials, were purified by recrystn or repptn until electrophoretically homogeneous.

Disodium Benzenesulfonamidonaphthalenedisulfonates (16, 18, 20, 21).—A mixt of aminonaphthalenedisulfonic acid or salt, $PhSO_2Cl$, and $NaHCO_3$ in H_2O was stirred at 25–50° for 16–24 hr. The soln was brought to pH < 2 by addition of 2 *M* HCl and then passed through Amberlite IR 120 (H^+). Compds **16** and **20** were converted into the Na salt and recrystd from H_2O.

Didosium Benzamidonaphthalenedisulfonates (13, 15, 17, 19).—Aminonaphthalenedisulfonic acid or salt was dissolved in a soln of $NaHCO_3$ in H_2O and BzCl was added. The soln was stirred at room temp for 1–6 hr, then brought to pH < 3 by addn of 2 *M* HCl, and extd with Et_2O. The aq soln was passed through Amberlite IR 120 (H^+) and the eluate was evapd. The acid was converted into the Na salt and usually recrystd from EtOH–H_2O.

Results and Discussion

Double Test System.—If the same type of virus was allowed to multiply in human cells of different origin, the compds tested were sometimes only effective in one kind of cells and not in the other, whereas other compds showed antiviral activity in both kinds of cells. Such results could be interpreted to indicate that certain compds interacted with mechanisms of adsorption, penetration, or synthesis of virus that are specific for the cell type in question. The purpose of these investigations has been to find antiviral agents not specific to only one type of cells, but as broad acting as possible. Therefore, when a substance showed an interesting activity in one type of cells it was also tested in another kind.

Mechanism of Action.—When certain of the active compds were mixed with virus and excess substance was removed by dialysis or by other means (see Table II), the remaining amount of virus had lost much of its infective properties as compared to a blank treated the same way but omitting the antivirals. Cells preincubated with active compds and then washed were in some cases also protected (Table II). These experiments indicated that the various sulfonic acids may have different mechanisms of action. Some substances seem to have large affinity for the virions, thereby probably preventing the entry of virus into cells. Other substances may protect by being absorbed to the cell surface or by entering the cells. Some compds may be active by dual mechanisms (*cf.* Vital new red, Table II).

TABLE II

EFFECT OF PRETREATMENT OF CELLS AND VIRUS WITH VARIOUS SULFONIC ACIDS[a]

Substance	Virus used	Loss of infectivity by pretreat of virus with substance, %	Loss of infectivity by pretreat of cells with substance, %
Chromotrop 2 R (11[b])	HSV	88	0
OAB 13401 (13)	Adeno 3	54	0
OAB 13401 (13)	Rhino 33342	76	31
Suramin (10)	HSV	0	8
Chromotropic acid (24)	HSV	42–100	0–53
Aminomethanesulfonic acid (47)	HSV	0	0
Vital new red (3)	HSV	75–88	17–75

[a] Virus and substance (50 μg/ml in phosphate buffered saline) were left at 22° for 1 hr. To remove excess of substance the virus–substance mixt was centrifuged 1 hr at 4° at 200,000*g* (OAB 13401) or the mixt was dialyzed overnight at 4° against phosphate-buffered saline (all other substances). Blanks without substances were treated the same way. Cells were pretreated with substance 2 hr prior to infection and then washed with medium and infected. [b] Numbers refer to Table III.

Effects against Influenza A2/Stockholm/10/63 *in Vivo*.—Only a very few compds showed protective effect against this virus *in vivo*. The few active compds, when tested on several influenza strains (Table IV), showed considerable inconsistency in activity. This, however, is a well-known phenomenon for all known antiinfluenza compds, including amantadine.

Intranasal instillation of the compds 15 min before infection with virus appeared to afford some protection (Table IV).

Effects against Herpes Simplex *in Vitro* **and** *in Vivo*.—The best antiviral effect was found with relatively large molecules. With the exception of aminomethanesulfonic acid, which, however, is only active in amnion cells, all effectively protective compds were naphthalene derivatives. Although the mechanism of action may vary between different compds in the series, it seems likely that capacity for undisturbed π bonding between the naphthalene ring and a receptor is a requirement for antiviral activity. Thus, Ph-substituted compds containing flexible amide or amine linkages between the benzene and naphthalene rings may be inactive because the Ph groups in these compds can be twisted and cause sterical hindrance for π bonding to the naphthalene ring. In large molecules like suramin (**10**), which contain amide linkages, the extensive substitution on the Ph hampers the twisting of that group.

