STUDIES ON EASTERN EQUINE ENCEPHALOMYELITIS

VI. Facilitation of Infection in the Mouse*

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Fixed strains of neurotropic viruses are characterized by relative inability to enter the central nervous system after peripheral inoculation. In previous communications (1) it was emphasized that a fixed strain of equine encephalomyelitis virus had very weak invasive powers when injected subcutaneously, intramuscularly, or intravenously. The incidence of infection after intramuscular inoculation could be sharply increased by the injection of 50 per cent glycerine intraperitoneally in adequate amounts (1). The present paper attempts to determine under what conditions this phenomenon is operative, whether it can be produced by other agents, and to gain some suggestions as to the mechanism involved.

Methods

In the present study the same strain of fixed E.E. virus used previously was employed. The material used for inoculations was infected mouse brain tissue, emulsified 1:10 in sterile buffered salt solution, and centrifuged to throw down the coarse particles. This stock suspension (10^0) or appropriate decimal dilution was used. For propagation of the virus albino mice of the Rockefeller Institute strain, the same as that employed in previous studies, was employed. For experimental animals, Swiss mice, purchased in uniform lots from a single dealer, were used. These animals were all 12 to 13 weeks of age, and had an average weight of about 22 gm. Their susceptibility to the virus determined by titration was no less than that of the strain used for previous publications. In all experiments in which virus was given intramuscularly, a standard dose of 0.25 cc. of suitable dilution was employed.

Facilitation Effect

In the original experiments leading to the observation of the facilitation effect, the 50 per cent glycerine was injected intraperitoneally. However, the same effect could be produced if glycerine were given intramuscularly, as illustrated in the first experiment in Table I. The dosage given is of considerable importance. It was previously shown (1 *a*) that too small doses had no effect whatever on the action of the virus. The optimal dose, as shown

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in Experiment 2, Table I, was found to be 0.30 to 0.35 cc. of a 50 per cent solution. Larger amounts resulted in a too great immediate mortality, that is, the injected animals died within a few hours, indicated in the tables by the symbol X.

It was of interest to determine the maximal dilution of virus with which this effect could be obtained. As seen in Table I, Experiment 3, in the control group only a small percentage died after the undiluted (or 10^0) suspension.

TABLE I

Experiment No.	Route of glycerine administration	Dose of glycerine	Dilution of virus suspension	Results
		cc.		
1	Intraperitoneal	0.25	10^0	3, 3, 3, 3, 4, 4, 4, X, X, X, X
	Intramuscular	0.25		2, 3, 3, 3, 4, 4, 4, 0
	None—control	None		0, 0, 0, 0, 0, 0, 0, 0
2	Intramuscular	0.30	10^0	2, 3, 3, 3, 3, 3, 3, 4, 4, 5
	Intramuscular	0.40		3, 3, 3, 4, X, X, X, X, X, X
	None—control	None		4, 6, 0, 0, 0, 0, 0, 0, 0, 0
3	Intramuscular	0.30	10^0	3, 3, 3, 3, 3, 4, 0, 0, 0, 0
	None—control			3, 4, 0, 0, 0, 0, 0, 0, 0, 0
	Intramuscular		10^{-1}	2, 3, 3, 3, 3, 4, 5, 0, 0, 0
	None—control			0, 0, 0, 0, 0, 0, 0, 0, 0, 0
	Intramuscular		10^{-2}	2, 3, 3, 0, 0, 0, 0, 0, 0, 0
	None—control			N. T.

2, 3, = mouse died within 48, 72 hours after inoculation (etc.).
0 = mouse survived.
X = mouse died within a few hours after inoculation. Death not due to virus action.
N. T. = not tested.

None at all died with the next higher decimal dilution. Animals receiving glycerine showed a significant mortality rate with suspensions of 10^0 and 10^{-1}, but in the next higher dilution the mortality was low, comparable to the 10^0 control group. In quantitative terms it can therefore be said that the glycerine treatment increased the effective titer not more than 100 times.

