TABLE I. The Ascorbic Acid of Urine, White Cells, and Serum of 4 Subjects Receiving 1000 mg Ascorbic Acid per Day.

Day of exp.	Urine, mg/day	White cells,* mg %	Serum after 400 mg test dose (mg %) 0	1 hr	3 hr	5 hr	7 hr
0		27 ± 2†	1.22 ± .09	1.78 ± .13	1.97 ± .11	1.80 ± .06	1.63 ± .04
5	817‡ ± 35†	28 ± 3	1.81 ± .09	2.45 ± .09	2.39 ± .08	2.14 ± .11	1.94 ± .04
21	804 ± 25	30 ± 1	1.79 ± .06	2.28 ± .11	2.35 ± .05	2.19 ± .11	2.03 ± .04
39	714§ ± 35	28 ± 1	1.82 ± .13				
55		28 ± 2	1.54 ± .08				
67		31 ± 1	1.93 ± .15	2.45 ± .17	2.48 ± .20	2.19 ± .24	2.09 ± .26
98	822 ± 47	28 ± 1	1.64 ± .10	2.24 ± .99	2.14 ± .08	1.83 ± .13	1.76 ± .06

* White blood cells + platelets. † ± Stand. error. ‡ 3rd day of exp. § 43rd day of exp.

persons, at least, no harmful effects whatever were observed during the 3 months with 1000 mg daily intake.

1. Abt, A. F., and Farmer, C. J., *J.A.M.A.*, 1938, v111, 1555.

2. Wilson, M. G., and Lubschez, R., *J. Clin. Invest.*, 1946, v25, 428.

3. Bessey, O. A., Lowry, O. H., and Brock, M. J., *J. Biol. Chem.*, 1947, v168, 197.

4. Lowry, O. H., Lopez, J. A., and Bessey, O. A., *J. Biol. Chem.*, 1945, v160, 609.

Received May 29, 1952. P.S.E.B.M., 1952, v80.

α-Aminosulfonic Acids and Viral Propagation.* (19624)

W. W. ACKERMANN. (Introduced by T. Francis, Jr.)
(With the technical assistance of Hilda Kurtz, Elaine Kleinschmidt, and B. W. Uhlendorf.)

From the Department of Epidemiology and Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor, Mich.

In the case of methionine(1-3) and tryptophane(4), it has been shown that metabolites as small as these amino acids are involved in the propagation of animal viruses and that analogs of these compounds will inhibit viral multiplication. A number of interesting analogs of amino acids have been prepared by McIlwain. *In vitro* these α-aminosulfonic acids are potent inhibitors of bacterial growth (5). Further α-aminophenylmethanesulfonic acid and α-aminoisobutanesulfonic acid which resemble in structure phenylalanine and valine have been reported to inhibit the multiplication of vaccinia virus in tissue culture(6). An analog of glycine, α-aminomethanesulfonic acid, will inhibit the propagation of a bacteriophage of *B. coli*(7). The mode of action of these compounds to the present has not been clearly explained, but there is evidence to show that they interfere with amino acid metabolism.

Three α-aminosulfonic acids reminiscent in structure of tyrosine or phenylalanine have been synthesized and tested as inhibitors of viral propagation in various biological systems. In the following, the results of these studies are reported, and their significance is discussed.

Virus and tissue. The PR8 strain of Type A influenza virus was selected for those studies in which the Warburg flask culture or the embryonate egg was employed. After isolation from man, this virus had undergone 7 passages in ferrets, 593 passages in mice, and 131 passages in eggs. For the experiments performed with mice, the WS strain of Type A influenza virus was used. This virus was cultured for several years in tissue culture and has undergone 99 passages in mice(8). The host cells for the Warburg culture were those of the chorioallantoic membrane and were

* Aided by a grant from the National Foundation for Infantile Paralysis.

TABLE I. Inhibition of the Propagation of Influenza Virus in Tissue Culture by Aminosulfonic Acids. Duration of each exp. approximately 26 hr; initial titer* in each exp. approximately 2.7 logs.

