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THE INHIBITING EFFECT *IN VIVO* OF BETA-PHENYLSERINE ON RABIES INFECTION IN RATS

by

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INTRODUCTION

The development of effective chemotheraputic agents against viruses has been hampered by the intimacy of the virus-host cell complex. It has been difficult to find an agent which will inhibit virus propagation and not have an adverse effect on normal cells. Before the problem can be approached critically it will be necessary to be aware of the changes in the metabolism of the host when infected with a virus, so that inhibitors of metabolic pathways peculiar to the infected cells can be applied. Moreover, an understanding of the pathogenesis of the diseases must necessarily precede an investigation into the metabolic schemes involved. Our understanding of the pathogenesis of rabies is still nebulous.

Although early in the nineteenth century, Zinke and others showed that rabies virus is present in the saliva of infected dogs, and that the bite of an infected animal could transmit the virus, the route by which the virus reaches the central nervous system is still unknown. At the present time most workers believe that the virus gains access to the central nervous system by passage along the neuroaxons leading from the site of introduction of the virus. Although this is the accepted theory, some workers have reservations since most of its postulates are based on work done many years ago and believe that a reexamination of the phenomenon is in order.

Evidence for the neural transmission of rabies virus was obtained when Goodpasture (1925) showed that the injec-

tion of the virus into the masseter muscle of rabbits resulted in the spread of the virus along the fifth cranial nerve as evidenced by histological changes in the gasserian ganglion. Once the virus reached the central nervous system it spread throughout the central nervous system, to the salivary glands, and to other tissue. Johnson (1952) has stated that the virus is rarely found in the blood, spleen, liver, or lymph nodes. This would bear out the contention that the virus reaches the central nervous system by a pathway not associated with the vascular system of the animal. This was considered the case with poliomyelitis until comparatively recently when it was found that the virus could be detected in the blood stream. Burnet (1955) stated that occasionally a higher concentration of rabies virus could be found in the submaxillary gland of an infected animal than in the brain and therefore extraneural propagation may occur.

Burnet's supposition that the virus may multiply in non-nervous tissue in the intact animal may be correct, since Kissling (1958) has shown the virus may propagate in hamster kidney cells. Kanazawa (1936) reported the propagation of a rabbit-fixed strain of rabies virus in a Maitland-type culture of embryonic rabbit brain and this was substantiated by Webster and Clow (1937). A few years later Kligler and Bernkopf (1941) stated that rabies virus could be propagated only in the presence of embryonic brains, either rat or mouse brain serving equally well; adult brain would not support virus propagation.

A study of the amino acids found in the nervous tissue of newborn and adult mouse-brain revealed that tissue from newborn mice has less glutamic and aspartic acids, less gamma-aminobutyric acid, less cystine, and more taurine and alanine than does adult tissue. Small amounts of arginine, tyrosine, and alpha-aminobutyric acid were found in newborn mouse brain but were not detected in the adult tissue. (Roberts, Frankel, and Harmon, 1950) In an attempt to correlate these findings with the observations of Kligler and Bernkopf, Hosley (1955) reasoned that those compounds found in the adult mouse-brain might exert an inhibitory effect on virus propagation while those compounds found in the infant mouse-brain might stimulate virus production. Using a Maitland-type culture of infant mouse-brains he discovered that while none of the amino acids reported in the adult brain and not in the infant brain would inhibit virus propagation, tyrosine could significantly increase the yield of virus. Further studies lead to the observation that the analogue of tyrosine and phenylalanine, β-phenylserine would markedly inhibit the production of virus. Attempts to demonstrate an in vivo effect were equivocal.

The work to be presented in this study was initiated to determine whether β-phenylserine could inhibit the propagation of rabies virus in the intact animal, and if so, to characterize so far as possible, its mode of action. Such an effect would be more than a biological curiosity. The disadvantages associated with the Pasteur treatment are

well known and an effective chemoprophylactic agent would obviate the need for this long and uncomfortable treatment. Since so little is known of the basic mechanisms involved in virus-host cell interaction in the living animal, a chemical inhibitor of virus propagation in vivo would provide a system in which these could be subjected to study. Although the majority of the work reported was concerned with the prophylactic powers of β-phenylserine, an attempt was made to determine the means by which this protection was achieved.