Mice after receiving glycerine show a fairly definite picture, consisting first of restlessness, followed by a period of apathy in which they sit with markedly ruffled fur. This stage is generally succeeded by definite prostration, interrupted by convulsive jerks and twitchings and sometimes frank generalized convulsions. Some animals succumb in this period but the others gradually recover. Those that survive 18 hours are indistinguishable from normal animals until the action of the virus makes itself

manifest. Too small a dose of glycerine not leading to obvious signs, does not facilitate virus action.

When the glycerine is injected into one of the hind legs, a great edematous swelling of the affected leg occurs, in the course of ½ to 1 hour. Some measure of the changes going on within the animal was sought. Since the edema clearly indicated a redistribution of fluid, attempts were made to excise in symmetrical fashion the edematous and control legs, and by weighing determine the amount of the fluid exudations. After numerous trials, however, this method was discarded as not sufficiently accurate for use in such a small animal.

Blood Changes

Since the variations in the red blood count have yielded valuable information in numerous experimental studies on shock, large numbers of determnations were made on mice, but this method, too, was not satisfactory. Blood was drawn from the tail

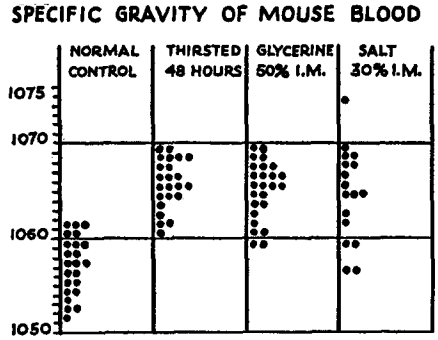

FIG. 1

veins, before and after administration of glycerine. The red count of untreated mice, given free access to water, varied between 8 and 12 million cells. After the administration of glycerine, in the majority of instances blood would not flow from the tail vein at all. At first a brachial vein was exposed and incised, under ether anesthesia, and the freely flowing blood utilized. However, comparative counts on the same animal, using blood simultaneously from the tail and from the brachial vein, showed poor correlation. Invariably the blood from the tail was more concentrated, ranging from 250,000 to 2,000,000 more red blood cells per c.mm. and having a specific gravity 0.002 to 0.007 greater than blood flowing freely from the brachial vein. Blood from the tail vein was not considered satisfactory and enumeration of red blood cells was not deemed sufficiently accurate in the mouse.

One measure of fluid change within the mouse was the determination of specific gravity of whole blood, by the falling drop technique of Barbour and Hamilton (2). The blood was taken from the brachial vein, since tail blood was not a satisfactory index. Multiple successive tests on the same animal before and after the injection of glycerine could not be performed. Consequently whole groups of mice were examined, each mouse serving for a single determination. Results are expressed in Fig. 1.

In normal mice with free access to water, the range of specific gravity varied from 1.051 to 1.061. In another group receiving 0.35 cc. of 50 per cent glycerine, intramuscularly, the blood showed a specific gravity range of 1.059 to 1.069. The examinations were carried out from ¾ hour to 3 hours after the injection, at such a time when the animals showed some symptoms of tremor or other systemic disturbance. The slight time variation following inoculation had no bearing on the result.

It is evident that concentration of the circulatory blood volume is a correlate of the action of glycerine. There are other less drastic modes, however, of producing this degree of concentration. If mice are deprived of water for 48 hours, the blood becomes more concentrated and the specific gravity increases to a range of 1060 to 1069, practically identical with the increase produced by