Inhibitor	α-amino-*p*-methoxyphenylsulfonic acids					
Molar conc. on $\times 10^4$	0	1.4	2.8	5.7	8.5	14
Final egg infectivity titer*	8.5	7.7	6.5	5.5	3.5	2.7
Inhibitor	α-aminophenylmethanesulfonic acid					
Molar conc. $\times 10^4$	0	.8	1.6	3.2	6.4	9.6
Final egg infectivity titer	8	7.3	6.5	6	5.5	2.3
Inhibitor	α-amino-β-phenylethanesulfonic acid					
Molar conc. $\times 10^4$	0	.7	1.5	3	6	9
Final egg infectivity titer	9	7.7	6.3	5.8	3.7	1.5

* Titers expressed as the log of the reciprocal of the dilution.

TABLE II. Inhibition of Propagation of Influenza Virus in the Embryonate Egg by Aminosulfonic Acids. Duration of the experiment was 28 hr. Each egg received an inoculum of .1 ml of virus of titer‡ 2.

		Inhibitor conc., mg/egg									
		0	2	4	6	8	12	16	20	24	28
A*	Fraction of eggs dead	0/10	0/10	0/10	0/10	0/10	0/10	1/10	1/10	0/10	3/10
	" " " with RBCT†	10/10	4/10	4/10	1/10	0/10	0/10	0/10	0/10	0/10	0/10
	Egg infectivity titer‡	9.2	8	7.6	6.4	.5	0	0	0	0	0
B*	Fraction of eggs dead	0/10	0/10	0/10	1/10	0/10	1/10	3/10	3/10	3/10	4/10
	" " " with RBCT†	10/10	9/10	7/10	3/10	0/10	0/9	0/10	0/10	0/10	0/10
	Egg infectivity titer‡	9.7	9.5	9.5	8.5	1.5	.5	2.3	0	0	0
C*	Fraction of eggs dead	1/10	0/10	1/10	2/10	0/10	4/10	5/10	7/10	8/10	9/10
	" " " with RBCT†	9/10	5/10	5/10	1/10	0/10	0/10	0/10	0/10	0/10	0/10
	Egg infectivity titer‡	9.7	9.7	9.3	8.3	4.8	0	0	—	—	—

* A = α-amino-*p*-methoxyphenylmethanesulfonic acid. B = α-aminophenylmethanesulfonic acid. C= α-amino-β-phenylethanesulfonic acid.
† RBCT—positive hemagglutinin. ‡ Titer expressed as the log of the reciprocal of the dilution.

TABLE III. Effect of α-amino-*p*-methoxyphenylmethanesulfonic Acid on Respiration of the Chorio-Allantoic Membrane. Duration of exp. 28 hr.

Molar conc. of aminosulfonic acid $\times 10^4$	Oxygen uptake*	Final virus titer egg infectivity† (initial = 3)
0	21.6	7.3
2.8	20.9	5.7
5.7	18.9	4.7
8.5	19.8	2.7

* μl of oxygen/200 mg of tissue/30 min.
† Titer expressed as the log of the reciprocal of the dilution.

obtained from 14-day-old embryonate chicken eggs. *Warburg flask culture.* In some experiments the host-virus system was maintained in the flasks of a Warburg apparatus using a modified Simms' medium. The details of this method have been reported previously(1,9). Each flask contained 200 mg of chorioallantoic membrane which was used intact without mincing. The cultures were incubated at 37°C with shaking, and the duration of each experiment is given in the corresponding tables. The inoculum was prepared by making suitable dilutions of infected allantoic fluid in Simms' solution. In all instances 0.3 ml of inoculum was used per flask, and the final volume of fluid and tissue was 3.0 ml. *Virus titer.* The amount of virus was estimated by determining the infectious titer for eggs. For this purpose 10-fold serial dilutions of the virus were prepared in broth and 4 eggs were inoculated with 0.1 ml of each dilution. After 3 days of incubation at 37°C samples of allantoic fluid were removed from each egg. These fluids were tested for virus by the addition of red blood cells. The 50% infectious titer was calculated using the method of Reed and Muench(10). Virus titers were also estimated by red cell agglutination using a pattern method(11). *Oxygen uptake.* The respiration of the cultures was measured manometrically in the conventional manner with the Warburg apparatus. *Compounds tested.* α-Aminophenylmethanesulfonic acid was prepared by the method of McIlwain(5). α-

