AN ANTIVIRAL SUBSTANCE FROM PENICILLIUM FUNICULOSUM

IV. Inquiry into the Mechanism by which Helenine Exerts its Antiviral Effect

By RICHARD E. SHOPE, M.D.

(*From The Rockefeller University*)

(Received for publication 22 September 1965)

In earlier papers (1, 2) it was shown that a substance, helenine, derived from *Penicillium funiculosum*, possessed activity against Semliki Forest virus and Columbia SK encephalomyelitis virus infections in mice provided treatment was instituted within a few hours following infection. The substance also proved to be active against poliomyelitis virus infections in monkeys (3) and Eastern equine encephalomyelitis virus infections in mice (4). Evidence has been presented that, chemically, helenine is a ribonucleoprotein (5).

The exact mechanism by which helenine exerted a favorable effect against the various virus infections for which it was found active was not established, but in a paper published in 1953 (6) a possible mechanism by which it might act was discussed. This was based on the observation, made in study of its activity against Semliki Forest and SK Columbia viruses, that its effect plateaued, in that beyond a certain level increasing the dose of helenine did not improve its antiviral effectiveness. It was concluded that "the 'plateauing' would suggest that helenine acts only indirectly through some antiviral function of the host itself. The fact that very large doses or repeated doses frequently do not improve the therapeutic result obtained with smaller, optimal doses of helenine can perhaps be best explained on the basis of a 'triggering' function for helenine—a heavy pull on the trigger of a gun does not fire it any more completely or powerfully than does a small but adequate pull" (6).

Also in the same studies, the observation was made that animals saved by treatment with helenine were frequently not immune to subsequent challenge with the virus from which they had been spared. From such findings, it was concluded that "helenine therapy against minimal doses of virus exerts its effect, directly or indirectly, on the virus, destroying not only its infectivity, but its antigenicity as well." An alternative speculation, resulting from comparison with the action of viral antibody, was that this effect of helenine therapy took place at "a stage before the developing virus has acquired the capacity to elicit an immune response."

One concept of the mechanism of antiviral activity of helenine put forward in our original publication was that it possibly exerted its effect indirectly through triggering or inducing the host itself to produce a substance that was

transiently effective in preventing virus replication. Furthermore, this substance, whatever it was, either interfered with the acquisition of viral immunity or acted at a stage in virus replication prior to the time that immunizing antigen was produced. This concept of the possible mechanism of helenine antiviral activity was formulated during work carried out between 1946 and 1952 at a time when our knowledge of the mechanisms of virus replication and of interference with viral activity was not as advanced as it is today. Because so much new knowledge concerning those phases of virus activity seemingly implicated in the earlier studies has emerged since publication of the helenine work in 1953, it has seemed worthwhile to reopen these studies, and to consider the findings in the light of our newer virological knowledge.

The findings to be outlined in the present paper support and extend the concept presented earlier that the antiviral activity of helenine results not from direct action of helenine upon the virus itself but rather through an antiviral substance that it induces the treated host to elaborate. The findings furthermore extend the earlier evidence that the sparing effect of helenine in viral infections treated by it is effected without the induction of viral immunity.

Materials

Mice.—The Rockefeller University strain of Swiss mice, free of known specific pathogens, were used in the present work. They were ordinarily about 4 wk of age at the outset of individual experiments.

Virus.—The Semliki Forest virus used was the same strain as that employed in our earlier experiments (2) and was initially isolated by Smithburn and Haddow (7). It is lethally pathogenic for our mice in high titer when administered either subcutaneously or intraperitoneally. In the present experiments, infections were induced by the subcutaneous inoculation, under the loose skin of the back, of 0.5 cc of a dilution of virus containing from 10 to 1000 times the amount that regularly killed all untreated control animals. This ordinarily amounted to a 10^{-6} to 10^{-8} dilution of infected mouse brain suspended in veal infusion broth pH 7.4. Pools of brains from about 15 intracerebrally infected mice, harvested when some were dead and the remainder showed characteristic signs of infection, were prepared in 10% suspension in broth and divided among a number of screw-capped vials for storage frozen under CO_2 until ready for use. Kept in this way, the virus retained an adequate potency and a single batch was sufficient for about 6 months of experimental work. Dilution of the virus suspension to the concentration desired in an individual experiment was made in broth just prior to use and the diluted virus was kept in an ice bath during the time of its administration.

