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THE STRUCTURE-ACTIVITY RELATIONSHIPS OF THE ANTIVIRAL CHEMOTHERAPEUTIC ACTIVITY OF ISATIN β-THIOSEMICARBAZONE

BY

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As part of an investigation devoted to the development of new antiviral agents a compound of established antiviral activity has been subjected to systematic structural modification. The structure-activity data so obtained have been used in the design of new compounds, some of which are described. The compound chosen was isatin β-thiosemicarbazone, which has high activity against neurovaccinia infection in mice, and a 4-point parallel-line assay of *in vivo* chemotherapeutic activity has been developed, which has enabled the activity of the derivatives to be determined against isatin β-thiosemicarbazone as a standard. The overall dimensions of the isatin β-thiosemicarbazone molecule appear to be nearly maximal for the retention of high activity, as all substituents in the aromatic ring decrease the activity irrespective of their nature or position. The projection of the $-CS.NH_2$ group in relation to the ring nitrogen was found to be critical, as the α-thiosemicarbazone was inactive. A number of modifications of the side-chain were investigated: all led to reduction or loss of antiviral activity. The antiviral activity showed a positive correlation with chloroform solubility over a considerable range. The most active compound encountered was 1-ethylisatin β-thiosemicarbazone, with an activity of 286 (isatin β-thiosemicarbazone≡100). Isatin β-thiosemicarbazone showed no activity against 15 other viruses, and 20 related compounds showed no activity against ectromelia.

The antiviral effect of isatin β-thiosemicarbazone (I) against vaccinia virus was first observed by Thompson, Minton, Officer, and Hitchings (1953), who found that mice receiving a diet containing 0.6% of the compound were protected against intracerebral infection with about 100 LD50 of the virus. Their experiments were not designed to demonstrate the maximum effect of the compound, and it was subsequently shown (Bauer, 1955) that mice treated with isatin β-thiosemicarbazone by subcutaneous injection of 2 doses of 125 mg./kg. each day throughout the incubation period would survive intracerebral infection with about 10,000 LD50 of vaccinia virus without showing signs of illness. The compound also gave complete protection against 100 to 1,000 LD50 of virus when given in a single dose of 25 mg./kg. 18 hours after infection.

The activity of isatin β-thiosemicarbazone against vaccinia infection is thus fully equal to the effect of the sulphonamides and tetracyclines against the agents of the psittacosis-lymphogranuloma group. These organisms have long been described as viruses, but present opinion assigns them to the Rickettsiales, and isatin β-thiosemicarbazone thus remains the only true example of an antiviral agent active in experimental animals which confers a protection against infection which is sufficiently powerful to be of clinical interest.

It is therefore important to investigate the relationship between structure and antiviral activity, since the information gained, apart from its intrinsic importance in the chemotherapy of pox virus infections, may make it possible to establish principles governing activity which can be applied to other classes of antiviral agents yet to be discovered. The first steps in this direction were taken by Thompson *et al.* (1953), who considered that the antiviral activity required both the thiosemicarbazone side-chain and a cyclic component. In the present work the relationship of structure to activity has been studied in much greater detail. A number of related compounds

and derivatives of isatin β-thiosemicarbazone have been prepared and tested, the relationship between structure and activity has been fully elucidated, and some of the derivatives have been found to possess a therapeutic activity considerably greater than that of the parent compound.

METHODS

Synthesis of Compounds

The melting points and analytical data of new substituted isatin β-thiosemicarbazones prepared in the present investigation are given in Table I, together with literature references to the preparation of those isatins which are already known.

Ring-substituted Compounds.—These were prepared by refluxing equimolar quantities of thiosemicarbazide with the appropriate substituted isatin in 50% aqueous ethanol for several hours. The products which separated on cooling were collected and washed well with hot water. Crystallization from butanol afforded samples for analysis. *5-Carboxymethylisatin:* prepared from *p*-aminophenylacetic acid by the Sandmeyer synthesis, orange needles, m.p. 224°; found: C, 59.4; H, 3.5; N, 7.3; Calc. for $C_{10}H_7O_4N$: C, 59.9; H, 3.5; N, 7.0. *5-Ethoxycarbonylmethylisatin:* esterification of 5-carboxymethylisatin with ethanol and concentrated sulphuric acid gave 5-ethoxycarbonylmethylisatin as orange plates, m.p. 132°.

N-*substituted Compounds.*—*1-Pentylisatin:* equimolar quantities of 1-sodioisatin and pentyl bromide were heated under reflux for 48 hr. The mixture was filtered and the filtrate was extracted with a small volume of 2N sodium hydroxide, which on acidification with concentrated hydrochloric acid gave 1-pentylisatin as red plates, m.p. 47°. 1-Isopropyl- and 1-isobutylisatin were prepared similarly. *1-(2-Hydroxyethyl)-isatin:* equimolar quantities of 1-sodioisatin and 2-chloroethanol were heated under reflux for 48 hr. The mixture was filtered, the filtrate was reduced to small volume and the product separated on the addition of 60/80° petroleum ether. Crystallization from aqueous methanol gave orange needles, m.p. 118°. *5-Carboxymethyl-1-methylisatin:* methylation of 5-carboxymethylisatin with dimethyl sulphate in 2N sodium hydroxide gave 5-carboxymethyl-1-methylisatin as red needles, m.p. 180°. *5-Ethoxycarbonylmethyl-1-methylisatin:* esterification of 5-carboxymethyl-1-methylisatin with ethanol in concentrated sulphuric acid gave 5-ethoxycarbonylmethyl-1-methylisatin as bright orange needles, m.p. 143°. *1-Methyl-4-trifluoromethylisatin:* methylation of 4-trifluoromethylisatin (Sadler, 1956) with dimethyl sulphate in 2N sodium hydroxide gave 1-methyl-4-trifluoromethylisatin as large orange plates, m.p. 156°.

