compounds, and physical and chemical properties of these chromones were characterized: 2-Methyl-8-methoxy-5, 6-benzochromone (Ⅳ), 2-methyl-7-methoxy-5,6-benzochromone (X), 2-methyl-7,8-dimethoxy-5,6-benzochromone (XIV), 2-methyl-3-acetyl-8-methoxy-5,6-benzochromone (XXV), 2-methyl-3-acetyl-7-methoxy-5,6-benzochromone (XXII), and 2-methyl-3-acetyl-7,8-dimethoxy-5,6-benzochromone (XXIII).

Hydroxylation of 2-methyl-8-hydroxy-5,6-benzochromone (Ⅲ) or 2-methyl-7-hydroxy-5,6-benzochromone (V) gave 2-methyl-7,8-dihydroxy-5,6-benzochromone (XX) from both.

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22. Hidetaka Yuki, Fumihiko Sano, Shin-ichi Takama, and Seikichi Suzuki: Studies on Antiviral Agents. I. Relationship between Chemical Reactivity of Sulfhydryl Reagents and Their Inactivating Activity of Adenovirus Type 5.

(*Research Laboratories, Chugai Pharmaceutical Co., Ltd.*[*1])

For a number of viral diseases, only three antiviral chemotherapeutic agents are available at present; iodouracil deoxyriboside (IUDR)[1,2] for herpes simplex virus, N,N′-anhydrobis(2-hydroxyethyl)biguanide (ABOB)[3] for influenza virus, and N-methyl isatine β-thiosemicarbazone[4,5] for pox viruses. As viral diseases are increasing in frequency and virulency, various kinds of works have been carried out hoping an appearance of effective agent.

Several workers reported that sulfhydryl reagents could inactivate the various viruses, suggesting one of the directions towards the investigation of the antiviral agents. As typical sulfhydryl reagents, *p*-chloromercuribenzoic acid (PCMB) and other organo-mercuric compounds have mostly been studied, and it was found that these reagents inactivate streptococcal bacteriophage,[6] enteroviruses[7] and many others.[8,9]

Buckland[10] reported that PCMB inactivated hemagglutinating activity of various viruses, and Allison and co-workers[9] found that PCMB and iodoacetamide could reduce the virus infectivity of the thirty-six viruses including adenovirus type 5.

Considerable part of human respiratory and eye diseases is caused by adenoviruses, but little work has been known on the study of chemotherapeutic agents against these viruses.

In order to approach towards the investigation of the antiviral agents, a relationship between chemical reactivities of sulfhydryl reagents and their inactivating activities against adenovirus type 5 was examined. These results are reported below.

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Materials and Methods

Synthesis of Methyl N-Acetylcysteinate (MAC) (I)——A mixture of dimethyl N,N′-diacetylcystinate 2.0 g., Zn-powder 6.0 g. and acetic acid 25 ml. was stirred at 50° for 3 hr. To the filtrate of the reaction mixture H_2S was babbled through for 5 min., then ZnS precipitated was seperated by centrifugation. The supernatant was evaporated to dryness in a reduced pressure at room temperature, and the residue was kept in an atmosphere of nitrogen at −10°, resulting in crystallization. Recrystallization from a mixture of AcOEt and petr. ether gave colorless crystals. m.p. 81~81.5°. *Anal.* Calcd. for $C_6H_{11}O_3NS$: C, 40.66; H, 6.26; N, 7.90. Found: C, 40.55; H, 6.38; N, 7.97.

Reaction Condition of MAC with Sulfhydryl Reagents——MAC solution: $2\times10^{-4}M$ in $M/100$ phosphate buffer solution of pH 7.2. Sulfhydryl reagent solutions: $2\times10^{-3}M$ or $2\times10^{-4}M$ in $M/100$ phosphate buffer solution of pH 7.2. When the following method (A) is applied, small volume of water-miscibie organic solvent could be used to dissolve the reagents, though the readjustment of the pH value may be required. Equal volumes of the both solutions were mixed, and the mixture was kept standing for 30 min. at room temperature, then the unreacted SH value was determined by the following method.

Method (A)——Liddel's method[11] for the colorimetric determination of cystein was applied.

Method (B)——Kolb's method[12] for the microdetermination of sulfhydryl group by phosphotungstate was applied.

Reactivity was expressed as

$$\frac{\text{SH value (mole) after the reaction}}{\text{SH value (mole) before the reaction}}\times 100\ (\%)$$

Cell Cultures——HeLa cells were grown serially by tissue culture in Gey's solution with 0.5% lactoalbumin hydrolysate, 0.1% yeast extract and 20% calf serum, and 5.0×10^4 cells were inoculated into each tube. All cell cultures were incubated at 37°.

