THE CHEMOTHERAPY OF ECTROMELIA INFECTION WITH ISATIN β-DIALKYLTHIOSEMICARBAZONES

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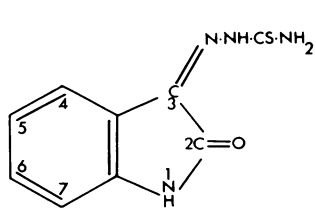
ISATIN β-thiosemicarbazone (I) and certain of its derivatives have been found to possess a high degree of antiviral activity in animals infected with vaccinia, rabbitpox, alastrim, variola major, and white cowpox viruses (Thompson, Minton, Officer and Hitchings, 1953 ; Bauer, 1955 ; Bauer and Sheffield, 1959 ; Bauer and Sadler, 1960*a* ; Bauer, Dumbell, Fox-Hulme and Sadler, 1962 ; Bauer, 1961). Isatin β-thiosemicarbazone itself, however, was found to be inactive against



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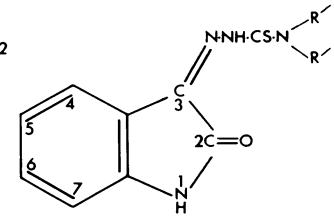

II

ectromelia virus (Bock, 1957). This observation was confirmed by Bauer and Sadler (1960*b*), who also found no activity against ectromelia among 19 derivatives of the compound. When the hydrogen atoms of the terminal amino group of the side-chain were replaced by two alkyl groups (II), however, high activity against ectromelia appeared and activity against vaccinia and the other viruses was abolished (Bauer and Sadler, 1961). Thus *N*-methylisatin β-4′ : 4′-dimethylthiosemicarbazone (II, R′ = Me) administered subcutaneously in a repeated dose of 50 mg./kg. would protect mice against death after intraperitoneal infection with 1000 LD_{50} of the Sandom (pseudolymphocytic choriomeningitis) strain of ectromelia virus.

In further work, which is the subject of the present communication, a number of other related compounds were synthesized and tested in order to establish the structure-activity relationships of the anti-ectromelia activity, and in particular to ascertain whether they are the same as those already established for the activity of isatin β-thiosemicarbazone against vaccinia infection. The effect of chemotherapeutic treatment upon the multiplication of ectromelia virus in mice was also investigated, and evidence is presented that the active compounds, in addition to protecting against death, also inhibit the multiplication of the virus.

MATERIALS AND METHODS

Viruses

Ectromelia.—The Sandom (pseudolymphocytic choriomeningitis), Hampstead and Moscow strains.

Vaccinia.—The I.H.D. strain.

All the viruses were adapted to intracerebral passage in mice. At the onset of symptoms the brains were removed, ground with tissue culture medium (Sheffield, Bauer and Stephenson, 1960) and mixed with 1/3 volume of glycerol to make a stock suspension with a final content of brain material of 10 per cent. The stock suspensions were stored at $-25°$; for use in the experiments serial decimal dilutions were prepared in tissue culture medium and were left in ice while inoculations were being carried out.

Virus growth curve

Two groups of 12 mice were infected intracerebrally with a selected dilution of virus. One group was used as a control. The mice of the other group were dosed with the compound under test. This was ground and suspended in 5 per cent gum arabic solution in such a concentration that the dose selected was contained in 0·1 cc.; this volume of the suspension was injected subcutaneously twice daily for 4 days, the first dose being given 6–7 hr. after infection. On the 1st day after infection and at daily intervals thereafter 2 mice were selected at random from each of the groups; the brains were removed and ground together with 8 cc. of tissue culture medium so as to give a 10 per cent suspension. Coarse particles were removed by low-speed centrifugation and each supernatant fluid was injected intracerebrally into a group of 12 mice, which were kept under observation for 14 days. The survival time of the mice which died was recorded to the nearest half-day.

The survival times were converted into reciprocals, the value for survival beyond the period of observation being taken as zero, and the mean reciprocal survival time for each group was converted into a titre in D_0 units (Bauer, 1960) by dividing by $-0·033$ (the regression coefficient of the dose-response curve of the Sandom strain of ectromelia virus on intracerebral infection) and changing the sign.