Compounds Substituted in the Side-chain.—*Isatin β-2-phenylthiosemicarbazone:* equimolar quantities of 2-phenylthiosemicarbazide (Mautner and Kumler, 1956) and isatin were heated under reflux in ethanol for 2 hr. The product was removed by filtration and washed with ethanol. Crystallization from butanol gave yellow plates, m.p. 210° (yield 80%). Found: C, 60.9; H, 4.2; S, 10.7; Calc. for $C_{15}H_{12}ON_4S$: C, 60.8; H, 4.1; S, 10.8. *1-Methylisatin β-2-phenylthiosemicarbazone:* equimolar quantities of 2-phenylthiosemicarbazide and 1-methylisatin were heated under reflux for 4 hr. in 50% aqueous ethanol. The ethanol was removed by distillation and the product which crystallized on cooling was recrystallized from 50% aqueous methanol: yellow needles, m.p. 175° dec. (yield 70%). Found: C, 61.9; H, 4.6; S, 10.3; Calc. for $C_{16}H_{14}ON_4S$: C, 61.7; H, 4.5; S, 10.3. The following compounds were prepared similarly. *Isatin β-4-methylthiosemicarbazone:* yellow needles from ethanol, m.p. 260° (decomp.). Found: C, 51.3; H, 4.4; N, 23.8; S, 13.4; Calc. for $C_{10}H_{10}ON_4S$: C, 51.3; H, 4.3; N, 23.9; S, 13.7. *Isatin β-4-allylthiosemicarbazone:* yellow needles from ethanol, m.p. 211°. Found: C, 55.5; H, 4.7; N, 20.7; S, 11.9; Calc. for $C_{12}H_{12}ON_4S$: C, 55.3; H, 4.7; N, 21.5; S, 12.3. *Isatin β-4-phenylthiosemicarbazone:* yellow needles from ethanol, m.p. 255° (decomp.). Found: C, 60.9; H, 4.2; N, 18.6; S, 10.5; Calc. for $C_{15}H_{12}ON_4S$: C, 60.8; H, 4.1; N, 18.9; S, 10.8. *Isatin β-S-methylthiosemicarbazone:* yellow needles from ethanol, m.p. 257° (decomp.). Found: C, 51.3; H, 4.4; N, 23.5; S, 13.9. Calc. for $C_{10}H_{10}ON_4S$: C, 51.3; H, 4.3; N, 23.9; S, 13.7.

Miscellaneous Compounds.—*Isatin α-thiosemicarbazone* (prepared by Dr. C. G. Raison). Hot solutions of isatin α-anil (2.22 g. in 10 ml. alcohol) and thiosemicarbazide (0.91 g. in 10 ml. water) were mixed and boiled for 1 hr. When cold, the separated crystals were recrystallized from aqueous dimethylformamide. The compound formed brick-red crystals, m.p. 216–217° (decomp.). Found: C, 48.8; H, 3.8; S, 14.4. Calc. for $C_9H_8ON_4S$: C, 49.1; H, 3.7; S, 14.5. *3-Formyl-1-methyloxindole thiosemicarbazone:* equimolar quantities of 3-formyl-1-methyloxindole (Julian, Pikl, and Boggess, 1934) and thiosemicarbazide were refluxed in aqueous ethanol. The product, which separated on cooling, was recrystallized from aqueous ethanol, yellow plates, m.p. 219°. Found: C, 53.6; H, 4.5; S, 12.3; Calc. for $C_{11}H_{12}ON_4S$: C, 53.2; H, 4.8; S, 12.9. *1-Acetylindoxyl thiosemicarbazone:* equimolar quantities of 1-acetylindoxyl (Vorländer and Drescher, 1901) and thiosemicarbazide were refluxed in aqueous ethanol. The product separated from the hot reaction mixture and was recrystallized from a large volume of ethanol, white plates, m.p. 242°. Found: C, 53.2; H, 4.9; S, 12.9; Calc. for $C_{11}H_{12}ON_4S$: C, 53.2; H, 4.8; S, 12.9. *β-1-Benzimidazolylpropionthioamide:* a solution of 0.1 mole of β-1-benzimidazolylpropionitrile (Efros, 1953) in 20 ml. of 30% (w/v) ammoniacal ethanol was saturated with hydrogen sulphide and allowed to stand for 2 days. The ethanol was removed under reduced pressure and the residue was crystallized several times from hot water giving white needles, m.p. 151° (yield 25%). Found: C, 59.1; H, 5.3; S, 15.5. Calc. for $C_{10}H_{11}N_2S$: C, 58.5; H, 5.4; S, 15.6.