Diluents——The phosphate-buffer saline solution (PBS) containing $0.1181M$ KH_2PO_4, $0.0015M$ Na_2HPO_4, $0.137M$ NaCl, $0.0027M$ KCl, $0.0009M$ $CaCl_2$ and $0.005M$ $MgCl_2\cdot6H_2O$ was used throughout the experiments.

Virus——Adenovirus type 5 has been propagated by serial passage on HeLa cell cultures. Stock virus was prepared as follows: intracellular virus of infected HeLa cell cultures was liberated by freezing and thawing six times, and the supernatant was treated with fluorocarbon Daifron S_3 (Osaka Kinzoku Co.) twice and preserved at −25° for the experimental use.

For the determination of inactivating activity of reagents against adenovirus type 5, virus stock solution ($10^{7.25}$ $TCID_{50}$/ml.) was diluted to 1:5 with PBS and it was mixed with an equal volume of reagents dissolved in PBS containing 0.1% carboxy-methyl cellulose, so as to make the final concentration of the reagents in a reaction mixture $5\times10^{-4}M$ and the final infectivity $10^{6.25}$ $TCID_{50}$ per ml. The mixtures were incubated at room temperature for 60 min., and made to 10^{-1} and 10^{-2} dilutions with PBS. Two tenths ml. of each diluent was inoculated on HeLa cell culture of three or four tubes. After an adsorption period of 120 min. at 36°, those cell cultures were washed twice with 3 ml. of PBS, and to this 1 ml. of maintenance medium was added. All of the inoculated tubes were incubated at 36°. The cytopathic effects were observed daily. Then, the inactivating activity of the reagents were determined as below.

HS-CH_2-CH-$COOCH_3$ I
CH_3-CO-NH

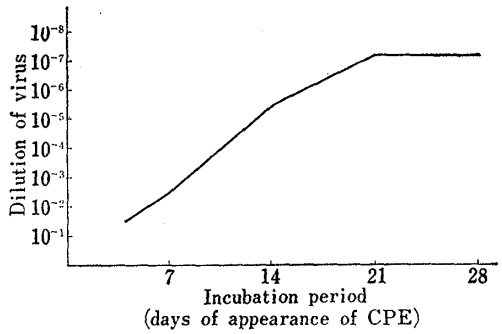

Fig. 1.

The serial ten-fold dilutions of adenovirus type 5 were inoculated to the test tubes in which HeLa cells had been grown, then these tubes were incubated at 36°. Microscopic observations were done, and the days of CP-appearance were recorded daily. Dilutions of virus and days of CP-appearance were shown on the vertical line and horizontal line respectively.

Adenovirus type 5 requires such a long period of incubation as 3 to 4 weeks for the determination of final infectivity. However, infective dose ($TCID_{50}$) and the incubation period, which begins to show viral cytopathic effect, indicates the linear relation in the initial three weeks, and a decrease of every one tenth of virus amount lengthens the additional three days in the incubation period, as is seen in Fig 1. Based upon these results, the remaining virus infectivity of the tubes treated was estimated briefly from the prolonged days of appearance of viral cytopathic effect comparing with those of the control tubes. Thus virus inactivating activity of sulfhydryl reagents was determined.

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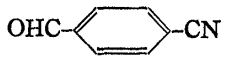
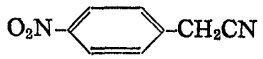
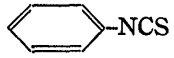
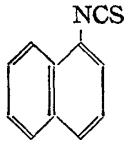
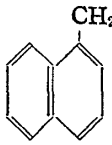
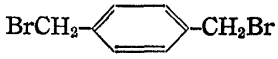
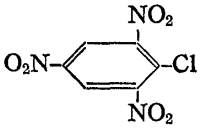
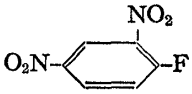
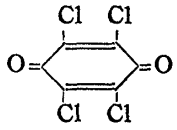
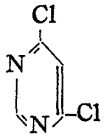
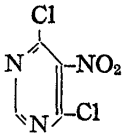
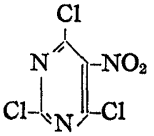
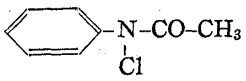
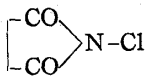
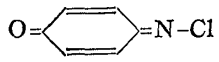
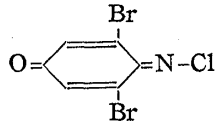
Table I. Inactivation of Adenovirus Type 5 and Chemical Reaction with Methyl N-Acetylcysteinate (MAC) of Sulfhydryl Reagents

