THE ANTIRICKETTSIAL EFFECT OF THIONINE DYES

I. THE USE OF METHYLENE BLUE AND TOLUIDINE BLUE TO COMBAT EXPERIMENTAL TSUTSUGAMUSHI DISEASE (SCRUB TYPhUS)

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Since the outbreak of the recent war a number of contributions have been made to the chemotherapy of rickettsial diseases. Forbisen (4-4'-bis-antipyrine) and toluidine blue (TB) were shown to be active against murine typhus infections in mice (1). Although found to be effective against pulmonary infections in mice with murine and epidemic rickettsiae, p-sulfamidobenzamidine and p-sulfamidobenzamidoxine proved to be both toxic and ineffective in human cases of epidemic typhus (2). Against murine typhus infections of mice and of chick embryo yolk sacs, penicillin was found effective (3, 4), but when tested against Rocky Mountain spotted fever in guinea pigs, it showed no activity (5). Furthermore, in limited trials against human cases of scrub typhus (Tsutsugamushi disease) (6) and of epidemic typhus (8), penicillin was found to be of uncertain value. A growing body of evidence testifies to the effectiveness against both experimental and naturally occurring infections with various members of the rickettsial group of still another agent, para-

Because of the observations made in this laboratory concerning the effectiveness of toluidine blue (TB) in combatting murine typhus infections (1), trials of this dye were made against experimental scrub typhus when strains became available. Soon thereafter, stimulated by word that the closely related dye, methylene blue (MB), had also been found active against murine infections (17), investigation of the activity of MB against scrub typhus was begun. The demonstration of the chemotherapeutic activity of both TB and MB in infections with Rickettsia orientalis was reported in preliminary form (18) almost coincidentally with a report of similar content from an independent source (19). As the result of these reports, therapeutic trials of MB in human cases of scrub typhus were conducted in Burma (20), and these revealed that effective dosage in man was accompanied by serious toxic manifestations.

In spite of the unsatisfactory outcome of the human trials, the antirickettsial activity of these dyes is so striking in experimental tsutsugamushi disease as to provoke continued interest. The present paper extends the previously
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reported observations as to the results observed in the treatment of the experimental disease with MB and TB (18). In addition, the occasion has been taken to describe briefly, in the section on materials and methods, certain problems related to the experimental disease and the techniques of handling R. orientalis.

Materials and Methods

Rickettsial Strains.—Four strains of R. orientalis—Raub, Seerangayee, Queensland, and Karp—were used in this study. The majority of the experiments were done with the last-named strain, which was isolated in Australia by Dr. F. M. Burnet from a soldier who presumably had contracted the infection in New Guinea (21). The Queensland strain was isolated from a bandicoot in Australia by Dr. W. G. Heaslip. The Raub and Seerangayee strains were isolated from human cases by Dr. R. Lewthwaite in Malaya.1 The various strains had previously been carried in continuous passage in mice, guinea pigs, or in the anterior chamber of the rabbit’s eye for periods of months or years. In this laboratory they have been maintained by serial passage in cotton rats or, later, in the yolk sacs of developing eggs.

Sources of Infectious Material.—The preliminary observations were made with infectious material which consisted of freshly prepared suspensions of tissues, usually liver, of mice or cotton rats infected by the intraperitoneal route and sacrificed when obviously ill. This use of fresh material was necessitated by our inability to maintain infectious material in the frozen state (−76°C.) without marked loss in infectivity. Later, as had been suggested by Dr. N. H. Topping, it was found that such material could be stored with little loss of titer for several months if skim milk was employed as the suspending medium. Meanwhile these strains had been satisfactorily adapted to serial passage in the yolk sac of the developing chick embryo, the infective titers of which regularly surpassed those of the animal tissues. In all of the later work, therefore, the infectious material employed consisted of 20 per cent suspensions of pooled yolk sac made in skim milk and stored at −76°C. until used. Embryos 6 or 7 days old were inoculated by the yolk sac method (22) with 0.5 cc. of an approximately 1:50 dilution of infected yolk sac of the preceding passage. After 9 to 12 days' additional incubation at 35°C., the yolk sacs of the surviving embryos (usually from 50 to 65 per cent of those inoculated) were harvested, suspended in milk, homogenized in a Waring blender, and stored in flame-sealed glass ampoules in a dry ice chest. The infective titer of such yolk sac pools has been on the order of 10⁻⁸.