In the compds containing the rigid azo group between the naphthalene and the Ph, movements of the benzene ring are obviously very limited. Compds having 3 or less aromatic rings are active when at least 2 SO_3H groups and two H bonding sites (OH groups) are present on the naphthalene residue.

The antiviral effect of some of the most efficient compds in tissue culture experiments has also been evaluated in an *in vivo* test system using mice. When intracerebrally injected with herpes simplex virus the animals develop encephalitis and die of the infection after 1–2 weeks. Experiments were carried out in which virus and antiviral substances were mixed and injected intracerebrally into groups of 10–12 mice. Two control groups received virus alone or PBS buffer, respectively. All control animals receiving only PBS buffer survived, the other results are given in Table V.

Experiments have also been carried out in which suramin, chromotropic acid, and $N_2NCH_2SO_3H$ were injected intracerebrally before or after virus inoculation. No protective effect could be demonstrated in these experiments. Ip or oral administration of the substances 15 min prior to virus inocculations was also without protective effect.

The well-known anti-herpes-virus substance, 2-iododeoxyuridine (IDU), was completely ineffective when mixed with virus and tested in this system.

Effects against Adeno Virus *in Vitro*.—The large sulfonic acid molecules (1–12, Table III) are invariably

TABLE III

THE RESULTS OF *in Vitro* AND *in Vivo* TESTING OF 49 COMPDS

No.	Substance	% protection against: Cell type[b]	HSV	Cell type[b]	Rhino 33342	Cell type[b]	Adeno 3	Dose, mg/mouse ip	Influenza virus A2/Stockholm/10/63, % surviving mice (% survivors in control groups)
				Dyes etc.[a]					
1	Cloth blue R	A	0-0-tox	A	0-0-0	A	0-0-0	0.5	0 (7)
		C	0-0-0	L	0-0-10	L	0-0-0		
2	Chlorazol paper brown	A	0-0-0	A	0-0-0	A	0-0-0	0.5	20 (7)
		C	0-0-0	L	0-0-0	L	0-0-0		
3	Vital new red	A	0-20-100	A	0-0-0	A	0-10-0	0.5	0 (7)
		C	x-100-100	L	0-20-100	L	0-0-20		
4	Chlorantine fast red	A	0-0-40 (tox)	A	0-0-45	A	0-0-0	0.5	20 (7)
		C	0-0-90	L	0-0-100	L	10-15-20		
5	Trypan blue	A	0-0-100	A	0-0-0	A	0-0-0	0.5	43 (20)
		C	30-100-100	L	0-0-30	L	0-0-20		
6	Benzopurpurine	A	0-10-100	A	0-0-0	A	0-0-0	0.5	20 (7)
		C	x-30-100	L	0-10-100	L	10-10-10		
7	Evans blue	A	10-80-90	A	0-0-0	A	0-0-0	0.5	10 (7)
