THE TUBERCULOSTATIC ACTIVITY OF SOME THIOSEMICARBAZONES

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During the past five years, there has been a marked increase in the search for substances of possible value for the treatment of tuberculosis. The work has resulted in a large number of publications describing a number of compounds which show activity *in vitro*, in animals and, in some instances, in humans.

The discovery by Lehmann[1], and confirmation by others[2,3,4] that *para*-aminosalicylic acid possesses marked activity in the experimental animal and in humans prompted a detailed examination of a relatively large number of related substances. Reports which have been made[5,6,7,8] suggest that the particular configuration displayed by *para*-aminosalicylic acid is specific for activity.

Reports from Germany that certain benzaldehyde thiosemicarbazones, particularly those which are substituted in the aromatic nucleus in the 4-position, possess a high degree of activity both in the animal and in the human[9], suggested an investigation of a group of benzaldehyde thiosemicarbazones bearing some chemical relationship to *para*-aminosalicylic acid. The German workers have chosen from a number of thiosemicarbazones, 4-acetamidobenzaldehyde thiosemicarbazone (Thiacetazone, T.B.1, Conteben) as being the most useful substance.

The compounds which prompted the present study were thiosemicarbazones of derivatives of salicylaldehyde. The high *in vitro* activity of 4-aminosalicylaldehyde thiosemicarbazone reported by some of us in an earlier paper[8] stimulated a detailed investigation of a series of related substances.

Table I lists the substances studied. Compounds 11, 12 and *para*-aminosalicylic acid were included for purposes of comparison as Nos. 11 and 12 had previously been the subject of work by Domagk and his collaborators[9,10].

Experimental

(a) *Tuberculostatic Activity.* The *in vitro* activities of the compounds were determined by the technique previously described[6,7].

The *in vivo* activities were determined by two methods, firstly, the method of Rees and Robson[11], based on the effect of the drug on the development of tuberculous lesions in the mouse cornea, referred to in Table II as the " corneal test." The compounds were administered orally

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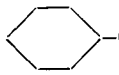
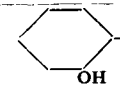
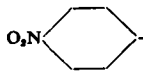
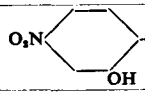
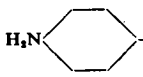
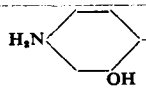
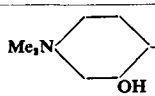
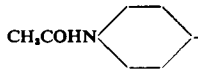
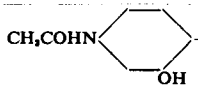
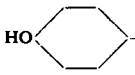
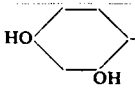
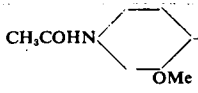
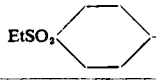
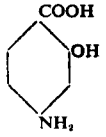
TABLE I

Name	Formula	Activity *in vitro* against *Mycobacterium tuberculosis* H37RV (Dubos type medium; inoculum 0·001 mg./ml.) Inhibitory concentration mg./100 ml.		Acute toxicity (Mouse LD50, g./kg.)	n
		In absence of serum	In presence of 10 per cent. of human serum		
dehyde semicarbazone		0·0121—0·0060	0·195—0·0975	0·1—0·2 (oral)	
ldehyde semicarbazone		0·195—0·0975	1·56—0·78	4 (subcutaneous) >2·5 (oral)	
benzaldehyde semicarbazone		0·39—0·195	1·56—0·78	2 (oral)	
salicylaldehyde semicarbazone		0·195—0·0975	0·78—0·39	>10 (oral)	
nobenzaldehyde semicarbazone		0·195—0·0975	0·78—0·39	0·5—0·6 (oral)	
nosalicylaldehyde semicarbazone *		0·0121—0·006†	0·39—0·195	3 (oral)	
ethylamino- ylaldehyde semicarbazone		0·0121—0·006	1·56—0·78	>10 (oral)	

hibitory concentration of this compound was reported in a previous paper as being 0·003 to 0·0015. We have not been able during the course of
isfactory explanation for this discrepancy. † Hoggarth, *et. el. Brit. J. Pharmacol, 1949*, 4, 248.