Diluents.—Because of evidence obtained in this laboratory with typhus rickettsiae (23), nutrient broth was employed as the diluent in early experiments. Although this proved satisfactory for work, which could be performed rapidly, it was found to be inadequate for the maintenance of the infectivity of R. orientalis during large scale experiments. Tests for the maintenance of infectivity over a 24 hour period (5°C.) of material with an original infective titer of approximately 10⁻⁸ revealed losses in titer of one log when the diluents were skim milk or 2 per cent normal yolk sac in saline, of 2 logs with 10 per cent human serum in saline, of 3 logs with nutrient broth, and of 4 logs with saline. When distilled water was employed infectivity was completely lost. In the later and by far the greater part of the experiments herewith reported, dilutions were made only with sterile skim milk. As an added precaution, all inocula were kept chilled in an ice water bath from the time of dilution until inoculations were completed.

Experimental Animals.—The majority of the experiments were performed with albino Swiss mice obtained from one or another of two commercial breeders. The remaining work was done with Eastern cotton rats (Sigmodon hispidus hispidus), also commercially bred.

1 All four of these strains were acquired by us through the courtesy of Dr. R. Lewthwaite.
Inoculation.—Animals usually were inoculated by the intraperitoneal route with a volume of 0.25 cc. Exceptionally, as indicated in the text, inocula of similar volume were given subcutaneously or intravenously, or a volume of 0.03 cc. was given intracerebrally.

Notes on the Mouse Disease.—In experiments with the Karp strain it was observed that mice are susceptible, with some uniformity, to lethal infection when inoculated by the intraperitoneal, intravenous, or intracerebral routes, but are only irregularly susceptible to disease induced by subcutaneous infection. The intranasal route was not employed. When comparable series of mice were inoculated by the several routes and observed for lethal outcome it was found that the maximum lethal end point followed intraperitoneal inoculation, and that the end points in the intravenously and intracerebrally inoculated animals were respectively about 2 and 4 logs less. In subcutaneously inoculated mice death ensued irregularly over a fairly wide range of dilutions, but with no dose did a majority of the mice die.

Challenge of the surviving mice at 30 days after inoculation, employing a certainly lethal intraperitoneal inoculum ($10^6$ to $10^4$ LD₅₀), revealed an absolute coincidence of the lethal and immunizing end points in the intraperitoneally inoculated series. This observation has been repeated a number of times, and the only mice surviving the challenge inocula were occasional single mice which survived infection with dilutions below that of the lethal end point. For this reason we believe that the mouse is essentially completely susceptible to intraperitoneal infection with the Karp strain. In contrast, challenge of the survivors of series inoculated by other routes than the intraperitoneal revealed a well demarcated zone of resistance beyond the original lethal end point and varying in extent with the route of the original infection. In each case, however, the challenge results permitted the calculation of an immunizing end point which did not differ significantly from the lethal end point observed in mice inoculated in parallel by the intraperitoneal route.

Weight and age of the mice did not appear to be factors of importance in influencing susceptibility. Increase in ambient temperature has been reported to effect a great increase in the resistance of mice to murine typhus (24). However, others have found the similarly induced resistance of mice to scrub typhus to be of a low order of magnitude (19), and in our own experience the warmer weather of the summer months did not interfere with the conduct of experiments.

In intraperitoneally inoculated mice the disease became apparent after from 4 to 17 days, depending on the dilution of inoculum employed, and most of the deaths occurred by the 21st day. Cardinal signs of the disease were roughening and matting of the fur, loss of appetite, weakness and reduction of activity, bloating of the abdomen and, often, the protrusion of an inaudiated stool from the rectum. Following other routes of infection, somewhat longer incubation periods were noted and eventual recovery of obviously sick mice occurred with some frequency. Mice infected intracerebrally usually manifested signs of cerebral irritation and often died during an attack of extensor spasm.

Notes on the Cotton Rat Disease.—Like mice, cotton rats were found to be more susceptible to intraperitoneal infection, less so to intracardial and intracerebral infection, and relatively resistant to subcutaneous infection. The susceptibility of these animals was also found to be influenced to some extent by size or age and to a greater extent by season. Larger rats were

*Much less detailed studies suggest that this conclusion is also valid with respect to the other three strains employed in these studies; i.e., Raub, Seerangayee, and Queensland. Certain strains of R. orientalis which have come into our hands more recently, however, appear to be less virulent for mice, in that even relatively large intraperitoneal infecting doses are not uniformly lethal and small doses ordinarily produce only an immunizing infection. The Calcutta and Gilliam strains, yolk-sac adapted, when received by us, were of this type. In both these cases, however, it has been possible to increase the virulence for mice to some degree by continued serial passage in these animals.
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more resistant than smaller ones and, in marked contrast to the behavior of mice, all rats became highly resistant during warm weather. Even in the winter, however, the susceptibility of cotton rats was not as great as that of mice.