Helenine.—The helenine preparations used were prepared essentially as described for earlier work with Columbia SK encephalomyelitis virus (1). The important features of this preparation were as follows.

The mold, *Penicillium funiculosum*, was grown in stationary cultures on the surface of a medium previously described (1) in 32 oz Blake bottles. Each bottle contained 120 cc of medium and, after inoculation with the mold spores, was placed flat on its side and incubated undisturbed at room temperature (±26°C) for from 6 to 10 days. By the end of this time, a heavy fleshy pellicle of surface growth had taken place. The pellicles, together with remaining culture fluid, were poured onto a double layer of surgical gauze in a large filter funnel and the retained pellicles were squeezed almost free of fluid by manual twisting of the gauze in which they were held. Although the fluid portions of the cultures were known from earlier work (1)

to contain some helenine activity, this was of such a low order that in the present experiments it was discarded and only the pellicles were used as a source of helenine.

The pellicles were placed in a Waring Blendor and ground for about 4 min, adding 10 to 15 cc of phosphate-buffered saline (pH 7.0), containing 0.005 M magnesium sulfate, for each pellicle ground. The resulting gruel-like suspension was then centrifuged at 2000 RPM at 5°C for 30 min in an International refrigerated centrifuge. The supernatant containing most of the helenine activity was decanted for further processing. It was measured and an equal volume of chilled acetone was added to it with stirring. A copious, gummy, sticky precipitate containing all of the antiviral activity resulted. The precipitate, part of which floated, was collected by skimming and by centrifugation at 2000 RPM at 5°C for 30 min. The clear supernatant was decanted and discarded and the brown amorphous gummy precipitate was scraped from the bottom and sides of the centrifuge bottles with a spatula using small amounts of phosphate-buffered saline containing 0.005 M magnesium sulfate to facilitate its removal. Although the precipitate would eventually all suspend or dissolve in the buffered saline by triturating it with a spatula in small amounts of fluid, the process of dissolving it could be facilitated by homogenizing the precipitate with the desired amount of solvent for about 1 min in a Waring Blendor. Ordinarily, in order to achieve a 2- or 3-fold concentration in helenine activity, the precipitate was taken up in a volume of buffered saline amounting to one-half or one-third of the volume of fluid from which it had originally been precipitated by acetone. The resulting concentrated solution of the acetone precipitate was a brown turbid fluid high in helenine antiviral activity. This could be clarified without significant loss of activity by slow freezing and thawing. In practice, the turbid solution containing helenine was frozen overnight in a mechanical freezer at about −15°C. It was subsequently placed in an incubator at 37°C and thawed without agitation. A clear amber fluid containing a flocculent precipitate resulted. This was centrifuged at 2000 RPM at 5°C for 30 min. The clear or slightly opalescent amber-colored supernatant which contained essentially all of the helenine activity was saved and the amorphous precipitate was discarded. It is this clear solution, prepared as just described, that has been used as helenine in the present experiments.

Such solutions of helenine are stable, so far as antiviral activity is concerned, for periods of at least as long as 3 months, if stored frozen in screw-capped vials in a mechanical freezer at about −15°C. In practice, we ordinarily distribute each freshly prepared batch of helenine in ½ oz vials to be stored frozen until needed for use. For reasons that are not presently apparent, freezing at the temperature of solid CO_2, it has been found, is not a satisfactory method for preserving the full activity of helenine. This is contrary to a statement made earlier (6).

Methods of Tests

In previous work (1, 2), study of the effectiveness of helenine in viral infections was limited largely to tests of its capacity to act therapeutically and little or no consideration was given to its ability prophylactically to modify viral activity. In view of the fact that earlier work had indicated that the activity of helenine might result from its stimulation of the host to produce an antiviral material, it seemed possible that its mechanism of action might be more clearly defined if its prophylactic effectiveness were to be studied. The present experiments deal largely with the prophylactic activity of helenine, its action when given prior to the administration of virus. In these experiments, helenine has been routinely injected intraperitoneally and the virus by the subcutaneous route. In each experiment, the animals have been kept under observation for 14 days since we know from experience that all deaths from Semliki Forest virus infection will occur within that interval. At the end of this period of observation, the results were tabulated and the animals either sacrificed and discarded or were tested

for the acquisition of immunity by challenge with the same dosage of virus that they had previously survived.

