*Brit. J. Pharmacol.* (1952), 7, 455.

# THE PREVENTION OF ENCEPHALITIS DUE TO THE VIRUSES OF EASTERN EQUINE ENCEPHALOMYELITIS AND LOUPING-ILL: EXPERIMENTS WITH TRYPAN RED, MEPACRINE, AND MANY OTHER SUBSTANCES

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(Received March 10, 1952)

Cobb, Cohen, and Ney (1938) observed that intraperitoneally administered brilliant vital red and neutral red increased the resistance of mice and rabbits to various convulsant drugs (cocaine, strychnine, picrotoxin, etc.), but not to electrically-induced convulsions. Aird (1939) and Aird and Strait (1944) confirmed this and suggested that the effect was due to decreased permeability of the blood-brain barrier, since spectrophotometric determinations showed a reduction by 30–40 per cent in the amount of cocaine passing into the brain and cerebrospinal fluid of dogs and cats treated with brilliant vital red or trypan red. Aird and Strait found that the maximum effect with trypan red, a dye closely related to brilliant vital red, occurred after three or more daily doses. Hurst and Davies (1950) were able partly to confirm these observations.

Wood and Rusoff (1945) studied the effect of similar pre-treatment on the incidence of encephalitis resulting from injection of " M M " virus into mice and cotton rats. In mice, one to three daily intraperitoneal doses of trypan red conferred increasing degrees of protection against small doses of virus administered by the same route on the 4th day. A degree of protection lasted for at least 29 days after the last injection of dye. The effect in cotton rats was less definite. Trypan red did not inactivate virus *in vitro* in 36 hours at 37° C. It did not have an effect when given simultaneously with virus. Against " M M " virus injected intracerebrally preliminary treatment with trypan red was without action, but with the Lansing virus thus introduced deaths were significantly reduced ($P=0.03$). Congo red and brilliant vital red were rather less effective; trypan blue, acid fuchsin, alizarin red, superchrome violet B, neutral red, and bismarck brown ineffective. Wood and Rusoff were cautious in interpreting these results in terms of altered permeability of the blood-brain barrier.

Hammon, Aird, and Sather (1948) failed to induce significant protection against the virus of Russian spring-summer encephalitis or " M M " virus when trypan red was injected subcutaneously and virus intraperitoneally; they interpreted Wood and Rusoff's results as due to non-specific peritoneal protection afforded by the injection, prior to virus, of inert particles. Murray, Scrugham, and Foter (1949) concluded that congo red gave apparent protection against " M M " virus but that the degree of protection was not significant.

Our own observations, made at intervals during the last four years, agree largely with those of Wood and Rusoff in showing that some dyes afford partial protection against *certain* neurotropic viruses. So also do some other chemical substances of wholly dissimilar nature. Preliminary experiments with a number of infections showed that the effect was probably most marked against the arthropod-borne viruses of Eastern equine encephalomyelitis and louping-ill, and it is with these alone that the present report is concerned; a companion paper deals with the effect of various substances in other virus diseases. It is pertinent to what follows to recall briefly the course of the diseases under consideration. Injected peripherally, a small dose of virus multiplies greatly and circulates in the blood stream, without necessarily reaching the nervous tissues and causing encephalitis (Hurst, 1936). The systemic phase of infection is accompanied by few or no signs of illness and, if encephalitis does not supervene, the animal survives immune to peripheral re-injection of virus but seldom immune to intracerebral inoculation. The proportion of animals developing encephalitis is determined by several factors of which, as Sabin and Olitsky and others have demonstrated, the most important is age (see Hurst, 1950); Hurst produced evidence that one of the factors contributing to relative resistance of the older animal is the more restricted multiplication of virus and its more evanescent appearance in the blood stream, combined with a rather lower susceptibility of the central nervous system. As far as the laboratory mouse is concerned, involvement of the central nervous system is always (equine encephalomyelitis) or nearly always (louping-ill) fatal, so that for practical purposes the number of deaths indicates the number of animals in which the nervous system has been invaded.

## EXPERIMENTAL

### *The viruses and methods used in this work*

The strains of virus were those used in previous investigations (Hurst, 1936, 1950). They were titrated before experiments and a dose given which, we hoped, would lead to between 25 and 28 deaths in groups of 30 untreated mice. Replicate control groups usually suffered remarkably similar mortalities. Thus on one occasion the mortalities in 4 groups of 30 mice infected with the same virus by different operators were 29, 27, 28, and 29 in mean periods of 5.1, 5.0, 5.7, and 4.9 days respectively. Other similar observations could be cited. Nevertheless, we did not always succeed in achieving the desired mortality, which failure we are inclined to attribute to lack of control over the age of the animals rather than to the virus. As we have said, the development of encephalitis is by no means synonymous with infection of the animal, and the most important variable relating the two is age. Although we used randomized mice of uniform weight (17–18 g. when dosing first began), weight is, of course, only an approximate indication of age, since animals from small litters grow more quickly than those from large. Throughout, therefore, we impressed upon our supplier of animals that the mice must all come from small litters, but we had no means of checking that these instructions were always obeyed.

A further source of variation over the years were the different samples of virus used to initiate infection. A study of our records showed that with a virus which had been passed at rather infrequent intervals, while the preliminary titration and choice of a suitable infecting dose might ensure the desired mortality, the deaths occurred later and over a longer interval of time than with a virus passed several times in rapid succession. The mean period of survival was therefore longer. This difference in the intrinsic " virulence " of the virus apparently influenced the result of treatment with some of the weaker therapeutic agents, which often produced an effect only when tested against a less rapidly acting sample

of virus, or produced a better effect under these conditions. This fact explains much of the variation between successive tests, noted particularly in Tables V and VI. In recent work we have minimized such variation by continual return to our lyophilized " master " virus, and in many instances have repeated earlier observations to verify conclusions previously drawn. One example of the kind of observation made in this connection appears in the companion paper.

In most experiments we observed the animals for 30–35 days, that is to say for a much longer period than is necessary when no therapy is attempted. Elsewhere (Hurst, 1948; Hurst, Peters, and Melvin, 1950) we have pointed out that the effects of various remedies on experimental psittacosis and lymphogranuloma venereum can be accurately compared only if adequate time is allowed for delayed deaths to occur, and the same is true of some of the effects described below. The significance of the results was assessed with the aid of the tables of Loewenthal and Wilson (1939).

Initial doses of the various compounds were determined by preliminary toxicity tests as those which, under conditions comparable with those obtaining in the therapeutic tests, caused no mortality and also allowed the mice to gain weight as fast as, or nearly as fast as, undosed controls. In a few instances it was found that this " maximum tolerated dose " was not necessarily as effective as a smaller dose, an observation with which we were already familiar in the chemotherapy of psittacosis and lymphogranuloma venereum. On the other hand, subeffective doses of some weakly active compounds often resulted in a mortality heavier than that in untreated mice. Many drugs were administered intraperitoneally to avoid possible vagaries of absorption from the alimentary canal, and substances of very high molecular weight were injected intravenously to ensure as wide a distribution as possible; on these occasions control mice received a comparable volume of water intraperitoneally or intravenously, although *ad hoc* tests on several occasions revealed no difference in mortality between mice given no treatment and those receiving water intraperitoneally or intravenously in the amounts used. Where twice-daily dosing is mentioned in this paper, it should be understood that only single doses were given on Saturdays and Sundays.

*Preliminary tests with trypan red*

We stained mice with four daily intraperitoneal doses of 1 mg. trypan red (C.I. 438), infected them with virus on the 5th day, and continued dosing with trypan red on the 7th, 10th, 13th, and 16th days. This treatment clearly reduced mortality following intramuscular injection of equine encephalomyelitis virus; in two of three experiments the results were highly significant ($P = < 0.005$). With louping-ill no individual result attained significance ($P = 0.05$), but the trend of five experiments was unmistakable and the combined results significant. With neither virus was treatment effective against intracerebral infection.