The signs and course of the disease in cotton rats were similar to those in mice, although, especially in the case of larger rats, death occasionally was not preceded by detectable signs of illness.

Chemotherapeutic Agents. Toluidine Blue.—Most of the experiments with TB were carried out with the zinc salt produced by the National Aniline Division of the Allied Chemical and Dye Corporation, a product stated to contain 58 per cent dye. Later, through the courtesy of Mr. J. D. Nantz of that organization, a partially purified preparation was obtained, free of zinc, and containing 85 per cent dye in chloride form.

Methylene Blue.—Except for one or two preliminary experiments done with a Grüber's preparation all work with MB was carried out using the medicinal grade marketed by the Merck Company.

Para-Aminobenzoic Acid.—A PABA preparation of the Eastman Kodak Company was employed in the acid form, or, in one experiment, converted to the sodium salt by the addition of a calculated equivalent of sodium bicarbonate.

Forbisen.—Forbisen (4-4' bis-antipyrine) was supplied by Mr. W. W. Allen of the Dow Chemical Company.

Administration of Drugs.—In most instances, and unless otherwise stated, drugs were fed to mice by incorporating them in a determined concentration, usually 0.25 per cent by weight, in ground fox chow. This mixture was made into a mash by the addition of water and placed in the cages in quantities calculated to give each mouse 3 gm. by dry weight of food per day. Additional water was supplied by bottles. Unless otherwise stated, feeding was begun immediately after inoculation and continued for from 14 to 16 days.

Presentation of Results.—The routine period of observation was 21 days, and the computation of results was based upon the number of mice alive at the end of this period. End point determinations have been made by the method of Reed and Muench (25). Results otherwise have been expressed as survival ratios, which are defined as the ratio of the number of mice surviving to the number inoculated and surviving through the incubation period (deaths from intercurrent infections were thus in some part excluded). In some instances the presentation of results in the form of such ratios has been supplemented by the calculation of the average survival time of the group. In such calculations, mice surviving have been given the arbitrary survival time of 21 days.

EXPERIMENTAL

Certain facts were rapidly established during preliminary experiments which will not be described in detail. The activity of TB (0.5 per cent in the diet) against a minimal Karp strain infection in mice was found to exceed greatly that of forbisen (1.5 per cent) and of PABA (tested only in 2 per cent concentration in the diet). Toluidine blue was also shown to be active against infections in cotton rats with three additional strains of R. orientalis—the Seerangayee, Queensland, and Raub strains. Subsequently, it was observed that MB also was active against Karp strain infections in mice.

These observations made it clear that the antirickettsial activity of TB, and presumably of MB as well, could be elicited in at least one animal species other than the mouse, and against several rickettsial strains. To avoid the introduction of unnecessary variables, the work to be reported was restricted to mice infected with R. orientalis of the Karp strain.
The Quantitative Limits of Activity of Orally Administered Agents

The results of the preliminary observations warranted a more precise assay of the activity of TB and MB. Additional experiments with PABA also were performed in view of the striking results achieved with that agent in treating scrub typhus infections in gerbilles (14, 15). Important points to determine were the maximum doses of these drugs which could be administered and their relative effectiveness in such concentrations.

The Tolerance of Mice to Oral Drug Administration.--In the work with TB in murine typhus infections it was possible to feed mice concentrations of dye as great as 1.5 per cent for a 5- to 8-day feeding period. In the present work, however, drug administration was usually continued for a minimum of 14 days and the maximum concentration of dye tolerated was much lower. Although in a few experiments mice tolerated TB in 0.5 per cent concentration, consistent tolerance was observed only with concentrations of 0.25 per cent or less. Even when the dye used was the zinc-free chloride, higher concentrations proved toxic.

Essentially the same limitations were found to apply also in the case of MB. Groups of 16 mice were placed on diets containing 1.0, 0.75, and 0.5 per cent dye and observed for 16 days. At the end of this period the only surviving animals were 5 mice in the group fed 0.5 per cent dye. With a level of 0.25 per cent MB, however, little evidence of toxicity was observed, even when feeding was prolonged for 37 days.

In the case of PABA mice readily tolerated concentrations of 3.0 per cent or of 3.5 per cent when the sodium salt was employed. With concentrations of 6.0 or 9.0 per cent, however, the mice refused to feed.