From the standpoint of tabulating the results, the findings with helenine have been complicated by their variability. In those experiments in which helenine was given at a time to achieve an optimal effect, it has seemed best to record the results on the basis of the actual number of survivors. However, in those experiments in which the timing of the administration of helenine was less favorable and in which only prolongation of survival time, without survivors, was achieved, some other means of recording results had to be resorted to. A comparison of the average survival times, in days, of groups of treated animals, with the average survival time of untreated control animals, was

TABLE I

Prophylactic Effect of Helenine Prepared from Cultures of Various Colony Isolates of Penicillium funiculosum and Failure of Surviving Mice to Develop Immunity

Helenine from culture No.*	Initial infection with Semliki Forest virus subcutaneously		Challenge infection with Semliki Forest virus subcutaneously‡
	Survival index	Survivors	Survivors
BC 17–5	3.7	39/39§	1/39
R 5–4	3.1	11/15	0/11
G 28–4	3.3	5/7	1/5
BC 64–4	3.7	7/7	0/7
R 5–5	3.6	6/7	1/6
Controls, average days survived = 3.8		0/18	

* Administered intraperitoneally 24 hr before subcutaneous inoculation with virus.

‡ Surviving mice challenged 2 wk after initial infection with same dose of virus.

§ $\frac{\text{Surviving mice}}{\text{No. of mice in group}}$

achieved by dividing the value for the treated groups by the corresponding value for the control group. The quotient of this division is a value that enables estimation of the therapeutic efficacy of a substance under test, and will be referred to as the survival index (SI). In the present paper, in appraising the antiviral efficacy of helenine, the results will be recorded both on the basis of the actual number of survivors as well as the survival index.

Mice that survived their initial infection with Semliki Forest virus for 14 days and that were then challenged to test for the acquisition of immunity were injected subcutaneously with the same dose of virus they had previously survived. Since, as will be shown later, mice almost never withstood rechallenge, the results will be recorded only on the basis of survivors.

Prophylactic Effect of Helenine against Semliki Forest Virus

Several samples of helenine prepared from cultures of *P. funiculosum* derived from individual colony isolates of the mold were tested for their capacity to protect mice against Semliki Forest virus when a single injection preceded the

virus infection by 24 hr. As shown by the results given in Table I, helenine saved 68 out of the 75 mice, to which it was given prophylactically, from a dose of Semliki Forest virus that killed all of 18 control mice in an average of just under 4 days. Of the 68 mice saved by helenine, only 3 survived infection 2 wk later

TABLE II

Prophylactic Effectiveness of Helenine against Semliki Forest Virus Infection in Mice

Time of intraperitoneal administration of helenine	Initial infection with Semliki Forest virus subcutaneously		Challenge infection with Semliki Forest virus subcutaneously*
	Survival index	Survivors	Survivors
4 days before infection	1.3	1/27‡	0/1
3 " " "	2.2	9/25	0/7
2 " " "	4.0	25/27	0/22
1 day " "	3.8	25/29	0/25
12 hr " "	3.8	25/29	0/25
6 " " "	3.3	20/29	0/20
3 " " "	2.1	1/12	0/1
2 " " "	1.8	0/22	
1 " " "	1.7	0/12	
1 hr after infection	1.8	1/15	1/1
2 " " "	1.5	0/15	
3 " " "	1.4	0/12	
4 " " "	1.6	1/10	1/1
6 " " "	1.1	0/7	
12 " " "	1.2	0/7	
18 " " "	1.1	0/7	
24 " " "	1.1	0/7	
Controls, average days survived = 3.4		0/39	

* Surviving mice challenged 2 wk after initial infection with same dose of virus.

‡ $\frac{\text{Surviving mice}}{\text{No. of mice in group}}$

when inoculated with the same dose of virus from which they had initially been spared by helenine. This experiment demonstrated that helenine exerts a marked prophylactic effect against Semliki Forest virus infection and that the mechanism by which it exerts its antiviral activity is one which achieves its effect before viral immunity is acquired.

Influence of Time of Administration on Antiviral Effectiveness of Helenine

In order to determine the period of time preceding infection during which helenine might be effective in protecting mice from death by Semliki Forest

virus, an experiment in which it was administered at various periods before and after virus infection was conducted. The results are recorded in Table II.