Group	No.	Compound	Virus inactivation	Reaction with MAC (%)	Concns. tested (*M*)	Method of SH-analysis
A	1		—	0	10^{-3}	A
	2		—	0	10^{-4}	〃
	3		—	20	10^{-4}	〃
	4		++	45	10^{-3}	〃
B	1	$ClCH_2CONH_2$	—	0	10^{-3}	〃
	2	$Cl_2CHCONH_2$	—	10	10^{-3}	〃
	3	ICH_2COOH	—	25	10^{-3}	〃
				0	10^{-4}	〃
	4	ICH_2CONH_2	—	90	10^{-3}	〃
				35	10^{-4}	〃
	5		—	0	10^{-4}	〃
	6		—	40	10^{-3}	〃
				0	10^{-4}	〃
	7		+++	95	10^{-3}	〃
				80	10^{-4}	〃
	8		—	100	10^{-3}	〃
				100	10^{-4}	〃
	9		+++	85	10^{-3}	〃
				85	10^{-4}	〃
	10		—	0	10^{-3}	〃
	11		++	95	10^{-3}	〃
				20	10^{-4}	〃
	12		+++	100	10^{-3}	〃
				95	10^{-4}	〃

	13		+++	100	10^{-3}	B
				100	10^{-4}	〃
	14		+++	100	10^{-3}	A
				90	10^{-4}	〃
	15		+	80	10^{-3}	〃
				55	10^{-4}	〃
	16		+++	65	10^{-3}	〃
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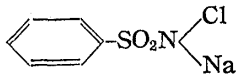
	17		+++	100	10^{-3}	B
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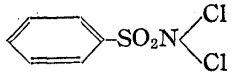
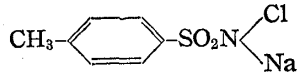
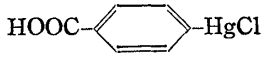
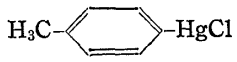
	18		+++	100	satd.	〃
	19		+++	100	10^{-3}	〃
				100	10^{-4}	〃
C	1		++	100	10^{-4}	A
	2		++	100	satd.	B
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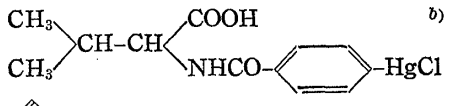
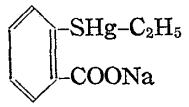
	3	 b)	++	100	10^{-4}	〃
	4		+	10	10^{-3}	A
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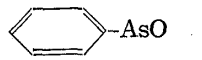
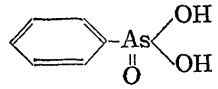
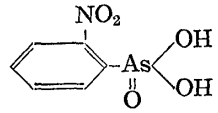
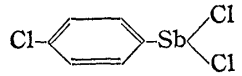
	5		−	70	10^{-4}	〃
	6		−	0	10^{-3}	〃
	7		−	0	10^{-3}	〃
	8		−	10	10^{-3}	〃
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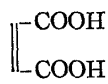
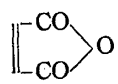
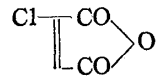
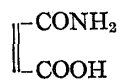
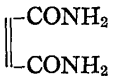
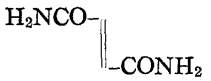
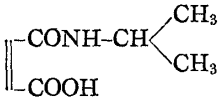
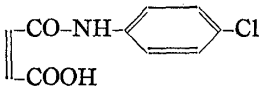
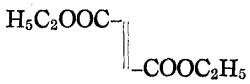
D	1		−	0	10^{-3}	B
	2		−	0	10^{-3}	〃
	3		−	0	10^{-3}	〃
	4		−	0	10^{-3}	〃

