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ESTIMATION AND PHOTOLABILITY OF SOME THIOSEMICARBAZONES

BY

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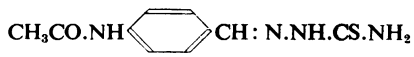
The author (1949) has already described a spectrophotometric method of estimating three benzaldehyde thiosemicarbazones in blood, among them the highly active *p*-ethylsulphonyl derivative (8388, "Berculon B,"* Hoggarth, Martin, Storey, and Young, 1949). Since then it has become known that the *p*-acetylamino derivative (I, " Conteben," 9311, " Berculon A,"* " Tibione ") has received extensive therapeutic trials in Germany, and numerous reports, usually claiming varying degrees of



I

effectiveness, have appeared. These reports and other, previously unpublished, information have recently been summarized by Hinshaw and McDermott (1950). This paper presents modifications of the earlier method that permit simultaneous estimation of berculon A and the corresponding unacetylated amine 6198 (Hoggarth, Martin, Storey, and Young, 1949), and other changes that raise its sensitivity. Increased sensitivity is necessitated by the low doses of berculon A used clinically. A dose of 200 mg. daily gives a maximum concentration of about 0.1 mg./100 ml. blood (Martin and Spinks, unpublished data).

The photolability of the thiosemicarbazones is also described: it cannot be disregarded if an accurate analysis is to be made.

Experimental Section

Estimation of thiosemicarbazones

p-Acetylaminobenzaldehyde thiosemicarbazone (berculon A) can be hydrolysed to a diazotizable amine, and a colorimetric method based on this property has been described, although not in detail, by Behnisch, Mietzsch, and Schmidt (1950). This method was studied independently, but was abandoned because the thiosemicarbazone group proved to be labile under the conditions of hydrolysis used, and because a spectrophotometric procedure seemed to offer greater specificity and almost equal sensitivity. When it was found that the therapeutic concentrations of berculon A were very low, attempts were made to develop an alternative method by converting the drug to a coloured phenylhydrazone, for example, by acid hydrolysis in the presence of 2: 4-dinitrophenylhydrazine. It was hoped eventually to use a fluorescent

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hydrazine. So far no useful method has been developed, but the possibility is still being studied. Another type of procedure that has been tested is the conversion of the thiosemicarbazones into stable metallic salts by reaction with a known amount of a metallic ion, usually bivalent mercury, followed by colorimetric estimation of the excess mercury with dithizone. The thiosemicarbazone-mercury complexes, although closely related structurally to the red dithizone-mercury complexes, are colourless. Berculon A can be estimated by this technique, but the sensitivity is low.

Only the spectrophotometric methods will be described in detail. There are three of these. The spectrophotometric method of estimating berculon B (Spinks, 1949) was first modified to allow simultaneous estimation of berculon A and *p*-aminobenzaldehyde thiosemicarbazone, which could possibly be formed *in vivo* by deacetylation. Although later work showed that this conversion could occur in mouse and dog only to a slight extent, if at all, the method will be described because species differ greatly in the extent to which acetylation or deacetylation of sulphonamides predominates (Krebs, Sykes, and Bartley, 1947), and others might be able to deacetylate berculon A more readily. The second method is almost identical with that previously described for the estimation of berculon B (Spinks, 1949); it is used after the administration of high doses to species in which deacetylation has been shown not to occur. The third method is suitable for the estimation of 0.5 to 5.0 μg. of drug in 3.0 ml. of blood.

All methods must be performed in weak unfiltered tungsten light, of about the strength that can be used in photographic work for handling contact paper.

Method I. Simultaneous estimation of p-*aminobenzaldehyde thiosemicarbazone* (6198) *and* p-*acetylaminobenzaldehyde thiosemicarbazone* (*berculon A*)

Details of technique and reagents are the same as those prescribed for the estimation of berculon B (Spinks, 1949), with the addition of 1 mg./100 ml. standard aqueous solutions of 6198 and berculon A, freshly prepared by diluting 50 mg./100 ml. solutions in methanol. These solutions must be stored in the dark.

Procedure.—Shake 2 ml. of blood and 2 ml. of 0.2 M-disodium hydrogen phosphate with 40 ml. of chloroform (*B.P.*) for 5 minutes, and filter the extract into a 10-cm. cell. Read the optical densities at 320 and 342 mμ against a blank (extract of 2 ml. of water): let the readings be u_1 and u_2 respectively. Also read the optical densities (a_1 and a_2) at the same wavelengths of a standard prepared by extracting 2 ml. of a 1 mg./100 ml. solution of 6198, and those (b_1 and b_2) of a corresponding standard extract of berculon A. Let the concentrations of 6198 and berculon A in the unknown be x and y mg./100 ml. respectively. Then, assuming linearity of the concentration-optical density graphs, it follows that

$$u_1 = xa_1 + yb_1 \text{ and } u_2 = xa_2 + yb_2$$

$$\text{hence} \quad x = \frac{b_1u_2 - b_2u_1}{a_2b_1 - a_1b_2} \text{ and } y = \frac{a_2u_1 - a_1u_2}{a_2b_1 - a_1b_2}$$

Notes.—This method is based on one previously described for the simultaneous colorimetric estimation of *p*-methylthioaniline and its metabolite, *p*-methylsulphonylaniline (Spinks, 1948). It depends on dissimilarity of the absorption spectra of 6198 and berculon A (Fig. 1) and on linearity of the concentration-optical density graphs of both compounds at both wavelengths. This was established directly and is also demonstrated by the recovery of the two compounds shown in Table I. Neither was recovered quantitatively from