TABLE I

SUBSTITUTED ISATIN β-THIOSEMICARBAZONES

Literature references are to the preparation of the corresponding isatins.

Substituent	Literature Reference	m.p.°	Formula	Required %			Found %		
				C	H	S	C	H	S
5-Fluoro ..	Holt and Sadler (1958)	275	$C_9H_7ON_4SF$	45·4	2·9	13·4	46·0	3·2	13·2
6-Fluoro ..	Sadler (1956)	257	$C_9H_7ON_4SF$	45·4	2·9	13·4	45·4	2·9	13·8
7-Fluoro ..	Holt and Sadler (1958)	263	$C_9H_7ON_4SF$	45·4	2·9	13·4	45·6	3·0	13·4
4-Chloro ..	Sadler (1956)	288	$C_9H_7ON_4SCl$	42·4	2·7	12·6	42·4	2·2	12·7
6-Chloro ..	,,	270	$C_9H_7ON_4SCl$	42·4	2·7	12·6	42·5	2·4	12·7
7-Chloro ..	Holt and Sadler (1958)	272	$C_9H_7ON_4SCl$	42·4	2·7	12·6	42·2	2·7	12·2
4-Bromo ..	Sadler (1956)	294	$C_9H_7ON_4SBr$	36·1	2·3	10·7	36·2	2·2	10·5
6-Bromo ..	,,	290	$C_9H_7ON_4SBr$	36·1	2·3	10·7	36·2	2·3	10·9
7-Bromo ..	Holt and Sadler (1958)	275	$C_9H_7ON_4SBr$	36·1	2·3	10·7	36·5	2·3	10·3
4-Iodo ..	Sadler (1956)	281	$C_9H_7ON_4SI$	31·2	2·0	9·2	32·0	2·3	9·7
5-Iodo ..	Borsche *et al.* (1924)	270	$C_9H_7ON_4SI$	31·2	2·0	9·2	31·5	2·2	9·0
6-Iodo ..	Sadler (1956)	275	$C_9H_7ON_4SI$	31·2	2·0	9·2	31·7	2·2	9·1
7-Iodo ..	Holt and Sadler (1958)	263	$C_9H_7ON_4SI$	31·2	2·0	9·2	30·7	1·6	9·0
6-Methyl ..	Sadler (1956)	270	$C_{10}H_{10}ON_4S$	51·3	4·3	13·7	51·1	4·4	13·5
7-Methyl ..	Holt and Sadler (1958)	275	$C_{10}H_{10}ON_4S$	51·3	4·3	13·7	51·1	4·4	13·6
1-Methyl ..	Hantzsch (1921)	245	$C_{10}H_{10}ON_4S$	51·3	4·3	13·7	50·5	4·5	13·7
1-Ethyl ..	Michaelis (1897)	201	$C_{11}H_{12}ON_4S$	53·2	4·8	12·9	53·1	5·1	12·8
1-Propyl ..	,,	193	$C_{12}H_{14}ON_4S$	54·9	5·4	12·2	55·1	5·1	12·1
1-Isopropyl ..	—	225	$C_{12}H_4ON_4S$	54·9	5·4	12·2	54·9	5·3	12·1
1-Pentyl ..	—	184	$C_{14}H_{18}ON_4S$	57·9	6·2	11·0	57·7	6·0	11·0
1-Hydroxymethyl	Reissert and Handeler (1924)	230	$C_{10}H_{10}ON_4S$	48·0	4·0	12·8	48·1	4·3	13·2
5-Amino ..	Giovannini and Portmann (1948)	>320	$C_9H_9ON_5S$	45·9	3·8	13·6	46·0	4·4	12·9
5-Hydroxy ..	,, ,,	284	$C_9H_8O_2N_4S$	45·8	3·4	13·5	45·5	3·4	13·4
1-Benzyl ..	Antrick (1885)	268	$C_{16}H_{14}ON_4S$	61·9	4·5	10·3	62·5	4·5	9·4
1-(2-Hydroxyethyl)	—	247	$C_{11}H_{12}O_2N_4S$	50·0	4·5	12·1	49·9	4·6	12·2
1-Carboxymethyl	Langenbeck (1928)		$C_{11}H_{10}O_3N_4S$	47·5	3·6	11·5	47·7	3·9	11·0
5-Carboxymethyl	—	273	$C_{11}H_{10}O_3N_4S$	47·5	3·6	11·5	47·1	3·6	11·1
1-Acetyl ..	Suida (1878)	244	$C_{11}H_{10}O_2N_4S$	50·4	3·8	12·2	50·8	4·1	12·3
1-Ethoxycarbonylmethyl	Ainley and Robinson (1934)	183	$C_{13}H_{14}O_3N_4S$	51·0	4·5	10·5	51·0	4·6	10·4
1-Diethoxycarbonylmethyl	,, ,,	200	$C_{16}H_{18}O_5N_4S$	50·8	4·8	8·5	50·6	4·4	8·4
1-(2-Cyanoethyl)	Carlo and Lindwall (1945)	234	$C_{12}H_{11}ON_5S$	52·7	4·0	11·7	52·6	4·0	11·8
4,5-Benz ..	Martinet (1919)	275	$C_{13}H_{10}ON_4S$	57·8	3·7	11·9	57·8	3·7	11·9
6,7-Benz ..	,,	280	$C_{13}H_{10}ON_4S$	57·8	3·7	11·9	57·3	3·7	11·7
5-Ethoxycarbonylmethyl	—	215	$C_{13}H_{14}O_3N_4S$	51·0	4·5	10·5	51·3	4·6	10·3
5-Carboxymethyl-1-methyl	—	264	$C_{12}H_{12}O_3N_4S$	49·3	4·1	10·9	49·6	3·8	10·5
5-Ethoxycarbonylmethyl-1-methyl	—	183	$C_{14}H_{16}O_3N_4S$	52·6	5·0	10·0	53·3	5·3	9·8
1-Methyl-4-trifluoromethyl	—	263	$C_{11}H_9ON_4SF_3$	43·7	3·0	10·6	44·6	3·1	10·7
5-Carboxy ..	Giovannini and Portmann (1948)	273	$C_{10}H_8O_3N_4S$	45·5	3·0	12·1			
7-Carboxy ..	,, ,,	296	$C_{10}H_8O_3N_4S$	45·5	3·0	12·1	45·3	2·9	12·2