As shown by the findings outlined in this table, helenine exerts very little antiviral effect when a period of 4 days elapses between its administration and virus infection. The animals in this group survived somewhat longer than the untreated controls but all except one out of the 27 in the group succumbed. When 3 days intervened between helenine administration and infection with virus, the treated animals lived on an average of over twice as long as the controls and 9 out of the 25 in the group survived. It was apparent from this result that considerable antiviral effect persisted for at least 3 days after helenine administration. Helenine administered 2 days, 1 day, and 12 hr prior to virus infection exerted a maximal antiviral effect and most of the treated mice survived. So far as could be told from these results, the helenine effect persisted in its full strength for a period extending between 12 and 48 hr after its administration. This constituted a plateau of full effectiveness of approximately 36 hr duration beginning 12 hr after administration.

Helenine given 6 hr before virus still exerted a rather marked antiviral effect as evidenced by the survival of about two-thirds of the mice in this group. However its effectiveness diminished rapidly after this and when it preceded infection by only 3 hr, it no longer saved the lives of most of the mice in the group though it still doubled their survival time. Among the mice treated with helenine at intervals from 2 hr before infection to 4 hr after infection, there were essentially no survivors although all groups showed a significant increase in survival time as compared with the untreated controls. Helenine given 6 hr or longer after infection was completely without antiviral effect in the present experiment. It should perhaps be pointed out that the dose of Semliki Forest virus used in testing the antiviral efficacy of helenine in this experiment was a particularly potent one as evidenced by its killing all of the 39 control mice in an average of 3.4 days. Had the infectivity test been a less rigorous one, there would undoubtedly have been animals surviving in some of those groups which in the present experiment exhibited only prolongation of life. Even with this relatively severe infectivity test, however, the results shown in Table II indicate clearly that a certain rather definite period of time must intervene after helenine administration for the development of effective antiviral action. The indication is strongly apparent from these results that helenine itself is not the substance directly responsible for the antiviral effect under study. Rather the ultimate material responsible would appear to be something generated by the host itself which reaches full effectiveness between 6 and 12 hr after helenine administration and then persists for about 36 hr. This material, whatever its nature may be, begins to decrease sometime between 2 and 3 days after its induction by helenine and has largely disappeared by 4 days.

As in the earlier experiment, all of the survivors in the present experiment that had received helenine prophylactically succumbed when later challenged

on the 14th day with a dose of virus from which they had been initially spared. Both of the 2 mice that survived from among the groups treated with helenine after infection survived the challenge dose of virus. This observation is probably of doubtful significance since experience with larger numbers of animals surviving in similar experiments has indicated that animals saved by helenine are usually still fully susceptible to virus infection regardless of whether the helenine responsible for sparing them initially was administered before or shortly after virus infection.

The orientation of the time of effectiveness of helenine with the time of administration of virus, as has been done, does not, in all likelihood, accurately represent the true period of time required for the induction of the antiviral substance by the host because it does not take into account the exact point in the viral infection cycle at which the material acts. Study of the time of first appearance of detectable Semliki Forest virus in various organs of mice infected subcutaneously with a dose of virus the same as that ordinarily employed in the helenine experiments has shown that a period of between 7 and 9 hr elapses between inoculation and the first appearance of detectable virus. In such experiments, approximately 5% tissue suspensions of thymus, lung, liver, spleen, kidney, and brain prepared from pairs of mice killed 1, 2, 3, 4, 5, 7, 9, 10, 11, 13, and 15 hr after subcutaneous infection were tested for the presence of virus by mouse inoculation. No virus was detectable by this procedure for the first 7 hr after inoculation. At the 9th hr after inoculation, virus was present, in small amounts as judged by the prolonged survival of mice receiving the organ suspensions, in the lungs, liver, and spleen, but not in the thymus, kidney, or brains. From the 10th hr onward, virus was present in all organs tested as well as in the brain.

Assuming from these findings that virus given subcutaneously, in the dosage under study, has gone through one complete replicative cycle sometime between 7 and 9 hr after administration and that mice saved after this period should therefore be immune to subsequent infection with the virus, it must be concluded, since helenine-spared mice are ordinarily not immune, that helenine acts at a point in viral replication sometime prior to the 9th hr postinoculation. The mechanism through which the antiviral substance induced in mice by helenine might act to interfere with the development of virus and the relationship of this point of action to the timing of the period of effectiveness of helenine will be discussed later.