Determination of the Relative Effectiveness of Drugs.--Administration of all drugs was begun immediately after infection and continued for 16 days in the case of MB and TB and for 21 days in the case of PABA. In some experiments mice were fed the same drug in different concentrations; in others 2 or more drugs were compared; in all of the experiments drug activity was measured by employing groups of mice infected with serial tenfold dilutions of infected yolk sac and comparing the lethal end points obtained in treated animals with those obtained in untreated controls.

The results of a number of experiments have been presented in Table I. The survival ratios observed afford a basis for appreciation of the protection achieved in each range of infective dose. In addition, the efficacy of the drugs has been given mathematical expression as the log LD$_{50}$ of the infectious dose against which the agents were effective. In spite of variations of considerable magnitude between the indices of effectiveness observed in different experiments for the same drug employed in the same concentration, these experiments permit certain conclusions to be drawn.

Both MB and TB possess a remarkably high order of activity when employed in 0.25 per cent concentration. The activity of MB, which is clearly greater than that of TB, was never fully gauged, since the protective effect of the dye was not overridden by the maximum infecting doses employed; i.e., about $10^8$ LD$_{50}$. The concentration of dye fed also is important. By reducing the concentration to 0.10 per cent the protective action was lowered more than a hundredfold.

Under the conditions of these experiments the activity of PABA proved to
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be slight. Experiment 6 indicates that PABA, even when administered as the sodium salt, is some 37 times less active than MB employed in 0.10 per cent concentration. The recently reported work in gerbilles, however, has emphasized the importance of maintaining adequate blood levels of PABA by supplementary parenteral injection (14, 15). Furthermore, work now in progress indicates that a greatly improved effect against scrub typhus in mice can be

### TABLE I

The Relative Effectiveness of Toluidine Blue, Methylene Blue and Para-Aminobenzoic Acid When Fed to Mice Infected with Graded Doses of Karp Strain Rickettsia

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (per cent) of diet</th>
<th>Survival ratios of mice infected with approximate number of LD$_{50}$ indicated</th>
<th>Infectious dose protected against (log LD$_{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Not infected</td>
<td>ca. 10$^9$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10/19</td>
</tr>
<tr>
<td>1</td>
<td>TB*</td>
<td>0.25</td>
<td>10/19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>0/20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>3/17</td>
</tr>
<tr>
<td>2</td>
<td>TB§</td>
<td>0.25</td>
<td>27/34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0/17</td>
</tr>
<tr>
<td>3</td>
<td>MB</td>
<td>0.25</td>
<td>19/20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>20/20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18/19</td>
<td>14/20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0/20</td>
</tr>
<tr>
<td>4</td>
<td>MB</td>
<td>0.10</td>
<td>28/30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0/24</td>
</tr>
<tr>
<td>5</td>
<td>PABA (acid)</td>
<td>3.0</td>
<td>0/19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0/8</td>
</tr>
<tr>
<td>6</td>
<td>MB Sodium PABA (acid)</td>
<td>3.5</td>
<td>0/8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0</td>
<td>1/8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0/7</td>
</tr>
</tbody>
</table>

* Toluidine blue in form of zinc salt, 58 per cent dye.

§ In each experiment the dilutions in the untreated control series were carried beyond the lethal end point, but the results observed in dilutions containing about 1 LD$_{50}$ or less have been omitted from the table.

§ Toluidine blue in form of chloride, 85 per cent dye.
achieved by supplementing the sodium salt of PABA in the diet with 1 per cent sodium PABA, carefully adjusted to pH 7.0, in drinking water supplied ad libitum (26).

Activity of Methylene Blue against Cerebral Infection.—The neural localization of the infection with \textit{R. orientalis} in man is not uncommon. To determine the effectiveness of MB against a neural infection, a single experiment was carried out which differed from those just reported only in that the mice were inoculated by the intracerebral route. The results are presented in Table II. In spite of the lesser virulence of the rickettsiae for mice when injected intracerebrally, the dye is much less effective in combatting the resulting primarily neural infection. None the less, it is clear that a significant protective effect was obtained.