Physical Investigations

Infra-red Absorption Spectra. — Spectra were determined by using a Perkin-Elmer 21 double-beam recording spectrometer fitted with a rock salt prism.

Water/lipid Partition Coefficients.—Solubilities in chloroform and partition coefficients of the compounds between chloroform and water were determined spectroscopically at 25° using matched quartz cells in a Hilger Uvispek.

Biological Methods

Viruses.—The IHD strain of neurotropic vaccinia virus adapted to intracerebral passage in mice was used. A stock suspension was prepared from an infected mouse brain homogenized in normal horse serum or tissue culture medium (10% bovine serum, 0.25% lactalbumin hydrolysate, 5% papain digest broth, 80% Earle solution) and mixed with 3 volumes of glycerol. The stock preparation was stored at −20°. Mice were inoculated intracerebrally with a suitable dilution of this preparation in tissue culture medium, and the time elapsing between infection and death was recorded to the nearest half-day. Animals which were alive and well at the end of 14 days were considered to have survived indefinitely.

The origin of the other neurotropic viruses used has been described previously (Bauer and Bradley, 1956); some were insufficiently stable as glycerol suspensions, and inoculations were made from intact brain tissue stored at −20° and homogenized at the time of use.

Treatment.—Most of the test compounds were only slightly soluble in water, and were made up as suspensions. The dose chosen was inoculated subcutaneously in a volume of 0.1 ml. twice daily for 5 days, the first dose being given 2 to 5 hr. after infection.

Preliminary Test of Antiviral Activity.—In previous work (Bauer, 1958) it has been shown that the mean reciprocal survival time of mice infected intracerebrally with neurovaccinia virus has a linear regression upon the logarithm of the dilution of virus used for infection, and that this relationship can be used as the basis of a test of chemotherapeutic activity, since treatment with an active compound will cause a significant reduction of the mean reciprocal survival time over a very wide range of virus doses. The compounds examined in the present work received a preliminary evaluation in a test of this nature, in which groups of 6 mice infected intracerebrally with about 1,000 LD50 of neurovaccinia virus were treated with doses of 125 mg./kg. and the survival times were compared with those of a control group of 6 mice which were similarly infected but left untreated. Compounds which gave no significant ($P>0.05$) reduction of the mean reciprocal survival time at this dose were considered to be inactive.

Determination of Dose-response Curves. — Compounds which showed activity in the preliminary test were examined further at lower doses. It was found that the mean reciprocal survival time gave a linear regression on the logarithm of dose of compound between the minimum dose producing an observable effect and the dose giving indefinite survival of all animals. The curve obtained with 1-propylisatin β-thiosemicarbazone is shown in Fig. 1, in which the response obtained at doses of 0.25, 0.5, and 2.5 mg./kg. is shown in comparison with the responses observed in the corresponding control groups.

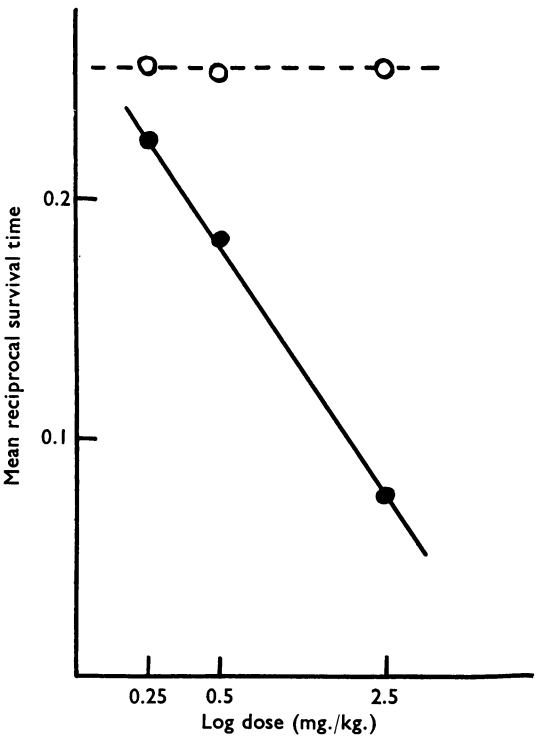


FIG. 1.—Dose-response curve of antiviral activity of 1-propylisatin β-thiosemicarbazone in mice infected intracerebrally with about 1,000 LD50 of neurotropic vaccinia virus. Ordinate, mean reciprocal survival time ; abscissa, log dose of compound. Treated ●——●; controls, O— — —O.