HISTORICAL

Since antiquity, man has been familiar with the etiology of rabies. It is now well established that the deposition of the virus on a traumatized area is necessary for infection to occur. However, the method by which the virus is transported to the central nervous system is still open to question. The viruses of rabies and poliomyelitis were long considered strict neurotropic viruses, that is, they were though to multiply only in central nervous system tissue; however, Horstman (1952) showed that there is a viremia in the early stages of poliomyelitis in orally-infected monkeys, and this was also found to be the case in human infections.

When it was found that poliomyelitis virus was able to multiply in the cells of the small intestines and then to be transported to the central nervous system via the blood stream, the concept of the strict neurotropism of poliomyelitis was dealt a severe blow. Although Burnet (1955) and Field (1952) do not believe that axonal transport alone accounts for the presence of rabies virus in the central nervous system, the fact still remains that no one has shown conclusively that the virus can be detected in the vascular system of the animal.

In 1941, Bodian and Howe were able to measure the rate at which poliovirus travels in an axon. They reported that the rate of progression up the sciatic nerve in monkeys was 2.4 mm /hour and in mice 0.2 mm /hour. This work and the histopathological studies of Goodpasture (1925)

are good indications that axonal transport does occur. Field (1952) theorized that at the site at which a neurotropic virus gains entry into the body a small focus of infection is established in the nerve endings. The damaged neurons then activate an area in the central nervous system which renders this part of the motor arc more susceptible to the virus which is being carried in the blood.

The ideal antiviral chemotheraputic or chemoprophylactic agent is one which will interfere with virus propagation while not seriously affecting the host cells. A great deal of investigative work has been devoted to finding inhibitors of virus nucleic acid synthesis. A large number of inhibitors have been tested in tissue culture systems and although a few have been found to be effective, the majority are unsatisfactory. Unfortunately even the most effective inhibitors of virus multiplication in vitro do not protect against the virus in vivo (Thompson, et. al., 1950).

Tamm and his co-workers (1956) have found that the N'-B-D-ribofuranoside derivative of chlorinated benzimidazoles inhibited the multiplication of influenza virus in an in vitro culture of chick chorioallantoic membranes. However, these compounds did not affect vaccinia virus, and so they postulated that the ribofuranoside moiety of the inhibitor interfered with the production of the influenza RNA but not with the production of vaccinia DNA.

In general, inhibitors of carbohydrate metabolism have not been found to be effective in inhibiting virus synthesis. An exception to this is found in the work of Ackermann (1951) and others with sodium fluoroacetate. The intraperitoneal injection of sublethal doses of this compound increased the concentration of citrate in the mouse lung and blocked the citric acid cycle. The development of influenza virus in the mouse lung was inhibited, but no *in vitro* effect on the infectivity or hemagglutinating property of the virus could be shown. Mathews and Smith (1955) suggested that the inhibitory effect observed *in vivo* was due to the chelating activity of the accumulated citrate which deprived the virus of metals necessary for multiplication.

A greater measure of success has been attained in the use of amino acid analogues as inhibitors of virus replication. Brown and Ackermann (1951) found that DL-ethionine inhibited the growth of the Lansing strain of poliomyelitis virus grown in minced brain tissue from human embryos, and that the inhibition could be abolished by methionine. Ackermann (1952) showed that α-amino-p-methoxyphenylmethanesulfonic acid would protect mice injected with the WS strain of influenza. This protection could not be annulled by phenylalanine or tyrosine. Thompson and Wilkin (1948) reported that β-2 thienylalanine would inhibit the multiplication of vaccinia virus in Maitland-type cultures of chick embryo tissue and that this inhibition could be reversed by phenylalanine. In 1952, Brown demonstrated in tissue culture that β-2 thienylalanine

inhibition of MEF1 strain of Lansing type poliomyelitis could be annulled by phenylalanine. Morgan (1954) showed that β-2 thienylalanine could also inhibit the propagation of Theiler's GDVII mouse encephalomyelitis virus in minced mouse brain and also the Lansing strain of poliomyelitis virus, and vaccinia virus in tissue culture. Heggie and Morgan (1956) found that tryptophane and phenylalanine were essential for the proliferation of psitticosis virus in chick embryo tissue grown in vitro and that 6-methyltryptophane and β-2 thienylalanine would inhibit this propagation. In most cases where an amino acid analogue is active in preventing virus multiplication its action is specific in that the inhibition can be reversed by the appropriate natural amino acid.