When we administered dye intravenously instead of intraperitoneally, the mice tolerated daily doses of only 0.5 mg. At this level of dosage beneficial effects were very much less pronounced, and sometimes heavier mortalities occurred among the vitally stained than among control mice. When also the intraperitoneal dose was lowered to 0.5 mg., the protective effect largely disappeared. When given after virus the higher doses of dye produced no beneficial effect. Finally, when the " virulence " of the equine encephalomyelitis virus was exalted to a point at which recipient mice died after a mean period of less than about five days, the effect of trypan red was greatly diminished or even absent.

Histological examination of groups of 10 treated and 10 untreated mice at daily intervals after intramuscular injection of equine encephalomyelitis virus disclosed

no qualitative or topographical difference in the neural response in the two groups. At the earliest stage of nervous involvement in this experiment, 72 hours after infection, 4 of 10 control animals showed early lesions while none of 10 treated mice did so. Invasion of the nervous system thus seemed to be retarded in treated animals, with the implication perhaps that some mice which normally would have developed encephalitis at a later stage failed to do so when dosed with trypan red.

Treated mice surviving equine encephalomyelitis without showing signs of nervous involvement were usually immune to intramuscular injection of large doses of virus, but seldom resisted intracerebral injection. In this respect they resembled untreated animals which escape obvious nervous involvement, and in which there is no indication that virus has ever gained access to the central nervous system (Hurst, 1950). In other words the evidence does not suggest that trypan red acts by converting an apparent into a subclinical infection of the nervous system.

These preliminary tests demonstrated that doses of trypan red near the maximum tolerated protected from death a proportion of mice infected intramuscularly, but not intracerebrally, with equine encephalomyelitis or louping-ill. At the dosage used the effect was evident only when administration of dye preceded infection with virus. The subsequent immunity of survivors to intramuscular re-injection of virus showed that systemic infection had not been prevented. The absence of cerebral immunity in survivors, and the lack of effect of trypan red when virus was injected directly into the brain, suggested that the dye acts by preventing virus from reaching the central nervous system rather than by modifying an established nervous infection.

*Examination of other dyes, whitening agents, and suramin*

The behaviour of trypan red suggested that other vital dyes might prevent mice from dying of equine encephalomyelitis. Accordingly we tested a number of dyes of different chemical type and biological behaviour. The bis-azo bismarck brown (C.I. 331), the azine neutral red (C.I. 825), and the anthraquinone new wool blue (an experimental dye) are basic, and the triphenylmethane " Lissamine " green (C.I. 735) is a zwitterion; all these readily pass the blood-brain barrier and stain the nervous substance. The bis-azo dyes trypan red, trypan blue (C.I. 477), and pontamine sky blue (C.I. 518) and the triphenylmethane isamine blue (C.I. 710) are acidic; they colour the meninges and the choroid plexuses, but do not penetrate the barrier to an appreciable extent and leave the nervous tissues proper uncoloured; microscopically, with the first three, particles of dye may be found in capillary endothelial cells and sometimes in mesodermal cells closely applied to the walls of the capillaries.

Of these dyes only trypan red, trypan blue, and pontamine sky blue showed a clear effect against equine encephalomyelitis. Chemically their molecules are characterized by possessing 5, 4, and 3 sulphonic acid groups respectively. It seemed possible that if these dyes were adsorbed on to membranes they might impart to these an electrical charge tending to repel particles of virus, and thus interfere at some stage with the passage of virus into the central nervous system. Were this so, the effect should vary according to the number of acid groups present. Accordingly we examined other substances containing in the molecule various numbers of —SO·H (or other acid) groups. Many of these were bis-azo dyes

fairly closely related to trypan red and trypan blue and to one another; as a class they are cotton-substantive (that is, they show a selective adsorption to cotton). Further substances chosen for test were diacyldiaminostilbene sulphonic acids (D 51627, etc.), again related closely among themselves. They also have an affinity for cotton, and are used in the textile industry as whitening agents. Finally, we examined suramin (" Antrypol "), also weakly cotton-substantive and containing six $-SO_3H$ groups.

The tests set out in Table I suggest that, within these groups of compounds, the protective action against nervous involvement with the virus of equine encephalo-

TABLE I

EFFECT OF VARIOUS DYES, WHITENING AGENTS, AND SURAMIN ON EQUINE ENCEPHALOMYELITIS IN MICE

Because of its persistence in the body suramin was given intravenously once, 24 hours before virus ($10^{-5}$). The other compounds were given intraperitoneally twice daily beginning 4 days before and ending 8 days after virus. The substances were commercial products except for those marked with an asterisk; these were purified from products manufactured by Messrs. Imperial Chemical Industries, Limited. The figures in parentheses in this and subsequent tables are the mean periods of survival of fatal cases calculated from the time of administering virus. Animals were observed for 30–35 days

| Compound | Number of acid groups in molecule | Dose in mg./18 g. | Mortalities in groups of 30 mice | | |
|---|---|---|---|---|---|
| | | | (i) | (ii) | (iii) |
| Water | — | 0.2 (c.c.) | 26 (5.1) | 30 (5.1) | 30 (4.6) |
| Trypan red (C.I. 438) | 5 | 0.5 | 19 (5.6) | 17 (12.6) | 23 (16.2) |
| Trypan blue (C.I. 477) | 4 | 0.75 | 16 (12.3) | 15 (11.5) | 18 (6.1) |
| | | 0.5 | — | — | 26 (6.8) |
| Pontamine sky blue (C.I. 518) | 3 | 2 | 19 (6.2) | — | — |
| Brilliant vital red (C.I. 456) | 3 | 1 | — | 26 (5.0) | — |
| Chlorazol violet WBS (C.I. 387)* | 3 | 0.75 | — | 28 (4.1) | — |
| Chlorazol violet R (C.I. 388)* | 3 | 1.5 | — | 23 (5.2) | — |
| Chlorazol violet N (C.I. 394)* | 2 | 1 | — | 30 (5.1) | — |
| Congo red (C.I. 370) | 2 | 2 | — | 25 (6.3) | — |
| Chlorazol corinth GWS (C.I. 375)* | 2 | 0.75 | — | 29 (6.1) | — |
| Chlorazol brown MS (C.I. 420)* | 2 | 1.5 | — | 27 (4.5) | — |
| Benzopurpurine (C.I. 448) | 2 | 2 | — | 27 (5.6) | — |
| Benzopurpurine 4B (C.I. 448)* | 2 | 1.5 | — | 27 (4.5) | — |
| D 51627* | 6 | 0.5 | — | 16 (4.6) | 26 (4.4) |
| D 51554* | 4 | 0.5 | — | 24 (4.7) | 30 (4.9) |
| D 51904* | 2 | 1.5 | — | 24 (4.8) | — |
| | | 0.5 | — | — | 30 (4.7) |
| Suramin (" Antrypol ") | 6 | 2 | — | 24 (5.0) | 25 (4.8) |
| | | 1 | — | 28 (4.8) | — |

myelitis is related to the number of acid groups in the molecule; for example, with the exception of pontamine sky blue the dyes containing two and three acid groups showed up poorly, even though in many instances the maximum tolerated dose was higher than that of trypan red or trypan blue with five and four acid groups respectively. It will be noted that in these experiments both trypan red and trypan blue usually lengthened the mean period of survival in animals ultimately dying, owing to many of the deaths occurring at a late stage after dosing had stopped. On the other hand, with D 51627 and suramin all the animals which died did so during the period in which controls succumbed.