Amino-*p*-methoxyphenylmethanesulfonic acid was prepared in this laboratory from anisaldehyde using the procedure described by McIlwain for the synthesis of α-aminoisobutanesulfonic acid. The product was recrystallized from ethanol and water. Analysis calculated for the monohydrate: $C_8H_{11}O_4SN \cdot H_2O$

	Calculated	Found
C	40.84	41.76
H	5.58	5.58
N	5.95	5.87

The author is indebted to Parke, Davis and Company for supplying a sample of α-aminophenylethanesulfonic acid and for supplying amounts of the other analogs in sufficient quantity for animal testing.

Results. Effect of aminosulfonic acids on influenza virus in vitro. Prior to testing the effect of the aminosulfonic acids on the host-virus system, it was desirable to know what interaction such organic structures might have with the virus alone. Since these compounds act similarly and have the same reactive groups, only data for α-amino-*p*-methoxyphenylmethanesulfonic acid will be presented. Various concentrations of this sulfonic acid were prepared in 0.15 M saline. After the pH had been adjusted to 7.0, the preparations were added to equal volumes of allantoic fluid containing influenza virus. Control samples were made by mixing equal amounts of infected allantoic fluid and isotonic saline. Some of the mixtures were incubated for 18 hours at 4°C and others for the same duration at 37°C. At the end of the incubation period, the titers of infectivity for eggs and of hemagglutination for red blood cells were determined. The maximum concentration of inhibitor used, 8.5 x 10^{-3} molar, was found to have no effect on the infectivity or hemagglutinating properties of the virus. This demonstrates that the sulfonic acid is not virucidal under these conditions in that it does not destroy influenza virus on contact.

Effect of aminosulfonic acids on the propagation of influenza virus in tissue culture. In 3 separate experiments the multiplication of influenza virus in Warburg flask cultures was estimated in the presence and absence of various concentrations of aminosulfonic acids. The inhibitors, the virus, and the tissue were added in that order, while the flasks were immersed in an ice bath. When all additions were completed, the flasks were transferred to the water bath at 37°C. Each of the compounds was found to be inhibitory at approximately the same concentration. α-amino-β-phenylethanesulfonic acid produced inhibition at slightly lower concentrations than did the other analogs. This sulfonic acid at a 9.0 x 10^{-4} molar concentration completely suppressed viral multiplication and showed some inhibitory effect at concentrations as low as 0.7 x 10^{-4} molar (Table I). The ability to recover from a completely inhibited culture the virus which was added in an inoculum (Table I), is further evidence that the action of these sulfonic acids is not virucidal.

Effect of aminosulfonic acids on influenza virus propagation in the embryonate egg. In view of the antiviral activity of these inhibitors in tissue culture (Table II), their effect on the propagation of virus in the embryonate egg was studied. Ten eggs, which were employed for each concentration of inhibitor tested, were inoculated with influenza virus via the allantoic route. Fifteen minutes later appropriate dilutions of the aminosulfonic acids, prepared at pH 7.0 in saline, were injected at the same site. After an incubation period of 28 hours, the eggs were chilled and samples of allantoic fluid were removed. The fraction of eggs which died during the incubation period and the fraction with hemagglutinin were recorded. The viral infectivity for eggs was determined on samples of allantoic fluid pooled from each group. These data for each aminosulfonic acid are recorded in Table II. The 50% lethal dose per egg of α-amino-*p*-methoxyphenylmethane sulfonic acid was approximately 28 mg. Concentrations of this analog as low as 4 mg per egg showed some inhibitory effect on the yield of virus produced. When 8 mg of the compound were employed, complete inhibition of the formation of hemagglutinin in each egg was observed. Under the same conditions, the infectivity titer was $10^{-0.5}$, while that of the control was $10^{-9.2}$. This compound is of particular interest, since the amount which shows toxicity for the embryo is about 6 times that which