<table>
<thead>
<tr>
<th>TABLE II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene Blue in Treatment of Intracerebrally Infected Mice</td>
</tr>
<tr>
<td>Dilution of Karp-infected yolk sac</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>$10^{-1.3}$</td>
</tr>
<tr>
<td>$10^{-1.1}$</td>
</tr>
<tr>
<td>$10^{-2.3}$</td>
</tr>
<tr>
<td>$10^{-2.1}$</td>
</tr>
<tr>
<td>$10^{-2.9}$</td>
</tr>
<tr>
<td>$10^{-2.7}$</td>
</tr>
<tr>
<td>Mortality end points</td>
</tr>
</tbody>
</table>

The Time and Duration of Dye Administration

The undeniably great antirickettsial activity so far demonstrated for TB and MB must be considered prophylactic rather than therapeutic, since treatment was begun promptly after infection. The effectiveness of delayed treatment and the minimum necessary period of treatment remained to be investigated.

Effectiveness of Dyes When Administration Is Delayed.—In Table III are presented the results of experiments with both TB and MB in which feeding of the respective dyes was initiated at various intervals after infection. Against infection with $10^{4.48}$ LD$_{50}$ of Karp strain rickettsiae, TB in 0.25 per cent concentration was significantly effective when first administered as late as 6 days after infection. When the dye was fed in 0.5 per cent concentration, a similar result was obtained. By the 8th day the mice, already somewhat sick, had nearly stopped eating and so ingested little or no dye. In the second experiment a larger infecting dose ($10^{4.2}$ LD$_{50}$) was employed with a consequent short-
enching of the incubation period. Since some mice were already slightly sick by the 6th day post infection, the feeding of MB begun at this time was of diminished but still significant effect. In another experiment, not included in the table, 15 mice infected with $10^5 \text{LD}_{50}$ were fed upon an MB diet for the first time on the 10th day post infection when 12 already showed signs of illness. Five of these mice survived, although all of 12 controls succumbed. Attempts to initiate treatment at still later stages in the disease, either by stomach tube or by parenteral means, were uniformly unsuccessful.

The importance of the results obtained in Experiment 2, Table III, is enhanced by supplementary observations made as to the development of the infection in untreated control animals, some of which were sacrificed at intervals after infection. By 4 days post infection the blood was clearly infectious and the infectivity titer of the liver was $10^{-4.48}$; by the 7th day the liver titer had risen to $10^{-6.3}$. It thus is evident that treatment with MB, and presumably also with TB, was effective when initiated after the development of systemic infection. These dyes accordingly may safely be said to possess therapeutic activity.

**Determination of the Minimum Necessary Period of Dye Administration.**—The period of administration of MB at the maximum tolerated level necessary to afford a maximum therapeutic result in experimental infections must be determined by the size of the infecting dose and the time of initiation of treatment. A survey of the interrelation of these factors should yield information directly bearing on the treatment of the human disease.

Of the several experiments performed, the three which are outlined in Table IV afford results which are directly comparable. The mice employed were from the same source and of approximately the same age, and the technique of dye administration, 0.25 per cent MB in
ground fox chow, was identical in all. In the first experiment mice were inoculated with a large \((10^{4.45} \text{LD}_{50})\) or a small \((10^{4.48} \text{LD}_{50})\) infecting dose and placed on treatment at once. In the second experiment a single infecting dose \((10^{4.48} \text{LD}_{50})\) was employed, but the mice were not given MB until the 3rd or the 6th day. In the third experiment treatment was withheld until the 7th day after an infecting dose which, on the basis of the control titration, was \(10^{4.78} \text{LD}_{50}\) but which, on the basis of the average survival time of the untreated control mice, behaved as a slightly smaller dose than that employed in the second experiment. In these latter two experiments supplementary observations as to the development of infection prior to treatment were made by sacrificing representative mice in groups of four and determining the presence of the infecting agent in the blood (at daily intervals) and the actual infective level of the liver (on the days that treatment was begun). At various intervals up to the 17th day post infection, groups of 20 mice in each treated series were changed to normal diet. The results are presented as the survival ratios determined on the 28th day post infection. For

**TABLE IV**

*Effect of Systematic Withdrawal of Methylene Blue from Mice Infected with Different Doses of Karp Strain Rickettsiae or Placed on Dye Treatment at Intervals after Infection*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Intraperitoneal infecting dose (\text{LD}_{50})</th>
<th>Day MB diet was begun</th>
<th>Infective titer of livers* on day MB diet was begun</th>
<th>Survival ratios of mice changed to normal diet on indicated day post inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(3 \times 10^4)</td>
<td>0</td>
<td>(0/20)</td>
<td>0/20 5/20 14/20 18/19 16/17 16/17</td>
</tr>
<tr>
<td>2</td>
<td>(3 \times 10^4)</td>
<td>0</td>
<td>(0/20)</td>
<td>5/20 3/20 4/19 12/18 15/20 17/18 17/17</td>
</tr>
<tr>
<td>3</td>
<td>(6 \times 10^4)</td>
<td>3 10^{-1}</td>
<td>0/20</td>
<td>12/19 10/20 17/19 19/19 18/20 18/20 18/20</td>
</tr>
<tr>
<td></td>
<td>(5 \times 10^4)</td>
<td>6 10^{-1.44}</td>
<td>0/20</td>
<td>2/19 12/19 15/19 16/19 17/19 18/20 18/20</td>
</tr>
<tr>
<td></td>
<td>(6 \times 10^4)</td>
<td>7 10^{-4}</td>
<td>0/20</td>
<td>0/20 4/19 7/19 12/20 14/20 14/20</td>
</tr>
</tbody>
</table>