It thus appeared that several dyes or whitening agents, possessing an affinity for cellulose and containing in the molecule a sufficient number of acid groups, were able to reduce the incidence of nervous involvement in mice infected with equine encephalomyelitis virus. The cotton-substantive drug suramin also had some action in this sense.

*The effect of mepacrine and a comparison with that of trypan red*

Although so far the facts accorded well with the hypothesis advanced above, we now made an apparently contradictory discovery. Hurst (1948) demonstrated the therapeutic activity of Nitroakridin 3582 against psittacosis and lymphogranuloma, but found it inactive against equine encephalomyelitis and louping-ill. In similar work Eaton, van Allen, and Wiener (1947) had noted that substitution of —Cl for $—NO_2$ in the Nitroakridin molecule radically modifies its properties. It seemed worth while, therefore, to examine some other acridine derivative, and we tested mepacrine, which is, of course, strongly *basic*. As will be seen later, the choice of mepacrine as the acridine derivative to be tested was a lucky one.

Table II illustrates the protective action of mepacrine against nervous involvement in equine encephalomyelitis and louping-ill. On repeated administration the most effective doses were near the toxic limit and some, but not all, groups of mice

TABLE II

EFFECT OF MEPACRINE ON EQUINE ENCEPHALOMYELITIS AND LOUPING-ILL IN MICE

Drug was given orally (*a*) twice daily beginning 4 hours before virus injected intramuscularly or 24 hours before virus injected intracerebrally, or (*b*) as a single large dose 24 hours before virus injected intramuscularly

| Virus | Infecting dilution and route of inoculation | Dosage/18 g. | Mortalities in groups of 30 mice | |
|---|---|---|---|---|
| | | | Treated | Untreated |
| Equine encephalomyelitis | $10^{-4}$ intramuscular | 2 mg. b.i.d. 3 days, 1 mg. b.i.d. 9 days | 9 (10.4) | 28 (5.4) |
| | ,, | 1 mg. b.i.d. 12 days | 20 (5.8) | |
| | ,, | 0.5 mg. b.i.d. 12 days | 26 (5.3) | |
| | $10^{-6.5}$ intracerebral | 2 mg. b.i.d. 3 days, 1 mg. b.i.d. 7 days | 15 (4.1) | 25 (2.8) |
| | $10^{-2}$ intramuscular | One dose 10 mg. | 8 (8.4) | 29 (4.5) |
| | $10^{-3}$ ,, | ,, ,, 10 ,, | 3 (6.0) | 29 (4.4) |
| | $10^{-4}$ ,, | ,, ,, 10 ,, | 3 (11.3) | 30 (4.6) |
| | $10^{-5}$ ,, | ,, ,, 10 ,, | 4 (14.7) | 25 (4.6) |
| | $10^{-6}$ ,, | ,, ,, 10 ,, | 0 | 6 (10.3) |
| Louping-ill .. | $10^{-4}$ intramuscular | 2 mg. b.i.d. 3 days, 1 mg. b.i.d. 15 days | 14 (19.2) | 23 (9.2) |
| | ,, | 1 mg. b.i.d. 18 days | 15 (11.6) | |
| | ,, | 0.5 mg. b.i.d. 18 days | 21 (10.6) | |
| | $10^{-6}$ intracerebral | 2 mg. b.i.d. 3 days, 1 mg. b.i.d. 13 days | 24 (8.2) | 28 (8.3) |
| | $10^{-2}$ intramuscular | One dose 10 mg. | 29 (9.6) | 30 (7.8) |
| | $10^{-3}$ ,, | ,, ,, 10 ,, | 27 (12.2) | 27 (9.6) |
| | $10^{-4}$ ,, | ,, ,, 10 ,, | 18 (13.9) | 26 (10.6) |
| | $10^{-5}$ ,, | ,, ,, 10 ,, | 18 (15.0) | 26 (10.6) |
| | $10^{-6}$ ,, | ,, ,, 10 ,, | 8 (14.4) | 22 (11.3) |

receiving them gained weight only slowly during the first few days; when dosing ended the mice very quickly caught up with the controls. In general, mepacrine was most effective against equine encephalomyelitis virus injected intramuscularly, less effective against this virus injected intracerebrally or louping-ill virus given intramuscularly, and only slightly active against the latter given intracerebrally. A single large dose of mepacrine was highly active against a wide range of dilutions of equine encephalomyelitis virus injected intramuscularly, but less successful against the bigger infecting doses of louping-ill virus. Unlike trypan red, the strongly basic mepacrine readily passes the blood-brain barrier, which no doubt accounts for its activity against virus subsequently injected into the brain. Comparative experiments showed that against virus injected intramuscularly mepacrine was much more active than trypan red, especially against over-virulent virus when the effect of trypan red tended to be swamped.

The striking success of mepacrine in diminishing mortality in equine encephalomyelitis of mice naturally led to some thought of what would be required of a drug to be used against this virus in the field. As stated previously, equine encephalomyelitis and louping-ill are arthropod-borne diseases, and in consequence are of seasonal occurrence when the appropriate vector is available. They are also primarily diseases of animals, and the symptoms of infection are so indefinite, unless the central nervous system has been invaded, that in practice diagnosis is not made until this event has occurred, by which time neither trypan red nor mepacrine has any power to modify the result. Treatment would, therefore, need to be prophylactic at the beginning of the season of risk, and since repeated dosing would not be feasible a drug would succeed only if it were active on administration of a single dose, and if the effect of that dose persisted for a number of weeks. While protecting the animal against invasion of the nervous system by the virus, the ideal remedy would not prevent entirely the systemic infection; thus the animal would acquire a lasting immunity to further infection by a peripheral route. These considerations, together with the hope of defining further the mechanism by which trypan red and mepacrine protect many animals against encephalitis, account for the experiments described below.

As we have seen above, a single large dose of mepacrine is effective against virus injected subsequently, and other experiments showed that it exercises a considerable though diminishing effect when administered 1, 24, or 48 hours after infection. On the other hand, it is useless when given 72 hours after infection, when nervous invasion has taken place. Under similar conditions a single dose of trypan red was only very slightly, if at all, active at any stage.

The duration of the effect of a single dose of mepacrine or trypan red is not easily determined in a short-lived and rapidly maturing animal such as the mouse, in which by the age of 6 weeks susceptibility to involvement of the central nervous system has fallen very considerably in the absence of any treatment (see Hurst, 1950). If the experimenter begins with newly-weaned mice which will tolerate only small quantities of drug, that which persists in the tissues is rapidly diluted owing to the increasing size of the mouse. If, on the other hand, he starts with animals of 16–18 g., these soon become fully-grown adults of which a relatively small percentage can be expected to develop encephalitis. In fact, we have carried out tests with both newly-weaned mice and animals of 16–18 g., increasing the

infecting dose of virus steeply with age to compensate as far as possible for the declining susceptibility to encephalitis. The results with newly-weaned mice suggested persistence of a possible slight effect of mepacrine against equine encephalomyelitis at 8 days and of trypan red at 8 and 14 days. Against louping-ill no effect was noted at either time. In the experiments with older mice we also included suramin because of its known persistence in the animal body and the apparent slight protection obtained with it (Table I). Immediately after treatment mepacrine induced significant protection against louping-ill and very highly significant protection against equine encephalomyelitis; at 3 weeks hints of an effect were seen against both viruses, and none thereafter. Trypan red afforded significant protection against equine encephalomyelitis but not against louping-ill one day after dosing, and none subsequently. On this occasion the protection afforded against equine encephalomyelitis by suramin did not attain significance and there was none against louping-ill. These experiments indicate that not even mepacrine will meet the challenge offered by conditions obtaining in the field.