will inhibit the multiplication of influenza virus in the embryonate egg (Table II). The other aminosulfonic acids produced similar results. However, α-amino-β-phenylethanesulfonic acid was definitely more toxic to the embryo than either substituted methanesulfonic acid. The increased toxicity for the host was not paralleled by increased anti-viral activity (Table II).

Effect of aminosulfonic acids on influenza virus propagation in the brains of mice. Eighty mice were inoculated intracerebrally with the WS strain of Type A influenza virus. Three groups of 20 mice were inoculated by the same route one hour later with 300 μg of one of the 3 aminosulfonic acids, while one group was inoculated with saline. Eighty-five per cent of the control animals died, and similar results were obtained with 2 of the analogs. Of the group of mice receiving α-amino-β-phenylethanesulfonic acid only 45% succumbed. In a series of 3 similar experiments 15, 13, and 20% of the control animals survived, while 55, 52, and 56% of those treated with the ethanesulfonic acid derivative survived. The compound was without effect when administered intraperitoneally or when incorporated into the diet. It did not affect the survival of animals receiving an inoculum of virus which produced an incidence of death much higher than 85%.

Concerning the mode of action of the aminosulfonic acids. The α-aminosulfonic acids are not stable structures in aqueous solvent at elevated temperatures(5). Hence, it was considered that their antiviral activity might be due to the aldehydes which are formed by decomposition. Anisaldehyde, which results from the decomposition of α-amino-*p*-methoxyphenylmethanesulfonic acid, was tested in tissue culture and found to be inactive at 12 x 10^{-4} molar concentration. This amount of the corresponding aminosulfonic acid will produce complete inhibition (Tables I and II). Further, the aldehyde which showed no antiviral activity was found to be quite toxic for the embryonate egg. Ammonium sulfate and sodium bisulfite were both tested in eggs and found to be inactive at concentrations corresponding to effective inhibitory levels of the aminosulfonic acids. Thus, the decomposition products cannot account for the antiviral activity of this sulfonic acid. However, their instability may explain the difference in toxicity of the 3 aminosulfonic acids for the host, since the most toxic of the compounds (α-amino-β-phenylethanesulfonic acid) also appears to be the least stable of the 3 inhibitors.

In view of the structural relation of the sulfonic acids to phenylalanine and tyrosine, attempts were made to prevent or reverse their inhibitory action in tissue culture by the use of the corresponding aromatic amino acids. All attempts in this direction were without success. The action of 5.7 x 10^{-4} molar α-amino-*p*-methoxyphenylmethanesulfonic acid could not be prevented by the addition of 10 times that amount of tyrosine or phenylalanine.

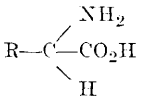
A number of compounds which interfere with the oxidative processes of the Krebs cycle inhibit viral multiplication(9,12). These aminosulfonic acids apparently do not act via that principle, since the respiration of the chorioallantoic membrane is unaltered by concentrations of α-amino-*p*-methoxyphenylmethanesulfonic acid which produced complete inhibition of viral propagation (Table III). Since the endogenous respiration of tissue will not continue in the medium if the cellular membranes are disrupted, no gross damage to the host cells seems to be involved when they are maintained in the presence of the compound. This conclusion is further supported by the low toxicity *in vivo* of the sulfonic acid for the embryonate egg (Table II).

Discussion. The action of these α-aminosulfonic acids is not virucidal. Rather, the effect is directed toward some site of the host cell which is essential for the viral synthetic process. That this is a desirable site for chemotherapeutic action is indicated by the low toxicity of amounts of these compounds which cause inhibition of viral propagation in the egg (Table II).