In Hosley's (1955) studies of the propagation of rabies virus in Maitland-type cultures of foetal and neonatal mouse brains, phenylalanine enhanced the propagation of the virus and contrariwise β-phenylserine caused a decrease in its propagation. However, the inhibition by β-phenylserine could not be reversed with tyrosine and tyrosine alone increased the yield of virus. Although β-phenylserine has not been used as extensively as β-2 thienylalanine as an inhibitor of virus replication in vitro, it is an effective inhibitor of phenylalanine metabolism in bacteria.

Beerstecker and Shive (1946) have reported that β-phenylserine inhibits phenylalanine utilization in E. coli, Lactobacillus arabinosus 17-5, and Streptococcus

faecalis R. Others have shown that β-phenylserine inhibits the hydroxylation of phenylalanine to tyrosine and that the analogue is not converted to either phenylalanine or tyrosine in bacteria. Furthermore, it has been established that this inhibition can be nullified by increasing the concentration of phenylalanine in the system. (Malho and Malho-Lacroix, 1952)

An examination of the metabolic utilization of phenylalanine and tyrosine by three human cell strains (cervical carcinoma (Hela), normal liver, normal conjunctiva) grown in tissue culture showed that both amino acids and in all three cell lines the compounds were incorporated intact almost exclusively into protein. (Eagle, 1957) Furthermore, the cell lines were not able to convert phenylalanine to tyrosine, which indicates the inhibition which results from β-2 thienylalanine or β-phenylserine is not due to the inhibition of phenylalanine hydroxylase but to some other factor. This other factor may be the incorporation of the analogue into protein which is then rendered non-functional.

A perusal of the literature indicates that the effect of β-phenylserine or β-2 thienylalanine on the intact animal has received limited attention. Armstrong (1950) showed that the rat is incapable of converting β-phenylserine into phenylalanine or tyrosine. Younathan, et al. (1956) reported that both DL-propargylglycine and β-2 thienylalanine were capable of lowering the xanthine oxidase activity of the adult rat's liver and that this inhibition could be reversed by phenylalanine. The effect

of β-2 thienylalanine on xanthine oxidase became of especial interest after Bauer and Bradley (1956), found that when mice were infected with different neurotropic viruses the xanthine oxidase activity of the brain increased; furthermore, the extent of this increase was a characteristic of the virus employed. The role of xanthine oxidase in the formation of new virus or the relationship between β-2 thienylalanine and this enzyme has not been established as yet.

MATERIALS AND METHODS

Virus. A strain of rabies virus was isolated from a routine specimen submitted for diagnostic study to the Pasteur Institute of the University of Michigan. Before a virus pool was formed, the virus had been passed intracerebrally 5 times in Swiss albino mice (weight 10-14 grams) and two passages had been made in rats inoculated into the masseter muscle. A virus pool was prepared by injecting a 10 per cent suspension of infected mouse-brain into a number of Swiss albino mice (weight 10-14 grams). These mice were sacrificed when prostrate with rabies. The brains were removed aseptically and ground in a morter, using alumdum or sand as the abrasive. A 20 per cent suspension was prepared by the addition of distilled water. After removing coarse particles of tissue by low speed centrifugation, the supernatant fluid was distributed in screw cap vials in 1.3 ml amounts and stored at -45C until needed. In the course of these investigations it was necessary to prepare seven such virus pools. Each pool prepared by this method had a titer of approximately $1 \times 10^{-4.5}$ when injected intracerebrally into Swiss albino mice.

Virus Titrations. Titrations were carried out using serial ten-fold dilutions of pool virus, injecting six mice at each dilution level intracerebrally with 0.03 ml of virus or injecting six to ten rats at each dilution into the masseter muscle with 0.03 ml of virus. The dilutions were made in buffered gelatine saline (5.00 grams of Bacto-Gelatine in

phosphate-buffered saline) and kept in an ice bath until all the animals were inoculated. The LD_{50} endpoints were calculated by the method of Reed and Muench (1938). The death of an animal within 96 hours after injection was considered non-specific and did not enter into the calculations.

Compounds. Organic compounds used were obtained from the following sources: (All amino acids and their derivatives are in the DL-form.)

β-phenylserine, serine, Hypoxanthine, tyrosine, sodium phenylpyruvate, and phenylpropionic acid from the Nutritional Biochemicals Corp.; β-hydroxypropionic acid from the Aldrich Chem. Corp.; sodium β-glycerophosphate, and phenylglycine from the Eastman Kodak Corp.; β-2 thienylalanine from the California Institute for Biochemical Research.

