*Brit. J. Pharmacol.* (1952), 7, 494.

# THE ANTIFILARIAL ACTION AND TOXICITY OF METHYLENE VIOLET AND OF OTHER COMPOUNDS OF THE PHENOSAFRANINE SERIES

BY

F. HAWKING, W. E. ORMEROD, J. P. THURSTON, AND W. A. F. WEBBER

*From the National Institute for Medical Research, London, N.W.7, and the Medical Research Council Field Station, Fajara, Gambia, W. Africa*

(Received April 30, 1952

These papers describe an investigation of the antifilarial action of methylene violet and its derivatives, which culminated in a clinical trial of methylene violet. Unfortunately, no effect was produced against *W. bancrofti* or *A. perstans* in man. Methylene violet RRA (NN-dimethylphenosafranine) belongs to the phenosafranine series, and is the 3-amino-7-dimethylamino-derivative of the basic structure shown at the head of Table I.

## I. ANTIFILARIAL ACTION AGAINST *Litomosoides carinii*

(by W. A. F. W. and F. H.)

The compounds of the phenosafranine series are difficult to purify, so that figures obtained for their activity are only approximate, whereas those for the toxicity may be much increased by the presence of toxic impurities. Accordingly, the true toxicity of the different derivatives may be less than those recorded here, whereas the activity may be greater or less.

### METHODS

The drugs were obtained from several sources, especially from Professor W. Bradley to whom our thanks are due. The antifilarial action of the compounds was tested in cotton rats infected with *Litomosoides carinii* as described by Sewell and Hawking (1950). Briefly, the drug was injected intraperitoneally once daily for six successive days; six days after the last dose, the rat was killed and the worms present in the pleural cavities were examined to see if they were alive or dead, attention being concentrated mostly on the female worms. The microfilariae in the blood were also examined, but no action upon them *in vivo* was detected. The result is expressed as the minimum effective dose (M.E.D.) i.e., the dose which cures (i.e., kills most of the worms) in more than three-quarters of the rats treated. This expression and that for the maximum tolerated dose have only an approximate value, but they are adequate for preliminary screening. The chronic toxicity of each compound was estimated by intraperitoneal injections into mice once daily for four days. It was reckoned empirically (on the basis of our previous experience) that the maximum dose (mg. per kg.) tolerated by cotton rats for six doses was 40 per cent of the dose (mg. per kg.) tolerated by mice for four doses. The result is expressed as the maximum tolerated dose (M.T.D.), i.e., the maximum dose tolerated by more than three-

quarters of the animals. A study was also made of the antifilarial action of the drugs *in vitro* upon adult worms and upon microfilariae, using the methods described by Hawking, Sewell, and Thurston (1950). The microfilariae were suspended in a mixture of serum and Tyrode solution at 37° C. They lived well for several days. The adult worms were suspended in a similar medium in Carrel flasks at 37° C.; in the absence of drug they survived more than three days.

## RESULTS

The results of these tests are shown in Table I. The various redox-potential indicators were studied to see if activity could be correlated with redox-potential. The $E_3$ (i.e., oxidation-reduction potential) of phenosafranine is –0.252 v. at *p*H 7.0. As shown in the Table, all of them were inactive. Four compounds were tested upon adult worms *in vitro* at 37° C. The minimum concentration lethal in 1–2 days of methylene violet, janus green, or C.I. 836 was 5 $\mu$g. per c.c., that of janus blue was more than 20 $\mu$g. per c.c. The phenosafranine compounds act upon the adult worms and not to any significant extent upon the microfilariae. Moreover, there is no parallelism between the action upon microfilariae *in vitro* and that upon the adult worms *in vivo* (cf. compounds 840 and 854 in Table I). At autopsy 6 days after the end of treatment the worms were often found bound together with fibrin (as happens after treatment with other types of filaricidal drug); they were usually stained faintly with the dye, but not more than the adjacent tissues were.

In addition, compounds of several related series which were examined at an earlier stage of this work have been reported by Sewell and Hawking (1950). Thus six dyes of the thiazine type were tested, viz. Thionin (Colour Index 920*), methylene green (C.I. 924), toluidine blue (C.I. 925), methylene blue, and new methylene blue (C.I. 927). The maximum tolerated dose of these compounds was between 20 and 100 mg. per kg. Among oxazine dyes tested, brilliant cresyl blue (C.I. 877) and nile blue sulphate (C.I. 913) had an M.T.D. of 20 mg. per kg.; three phenazonium compounds tested, viz., gallocyanin (C.I. 883), gallamine blue (C.I. 894), and resorcin blue (C.I. 908), had an M.T.D. of 50–500 mg. per kg. Among phthalein dyes erythrosin (C.I. 773) and the corresponding bromine compound, eosin, were tested; the M.T.D. was 100–200 mg. per kg. Acriflavine was also examined; the M.T.D. was 4 mg. per kg. None of the above dyes showed antifilarial action *in vivo*. Three of the compounds in Table I, viz., janus green, diethyl safranin, and safranin T (C.I. 841), have also been examined by Peters, Bueding, *et al.* (1949); they found no activity *in vivo* and only a slight activity upon adult worms *in vitro*. Methylene violet was found to be lethal to *Litomosoides in vivo* by Brown and Hussay (1947).

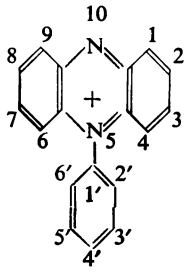
### *Relation of activity to structure*

Although the number of compounds examined was too small to allow of generalization, an aryl group attached to a quaternary nitrogen (5) is apparently essential for activity, for no action was discovered in the substances lacking this group. All the active compounds have a $-NMe_2$ or -NHMe group in the 3 or 7 position, but too few compounds without this group were examined to say whether this is

* Reference number in the *Colour Index* of the Society of Dyers and Colourists (1924), edited by F. M. Rowe; published by the Society, Bradford.