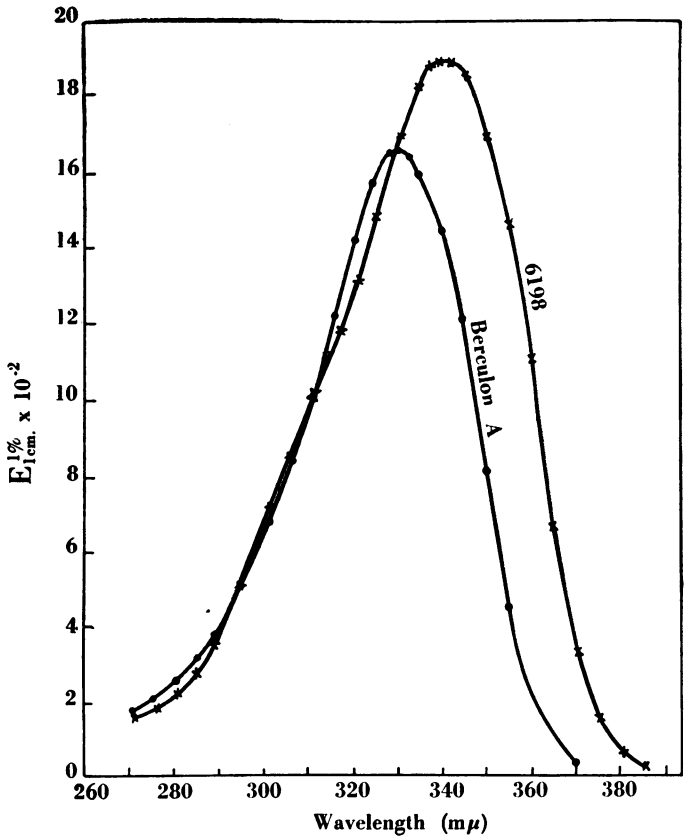


FIG. 1.—Absorption spectra of 6198 and berculon A in chloroform.

blood, and results must therefore be multiplied by a recovery factor. The blank in normal blood is low, of the order of 0.05 to 0.1 mg./100 ml. for berculon A, and —0.05 to —0.01 mg./100 ml. for 6198; the negative sign arises from the fact that the ratios of optical densities, *d*320/*d*342, are about 1.4, 1.05, and 0.69 for normal blood, berculon A, and 6198 respectively. The apparent concentration of 6198 found in normal blood before dosing is therefore added to the values obtained after dosing.

Concentrations of the two compounds found in mouse blood after the administration of each alone are shown in Figs. 2 and 3. 6198 was not detected with certainty after the administration of berculon A, and was rapidly acetylated to give berculon A when it was itself administered. Indeed, 100 mg. 6198/kg. gave higher concentrations of berculon A than 500 mg. of the latter did. Presumably berculon A is poorly absorbed and 6198 well absorbed. Compound 6198 is more toxic than berculon A and its administration in place of berculon A is probably inadvisable.

The concentrations of berculon A in Fig. 3 are about a quarter of those given by berculon B under the same conditions (Spinks, 1949). A ratio of this order has been established also for other doses in mice and for the dog (cf. Fig. 5) and monkey (Francis, Spinks, and Stewart, 1950).

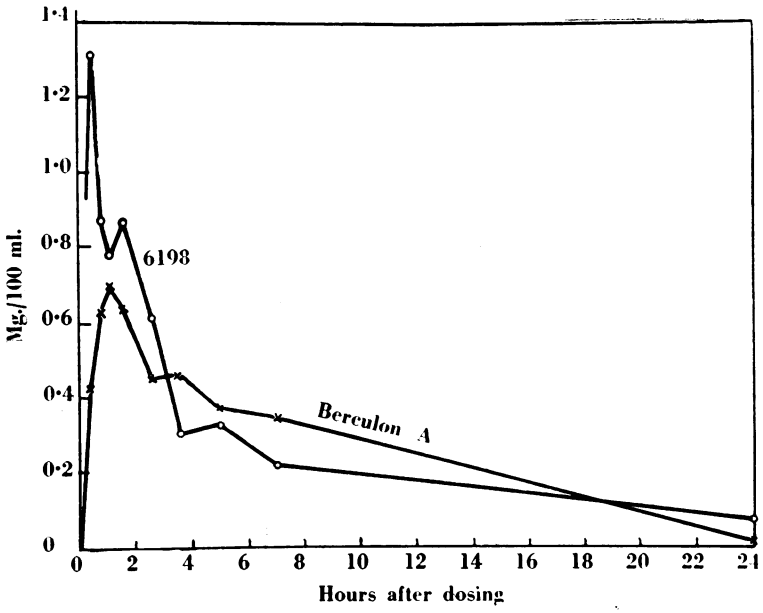


FIG. 2.—Concentrations of 6198 and berculon A in the blood of mice receiving 100 mg. 6198 per kg.

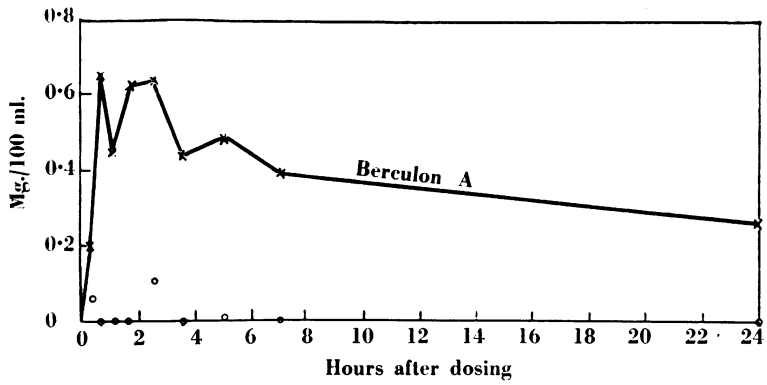


FIG. 3.—Concentrations of 6198 (O) and berculon A (×) in the blood of mice receiving 500 mg. berculon A per kg.