Attempted Exhaustion of the Helenine Effect

With the acquisition of evidence that helenine exerted its effect by inducing the host to which it was administered to produce an antiviral substance, it seemed worthwhile to try to determine whether this capacity of the host could be exhausted by repeated injections of helenine.

To do this, injections of helenine were spaced at 2- or 3-day intervals, the one succeeding

the previous one just when the full effectiveness of the preceding helenine injection, to judge from the findings recorded in Table II, should be near its peak or beginning to wane. In an initial experiment, individual groups of mice were given from 1 to 7 injections of helenine intraperitoneally at these intervals and the last injection in each instance was administered 24 hr before the animals were inoculated with Semliki Forest virus subcutaneously at a 10^{-8} dilution of infected mouse brain.

The results of this first experiment were irregular in that they failed to yield an end point indicating complete exhaustion of the capacity of the host to react

TABLE III

An Attempt to Exhaust the Helenine Effect by Multiple Repeated Injections

Helenine intraperitoneally at 2- or 3-day intervals	Infection with Semliki Forest virus subcutaneously	
	Dilutions of infected mouse brain injected in 0.5 cc dosage	
	10^{-8}	10^{-7}
No. of injections*	Survivors	Survivors
9		0/14
7	3/8‡	0/14
6	4/8	
5	2/7	3/13
4	2/7	
3	3/7	
2	4/6	
1	5/7	12/14
Controls, untreated	0/7	0/15

* Last injection of helenine in each group 24 hr before infection with virus.

‡ $\frac{\text{Surviving mice}}{\text{No. of mice in group}}$

favorably to a prophylactic injection of helenine given 24 hr prior to infection. As shown in the second column of Table III, a few of the mice that had had from 2 to 6 injections of helenine preceding the last prophylactic dose withstood a 10^{-8} dilution of virus. This indicated that, even though multiple preceding injections of helenine may have diminished the ability of certain of the mice to respond to the final injection of helenine, some remained capable of responding sufficiently to protect them from a fatal outcome with the dilution of virus employed to challenge them. Since it seemed possible that a stiffer virus challenge in the experiment might have resulted in a more definitive demonstration of exhaustion of the capacity to respond to helenine, a second experiment was conducted with the view in mind of challenging these animals with a greater concentration of virus.

In this second experiment, one group of animals received 9, a second group 7, and a third group 5, injections of helenine spaced at 2- or 3-day intervals, and in each group the final injection of helenine was administered 24 hr before infection. A fourth group of mice received only a single injection of helenine 24 hr before infection. Virus challenge in this experiment was with a 10^{-7} dilution of infected mouse brain, a dose of virus ten times greater than had been employed in the first experiment.

As shown in the third column of Table III, all of the mice in the groups receiving either 6 or 8 injections prior to the final prophylactic dose of helenine died when challenged with the 10^{-7} dilution of virus. In like manner, 10 of the 13 mice in the group receiving four injections of helenine prior to the final

TABLE IV

An Attempt to Demonstrate the Acquisition of Immunity to the Helenine Effect

Helenine intraperitoneally		Initial infection with Semliki Forest virus subcutaneously		Challenge infection with Semliki Forest virus subcutaneously*
No. of injections	Interval before infection	Survival index	Survivors	Survivors
1	24 hr	2.5	7/7‡	0/7
3	2, 3, and 4 wk	0.92	0/14	
4	2, 3, and 4 wk and 24 hr	2.41	12/13	0/12
Controls, average days survived = 5.6			0/7	

* Surviving mice challenged 2 wk after initial infection with same dose of virus.

‡ $\frac{\text{Surviving mice}}{\text{No. of mice in group}}$

prophylactic dose also succumbed. In contrast, only 2 of the 14 mice in the group receiving but a single injection of helenine 24 hr before inoculation with virus died. All of 15 control mice in the experiment succumbed. As in the preceding experiments, surviving mice developed no immunity as a result of their initial exposure to virus and were susceptible when rechallenged 14 days later with the same dose of virus that they had earlier withstood.

It seems clear that the results recorded in Table III indicate that the prolonged administration of helenine at 2- or 3-day intervals markedly weakens, and in some instances exhausts, the capacity of the mouse to respond favorably to a prophylactic dose of helenine given 24 hr before virus infection. These findings further support the view that helenine does not act directly on the virus but rather exerts its antiviral effect by inducing the host to elaborate a material which is responsible for the antiviral effect. This material, whatever its character, seemingly does not exist in the host in unlimited amount as evidenced by the failure of the host to continue its elaboration indefinitely when repeatedly

stimulated by helenine injections spaced at intervals favorable to its continuous elaboration.