TABLE I

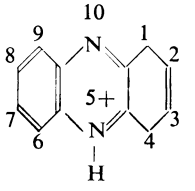
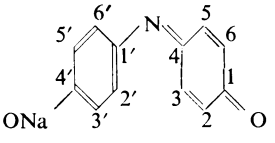
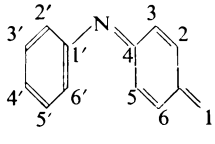
THE ANTIFILARIAL ACTION OF VARIOUS DYES *in vivo*




| Colour Index No. or name of drug | Substituents | M.T.D.* (mg./kg.) | M.E.D. (mg./kg.) | Therapeutic efficiency | M.E.D. *in vitro* microfilaria μg./c.c. |
|---|---|---|---|---|---|
| 828 | 7-$NHC_6H_5$-8 : 9-benz | 50 | >50 | 0 | >40 |
| 830 | 1 : 2-benz-3-(=O) | 500 | >500 | 0 | >40 |
| 832 | 1 : 2-benz-7-$N(CH_3)_2$ | 20 | >20 | 0 | 2.5 |
| 833 | 1 : 2-benz-3-$NHC_6H_5$-7-$NR_2$, where R=$CH_3$ or $C_2H_5$ | 100 | >100 | 0 | >40 |
| 836 | 1 : 2-4″-NH(*p*-$CH_3C_6H_5$)benz-7-$N(CH_3)_2$-4′-$CH_3$ | 1.0 | 0.5 | 2 | 2.5 |
| 840 | 3 : 7-di$NH_2$ | 10 | 10 | 1 | 1.5 |
| 841 | 2 : 8-di$CH_3$-3 : 7-di$NH_2$-1R. R=H or $CH_3$ | 5.0 | 5.0 | 1 | |
| T.3 | 3-$NH_2$-7-$NHCH_3$ | 3.0 | 2.0 | 1 | |
| 842 | 3-$NH_2$-7-$N(CH_3)_2$ | 3.5 | 0.5 | 7 | 2.5 |
| 844 | 2-$CH_3$-3-$NH_2$-7-$N(CH_3)_2$ | 5.0 | 5.0 (slight action) | 1 | 10 |
| 847 | 3 : 7-di$N(C_2H_5)_2$ | 100 | >100 | 0 | >40 |
| 848 | 2 : 8-di$CH_3$-3-$NHCH_3$-7-$NHC_2H_5$ | 1.0 | 0.5 | 2 | 2.5 |
| T.4 | 3-$NH_2$-7-$NHC_6H_5$ | 5.0 | 2.0 | 2 | |
| 849 | 3-$NHC_6H_5$-7-$N(CH_3)_2$ | 2.0 | 0.35 | 6 | 20 |
| 850 | 3-NH(2′-$CH_3C_6H_4$)-7-$N(CH_3)_2$-1-$CH_3$ | 50 | 50 (slight action) | 1 | >40 |
| 851 | 3-NH(4′-$CH_3C_6H_4$)-7-$N(CH_3)_2$ | 2.0 | 0.35 | 6 | 10 |
| 854 | 3 : 9-di$N(CH_3)_2$-6-$CH_3$-3′ : 4′benz | 2.0 | 0.5 | 4 | 40 |
| 861 | 2 : 3 : 7 : 8-tetra $NHC_6H_5$ | 50 | >50 | 0 | >40 |
| Rosinduline | 1 : 2-benz-3-$NHC_6H_5$ + two sulphonic acid groups | 100 | >100 | 0 | |
| Janus blue | 2 : 8-di$CH_3$-3-$NH_2$-7(−1″)-N : N-$C_{10}H_6$.OH(2″) | 100 | 100 (some action) | >1 | 20 |
| Janus green | 3-$N(C_2H_5)_2$-7-N : N-$C_6H_5$. $N(CH_3)_2$(*p*) | 2.0 | 0.5 | 4 | 2.5 |

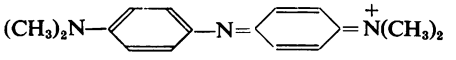
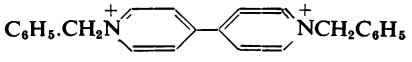
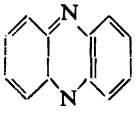
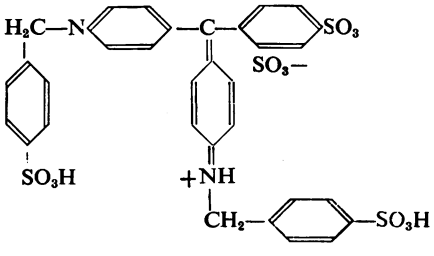
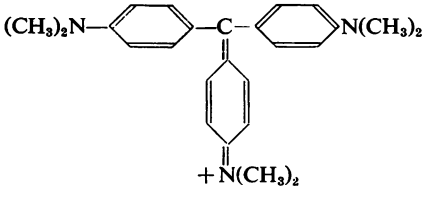
* The M.T.D. (maximum tolerated dose) is the dose for rats calculated from the dose tolerated by mice, as described on p. 494.