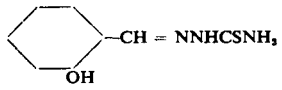
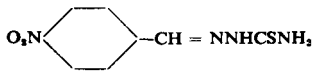
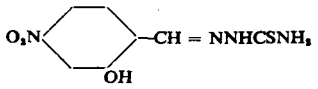
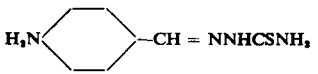
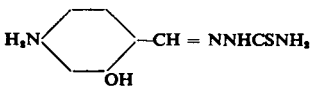
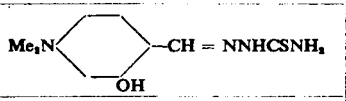
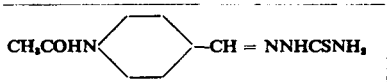
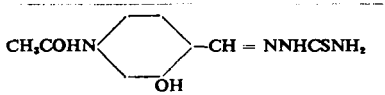
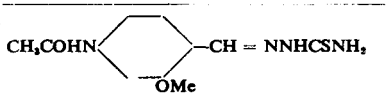
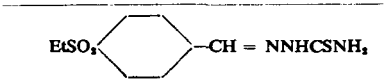
TABLE I (continued)

Name	Formula	Activity *in vitro* against *Mycobacterium tuberculosis* H37RV (Dubos type medium; inoculum 0·001 mg./ml.) Inhibitory concentration mg./100 ml.		Acute toxicity (Mouse LD50, g./kg.)
		In absence of serum	In presence of 10 per cent. of human serum	
amidobenzaldehyde semicarbazone acetozone; Domagk's l Conteben)		0·0121—0·006	0·048—0·024	1—2 (subcutaneous) >20 (oral)
amidosalicylaldehyde semicarbazone		1·56—0·78	1·56—0·78	>10 (oral)
roxybenzaldehyde semicarbazone		>0·006	0·39—0·195	4—5 (oral)
roxysalicylaldehyde semicarbazone		0·39—0·195	———	>3 (oral)
amido-2-methoxy-aldehyde semicarbazone		0·78—0·39	3·125—1·56	>5 (oral)
lsulphonyl-aldehyde semicarbazone magk's T.B.3, 8338†)		0·0976—0·0487	———	>10 (oral)
-Aminosalicylic Acid		0·0487—0·0243	0·0975—0·0487	4 (subcutaneous) 4 (oral)

Hoggarth *et. al. Brit. J. Pharmacol.*, 1949, 4, 248.

in admixture with the diet and a compound shown as " active " in Table II is one which after 28 days' administration prevented the development of a corneal lesion, as compared with the controls which all developed tuberculous lesions by the 14th day.

TABLE II

Compound Number	Formula	Survival test activity (a) (b)	Corneal test activity (a)
10		——	Inactive
32		Active	Active
30		——	Inactive
24		Active	Active
5		Inactive	Inactive
7		Inactive	Inactive
11		Active	Active
6		——	Inactive
51		——	Inactive
12		Active	Active

(a) The compounds were administered at approximately the maximum tolerated dose.
(b) Administered by gastric tube in suspension in gum acacia.

The second test employed was the mouse survival test described by several authors[12,13,14] which is based on the effect of the drug on the survival time of mice after intravenous injection with *Mycobacterium tuberculosis*. By the " survival test " compounds in Table II are listed as " active," if they give a significant increase in the survival time of a group of 10 mice over a group of controls.

(b) *Toxicity.* The approximate acute LD50 of each compound was determined in the usual way, using the oral route in all cases, supplemented by subcutaneous administration in a few instances. Chronic toxicities were determined by daily oral doses given over a period of 28 days.

(c) *Synthesis.* The syntheses of the compounds not previously described are herewith reported:—

4-Nitrosalicylaldehyde Thiosemicarbazone (*4-nitro-2-hydroxybenzaldehyde thiosemicarbazone—Compound No. 30*). 4-Nitro-2-acetoxytoluene (40 g.) was dissolved in a mixture of acetic anhydride (600 ml.) and acetic acid (600 ml.). The solution was cooled to 0°C. and sulphuric acid (96 ml.) was added with stirring. To the cold acid solution chromium trioxide (112 g.) was added in portions over 30 minutes. The mixture was finally stirred for a further 3 hours, maintaining the temperature between 5° and 10°C. On pouring into iced water (5 l.) the precipitate which separated was filtered, dried and crystallised from benzene-ligroin to yield 4-nitro-2-acetoxybenzal diacetate (20·5 g.) as prisms, m.pt. 79°C. Found: C, 50·3; H, 4·2; N, 4·8. $C_{13}H_{13}O_8N$ requires: C, 50·2; H, 4·2; N, 4·5 per cent.