Xanthine Oxidase Determinations. The method of Bauer and Bradley (1956) was used to determine the amount of xanthine oxidase in rat brains. Albino rats of approximately the same age and weight as the test animals ($\pm$ 5 grams) were selected. At the proper times the animals were stunned by a sharp blow on the thoracic vertebrae and then decapitated. The brains were removed and placed in a chopped-ice bath. After weighing, the brains were ground in a mortar with sand (1 gram of sand per gram of brain tissue) and borate buffer (pH 9.0) added to give a 20% suspension. Large particles were removed by low speed centrifugation and the supernatant

fluid was poured into a test tube to insure accurate sampling of the brei. Three-tenths ml of the rat brain suspension was placed in a test tube containing 0.25 ml of a solution of 2:3:5-triphenyltetrazolium chloride (20 mg/ml) prepared fresh each third day and stored in the dark; 0.5 ml of borate buffer (pH 9.0); 0.25 ml of a solution of hypoxanthine (0.26 mg/ml); and sufficient water to bring the total volume to 2.25 ml. Blanks, which contained no substrate, were also prepared. The reaction vessels were shaken and placed in an opaque glass vacuum desicator which was sealed, evacuated, and placed in the 37C incubator for 19 hours. At the end of the incubation period the red formazan was dissolved in 5 ml of glacial acetic acid and then extracted with 3 ml of toluene and 2 ml of acetone. The optical density at 495 mμ was determined in a Beckman DU spectrophotometer and the difference between the test and blank values gave a measure of the enzyme's activity. Determinations were made in triplicate and the values obtained were divided by the dry weight of the brain to give the value—optical density/gram of brain (dry weight).

The optimal substrate concentration was determined by varying the substrate concentration in the presence of a homogenate made from three rat brains. The results are given in Figure 1.

The accuracy of the method was checked by preparing a number of samples in quadruplicate. The formazan was extracted from two of a set of four tubes while the other two samples were divided in half, one portion diluted 1:1