TABLE I—*continued*

| Colour Index No. or name of drug | Substituents | M.T.D.* (mg./kg.) | M.E.D. (mg./kg.) | Therapeutic efficiency | M.E.D. *in vitro* microfilaria $\mu$g./c.c. |
|---|---|---|---|---|---|
| |  | | | | |
| 825 | 3-$N(CH_3)_2$-7-$NH_2$-8-$CH_3$ | 100 | >100 | 0 | >160 |
| 827 | 1 : 2-benz-3-$NH_2$-5-$C_2H_5$-8-$CH_3$ | 10 | >10 | 0 | 10 |
| 838 | 2 : 8-di$CH_3$-3 : 7-di$NH_2$-5-$CH_3$ | 5.0 | >5 | 0 | >40 |
| FIL 10 | 5-$CH_3$ | 2.0 | >2 | 0 | |
| |  | | | | |
| | | | | | *E'o at* p*H* 7.0 |
| *o*-Chlorophenol indophenol | 2-Cl | 10 | >10 | 0 | +0.233 |
| *o*-Bromophenol indophenol | 2-Br | 500 | >50 | 0 | +0.230 |
| Thymol indophenol | 6-$CH(CH_3)_2$-3-$CH_3$ | 100 | >100 | 0 | +0.174 |
| 1-Naphthol-2-sodium sulphonate indophenol | 2-$SO_3Na$-5 : 6-benz | 50 | 50 very slight action | 1 | +0.123 |
| *m*-Cresol indophenol | 3-$CH_3$ | 20 | >20 | 0 | +0.208 |
| *o*-Cresol indophenol | 2-$CH_3$ | 50 | >50 | 0 | +0.191 |
| Guiacol indo-2 : 6 dibromophenol | 2 : 6-diBr-3'-$OCH_3$ | 5 | >5 | 0 | +0.159 |
| Phenol indophenol | None  | 100 | >100 | 0 | +0.227 |
| Toluylene blue | 1-$N(CH_3)_2Cl$-3'-$CH_3$-4'6'-di$NH_2$ | 50 | 50 | 1 | +0.115 |
| Phenol blue | 1=0-4'-$N(CH_3)_2$ | 20 | >20 | 0 | +0.224 |
| *m*-Toluylene diamine indophenol | 2 : 6-di$NH_2$-5-$CH_3$-4'-ONa | 50 | >50 | 0 | −0.125 |

* The M.T.D. (maximum tolerated dose) is the dose for rats calculated from the dose tolerated by mice, as described on p. 494.

TABLE I—*continued*

| Colour Index No. or name of drug | Formula | M.T.D.* (mg./kg.) | M.E.D. (mg./kg.) | Therapeutic efficiency |
|---|---|---|---|---|
| Bindschedler's green |  | 10 | >10 | 0 |
| Benzyl viologen $E'_0 - 0.359$ |  | 0.5 | >0.5 | 0 |
| 855 |  | 50 | >50 | 0 |
| Erioglaucine |  | 500 | >500 | 0 |
| Gentian violet |  | 2 | >2 | 0 |

* The M.T.D. (maximum tolerated dose) is the dose for rats calculated from the dose tolerated by mice, as described on p. 494.

significant; certainly not all compounds with this group were active. Besides 842 (3-$NH_2$) the most potent compounds are Nos. 849 and 851 in which there is a 3-arylamino group. But this activity is destroyed by inserting methyl groups in the *ortho*-positions on the two benzene rings, possibly because these groups block the *para*-positions which could otherwise be attacked to form quinonoid derivatives. Other active compounds are 835, 848, and 854. The inactivity of 844 and 847 is remarkable.

According to Dickens (1936) phenosafranine inhibits the Pasteur reaction in tissues, i.e., the oxidation of lactic acid which normally occurs during aerobic glycolysis; probably this is due to the blocking of certain enzyme systems. Pheno-

safranine also inhibits the breakdown of co-zymase by brain tissue (McIlwain, 1949). A similar action is exerted by various pyridine, acridine, and quinoline derivatives which contain a pentavalent nitrogen atom and a conjugated chain terminating usually in a basic group, and which are used in photography as photosensitizers and desensitizers. It is not known whether the lethal effect upon filariae is produced by this or some other means. This type of action seems similar to that of the cyanine dyes, studied by Peters, Bueding, *et al.* (1949) which were active against *Litomosoides* in rats but not against *Wuchereria* in man; these compounds were very potent in inhibiting the oxygen consumption of *Litomosoides*, but had no effect upon anaerobic glycolysis.

*Conclusion*

It was concluded that some compounds in this series possessed chemotherapeutic activity against the experimental infection in cotton rats in a sufficiently high degree to warrant a clinical trial against human filariasis. One of them, viz., methylene violet (842), was accordingly studied in more detail.

## II. Pharmacology of Methylene Violet

(by W. E. O., J. P. T., W. A. F. W., and F. H.)

The work described in the previous section had shown that the most promising members of the series were compounds 849, 851, and 842 (methylene violet). The only one for which a large and relatively pure sample could be obtained was methylene violet. Accordingly, this was chosen for more detailed study leading up to a clinical trial which would indicate whether this type of compound acted against *Wuchereria bancrofti* in man as well as against *Litomosoides* in cotton rats.

*Specimens*

Most of the specimens of the phenosafranine dyes are really mixtures of the nominal compound with various other derivatives, so that a knowledge of the composition of the specimens under study is of fundamental importance. The following specimens of methylene violet were studied:

A.—A sample supplied by Gurr Ltd., as a biological stain. This was impure and it was not used after the initial results had been obtained.

B.—A small pure sample, kindly provided by Professor Bradley, which was valuable as a standard of reference but was too scanty for clinical work.

C.—A sample of about 500 g. supplied by Farbwerke Hoechst which was fairly pure and which was used for most of the present work.

D.—A sample of 200 g. supplied by Allied Chemical and Dye Corporation, New York, without knowing that it might be required for biological use; they stated that it contained lead, arsenic, and other heavy metals in significant amounts.