		C	x-100-100	L	0-0-20	L	10-0-0		
8	Indigocarmine	A	0-0-0	A	0-17-0	A	0-0-0	0.5	0 (7)
		C	0-0-0	L	0-0-0	L	0-0-0		
9	Pontamine sky blue	A	0-0-100	A	0-0-0	A	0-0-0	0.5	20 (7)
		C	x-30-100	L	0-0-20	L	0-0-0		
10	Suramin	A	0-47-100	A	20-40-tox	A	0-0-10	0.5	22 (20)
		C	10-70-100	L	x-0-100	L	0-0-0		
11	Chromotrop 2R	A	0-10-43	A	x-16-32	A	0-5-20	2.0	20 (13)
		C	x-90-100	L	50-90-90	L	10-20-25		
12	Paraorange	C	0-0-0	L	20-10-10	L	0-0-0		See Table IV
				Naphthalene derivatives					
13	1,8-$(OH)_2$-2-C_6H_5CONH-3,6-$(SO_3Na)_2$	A	5-10-0	A	5-18-85	A	60-60-83	4.0	0 (7)
		C	x-0-30	L	x-30-66	L	x-90-90		
14	1-OH-8-C_6H_5-CONH-3,6-$(SO_3Na)_2$	A	0-33-10	A	0-5-40	A	0-0-0	2.0	50 (47)
		C	x-0-10	L	x-20-26	L	x-20-20		
15	1-C_6H_5CONH-3,6-$(SO_3Na)_2$	A	0-5-0	A	0-0-0	A	0-0-0	4.0	20 (10)
		C	x-0-0	L	x-10-10	L	x-0-25		
16	1-$C_6H_5SO_2NH$-3,6-$(SO_3K)_2$	A	0-0-0	A	0-5-15	A	0-0-0	4.0	20 (10)
		C	x-0-25	L	0-0-33	L	23-10-x		
17	2-C_6H_5CONH-6,8-$(SO_3Na)_2$	A	15-33-45	A	0-0-8	A	0-0-0	4.0	0 (10)
		C	0-0-10	L	x-50-63	L	0-0-0		
18	2-$C_6H_5SO_2NH$-6,8-$(SO_3Na)_2$	A	0-0-10	A	20-20-0	A	0-0-0	4.0	0 (7)
		C	0-0-0	L	0-10-15	L	x-10-x		
19	1-C_6H_5CONH-4,8-$(SO_3Na)_2$	A	0-0-0	A	0-0-5	A	0-0-0	4.0	0 (7)
		C	0-0-0	L	x-63-20	L	x-0-30		
20	1-$C_6H_5SO_2NH$-4,8-$(SO_3Na)_2$	A	0-25-30	A	20-43-10	A	10-0-0	4.0	0 (7)
		C	0-0-0	L	30-60-68	L	0-0-0		
21	1-$C_6H_5SO_2NH$-3,6,8-$(SO_3H)_3$	A	25-0-0	A	0-0-0	A	0-0-x	4.0	11 (7)
		C	36-50-tox	L	20-50-80	L	x-60-90		
22	1,8-$(OH)_2$-2-NH_2-3,6-$(SO_3Na)_2$	A	0-0-tox	A	30-40-tox	A	0-23-tox	4.0	10 (8)
		C	x-0-30	L	x-0-33	L	65–60–90		
23	1-OH-2-NH_2-3,6-$(SO_3H)_2$	A	0-0-55	A	0-0-0	A	0-0-40	4.0	0 (7)
		C	0-0-0	L	55-65-65	L	10-90-x		
24	1,8-$(OH)_2$-3,6-$(SO_3Na)_2$	A	0-0-80	A	0-30-50	A	0-40-50	2.0	10 (20)
		C	0-10-100	L	10-30-10	L	0-20-40		
25	1-OH-8-NH_2-5,7-$(SO_3H)_2$			A	0-0-tox	A	20-0-40 (tox)	2.0	0 (14)
		C	0-0-0	L	60-80-80	L	x-20-x		
26	1-OH-6-NH_2-3,5-$(SO_3H)_2$	A	0-0-0	A	18-14-10	A	0-0-0	4.0	0 (7)
		C	0-0-0	L	55-70-70	L	30-50-x		
27	1-OH-3,6-$(SO_3Na)_2$	A	5-0-0	A	10-14-20	A	0-0-0		See Table IV
		C	0-0-0	L	10-30-x	L	x-43-x		
28	1-NH_2-3,6-$(SO_3H)_2$	A	0-0-0	A	5-5-10	A	0-0-0	4.0	0 (7)
		C	0-0-0	L	x-50-55	L	0-0-0		
29	1,3,6-$(SO_3Na)_3$	A	0-0-20	A	5-10-10	A	0-0-0	2.0	30 (20)
				L	10-5-15	L	0-0-80		
30	2-OH-3,6$(SO_3Na)_2$	A	0-0-0	A	0-0-0	A	0-0-0	2.0	33 (20)
		C	0-0-0	L	x-10-70	L	x-43-x		

