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THE ESTIMATION OF SOME THIOSEMICARBAZONES AND THEIR BLOOD CONCENTRATIONS IN EXPERIMENTAL ANIMALS

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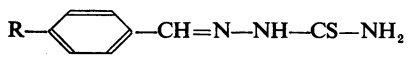
Thiosemicarbazones of some aromatic aldehydes have been shown to possess appreciable antituberculous activity (Domagk, Behnisch, Mietzsch, and Schmidt, 1946; Domagk, 1948; Hoggarth, Martin, Storey, and Young, 1949). Among the compounds described by Hoggarth *et al.* (1949) the most promising were *p*-anisaldehyde thiosemicarbazone (6057; I, R = CH_3O), *p*-hydroxybenzaldehyde semicarbazone (6082; I, R = OH) and *p*-ethylsulphonylbenzaldehyde semicarbazone (8388; I, R = $C_2H_5SO_2$). The methods described here were



6057: R = CH_3O (I)
6082: R = OH
8388; R = $C_2H_5SO_2$

developed so that the absorption and excretion of these three compounds could be studied in relation to their therapeutic effects in mice.

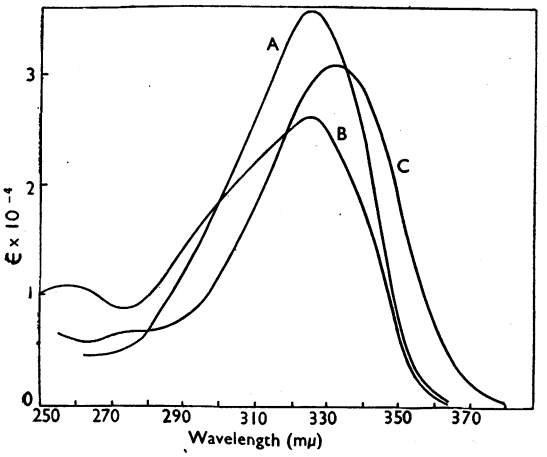


FIG. 1.—Absorption spectra of 6057 (A), 6082 (B) and 8388 (C) in chloroform. Ordinates: $\epsilon \times 10^{-4}$; abscissae: wavelength (mμ).

EXPERIMENTAL SECTION

It is known that certain unsaturated thiosemicarbazones show intense absorption of ultraviolet light (Heilbron, Johnson, Jones, and Spinks, 1942). The absorption spectra of the three compounds in chloroform were therefore examined by means of the Beckman photoelectric spectrophotometer (Fig. 1). They were quite suitable for the purpose of estimation provided that a method of extraction from biological fluids could be devised. It was found that 6057 and 8388 could be extracted by the same simple procedure but that 6082 could not. Descriptions of the methods and of their use in animal experiments follow.

1. *Estimation of* p-*anisaldehyde thiosemicarbazone* (6057) *and* p-*ethylsulphonylbenzaldehyde thiosemicarbazone* (8388) *in blood*

Reagents

1. 0.2*M*-disodium hydrogen phosphate, preserved by adding a trace of chloroform.
2. B.P. chloroform.
3. 0.2 g./100 ml. stock solution of 6057 or 8388 in methanol (stable in the dark).
4. 1 mg./100 ml. standard solution, prepared just before use by diluting the stock solution with water.

Procedure

Pipette 2 ml. of blood into 4 ml. of 0.2*M*-disodium hydrogen phosphate in a 60 ml. glass-stoppered bottle. Add 40 ml. of chloroform and shake for 5 min. An emulsion rarely forms, and can always be resolved by centrifuging the bottle. Withdraw most of the lower layer and clarify it by filtration through Whatman No. 1 or similar semi-fine paper, or by shaking it in a clean dry bottle with about half a gram of anhydrous sodium sulphate, A.R. Transfer it to a 10 cm. silica spectrophotometer-cell, and read the optical density at 325 mμ (6057) or 332 mμ (8388) against a blank. Let the reading be "*a*." Prepare the blank and a standard at the same time as the unknown by substituting 2 ml. of distilled water, and 2 ml. of the standard solution for blood in

the above procedure. Read the standard also against the blank. Let the reading be "*b*." Then the concentration of 6057 or 8388 in the unknown is *a*/*b* mg./100 ml. Normal blood gives a reading equivalent to about 0.05 mg./100 ml., which must be subtracted from the experimental results.