The mechanism by which mepacrine produces its effect is naturally of interest. Our first indication of an action on the growth of virus came from immunity tests in mice surviving as the result of treatment. Injected intramuscularly with equine encephalomyelitis virus, untreated mice may survive either because they have escaped infection altogether, or because while they have sustained a systemic infection the nervous system has not suffered invasion. These alternatives may readily be distinguished. In the former event the mice are not immune to intramuscular re-injection of virus, although by reason of advancing age a proportion may be resistant to encephalitis and, unless blood be examined daily for the presence of virus (which we did not do), re-inoculation may in some animals lead to no apparent result; this proportion can be minimized by using very large challenge doses of highly virulent virus. In the second event the mice are solidly immune to virus re-injected intramuscularly. Examining many animals from our therapeutic tests in this way, we found that 11 of 58 (19 per cent) surviving controls died from encephalitis after re-inoculation with a 10 per cent suspension of very virulent virus. Of 364 mice subjected to miscellaneous treatments which had had little or no effect on mortality 69 died of encephalitis—exactly the same percentage as among the controls. Of 281 mice, infected with the usual dose of virus and treated with mepacrine, 112 (40 per cent) died on challenge, but among those surviving treatment with mepacrine and infection with larger doses of virus (Table II) the percentage of resistant animals was much the same as among controls. These figures suggest that, provided the initial dose of virus is small, mepacrine can prevent a certain number of animals from becoming infected.

The best index of extraneural growth of equine encephalomyelitis virus is the daily titre of virus circulating in the blood (Hurst, 1936, 1950). We therefore titrated virus daily in the pooled blood or brains of groups of mice under treatment or not, with the results assembled in Table III. The 50 per cent end-points given are not 50 per cent fatalities, but were derived on the principles outlined previously (Hurst, Peters, and Melvin, 1950). It appeared that treatment with mepacrine greatly reduced the amount of equine encephalomyelitis virus circulating in the blood stream after intramuscular injection, even on occasion to the point where virus was no longer detectable. By contrast, trypan red had comparatively little

TABLE III

DAILY TITRES OF EQUINE ENCEPHALOMYELITIS OR LOUPING-ILL VIRUS IN THE BLOOD OR BRAINS OF MICE TREATED OR UNTREATED WITH MEPACRINE OR TRYPAN RED

Equal volumes of defibrinated blood from each of 6 mice infected intramuscularly, or the whole brains of each of 4 mice infected intracerebrally, were pooled and titrated at half-logarithmic intervals intracerebrally in groups of 6 mice. The dilutions of virus giving 50 per cent end-points in the passage mice were derived as stated in the text. The $\log_{10}$ dilutions are given below as positive quantities. N.V. = no virus detected. Tr. = virus present in small amount, but the degree of extrapolation needed was too great to permit a reasonably accurate estimate of titre

| Virus and route of inoculation | Treatment | Days after infection | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| Equine encephalomyelitis $10^{-4.5}$ i.m. | None | 1.6 | 3.0 | 1.8 | Tr. | — |
| | Trypan red 5 mg. i.p. 24 hr. before virus | 1.6 | 1.7 | 1.0 | Tr. | — |
| | Trypan red 0.5 mg. i.p. b.i.d. beginning 2 days before virus | 2.3 | 1.9 | 1.3 | Tr. | — |
| | Mepacrine 10 mg. orally 4 hr. before virus | Tr. | 0.4 | N.V. | N.V. | — |
| | Mepacrine 2 mg. orally b i d. for 3 days then 1 mg. b i.d. beginning 24 hr. before virus | Tr. | 0.3 | N.V. | N.V. | — |
| Equine encephalomyelitis $10^{-4.5}$ i.m. | None | 2.2 | 2.7 | 0.6 | N.V. | — |
| | Trypan red 1 mg. i.p. daily for 4 days before virus then once every 3 days | 1.8 | 1.6 | Tr. | N.V. | — |
| Equine encephalomyelitis $10^{-5}$ i.m. | None | 0.4 | 3.0 | 2.1 | — | — |
| | Mepacrine 10 mg. orally 24 hr. before virus | N.V. | N.V. | N.V. | — | — |
| $10^{-2}$ i.m. .. .. | None | 1.9 | 2.3 | Tr. | — | — |
| | Mepacrine 10 mg. orally 24 hr. before virus | N.V. | 0.9 | Tr. | — | — |
| Equine encephalomyelitis $10^{-5.5}$ i.c. | None* | 3.1 | 8.5 | 7.0 | 7.7 | — |
| | Mepacrine 10 mg. orally 24 hr. before virus* | N.V. | 7.3 | 7.3 | N.V. | — |
| | Mepacrine 2 mg. and 1 mg. b.i.d. as above* | N.V. | 7.5 | 7.3 | N.V. | — |
| Louping-ill $10^{-4}$ i.m. | None | Tr. | 0.4 | Tr. | Tr. | — |
| | Mepacrine 10 mg. orally 24 hr. before virus | N.V. | N.V. | Tr. | Tr. | — |
| $10^{-2}$ i.m. .. .. | None | 0.5 | 0.3 | 0.1 | Tr. | — |
| | Mepacrine 10 mg. orally 24 hr. before virus | Tr. | Tr. | 0.4 | 0.5 | — |
| Louping-ill $10^{-6}$ i.c. | None† | — | 2.1 | 4.7 | 6.4 | 6.3 |
| | Mepacrine 10 mg orally 24 hr. before virus† | — | 1.8 | 3.9 | 5.1 | 6.6 |

* In duplicate groups mortalities 25, 10, and 15 out of 30 respectively. † In duplicate groups mortalities 30 and 25 out of 30 respectively.

effect on titre. Against small intramuscular inocula of louping-ill virus mepacrine exercised some inhibitory effect, but with larger infecting doses it merely retarded the rise to full titre in the blood. Against equine encephalomyelitis given intracerebrally mepacrine exerted a definite effect; with louping-ill it again only retarded full development of the virus, though this action was reflected in a slightly diminished mortality. Taking the observations as a whole, it is evident that they harmonize with the figures for mortality in the various experiments previously described. The action of mepacrine on virus is one which is seen only in the mouse; it has no parallel *in vitro*. A 2 per cent solution does not inactivate an equal volume of a $10^{-5}$ dilution of equine encephalomyelitis virus within 24 hours at room temperature.

In the literature dealing with the mechanism of poisoning of various lowly organisms with mepacrine, a number of vitamins and other substances essential to metabolism have been reported to inhibit its action. We tested some of these and other substances important metabolically to see whether they would counteract the action of mepacrine on equine encephalomyelitis virus; except for biotin the doses used were near the largest tolerated in previous toxicity tests. It can be seen from Table IV that a number of these substances tended to diminish the effect of

TABLE IV

ATTEMPTS TO INHIBIT THE ACTION OF MEPACRINE AGAINST THE VIRUS OF EQUINE ENCEPHALOMYELITIS

Mice were or were not given a single oral dose of mepacrine (10 mg./18 g.) 24 hours before virus. The other substances listed were given by various routes twice daily for 5 days beginning 4 hours before virus ($10^{-5}$) intramuscularly to groups of 20 mice