The above compound (1 g.) was hydrolysed by boiling with aqueous ethyl alcohol (50 per cent.) (4 ml.) containing sulphuric acid (0·25 ml.) for 30 minutes. On cooling to 0°C. 4-nitrosalicylaldehyde (0·5 g.) separated and was recrystallised from aqueous ethyl alcohol from which it was obtained as pale yellow plates, m.pt. 133° to 134°C. undepressed by a specimen prepared by Seggesor and Calom[15]. Found C, 50·5; H, 3·1; N, 8·0. $C_7H_5O_4N$ requires: C, 50·3; H, 3·0; N, 8·4 per cent.

The theoretical yield of the thiosemicarbazone was obtained by treating the above aldehyde in alcohol with an aqueous solution of thiosemicarbazide. 4-Nitrosalicylaldehyde thiosemicarbazone was obtained as yellow needles, m.pt. 230°C. (decomp.) from aqueous dioxan. Found: C, 40·0; H, 3·35; N, 23·0; S, 13·45. $C_8H_8O_3N_4S$ requires: C, 40·0; H, 3·33; N, 23·3; S, 13·33 per cent.

4-Aminosalicylaldehyde Thiosemicarbazone (*4-amino-2-hydroxybenzaldehyde thiosemicarbazone—Compound No. 5*). 4-Nitro-2-acetoxybenzal diacetate (2·5 g.) was reduced in ethyl acetate solution (20 ml.) by means of hydrogen in the presence of Adam's catalyst. After filtration and removal of the solvent *in vacuo* 4-amino-2-acetoxybenzal diacetate (2·1 g.) was obtained, which on crystallisation from benzene-ligroin separated as pale yellow prisms, m.pt. 101°C. (decomp.). Found: C, 55·8; H, 5·25; N, 5·2. $C_{13}H_{15}O_6N$ requires C, 55·5; H, 5·33; N, 5·0 per cent.

The above compound (8 g.) on adding to a boiling solution of thiosemicarbazide (8 g.) in water (130 ml.) containing ethyl alcohol (10 ml.) and 2N hydrochloric acid (10 ml.) was converted to the crude thiosemicarbazone. 4-Aminosalicylaldehyde thiosemicarbazone was obtained as yellow needles, m.pt. 217°C. (decomp.), from aqueous propylene glycol.

Found: C, 45·7; H. 4·7; N, 26·7; S, 15·4. $C_8H_{10}ON_4S$ requires: C, 45·7; H, 4·8; N, 26·7; S, 15·2 per cent.

4-Acetamidosalicylaldehyde Thiosemicarbazone. (4-acetamido-2-hydroxy benzaldehyde thiosemicarbazone—Compound No. 6). 4-Nitro-2-acetoxybenzal diacetate was reduced in ethyl acetate solution by means of hydrogen in the presence of Adams catalyst. The resultant 4-amino-2-hydroxybenzal diacetate was acetylated without purification by means of acetic anhydride. The resultant tetracetate was isolated by precipitation with a large volume of water. 4-Acetamido-2-acetoxybenzal diacetate separated in lustrous plates from ethyl acetate-ligroin, m.pt. 135°C. Found: C, 55·7; H. 5·38; N, 4·40. $C_{15}H_{17}O_7N$ requires C, 55·7; H. 5·26; N, 4·35 per cent.

4-Acetamido-2-acetoxybenzal diacetate (1 g.) was dissolved in ethyl alcohol (5 ml.) and 20 per cent. sodium hydroxide solution (5 ml.) and allowed to stand for 2 hours at room temperature. Acidification with 5N sulphuric acid gave crude aldehyde (0·4 g.). 4-Acetamidosalicylaldehyde separated with 1 molecule of water as pale yellow needles from aqueous alcohol, m.pt. 186°C. (decomp.). Found: C, 54·7; H, 5·56; N, 7·11. $C_9H_9O_3N,H_2O$ requires C, 54·8; H, 5·58; N, 7·10 per cent.

Drying at 100°C. 1 mm. gave the anhydrous aldehyde. Found: C, 60·1; H, 5·00; N, 7·8. $C_9H_9O_3N$ requires C, 60·3; H, 5·03; N, 7·8 per cent.