Notes

It was established that the graph of concentration against optical density was a straight line: a single standard was therefore adequate. Disodium hydrogen phosphate was added because previous experience with similar methods (Spinks, 1946) had shown that the extraction of interfering materials from blood was reduced when the blood was buffered to weak alkalinity. At first this caused difficulty because the disodium hydrogen phosphate used contained mercuric chloride as preservative. Extraction of 6057 was completely prevented, presumably because it formed a solvent-insoluble mercury salt. When a trace of chloroform was substituted for mercuric chloride recovery from water and blood was quantitative (Table I). The recovery from

TABLE I

RECOVERY OF 6057 AND 8388 FROM BLOOD (2 ML.)

μg. added		μg. found		% recovery	
6057	8388	6057	8388	6057	8388
2	5	1.83	4.96	91	99
4	10	3.97	9.25	99	92
6	15	5.88	15.1	98	101
10	25	9.50	25.0	95	100
14	35	13.3	34.3	95	98
20	50	20.0	46.2	100	92

plasma and urine was also quantitative. However, concentrations of 6057 in urine were later found to be very low, so that its presence had to be confirmed, and its amount assessed, by constructing a full absorption spectrum of each extract. Concentrations of 8388 in urine were much higher, and the method described could be used without modification.

2. *Estimation of* p-*hydroxybenzaldehyde thiosemicarbazone* (6082) *in plasma*

Reagents

1. 4*M*-sodium dihydrogen phosphate.
2. B.P. chloroform.
3. 1 g./100 ml. sodium carbonate.

4 and 5. Solutions of 6082 as described for 6057 and 8388.

Procedure

Pipette 2 ml. of plasma (which must not be contaminated by haemolysis) into a bottle. Add 2 ml. of 4*M*-sodium dihydrogen phosphate and 40 ml. of chloroform. Shake vigorously for 5 min. and filter the lower layer into a measuring cylinder. Transfer an aliquot of 30 ml. to a dry bottle. Add 8 ml. of 1 per cent sodium carbonate and shake vigorously for 3 min. Decant the upper layer into a test tube, centrifuge it for 5 min., and transfer it to a 2 cm. spectrophotometer cell. Read the optical density ("*a*") against a blank at 330 mμ. The blank and a standard are prepared by substituting 2 ml. of water and 2 ml. of a 1 mg./100 ml. standard solution of 6082 for plasma in the above procedure. If the reading of the standard is "*b*" the concentration of 6082 in the unknown is *Fa*/*b* mg./100 ml., where *F* is a recovery factor derived from data such as those of Table III.

Notes

Only a trace of 6082 was extracted from water or blood under the conditions used for 6057. This was shown to be due to two effects: first, 6082 was a stronger acid (*p*Ka about 9.5) than its phenolic structure had suggested (Table II); and second, it could not be fully extracted even at low *p*H values unless salted out by a high concentration of buffer (Table II). Further, when a *p*H of 4.8 and a high

TABLE II

EFFECT OF *p*H OF ADDED BUFFER, AND MOLARITY OF ADDED NaH_2PO_4 ON THE EXTRACTION OF 6082 FROM WATER INTO CHLOROFORM

*p*H of 0.2 *M*-buffer	% Extraction	Molarity of NaH_2PO_4 (*p*H 4.8)	% Extraction
5.0	48	4.0	101
6.0	45	2.0	88
7.0	46	1.0	70
8.0	41	0.5	60
8.9	32	0.2	52
10.6	6.5		
10% Na_2CO_3	0		

buffer concentration were used in the extraction of blood very large amounts of interfering material, equivalent to several mg. of 6082/100 ml., were extracted; and even then the recovery of 6082 was only about 20 per cent. Consequently, plasma was analysed instead. Interfering material was again removed, but in less amount, and when the 6082 was re-extracted into 1 per cent sodium carbonate (cf. Table III) the blank was reduced almost to zero. The recovery of 6082 from plasma was incomplete but adequate (Table III). The recovery from urine was theoretical; but 6082, like 6057, reaches only low concentrations in urine, and full absorption spectra of extracts must be constructed.

TABLE III
RECOVERY OF 6082 FROM PLASMA

Added, μg.	Found, μg.		% Recovery (Na_2CO_3)
	1st extraction ($CHCl_3$)	Re-extraction (1% Na_2CO_3)	
0	6.17	0.07	—
4	9.75	3.00	73
10	14.0	7.24	72
20	19.3	14.9	74
30	26.2	23.2	77
40	32.4	31.2	78
0	4.25	0	—
20	18.9	12.8	64
40	39.0	29.0	72
		Mean:	73

A disadvantage of analysing 6082 in plasma was that much data had already been obtained on the concentration of 6057 in blood. However, the validity of comparing blood with plasma concentrations in this series is indicated by the virtual identity of concentrations of 6057 in blood and plasma and by the fact that concentrations of 8388 in plasma are about 70 per cent of those in blood (results not shown). Another important point is that the major difference between the compounds is in rate of elimination from the blood; its apparent value could hardly be affected by analysing plasma instead of blood.