* Observations made on pooled tissues of 4 mice selected at random for sacrifice just prior to institution of MB diet.

\(\downarrow\) Indicates time treatment with MB was initiated.

From the first experiment it appears, somewhat surprisingly, that the period of dye administration necessary to insure maximum protection against the smaller dose (from 12 to 14 days) is significantly longer than the period necessary (8 days) in the case of the dose ten thousand times more infective. From Experiments 2 and 3, in which nearly identical infecting doses were employed, it appears that the time of initiation of treatment is more important than the time of withdrawal in determining the therapeutic effectiveness of MB. Treatment continued beyond the 10th or 12th day did not result in an increased survival rate in any series even though the time of initiation of treatment varied between the 3rd and the 7th day. On the other hand the maximum survival rates achieved varied inversely with the day on which dye was first...
administered. Thus, the survival rates among mice continued on treatment to the 17th day were 97, 50, and 36 per cent when treatment was begun on the 3rd, 6th, and 7th day, respectively. The associated observations as to the infectivity of the blood (constant from the 3rd day on) and of the liver (shown in Table IV) indicate in each case that the infection was systemic at the time treatment was initiated.

The results just described suggest that the safe withdrawal of therapy depends not upon its previous duration but upon some aspect of the infection which evolves more rapidly when the infecting dose is large than when it is small. As shown in the following paper, the evolution of the infection proceeds in the face of adequate treatment. The results of an experiment not otherwise of present interest indicate that the immune reaction develops more rapidly following a larger infecting dose. A reasonable hypothesis, therefore, is that treatment may safely be discontinued after the onset of the immune reaction. In the absence of a satisfactory means of determining this point in the human infection, the practical conclusion is that unconsidered prolongation of dye therapy, at least beyond the usual duration of the disease, cannot be expected to contribute to the cure.

Parenteral Administration of Methylene Blue

The oral route of dye administration had been chosen and subsequently adhered to because of its simplicity, its remarkable effectiveness, and the great degree of tolerance exhibited by mice to dye so administered. Human treatment by this method was envisioned, although calculations on a relative weight basis suggested that the indicated human dose would be huge; i.e., over 20 gm. a day. As the result of a single observation within our own experience which was soon more than confirmed by field trials in a series of 24 cases (20), it appeared that the capacity of MB to irritate the gastrointestinal tract, as manifested by the stimulation of vomiting and diarrhea, limited the daily tolerated intake to doses of less than 3 gm. even when taken in small portions at intervals of 2 hours. This dose appeared to exert no significant influence on the course of the human disease. Our attention, therefore, was turned to investigating the effectiveness of dye administered parenterally.

Observations on the Toxicity of Parenterally Administered Methylene Blue.—Preliminary tests of the acute toxicity of MB for normal mice of 12 to 15 gm. indicated the approximate
LD₅₀ to be 2.5 mg. administered subcutaneously and slightly over 1.25 mg. given intravenously. Because of its greater convenience the subcutaneous route was selected for experimental purposes. With the maximum tolerated daily dose of approximately 1 mg. (determined for a 10- to 12-day period of administration) few tissue sloughs were observed when the site of inoculation was sufficiently varied from day to day. In these and in all subsequent experiments the amount of dye administered was contained in a single inoculum of 0.25 ml.

The possibility that susceptibility to the toxic action of the dye may increase as the infection evolves also was investigated because of its obvious pertinence to the problem of delayed therapy. In two separate experiments employing relatively large groups of infected mice, susceptibility to the toxic effects of MB was shown to increase, on the order of twofold, in the terminal 2 days of infection.