TABLE III (*Continued*)

No.	Substance	% protection against: Cell type[b]	HSV	Cell type[b]	Rhino 33342	Cell type[b]	Adeno 3	Dose, mg/mouse ip	Influence virus A2/Stockholm/20/63, % surviving mice (% survivors in control groups)
31	2-NH_2-6,8-$(SO_3H)_2$	A	0-0-0	A	7-0-10			4.0	0 (7)
		C	0-0-0	L	x-55-70	L	0-0-0		
32	3,6-$(SO_3Na)_2$	A	0-0-10	L	10-10-0	L	10-10-0	4.0	10 (10)
33	1-OH-4-SO_3Na	A	0-0-0	A	0-0-15				
		C	0-0-0	L	x-63-x	L	0-0-0	2.0	20 (20)
34	1-OH-3-SO_3Na	A	0-0-0	A	0-0-0				
		C	0-0-0	L	x-44-x	L	0-0-0	2.0	50 (20)
35	1-OH-5-SO_3Na	A	0-5-10	A	0-0-0	A	0-0-0		
		C	0-0-0	L	0-0-0	L	0-0-0	2.0	20 (20)
36	2-NH_2-6-NO_2-8-SO_3Na	A	0-0-0	A	0-5-0	A	0-0-0		
		C	0-0-tox	L	30-60-10	L	10-45-30	2.7	40 (20)
37	1-OH-2,4-$(NO_2)_2$-7-SO_3Na	A	0-0-0	A	0-0-0	A	0-15-tox		
		C	20-20-tox	L	20-10-40	L	0-10-10	3.0	0 (20)
				Benzene derivatives					
38	1-OH-3-SO_3Na	A	19-10-45	L	0-0-0	L	0-0-0	2.0	10 (7)
39	1-OH-4-SO_3Na	A	0-10-20	L	10-0-0	L	0-0-0	2.0	10 (14)
40	1-NH_2-2-SO_3H	A	20-0-10	L	10-10-10	L	0-0-40	4.0	0 (0)
41	1-NH_2-3-SO_3H	A	33-17-17	L	0-0-0	L	0-0-0	4.0	10 (20)
42	1-NH_2-4-SO_3H	A	40-10-10	L	0-0-0	L	0-0-0	2.0	0 (15)
43	1,2-$(SO_3K)_2$	A	0-0-0	L	0-10-0	L	0-0-0	2.0	40 (13)
44	1-OH-2-COOH-4-SO_3Na	A	0-0-20	A	0-0-0	A	0-10-25		See Table IV
				L	0-0-0	L	0-5-30		
45	1,2-$(COOH)_2$-4-SO_3Na	A	0-0-10	L	0-0-0	L	0-0-0	1.0	20 (20)
				Aliphatic derivatives					
46	NH_2SO_3H	A	0-0-0	L	0-0-0	L	0-0-0	1.0	10 (20)
47	$NH_2CH_2SO_3H$	A	10-70-tox	A	40-50-tox				
		C	0-0-tox	L	65-90-x	L	0-0-x	0.5	10 (27)
48	$NH_2CH_2CH_2SO_3H$	A	0-0-0	L	15-10-10	L	10-10-0	2.0	10 (20)
49	$HOCH_2SO_3Na$	A	0-70-tox	L	0-0-tox	L	0-0-tox	0.5	30 (20)

[a] The tissue culture experiments were carried out with 5, 10, and 50 μg of substance per ml. In the table the degree of protection (%) is given at these concns. [b] Cell types used were: A = human amnion AV 3 cells, C = human conjunctiva cells, and L = human embryonic lung cells (x = not done; tox = toxic concn). [c] The dye names used are those in E. Gurr, "Encyclopaedia of Microscopic Stains," L. Hill Ltd, London, 1960. This reference also gives full structural formulas of the dyes.

TABLE IV

RESULTS FROM *in Vivo* STUDIES ON DIFFERENT INFLUENZA STRAINS

Influenza strain	5-Sulfosalicylic acid: Dose, mg ip	% survivors[a]	1-Naphthol-3,6-disulfonic acid: Dose, mg ip	% survivors[a]	Paraorange: Dose, mg ip	% survivors[a]	Amantadine: Dose, mg ip	% survivors[a]
A2/Stockholm/10/63	1	70 (20)	1 in	22 (0)	1 in	30 (0)	1 in	14 (0)
A2/Stockholm/10/63	1	0 (7)	2	67 (20)	2	60 (20)	1	70 (8)
A2/Stockholm/10/63	1	50 (20)	1	20 (8)	2	10 (7)		
A2/Stockholm/10/63			1	30 (20)	2	40 (20)		
A2/Hongkong/1/68	1	30 (8)	1	30 (8)	2	20 (8)	1	10 (8)
A2/Japan/305/57	1	20 (13)	1	30 (13)	2	40 (13)	1	50 (13)
A2/England/2/64	1	100 (87)	1	60 (87)	2	80 (87)	1	90 (87)
A2/Singapore/1/57 (Frankfurt)	1	60 (60)	1	90 (60)	2	100 (60)	1	60 (60)
A2/Taiwan/1/64	1	20 (33)	1	10 (33)	2	50 (33)	1	70 (33)

[a] The figures within parentheses represent percentage survivors in the control groups. All substances were administered 15 min before intranasal infection of the animals.

without effect in both the amnion and the lung cell systems. Activity is found only in compds related to chromotropic acid (**13**, **22**, **24**). There may, therefore, be a factor of sterical hindrance involved which only allows for molecules of a certain maximum size to be active. Active compds contain at least 2 H-bonding sites (OH, NH_2, or amide groups) as well as SO_3H groups. π bonding between a receptor and the naph-

TABLE V

EFFECT OF SULFONIC ACIDS ON HERPES SIMPLEX VIRUS INJECTED INTRACEREBRALLY INTO MICE (FOR DETAILS SEE EXPERIMENTAL SECTION)

Substance	Injected amount, μg	% survivors of virus and substance	% survivors of virus
Vital new red	10	30	13
	20	90	67
	30	67	42
Evan's blue	10	40	13
	20	100	67
	30	92	42
Suramin	10	55	10
	20	100[a]	67
Chromatropic acid	50	40	10
	50	100	67
Aminomethanesulfonic acid	50	50	10
	50	80	67
	10	0	13

[a] 40% of the animals died of toxic symptoms during the first few days. All the remaining animals survived.

thalene ring does not seem to be as important as with herpes simplex virus.