Dose-response curve of antiviral activity

Six groups of 6 mice were infected intracerebrally with about 1000 LD_{50} of virus. The animals of the first and last groups were left untreated as controls. Four dose-levels of the test compound increasing in a 2-fold ratio and generally covering a range of effect from minimal to complete protection were selected on the basis of the results of rough preliminary tests of activity, and each dose-level was assigned to the treatment of one of the remaining groups of mice. The compound was administered subcutaneously 6 hr. after infection and twice daily thereafter for 4 days. The mice were observed for 14 days and the survival times were recorded to the nearest half-day. The mean reciprocal survival time for each group of treated mice was plotted against the logarithm of the dose of compound to the base 2, and a line was fitted to the points by the method of least squares. From the abscissa of this line at the point corresponding to the mean ordinate for the 2 control groups the E_0 (Bauer, 1961), or zero effect dose, was calculated, giving the required numerical measure of the antiviral effect.

Compounds

Some of the compounds have been described previously (Bauer and Sadler, 1961); the remainder were synthesized by Dr. P. W. Sadler and Mrs. C. Webley in the Courtauld Institute of Biochemistry, London.

RESULTS

Effect of Treatment on Growth of Virus

Mice were infected intracerebrally with 1000 LD_{50} of the Sandom strain of ectromelia virus and treated with isatin β-4′ : 4′-pentamethylenethiosemicarbazone in a dose of 2 mg.; the curve of growth of the virus in the brains of the treated animals and in a control group of infected and untreated animals was then obtained by the method described, and is illustrated in Fig. 1. In the absence of chemo-

therapeutic treatment (upper curve) the virus multiplies exponentially, increasing in titre by about 4 log units over a period of 4 days. In the treated animals (lower curve) no evidence of any appreciable rise in titre could be obtained, and after 4 days of treatment the brains contained about 1/1000th of the amount of virus present in the untreated animals. The compound thus has a virustatic action, being able to inhibit the multiplication of the virus in experimental animals.

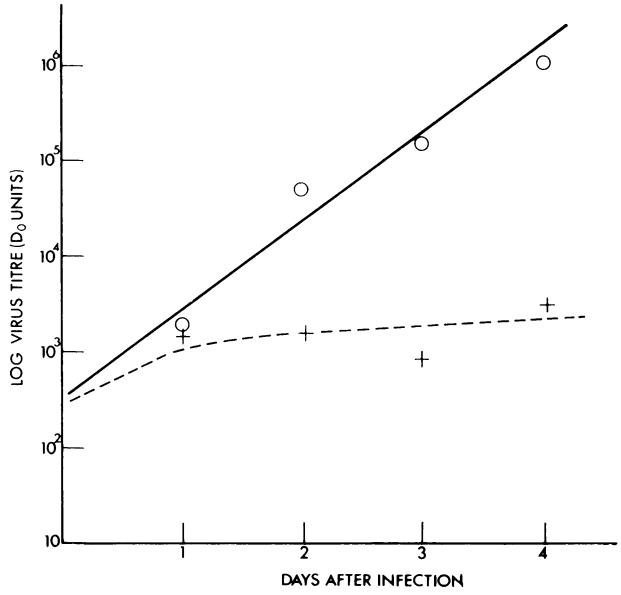

FIG. 1.—Effect of isatin β-4′ : 4′-pentamethylenethiosemicarbazone on the multiplication of the Sandom strain of ectromelia virus. Upper curve : growth of virus in the brains of control mice. Lower curve : growth of virus in the brains of treated mice.

Similar results were obtained in mice infected intracerebrally with about 1000 LD_{50} of the Hampstead strain of ectromelia virus and treated with isatin β-4′ : 4′-tetramethylenethiosemicarbazone according to the same dosage schedule. The control animals all died after 5 days, whereas the brains of the treated mice only contained 1/1000th of the amount of virus at this time, and virus could no longer be detected in the brain after 9 days (Fig. 2).