E.—A sample from the same Corporation, which had been purified for clinical trial. Its apparent toxicity in mice was about the same as that of sample C, and it was slightly less toxic than C in rabbits. It was received near the end of this work, too late for use in West Africa.

Chromatographic analysis of sample C revealed two main fractions F and G (totalling about 87%) which were violet and eluted with alcohol; a subsidiary fraction H (about 5.8%) red, eluted with chloroform; a second subsidiary fraction blue, eluted with acetone; and several other bands of negligible amounts. Somewhat similar fractions were demonstrated in samples A and D.

F.—This was pure methylene violet with a maximum light absorption band at 250–255 mμ; it cured two infected cotton rats with doses of 0.3 mg. per kg.

G.—This had a maximum absorption band at 235–240 mμ. It was apparently produced by oxidation of methylene violet under catalysis by the alumina of the chromatographic column; it could also be prepared by the action of chromic oxide on methylene violet dissolved in glacial acetic acid at 100° C. It was less soluble in moist butanol than methylene violet and sparingly soluble in dry butanol. It could easily be separated from methylene violet by adsorption of the two substances on to activated charcoal; methylene violet could be eluted with ethanol, but the oxidized form was completely retained. Probably it is a compound of the safraninone type with a carbonyl oxygen in position 3′. It shows the general properties described for other members of this group by Jaubert (1895), but no detailed characterization was performed, since it showed no activity against cotton rat filariasis in a dose of 5.0 mg. per kg. (2 rats). This derivative also appears in the urine of animals treated with methylene violet.

Chemical analyses of two of these samples (Weiler and Strauss) showed the following. percentage composition.

Sample C—C, 63.36; H, 5.72; Cl, 13.9; N, 14.8. Total 97.78%
Sample D—C, 58.78; H, 5.55; Cl, 15.2; N, 13.2. Total 94.67%
$C_{20}H_{20}N_4Cl$ requires C, 68.4; H, 5.7; Cl, 10.0; N, 15.9. Total 100%

Tests for zinc were negative. The solubility in water at room temperature (20° C) of sample C, after grinding, was approximately 5 mg. per ml. The *p*H of a 1 : 1,000 solution in water was 4.55 (sample B) or 4.65 (sample C).

The therapeutic potencies and toxicities of the first four specimens are given in Table II.

TABLE II

| Specimen | M.T.D. (mg./kg.) | M.E.D. (mg./kg.) | Therapeutic index |
|---|---|---|---|
| A | 10 (infected rats) | 2.0 | 7 |
| B | 5 (clean rats)<br>3.5 (infected rats) | 0.2 | 17–25 |
| C | 5 | 0.5 | 10 |
| D | 5 | 1.0 | 5 |

The M.T.D. is the maximum dose tolerated by three-quarters of the animals; it was determined on cotton rats, which were inoculated intraperitoneally daily for 6 days; infected rats tolerate less than clean rats. The M.E.D. is the minimum dose effective in killing the worms in three-quarters of the animals. The numbers of rats used for each dose was small (2–4 for each specimen) so that the results are suggestive rather than conclusive. According to these results, the toxicity varied a moderate amount, but the therapeutic efficiency varied greatly.

Sample B had the best therapeutic index (?17), but sample C was also fairly high (10). When given by mouth 20 mg. per kg. of methylene violet (sample A) had little antifilarial action. In order to test the action on immature worms, four rats were treated with 3 mg. per kg. of sample C 20–26 days after exposure to infected mites; two rats out of four developed microfilariae in the blood while four out of five control untreated rats became infected; apparently action on immature worms was less effective than on mature ones.

The bactericidal power of methylene violet (kindly tested by Dr. A. T. Fuller) as indicated by the concentrations required to kill organisms in broth overnight at 37° C. was as follows: Haemolytic streptococcus (Richards) 0.5 mg. per 100 c.c., *Staph. aureus* 2 mg.; and *E. coli* 50 mg.

*Toxicity of methylene violet*

*Mice.*—When given intravenously as a single dose the LD50 of sample D was 21 mg. per kg. When given intraperitoneally daily for 4 days, the LD50 was 11.5 mg. per kg. for sample C and 9 mg. for sample D.

*Cotton rats* (uninfected).—When given intraperitoneally daily for 6 days the maximum tolerated dose of samples A, B, C, and D was between 5–10 mg. per kg. (Table II). When given by mouth, the maximum tolerated dose was about 150 mg. per kg. for sample B.

*Rabbits.*—The toxicity is shown in Table III. Briefly, most but not all rabbits tolerated 10 successive intravenous doses of 5 or 10 mg. per kg. of sample C

TABLE III

TOXICITY OF METHYLENE VIOLET FOR RABBITS, GIVEN IN 10 SUCCESSIVE DAILY DOSES, UNLESS OTHERWISE STATED

| Dose (mg./kg.) | Sample C | | Sample D | |
|---|---|---|---|---|
| | No. dead / No. treated | Remarks | No. dead / No. treated | Remarks |
| | | *Intravenous administration* | | |
| 2.5 | 0/4 | 2 lost weight | 0/2 | Both lost weight slightly |
| 5 | 2/5 | 2 died after 7 and 11 doses<br>2 lost weight | 2/2 | 2 deaths after 2 and 4 doses |
| 10 | 1/5 | 1 died after 6 doses<br>3 lost weight | 1/1 | After 1 dose |
| 20 | 3/3 | Deaths after 1, 2, and 10 doses | 1/1 | After 2 doses |
| 25 | 1/1 | One dose | | |
| 30 | 0/1 | One dose | | |
| 50 | 1/1 | One dose | | |
| | | *Oral administration* | | |
| 20 | 0/2 | 11 and 13 doses | 0/1 | 4 doses |
| 30 | 0/2 | 8 doses | 0/1 | 9 doses |
| 40 | 0/1 | 8 doses | 0/1 | 8 doses |
| 80 | 0/2 | 2 and 4 doses | 0/1 | 3 doses |
| 100 | 0/4 | 2 doses | 0/2 | 2 doses |
| 150 | 1/2 | 1 death after 4 doses<br>1 tolerated 1 dose | 0/1 | 1 dose |