TABLE II

Effect of Thirst and Glycerine in Relation to Intramuscular Virus

Experiment No.	Treatment	Dilution of virus suspension	Results
1	Thirsted 48 hrs., no glycerine	10^{0}	7, 8, 8, 0, 0, 0, 0, 0, 0, 0
	Controls, no thirst or glycerine		3, 3, 4, 0, 0, 0, 0, 0, 0, 0
	Thirsted 24 hrs., plus glycerine		2, 2, 2, 3, 7, X, X, X, X
	Glycerine, but not thirsted		3, 3, 3, 3, 3, 4, 4, 5, 0, X
2	Thirsted 48 hrs., no glycerine	10^{-1}	4, 4, 0, 0, 0, 0, 0, 0, 0, 0
	Controls, no thirst or glycerine		4, 4, 7, 0, 0, 0, 0, 0, 0, 0
	Thirst 48 hrs., no glycerine	10^{0}	3, 3, 4, 4, 6, 0, 0, 0, 0, 0
	Controls, no thirst or glycerine		3, 4, 4, 4, 5, 0, 0, 0, 0, 0

Animals thirsted for 48 hours received water 14 hours after virus inoculation.

glycerine. Such thirsted mice show none of the behavior changes seen after administration of glycerine. When such thirsted animals are inoculated with virus, the rate of death is almost exactly the same as controls, although mice receiving glycerine showed the usual facilitation effect (Table II). In the first experiment it is seen that animals receiving glycerine were much more susceptible to virus than those that did not, regardless of whether the latter were thirsted or not. One group of mice was deprived of water for 24 hours and then given glycerine. In this condition of partial dehydration the glycerine produced a greater initial mortality, but those that survived the initial period succumbed to the virus with greater rapidity. A partial dehydration through deprivation of drinking water renders the facilitation effect from glycerine more striking.

In Experiment 2 of Table II further evidence is presented that thirsted animals not receiving glycerine behave no differently toward the virus than well watered controls.

Evidently the disturbance of which blood concentration is an index must be produced suddenly as by glycerine, since the same degree of hemal concentration produced gradually by thirst is ineffective in facilitating virus action.

Dehydration of the Nervous System

Since the degree of hemal concentration may be increased to identical levels by two different procedures, one of which (glycerine) facilitates virus action while the other (thirst) does not, some other differentiating point must be sought. The hypothesis was adopted that changes in the water content of the brain might be of significance.

To test this hypothesis the following procedure was adopted.

Mice were anesthetized with ether, and exsanguinated by section of the brachial arteries and then of the heart. Immediately the brain was removed, and weighed in a crucible whose weight had just been determined. The brain was then dried to constant weight in a hot air oven whose temperature ranged from 90° to 100° but did not go above the latter figure. Tissue remained in the oven at least 48 hours. The weight of the dry brain in normal controls as well as in various experimental groups, ranged generally from 0.0800 to 0.0975 gm. depending on the amount of tissue removed from the skull. The wet weight of the normal brain varied from 0.3610 to 0.4463 gm. After administration of glycerine, or, as is shown subsequently, of strongly hypertonic sodium chloride, the initial (wet) weight of the brain was sharply reduced, to as low as 0.3105 gm. After determination of the dry weight, the results were expressed in the ratio of dry to wet brain, that is, the percentage of solids.

The solids content of the brain of the normal control mice which had been allowed water *ad libitum* averaged 22.1 per cent (Fig. 2). Brains of mice deprived of all water for 48 hours and then treated identically with the controls, averaged 22.6 per cent. As seen in Fig. 2, the values are quite closely bunched and the spread not great. The animals into which 50 per cent glycerine was injected intramuscularly, exhibited a much greater spread, with a mean value of 24.8 per cent solids. The great majority of these animals showed typical symptoms of the type described above, and there was a high correlation between the severity of the symptoms, in regard to the tremors and prostration, and the degree of cerebral dehydration. Animals with a percentage solids of 26 per cent or greater undoubtedly would have succumbed had they been so allowed. Mice which proved to have a lower percentage of solids (*i.e.*, greater amount of water) would probably have survived.