Effect of Substitution on Antiviral Activity

Introduction of methyl groups in the 4′-position

The dose-response curve of isatin β-thiosemicarbazone in mice infected with about 1000 LD_{50} of the Sandom strain of ectromelia virus is shown in Fig. 3 (curve *a*). The slope of the curve does not differ significantly from the mean ordinate of the control group (dotted line), and the compound is therefore devoid of antiviral effect in doses ranging from 0·5 to 4 mg. (25–200 mg./kg.). A similar dose-response curve was obtained with isatin β-4′-methylthiosemicarbazone (Fig. 3, *b*). With isatin β-4′ : 4′-dimethylthiosemicarbazone, however, a dose-response curve indicating high antiviral activity was obtained (Fig. 3, *c*). The E_0 lay

around 0·05 mg., and complete protection against death (reciprocal survival time of zero) is attained with a dose of 0·8 mg. (40 mg./kg.).

With vaccinia virus the same compounds gave the same sequence of dose-response curves but in reversed order (Fig. 4). Here, the dose-response curve indicative of high antiviral activity was given by isatin β-thiosemicarbazone

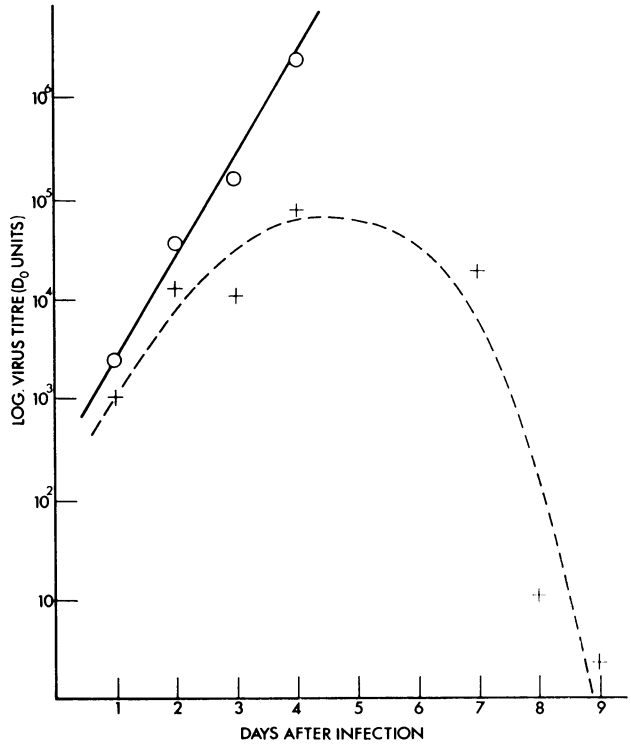

FIG. 2.—Effect of isatin β-4′ : 4′-tetramethylenethiosemicarbazone on the multiplication of the Hampstead strain of ectromelia virus. Upper curve : growth of virus in the brains of control mice. Lower curve : growth of virus in the brains of treated mice.

(curve *a*), and the other 2 compounds gave horizontal curves not differing significantly from the control values. The introduction of a methyl group into the 4′-position of the side-chain is thus sufficient to abolish activity against vaccinia, but two methyl groups are necessary for the appearance of activity against ectromelia, one group being insufficient.

Effect of higher alkyl groups in the 4′-position

After establishing that the introduction of two methyl groups into the 4′-position of the side-chain of isatin β-thiosemicarbazone conferred activity against ectromelia, the effect of introducing higher alkyl groups was next investigated. Fig. 5 shows dose-response curves obtained against the Sandom strain of ectromelia virus with isatin β-4′ : 4′-dimethylthiosemicarbazone (curve *a*), and the next 3 higher homologues. The antiviral activity of isatin β-4′ : 4′-diethylthiosemicarba-

zone (curve *b*) and isatin *β*-4′ : 4′-dipropylthiosemicarbazone (curve *c*) are approximately equal, since their dose-response curves coincide, and less than that of the dimethyl compound, since the curves are displaced away from the origin. Isatin *β*-4′ : 4′dibutylthiosemicarbazone (curve *d*) is still less active. Increasing the chain length of the alkyl substituent in the 4′-position thus reduces the activity against ectromelia virus.