By mouth, four rabbits tolerated two daily doses of 100 mg./kg. of sample C and one rabbit tolerated a single dose of 150 mg./kg. A rabbit which was given repeated daily doses of 150 mg./kg. died after the fourth dose. Probably little of the drug was absorbed from the alimentary canal. Sample D was a little more toxic, the maximum tolerated intravenous dose being 2.5 mg. per kg. Rabbits which died after an acutely fatal intravenous dose died in convulsions about 15 minutes later. The symptoms of chronic toxicity consisted of general malaise, loss of weight, poor

condition of the fur, and anorexia. Shortly before death there were tremors, convulsions, and (in one case) paralysis of the hind legs. Histological changes are described below.

TABLE IV

THE TOXICITY OF METHYLENE VIOLET FOR MONKEYS

| Monkey | Dosage | Remarks |
|---|---|---|
| | SAMPLE C | |
| 199 .. .. | *By mouth*<br>10 mg./kg. × 2<br>20 mg./kg. × 5 } days<br>30 mg./kg. × 2 | Insignificant loss in weight |
| 203 (previous sample D i.v.) .. .. | 10 mg./kg. × 15 in 21 days | No loss of weight |
| 205 .. .. .. | 10 mg. × 1<br>20 mg. × 3 } in 8 days<br>40 mg. × 2 | Monkey had diarrhoea (which may not have been due to drug). Lost weight and died 3 days after last dose |
| 206 .. .. .. | *Intravenously*<br>2.5 mg. × 15 in 19 days | No symptoms, no loss of weight |
| 207 .. .. .. | 2.5 mg. × 15 in 19 days | Gained weight |
| 199 (previously dosed by mouth) | 5 mg./kg. 3 in 3 days | Monkey became very depressed and was killed when moribund 6 days later |
| 198 .. .. .. | 5 mg. × 4<br>10 mg. × 4 } in 9 days | Died 2 days after last dose with few symptoms |
| | SAMPLE D | |
| 203 .. .. | *Intravenously*<br>5 mg./kg. × 3 in 3 days | Monkey became very violet and depressed. Lost weight 6.1→5.3 kg. Recovered |

*Monkeys.*—The effects are shown in Table IV. It was concluded that sample C was well tolerated in intravenous doses of 2.5 mg. per kg. but probably not in doses of 5 mg. per kg. When given orally a dose of 30 mg. per kg. was tolerated. The symptoms of toxic action included vomiting, loss of weight, general depression and loss of appetite. The skin of most of these monkeys became stained violet, but recovered its natural colour in 1–2 weeks after the drug was stopped. In one monkey (198) there was subcutaneous haemorrhage at a site of old tattooing. Albuminuria and the passage of casts indicated damage to the kidneys. There were no significant changes in the numbers of the cells in the blood.

Examination *post mortem* of monkeys and rabbits dying from methylene violet showed the skin and subcutaneous tissue to be stained mauve; in animals treated by mouth, the lining of the stomach was also mauve. The kidney showed the most significant lesions, viz., necrosis and calcification of some of the tubules especially of Henle's loop; the other tubules were not much affected; the glomeruli were sometimes swollen or infiltrated by polymorphs. The liver often showed fatty degeneration. The bone marrow and other tissues appeared normal. These

findings agree with those of Ginzler (1946), who reported that in animals treated with safranin 0 (C.I. 841) the chief lesion was damage to the kidney tubules.

*Absorption, distribution, and excretion*

*Methods of estimation.*—Methylene violet was extracted from blood and urine by shaking with *n*-butanol saturated with water; alcohol-water partition for methylene violet was high and practically all the dye was extracted when the volume of butanol was greater than that of the blood or urine. The butanol layer was separated by centrifugation and the density of the colour was estimated in a Duboscq colorimeter by comparison with a standard concentration of methylene violet in absolute *n*-butyl alcohol. The standard was stable over a period of three months. By this relatively crude method, concentrations of the order of 1 μg./c.c. could be estimated, and lower concentrations could be detected. Tissues were ground in a mortar with sand and extracted in the same way. Intense colour was found in extracts of faeces from animals that had received methylene violet by mouth, but the colour could not be matched, since coloured faecal products were also soluble in butanol. Some difficulty was found in matching the extracts from urine and organs of monkeys that had been dosed with methylene violet for a week or more. These extracts contained a yellowish substance, which proved to be the oxidized derivative of methylene violet described above as fraction G.

*Absorption.*—When given by mouth, much of the dye can be recovered from the faeces. A small amount is absorbed in the intestine, since about 1 per cent or less appears in the urine. When given intravenously to rabbits, methylene violet rapidly leaves the blood, and 5–10 minutes later it is difficult to demonstrate in the plasma.