| Substances tested | Mortalities in groups of 20 mice | | | | | |
|---|---|---|---|---|---|---|
| | Without mepacrine | | | With mepacrine | | |
| | (i) | (ii) | (iii) | (i) | (ii) | (iii) |
| None .. .. .. | 15 (5.4) | 17 (5.8) | 17 (5.8) | 0 | 4 (9.0) | 5 (11.4) |
| Adenosine triphosphate, 10 mg. i.p. .. .. | 20 (5.3) | — | — | Toxic* | — | — |
| Adenosine triphosphate, 5 mg. i.p. .. .. | — | 12 (5.6) | — | — | 9 (6.4) | — |
| Ascorbic acid, 5 mg. i.v. .. | 19 (5.5) | — | — | 1 (10.0) | — | — |
| Biotin, 2.5 μg. i.v. .. | — | — | — | — | 6 (11.0) | — |
| Calcium pantothenate, 10 mg. i.p. .. .. | 19 (5.4) | 15 (5.7) | — | 1 (7.0) | 9 (6.2) | — |
| Folic acid, 0.5 mg. i.v. .. | — | 17 (5.5) | 16 (5.0) | — | 0 | 2 (10.0) |
| Glutathione, 10 mg. i.p. .. | 19 (5.4) | 19 (5.8) | — | 8 (8.0) | 11 (7.5) | — |
| 2 mg. i.p. .. .. | — | 19 (5.6) | — | — | 8 (7.0) | — |
| Nicotinic acid, 10 mg. oral | 19 (5.7) | 15 (6.2) | — | 5 (11.9) | 6 (8.3) | — |
| Pyridoxine, 10 mg. i.v. .. | — | — | 17 (5.4) | — | — | 10 (11.8) |
| 2 mg. i.v. .. .. | — | — | — | — | — | 6 (9.8) |
| Riboflavine, 10 mg. oral .. | 19 (5.3) | 18 (5.7) | — | 4 (11.8) | 5 (8.0) | — |
| "Synkavit" (Roche), 10 mg. oral .. .. .. | — | 16 (6.0) | 18 (5.3) | — | 1 (11.1) | 6 (12.4) |
| Thiamine, 1 mg. i.v. .. | 20 (5.7) | 15 (5.7) | — | 5 (8.4) | 5 (6.2) | — |
| None .. .. .. | 16 (5.7) | 15 (5.6) | — | — | 5 (10.6) | — |

* Animals given mepacrine combined with the larger dose of adenosine triphosphate all died.

the drug; they were so dissimilar in nature, however, that it is doubtful whether any useful conclusions can be reached concerning the site or mode of action of mepacrine. Folic acid may have slightly enhanced the therapeutic activity of mepacrine.

A further series of experiments attempted to determine whether or not electrical charge is important in contributing to the effect with mepacrine or with trypan red. If it is, it seemed possible that simultaneous exhibition of both drugs might destroy the effect due to either. Accordingly, we administered single doses of mepacrine orally and trypan red intraperitoneally, in stoichiometric proportions, four hours before infecting mice with equine encephalomyelitis virus. On two occasions the trypan red was given half an hour before mepacrine, and on two others half an hour after. In three of these experiments the effect of the combined treatment on mortality was the same as that obtained with mepacrine alone; in one experiment the mortality in the doubly medicated group was significantly higher than in the group treated only with mepacrine, though it was not nearly as high as in that receiving only trypan red (11, 2, and 25 out of 30 mice respectively—controls 28 out of 30). We cannot hold that these experiments demonstrate conclusively that the electrical charge of one drug is capable of counteracting the effect of the opposite charge of the other.

*The effect of mepacrine on louping-ill in its natural host and on equine encephalomyelitis in the chick-embryo*

We have seen that of the two diseases louping-ill is more difficult to influence beneficially than is equine encephalomyelitis. When in addition we found mepacrine to be tolerated in much smaller doses in the sheep than in the mouse, we did not begin therapeutic experiments in lambs with unrestrained optimism. The lambs were between 5 and 6 months old and their sera had been shown to be free from antibodies to louping-ill. Three received 50 mg./kg. of mepacrine on alternate days beginning 24 hours before virus, and three the same dose beginning 6 days before virus ($10^{-5}$ intramuscularly). Infections were more severe in the treated animals than in an equal number of controls; the average daily titres of virus in the blood were higher, and five of six treated animals developed encephalitis and died as against two of six controls.

Mepacrine proved equally disappointing as a therapeutic agent against equine encephalomyelitis in the chick-embryo. Applied directly to the chorio-allantoic membrane 24 hours before virus, it killed all embryos receiving 1 mg. or more and many of those receiving 0.1–1 mg. In the doses tolerated it did not protect against a single LD80 of virus. Embryos tolerated doses of 1.25 mg. given into the yolk-sac, but these also did not protect against the small dose of virus placed several hours or 24 hours later on the chorio-allantoic membrane.

*Examination of other substances tending to be stored intracellularly for a prophylactic effect in equine encephalomyelitis*

The comparable behaviour noted above prompted consideration of the characteristics shared by such apparently dissimilar compounds as trypan red (or trypan blue) and mepacrine. Both carry strong electrical charges, though these are of opposite sign, hence the differences in the behaviour of the compounds *vis-à-vis* the blood-brain barrier. Mepacrine, like trypan red, is cotton-substantive. Therapeutically, both substances possess antiprotozoal activity, mepacrine against malaria parasites, trypan red against trypanosomes. Finally, both substances tend to be stored in cells. Vital staining is, of course, the expression of this property in trypan red, whereas mepacrine is concentrated in the tissues, as opposed to the plasma, to a very high degree. This last consideration led to examination of other substances known or expected to accumulate in cells.

We chose for examination the compounds listed in Table V for the following reasons: Nos. 1–5 are antimalarials all of which produce high concentrations in the tissues. The same applies to the trypanocidal drugs Nos. 6–8 and to No. 9. No. 10 has been used to visualize the vascular endothelium (Schlegel, 1949), and a metabolite of No. 11 stains liver cells more or less permanently (Hurst, 1952). Nos. 12–14 are wool dyes, that is to say they possess a strong affinity for protein. They are not very satisfactory in use; if given intraperitoneally they cause intense local staining but do not go elsewhere in appreciable amounts, while a single intravenous injection colours the mouse's tail so deeply that usually the operator cannot find the veins for a second injection. Nos. 15–20 are pairs of closely related chemicals, one member of which does not localize in the tissues while the other does. Hogan and Eagle (1944) have discussed the behaviour of Nos. 15 and 16. The other pairs

TABLE V

EFFECT OF VARIOUS DRUGS TENDING TO LOCALIZE IN THE TISSUES ON EQUINE ENCEPHALOMYELITIS IN MICE

Single doses were given 4 or 24 hours before virus; repeated doses were begun 3 days before and ended 8–11 days after administration of virus ($10^{-5}$ or $10^{-5.5}$ intramuscularly)