4-Acetamidosalicylaldehyde (0·1 g.) in ethyl alcohol (3 ml.) was added to a hot solution of thiosemicarbazide (0·06 g.) in water (5 ml.) and two drops of acetic acid and boiled for 1 minute. 4-Acetamido-salicyaldehyde thiosemicarbazone separated as golden plates, m.pt. 252°C. (decomp.). Found: C, 47·8; H, 4·9; N, 21·9; S, 12·7. $C_{10}H_{12}O_2N_4S$ requires: C, 47 6; H, 4·8; N, 22·2; S, 12·7 per cent.

4-Dimethylaminosalicylaldehyde Thiosemicarbazone (4-dimethylamino-2-hydroxybenzaldehyde thiosemicarbazone—Compound No. 7), 4-Dimethylaminosalicylaldehyde (4·0 g.) was dissolved in ethyl alcohol and added to a boiling solution of thiosemicarbazide (2·6 g.) in water (50 ml.) containing acetic acid (2 ml.). After refluxing for 5 minutes 4-dimethylaminosalicylaldehyde thiosemicarbazone separated and was purified by recrystallisation from a mixture of dioxan and alcohol, from which it was obtained as yellow plates m.pt. 235°C. (decomp.). Found: C, 50·60; H, 5·95; N, 23·5; S. 13·4. $C_{10}H_{14}ON_4S$ requires: C, 50·42; H, 5·88; N, 23·5; S, 13·4 per cent.

4-Hydroxysalicylaldehyde Thiosemicarbazone. (2:4-Dihydroxybenzaldehyde thiosemicarbazone—Compound No. 9). 2:4-Dihydroxybenzaldehyde (2 g.) was dissolved in hot water (10 ml. and added to a solution of thiosemicarbazide (1·6 g.) dissolved in hot water (20 ml.) containing acetic acid (1 ml.). The thiosemicarbazone, which separated almost immediately, was purified by crystallisation from aqueous alcohol whereby 4-hydroxysalicylaldehyde thiosemicarbazone was obtained as needles,

m.pt. 235°C. Found: C, 45·7; H, 4·25; N, 19·7; S, 15·16. $C_8H_9O_2N_3S$ requires: C, 45·5; H, 4·26; N, 20·0; S, 15·2 per cent.

4-Acetamido-2-methoxybenzaldehyde Thiosemicarbazone (*Compound No. 51*). 4-Amino-2-methoxybenzaldehyde prepared by a modification of the method of Hodgson[16] was acetylated by refluxing with acetic anhydride. 4-Acetamido-2-methoxybenzaldehyde was isolated by pouring the reaction mixture into iced water and, when recrystallised from hot water, it separated as needles, m.pt. 145° to 146°C. 4-Acetamido-2-methoxybenzaldehyde (25 g.) dissolved in boiling water (250 ml.) was added to a solution of thiosemicarbazide (12·3 g.) in boiling water (100 ml.) and 2 drops of acetic acid added. The thiosemicarbazone separated as a pale yellow solid which was filtered and washed with hot water, (36 g.). 4-Acetamido-2-methoxybenzaldehyde thiosemicarbazone crystallises from aqueous alcohol as pale yellow needles, m.pt. 232°C. (decomp.). Found: N, 20·6. $C_{11}H_{14}O_2N_4S$ requires N, 21·05 per cent.

Results

From Table I, it will be seen that most of the compounds possess *in vitro* activities of a high order, which (in those tested) is not markedly affected by the presence of human serum. From the same table, it is also clear that substitution of an hydroxyl group in the 2-position results, generally, in a decreased toxicity.

Table II shows that the 2-hydroxyl substitution results in a complete loss of *in vivo* activity and the same table shows a satisfactory degree of correlation between the two tests for tuberculostatic activity.

Discussion

The above results illustrate once again that the incorporation of the chemical characteristics of two active compounds into one molecule cannot be relied upon to produce an active resultant. Compounds Nos. 5, 6 and 7 are examples of substances containing characteristics of both thiacetazone (TB1) and para-aminosalicylic acid and are themselves inactive *in vivo*.