Effect against Rhino Virus 33342 *in Vitro*.—A rather striking difference is seen between the protective effect of a number of substances in the two *in vitro* test systems used. For instance, several of the dyes give excellent protection in the lung cell system, whereas they are completely inactive or almost inactive in the amnion cell system.

Several of the compds being protective in both test systems (**11, 13, 14, 20, 22, 24**) are closely related to chromotropic acid.

It is noteworthy that the one-carbon compd, H_2N-CH_2SO_3H, is active in both test systems. The distance between the H_2N and SO_3H groups in this type of compd is crucial (*cf.* **47, 48, 49**).

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Synthesis of Potential Anticancer Agents. 38. *N*-Nitrosoureas. 4.[1] Further Synthesis and Evaluation of Haloethyl Derivatives[2]

THOMAS P. JOHNSTON,* GEORGE S. MCCALEB, PAMELA S. OPLIGER, W. RUSSELL LASTER, AND JOHN A. MONTGOMERY

Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Alabama 35205

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Additional congeners of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) were synthesized with special emphasis on alicyclic and heteroalicyclic analogs of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), which further exemplified steric control of nitrosation. Steric control of nitrosation by noncyclic tertiary branching was also demonstrated. Attempted modifications of the nitrosoureido function were successful only in the case of 1-(2-chloroethyl)-1-nitroso-3-(*p*-tolylsulfonyl)urea (**51**), isolation of characterizable nitroso derivatives of (methylsulfonyl)-, thio-, and alkoxyureas and a nitronitrosourea being thwarted by instability. Activities of the new 2-chloroethyl- and 2-fluoroethylnitrosoureas against both intraperitoneally (ip) and intracerebrally (ic) inoculated murine leukemia L1210 were compared, in terms of the chemotherapeutic indices ED_{50}/LD_{10} and ED_{90}/LD_{10}, with BCNU, CCNU, and the isomeric mixture **6** derived by nitrosation of 1-(2-chloroethyl)-3-(2-fluoroethyl)-urea. The most effective compound against these two forms of leukemia L1210 was found to be the isomeric mixture **6** with 1-(2-fluoroethyl)-1-nitroso-3-(tetrahydro-2*H*-thiopyran-4-yl)urea *S,S*-dioxide (**25**), 1-(2-fluoroethyl)-1-nitroso-3-(tetrahydro-2*H*-thiopyran-4-yl)urea (**23**), and 3-(4-acetoxycyclohexyl)-1-(2-chloroethyl)-1-nitrosourea (**47**) being almost as active. High activity against the ip disease and slight activity against the ic disease were shown by **51**, which is another example of structural limitation to crossing the blood–brain barrier.

The synthesis of numerous congeners of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a clinically promising antineoplastic agent,[3] led to a definition of structural requirements for exceptional activity against murine leukemia L1210, implanted both intraperitoneally and intracerebrally.[4] Such activity was limited, for the most part, to 1-(2-haloethyl)-1-nitrosoureas substituted in the 3 position by a 2-haloethyl or an alicyclic group, the halogen atom being either Cl or F; for example, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU),[4] which is also undergoing clinical trials, was particularly effective against both forms of leukemia L1210.

Chemistry.—Further synthesis in this area made available the additional haloethylnitrosoureas (from haloethylureas of Tables I and II) of Tables III and IV for comparative evaluation against experimental animal tumor systems. Nitrosations were carried out in undiluted HCO_2H with $NaNO_2$, a system known to minimize random nitrosation of chloroethylureas substituted at the 3 position by cyclic groups.[4] Such steric control was apparently operative also in the nitrosation of 1,1′-(2-chlorotrimethylene)bis(3-cyclohexylurea) (1), since decomposition of the product with cyclohexylamine gave 1,3-dicyclohexylurea, the product expected from structure **2**.[5] The conversion of 1-(2-chloroethyl)-3-(α,α-dimethylphenethyl)urea (**3**)

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(5) The structure of **2** could not be definitely decided by pmr spectroscopy[4] because of overlapping of signals, but the NH protons appeared to be split by single (1-cyclohexyl) protons and not by two (CH_2) protons.