Tests with Other Substances and Modes of Administration

The above evidence suggests than an acute dehydration of the brain is a differentiating factor which controls the susceptibility to virus. Thirsted mice, in spite of a high concentration of the blood, reacted identically with controls in reaction to the virus. These animals showed only a slight diminution in water content of the brain. Mice receiving glycerine, on the other hand, exhibited a

greater drop in water content, expressed in Fig. 2 as the percentage solids. As already demonstrated, these mice had a considerably enhanced susceptibility to virus.

The importance of acute dehydration could be checked in various ways. The possible local action of glycerine on the tissues at the site of inoculation could be eliminated by intravenous administration of glycerine. This is an heroic pro-

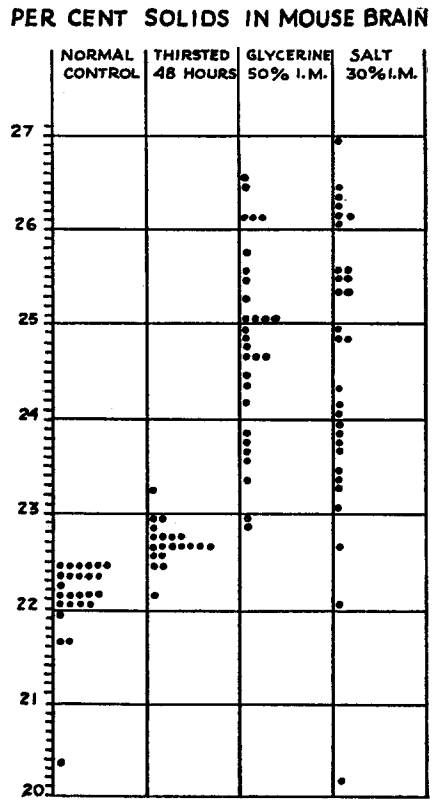

FIG. 2

cedure, resulting in immediate death of injected mice in a very high percentage of animals, even with a dose of only 0.15 cc. of 50 per cent glycerine. However, in preliminary tests, those that survived the first few hours survived indefinitely. The mice that received glycerine intravenously showed a markedly increased susceptibility to virus given intramuscularly. This is shown in Table III. The treated animals died of encephalitis not only in greater numbers than the controls, but after a shorter incubation period.

Acute dehydration of the tissues can be produced by any strongly hypertonic solution parenterally injected. Experiments were carried out with a 30

per cent solution of sodium chloride, injected in a dose of 0.30 cc. intramuscularly. Virus was given simultaneously to these and also to control animals. The injection of the salt caused a high immediate mortality, within a few hours. The survivors however all succumbed to encephalitis. In two experiments, 9 of 9 treated animals died of encephalitis with typical and unmistakable signs, while only 1 of 20 controls died. These data are presented in Table IV. Additional data are given in Table V.

The intramuscular injection of strong salt solution resulted similarly to glycerine in its effect on the specific gravity of the blood and the water content of the brain. In Figs. 1 and 2 are presented the results. The animals treated

TABLE III

Intravenous Administration of Glycerine in Relation to Intramuscular Injections of Virus

Experiment No.	Mice with glycerine	Controls
1	3, 3, 3, 3, 3, 4, 4, 4, 0	4, 0, 0, 0, 0, 0, 0, 0, 0, 0
2	2, 2, 2, 3, 3, 3, 4	3, 3, 4, 4, 5, 0, 0, 0, 0, 0

Animals dying immediately after the glycerine injection are not recorded.

TABLE IV

Intramuscular 30 Per Cent Sodium Chloride in Relation to Intramuscular Virus

Experiment No.	Dilution of virus	With salt	Controls
1	10^{0}	3, 3, 4, 4	4, 0, 0, 0, 0, 0, 0, 0, 0, 0
2	10^{-1}	3, 3, 3, 3, 4	0, 0, 0, 0, 0, 0, 0, 0, 0, 0

Animals dying immediately after the salt injection are not recorded.

with salt showed somewhat greater scatter than those receiving glycerine. Possible reasons for this are commented on in the discussion.

