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140. Hidetaka Yuki,[*3] Torahiko Kishikawa, Yasuo Tohira, Seikichi Suzuki, Fumihiko Sano, and Shigeru Hayakawa : Studies on Antiviral Agents. V.[*1] Synthesis and Antiviral Activity of N-Chloro Compounds.

(*Research Laboratories, Chugai Pharmaceutical Co., Ltd.*[*2])

N-chloro compounds were synthesized and their virus inactivating activity and probable mechanism of the action were investigated. It is likely that the N-chloro compounds oxidize the sulfhydryl groups of the viral protein coat to form disulfide, disulfoxide, and sulfinic acid resulting in inactivation of the virus. This action is nonspecific and irreversible, and is lost in the presence of serum proteins.

(Received May 19, 1966)

In the part I[1] of this series, it has been shown that sodiun N-chloro-*p*-toluenesulfonylamide (chloramine T) and other N-chloro compounds have marked antiviral activity against adenovirus type 5. Up to date, several papers dealing with virus inactivating activity of chloramines have been presented. Bucca[2] reported that 2 p.p.m. of available chlorine liberated from calcium hypochlorite or chloramine T resulted in 4 logs decrease of infectivity of eastern equine encephalomyelitis virus after one hour contact period. Vaccinia virus was also killed within thirty minutes by the action of 3% chloramine solution.[3] Lund[4] discussed the inactivating effect of chloramine from a view point of its oxidizing power, and Cook, *et al.*[5] referred to the biological activity of chlorine-liberating compounds. To shed further light on these, a variety of N-chloro compounds were synthesized, and their antiviral activity and its probable mechanism were investigated. These are reported below.

Synthesis of N-Chloro Compounds

Chattaway synthesized a number of N-chloro compounds by reaction of the corresponding N-H compounds with bleaching powder or sodium hypochlorite in a sodium bicarbonate solution[6,7] or in glacial acetic acid.[8] These synthetic methods were essentially followed by us. In the first place, the N-chloro compounds of acetanilide (I-1), benzanilides (I-2～6) and, 4,4′-dibenzamidodiphenylsulfone (II) were synthesized, but the chlorines of these aromatic amides, as Chattaway described,[6] were so labile that they were shortly rearranged to ortho or para-position of the benzene ring. So, the biological tests of these compounds had to be done shortly after the preparation. Thereafter, preparation of the N-chloro compounds was oriented

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[*1] Part IV : This Bulletin, **15**, 1120 (1967).

[*2] Takadaminami-cho, Toshima-ku, Tokyo (由岐英剛, 岸川虎比古, 東平靖雄, 鈴木清吉, 佐野文彦, 早川繁).

[*3] Present Address : Faculty of Pharmaceutical Sciences, Osaka University, Toneyama, Toyonaka-city, Osaka.

1) This Bulletin, **14**, 139 (1966).
2) M.A. Bucca : J. Bacteriol., **71**, 491 (1956).
3) St. Angeloff, *et al.* : Arch. Exptl. Veterinarmed., **10**, 365 (1956).
4) E. Lund : Acta Pathol. Microbiol. Scand. Suppl., **154**, 349 (1962).
5) A.M. Cook, W.R. Brown : J. Pharm. Pharmacol., **16**, 611 (1964).
6) F.D. Chattaway, K.J.P. Orton : J. Chem. Soc., **75**, 1046 (1899).
7) *Idem* : *Ibid.*, **79**, 274 (1901).
8) F.D. Chattaway : *Ibid.*, **87**, 381 (1905).