*Distribution.*—The distribution of methylene violet in the organs after death was investigated in rabbits and monkeys. Table V shows the amount of methylene violet extracted from the livers and kidneys of seven rabbits which died within two days of the last dose. In three other rabbits which died three days after receiving 10 daily doses of 10 or 20 mg. per kg. no dye could be found in these organs. In monkey 198, which died two days after the last dose of 10 mg. per kg. i.v., the concentration (in μg. per g.) in the different organs and the percentage (in parentheses)

TABLE V

DISTRIBUTION OF METHYLENE VIOLET IN RABBITS

| Rabbit | Dose schedule | Days since last dose | Liver concentration μg /g. (% of total) | Kidney concentration μg./g. (% of total) | Total weight, mg. |
|---|---|---|---|---|---|
| 137 | 80–150 mg. by mouth | Immediate | 70 (80%) | 85 (20%) | 4.5 |
| 135 | 20 mg. i.v. once | Immediate | 56 (48%) | 300 (52%) | 5.8 |
| 132 | 20 mg. i.v. once | 1 | 5 (75%) | 3.5 (25%) | 0.27 |
| 130 | 20 mg. i.v. × 2 | 1/2 | 89 (70%) | 180 (30%) | 7.5 |
| 119 | 5 mg. i.v. × 4 | 1 | 16 (54%) | 65 (46%) | 1.3 |
| 120 | 5 mg. i.v. × 2 | 2 | Trace | 19 | 0.17 |
| 118 | 10 mg. i.v. once | 2 | 10 (86%) | 10.6 (14%) | 0.6 |

NOTE.—Rabbit 118 showed toxic symptoms of head drop, jerking, retching, and depression for 24 hours before death.

of the total amount recovered (19 mg.) was as follows: liver 37 (30%), kidney 121 (21%), lungs 8.5 (2.7%), spleen 4 (0.05%), heart 10.4 (2.7%), muscle 3.5 (42%), brain 0. In monkey 205, which died three days after the last dose of 40 mg./kg. by mouth, and in monkey 206, which died eight days after 2.5 mg. i.v., no appreciable amount of dye was found in the organs. According to these results the greatest concentration per gramme of tissue was found in the kidney and the next greatest in the liver. However, the total amount of drug in the liver was greater than that found in the kidney. The concentration in skeletal muscle was low, but the total amount was large. The dye is deposited in the kidney more quickly than in the liver and is also excreted from it more quickly. Immediately after intravenous injection about a quarter of the dose can be recovered from the kidney and liver; after three days only a trace or none remains in rabbits. Sheehan (1931) found that 20 per cent of the dye injected intravenously was immediately deposited in the kidney. In one of our patients a sample of cerebrospinal fluid was withdrawn five hours after the eighth dose (1.5 mg. per kg.) of methylene violet; there was no trace of dye in the fluid.

*Metabolism.*—Some of the methylene violet, which appears in the urine, has undergone a metabolic change (probably by oxidation) into a derivative described above as fraction G.

*Excretion.*—Once methylene violet has been absorbed into the body the chief route of excretion is via the urine. Traces of it appear in the urine of man in less than half an hour after intravenous injection. Much of the excreted material consists of the oxidized derivative, fraction G. If the urine is allowed to stand so that bacteria multiply in it (causing reduction) the colour fades; it can be readily restored by shaking with air (oxidation). After repeated treatment of monkeys, excretion in the urine persists for two to six days.

When 15 mg. were injected intravenously into a volunteer (F. H.) the excretion of dye in the urine followed the course in Fig. 1. Altogether 1.13 mg. was recovered from the urine, i.e., about 7 per cent of the dose. In patients treated intravenously at Fajara with a total dose of about 500 mg. during 14 days, the amount excreted in the urine was 13–30 per cent of the dose (average 20); the concentration in the urine was usually less than 10–20 mg. per litre, but once it rose as high as 50 mg.;

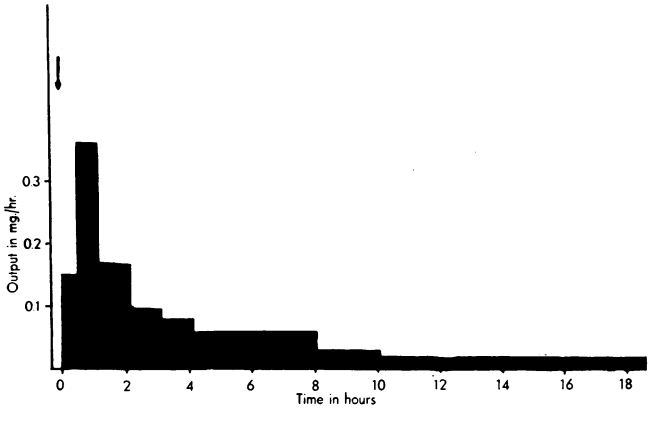



FIG. 1.—The hourly output of methylene violet in the urine, after the intravenous injection of 15 mg. (indicated by arrow) into a volunteer weighing 82 kg.

when administration ceased, measurable amounts continued to appear in the urine for at least three days. In a patient treated with 1.5 g. per day (26 mg. per kg.) by mouth, the amount excreted in the urine remained small (3–8 mg. per day or 3 mg. per litre), while the faeces were stained deep purple. The compound is not absorbed sufficiently well from the alimentary canal for this method of administration to be worth while.

## III. Methylene Violet and Human Filariasis
(by F. H.)

The clinical trial of methylene violet on patients infected with *Wuchereria bancrofti* was carried out at the Medical Research Council Field Research Station, Fajara, Gambia, West Africa. The patients who received methylene violet may be divided into four groups.

(*a*) *Patients without microfilariae in the blood.*—These were treated, mainly to determine the maximum tolerated dose. In the first place a European volunteer (F. H.), weighing 80 kg., received 75 mg. and 150 mg. respectively of the compound by mouth on two successive days. Later he received 3 mg., 7.5 mg., and 15 mg. respectively intravenously on alternate days. No ill effects were experienced. Three in-patients were then given daily intravenous doses which were gradually increased. Doses of 0.8 mg. per kg. daily were well tolerated, but doses of 1.3 mg. per kg. were followed in some cases by mild albuminuria. It was concluded provisionally that the maximum tolerated intravenous dose was probably about 50 mg. daily for an average patient weighing 50–60 kg.