	5		—	0	10^{-3}	B
	6		—	0	10^{-3}	〃
	7		—	0	10^{-3}	〃
	8		—	0	10^{-3}	〃
	9		—	60	10^{-3}	〃
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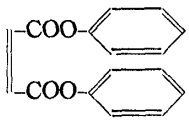
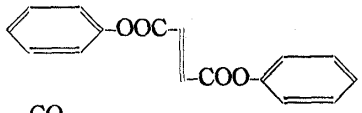
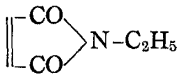
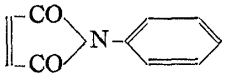
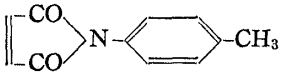
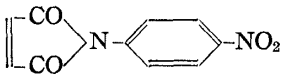
	10		+	10	10^{-3}	〃
	11		—	0	satd.	〃
	12		—	100	10^{-3}	〃
				100	10^{-4}	〃
	13		+	60	10^{-4}	〃
	14		—	0	10^{-4}	〃
	15		—	10	10^{-3}	〃
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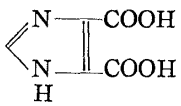
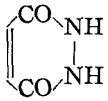
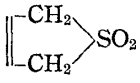
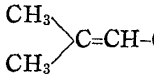
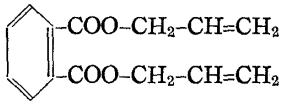
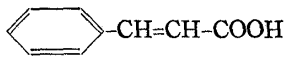
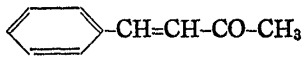
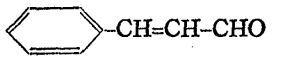
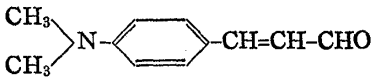
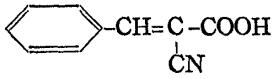
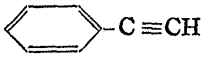
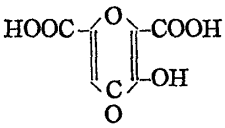
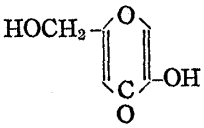
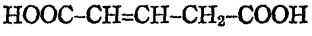
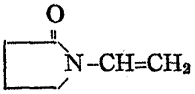
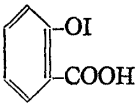
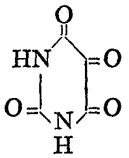
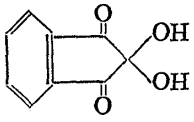
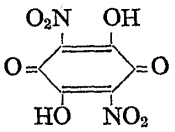
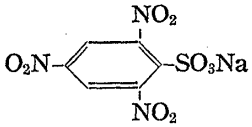
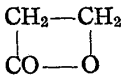
	16		—	40	10^{-3}	〃
				10	10^{-4}	〃
	17		—	0	10^{-4}	〃
E	1	$CH_2=CH-CH_2-SO_3Na$	—	0	10^{-3}	〃
	2	$SO_2(CH=CH_2)_2$	—	100	10^{-3}	〃
				25	10^{-4}	〃
	3		—	0	10^{-3}	〃
	4	$CH_3-CH=CH-COOC_2H_5$	—	0	10^{-3}	〃
	5		—	0	10^{-3}	〃
	6	$O=P(O-CH_2-CH=CH_2)_3$	—	0	10^{-3}	〃
	7		—	0	10^{-3}	〃
	8		—	0	10^{-3}	〃

	9		—	0	10^{-3}	B
	10		—	10	10^{-3}	〃
				0	10^{-4}	〃
	11		—	0	satd.	〃
	12		—	40	10^{-3}	〃
				0	10^{-4}	〃
	13		—	0	10^{-3}	〃
	14		—	0	10^{-3}	〃
	15		—	0	10^{-3}	〃
	16		—	0	10^{-3}	〃
	17		—	0	10^{-3}	〃
F	1		—	100	10^{-3}	A
				100	10^{-4}	〃
	2		—	0	10^{-3}	〃
	3		—	65	10^{-3}	〃
				45	10^{-4}	〃
	4		—	0	10^{-3}	〃
	5		+++	95	10^{-3}	〃
				60	10^{-4}	〃
	6		—	0 (pH 6.2)	10^{-3}	B
				5 (pH 7.2)	10^{-3}	〃
				40 (pH 8.2)	10^{-3}	〃

a) No prolongation of appearance of cytopathic effect was expressed as (−); prolongation of 3 days or less, (+); prolongation of 6 days or less, (++); prolongation of more than 6 days, (+++).
b) Synthetic method will be reported at a later opportunity.

Results and Discussion

The cystein residue of viral protein coat is supposed to be responsible for the reaction with sulfhydryl reagents. As the amino and carboxyl groups of amino acids in proteins exist as peptide linkages, MAC has been chosen as a model compound of cystein residue in the viral protein because preparation of its amide type compound was unsuccessful. Chemical reactions of MAC with the sulfhydryl reagents have been carried out in the same conditions as in the biological system. Both biological and chemical activities of the reagents were listed in the table.

Sulfhydryl reagents to be tested were classified into six groups. Group A, nitrile and isothiocyanate; group B, active halogen compounds; group C, metal compounds; group D; maleic acid derivatives; group E, other active double bonded compounds; and group F, others.