Method II. Estimation of berculon A alone in high concentration

Reagents and conditions are as prescribed for the estimation of berculon B (Spinks, 1949) with the addition of a standard solution of berculon A. Extracts are read at λ_{max} 330 mμ.

Notes.—The recovery of berculon A alone from blood is given in Table II. The mean recovery did not differ significantly from that given in Table I. This method is suitable for estimating 5 μg. and more of berculon A in 2 ml. of blood.

Method III. Estimation of berculon A in low concentration

Three ml. of blood are shaken with 40 ml. of chloroform for 5 minutes. The extract is filtered into a 10-cm. spectrophotometer cell, and the optical density is read every 5 mμ from 280 to 380 mμ. The absorption spectrum is drawn (Fig. 4), using the largest con-

TABLE I

RECOVERY OF BERCULON A AND 6198 FROM BLOOD (2 ML.) BY SIMULTANEOUS SPECTROPHOTOMETRIC ESTIMATION AT λ_{max} 320 AND 342 Mμ
(Method I)

Added (μg.)		Found (μg.)		Recovery (%)	
6198	Berculon A	6198	Berculon A	6198	Berculon A
0	0	(0)	(1.10)	—	—
20	0	16.2	(3.56)	81	—
0	20	(0.72)	18.3	—	86
4	4	3.66	4.72	91	90
10	10	9.03	8.83	90	77
10	20	8.38	19.0	84	89
20	10	17.8	9.13	89	80
0	0	−0.41	2.14	—	—
4	4	3.28	5.44	92	82
12	12	9.70	13.4	84	94
20	20	16.3	17.9	83	79
28	28	26.3	23.1	95	75
40	40	34.0	33.4	86	78
20	0	18.1	(2.10)	92	—
0	20	(1.50)	16.7	—	73
			Mean	88	82
			Limit of error of mean (P = 0.05)	±3.0	±4.5

venient scale. If berculon A is present the curve will show a hump, with a maximum near 330 mμ. On either side of this hump there will be a point of sharp curvature. Join the points of sharp curvature by a straight line meeting the curve tangentially at both points. Then if the absorption spectrum of a normal extract is approximately linear (Fig. 4) the part of the curve above the tangent will not include any absorption due to normal constituents. Record the optical density increment (a) above the tangent at 330 mμ. Now note the wavelengths at which the tangent meets the curve. Join the same wavelengths on the absorption spectrum of an extract of 3 ml. of a 0.1 mg./100 ml. solution of berculon A by a straight line. Let the density increment at 330 mμ above the line be a' and that below b'. Then the approximate optical density of berculon A in the unknown blood is $F(a'+b')a/a'$, and the concentration $Fa/10a'$ mg./100 ml., where F is the recovery factor obtained from data such as those of Table III.

TABLE II

RECOVERY OF BERCULON A FROM BLOOD (2 ML.) BY ESTIMATION AT λ_{max} 330 Mμ
(Method II)

Added (μg.)	Found* (μg.)		Recovery (%)	
0	0.23	0.90	—	—
5	3.81	4.48	72	72
10	8.08	8.20	78	73
15	13.0	12.8	85	79
25	20.2	19.1	80	73
35	28.7	29.9	82	83
50	42.0	40.5	84	79
		Mean	78	
		Limit of error of mean (P=0.05)	±3.1	

* Two separate experiments.

TABLE III

RECOVERY OF BERCULON A FROM MOUSE BLOOD (3 ML.) BY THE GRAPHICAL METHOD (Method III)

Added (μg.)	Found (μg.)	Recovery (%)
0	0	—
0.5	0.328	66
1.0	0.600	60
1.0	0.641	64
1.5	0.975	65
1.5	0.992	66
1.5	1.08	72
2.5	1.58	63
5.0	3.46	69
	Mean	66
	Limit of error of mean (P=0.05)	±3.1

Notes.—The recovery of berculon A from blood by this procedure is given in Table III. Detailed work on the method shows that the low recovery is due to two factors: first, the incomplete (80 per cent) extraction of berculon A from blood (Tables I and II); second, an apparent loss (20 per cent) of berculon A due to the departure of the normal curve from linearity (Fig. 4). The normal curve is always concave with respect to the tangential straight line, and the increment *a* is therefore smaller than it would be were the curve approximately linear. It might be supposed at first sight that this concavity would introduce a constant error, and therefore that the recovery of different amounts would be affected disproportionately. This is not so, because the tangential straight line is shorter for smaller amounts of berculon A; it therefore cuts off a smaller part of the normal curve and the error due to the concavity is consequently less. Different amounts may therefore be estimated without serious error using a single recovery factor *F*. However, *F* should be established for blood of the species to be studied experimentally. The method is suitable for the estimation of from 0.5 to 5.0 μg. of berculon A. The curves shown in Fig. 4 illustrate the estimation of 3 μg. An amount of this order is found in 3 ml. of blood in therapeutic experiments. Less than 5.0 μg. of berculon A cannot be estimated directly by method II because such an amount absorbs no more strongly than an extract of normal blood, and the absorption of such an extract varies from animal to animal, and in the same animal at different times. For example, the absorption at 330 mμ of an extract of human blood increased by nearly 150 per cent half an hour after the subject had drunk half a pint of milk.

The method can be applied to berculon B, the recovery of which from blood is shown in Table IV. The whole of the apparent loss of berculon B, which is extracted quantitatively (Spinks, 1949), is due to the departure of the normal curve from linearity.

The application of the method to the measurement of low concentrations of berculon A and berculon B in dog blood is shown in Fig. 5. As in the mouse berculon B gave concentrations 4–5 times higher than those of berculon A.