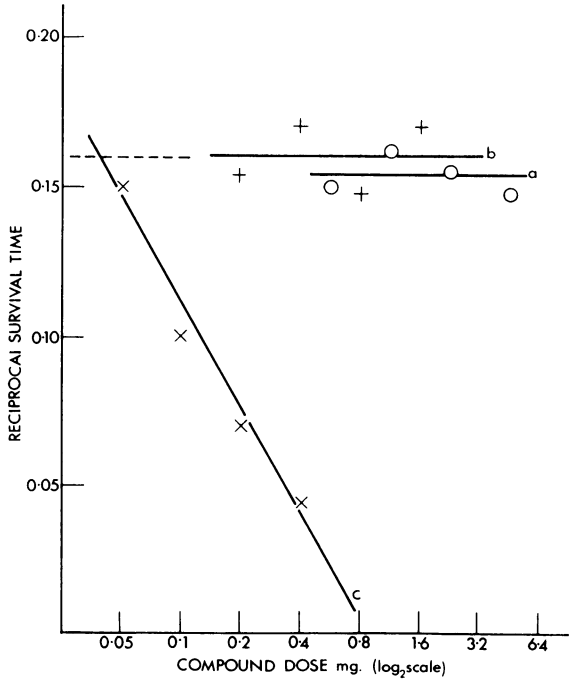

FIG. 3.—Dose-response lines of the action of (*a*) isatin *β*-thiosemicarbazone, (*b*) isatin *β*-4′-methylthiosemicarbazone and (*c*) isatin *β*-4′ : 4′-dimethylthiosemicarbazone in mice infected intracerebrally with the Sandom strain of ectromelia virus. The dotted line indicates the mean value of the ordinate obtained in the absence of treatment.

Effect of substitution in the 1*-position*

Taking isatin *β*-4′ : 4′-dimethylthiosemicarbazone as the reference compound, the effect of substitution in the 1-position was next investigated (Fig. 6). Substitution of a methyl group displaces the dose-response curve from the position characteristic of the unsubstituted compound (curve *a*) towards higher dose-levels (curve *b*), and a still further displacement is produced by substitution of an ethyl group (curve *c*). Alkylation in the 1-position therefore reduces antiviral activity against ectromelia ; this is in contrast to the results obtained with vaccinia (Bauer and Sadler, 1960*b*) and variola major viruses (Bauer *et al.*, 1962), in which 1-alkylation causes an increase in antiviral activity.

The effects of substitution in the 4′-position of the side-chain and in the 1-position, which have been illustrated graphically in Fig. 3, 4, 5 and 6, are presented in a numerical form in Table I, in which the values of the E_0 determined against the Sandom strain for the various derivatives are presented.

TABLE I.—*Effect of Substitution in the* 1- *and* 4′-*Positions on the Anti-ectromelia Activity of Isatin β-*4′ : 4′-*dimethylthiosemicarbazone.*

Substituent in 1-position	Substituent in 4′-position and value of E_0					
	Dimethyl	Diethyl	Dipropyl	Dibutyl	Methylbutyl	Diallyl
None	0·025–0·05	0·05	0·02–0·04	<0·25	0·05–0·1	0·1
Methyl	0·025–0·05	0·05	0·05	0·1·012	–	–
Ethyl	0·05	0·05–0·1	0·2	~1·0	–	–