(*b*) *In-patients.*—Five patients were treated with intravenous doses up to 75 mg. per day (1.3 mg./kg.) for 12–14 days. One patient with onchocerciasis was also treated (see below).

(*c*) *Out-patients at Village A.*—Village A is approximately 60 miles east of Bathurst; there is a high incidence of filariasis. Twenty-one persons were treated as out-patients with 10–14 daily injections up to 75 mg. per patient, so that the total dose ranged from 10–23 mg. per kg. (average 12.6 mg. per kg.).

(*d*) *Out-patients at Village B.*—Village B is 11 miles east of Fajara. Nineteen persons were treated as out-patients with 9–11 daily intravenous doses up to 55 mg. each, so that the total dose ranged from 5.8–10 mg. per kg. (average 7.9 mg. per kg.). All these patients contained many microfilariae of *W. bancrofti* (20 to 1,000 Mf. per 20 cu.mm. of blood) and some also contained microfilariae of *A. perstans.*

*Therapeutic effect.*—There was no immediate effect upon the microfilarial count (and none was expected). The patients at Village A were examined 10 months later (16 patients) and those at Village B at 4, 7 (17 patients), and 10 months later (16 patients). In only one patient had the blood ceased to contain microfilariae. In all the other cases there had been no significant change in the numbers of *Mf. bancrofti* or of *Mf. perstans* at the later periods of observation. It was concluded that the treatment had failed to kill any appreciable number of the adult filarial worms of both types present in these patients.

*Treatment of onchocerciasis.*—One case of onchocerciasis, discovered in an African who had come from the Futa Jalo region, was treated. The details are as follows:

Man aged 25 years, weight 55 kg. Small onchocercal nodules on the left hip and great trochanter. Skin clips from the left hip near the nodules showed many microfilariae of *O. volvulus.* From January 19 to 30 he was given methylene violet intravenously in

daily doses of 25–50 mg., the total dose being 425 mg. in 14 days, i.e., 7.7 mg. per kg. The doses had to be restricted because a trace of albumin occurred in the urine. On February 1 and March 10 microfilariae were still present in skin clips taken from the left hip. On March 13 two small nodules were excised from the left hip. When opened in Ringer's solution and warmed gently to 39° C., one male worm was seen moving actively and so was a coil which appeared to be part of a female worm. Both nodules contained great numbers of living microfilariae at all stages of development from the early ovum to the mature larva, thus indicating the presence of living females with active gestation. It was concluded that in this patient the treatment with methylene violet had failed to kill the adult worms.

*Toxicity.*—In the nine in-patients (groups *a* and *b*) counts were made of the red and white cells in the blood, together with differential counts of the leucocytes. No significant changes were observed during the course of treatment. The only common toxic effect observed was albuminuria. This was present in a few apparently healthy Africans even before treatment and its significance was sometimes doubtful. In 28 patients (groups *a*, *b*, and *c*) whose urine was examined daily, albuminuria occurred in 10, usually after doses of 75 mg. (In these cases the dose was omitted or reduced for one or two days and the albuminuria soon subsided.) Usually the amount of albumin was small, i.e., a faint cloud on boiling in the presence of acid (less than 20 mg. per 100 c.c. when measured in an albuminometer); in only one case was there a dense precipitate. The occurrence of albuminuria was the limiting factor which prevented higher doses of methylene violet being given. In addition, the treatment was probably mildly depressing, but this was difficult to prove or evaluate. Retching occurred in one patient 5 minutes after injection. With the exception of a curious lesion in the thumb nails, to be described below, there were no other toxic effects that were definitely due to the systemic administration of the drug. Thus, among all the 49 patients treated, there were complaints of pain in abdomen, colic, after the 10th or more doses (2), diarrhoea (2), pain in hip, spreading all over the body after 9th dose (1), and papular rash on body, which quickly subsided although treatment was continued (1); but these complaints were probably due to other intercurrent causes. In a few patients, a small amount of drug was injected into the arm outside the vein. This seldom caused more than a mild localized inflammation which subsided in 1–2 days. In one man there was a firm hard tender swelling of the forearm 2 weeks after the last dose.

In 3 of the 21 patients treated at Village A there was a curious affection of the thumb nails. (These lesions were not seen by the writer, but were reported by Dr. I. McGregor, who revisited the village 16 days after the last dose.) One man (total dose 12.2 mg./kg.) had complained of pain 7 days after the last dose, but there had been no visible lesion; sixteen days after, both thumb nails were floating on a bed of pus, a thin purulent discharge came away from the top of the nail, and there was tenderness and pain; 33 days after, the condition was the same. A second man (total dose 12.2 mg. per kg.) showed a similar picture and there was also purple pigmentation at the tip of the thumb beyond the nail margin. In a boy (total dose 23.8 mg./kg.) the nails had separated by the 16th day, and the thumbs were dry; by the 33rd day the condition was unchanged. Similarly, at Village B, 3 weeks after the last dose, 10 of the 19 patients were suffering from toxic effects on the thumb and finger nails; in 7 it was mild to moderate, and in 3 it was severe. Also in 1 of the 5 in-patients 4 weeks after treatment there was profuse suppuration

under all finger nails and 1 toe nail; this cleared up in a week without the loss of any nails. When all these patients were examined 9 months after treatment, the finger nails appeared quite normal.

## Discussion

This work shows that although methylene violet is effective in killing the adult worms of *Litomosoides* in cotton rats it has no significant action upon those of *Wuchereria bancrofti*, *Acanthocheilonema perstans*, or (probably) *Onchocerca volvulus* in man. The dose given to the patients is comparable to that which was effective in cotton rats; moreover chemotherapeutic compounds are usually much more active and more toxic, weight for weight, in larger animals or man than in small laboratory animals. Consequently, the failure to act is probably due to a difference of susceptibility between the human worms and those of cotton rats, and it is unlikely that other members of the phenosafranine series (even if more active against *Litomosoides*) would be any more effective in man. It does not seem worth while to investigate the antifilarial action of this type of compound any further.