toward that of aliphatic amines. As the aliphatic amines, methyl- and cyclohexylamine, ethylenediamine, tri- and hexamethylenediamine were chosen. As N-chloro compounds of amides are more stable than that of amines, those amines were led to the amides by reaction with benzoyl chloride, benzenesulfonyl chloride, and its para substituted compounds, and then to the corresponding N-chloro compounds (V and VII). Two chlorines were introduced to IV-5 by the reaction with hypochlorite yielding V-5, while only one chlorine was introduced to IV-4 yielding V-4 under the same reaction condition. The compound (V-4) is insoluble in an alkaline solution indicating disappearance of N-H bond of sulfonamide. From this, the structure of the chlorinated compounds was determined as V-4. In the cases of N,N′-bis(*p*-acetamidobenzenesulfonyl)trimethylenediamine (VI-6) and hexamethylenediamine (VI-7), two chlorines were introduced for four N-H bonds, and the products (VII-6 and 7) were insoluble in an alkaline solution as in the case of V-4. Since the above-mentioned compounds are extremely insoluble in water, preparation of N-chloro compounds of amino acids was attempted, but the only compound obtained in a crystalline form was N-benzenesulfonyl-N-chloro-*β*-alanine (V-6).

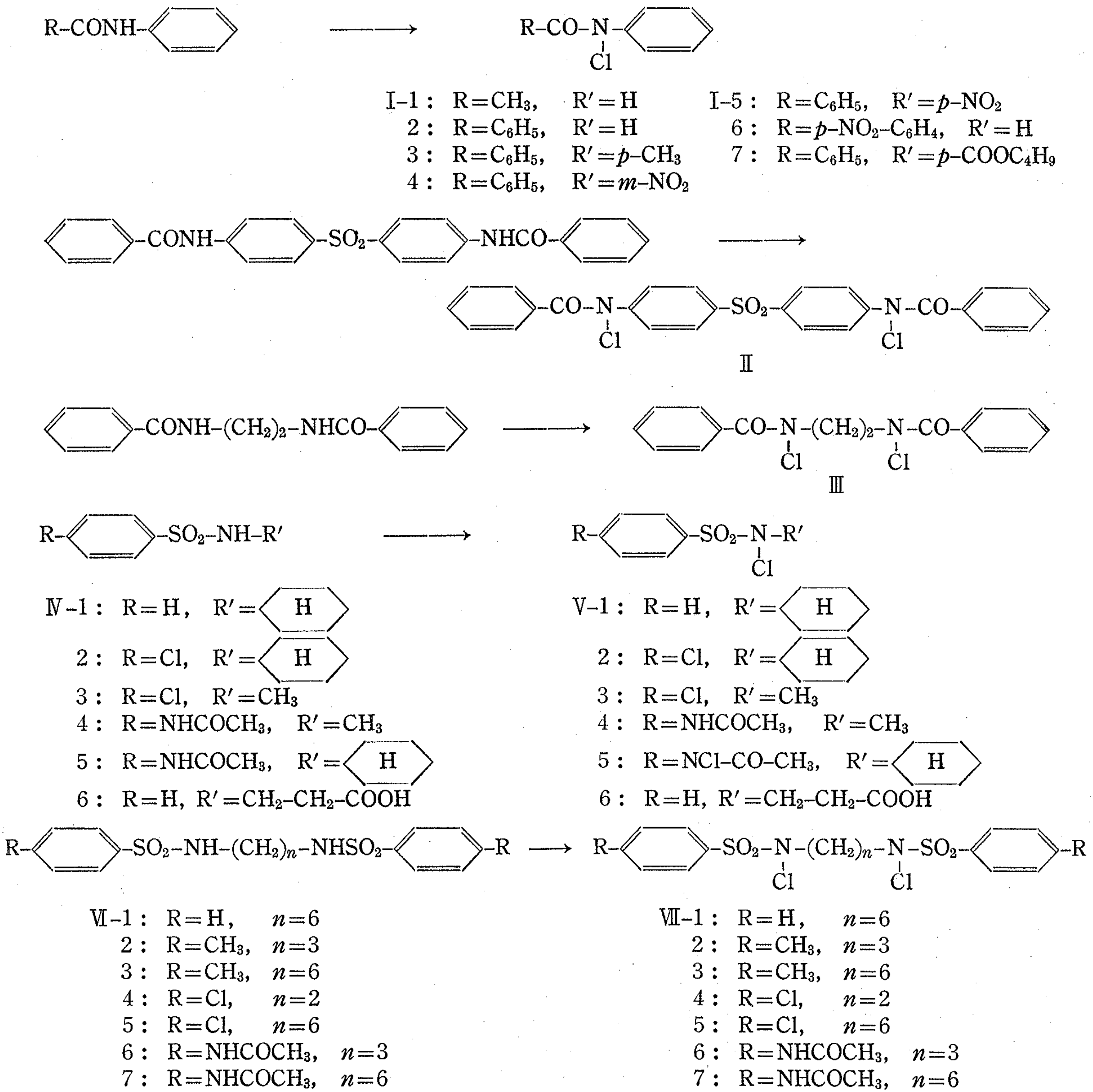

Chart 1.

Table I. N-Chloro Compounds

Compounds	Synthetic method	m.p. (C°)	Analysis Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
I-2	A	78~80	67.42	4.32	6.05	67.71	4.60	5.72
I-3	A	101~104	68.46	4.89	5.70	68.38	4.85	5.51
I-4	A	99~102	56.45	3.25	10.12	56.89	3.41	10.42
I-5	A	98~103.5	56.45	3.25	10.12	56.75	3.29	9.87
I-6	A	152~155	56.45	3.25	10.12	56.25	3.39	10.34
I-7	A	76~82	65.19	5.43	4.22	64.99	5.37	4.14
II	A	167~169	59.45	3.42	5.33	59.25	3.32	5.22
III	B	162	57.01	4.06	8.11	57.37	4.14	8.13
V-1	B	42	52.65	5.85	5.23	52.79	5.85	5.41
V-2	B	94~99	52.17	5.47	5.10	51.58	5.58	4.87
V-3	B	60~62	35.01	2.94	5.83	35.34	2.95	5.58
V-4	B	140~141	41.30	4.21	10.71	40.64	4.02	10.45
V-5	B	136~137	50.82	5.79	8.47	50.64	5.97	8.74
V-6	A	153~154	40.99	3.82	5.31	41.33	4.04	5.51
VII-1	B	161~163	46.45	4.76	6.02	46.61	4.61	5.77
VII-2	B	99~103	45.23	4.47	6.21	45.19	4.78	6.05
VII-3	B	138~142	48.68	5.31	5.68	48.78	5.68	5.57
VII-4	B	141~142	35.02	2.94	5.83	35.24	2.65	5.90