Assay of Antiviral Activity.—The regression lines obtained for the dose-response curves clearly formed a suitable basis for an assay of antiviral activity based on the conventional 4-point design, in which the active compounds were assayed against isatin β-thiosemicarbazone, which was chosen as a standard. From the dose-response curve of a given compound 2 doses were selected, one being twice the other, which would give responses within the region of linearity when 1,000 LD50 of virus was used as the infecting dose. Two doses were similarly selected for isatin β-thiosemicarbazone ; 1.25 and 2.5 mg./kg. were found to be suitable, although in some assays 1 and 2 mg./kg. were used. Four groups each of 6 mice were inoculated intracerebrally with about 1,000 LD50

of virus; 2 groups were treated with the test compound and 2 with isatin β-thiosemicarbazone, both in the selected doses. The survival times were converted to reciprocals (indefinite survival giving a reciprocal of zero) and the joint regression coefficient (b) was calculated for the 24 responses. The relative potency (R) was then obtained in the usual way as a function of the horizontal distance between the individual regression lines for test compound and standard from the relation

$$\log R = \log x_S - \log x_T - (\bar{y}_S - \bar{y}_T)\, b^{-1}$$

where x_S is the mean dose of isatin β-thiosemicarbazone, x_T is the mean dose of test compound, y_S is the mean response with isatin β-thiosemicarbazone and y_T the mean response with the test compound. The value obtained for R was then multiplied by 100 to give the activity based on isatin β-thiosemicarbazone taken as 100, and the result was finally multiplied by the ratio of the molecular weights of test compound and standard to give the activity expressed on an equimolar basis. The results of a typical assay are shown graphically in Fig. 2. The upper line represents the response obtained with doses of 1.25 and 2.5 mg./kg. of isatin β-thiosemicarbazone, and the lower line the responses with 5 and 10 mg./kg. of 7-methylisatin β-thiosemicarbazone; b was found to be −0.039, $\bar{y}_S$ 0.2176 and $\bar{y}_T$ 0.0747, giving a value of 0.89 for R, equivalent to a relative activity on an equimolecular basis of 94 for the 7-methyl derivative in comparison with the parent compound.

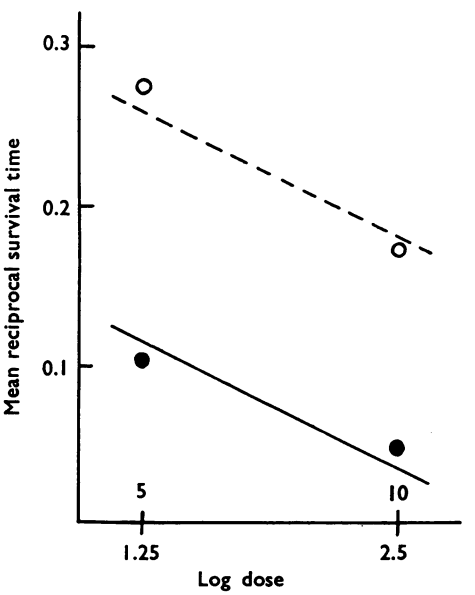


FIG. 2.—Four-point assay of antiviral activity of 7-methylisatin β-thiosemicarbazone in mice infected intracerebrally with about 1,000 LD50 of neurotropic vaccinia virus. Ordinate, mean reciprocal survival time; upper abscissa, log dose of test compound; lower abscissa, log dose of isatin β-thiosemicarbazone. ●——●, 7-methylisatin β-thiosemicarbazone; O— — —O, isatin β-thiosemicarbazone.

RESULTS

Effect on Antiviral Activity of Substitution in the Aromatic Ring

The introduction of substituents into the aromatic ring, as shown in Table II, usually results in reduction or total loss of activity. Substitution in the 5- position has a particularly marked effect, activity being lost except with substituents, such as fluorine, with small atomic or group radii. Activity is reduced to a lesser extent by substitution in the 4- and 6- positions, and some of the 7- substituted compounds still retain quite high activity.

An attempt was made to correlate the effect of substitution on antiviral activity with σ values, which are also given in Table II. The σ value, derived originally by Hammett (1949) from studies of the rates of hydrolysis of a series of substituted benzoic esters, affords a numerical measure of the

TABLE II

ANTIVIRAL ACTIVITY AND σ-VALUES OF RING SUBSTITUTED ISATIN β-THIOSEMICARBAZONES

(No substituent ≡ 100)