OPTIMAL SUBSTRATE CONCENTRATION

RAT BRAIN XANTHINE OXIDASE

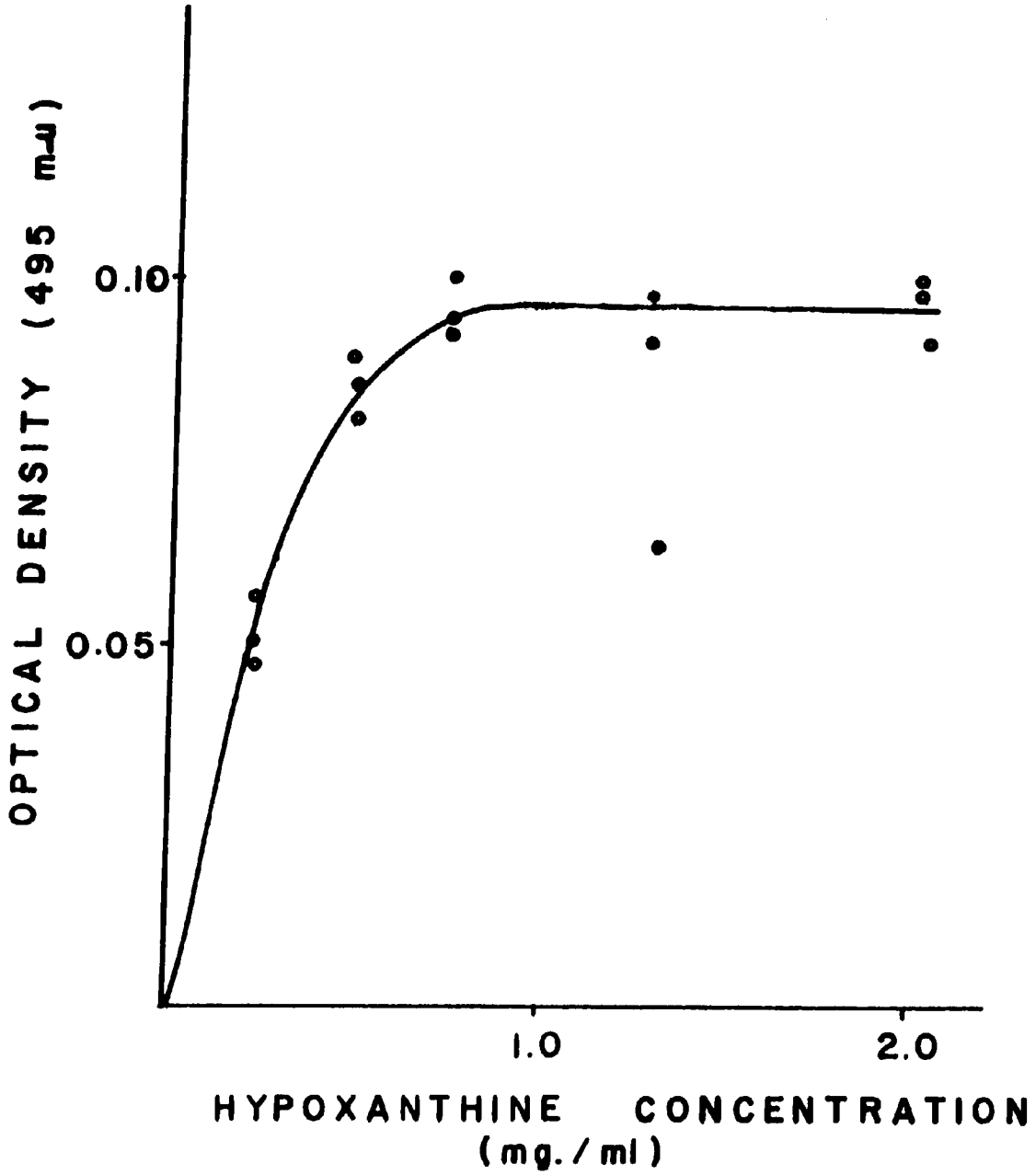

FIGURE 1

with buffer while the other was diluted 1:1 with the original brei. The difference between the optical densities of the samples diluted in buffer and those of the samples diluted in the original brei gave an estimate of the accuracy of the extraction procedure. It was possible to recover 92.8% ± 1.2% of the formazan.

Alkaline Phosphatase Determinations. The alkaline phosphatase activity of rat brains was determined by the method of Morton in Methods of Enzymology. Normal values were established by using adult albino rats of approximately the same age and weight. The animals were stunned by a blow on the thoracic vertebrae and immediately decapitated. The brains were removed and placed in a container with chopped ice. After weighing, the brains were ground in a mortar with sand (1 gram of sand per gram of brain) and 5 volumes of veronal buffer (pH 9.0). Low speed centrifugation removed large pieces of tissue. Five-tenths ml of this rat brain suspension was placed in a test tube containing 2.5 ml of veronal buffer (pH 9.0) and 0.5 ml of 0.01 M $MgCl_2$. After equilibrating for 10 minutes at 37C, 1.5 ml of a 0.015 M sodium B-glycerophosphate solution was added. The reaction was allowed to proceed for 60 minutes at 37C, after which 1 ml of 48% trichloroacetic acid was added and the precipitated protein removed by centrifugation. The inorganic phosphate formed by the reaction was determined by mixing 4 ml of the protein-free supernatant fluid with 1 ml of 5 N H_2SO_4 and 1 ml of 2.5% ammonium molybdate and then

adding 0.1 ml of sulfonic acid reagent and 3.9 ml of water. After standing 10 minutes the color was read at 660 mμ in a Klett-Summerson colorimeter. The sulfonic acid reagent was prepared by mixing 0.2 grams of 1-amino-2-naphthol-4-sulfonic acid with 1.2 grams of sodium bisulfite and 1.2 grams of sodium sulfite. When needed, 0.25 grams of this mixture was dissolved in 10 ml of water.

Observations were made in duplicate and the blank value was subtracted from the mean of the two experimental determinations to give a corrected reading. From these values the μM of organic phosphorus converted to inorganic phosphorus per hour per gram of brain (dry weight) were determined. The standard curve is shown in Figure 2. Two standards (K_2HPO_4) were included with each determination.