### TABLE V

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Infecting dose (LD₅₀)</th>
<th>Treatment*</th>
<th>Survival Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Route</td>
<td>Daily dose</td>
<td>Infected mice started on treatment on designated day post infection</td>
</tr>
<tr>
<td>1</td>
<td>Subcutaneous</td>
<td>1.125 mg</td>
<td>8/16 8/16 3/16 14/16</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>7.5 mg</td>
<td>24/27 22/27 17/18</td>
</tr>
<tr>
<td>2</td>
<td>Subcutaneous</td>
<td>1.0 mg</td>
<td>10/27 1/28 14/28</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>7.5 mg</td>
<td>24/27 22/27 17/18</td>
</tr>
</tbody>
</table>

*In all cases treatment was discontinued on the 12th day.

**The Therapeutic Effect of Parenterally Administered Methylene Blue.—** As a preliminary trial some of the infected mice surviving the tests of dye toxicity were carried on a daily regime of 0.625 mg. of MB given subcutaneously until the 13th day. Such treatment begun on the 3rd, 6th, and 8th days resulted in the respective survival of 17 in 36, 8 in 23, and 0 in 28 mice. Evidently some degree of therapeutic effect could be achieved by subcutaneous administration of MB.

Two experiments serve to illustrate the results of further exploration of parenteral therapy. The pertinent experimental details of these and the results are indicated in Table V. Apparently because the mice employed in Experiment 1 were 3 weeks older and correspondingly heavier than those usually used, both the tolerance to subcutaneous MB and the degree of ther-
apeutic effect achieved were greater than in any subsequent experiment. Experiment 2, in which added groups of mice were placed on oral treatment regimes for comparative purposes, yielded more typical results. The effectiveness of maximal tolerated oral dosages (7.5 mg. daily) clearly surpasses that of regimes based on subcutaneous administration of dye, even when toxic or near toxic dosages are employed. Certainly non-toxic subcutaneous dosages were of little or no therapeutic effect.

**DISCUSSION**

The evidence herewith presented conclusively demonstrates that, when incorporated in the diet in suitable concentrations, two dyes of the thionine series, toluidine blue (TB) and methylene blue (MB), possess a remarkable ability to protect mice against the usual lethal outcome of intraperitoneally induced infection with the Karp strain of *R. orientalis*. That this effectiveness is manifested in cotton rats and against three other strains of rickettsiae also has been indicated. Of the two dyes, MB possesses the greater activity and has proved capable of protecting a majority of mice infected with the maximal doses employed; i.e., about $10^3$LD$_{50}$. Furthermore, this activity is truly therapeutic, since treatment with either dye proved significantly effective when delayed until a systemic infection had become well established. These results are confirmed by similar observations with MB made independently by another group (19).

The observation that MB possesses activity against cerebral infection is of interest, since it suggests that one of the complications of the human disease, neural involvement, may be favorably influenced by treatment. Observations to be reported in the second paper of this series give this presumption additional support, since it was found that the localization, multiplication, and persistence of rickettsiae in the brain are greatly influenced by the administration of MB.

The early observations of the remarkable effectiveness of MB in treating experimental infections with *R. orientalis* led, naturally, to human trials (20) which at first were limited to oral administration. As already mentioned, the low tolerance of the human gastrointestinal tract to the irritating action of the dye limited the possible daily dose to less than 3 gm. or between 1/8 and 1/10 of the relative dose employed so successfully in mice. As the relative ineffectiveness of this small oral dose became apparent to the workers making these field trials, parenteral administration was investigated. The method employed was intravenous infusion of a dilute solution (0.1 per cent) with total daily doses of from 0.5 to 1.0 gm. Total doses given ranged from 1.7 to 7.0 gm., with an average dose of 4.2 gm. Although in 2 of 244 infusions immediate reactions occurred and in about half the cases some venous thrombosis resulted, the limiting factor to continued therapy was the marked reduction in red blood cell count and hemoglobin associated with the use of MB. Whole blood transfusions apparently were not employed to permit continued therapy. Also
of importance were the severity of the illness and the time of initiating treatment. Because of shortage of the dye, cases classed as mild when first seen were not included and treatment was reserved for moderately and severely ill patients. Study of the data suggests that the day of the disease on which the patient was first seen greatly influenced the classification of the patients into the above mentioned clinical groups, with the result that treatment appears to have been commenced much earlier in the moderately ill group (6 of 15 on the 3rd day and 10 by the 4th day) than in the severely ill patients (5 of 10 by the 6th day, one more on the 7th day, and all 10 by the 8th day). The field observers concluded that the moderately ill patients were significantly benefited by treatment but that the severely ill patients, because of the limits imposed by drug toxicity, were not able to tolerate enough dye to affect definitely the course of the disease. The mortality figures, 2 in 26 treated and 5 in 33 strictly comparable controls, were suggestive only. The available report of this experience does not contain sufficient original data to permit independent analysis but it would appear likely that a perhaps better correlation exists between time of beginning treatment and the effect observed; i.e., the earlier the better.