VII-5	B	128~130	40.46	3.77	5.24	39.96	3.76	5.31
VII-6	B	161~163	41.69	4.05	10.23	42.29	4.19	10.38
VII-7	B	205~208	45.59	4.85	9.68	45.17	4.86	9.49

The virus inactivating activity in phosphate buffer saline (PBS) solution of the compounds synthesized and other N-chloro compounds purchased was shown in the second column of Table II. Assay was carried out by the method described previously.1) Many compounds have a marked antiviral activity.

Lund4) reported that the inactivating effect of chloramine may be attributed to its oxidizing power, and Cook, *et al.*5) deduced that the site of the attack on phage by chloramine T would be the viral protein coat. The most sensitive functional group for the oxidizing agents in protein is the sulfhydryl group of cysteine moiety. Therefore, in order to deduce the mechanism of inactivation of virus by N-chloro compounds, chemical reaction of chloramine T and N-chlorosuccinimide with cysteine at the physiological condition was investigated. The reaction was carried out in the PBS solution of the physiological pH at room temperature. The reaction products were examined by paper electrophoresis, and the rates of their movement were compared with those of authentic samples. If N-chloro compound acts as an oxidizing agent, oxidized products such as cystine, cystinedisulfoxide, cysteine sulfinic acid, and cysteic acid would be produced. Bulmer and Mann9) found that benzylthiol, when treated with 0.5 mol. of chloramine T, is oxidized to dibenzyldisulfide (IX), but with 2.5 mols. is converted to benzyl *p*-toluenesulfonimidosulfine-*p*-toluenesulfonylimine (X), and that the disulfide (IX) when treated with an excess (4 mols.) of this reagent also furnished the compound (X). Therefore, there is a possibility to form the compounds of this type by the reaction of cysteine and chloramine T. The pattern of the electrophoresis, however, indicated formation of cystine, cystinedisulfoxide, and cysteinesulfinic acid after 30 minutes of the reaction, and any other product could not be detected. The spot of cystine disulfoxide disappeared after 45 minutes of the reaction. This means

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9) G. Bulmer, F.G. Mann: J. Chem. Soc., **1945**, 666.