Determination of Glucuronides in Rat Urine. The method of Disch (1947) was used to assay for glucuronides in rat urine. Five albino rats of approximately the same age and weight were kept in separate glass cages and fed a normal rat chow administered ad lib. Urine samples were collected at 24 hour intervals. After determining the volume of urine excreted it was diluted 1/25 for normal determinations and 1/50 when animals had been treated with B-phenylserine. After the diluted urine was chilled in an ice bath, 6 ml of concentrated H_2SO_4 were added. The solution was placed in a boiling-water bath for twenty minutes and then cooled to room temperature before adding 0.2 ml of an alcoholic solution of carbazole. The color was allowed to develop for 2

STANDARD CURVE FOR ALKALINE PHOSPHATASE DETERMINATIONS

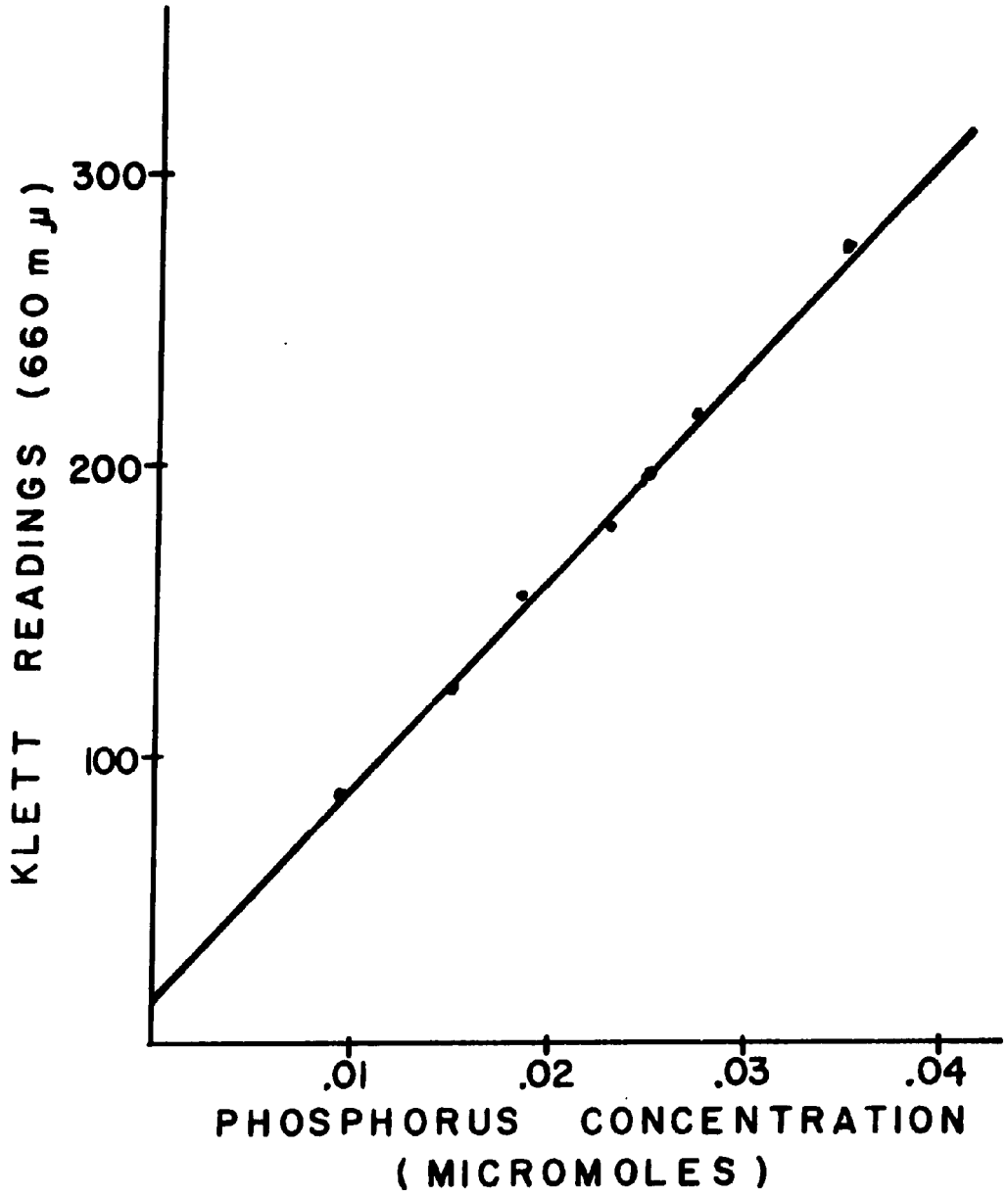

FIGURE 2

hours before reading in a Klett-Summerson colorimeter at 520 mμ. Figure 3 represents the standard curve.