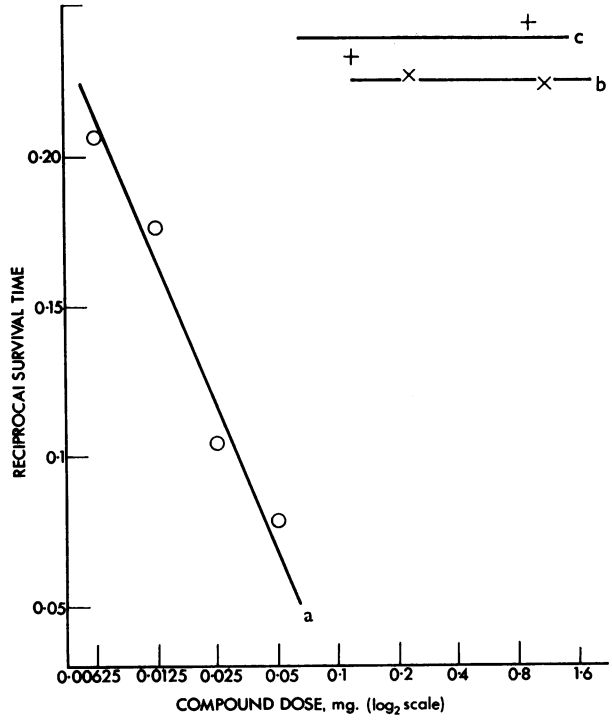

FIG. 4.—Dose-response lines of the action of (*a*) isatin β-thiosemicarbazone, (*b*) isatin β-4′-methylthiosemicarbazone, and (*c*) isatin β-4′ : 4′-dimethylthiosemicarbazone in mice infected intracerebrally with neurovaccinia virus.

TABLE II.—*Effect of Substitution in the Aromatic Ring and in the* 4′-*Position on the Anti-ectromelia Activity of Isatin β-*4′ : 4′-*dimethylthiosemicarbazone*

Substitution in aromatic ring		Substituent in 4′-Position and value of E_0	
Position	Substituent	Methyl	Ethyl
5	Chloro	~0·5	—
5	Methoxyl	0·05–0·1	0·1-0·2
6	Chloro	~0·1	~0·2
6	Methyl	~0·05	—
7	Methyl	~0·1	>0·2

Effect of substitution in the 5-position

With vaccinia virus substitution in the 5-position of isatin β-thiosemicarbazone was found to be particularly detrimental to antiviral activity (Bauer and Sadler, 1960*b*) ; it was therefore of interest to investigate the effect of this substitution on the activity of the isatin dialkylthiosemicarbazones against ectromelia virus, and some typical results are shown in Fig. 7 and Table II. Comparison of the dose

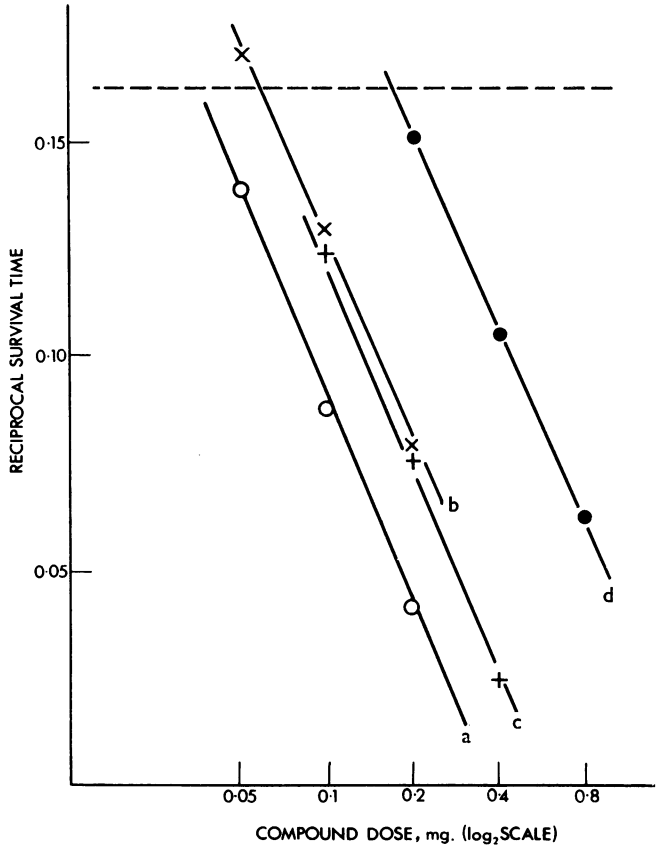

FIG. 5.—Dose-response lines of the action of (*a*) isatin β-4′ : 4′-dimethylthiosemicarbazone, (*b*) isatin β-4′ : 4′-diethylthiosemicarbazone, (*c*) isatin β-4′ : 4′-dipropylthiosemicarbazone and (*d*) isatin β-4′ : 4′-dibutylthiosemicarbazone in mice infected intracerebrally with the Sandom strain of ectromelia virus. The dotted line represents the mean value of the ordinate obtained in the absence of treatment.