Attempted Demonstration of Immunity to the Helenine Effect

In order to test the possibility that the antiviral material induced in mice might be antigenic, two groups of mice were given three intraperitoneal injections of helenine spaced at weekly intervals 2, 3, and 4 wk prior to infection subcutaneously with Semliki Forest virus. One of these groups, together with another group of animals, were injected intraperitoneally with helenine 24 hr before inoculation with virus.

As shown in Table IV, the mice that had had three injections of helenine at weekly intervals and then a prophylactic injection 24 hr before infection reacted to the infection just as did the group that received only the prophylactic injection of helenine and 12 of the 13 mice in this group survived. All 14 of the mice in the group that had had three weekly injections of helenine terminated 2 wk prior to infection and no prophylactic dose, died in roughly the same average number of days as did the controls. The outcome of this experiment indicated that mice in which the antiviral substance had been induced by helenine three times at weekly intervals did not become refractory to its effect as a result of these previous exposures. The indication was that the antiviral substance, whatever its character, was not antigenic for the host in which it was induced under the conditions of this experiment.

DISCUSSION

The findings in the present experiments have supported the tentative conclusions drawn from those of earlier work (6) that the antiviral effect of helenine is not due to a direct action of helenine itself but rather results indirectly through the stimulation by it of some antiviral function of the host itself. Evidence for this conclusion is supplied, so far as the present experiments are concerned, by the finding that a definite period of time must elapse between the administration of helenine to a host and the acquisition by that host of full resistance to viral infection. Were the antiviral effect a direct action of helenine itself, it would be anticipated that its greatest activity would be expressed within a short time after administration when the concentration of helenine in the host was presumably greatest and that it would then diminish.

It might perhaps be postulated that the observed lapse of time necessary for the host to acquire full protection against a virus, following helenine administration, corresponded to the period required for the host to convert, through some metabolic process, inactive helenine into a product possessing antiviral activity. Such a postulation would be easier to support were it not for the fact that the antiviral activity, once established in the host, persists in apparently full effectiveness for at least 36 hr. It would be very difficult to think that a metabolite of helenine would be maintained fully effective for so long. Furthermore,

if the antiviral effect of helenine resulted directly from a metabolic product derived from it, it would be anticipated that a dose response to it would be demonstrable.

The simplest and most readily supportable interpretation of the findings that have been described is that helenine induces the host to produce an antiviral substance and that this induction requires time to reach its maximum effectiveness. In the case of the virus employed in the present experiments, and at the virus dosages studied, the time lapse to assure complete protection of over half the animals, must be approximately 6 hr between helenine administration and virus inoculation. The antiviral substance reaches its height, to judge from its ability to spare the lives of virus-inoculated mice, in approximately 12 hr. It maintains its high level of activity until at least 48 hr after helenine administration and then gradually diminishes until by 96 hr it has largely disappeared. Its maximum activity is thus manifested during a plateau period of 36 hr between 12 and 48 hr after helenine administration.

A finding that has been very constant in the experiments described has been the failure of mice, that were spared by helenine, to develop immunity. Almost uniformly animals saved as a result of helenine administration have been subsequently fully susceptible when later challenged with the same dose of virus from which they had been spared. This observation confirms that made in earlier work (6) which at the time was interpreted as indicating either that helenine therapy acted, directly or indirectly, upon the virus, destroying not only its infectivity but its antigenicity as well, or that its effect occurred during a stage before the developing virus had acquired the capacity to elicit an immune response. Much more is known now than then about virus replication and the finding that helenine-spared mice are still susceptible can be definitely invoked as evidence that the action of the antiviral substance induced by helenine occurs at a stage in viral nucleic acid replication prior to that in which antigenic viral protein is produced. What is not made evident by the present experiments is whether the antiviral substance acts very early and prevents penetration of virus into cells in which it can develop or later at one of the stages of viral nucleic acid replication before antigenic viral protein is produced.