STANDARD CURVE FOR SODIUM GLUCURONATE

(CARBAZOLE METHOD)

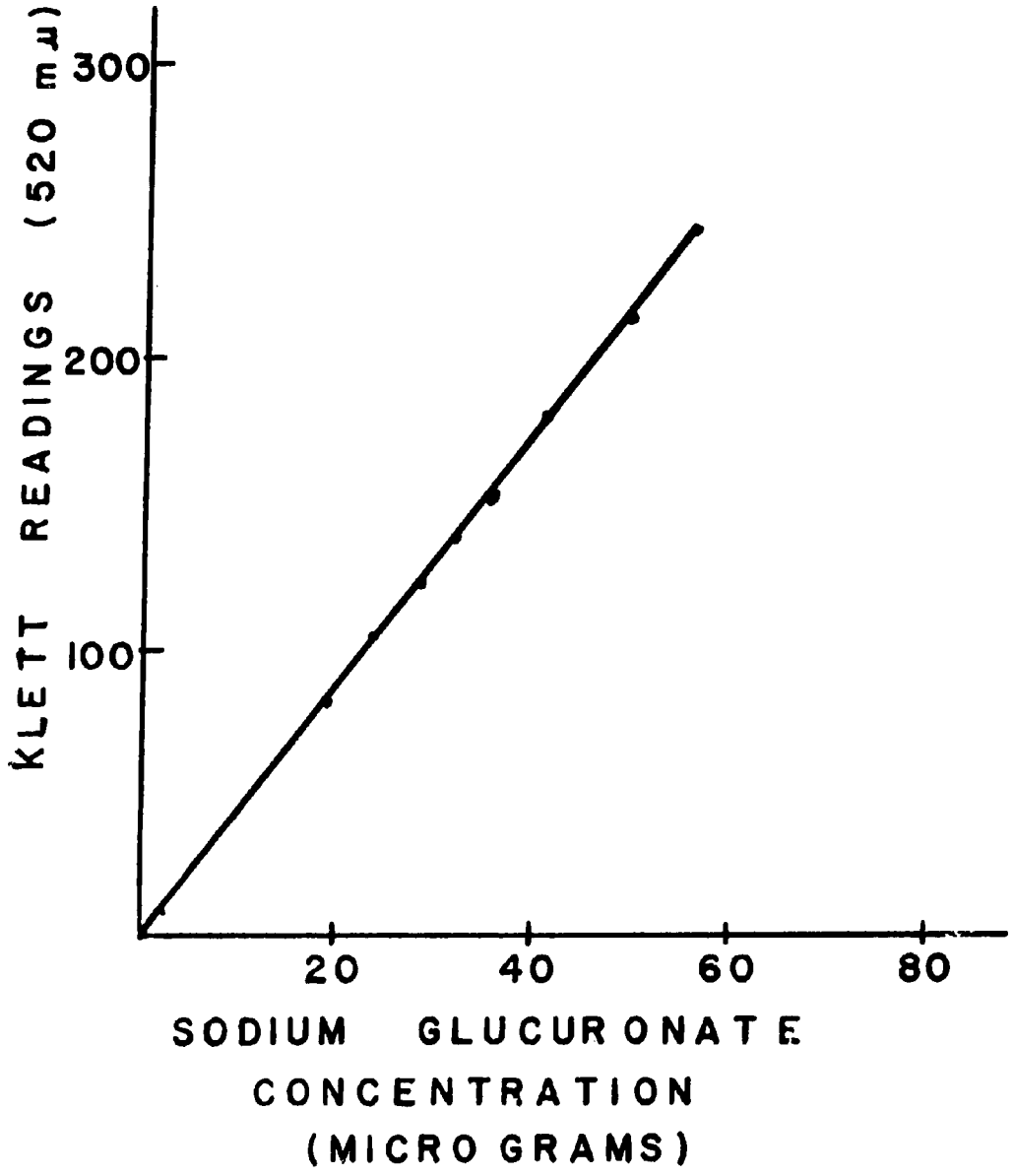

FIGURE 3

RESULTS

Selection of Test Animals and Route of Inoculation.

A system which would simulate the conditions of natural infection was considered advantageous in the evaluation of the chemoprophylactic effect of β-phenylserine. Although the intracerebral route of injection into mice is the most satisfactory when one wishes to assay rabies virus, it does not approximate the virus's natural mode of dissemination; furthermore, it was believed that employment of this route would be too severe a challenge for the procedure under investigation. Therefore, it was necessary to use a route by which one could (1) consistently produce infection after peripheral injection of the virus and (2) not overwhelm the test animal.