Profound alterations in the electrolyte content of the brain, without significant change of the total water content, may be induced by the injection of distilled water or 5 per cent glucose intraperitoneally. The latter method was extensively employed by Darrow and Yannet (3). There is a shift of electrolytes into the injected fluid, with, for practical purposes, a temporary loss of such electrolytes from the body. Preliminary studies showed that for a 22 gm. mouse, an inoculation of 4.0 cc. of either distilled water or of 5 per cent glucose was the maximum amount that could be tolerated intraperitoneally. Animals so treated showed in the course of a few hours some tremors and prostration, although the total picture was not identical with that produced by the injection of glycerine or salt.

Animals subjected to this treatment were inoculated with virus, together with suitable controls. Experiments were run using normal controls, glycerine-treated and salt-treated controls. The results are given in Table V. In both

the experiments there shown, only the groups receiving the strongly hypertonic solutions showed significant mortality. Considering the two experiments together, 22 of 27 mice treated with hypertonic solutions succumbed to virus, while of all other groups only 4 of 55 died. Determination of brain water content of mice receiving 5 per cent glucose intraperitoneally showed a strictly normal range, of 20.8 to 22.3 per cent solids, with a mean of 21.9 per cent in 10 determinations.

It is evident that the procedures accompanied by acute dehydration of the brain were correlated with enhanced susceptibility to virus action; while other

TABLE V

Comparison of Glycerine with Other Substances in Relation to Intramuscular Virus

Experiment No.	Substance injected	Place	Dose	Results
			cc.	
	Glycerine (50 per cent)	Intramuscular	0.35	3, 3, 3, 4, 4, 5, 5, 0, 0, 0
1	Glucose (5 per cent)	Intraperitoneal	4.0	0, 0, 0, 0, 0, 0, 0, 0
	Distilled water	Intraperitoneal	4.0	0, 0, 0, 0, 0, 0, 0
	None—control	—	—	6, 0, 0, 0, 0, 0, 0, 0, 0, 0
	Sodium chloride (30 per cent)	Intramuscular	0.30	3, 3, 3, 3, 3, 3, 3, 3 3, 3, 3, 3, 3, 4, 4, 0, 0
2	Glucose (5 per cent)	Intraperitoneal	4.0	0, 0, 0, 0, 0, 0, 0, 0, 0, 0
	Distilled water	Intraperitoneal	4.0	0, 0, 0, 0, 0, 0, 0, 0, 0
	None—control	—	—	3, 4, 4, 0, 0, 0, 0, 0, 0, 0, 0

In Experiment 1, undiluted virus suspension was used.

In Experiment 2, 10^{-1} dilution of virus suspension used.

Animals succumbing in a few hours to direct action of injected substances are not included in this table.

procedures wherein the brain was not dehydrated, even though profound electrolyte disturbances were present, showed no increased susceptibility to virus.

Other Modes of Virus Administration

The slight invasiveness, under normal conditions, of equine encephalomyelitis virus, does not apply to intranasal or intraocular injections. The effectiveness of these modes of inoculation has been previously described and discussed (1 *a*). The problem then arose, would the facilitation effect produced by glycerine apply to cases where virus could normally attain the nervous system quite readily.

Experiments were carried out, wherein virus suspensions in different dilutions were given into the eye or nose of control mice and of those receiving intramuscular glycerine, (0.35 cc. of 50 per cent solution). Since the controls would

ordinarily succumb, any facilitation effect would be evidenced principally in a shortened incubation period, as well as possibly a higher titration end point.

The results are seen in Table VI. The animals receiving glycerine appear almost identical with the controls. The facilitation effect is produced by glycerine when the virus is injected intramuscularly, but in cases of intranasal or intraocular injections no facilitating action can be detected.