A similar graphical technique was developed by Coggeshall and Glessner (1949) for the correction of background absorption in naphthalene estimation.

Photolability of thiosemicarbazones

All the methods described above must be carried out in dim light in order to prevent the rapid transformation of the thiosemicarbazones that occurs in strong light, including strong tungsten light. During early work on the development and

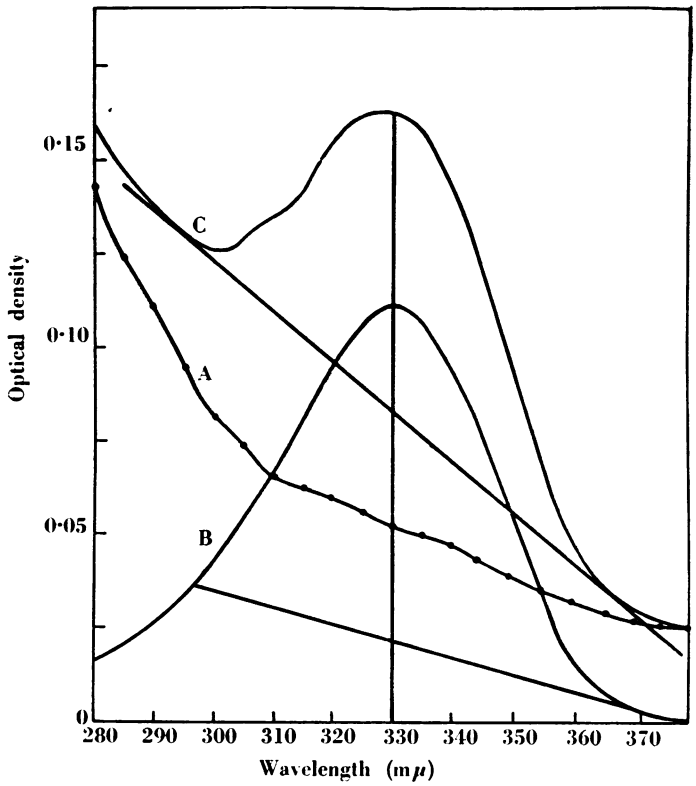


FIG. 4.—Estimation of berculon A by the graphical method: A, absorption spectrum of an extract of normal blood; B, absorption spectrum of an extract of 3 μg. berculon A; C, sum of A+B. For a full explanation, see text.

use of the methods described in a previous paper (Spinks, 1949) it was found that readings of standards remained normal and at about the values expected, although estimations were made in daylight. Later it was found that standards often gave low and erratic readings, and the results of several animal experiments had therefore to be abandoned. It was known that some thiosemicarbazones were photolabile; for example, *p*-dimethylaminobenzaldehyde thiosemicarbazone was slowly converted to a product with an entirely different absorption spectrum when irradiated in methanolic solution. The product was thought from its absorption spectrum to be a 1:3:4-thiadiazole formed by oxidative ring closure. Although a powerful ultra-violet lamp was used the transformation was very slow, and solutions of the compound in methanol did not appear to be labile in diffused daylight. Because of these findings no special precautions to avoid photodecomposition during estimations were taken until the erratic readings were observed. The problem was then re-examined and the difference between early and later findings was traced to three main factors. First, the photolability of the thiosemicarbazones is more marked in chloroform than in methanol, so that the work with methanol was misleading. Second, the early estimations were made in winter, at a time when fog

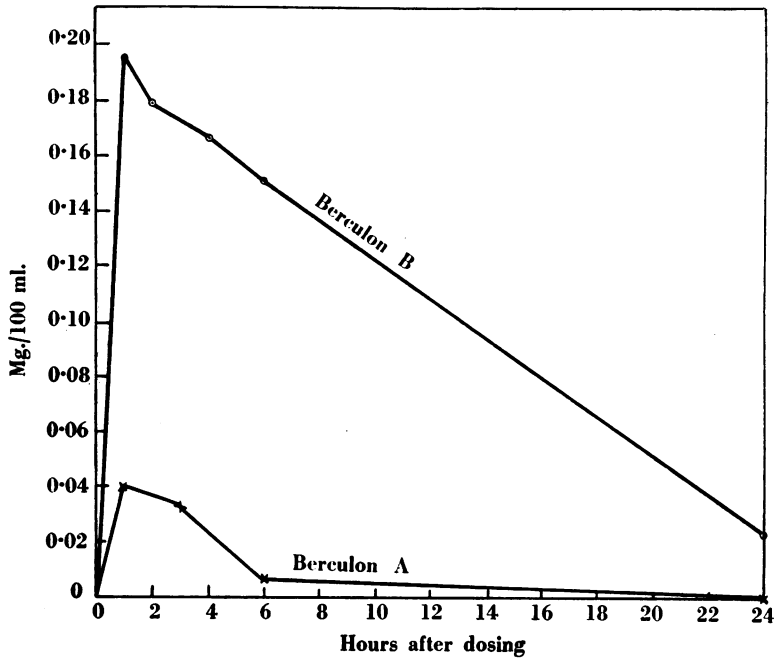


FIG. 5.—Blood concentrations of berculon A and berculon B in a dog after oral doses of 5 mg. per kg.

was very frequent. The later work was done during the exceptionally sunny weather of 1949. Third, the thiosemicarbazones undergo reversible photochange, the reversal occurring in the dark and therefore in the cell compartment of a spectrophotometer. A slight degree of photochange is consequently reversed before a reading can be taken. These findings were established qualitatively in daylight. Later quantitative

TABLE IV

RECOVERY OF BERCULON B FROM MOUSE BLOOD (3 ML.) BY THE GRAPHICAL METHOD (Method III)

Added (μg.)	Found (μg.)	Recovery (%)
0	0	—
0.5	0.459	92
0.5	0.444	89
0.5	0.428	86
1.0	0.735	73
1.0	0.722	72
1.5	1.09	73
1.5	1.28	85
2.5	2.14	86
5.0	3.94	79
5.0	4.28	86
	Mean	82
	Limit of error of mean (P=0.05)	±5.1

work with ultra-violet lamps showed that the behaviour of the thiosemicarbazones was even more complicated than had been thought. The findings are illustrated by the following experiments with *p*-aminobenzaldehyde thiosemicarbazone (6198). They were the first quantitative ones carried out.