The inability of ammonium ions, bisulfite ions, and the aldehyde precursor to inhibit demonstrates the essentiality of the intact α-aminosulfonic acid structure for anti-viral activity. In contrast the second substituent on the α-carbon can be varied considerably

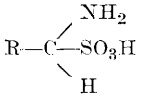
without a resulting quantitative alteration of the inhibition produced in "closed" biological systems (the embryonate egg and tissue culture).

The results obtained with these analogs are intelligible, if one assumes that their effect is directed toward an enzyme which has only a limited specificity for its substrate. Certain enzymes possess only limited specificity in that they act upon a number of different metabolites. For example the d- and l-amino acid oxidases are specific for α-amino acids, but their activity is not confined to any particular amino acid. Such enzymes may be quite specific for certain functional groups or parts of the substrate, presumably those groups which are common to all susceptible substrates. In the example of the α-amino acid oxidase, it is the α-amino carboxylic acid group which is required.



For this type of enzyme where the enzyme-specific grouping of the substrate is known, it should be possible to design a variety of inhibitors. They would be composed of 2 elements. One would have the geometric and electronic specifications necessary for enzymatic association and would be an analog of the enzyme-specific portion of the metabolite. The second element would be a non-enzyme-specific portion corresponding to the R moiety of the amino acid. Thus, modifications in the R grouping would lead to a number of inhibitors each capable of action at the enzyme site, but greatly differing in physical properties.

McIlwain reported the synthesis of a series of α-aminosulfonic acids which were found to be potent inhibitors of bacterial growth(5).



Further, the effect of these various analogs was specific only in that it was confined to action on the amino acids. Those which were most effective in alleviating the inhibition produced by any particular α-aminosulfonic acid did not necessarily correspond to the amino acids which bore the closest structural relation to the analog. These findings may be consistent with the concept outlined above. The α-aminosulfonic acids may act upon a single enzyme in the cell which normally has multiple substrates. It is not likely that such an inhibition would be completely reversed by any single substrate.

From the viewpoint of chemotherapy a useful inhibitor must possess, in addition to the proper geometry, a number of chemical and physical properties associated with permeability and solubility. Frequently, it is difficult to alter these physical properties by making structural changes without affecting the essential geometrical specifications. Hence, it may be more feasible to design useful inhibitors for those enzymes which possess only limited substrate specificity than for those with a higher degree of substrate selectivity.

Summary. Three α-aminosulfonic acids (α-aminophenylmethanesulfonic acid, α-amino-β-phenylethanesulfonic acid, and α-amino-*p*-methoxyphenylmethanesulfonic acid) were prepared and tested as inhibitors of the propagation of influenza virus. Each was found to cause marked inhibition of viral synthesis in tissue culture at a concentration of 5×10^{-4} molar. In the embryonate egg 8 mg of each compound completely inhibited the multiplication of influenza virus, although the 50% lethal dose for eggs of α-amino-*p*-methoxyphenylmethanesulfonic acid was approximately 30 mg per egg. Under special conditions, α-amino-β-phenylethanesulfonic acid afforded some protection to mice which were infected with the WS strain of Type A influenza virus. These sulfonic acids are not virucidal, and they do not affect the respiration of tissues cultured in the Warburg flask and are not reversed by tyrosine nor phenylalanine to which they are structurally related. Ammonium ions, bisulfite ions, and *p*-methoxybenzaldehyde did not affect viral propagation. A possible mode of action of these amino acid analogs is discussed.