When one considers the possible sites of action, in the virus replication cycle, of the antiviral substance induced by helenine, it becomes apparent that the findings recorded in Table II in this paper should all be increased by from 1 to 7 hr, depending on the point in the cycle at which this activity expresses itself. Since infective virus first becomes detectable in small amounts in animals inoculated subcutaneously, in about 9 hr after inoculation, it is apparent that by this time the virus has gone through at least one complete replicative cycle and that mice saved at such time should therefore be immune to later virus challenge. Since helenine-spared mice are not immune to subsequent infection, it must be assumed that the helenine-induced antiviral substance acts at a

time when a total cycle of virus replication has not yet been completed. Thus, if the antiviral substance acted at a point in virus replication just prior to its completion, the period of time required for the host to generate fully effective viral protection after helenine administration could be as long as 13 hr, i.e., the 7 hr elapsing between virus inoculation and the time just before infective virus became first detectable plus the 6 hr, shown from the results in Table II, that must elapse between helenine administration and the acquisition by the host of near-full viral protection. If, on the other hand, the antiviral substance acts to prevent virus penetration of susceptible cells, then its induction to almost full effectiveness within the host would be expected to be but an hour or two longer than the 6 hr indicated by the findings recorded in Table II. In either case, the time required for helenine to induce maximal protection against the fatal infection of mice by Semliki Forest virus should be calculated as from 1 to 7 hr longer than that shown in Table II, depending upon the stage in viral infection at which the helenine-induced antiviral substance actually exerts its life-sparing effect. In like manner, calculation of the period during which its activity persists at a high level of effectiveness would have to be altered correspondingly.

The nature of the helenine-induced antiviral substance cannot be determined from the findings reported. Of the known reactions that can be induced in a host to protect it against the deleterious effects of virus infection, the best studied is the interferon reaction of Isaacs and Lindenmann (8). Interferon is a substance elaborated by virus-infected cells which possesses definite chemical and physical properties dependent upon the host cell producing it. It modifies the cells of the species of host in which it is induced in such a manner as transiently to preclude the normal developmental cycle or the production of pathologic effect of various viruses in these cells. Interferon, responsible for this antiviral effect, is released from the cells producing it in a fully active form. More recently, Isaacs, Cox, and Rotem (9) have found that nucleic acids other than those of viral origin can stimulate cells to produce interferon. Also Kleinschmidt, Cline, and Murphy (10) have attributed the activity of the antiviral agent, statolon, to its capacity to induce interferon production in hosts treated with it. This observation is especially pertinent to our own studies with helenine because, though the chemical nature of statolon and helenine as published (11, 5) are quite different, the in vivo activities of the two materials against virus infections are remarkably similar. Whether, as in the case of statolon (10), the antiviral activity induced by helenine also represents an interferon or interferon-like effect is being studied by Dr. Michael Rytel of the Cornell University Medical College, and his findings will form the basis for a later paper.

At the time that the initial studies with helenine were carried out, Isaacs and Lindenmann had not yet made their pioneering studies of interferon so that

no basis as yet existed for comparison of the two antiviral activities. Even now the technical approaches used in the study of helenine and interferon have been so different that there is little basis for comparing the results obtained. The work with interferon has been done largely in embryonating eggs or cell culture and relatively little has been conducted in the intact host. Helenine studies, on the other hand, aside from those currently being carried out by Rytel, have dealt almost entirely with the antiviral activity of the substance in the intact host. Strangely enough, these two approaches have resulted in divergent findings so that aside from the fact that both interferon and helenine interfere with the progress of virus infections, the remaining findings have few facets that can be accurately compared. Thus though the period required for the induction of interferon, as tested in cell culture, by materials administered to intact hosts is known, little attention has been paid to the timing of the acquisition of full protection against viral infection by the host itself. Also though diligent search of the literature has been made, I have been unable to find a single record of any study in which animals saved by interferon have been subsequently tested for immunity to the same virus from which they were spared. Thus, in this respect, there is no knowing whether interferon, like the antiviral substance induced by helenine, spares the lives of virus-infected animals without their acquisition of immunity to the virus from which they were saved. If the antiviral substance induced by helenine is indeed interferon, the prolonged period during which its effect lasts (up to between 48 and 72 hr) as compared with the much briefer periods of its known persistence in the blood stream following stimulation by virus administration, would suggest its depot storage somewhere in the host long after it disappears from the blood stream. It is because of this complete lack of grounds for comparison between the known properties of interferon and the known activities of the helenine-induced antiviral substance that experiments with helenine, designed to study its capacity to induce interferon, have been initiated by Dr. Rytel.