A solution of 0.5 mg. 6198 in 100 ml. chloroform was prepared in a dark-room under weak diffused tungsten light (40-watt lamp in an opal glass globe at a distance of five metres). In a 2-cm. cell it had an optical density of 1.82 at λ_{max} 342 mμ. The cell was placed 20 cm. from an Hanovia U.V. lamp and irradiated for accurately timed periods, the density at 342 mμ being rapidly read at the end of each period. The findings were as follows (Fig. 6): the optical density dropped rapidly to 0.94 after 7 minutes; after 10 minutes it was 1.00. This slight rise was thought to be due to an error of observation, since reading was made difficult by the reversal of the photochange which began as soon as the cell was placed in the cell compartment. Irradiation was therefore discontinued and readings were taken in the dark every 5 minutes. The optical density rose until it almost reached its first value. In order to see whether this double change could be repeated the same solution was again irradiated (Fig. 6); this time the optical density fell for 3 minutes only, and then only to 1.04; it then rose during *continued irradiation*, and had reached 1.22

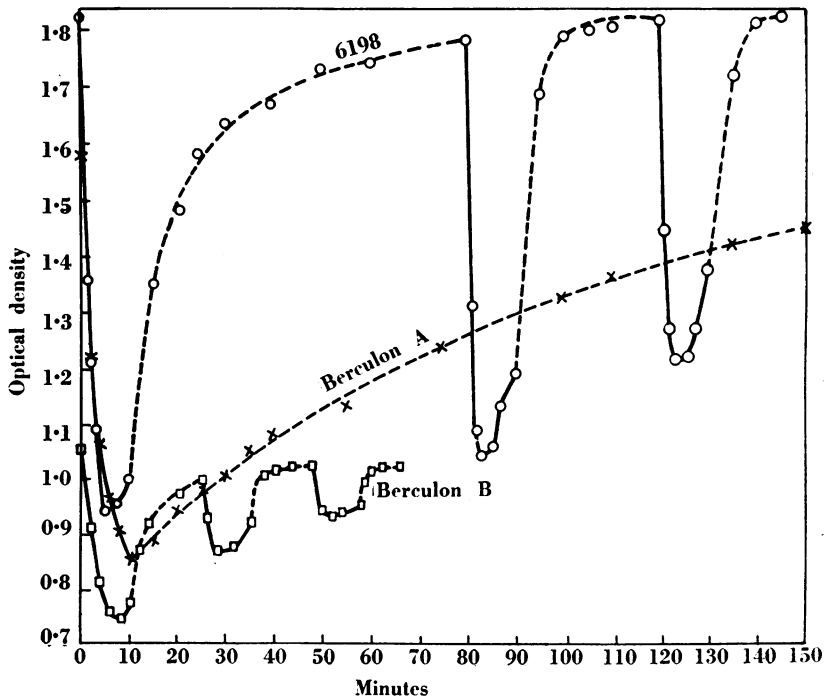


FIG. 6.—Change of optical density of chloroform solutions of 6198, berculon A, and berculon B during alternating periods of irradiation (continuous lines) and darkness (broken lines).

after 10 minutes. This rise continued during subsequent standing in the dark until the optical density was at its earliest value again. A third irradiation of the same solution confirmed the trend observed in the second. The minimum observed during this irradiation was higher than during the second, and the rise that occurred during irradiation was more marked (Fig. 6).

There was only one possible explanation of these results. The first photochange, which was reversible in the dark, was accompanied by a second photochange which was irreversible. The gradual accumulation of the product of this second change caused the trend shown by successive irradiations. Moreover, it was obvious that the reversible photochange was giving a product with a different absorption spectrum from the original compound, whereas the irreversible change was probably giving a product with a similar absorption spectrum. The last conclusion was confirmed by the following experiment. Another solution of 0.5 mg. 6198 in 100 ml. chloroform was prepared and the absorption spectrum was constructed. The solution was irradiated until no further change in optical density at λ_{max} 342 mμ occurred (Fig. 7). The optical density then remained constant during an