| No. | Compound | Dose in mg./18 g. and route | Frequency | Mortalities in groups of 30 mice | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | (i) | (ii) | (iii) | (iv) | (v) | (vi) | (vii) |
| — | None | — | — | 28 (5.1) | 30 (4.6) | 27 (6.1) | 28 (5.4) | 22 (8.1) | 29 (4.9) | 27 (5.7) |
| — | Mepacrine | 2 oral 3 days then 1 oral | b.i.d. | 3 (15.6) | 3 (8.3) | 2 (16.0) | 4 (13.3) | 5 (10.7) | 2 (6.6) | 0 |
| 1 | 2-(*p*.Chlorphenylguanidino)-4-β-diethylaminoethylamino-6-methylpyrimidine | 2 oral | b.i.d. | — | — | — | — | — | 29 (5.4) | — |
| | | 0.5 i.p. | b.i.d. | 21 (13.4) | 29 (5.0) | — | — | — | — | — |
| 2 | Pamaquin | 0.5 oral | b.i.d. | 26 (7.2) | — | — | — | — | 30 (4.9) | 25 (5.6) |
| 3 | Chloroquin | 1 oral | b.i.d. | 22 (6.3) | 28 (5.0) | — | — | — | 30 (4.7) | 30 (5.4) |
| 4 | Proguanil ("Paludrine") | 0.25 oral | b.i.d | — | 30 (5.7) | — | — | — | — | — |
| 5 | Quinine hydrochloride | 10 oral | Once | — | — | 18 (5.6) | — | — | 27 (4.9) | 28 (5.3) |
| | | 5 oral | b.i.d. | — | — | 17 (7.9) | — | 15 (6.5) | 30 (4.4) | 28 (5.3) |
| 6 | Stilbamidine isethionate | 1 i.p. | Once | — | — | — | 29 (5.2) | 18 (6.3) | — | 27 (5.4) |
| | | 0.25 i.p. | b.i.d. | — | — | — | 27 (5.4) | 16 (5.1) | — | 24 (6.4) |
| 7 | Pentamidine isethionate | 1 i.p. | Once | — | 29 (4.1) | 19 (6.0) | — | — | — | 28 (5.4) |
| | | 0.25 i.p. | b.i.d. | — | 28 (4.5) | 21 (6.3) | — | 15 (9.0) | — | 30 (5.6) |
| | | 0.1 i.p. | b.i.d. | — | — | — | — | 16 (8.4) | — | — |
| 8 | Antrycide* | 0.1 s.c. | b.i.d | — | — | 27 (6.9) | — | — | — | — |
| | | 0.05 s.c. | b.i.d. | — | — | 25 (6.7) | — | — | — | — |
| 9 | Benadryl | 2.5 oral | Once | — | 26 (4.5) | — | 27 (5.3) | 17 (8.4) | 28 (5.1) | 28 (5.2) |
| | | 1.25 oral | b.i.d | — | 29 (4.0) | 20 (6.7) | 29 (5.3) | — | 28 (5.1) | 25 (5.2) |
| 10 | Thioflavine S | 1 i.v. | Once | — | — | — | 23 (5.7) | 17 (6.8) | — | — |
| | | 0.15 i.v. | b.i.d | — | — | 20 (7.2) | 23 (5.7) | — | — | — |
| 11 | 2:6-Diamino-anthrapyrimidine | 4 oral | Once | — | — | — | 20 (5.6) | 18 (9.5) | — | 27 (5.2) |
| | | 0.2 oral | b.i.d. | — | — | — | 29 (4.7) | — | — | — |
| | | 0.05 oral | b.i.d. | — | — | — | 22 (5.0) | — | — | — |
| 12 | Carbolan brilliant blue 2R | 1 i.v. | Once | — | — | — | 27 (4.2) | — | — | — |
| | | 1 i.p. | b.i.d. | — | — | — | 28 (4.7) | — | — | — |
| 13 | Carbolan violet 2R | 2 i.v. | Once | — | — | — | 25 (5.4) | — | — | — |
| | | 1 i.p. | b.i.d. | — | — | — | 27 (4.6) | — | — | — |
| 14 | Carbolan blue B | 2 i.v. | Once | — | — | — | 28 (4.4) | — | — | — |
| | | 1 i.p. | b.i.d | — | — | — | 29 (4.6) | — | — | — |
| 15 | Tryparsamide | 20 oral | b.i.d | — | — | — | 23 (5.3) | — | — | — |
| 16 | Phenylarsenious acid | 0.01 i.p. | b.i.d | — | — | — | 29 (5.1) | — | — | — |
| 17 | *p*-Aminophenylethylsulphone | 2.5 i.p. | b.i.d | 25 (4.0) | — | — | — | — | — | — |
| 18 | *p*-Aminophenyl β-diethylaminoethylsulphone | 2.5 i.p. | b.i.d. | 30 (4.2) | — | — | — | — | — | — |
| 19 | 2:5-Diamino-4:6-dimethylpyrimidine | 2.5 i.p. | b.i.d | — | — | — | 22 (5.0) | — | — | — |
| 20 | 2-Amino-5-diethylaminoethyl-amino-4:6-dimethylpyrimidine | 2.5 i.p. | b.i.d. | — | — | — | 26 (5.8) | — | — | — |

* Also inactive in two tests against louping-ill.

were studied by my colleague Dr. A. Spinks (personal communication), who found that introduction into the molecule of a basic side-chain led to enhanced concentration in the tissues.

With some of these substances hints of activity appeared when they were tested against a less virulent virus in the sense already explained, but little or none when the virus was highly virulent. The activity of mepacrine stood out under all conditions. With the three pairs of compounds Nos. 15–20 any hint of activity present was in the member of the pair not accumulating in the tissues. Daily titrations of the blood in animals treated with pentamidine and stilbamidine disclosed no effect of these drugs on the growth of virus. Since Table V was compiled we have examined two more substances for activity with equally unencouraging results. One was the antimalarial drug "Nivaquine A" (Société des usines chimiques Rhône-Poulenc), the other "Vasoflavine" which Moses, Emery, and Schlegel

(1951) found more effective than thioflavine S for staining vascular endothelium. "Vasoflavine" is less toxic than thioflavine and can be given in approximately three times the dose, but whether given at this dose or at the same dose as thioflavine it has no greater activity in equine encephalomyelitis.

We examined the macromolecular substances collected in Table VI partly with the idea of persistence after a single dose, partly because some possessed properties (e.g., acidity) which at the time seemed to us of possible importance, and partly to test a hypothesis concerning the mode of action of these substances—a hypothesis which we shall mention in the discussion. Nos. 21–34 were polysaccharides and Nos. 35–42 synthetic polymers; Hurst and Stacey (1950) studied the influence of

TABLE VI

EFFECT OF VARIOUS MACROMOLECULES ON EQUINE ENCEPHALOMYELITIS IN MICE

To facilitate distribution all these compounds (except mepacrine) were given intravenously under conditions similar to those in Table V