Substituent	4-Position	6-Position		5-Position	7-Position	mσ Value for 5- and 7-Positions
	Activity	Activity	pσ Value	Activity	Activity	
Amino ..				0		−0·161
Methyl ..	3·6	0·3	0·170	0	94	−0·069
Ethyl ..				0	~50	−0·043
Carboxymethyl ..				0		−0·027
Ethoxycarbonylmethyl ..				0		
Hydroxy ..				0		−0·002
Methoxy ..		0	−0·268	0·03		0·115
Fluoro ..		43·1	0·062	35·5	⩾20	0·337
Iodo ..	0	3·9	0·276	0	75·3	0·352
Carboxy ..				0	0	0·355
Chloro ..	9·9	11·7	0·227	4·2	98·3	0·373
Bromo ..	67·3	10·5	0·232	3·1	16	0·391
Trifluoromethyl ..					34·5	0·415
Nitro ..				0	0	0·710

effect of a substituent upon the reactivity of a side-chain in an aromatic system. The 5- and 7-substituents are listed together with the *meta* σ values, as both positions are *meta* to the β-carbon atom. The 6- substituents, which are *para* to the β-carbon atom, are given *para* σ values. No σ factors are quoted for the *ortho* substituents in the 4- position, as both steric and field effects are superimposed, to varying extents, on polar effects, making precise evaluation of these factors difficult (O'Sullivan and Sadler, 1957a). The 5- and 7-substituents are arranged in order of increasing *meta* σ values.

Table II shows that there is no parallel between antiviral activity and the σ values of the substituents. Considerable discrepancies occur between the data for the 5- and 7- substituents although their σ factors are identical, and, although a fairly wide range of σ values is covered in the 5- substituted series, ranging from the low value with the amino group through the intermediate values with the halogen substituents to the high value with the nitro group, most of these show zero activity. However, two generalizations emerge. Firstly, the smallest substituent (fluorine) in both the 5- and 6-positions decreases the activity the least (all other substituents quoted have much greater atomic or group radii), and secondly, substituents in the 7-position exert a noticeably smaller effect. This suggests that steric effects are far more important than the inductive and mesomeric effects of the substituents. Supporting evidence for this is that neither the 4,5- nor the 6,7-naphthisatin β-thiosemicarbazone possesses antiviral activity (see below). The observation that most activity is retained in the 7- substituted derivatives might be related to the relatively high solubility of the parent isatins. These compounds probably owe their enhanced solubilities to steric hindrance of the normal hydrogen-bonded dimer (O'Sullivan and Sadler, 1956), which suggests that the *N*-alkyl derivatives might have greater activity as these cannot dimerize in this fashion.

Effect of N-substitution upon Antiviral Activity

The activity of a number of *N*-substituted isatin β-thiosemicarbazones is shown in Table III. Alkylation in this position produces a marked rise in activity, reaching a maximum with 1-ethylisatin β-thiosemicarbazone, which with a relative activity of 286 is the most active compound so far encountered. Activity falls off as the chain is lengthened further, and the 1-pentyl derivative is practically inactive. High activity is retained in the 1-(2-hydroxyethyl) compound, but most of the other substituents which are either large or strongly polar have only residual or zero activity. This supports the hypothesis that isatin β-thiosemicarbazone should be monomeric and not form *inter*molecular bonds if high activity is to be retained.

TABLE III

ANTIVIRAL ACTIVITY OF *N*-SUBSTITUTED ISATIN β-THIOSEMICARBAZONES

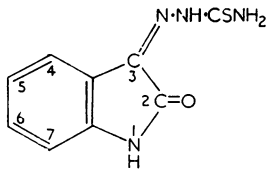
Substituent	Antiviral Activity
None	100
Methyl	202
Ethyl	286
Isopropyl	44
Propyl	28·5
Pentyl	3·4
Hydroxymethyl	42
1-Methyl-4-trifluoromethyl	48·4
2-Hydroxyethyl	204
Acetyl	87
Ethoxycarbonylmethyl	0
Diethoxycarbonylmethyl	0
2-Cyanoethyl	0
Carboxymethyl	0·02
5-Ethoxycarbonylmethyl-1-methyl	0
5-Carboxymethyl-1-methyl	0

Effect of Modifications in the Pyrrolidine Ring

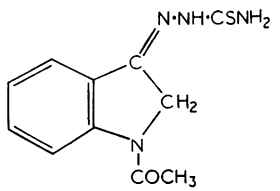
Modifications were next made at the 2- and 3-positions. The inactivity of 1-acetylindoxyl thiosemicarbazone (II) readily demonstrated that the α-carbonyl group was essential. Extension of the side-chain at the 3- position resulted in loss of activity, as 3-formyl-1-methyloxindole thiosemicarbazone (III) and 3-formylindole thiosemicarbazone were completely inactive. These and other similar results suggest that isatin β-thiosemicarbazone exerts its antiviral activity in the form of a six-membered, resonance-stabilized, hydrogen-bonded ring (IV), a type of bonding which has previously been demonstrated in the isatin β-oximes (O'Sullivan and Sadler, 1957b). Support for this hypothesis is provided by an analysis of the infra-red absorption data of isatin β-thiosemicarbazone and relevant reference compounds both in the solid state and in weakly polar solvents; however, the stability of such *intra*molecular bonds in an aqueous medium is uncertain. Isatin α-thiosemicarbazone may possess a similar *intra*molecular bonded structure (V), but it had no antiviral activity. This suggests not only that an all-planar configuration is

necessary but also that the orientation of the $-CS.NH_2$ group relative to the ring nitrogen is critical.