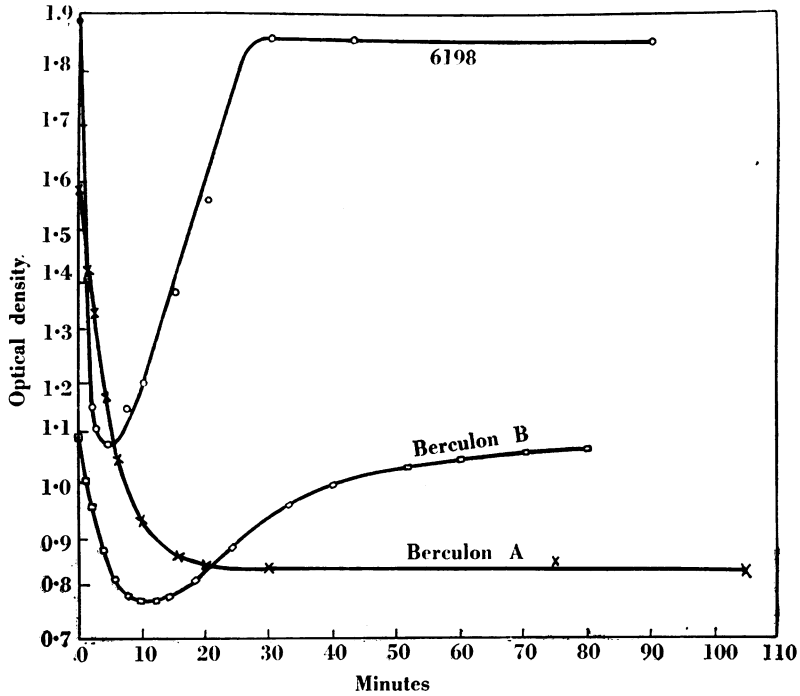


FIG. 7.—Change of optical density of chloroform solutions of 6198, berculon A, and berculon B during continued irradiation.

hour's irradiation and during an hour in the dark. The absorption spectrum was again constructed and was identical with the original one (Fig. 8).

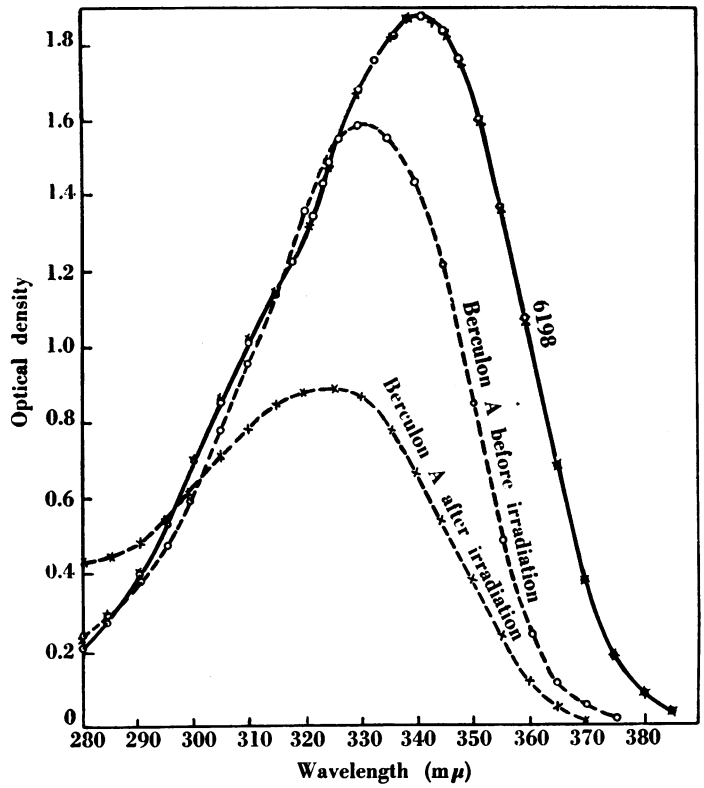
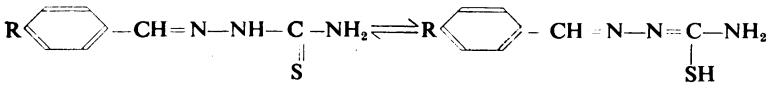


FIG. 8.—Absorption spectra of 6198 (continuous line) before (O) and after (×) irradiation; similar spectra of berculon A before and after irradiation until no further change occurred.

Before describing the transformations of berculon A it is convenient to summarize the behaviour of 6198 in the following diagram:

$$W \underset{\text{dark}}{\overset{\text{light}}{\rightleftharpoons}} Y \longrightarrow Z$$

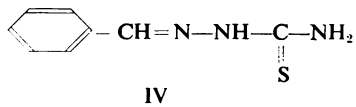
Compound *W* is the original one, *Y* is that formed initially in the light, *Z* is the product of continued irradiation. Compound *Z* may be formed from *W* instead of *Y*, although from the shape of the irradiation curve the route shown is the more likely. *W* and *Z* have identical spectra; that of *Y* is different. The reversibility of the *W*→*Y* transformation indicates that a simple tautomeric change is involved, and because a similar change is displayed by berculon B (see below) and *m*-methoxybenzaldehyde thiosemicarbazone (not described), this change must be independent of the nature and position of the substituent in the benzene nucleus, and must involve the thiosemicarbazone group. The obvious possibility is that shown:



II III

These tautomers would be expected to have different absorption spectra. Because of the instability of *Y* in the 6198 series it was impossible to obtain its absorption spectrum or to suggest structures for *W* and *Y*. It was therefore fortunate that berculon A behaved differently from 6198. The nature of the difference is shown in Figs. 6 and 7. When berculon A was irradiated under the standard conditions used for 6198, the change, $W \rightleftharpoons Y$, occurred as with 6198, but no evidence of any formation of *Z* was obtained. Moreover, the dark reaction, $Y \rightarrow W$, was slow enough to allow an approximate absorption spectrum of *Y* to be obtained. This absorption spectrum is given in Fig. 8. It showed marked differences from that of the original berculon A, the maximal absorption being much reduced and at a slightly lower wavelength. Berculon B, on the other hand, behaved like 6198 (Figs. 6 and 7): a form *Z* was produced, and its spectrum was identical with that of the original berculon B (not shown).