The discovery that a compound acts upon the experimental infection (*Litomosoides*), but not on the human one (*Wuchereria*), although disappointing, is a common occurrence in chemotherapeutic research. A close parallelism is given by the cyanine compounds, studied by Peters, Bueding, *et al.* (1949). This discrepancy does not indicate that *Litomosoides* should not be used as a test object, but merely that caution must be exercised in drawing conclusions from results obtained with it. In particular, any new type of compound which shows significant activity on the experimental infection should be given a small preliminary clinical trial as early as is possible; it may be inactive in man and waste of labour in further developing the series can be avoided, or it may exceptionally prove much more effective than had been expected and the compound is saved from being abandoned as having only mediocre activity.

The toxic action on the finger nails discovered during the clinical trials is interesting. In practice it would be a serious handicap to the use of these compounds (unless it were due only to an impurity). Theoretically it could be postulated that methylene violet interferes with some enzyme system responsible for the laying down of the nail (cf. Dickens, 1936).

## Summary

*Section I*

1. Compounds of the phenosafranine series were filaricidal when tested upon cotton rats infected with *Litomosoides carinii*. The compounds killed the adult worms, but did not affect the microfilariae *in vivo*. There was some action upon microfilariae *in vitro*, but this was not parallel with the action upon the adult worms *in vivo*.

2. The most active compounds of the series were Colour Index No. 842 (methylene violet RRA, 3-NH -7-N($CH_3$)$_2$), C.I. No. 849 (3-NH$C_6H_5$-7-N($CH_3$)$_2$), and C.I. No. 851 (3-NH($C_6H_5$*p*$CH_3$)-7-N($CH_3$) -3′-$CH_3$). Apparently an aryl group attached to a quaternary nitrogen atom is essential for this activity.

*Section II*

3. Methylene violet was examined for toxicity. The LD50 for mice, when the dye was given intraperitoneally daily for 4 days, was 11.5 mg. per kg. In cotton

rats the maximum tolerated dose, given intraperitoneally daily for 6 days, was 5–10 mg. per kg.; given orally, it was about 150 mg. per kg. Rabbits usually tolerated repeated daily intravenous doses of 5–10 mg. per kg. Monkeys tolerated daily intravenous doses of 2.5 mg. per kg.

4. The symptoms of toxic action in experimental animals consisted of vomiting, loss of weight, general depression, violet discoloration of skin, and albuminuria. At post-mortem examination the chief lesions were damage to the tubules of the kidney and fatty degeneration of the liver.

5. When given by mouth, methylene violet is poorly absorbed (probably about 5 per cent is absorbed) and most of the drug is excreted in the faeces. When given intravenously it passes rapidly out of the blood into the tissues. The highest concentrations occur in the kidney and liver, but the largest absolute amounts occur in the skeletal muscles, liver, and kidney. It does not penetrate into the cerebrospinal fluid.

6. Methylene violet, which has been absorbed, is excreted mainly through the kidney, about 20 per cent of the dose appearing in the urine. Much of the excreted material consists of an oxidized derivative which has no filaricidal activity.

*Section III*

7. Methylene violet was administered intravenously to about 50 patients infected with filariasis in the Gambia, West Africa. Ten to fourteen daily doses were given, ranging up to 75 mg. per patient. The average total dose was 8–12.6 mg. per kg.

8. These doses were generally well tolerated, the limiting untoward effect being the production of albuminuria. However, some of the patients showed a curious toxic effect upon the development of the finger nails three weeks after the end of treatment.

9. Methylene violet had no filaricidal action upon the microfilariae or adult worms of *Wuchereria bancrofti* or *Acanthocheilonema perstans*, as judged by the number of microfilariae in the blood nine months after treatment.

10. One patient with *Onchocerca volvulus* was treated, but no effect was seen upon the microfilariae or adults.

11. It is concluded that compounds of the phenosafranine series are unlikely to have value for the treatment of human filariasis.

Grateful acknowledgments are due to Professor B. S. Platt for clinical facilities at Fajara; to Dr. J. A. McFadzean, Dr. I. McGregor, and P. Sewell, Esq., Ph.D., for valuable co-operation; to Dr. H. King and Dr. J. Walker for advice; to Professor W. Bradley, Professor E. E. Turner, and Dr. H. King, and to Farbwerke Hoechst, and Allied Chemical and Dye Corporation, New York, for the supply of compounds; and to Mr. K. Broomfield for technical assistance.

REFERENCES

Brown, H. W., and Hussay, K. L. (1947). *J. Parasitol.*, **33**, 33.
Dickens, F. (1936). *Biochem. J.*, **30**, 1233.
Ginzler, A. M. (1946). *Proc. Soc. exp. Biol., N.Y.*, **61**, 231.
Hawking, F., Sewell, P., and Thurston, J. P. (1950). *Brit. J. Pharmacol.*, **5**, 217.
Jaubert, G. F. (1895). *Ber. dtsch. chem. Ges.*, **28**, I, 270.
McIlwain, H. (1949). *Biochem. J.*, **44**, xxxiii.
Peters, L., Bueding, E., Valk, A. D., Jr., Higashi, A., and Welch, A. D. (1949). *J. Pharmacol.*, **95**, 212.
Sewell, P., and Hawking, F. (1950). *Brit. J. Pharmacol.*, **5**, 239.
Sheehan, H. L. (1931). *J. Physiol.*, **72**, 201.