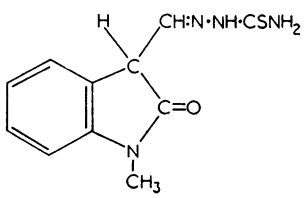
(I)



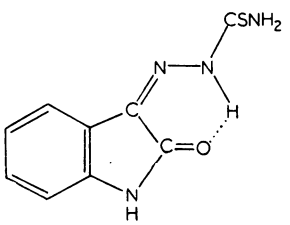
(II)



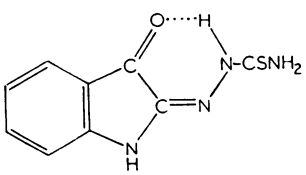
(III)



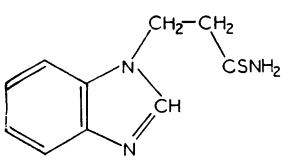
(IV)



(V)



(VI)



Effect of Substitution in the Side-chain

Finally, modifications were made to the thiosemicarbazone moiety, and the effects on antiviral activity are shown in Table IV. If *intra*molecular hydrogen bonding is of importance then replacement of the hydrogen atom on the nitrogen atom at the 2- position of the side-chain by another atom or group should result in loss of activity, as the molecule may no longer be held in configuration IV. Reference to Table IV shows this to be the case. The rest of the results presented indicate that an unsubstituted $-CS.NH_2$ group is essential for retention of activity.

TABLE IV

EFFECT OF SUBSTITUTION IN THE SIDE-CHAIN ON THE ANTIVIRAL ACTIVITY OF ISATIN β-THIOSEMICARBAZONE

R′–	R″=	R‴–	Antiviral Activity
H–	S=	H–	100
H–	O=	H–	0
H–	S=	C_6H_5	0
H–	HN=	H–	0
H–	S=	CH_3–	0
C_6H_5	S=	CH_3–	?
C_6H_5	S=	H–	0
H–	S=	CH_2=CH–CH_2–	4

R′–	R″–	R‴–	Antiviral Activity
—	CH_3–S–	H–	0

Miscellaneous Compounds

The following compounds were inactive: isatin, thiosemicarbazide, isatin β-amidinohydrazone, isatin β-hydrazone, di-β-isatinazine, 3-formyl-1-methyloxindole thiosemicarbazone, 1-acetylindoxyl thiosemicarbazone, α-naphthisatin β-thiosemicarbazone, β-naphthisatin β-thiosemicarbazone, isatin β-semicarbazone, isatin α-thiosemicarbazone, β-1-benzimidazolylpropionthioamide.

Activity of Isatin β-Thiosemicarbazone Against Other Viruses

In tests carried out in mice by the same method as the preliminary test of activity against neurovaccinia virus, isatin β-thiosemicarbazone in repeated doses of 100–125 mg./kg. had no therapeutic effect upon intracerebral infection with

poliomyelitis, influenza (NWS strain), rabies (Flury strain), Ilhéus, Wyeomyia, Zika, California, pseudolymphocytic choriomeningitis (Sandom strain, identical with ectromelia), Ntaya, Semliki, herpes, dengue 1, Anopheles A, Anopheles B or MM viruses.

Activity of Substituted Isatin β-Thiosemicarbazones and Related Compounds Against Ectromelia

In view of the close antigenic relationship between vaccinia and ectromelia viruses many of the compounds were tested against intracerebral infection with ectromelia (pseudolymphocytic choriomeningitis) virus. The following compounds showed no detectable activity when tested at doses ranging in some cases up to 100 mg./kg.: isatin β-semicarbazone, isatin β-4-phenyl thiosemicarbazone, isatin β-amidinohydrazone, isatin β-hydrazone, di-β-isatinazine, 3-formyl-indole thiosemicarbazone, and the β-thiosemicarbazones of the following substituted isatins: 5-fluoro-, 5-chloro-, 5-bromo-, 5-methoxy-, 5-ethyl-, 5-nitro-, 4-methyl-, 7-chloro-, 7-nitro-, 1-methyl-, 1-hydroxymethyl-, 1-(2-hydroxyethyl)- and 1-(2-cyanoethyl)-isatin.

Discussion

Isatin is a diketonic compound and it has many of the properties of a simple diketone. A number of dicarbonyl compounds have been reported to possess antiviral activity against Newcastle disease virus and the PR8 strain of influenza virus in fertile eggs (McLimans, Underwood, Slater, Davis, and Siem, 1957), but they were also virucidal *in vitro* and the effect was presumably due to the degradation of the amino-acids of the virus by the Strecker reaction. A mechanism of this kind cannot explain the activity of isatin β-thiosemicarbazone, since isatin has no activity against vaccinia virus *in vivo*, and the antiviral activities of the substituted isatin β-thiosemicarbazones show no correlation with the dehydrogenase activity of the corresponding isatins, as shown in Table V. The logarithms of the chloroform solubilities of the compounds shown in Table V show a positive correlation with antiviral activity (correlation coefficient 0.775, $P<0.01>0.001$). The increased activity of the simple *N*-alkyl derivatives may therefore result entirely from their improved lipid solubility, which might more readily enable them

TABLE V

σ-VALUES AND DEHYDROGENASE ACTIVITIES OF SUBSTITUTED ISATINS, WITH ANTIVIRAL ACTIVITIES AND PARTITION COEFFICIENTS OF THE CORRESPONDING β-THIOSEMICARBAZONES

Figures in column three are obtained from the reciprocals of the times required for the systems to decolorize methylene blue in 10^{-4} M solution of the substituted isatin and 0·05 M DL-alanine (O'Sullivan and Sadler, 1957c).