3. *Animal Experiments*

(*a*) *Mice.*—Each compound was given to groups of 4 mice in doses of 500 mg./kg., administered orally by syringe and blunt needle as a 2 per cent aqueous dispersion. Groups were killed at intervals after dosing by withdrawing heart blood under heavy chloroform anaesthesia. Blood from the 4 animals of a group was pooled for analysis. The concentrations found are shown in Fig. 2, which also includes the results of a similar experiment on 6057 in rats.

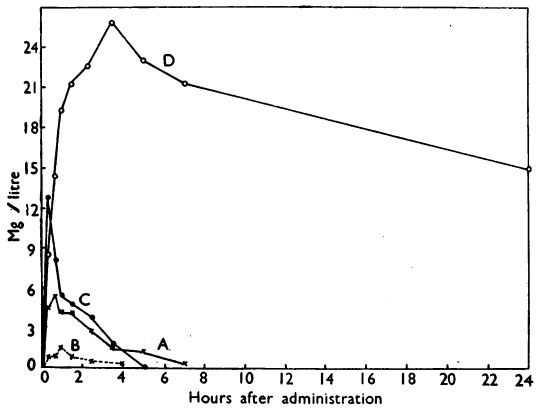


FIG. 2.—Concentrations of 6057 in mouse (A) and rat (B) blood, 6082 in mouse plasma (C) and 8388 (D) in mouse blood after the oral administration of 500 mg./kg. Ordinates: Milligrams per litre. Abscissae: Hours after administration.

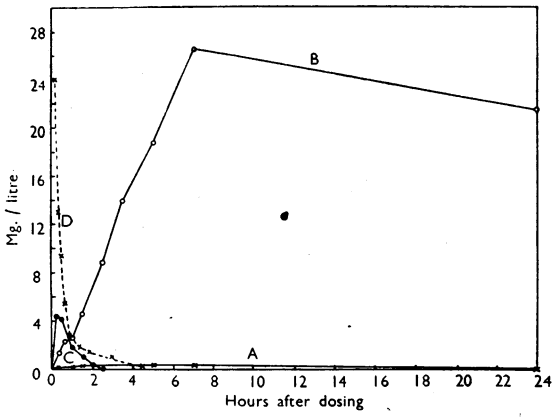


FIG. 3.—Concentrations of 6057 (A) and 8388 (B) in blood, and 6082 (C) in plasma, after oral doses of 250 mg./kg. in rabbits. Each graph is the average of three experiments. Concentrations of 6057 in blood (D) after i.v. administration of 40 mg./kg. to a single rabbit are also shown. Ordinates: Milligrams per litre. Abscissae: Hours after administration.

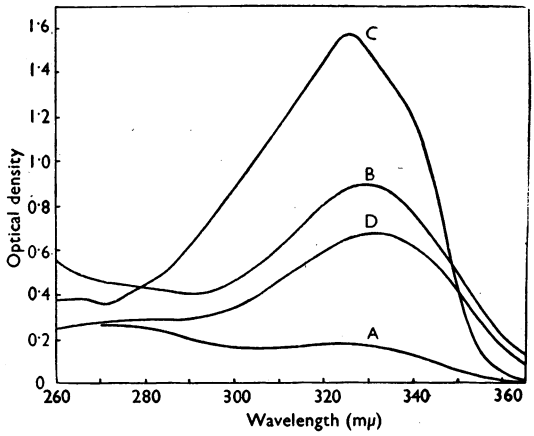


FIG. 4.—Absorption spectra of chloroform extracts of the first day's urine from rabbits receiving 6057 (A) and 8388 (B), and rats receiving 6057 (C), and of a 54 hour-blood sample (D) from a rabbit receiving 8388. Ordinates: Optical density. Abscissae: Wavelength (mμ).

(*b*) *Rabbits.*—Each compound was administered orally to rabbits as a 10 per cent aqueous dispersion, given by syringe and rubber catheter in doses of 250 mg./kg. 6057 was also administered intravenously as a 2 per cent dispersion in a dose of 40 mg./kg. Rabbits were bled from a marginal ear vein at intervals after dosing. The results are shown in Fig. 3. The identity of 8388 in a sample of rabbit blood taken 54 hours after dosing was confirmed spectrophotometrically (Fig. 4). 8388 could be detected in rabbit blood under these conditions for about a week.