Group A. A nitrile can react with an SH group yielding α-iminoalkyl sulfide (R-S-C(=NH)-R′) under the stronger condition, but this reaction did not take place under the biological conditions stated-above, consequently no virus inactivation was considered to be observed. An isothiocyanate generally reacts with an SH group to give dithiourethane (R-NH-CS-S-R′) even in the cold state. Actually, phenyl and naphthyl isothiocyanate (A-3, A-4) reacted with MAC and the latter showed significant antiviral activity.

Group B. The first six compounds are chemically active aliphatic halogeno compounds which reacted with MAC, but they did not reveal any virus inactivating activity. Even iodoacetamide (B-4), which has high reactivity with MAC, did not inactivate the virus.

The following six compounds (from B-7 to 12) are active aromatic halogeno compounds. Those biological and chemical activities are coincident, but not with 2,4-dinitrofluorobenzene (B-8).

Dichloropyrimidine (B-10) is inactive chemically and biologically. However, an introduction of the nitro group, which is strongly electron-attractive and is consequently able to give a stronger chemical reactivity to a chlorine atom at *o*- and *p*-positions, afforded a marked chemical activity along with a biological activity as is seen in compounds 11 and 12. All compounds, whose chlorine atom is directly linked to a nitrogen atom (B 13~19), are very active biologically and chemically.

Group C. All mercuric compounds are active in biological and chemical reactions, but not coincident with arsenic compounds.

Group D. Maleic acid derivatives have either weak or no virus inactivating activity although some of them react with MAC to a great extent. N-ethylmaleimide (D-12) reacted with MAC completely, but no inactivation of adenovirus was observed while (D-12) and (B-4) were reported to decrease the infectivity titer or enteroviruses at pH 8.6 and 9.0 in logarithmic orders.[13]

Group E. Active carbon-carbon double bonded compounds, which bear such a strong electron attracting group as CO and SO_2, react with an SH group to give a thioether compound. In the above-mentioned experimental conditions, however, only divinyl sulfon (E-2) and α-cyanocinnamic acid (E-12) reacted with MAC without showing any biological activity.

Group F. Only sodium picryl sulfonate (F-5) exhibited a marked virus inactivating activity and chemical reactivity. *o*-Iodosobenzoic acid (F-1) and ninhydrin (F-3) reacted only with MAC. As to the β-propiolactone (F-6), which is active against all viruses

13) L. Phillipson, P. W. Choppin : J. Exptl. Med., **112**, 455 (1960).

tested so far, the reaction rate with MAC increased as the pH rises, but virus inactivation could not be observed at pH 7.2 as it had been anticipated from the chemical reactivity at this pH.

In these experiments, all biologically active compounds have shown considerable chemical reactivity with MAC, but not vice versa. Every compound which possessed weak or no chemical activity could not exhibit the biological activity in this assay system. However, iodoacetamide, 2,4-dinitrofluorobenzene, phenylarsine oxide, N-ethylmaleimide, and divinylsulfone, which bear strong chemical activities, could not show the virus inactivating activity. This reason still remains undissolved. Phillipson and Choppin[8] reported that hemagglutinating activity of ECHO 11 virus was inactivated by $2\times10^{-2}M$ of N-ethyl maleimide and iodoacetamide at pH 8.6 and 7.2, respectively. The lack of virus inactivating activity of these two compounds in the present experiment may have resulted from either the weaker reaction conditions or the different natures of the viruses tested.

It has not been established yet whether the inactivation of adenovirus by the sulfhydryl reagents might be due to the inactivation of ability for viral adsorption or any other function, because certain inactivated virus particles are still able to adsorb and penetrate into the host cells without revealing infectivity.

The authors express their deep gratitude to Dr. T. Akiba, the director of this laboratories and Prof. Emeritus of Tokyo University, and Drs. Y. Nitta and H. Sano, the heads of the departments, for their kind advices and encouragements throughout this investigation. Thanks are also due to the members of microanalytical laboratory.

Summary

Seventy-one possible sulfhydryl reagents were examined on the chemical reactivity with methyl N-acetylcysteinate, as well as on the inactivating ability against adenovirus type 5.

It has been found that the compounds capable of inactivating the virus are always accompanied by the chemical reactivity with the SH group of methyl N-acetylcysteinate, and that the compounds incapable of reacting with the SH group are unable to exhibit the virus-inactivating activity either.

Above all, the compounds, whose chlorine atom is directly linked to a nitrogen atom, have shown a marked virus inactivating activity.

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