It was considered possible that this loss in activity might arise from a change in properties resulting from hydrogen bonding involving the 2-hydroxyl group. Supporting evidence for this postulation was supplied from the fact that the 2-hydroxylated compounds were invariably less soluble than their parent compound in water and alcohol. To test this suggestion, Compound No. 51, which contains a methoxyl group in the 2-position in place of a hydroxyl group, was synthesised. This compound, however, proved to be inactive and it appears, therefore, that an alternative explanation must be sought for the inactivity of the 2-hydroxylated thiosemicarbazones. This inactivity is probably a further illustration of the necessity for 4-substitution only for this type of compound, a fact which was pointed out by Behnisch, Mietzsch and Schmidt[17] and more recently by Hamre, Bernstein and Donovick[18]. It is noteworthy, however, that the former workers mention 3-substituted com-

pounds which were very active although they do not state whether they refer to *in vitro* or *in vivo* activity. Hoggarth, Martin, Storey and Young[19] also comment on the necessity for a 4-substituent for highest activity but report a few substituents in the 3-position confer activity.

A comparison of the *in vitro* and *in vivo* results affords another illustration of the fact, reported by other workers, that *in vitro* activity, even in the presence of serum, is no criterion of *in vivo* activity. It might be concluded that compounds active *in vivo* would, at least, show *in vitro* activity but this is not necessarily true since nicotinamide, admittedly a compound of a different type, has a marked effect *in vivo* by the survival test in high dosage but no *in vitro* activity.

The results obtained with the survival test appear to correlate well with those obtained by the mouse corneal test with the compounds studied. Further work is being carried out to determine whether this degree of correlation is applicable to other types of tuberculostatic substances.

Summary

1. Of a series of thiosemicarbazones substituted in the aromatic nucleus by a 2-hydroxyl group, several showed a high degree of *in vitro* activity coupled with a lower toxicity than the corresponding thiosemicarbazone un-substituted in the 2-position. The only compounds showing significant *in vivo* activity, however, were those substituted solely in the 4-position. The results confirm the findings of others that the ethylsulphonyl compound (Compound No. 12) is somewhat more active than the 4-acetamido compound.

2. There is no relationship between the *in vivo* and *in vitro* tuberculostatic activity within this series of substances.

3. A good correlation exists between the results obtained in this series of compounds by the mouse survival method and the mouse corneal method.

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DISCUSSION

The paper was read in abstract by MR. D. E. SEYMOUR.

MR. C. E. COULTHARD (Nottingham) said that he was very interested to see that the Rees and Robson test was now being applied to practical estimations in the laboratory. The guinea-pig test used a lot of material and took a long time. The egg test had not proved very successful in their laboratory and they had not tried Glover's aerosol test because it seemed dangerous. The administration of the tubercle bacilli intravenously, intraperitoneally and by other routes had been tried but variations in the bacterial strain and in the mice made it difficult to get standard results. More recently they had tried Youman's method in which the tubercle bacilli are injected intra-cerebrally. He asked whether the authors had had any difficulty in applying the corneal test (Rees and Robson) on the scale on which they had used it from the point of view of the susceptibility of the strains of mice. If one was going to use any of the other types of tuberculous infection of mice it was necessary to select suitable mice in order to get reproducible results.

PROFESSOR SPRING (Glasgow) said that the fact that the *ortho*methoxy group did not produce any effect must have been a great disappointment, but it was only one more example of the fact that one could not look at a formula and predict the result which would be obtained in these matters. The fact that the *ortho*methoxy derivative was not active was not of very great significance, unless it were established that the methyl ether of *para*-amino-salicylic acid was inactive also.

MR. D. E. SEYMOUR, in reply, said that he was sorry that Professor Robson, who carried out all the work on the corneal test, was not able to be present. Since the work described had been completed they had been doing the test themselves. With regard to the inactivity of compound No. 51 (the methyl ether), the methyl ether of *para*-aminosalicylic acid had been synthesised, though it had not been tested in animals, so that they could not come to any conclusion about it.

MR. E. M. BAVIN, dealing with the question of the variability of mice of different strains, said it was quite definite that there was this variation. He had started with a strain of Swiss mice which he had kept throughout, and had found, using the intravenous inoculation, that in 80 per cent. of the tests the survival time of the mice was about 20 to 25 days. Sometimes the mice lived for 40 or even 50 days and he did not know why that happened. There was some American work which showed that an increase of fat in the diet of the mice tended to increase their sensitivity to the bacteria. His own experience did not substantiate this.