TABLE VI

Intramuscular Glycerine in Relation to Intraocular and Intranasal Administration of Virus

Virus injected	Dilution	With glycerine	Control
Intraocularly	10^{-4}	3, 4, 4, 5, 5, 0, 0	4, 4, 4, 5, 6, 0, 0, 0
	10^{-3}	3, 3, 3, 4, 4, 6, 0	3, 3, 4, 4, 5, 5, 5, 0
	10^{-2}	3, 3, 3, 4, 4, 4, 4	3, 3, 3, 4, 4, 4, 4, X
	10^{-1}	3, 3, 4, 4, 4, 4, 4	3, 3, 4, 4, 4, 4, 4, 4
	10^{0}	N. T.	N. T.
Intranasally	10^{-4}	0, 0, 0, 0, X, X, X, X	0, 0, 0, 0, 0, 0, 0, 0
	10^{-3}	4, 7, 0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0, 0, 0
	10^{-2}	3, 4, 4, 4, 4, 0, 0, X	3, 3, 3, 3, 5, 5, 0, 0
	10^{-1}	3, 3, 4, 0, X, X, X	3, 3, 4, 4, 4, 4, 0, X
	10^{0}	N. T.	N. T.

DISCUSSION

The action of glycerine on the animal organism has been exhaustively reviewed by Deichman (4). The minimal lethal dose for mice has been worked out by Leake and Corbitt (5), whose findings are close to our own. They found that animals surviving 24 hours would survive indefinitely, which we have confirmed. No data in the literature really bear upon the cause of death from too much glycerine. The present studies indicate that excessive loss of water from the brain is a responsible factor. Animals with the most pronounced symptoms showed the most severe dehydration. A preliminary period of thirst (18 to 24 hours) rendered animals much more susceptible to glycerine, and incidentally, to the virus action following glycerine administration.

Hemoglobinuria is produced in mice by glycerine, but is not a causative factor in the phenomenon under investigation. The use of salt solution causes the same facilitation phenomenon, with the same degree of brain dehydration, but does not result in hemoglobinuria.

The use of 30 per cent sodium chloride renders most of the mice intensely thirsty. If water is not furnished until 3 to 4 hours after the injection, about 80 per cent of the animals die. Curiously enough, with water available, some of the animals show no inclination to drink, while some consume very large amounts. It is the latter which furnish the very low values in Fig. 2, and show no symptoms of shock or prostration.

Animals receiving glycerine exhibited far less thirst than the salt group. This finding is in agreement with the studies of Gilman (6) on the relative actions of sodium chloride and of urea.

The way in which the virus, intramuscularly injected, attains the nervous system, is not considered in the present paper. Sabin and Olitsky (7) have presented evidence for the mouse, while the author has considered the mechanism in the guinea pig (8). Just how the dehydration of the brain allows the virus to enter, whereas otherwise it would not, is not at present clear. The absence of facilitation when virus is instilled into the nose or injected into the eye, suggests a fundamentally different mechanism for these routes than for the intramuscular. These results are in complete harmony with the author's previous studies on herpes and pseudorabies viruses (1 *b*), and indicate a sharp difference between direct intraneural route of invasion and mediation of infection through the blood stream. The effectiveness of glycerine evidently applies only to the latter category.

SUMMARY

50 per cent glycerine injected intraperitoneally, intramuscularly, or intravenously, greatly enhances the activity of equine encephalomyelitis virus injected intramuscularly, increasing its virulence up to 100-fold. The same effect is produced by very concentrated sodium chloride. The result appears due to dehydration of the nervous system, suddenly produced. Gradual withdrawal of body fluids, produced by depriving animals of drinking water, results in sharp concentration of the blood, equal to that produced by glycerine or salt. But such deprivation of water alone does not result in significant dehydration of the brain, nor does it have any effect on virus action. The facilitation effect is not produced by drastic procedures involving shifts of electrolytes without loss of total water from the brain. Glycerine has no facilitating action when the virus is administered intranasally or intraocularly, suggesting a fundamental difference in pathogenesis between these routes and the intramuscular.

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