response curves of isatin β-4′ : 4′-dimethylthiosemicarbazone (curve *a*) and of its 5-methoxy derivative (curve *b*) shows that the latter still retains about 50 per cent of the activity of the unsubstituted compound, and a similar effect is noted with isatin β-4′ : 4′-diethylthiosemicarbazone (curve *c*) and its 5-methoxy derivative (*d*). In contrast, the substitution of a methoxyl group in the 5-position of isatin β-thiosemicarbazone reduces the activity against vaccinia virus by 97 per cent (Bauer and Sadler, 1960), and a further difference thus appears between the structure-activity requirements for vaccinia and ectromelia viruses.

The effect of substitution in the 6- *and* 7-*positions*

Substitution in the 6- and 7-positions of the aromatic ring was also consistent with the retention of the greater part of the antiviral activity. The values of the E_0 obtained against the Sandom strain with derivatives substituted in the aromatic ring are presented in Table II.

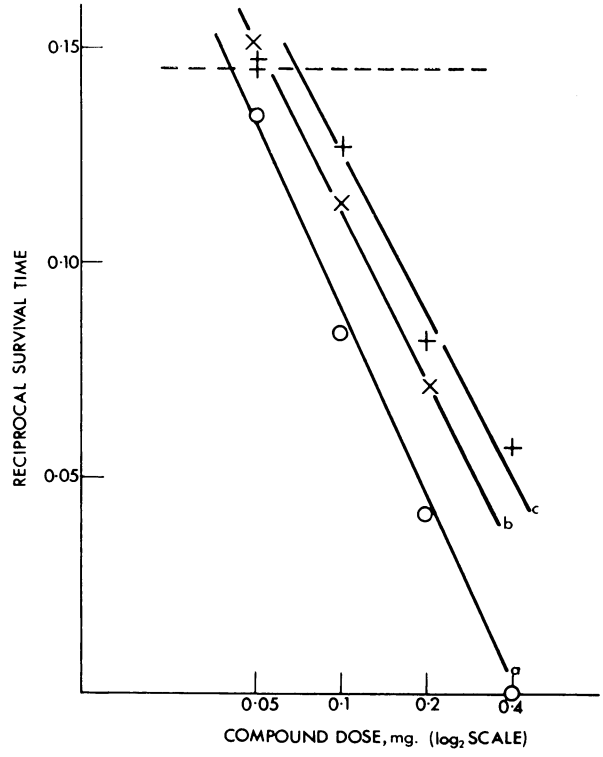

FIG. 6.—Dose-response lines of the action of (*a*) isatin β-4′ : 4′-dimethylthiosemicarbazone, (*b*) 1-methylisatin β-4′ : 4′-dimethylthiosemicarbazone, and (*c*) 1-ethylisatin β-4′ : 4′-dimethylthiosemicarbazone in mice infected intracerebrally with the Sandom strain of ectromelia virus. The dotted line represents the mean value of the ordinate obtained in the absence of treatment.

DISCUSSION

The results obtained show that the protection against death conferred by the isatin 4′ : 4′-dialkylthiosemicarbazones in mice infected with ectromelia virus is due to inhibition of the multiplication of the virus, and not to some unspecific effect such as suppression of the inflammatory response.

The results also provide further information on the chemotherapeutic sensitivity of ectromelia virus in comparison with that of vaccinia, variola major and alastrim viruses. There is a fundamental basic relationship between the sensitivities of the 2 groups of viruses in that the isatin molecule is required in both cases, but there is also a fundamental difference, in that, given the presence of the isatin molecule, the side-chain must be substituted for activity against ectromelia and

unsubstituted for activity against the vaccinia group, and that the antiviral activities of these 2 classes of compounds are mutually exclusive.