The finding that the prophylactic effect of helenine can be weakened or exhausted by repeated administration at 2- or 3-day intervals furnishes further evidence that the antiviral effect of helenine results not by its direct action on the virus but rather indirectly through some material that the injected host is induced to elaborate. This material seemingly either exists preformed in only limited amounts or is capable of elaboration by the host in only limited amounts under the stimulus of helenine. Its exhaustion in the experiments under discussion was not accompanied by any evident deleterious effect on the host, for the mice, even those that received as many as nine injections of helenine, appeared lively and grossly normal prior to infection with virus.

The failure to induce immunity to the antiviral substance, repeatedly induced by injections of helenine at weekly intervals, was evidenced by the continued prophylactic effectiveness of helenine in mice infected after such treatment.

Under the experimental conditions prevailing in the experiments described, the antiviral substance, whatever its character, seemingly was not antigenic for the host in which it was induced by helenine. This would suggest that it was either not protein in character or if protein, was one native to the host in which induced.

SUMMARY

1. Helenine injected intraperitoneally 24 hr prior to a regularly fatal dose of Semliki Forest virus saves most of the mice to which it is administered.

2. Mice saved by helenine develop no viral immunity and regularly succumb when rechallenged 2 wk later with the same dose of virus from which they were originally saved.

3. The time during which helenine is optimally effective in protecting mice from death by Semliki Forest virus covers a period of approximately 36 hr beginning after about 12 hr and extending to 48 hr before virus infection. When periods of less than 12 hr, or more than 48 hr, elapse between the time of helenine administration and virus inoculation, its protective effectiveness diminishes progressively.

4. Repeated injections of helenine at 2- or 3-day intervals, if continued long enough, exhaust the capacity of a host to respond favorably to helenine administered 24 hr before virus inoculation.

5. Helenine injections at intervals of 4, 3, and 2 wk before its administration 24 hr prior to infection do not decrease the effectiveness of this final dose in protecting mice from fatal infection by the virus.

The experimental results here reported indicate that, as suggested by the findings of earlier work, helenine does not act directly as an antiviral substance, but instead exerts its effect through some substance that it induces the host to elaborate. The nature of this induced antiviral substance is as yet unknown though, to judge from the failure of spared mice to acquire viral immunity, it appears to act at a stage in viral replication prior to that at which antigenic viral protein is produced.

The findings with helenine and those thus far reported for interferon afford no factual basis for judging the relationship of the two, if any.

BIBLIOGRAPHY

1. Shope, R. E., An antiviral substance from *Penicillium funiculosum*. 1. Effect upon infection in mice with swine influenza virus and Columbia SK encephalomyelitis virus, *J. Exp. Med.*, 1953, **97**, 601.
2. Shope, R. E., An antiviral substance from *Penicillium funiculosum*. II. Effect of helenine upon infection in mice with Semliki Forest virus, *J. Exp. Med.*, 1953, **97**, 627.
3. Cochran, K. W., and Francis, T., Jr., Antiviral action of helenine on experimental poliomyelitis, *Proc. Soc. Exp. Biol. and Med.*, 1956, **92**, 230.

4. Shope, R. E., unpublished data.
5. Lewis, U. J., Rickes, E. L., McClelland, L., and Brink, N G., Purification and characterization of the antiviral agent, helenine, *J. Am. Chem. Soc.*, 1959, **81,** 4115.
6. Shope, R. E., An antiviral substance from *Penicillium funiculosum.* III. General properties and characteristics of helenine, *J. Exp. Med.*, 1953, **97,** 639.
7. Smithburn, K. C., and Haddow, A. J., Semliki Forest virus. I. Isolation and pathogenic properties, *J. Immunol.*, 1944, **49,** 141.
8. Isaacs, A., and Lindenmann, J., Virus interference. I. The interferon, *Proc. Roy. Soc. (London), Ser. B,* 1957, **147,** 258.
9. Isaacs, A., Cox, R. A., and Rotem, Z., Foreign nucleic acids as the stimulus to make interferon, *Lancet,* 1963, **2,** 113.
10. Kleinschmidt, W. J., Cline, J. C., and Murphy, E. B., Interferon production induced by statolon, *Proc. Nat. Acad. Sc.*, 1964, **52,** 741.
11. Kleinschmidt, W. J., and Probst, G. W., The nature of statolon, an antiviral agent, *Antibiot. Chemotheraphy,* 1962, **12,** 298.