1. Ackermann, W. W., *J. Exp. Med.*, 1951, v93, 337.

2. Brown, G. C., and Ackermann, W. W., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 367.

3. Rafelson, M. E., Jr., Pearson, H. E., and Winzler, R. J., *Arch. Biochem.*, 1950, v29, 69.

4. Rasmussen, A. F., Jr., Clark, P. F., Smith, S. C., and Elvehjem, C. A., *Bact. Proc.*, 1951, 93.
5. McIlwain, H., *Brit. J. Exp. Path.*, 1941, v22, 148.
6. Thompson, R. L., *J. Immunol.*, 1947, v55, 345.
7. Spizizen, J., *J. Infect. Dis.*, 1943, v73, 212.
8. Francis, T., Jr., and Moore, A. E., *J. Exp. Med.*, 1940, v72, 717.
9. Ackermann, W. W., *J. Biol. Chem.*, 1951, v189, 421.
10. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.
11. Salk, J. E., *J. Immunol.*, 1944, v49, 87.
12. Ackermann, W. W., *J. Exp. Med.*, 1951, v93, 635.

Received May 16, 1952. P.S.E.B.M., 1952, v80.

Antimalarial Activities of Triazine Metabolites of Chlorguanide and Dichlorguanide.* (19625)

L. H. SCHMIDT, T. L. LOO, ROCHELLE FRADKIN, AND HETTIE B. HUGHES.
From The Christ Hospital Institute of Medical Research, Cincinnati.

During a study of the metabolic fate of the antimalarial drug chlorguanide, Carrington and coworkers(1) isolated a compound from the urine of humans and the feces of rabbits which was believed to be the hypothetical "active metabolite"(2-4) of the above drug. The isolated compound, tentatively identified as 2,4-diamino-1-p-chlorophenyl-1,6-dihydro-6,6-dimethyl-1,3,5-triazine (*CPT*), was reportedly 10 times as active as chlorguanide against infections with *Plasmodium gallinaceum* in the chick. These workers also described a second compound, 2,4-diamino-1-(3, 4-dichlorophenyl)-1, 6-dihydro-6, 6-dimethyl-1.3,5-triazine (*DCPT*), which was considered (5) to be the corresponding metabolite of dichlorguanide(6) and which was 50 times as active as the latter drug and 100 times as active as chlorguanide against infections with the above plasmodium(1,5).†

These findings were of considerable interest to this laboratory, which since 1946 has studied the metabolic fate of chlorguanide and related biguanides in man and rhesus monkey. Although this investigation(8,9) had pointed to the pattern of metabolic degradation described by Carrington, it had failed to provide any evidence for the presence in urine, feces, sera or tissues of metabolites more active than their parent drugs. Since this conclusion was based on assessments of activity in *P. cynomolgi* infections in the rhesus monkey(9), whereas that of Carrington and Davey was based on work with *P. gallinaceum*, the question arose whether the divergent findings were related to the use of different test objects. This led to a study of the activities of the triazines, *CPT* and *DCPT*, against infections with *P. cynomolgi*, the results of which are presented in this report.

Materials and methods. The comparative antimalarial activities of *CPT* and chlorguanide were measured in a group of 18 rhesus monkeys (3.5 to 4.8 kg), each of which was inoculated intravenously with approximately 500,000 trophozoites, obtained by suitable dilution in 0.85% saline of the blood of a passage monkey infected with the stock strain of *P. cynomolgi*.‡ Through use of conventional thick and thin blood films stained with Giemsa, parasitemias were followed daily until a density of approximately 100 parasites per 10,000 erythrocytes was attained. At this point, groups of 8 monkeys were treated either with *CPT* or with chlorguanide, the 2 remaining animals serving as untreated controls. Because previous studies(9) had shown that absorption of orally administered dihydrotriazines was uncertain, each drug was adminis-

* This study was supported in part by a grant-in-aid from the National Institutes of Health, U. S. Public Health Service.

† The activities of these triazines against *P. gallinaceum* infections have been confirmed recently by Coatney(7).

‡ The strain of *P. cynomolgi* was obtained June 1946 from Dr. R. J. Porter, University of Michigan. Since that time it has been maintained by serial intravenous trophozoite passages through untreated rhesus monkeys at intervals of 2 to 4 weeks.