The basic difference, which rests upon differences in the 4′-position of the side-chain, is also reflected in the minor differences which have been found in the structure-activity relationships of the 2 classes of compounds. Anti-ectromelia activity

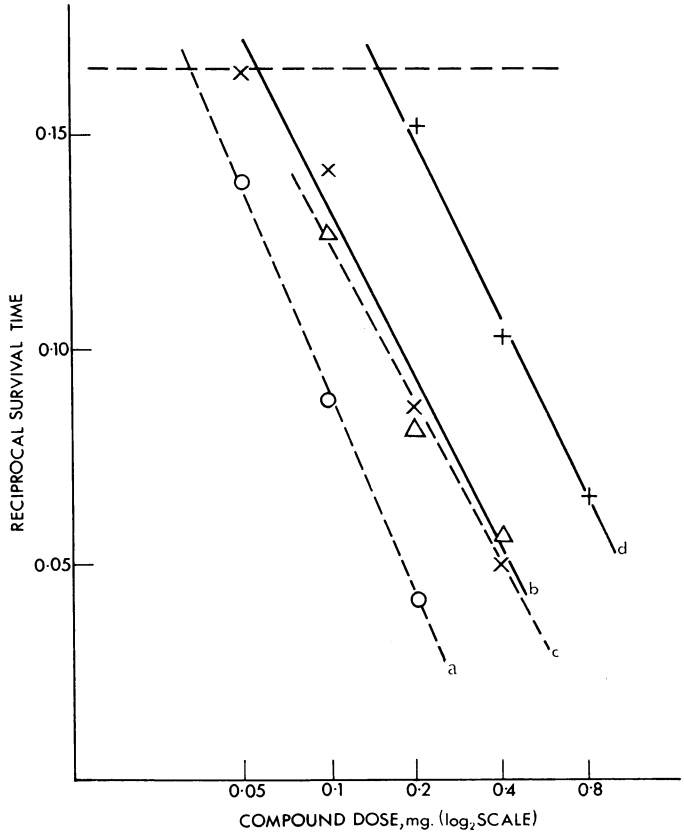

FIG. 7.—Dose-response lines of the action of (*a*) isatin β-4 : 4′-di'methylthiosemicarbazone, (*b*) 5-methoxyisatin β-4′ : 4′-dimethylthiosemicarbazone, (*c*) isatin β-4′ : 4′-diethylthiosemicarbazone, and (*d*) 5-methoxyisatin β-4′ : 4′-diethylthiosemicarbazone in mice infected intracerebrally with the Sandom strain of ectromelia virus. The horizontal dotted line represents the mean value of the ordinate obtained in the absence of treatment.

is reduced by alkylation in the 1-position, but is not greatly affected by substitution in the 5-position, whereas anti-vaccinia activity is increased by alkylation in the 1-position, and practically abolished by substitution in the 5-position.

These findings give further support to the hypothesis which has been propounded earlier, that the specificity of the antiviral agents of the isatin β-thiosemicarbazone series can only be explained by an action directed against the virus particle itself and not against a system in the host cell, and they further suggest that the differences in structure-activity relationships reflect differences at the molecular level in some constituent of the virus particle with which the compound interacts.

SUMMARY

The structure-activity relationships of the antiviral chemotherapeutic effect of isatin β-4′ : 4′-dialkylthiosemicarbazones in mice infected with ectromelia virus have been established. Evidence is presented to show that the compounds exert their protective effect by inhibiting the multiplication of the virus. The activity is reduced by increasing the chain length of the substituent in the 4′-position, and by substituting alkyl and other groups in the 1-position. Substitution in the 6- and 7-positions has relatively little effect. Substitution in the 5-position causes some reduction in activity, but this is not nearly so great as the effect of substitution in the 5-position on the activity of isatin β-thiosemicarbazone in infection with vaccinia virus. This and other differences in the structure-activity relationships of isatin β-thiosemicarbazone derivatives against vaccinia and ectromelia viruses are discussed, and it is concluded that they are a reflexion of differences in the constitution of the viruses at the molecular level.

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