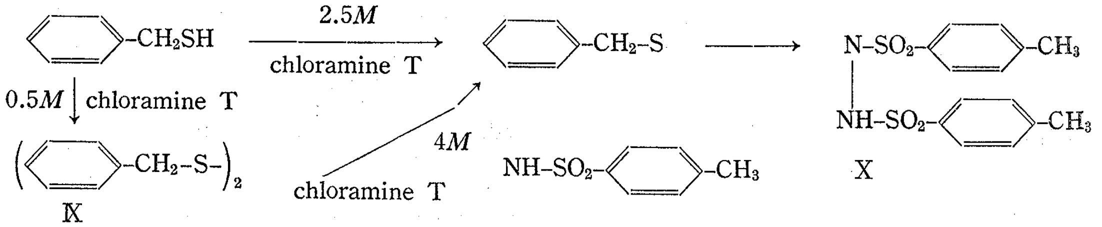


Chart 2.

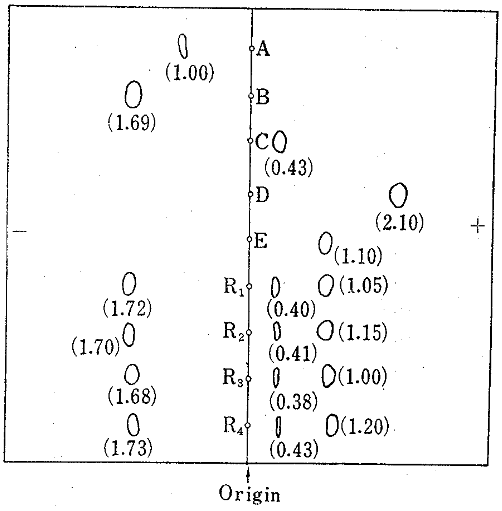


Fig. 1. Paper Electrophoretic Pattern of Cysteine, Cystine, Cystine Disulfoxide, Cysteic acid, Cysteine Sulfinic Acid and the Reaction Mixtures at 30 min. after the Beginning of the Reaction

A buffer solution of AcOH～HCOOH～water (15:5:80) (pH 1.5) was used at 50V/cm. for 25 min. Detection of the spots was made by the ninhydrin reagent. The migration rates are quoted relative to cysteine (R cyst=1.00). A, cysteine; B, cystine; C, cystine disulfoxide; D, cysteic acid; E, cysteine sulfinic acid; R_1, a reaction mixture of N-chlorosuccinimide ($2\times10^{-3}M$) and cysteine ($1\times10^{-3}M$) in PBS buffer, pH 7.2; R_2, a reaction mixture of chloramine T ($2\times10^{-3}M$) and cysteine ($1\times10^{-3}M$) in PBS buffer, pH 7.2; R_3, a reaction mixture of NaOCl or bleaching powder ($2\times10^{-3}M$) and cysteine ($1\times10^{-3}M$) in PBS buffer, pH 7.2; R_4, a reaction miture of N-chlorosuccinimide or chloramine T ($2\times10^{-3}M$) and cysteine ($1\times10^{-3}M$) in PBS buffer followed by treatment with sodium thiosulfate ($2\times10^{-2}M$) for 30 min.