Excretion of the three compounds in urine was examined using some of the above rabbits. Fig. 4 shows the absorption spectrum of an extract of the first day's urine of a rabbit receiving 6057. It was estimated from such curves that the amount of 6057 excreted in the urine did not exceed 1 per cent of that administered (Fig. 5); 6082 behaved similarly.

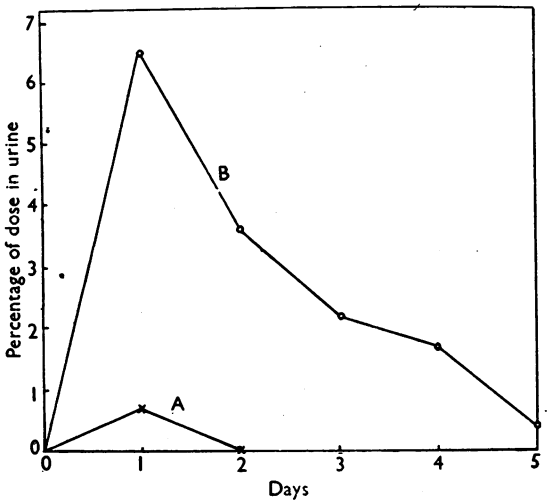


FIG. 5.—Excretion of 6057 (A) and 8388 (B) in the urine of typical individual rabbits after oral doses of 250 mg./kg. Ordinates: Percentage of dose in urine. Abscissae: Days.

Rats excreted about 2 per cent of an oral dose of 500 mg. of 6057/kg. Here the smaller urine volume permitted definite identification of the excreted material (Fig. 4). 8388 was readily identified even in rabbit urine (Fig. 4). Its behaviour was in sharp contrast to that of 6057 or 6082, since larger amounts appeared in the urine, and excretion continued over several days (Fig. 5). However, only about 10–14 per cent of the amounts administered to three rabbits were accounted for in the urine. Unfortunately, the analytical methods could not be applied to faeces, but it is improbable from analogy with other compounds of similar simplicity that much material was lost in the faeces. It may reasonably be assumed that most of each compound was degraded in the body (cf. Discussion).

DISCUSSION

The most important finding is that whereas 6057 and 6082 are rather rapidly eliminated, and attain only low blood or plasma concentrations, 8388 is very persistent and attains high blood concentrations. The marked difference between 8388 and the other two compounds is particularly evident when the areas beneath the blood concentration-time curves are measured. The figures for mice, expressed in arbitrary units, are: 6057, 2.4; 6082, 3.1; 8388 (to 72 hrs. after dosing) 154.

The results are in excellent agreement with the antituberculous properties of the three compounds, since 8388 is the least active *in vitro* (Martin, 1948) and the most active *in vivo* (Hoggarth *et al.*, 1949). It may reasonably be assumed that repeated administration during the therapeutic tests would lead to differences in blood concentration even more marked than those recorded here. The agreement between concentration and therapeutic effect suggests that the thiosemicarbazones act directly and not after conversion to metabolites.

It is important to consider the likely reasons for the difference between 8388 and 6057 or 6082, because of their possible bearing on the design of compounds that might attain blood concentrations similar to those of 8388 and have intrinsic activity comparable with that of 6057 or 6082. At present this consideration must necessarily be highly speculative. The nature of the blood concentration-time curves indicates that the main pharmacological difference between the compounds is in rate of elimination from the blood. Both 6057 and 6082 appear to be rapidly absorbed, probably more rapidly than 8388. Because most of each compound is degraded in the body it follows that the differences in the rates of elimination are mainly conferred by variation in the rate or manner of degradation. This is further supported by the fact that 8388, which is the most persistent compound, is nevertheless excreted in larger amount than the other two.

At first sight, the most likely point of chemical attack is the thiosemicarbazone group. If this be so it must be assumed that the group is readily attacked in 6057 and 6082, and only attacked with difficulty in 8388. Some weak indirect evidence in support of this assumption is available, in that Hoggarth (1948) has shown that a methoxy group in the *para* position of certain compounds related to benzaldehyde thiosemicarbazone renders the thiosemicarbazide

residue more susceptible to scission. However, a spectrophotometric search for metabolites in extracts of urine from rabbits receiving 6057 failed to indicate the presence of free or conjugated anisic acid, which is the most likely end-product of attack on the thiosemicarbazone group. Free or conjugated mercaptotriazole and thiodiazole also appeared to be absent. Both of these are potential cyclization products of thiosemicarbazones; the thiodiazole can be readily obtained *in vitro* by mild oxidation. The examination of the urine was not exhaustive, and other products may well have been present. In particular, *p*-hydroxybenzoic acid would probably not have been extracted by the solvents used.