| No. | Compound | Dose in mg /18 g. | Frequency | Mortalities in groups of 30 mice | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | (i) | (ii) | (iii) | (iv) | (v) | (vi) |
| — | Water | 0.1 (c.c.) | Once or b.i.d. | 28 (5.7) | 28 (6.6) | 28 (4.8) | 30 (4.6) | 19 (6.3) | 24 (6.8) |
| — | Mepacrine | 2 for 3 days 1 for 8–11 days | b.i.d. | 0 | 1 (9.5) | 5 (8.3) | 3 (9.3) | 0 | — |
| 21 | Dextran* | 12.5 | Once | 13 (5.3) | — | 25 (5.2) | 25 (5.1) | — | — |
| 22 | Dextran sulphate | 12.5 | Once | 18 (6.4) | — | 20 (5.2) | — | — | — |
| 23 | Pneumococcus Type II polysaccharide | 6 | Once | 22 (4.5) | — | — | — | — | — |
| 24 | Pneumococcus Type V polysaccharide | 50 | Once | 14 (6.2) | 22 (7.1) | — | — | — | — |
| 25 | Tragacanth | 0.05 | b.i.d. | — | 20 (5.3) | — | — | — | — |
| 26 | "Methocel" (cellulose methyl ether) | 5 | Once | — | — | — | 25 (4.8) | 13 (7.6) | 17 (6.8) |
| 27 | "Solvitose" (starch methyl ether) | 25 | Once | — | — | — | — | 24 (6.0) | — |
| 28 | Sodium carboxymethyl ether of starch, ratio 1 : 10 | 10 | Once | 28 (5.3) | — | — | — | — | — |
| 29 | ditto, ratio 1 : 11.8 | 2 | Once | — | — | 26 (4.8) | — | — | — |
| 30 | ditto, ratio 1 : 2.2 | 2 | Once | 24 (5.1) | — | — | — | — | — |
| 31 | Sodium carboxymethyl ether of amylopectin, ratio 1 : 1.0 | 10 | Once | — | — | 24 (4.9) | — | — | — |
| 32 | Sodium carboxymethyl ether of cellulose | 1 | Once | 28 (5.3) | — | — | — | — | — |
| 33 | Sodium carboxymethyl ether of amylose, ratio 1 : 1.3 | 10 | Once | — | — | 29 (4.9) | — | — | — |
| 34 | Oxycellulose | 4 | Once | — | 24 (6.2) | — | — | — | — |
| 35 | Polyvinyl alcohol, medium viscosity | 2.5 | Once | 24 (5.2) | 14 (5.1) | — | — | — | 24 (6.8) |
| | | 2.5 | b.i.d. | 19 (5.8) | — | — | — | — | — |
| | | 1 | b.i.d. | — | 23 (5.4) | — | — | — | — |
| 36 | Polyvinyl sulphuric ester* | 5 | Once | — | 18 (7.2) | 18 (5.4) | — | 7 (8.4) | — |
| | | 0.5 | b.i.d. | — | 20 (6.5) | 22 (13.0) | — | 15 (10.2) | — |
| 37 | Polyvinyl pyrrolidone | 50 | Once | — | — | 28 (5.3) | — | — | 22 (7.4) |
| | | 20 | b.i.d. | — | — | 28 (5.9) | — | — | — |
| 38 | Ammonium polymethacrylate | 0.5 | Once | — | 16 (9.3) | 14 (5.7) | 27 (4.7) | — | 12 (8.0) |
| | | 0.05 | b.i.d. | — | 22 (7.7) | 26 (4.6) | 28 (4.1) | — | — |
| 39 | Sodium polymethacrylate | 2.5 | Once | 24 (5.2) | — | 22 (6.1) | 23 (5.4) | — | 10 (8.2) |
| | | 0.1 | b.i.d. | 23 (5.0) | — | 27 (4.5) | — | — | — |
| 40 | Polyglyceryl methacrylate | 10 | Once | 21 (14.3) | — | 29 (4.7) | — | — | 26 (7.5) |
| | | 7 | b.i.d. | 22 (8.6) | — | — | — | — | — |
| | | 5 | b.i.d. | 28 (5.3) | — | — | — | — | — |
| 41 | Acrylamide - methacrylic acid interpolymer | 5 | Once | — | 21 (7.7) | — | — | — | — |
| 42 | Polyethylene polyamine | 2 | Once | — | 23 (5.8) | — | — | — | — |
| | | 0.5 | b.i.d. | — | 24 (5.7) | — | — | — | — |
| 43 | India ink (Windsor and Newton) 1 : 10 | 0.1 (c.c.) | b.i.d. | 26 (7.1) | 24 (8.3) | — | — | — | — |
| 44 | Pure carbon (Darco G 60), 2 per cent | 0.2 (c.c.) | b.i.d. | — | — | 23 (5.8) | — | 10 (6.5) | 12 (8.0) |

* In single tests against louping-ill these compounds increased the mean period of survival but not the number of survivors.

many of these substances on haemagglutination due to influenza viruses or to Nitroakridin 3582 and outlined the method of preparation of a number of them. Kaplan, Coons, and Deane (1950) have demonstrated the remarkable persistence in the body of pneumococcal polysaccharide, and, of course, others of these compounds are known to persist for various times after injection.

Once again, administration of some of these substances appeared to protect a proportion of mice against encephalitis due to a virus of lower virulence, and on the whole the effects were perhaps less completely swamped by a more " virulent " virus than were those in Table V. Sometimes, as with India ink, development of encephalitis was not so much prevented as materially delayed. Several of the apparently active substances did not carry a strong electrical charge, and even pure carbon seemed to show an effect. In experiments not listed in the Table, there was some suggestion that a preparation of carbon containing particles mainly between 0.1 and 0.3 $\mu$ in diameter was more effective than one in which they were only 0.05 to 0.1 $\mu$, but we did not have sufficient to confirm this. We could detect no constant difference between four dextrans ranging from 43,000 to 400,000 in molecular weight, or three polyvinyl alcohols of widely different viscosity. No substance consistently showed an effect anything like comparable with that of mepacrine.

Whenever in these experiments we obtained an appreciable difference in the death rate between treated and untreated mice we tested survivors for immunity. The percentage succumbing to re-injection of virus was always about the same as in surviving controls or among the survivors of ineffective treatments. In other words we received no suggestion that the growth of virus had been wholly prevented, as it may be by mepacrine. Nevertheless, it seemed of interest to follow the daily titres of virus in the blood of animals treated with some of these compounds, in the hope of shedding light on their mode of action. Some studies in this direction are outlined in Table VII, from which it would seem that the substances in question

TABLE VII

DAILY TITRES OF EQUINE ENCEPHALOMYELITIS OR LOUPING-ILL VIRUS IN THE BLOOD OR BRAINS OF MICE TREATED WITH VARIOUS MACROMOLECULES

For experimental details see Table III

| Virus and route of inoculation | Treatment | Days after infection | | | | | Mortality in duplicate groups of 30 mice |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 3 | 5 | |
| Equine encephalomyelitis $10^{-5.5}$ i.m. | None | 0.7 | 1.7 | 2.1 | 0.3 | — | 16 (5.7) |
| | Dextran 12.5 mg. i.v. 48 and 24 hr. before virus and 24 hr. after | Tr. | 0.8 | 0.4 | 1.4 | — | 13 (6.7) |
| | Dextran sulphate as preceding | Tr. | 0.7 | 3.1 | 1.6 | — | — |
| Equine encephalomyelitis $10^{-5}$ i.m. | None | 0.4 | 3.0 | 2.1 | — | — | — |
| | Polyvinyl sulphuric ester 5 mg. i.v. 24 hr. before virus | N.V. | N.V. | 3.7 | — | — | — |
| Equine encephalomyelitis $10^{-4.5}$ i.m. | None | 2.1 | 3.3 | 0.4 | Tr. | — | 29 (5.1) |
| | Carbon, 2 per cent, 0.2 c.c. i.v. b.i.d. beginning 3 days before virus | 0.4 | 2.1 | 2.2 | 0.9 | — | 24 (6.6) |
| | Sodium polymethacrylate 2.5 mg. i.v. 24 hr. before virus | N.V. | 2.1 | 1.4 | 0.6 | — | 13 (6.5) |
| Louping-ill $10^{-3.5}$ i.m. | None | 0.1 | 0.8 | 0.4 | 0.4 | Tr. | 26 (11.7) |
| | Polyvinyl sulphuric ester as above | N.V. | 0.4 | 0.4 | 1.4 | 0.4 | 26 (12.9) |
| $10^{-5}$ i.m. | None | Tr. | 0.1 | 0.9 | 0.8 | — | 18 (13.0) |
| | Polyvinyl sulphuric ester as above | N.V. | N.V. | Tr. | Tr. | — | 15 (14.1) |

have the common property of delaying the rise in titre in the blood stream, while usually allowing it ultimately to attain a level comparable with that in untreated animals. This delay is often associated with some lowering of mortality.

*Examination of a number of acridines and other substances more or less closely related structurally to mepacrine*

Thus far it seemed that, while hints of activity against equine encephalomyelitis had been encountered among a great variety of chemical compounds, nothing nearly as effective as mepacrine had emerged from a study of many substances which on a variety of grounds had been regarded as possible competitors. Of the antimalarial drugs tested, chloroquin and pamaquin both contain the same basic side-chain as mepacrine, attached in different positions to a quinoline, instead of an acridine nucleus. The poor showing of these drugs focused attention upon the acridine nucleus, and we tested a number of acridines and one structurally rather similar compound. Some of these compounds were chosen on the basis of their activity or otherwise in antibacterial tests (Albert, 1951). They included acridine, acridone, aminacrine, proflavine, and acriflavine, while 8-hydroxyquinoline served as a conveniently available substitute for 1-hydroxyacridine. Acridine orange (C.I. 792) and safranine T (C.I. 841) happened to be in the laboratory and were included. The remainder were synthesized by my colleague, Dr. G. Swain, to resemble mepacrine closely except that the substituent group in the 9-position differed from compound to compound. They were:

2-Methoxy-6-chloro-9-methylaminoacridine acetate
2-Methoxy-6-chloro-9-diethylaminoacridine
2-Methoxy-6-chloro-9-*iso*propylaminoacridine acetate
2-Methoxy-6-chloro-9-*n*-butylaminoacridine acetate
β-Diethylaminoethyl-*p*-(2-methoxy-6-chloro-9-acridylamino)benzoate dihydrochloride
6-Chloro-9-piperidino-2-methoxyacridine.