Substituent	σ	Dehydrogenase Activity Relative to DL-Alanine	Antiviral Activity	Solubility in Chloroform (mg./100 ml.)	Chloroform Water Partition
4-Methyl	—	11	3·4	8	0·063
6-Methyl	−0·170	30	0·3	18	0·15
7-Methyl	−0·069	55	88·8	16	0·185
None	0·0	100	100	32	0·33
6-Fluoro	0·062	50	39·8	16	0·48
5-Methoxy	0·115	18	0·03	3	0·077
4-Chloro	—	105	8·6	10	0·34
6-Chloro	0·227	180	4	21	0·69
4-Bromo	—	200	49·5	10	0·205
5-Fluoro	0·337	—	35·5	4	—
7-Carboxy	0·355	—	0	0	∞
7-Chloro	0·372	210	85	29	0·097
1-Methyl	—	—	190	160	0·083
1-Acetyl	—	—	87	255	—
1-Pentyl	—	—	0	>200	0·0
1-Diethoxycarbonylmethyl	—	—	0	>200	0·33
1-Ethyl	—	—	286	2,170	—
1-(2-Hydroxyethyl)	—	—	204	25	—
1-Butyl	—	—	28·5	1,600	—
1-Isobutyl	—	—	44	2,100	—

to pass the blood-brain barrier and so reach the neurotropic strain of vaccinia used as the test virus. This would also account for the inactivity of the compounds bearing strongly polar substituents. The reduction in activity produced by substitution in the aromatic ring suggests that steric factors are of importance, in that this region of the molecule must not exceed certain dimensions if activity is to be retained.

The general configuration of the isatin molecule resembles that of the purines and also tryptophan and its derivatives, but there is no evidence available as to whether isatin β-thiosemicarbazone acts as an antimetabolite to these compounds; in particular, an action against 5-hydroxytryptamine seems to be excluded by the lack of antiviral activity of 5-hydroxyisatin β-thiosemicarbazone.

The requirement for sulphur in the side-chain suggests that metal chelation may be of importance for antiviral activity, particularly of copper, and it is of interest to note in this connexion that vaccinia virus contains copper (Hoagland, Ward, Smadel, and Rivers, 1941), a circumstance which might enable it to take up isatin β-thiosemicarbazone selectively, and that salts of copper have a therapeutic effect against neurovaccinia infection in mice (Bauer, 1958). However, even if chelation is concerned, the configuration of the ligand is also critical, since, whereas all thiosemicarbazones chelate copper, only a very limited number have antivaccinial activity.

Several possibilities exist for the *in vivo* conversion of the compound into biologically more active substances. For instance, simpler substituted thioureas may be obtained, which bear resemblance to some of the phenylthiourea derivatives recently described by Doub *et al.* (1958). These compounds have marked tuberculostatic activity (Youmans *et al.*, 1958), but this is of little import as antiviral and tuberculostatic activities rarely run parallel (Hurst and Hull, 1956); benzthiouracils could also be obtained, and substituted phenoxythiouracils have considerable antiviral activity (Bauer, 1955), but this route is rendered unlikely as the suggested mechanism for the formation of the benzthiouracil seems chemically improbable, although it might occur biologically. Formation of a tricyclic compound by loss of the elements of water from the α-carbonyl group and the terminal amino group of isatin β-thiosemicarbazone is possible, and it has been clearly demonstrated that both these groups are essential for the retention of activity. However, the resulting structure is not sufficiently close to growth inhibitors such as 2-thiodenine or 6-thiopurine (Elion *et al.*, 1953) to be considered as a purine antagonist. The relationship to thioalloxazine is also somewhat remote, but the enhanced activity which results from 2-hydroxyethyl substitution in the 1-position (Table III) makes the synthesis of related glycosides of considerable interest as potential riboflavine antagonists.

The structures of these compounds seem too remote from that of nicotinamide to be considered as competitive inhibitors; some support is given by the fact that similar arguments have been used in the case of the tuberculostatic compound isoniazid (isonicotinic acid hydrazide) (Zatman *et al.*, 1953). Obviously other heteroaromatic thioamides containing more than one nitrogen atom per heterocycle are worthy of investigation, but it would seem to be essential for the thiocarbamyl group to be attached directly to one of the rings, as β-1-benzimidazolylpropionthioamide (VI) is inactive. The lack of activity of isatin β-thiosemicarbazone against other viruses, particularly ectromelia, is difficult to explain in accordance with the commonly held view that viruses control the metabolism of the cell in which they are multiplying, for this implies that the final common path in virus multiplication must be the same for all viruses. A possible explanation is that isatin β-thiosemicarbazone chelates with the copper present in vaccinia virus and thereby exerts a detrimental effect upon virus multiplication; if this is the case, then the lack of activity against other viruses implies that they do not contain copper, or, if they do, then the copper is either inaccessible or held in a chelate of higher stability constant.

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