A second possibility exists, since the methoxy and hydroxy *para* substituents of 6057 and 6082 are known to be capable of metabolic modification, whereas the ethylsulphonyl group of 8388 is thought not to be. For example, *p*-methylsulphonylacetanilide is a metabolic end-product of *p*-methylthioaniline in the mouse, rat, and rabbit (Rose and Spinks, 1948), and sensitive tests failed to detect sulphanilic acid derivatives in the urine of these animals. Similar results were obtained with the ethylsulphonyl homologue (Spinks, 1948). It is therefore probable that no further breakdown of the alkylsulphonyl group occurred. These considerations suggest that 6057 may be demethylated in the body to give 6082; 6082 would probably be conjugated with sulphuric acid or glucuronic acid. The conjugated derivatives when hydrolysed, would give *p*-hydroxybenzaldehyde which would almost certainly have escaped detection in the search for metabolites referred to above. These views do not directly explain the excretion of only a part of the 8388, but do so if the further assumption is made that the thiosemicarbazone group is slowly, and the *para* substituents of 6057 and 6082 are rapidly, attacked.

A third possibility is that each compound is hydroxylated in the body according to known metabolic reactions, and then conjugated: 6057 would give a guaiacol, and 6082 a catechol derivative; 8388 would probably be hydroxylated in the position *meta* with respect to the ethylsulphonyl group. It can be assumed that this *meta* hydroxylation would be less facile than the *ortho* hydroxylation of 6057 or 6082. Some support is available for the last two possibilities in that urine from rabbits receiving 6082 contains a water-soluble compound having an intense absorption spectrum with a sharp maximum at 315 mμ. The amount of this metabolite is estimated to be equivalent to about 60 per cent of the amount of 6082 administered, if similar intensities are assumed for the respective absorption spectra. This observation, made after the first draft of this paper had been written, shows, first, that 6082 is not excreted in the faeces to any marked extent, and, second, that the thiosemicarbazone group may well have remained intact, or may have been only partly degraded, say to the corresponding semicarbazone: simpler metabolites would be expected to absorb at shorter wavelengths. Otherwise there is little evidence in favour of any one of the theoretically possible methods of degradation. Indeed it is conceivable that all three could occur. However, they all suggest somewhat similar conclusions, i.e., that new antituberculous thiosemicarbazones should be sought among compounds having an unreactive *meta* directing group, or, less probably, an unreactive *ortho-para* directing group in the *para* position. It is possible that the requirements for high intrinsic activity and high blood concentrations may be incompatible; and the numerous examples of weakly active compounds belonging to one or other of these two suggested types (Hoggarth *et al.*, 1949) favour this view. It is hoped that a conclusion may be reached, first by studying the metabolism of the three compounds mentioned here, second by examining the blood concentrations of other compounds. A start has been made by determining the concentrations attained by *p*-acetamidobenzaldehyde thiosemicarbazone, which has been tested in man by Domagk's clinical collaborators (Moncorps and Kalkoff, 1947; Kuhlmann, 1948). So far it appears that this compound gives concentrations in mice similar to those of 6057 and 6082, and that it lacks the favourable persistence of 8388.

Summary

Spectrophotometric methods of estimating the thiosemicarbazones (6057, 6082, and 8388 respectively) of *p*-anisaldehyde, *p*-hydroxybenzaldehyde and *p*-ethylsulphonylbenzaldehyde have been devised.

6057 and 6082 are rapidly eliminated and attain only low blood or plasma concentrations in mouse and rabbit. 8388 is highly persistent and attains high blood concentrations. Only traces of 6057 and 6082 appear in rabbit urine. They are probably almost completely metabolized by the rabbit. 8388 is excreted unchanged in larger, but not theoretical, amounts.

The bearing of these results on the antituberculous activity of this group of compounds has been discussed.

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Note added in proof

The thiosemicarbazones were found to be photolabile during this work, but no evidence of photodecomposition was obtained during the estimations, which were carried out in winter. Repetition of some of them recently has shown that in summer decomposition by diffused daylight can occur, and estimations are now carried out in a darkroom, under diffused tungsten illumination. Readings of the standards indicate whether or not decomposition has occurred.