It remains to allot structures to forms *W* and *Y*, and to consider the possible nature of form *Z*. In order to do this it was necessary to obtain the absorption spectra of compounds possessing the unequivocal structures of tautomers II and III. The necessary compounds related to 6198, berculon A, or berculon B were not available, but those shown below (IV, V, VI) were. They were provided by



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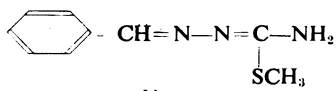

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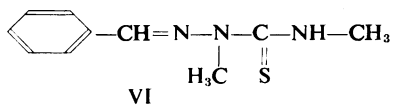


VI

Dr. Hoggarth, to whom the author's thanks are extended. The absorption spectra are shown in Fig. 9. Those of benzaldehyde thiosemicarbazone (IV) and its N^2: N^4-dimethyl derivative (VI) were similar and indicated that the former exists preponderantly in the form shown as II above. The maximal absorption of the S-methyl derivative (V) was much lower, and was at a lower wavelength. The absorption spectra of forms *W* (original) and *Y* (initial irradiation product) of berculon A (Fig. 8) corresponded well with those of benzaldehyde thiosemicarbazone and its S-methyl derivative respectively, although both curves were shifted to longer wavelengths. It is therefore probable that the original (*W*) form of the thiosemicarbazones has the thiocarbonyl (C=S) structure, or is a mixture in which this structure predominates, and that the first irradiation (*Y*) product has the sulphydryl (C–SH) structure.

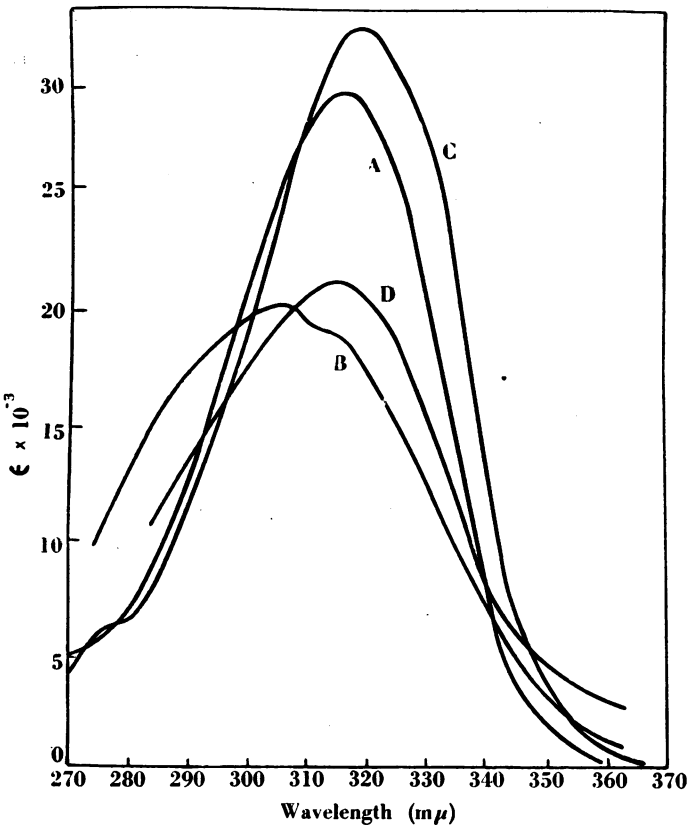
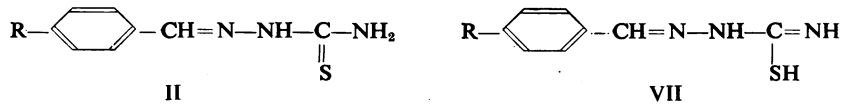
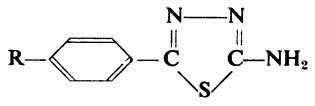


FIG. 9.—Absorption spectra, in chloroform, of benzaldehyde thiosemicarbazone (A), its S-methyl derivative (B), its N^2: N^4-dimethyl derivative (C), and its irradiation product (D).

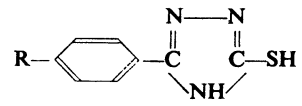
This view was strengthened by examining the photolability of benzaldehyde thiosemicarbazone. It behaved almost exactly like berculon A, and an approximate spectrum of form *Y* was obtained (Fig. 9). When allowance is made for a partial return to form *W* during the construction of the spectrum, and for the unpredictable effect of the methyl group on the spectrum of the S-methyl derivative, it must be concluded that forms *W* and *Y* of benzaldehyde thiosemicarbazone also have the C=S and C–SH structures respectively. Further evidence is provided by the stability to irradiation of the S-methyl (V) and N^2: N^4-dimethyl (VI) derivatives of benzaldehyde thiosemicarbazone. In them the tautomeric change, II→III, is impossible, although in (VI) the other possible change, II→VII, could still occur.



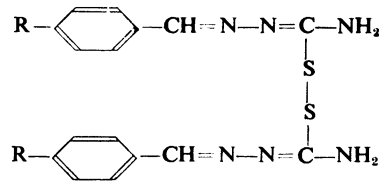
The nature of form *Z* has not been proved. The 1: 3: 4-thiadiazole (VIII) and 1: 2: 4-triazole (IX) which could be formed by oxidative ring closure are eliminated



VIII



IX



X

because they have very different spectra from the corresponding *W* form. The structure (X) would be expected to simulate (III) in spectrum, and so would the tautomer (VII). Another possibility is that *Z* is the initial form *W* stabilized in some way by continued irradiation. For example, a stabilizing substance might be formed from the solvent or from an impurity; or an impurity which stabilizes *Y* might be destroyed. Although at first sight this possibility does not seem very plausible it does explain the exact identity of the spectra of *W* and *Z*. If *W* and *Z* had different structures their spectra would be expected to differ at least slightly. It is also in accord with the behaviour of benzaldehyde thiosemicarbazone and berculon A. Their *Y* forms are more stable than those of 6198 and berculon B, in that they return only very slowly to *W* forms in the dark. Neither gave a *Z* form on continued irradiation. This is easily explained if *W* and *Z* are identical, because the return to *W* in the dark is then the same reaction as the production of *Z* on continued irradiation. Otherwise it is necessary to assume that two different reactions are equally affected by a single structural modification.