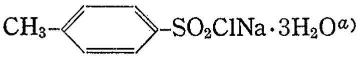
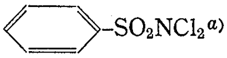
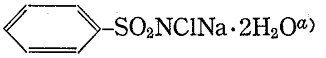
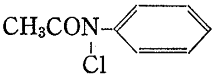
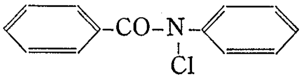
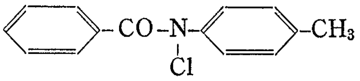
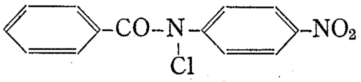
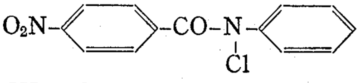
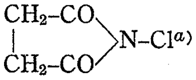
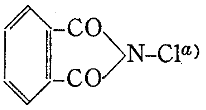
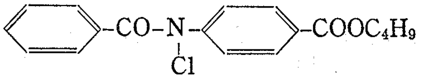
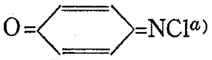
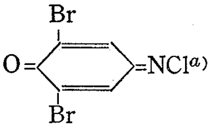
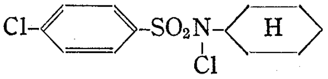
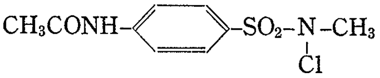
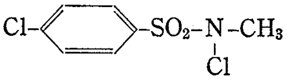
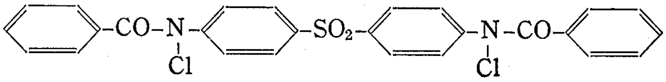
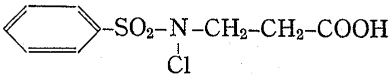
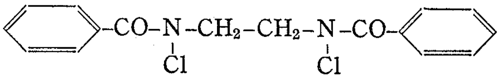
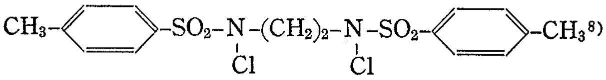
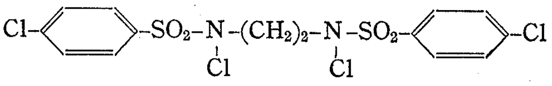
that N-chloro compounds acted only as an oxidizing agent, and did not undergo the exchange reaction to form a sulfonimidosulfine compound. The reaction products of cysteine with bleaching powder or sodium hypochlorite were identical with that of chloramine T or N-chlorosuccinimide supporting further that the virus inactivating activity of N-chloro compounds is due to their oxidizing action.

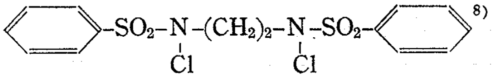
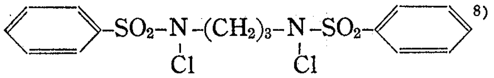
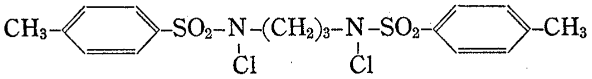
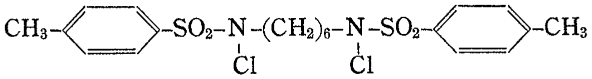
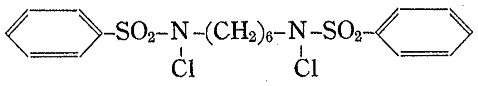
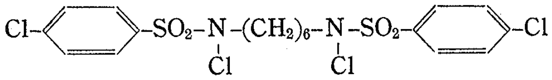
As long as the N-chloro compounds act as oxidizing agents, the inactivated virus may be reactivated by action of reducing agents. To examine this, sodium thiosulfate was added to the inactivated virus in the physiological condition and the activity of the virus was assayed, but no reactivation of the virus was observed. The pattern of an electrophoresis of the reaction mixture of sodium thiosulfate and the oxidized product of cysteine with chloramine T was exactly the same as the pattern of the reaction mixture before the addition of thiosulfate as was anticipated from the biological result (Fig. 1).

The antiviral assay so far mentioned was carried out at the protein-free conditions. It is important to test whether N-chloro compounds inactivate the virus in the presence of protein or not. Thus, the active compounds in the second column of Table Ⅱ were tested in the presence of 50% calf serum. Experimental results are shown in the last column of the table, which shows that N-chloro compounds are completely inactive in the presence of protein. This is probably because of the reaction of N-chloro compounds with SH group of the serum prior to the reaction with that of virus.