None of these substances showed more than hints of activity.

## Discussion

There have been those who feared that any medicinal interference with the progress of a virus disease must almost necessarily involve so much disturbance of the metabolic functions of the cells of the host as to poison the treated animal while restraining the onslaught of the virus. It is well recognized that often when an animal is brought to a poor physiological state its reaction to a virus is much less vigorous than usual, and if this poor physiological state is induced by administering a drug the difference in reaction is classed as a toxic rather than a true therapeutic effect. When, as with mepacrine, the experimenter can, at the cost of at most a temporary retardation of growth, protect the great majority of animals which would otherwise develop encephalitis, he can hardly be considered unjustified in regarding his results as a true therapeutic effect; it may well be that in virus diseases an attempt to distinguish sharply between toxic and therapeutic effects is as realistic or as unrealistic as the rather analogous attempts to separate " living " from " dead " which exercised the minds of workers with viruses a generation ago. If these points be conceded, it will be permissible for the present purpose to consider others of

the effects described in this paper as therapeutic, even though relative to that of mepacrine many of them were only slight and by themselves insignificant.

Therapeutic effects against the smaller viruses are not exactly numerous, and faced with a number of apparently similar effects in the course of this work we have tried to find some underlying unity among them. We need not dwell upon the obvious fact that unity cannot be established on the basis of chemical or physico-chemical properties common to the very heterogeneous compounds producing the effects. We may say at once that the only unity we have been able to discern is that all the substances clearly exhibiting activity are taken up by cells, and especially by reticulo-endothelial cells. Mepacrine is known to be concentrated in the spleen almost as much as in the liver; several other drugs giving hints of weak activity also concentrate in the tissues; trypan red and various macromolecular substances are ingested by cells of the reticulo-endothelium, where with suitable techniques some of them may be visualized histologically; and so on. There is evidence (Hurst, 1936), which need not be summarized here, that the virus of equine encephalomyelitis may grow particularly in cells of the reticulo-endothelial system, which perhaps gives point to our present hypothesis that it was by modifying these cells that the results in intramuscularly inoculated animals were obtained.

Whether or not this hypothesis is correct, we can discern no further unity between the various observations. Taken into the reticulo-endothelial cells, the macromolecular substances we have examined in detail only delay the appearance of virus in high titre in the blood stream; it is as though the cells are at first so preoccupied in storing polymer or carbon that the virus has to wait its turn before they can devote attention to its synthesis. If this is so, it is easier to explain the delayed onset of encephalitis after injection of India ink than the diminished mortality seen with some polymers. Trypan red ingested by the reticulo-endothelial cells does not seem markedly to influence growth of virus; in this instance we may, perhaps, imagine an additional action along lines suggested earlier in this paper. Mepacrine, on the other hand, strongly inhibits growth of equine encephalomyelitis virus so that much less is available in the blood stream to invade the nervous system, itself also protected by the presence of mepacrine which has passed the blood-brain barrier. The behaviour of mepacrine appears to be very narrowly specific, in that it is not shared to any extent by fairly closely related acridines or by substances with the same side-chain attached to the structurally not very dissimilar quinoline ring. How far purely pharmacological properties are responsible for this narrow specificity we have not yet had an opportunity to investigate.

Trypan red and such polymers as we have examined do not influence mortality resulting from intracerebral injection of virus, whereas mepacrine does. We must, no doubt, attribute this success to the ease with which mepacrine passes the blood-brain barrier, after which it may be expected to exercise the same action in the cells of the nervous tissues as in other circumstances it does in those of the reticulo-endothelial system. The effect against virus injected intracerebrally is weaker, however, than that against virus given intramuscularly, in that it can be obscured if too " virulent " a sample or too large a dose of virus is used for infection; this fact is no doubt related to the different concentrations of drug attained in the spleen and in the brain, and to the indubitable great " affinity " of the virus for nervous tissue.

Although in doses near the largest tolerated mepacrine has a definite effect on louping-ill in the mouse, its action on the disease in sheep is disappointing, possibly because the dose tolerated by this species is much lower. However this may be, the requirements for a drug to be useful in the field against equine encephalomyelitis or louping-ill are, as we have explained, highly exacting, and it is quite certain that mepacrine does not meet them. Possibly, in the event of another epidemic of equine encephalomyelitis in man similar to that in Massachusetts in 1938, large and regular twice-daily doses of mepacrine as a prophylactic in those at risk would merit trial, but particular notice should be taken of the observation frequently made in the course of the present work—that subeffective doses of a compound may lead to greater rather than to lower mortality. Apart from this possibility, unless we discover a drug with the activity of mepacrine in the mouse, and at the same time persisting in the body after a single dose, the outlook is not promising for the chemotherapy of diseases, predominantly of animals, in which the first indications of sickness are signs of an established encephalomyelitis.

## SUMMARY

Mepacrine, certain bis-azo dyes, and other substances containing in the molecule many sulphonic-acid groups, and some other drugs and macromolecules are able in varying degree to protect mice from nervous involvement by equine encephalomyelitis or louping-ill virus given intramuscularly. By far the most active substance is mepacrine. The action is always prophylactic; no action is seen when the drugs are administered after the nervous infection has become established. The action of the weaker agents is obscured by the use of too " virulent " a virus (as defined in the text), but that of mepacrine is seen under all circumstances and, in equine encephalomyelitis, against large infecting doses of virus. Other acridines examined have not shown activity comparable with that of mepacrine.

The active substances all appear to have one thing in common—they are taken up by cells of the reticulo-endothelial system, which is probably the chief site of multiplication outside the nervous system of the viruses under consideration. The macromolecular substances merely delay the appearance of virus in high titre in the blood stream, and trypan red has comparatively little effect on titre. Mepacrine, however, markedly inhibits the growth of equine encephalomyelitis virus and greatly reduces the titre in the blood, even though *in vitro* it does not inactivate virus; there is thus less virus available to invade the nervous system.

Mepacrine also influences the course of events when virus is inoculated intracerebrally. This effect can no doubt be ascribed to the ease with which the drug passes the blood-brain barrier, and to its exercising the same inhibitory action within the cells of the central nervous system as it does in the reticulo-endothelium. This action is, however, weaker and may be swamped by too " virulent " a virus or too large a dose.

Possibly because mepacrine is tolerated in smaller doses in the sheep than in the mouse, and because louping-ill is less easy to influence than is equine encephalomyelitis, no beneficial effect of mepacrine was noted against the former disease in its natural host.

The exacting requirements for a drug which would be useful in the field against the arthropod-borne encephalitides are considered at some length.

Experiments with mepacrine do not suggest that it will meet those requirements.

It is a pleasure to acknowledge the help of many people in the course of this investigation. Professor M. Stacey supplied some of the polysaccharides tested, Dr. Frank Fletcher, Medical Research Director of Benger Laboratories, Limited, some dextran fractions of various molecular weights, and Dr. Franz Bergel, Messrs. Roche Products, Limited, the "Synkavit." Others of the substances tested were supplied or synthesized by our colleagues Dr. A. D. Ainley, Mr. A. F. Fern, Dr. J. M. G. Pryce, Dr. A. Spinks, Dr. G. Swain, and Mr. R. W. Tomlinson. We have also profited by advice from and discussion with Drs. D. G. Davey, J. Madinaveitia, and F. L. Rose.

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