Since the isolation of the *Z* form of 6198 from chloroform would be difficult because of the low solubility of 6198, other solvents have been examined. It was hoped also that a solvent allowing analysis in daylight would be found. Fig. 10 shows the photolability of 6198 in methanol, butanol, ethylene dichloride, and tetrachlorethylene. The results should be compared with those in Fig. 6. Compound 6198 was fairly stable in methanol; it changed slowly in butanol, and rapidly in ethylene dichloride and tetrachlorethylene. In tetrachlorethylene it behaved like berculon A in chloroform, in that the *Y* form was stable to continued irradiation, and returned only slowly to the *W* form in the dark (not shown). In ethylene dichloride the *Y* form returned to *W* rapidly in the dark, as in chloroform. No formation of *Z* was observed in butanol, ethylene dichloride, or tetrachlorethylene. This finding provides some support for the theory that *Z* and *W* are identical, since it shows that *Z* is formed in a particular solvent only. A possible explanation (in part suggested by Mr. J. M. Thorp) is that the C–SH (*Y*) form is produced in light only in the presence of traces of water. The irradiation of chloroform would lead to the formation of phosgene ($CHCl_3 \rightarrow COCl_2 + HCl$) and dehydration of the solvent, with consequent stabilization of *W*. Yet another possibility (suggested by Dr. J. Madinaveitia) is that the HCl produced by irradiation forms a stable hydrochloride of *W*. Both these possibilities could readily be substantiated or

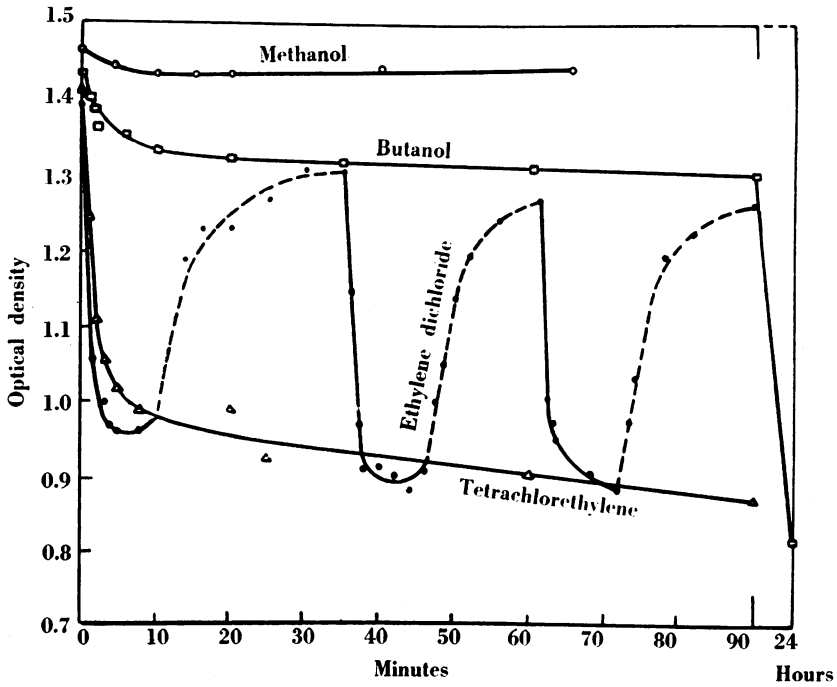


FIG. 10.—Change of optical density of solutions of 6198 in methanol, butanol, ethylene dichloride, and tetrachlorethylene during irradiation (continuous lines) or darkness (broken lines).

disproved by experiment. Unfortunately the author is at present unable to continue the study.

No solvent seemed likely to allow estimation of the thiosemicarbazones in daylight. Those of similar polarity to chloroform gave photolabile solutions of 6198; and methanol and butanol, in which 6198 was fairly stable, extracted much interfering material from blood.

SUMMARY

p-Aminobenzaldehyde thiosemicarbazone (6198) and *p*-acetylaminobenzaldehyde thiosemicarbazone (berculon A) have been estimated simultaneously by extracting them from blood with chloroform, reading the optical densities at 320 and 342 mμ, and solving simultaneous equations.

Compound 6198 is acetylated by the mouse to berculon A, but deacetylation of berculon A to 6198 has not been demonstrated in mouse, dog, or man. Berculon A can therefore be estimated directly in these species by reading optical density at λ_{max} 330 mμ only.

A graphical method of estimating berculon A or berculon B (8388; *p*-ethylsulphonylbenzaldehyde thiosemicarbazone) in blood in therapeutic concentrations of 0.02 to 0.2 mg./100 ml. is described. The direct technique cannot be used to estimate such small amounts because of the high and variable absorption of an

extract of normal blood. In the mouse and dog berculon A gives blood concentrations about a quarter as high as those of berculon B.

The photolability of the thiosemicarbazones is described. In chloroform the original form (*W*) of 6198 gives on irradiation a form *Y* which returns to *W* in the dark. Simultaneously there is produced a form *Z* which is stable in light and dark. The spectra of forms *W* and *Z* are identical, and suggest that *W* at least has the structure —CH=N—NH—C(=S)—NH_2. The *Y* forms of benzaldehyde thiosemicarbazone and berculon A return only slowly to *W* in the dark, and their spectra suggest that they have the structure —CH=N—N=C(-SH)—NH_2. The structure of form *Z* has not been proved, but it may be identical with *W*, stabilized in some way by continued irradiation. A possible manner of stabilization is discussed.

Because of their photolability thiosemicarbazones must be estimated in the dark-room. Weak, unfiltered tungsten light may be used.

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