From these facts, it might be concluded that N-chloro compounds formed hypochlorous acid, which oxidized the SH group of viral protein coat to disulfide, disulfoxide, and sulfinic acid resulting in inactivation of the virus. However, these reagents are incapable of inactivating the virus in the presence of serum proteins. After all, N-chloro compounds may be regaded as non-specific virus inactivating agents.

Table II. Antiviral Activities of N-Chloro Compounds

	Compounds	Inactivation of Adenovirus type 5 in PBS	Inactivation of Adenovirus type 5 in 50% calf serum
1		+++	–
2		+++	–
3		+++	–
4		+++	–
5		–	
6		–	
7		–	
8		–	
9		+++	–
10		–	
11		+++	–
12		+	–
13		+++	–
14		–	
15		–	
16		–	
17		++	–
18		–	
19		–	
20		+++	–
21		–	

22	 8)	−	
23	 8)	+++	−
24		+++	−
25		±	−
26		−	
27		−	

a) Commercial products.

Experimental

Synthesis of N-Chloro Compounds——General method (A): To a mixture of 50~100 ml. of satd. $NaHCO_3$ soln. and 20~50 ml. of NaClO soln. (10% available chloride) was added 2 g. of fine-powdered amide with vigorous stirring at room temperature and the stirring was continued for 1~3 hr. Solid material was collected by filtration and purified by reprecipitation from $CHCl_3$ solution by addition of ligroin or petr. ether. Yield, 60~80%.

General method (B) : To a suspension of 3 g. of amides in 150 ml. of AcOH was added 35 ml. of NaClO solution (10% available chlorine) with vigorous stirring. The undissolved material, if any, was filtered off, and a satd. $NaHCO_3$ soln. was added to neutralize the AcOH, and cooled. N-chloro compounds precipitated were filtered, and purified by reprecipitation as in the method (A). Yield, 30~60%.

Biological Tests

Determination of Inactivating Activity of N-Chloro Compounds against Adenovirus Type 5——Virus stock suspension ($10^{8.0}TCID_{50}$/ml.) was diluted to 1:5 in 0.01*M* PBS of pH 7.2 and it was mixed with an equal volume of PBS solution of the compounds so as to give the final concentration of the compounds of $5\times10^{-4}M$ in the reaction mixture.

The mixture was incubated at room temperature for 60 mins., and was made to 10^{-1} and 10^{-2} dilutions in PBS. Two tenths ml. of each dilution were inoculated on HeLa cell cultures of three or four tubes. After an adsorption period of 120 mins. at 36°, the cell cutures were washed twice with 3 ml. of PBS. To these 1 ml. of maintenance medium1) was added and they were incubated at 36°. Cytopathic effect was daily observed, and then the inactivating activity of the compounds was determined as described previously.1)

Determination of Inactivating Activity of N-Chloro Compounds against Adenovirus Type 5 in the Presence of Protein——Virus stock suspension ($10^{8.0}TCID_{50}$/ml.) was diluted by addition of 4 volumes of 0.01*M* PBS and 0.2 ml. of the dilution was mixed with 0.4 ml. of calf serum and 0.2 ml. of PBS solution of $10^{-3}M$ reagent so as to give the final infectivity of $5\times10^{6}TCID_{50}$/ml. After an incubation period of 60 mins. at room temperature, assays were carried out as previously described.

Attempt to Reverse the Viral Activity after Inactivation with N-Chloro Compounds——Two tenths ml. of dilution of virus ($5\times10^{7}TCID_{50}$/0.5 ml.) were inactivated with 0.5 ml. of $5\times10^{-4}M$ chloramine T or N-chlorosuccinimide at room temperature for 60 min. One ml. of $10^{-2}M$ of sodium thiosulfate in PBS was added to the inactivated virus suspension, and the mixture was kept at room temperature for 60 mins. The activity of the virus was then assayed. No reverse of the viral activity was observed.

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