Results of the experimental treatment of mice with MB administered subcutaneously serve to emphasize the difficulties imposed by dye toxicity. They further suggest that the chronic toxicity of parenterally given dye is relatively greater than that of dye administered orally, since, without consideration of the proportion of oral dye actually absorbed, it is clear that tolerated oral dosages are enormously effective, whereas clearly non-toxic parenteral doses afford little protection against lethal outcome. Finally, the apparent increase in susceptibility to the acute toxic effect of the dye in the late stages of the disease carries obvious practical implications.

It is evident from the foregoing that the practical application of MB as a therapeutic agent is seriously impeded by its toxicity, although the same evidence does suggest that early treatment with tolerated doses is effective. Both the human experience (20) and careful animal studies (27) indicate that the principal toxic effect of chronic nature is red blood cell destruction, perhaps due to a hemolytic effect. That this can be successfully combatted by whole blood transfusions is suggested by observations made in the case of an infection acquired in the laboratory. First treated on the 8th day of illness, the patient was maintained on a regime of parenteral MB for 8 days with a total dose of 5.6 gm. in spite of severe drops in the hematocrit which necessitated four transfusions of whole blood. However, a chemotherapeutic agent which requires such a drastic adjuvant procedure as repeated blood transfusions leaves more than a little to be desired.

\[\text{Case seen in the New York Hospital under care of Dr. Walsh McDermott. Temperature decline began 2 days after MB treatment was started; patient was afebrile on the 13th day of illness.}\]
Although both the clinical and experimental application of parenterally administered dye have been essentially discouraging in result, the potency of MB administered orally in the experimental disease is of such a high order of magnitude that further efforts to overcome or avoid the toxic effects are indicated. One hope lies in the study of related compounds which might possess a similar degree of antirickettsial activity and less or no toxicity. A number of observations of this nature will be reported in the succeeding paper. Another possible method, not yet put to test, would be to conjugate MB in various ways in the hope of suppressing the toxic factor and not the factor responsible for its chemotherapeutic effect. That these two factors may be differentiated is suggested by the markedly differing ratios of toxicity to effectiveness seen when the oral and subcutaneous routes of administration are employed. The use of an antidote constitutes a third approach. It was recently demonstrated that sodium nitroprusside has a marked antidotal action against the acute toxicity of MB (27). We have investigated its possible activity against the chronic toxicity and have found, unfortunately, that only a minimal and questionably significant antidotal effect was achieved.

Although in the experiments reported in this paper and also in those of McLimans and Grant (19) PABA was revealed as a far less potent agent than MB, improved methods of administration have resulted in the demonstration of a much greater effect (26). This improvement was apparently due to better maintenance of blood levels. Also, clinical trials of orally administered PABA in human cases of scrub typhus in Burma have given very encouraging results (16). In this paper it is not proposed to discuss the mode of action of the drugs examined but it may be stated that existing evidence suggests that the two agents, MB and PABA, operate by different mechanisms. The possibility of using these agents in combination, i.e. supplementing the activity of PABA with non-toxic doses of MB, presents itself and is under investigation with, so far, encouraging results.

CONCLUSIONS

Methylene blue (MB) and toluidine blue (TB) when administered in maximum tolerated oral doses to mice and to cotton rats are highly effective in preventing the usual lethal outcome of intraperitoneally induced infections with R. orientalis. This activity is manifest even when dye administration is delayed until a systemic infection has been well established. Methylene blue is also effective in cerebral infections in mice.

The toxicity of MB, however, limits parenteral (subcutaneous) administration of the dye to dosage levels which are much less effective than the maximum tolerated oral levels. The inability of mice to tolerate an adequately effective parenteral dose of MB suggests that the properties of the dye responsible for its
toxicity may be separated from those upon which its antirickettsial effect
depends.

The relationship between the response of mice to oral treatment with MB
and such factors as the size of the infecting dose and the times of initiation and
of withdrawal of treatment may be summarized as follows:
1. With a constant infecting dose, the time of initiation of treatment largely
determines the degree of therapeutic effect.
2. The interval after infection beyond which further treatment does not
increase the survival rate depends not upon the previous duration of treatment
but upon the size of the infecting dose. Paradoxically, treatment can be
discontinued sooner after a massive infecting dose than after a smaller one.

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