In an attempt to satisfy these criteria, groups of Swiss albino mice (weight 10-14 grams) and groups of albino rats (weight 130-150 grams) were injected with 0.03 ml of various virus dilutions into the masseter muscle, the gluteus muscle, or the gastrocnemius muscle. From the results summarized in Table 1 it is clear that rats injected into the masseter muscle with 0.03 ml of virus proved the most satisfactory.

Toxicity of B-phenylserine.

In order to determine the toxicity of the compound, various concentrations were administered to albino rats weighing approximately 150 grams. It was found that a

TABLE 1

EFFECT OF THE ROUTE OF INOCULATION OF RABIES VIRUS ON THE INCUBATION TIME AND INFECTIVITY

DILUTION	MASSETER		GLUTEUS		GASTROCNEMIUS	
Rats	D/I	M.I.T.	D/I	M.I.T.	D/I	M.I.T.
10^{-1}	6/6	20	5/6	22	4/6	24
10^{-2}	6/6	18	3/6	28	3/6	28
10^{-3}	1/6	23	0/6	--	0/6	--
Mice						
10^{-1}	1/6	10	2/6	11	2/5	11
10^{-2}	1/6	12	0/6	--	1/6	13
10^{-3}	0/6	--	0/6	--	0/6	--

D/I ---- Number dead/Number inoculated

M.I.T. - Mean Incubation Time (days)

single dose of 200 mg of β-phenylserine administered intraperitoneally would kill all the animals. When the animals received 20 mg of β-phenylserine per day they all died in 9-10 days. Administration of 10 or 15 mg per day for 1 month had no overt effect on the rats. On the basis of this information 1 ml of a 10 mg/ml solution of β-phenylserine administered daily was chosen as the dosage to be used.

Effect of β-Phenylserine on Rats Injected with Rabies Virus.

Pool virus with an intracerebral LD_{50} of 4.5 in mice and 4.3 in rats, and an intramuscular LD_{50} of 2.5 in rats was used in most of the test systems.

Each of three serial ten-fold dilutions of virus was injected (0.03 ml into the masseter muscle) into five rats of approximately the same age and weight (150 grams). Intraperitoneal administration of β-phenylserine was begun four days prior to injecting the animals, so that the fourth injection of the compound and the virus were given simultaneously. The experiment was composed of four groups: (1) rats injected with the virus and also with 1 ml of a 10 mg/ml solution of β-phenylserine daily until the first animal in the group died of rabies; (2) rats injected only with virus; (3) rats injected with virus and also with 1 ml of sterile water intraperitoneally each day; (4) five rats not injected with virus but with the same amount of β-phenylserine as group 1. The five animals in group 4 were all normal at the termination of the experiment. In

this and subsequent experiments, all surviving animals were observed for 60 days before considering them negative. The results of this trial, summarized in Table 2, indicated that a significant degree of protection was obtained with β-phenylserine in rats injected with rabies virus.

In subsequent experiments groups 3 and 4 were omitted since treating the animals with water did not alter the course of the infection and the dosage of β-phenylserine used was not toxic for the animals. However, another set of animals was included in addition to the control group and the group composed of animals which had received β-phenylserine four days prior to the injection of the virus. This additional group began receiving the compound 24 hours after injection of the virus to determine whether the compound was active after the animals were exposed to the virus. The results of these experiments are summarized in Table 3.

It is to be noted that although the protection demonstrated in experiment 2 is, at best, equivocal, there was a significant degree of protection observed in experiments 3 and 4. In experiment 2 six animals were used at each dilution level; however in experiments 3 and 4 and in all ensuing experiments 10 animals were used at each dilution level. This was done to increase the accuracy of the statistical analysis of the data. The data from experiments 3 to 6 was combined and is represented graphically in Figure 4. It can be seen that by the nineteenth day more than 50% of the animals in the control group were dead while the cumulative per cent mortality of the pre- and post-treated animals never

TABLE 2

THE EFFECT OF β-PHENYLSERINE ON RABIES INFECTION IN RATS

DILUTION	PHENYLSERINE TREATED		WATER CONTROL		CONTROL	
	D/I	M.I.T.	D/I	M.I.T.	D/I	M.I.T.
10^{-1}	1/5	31.0	5/5	20.0	5/5	17.0
10^{-2}	2/5	26.0	5/5	16.4	5/5	18.4
10^{-3}	0/5	--	0/5	--	1/5	17.0
LD_{50}	$10^{-0.87}$		$10^{-2.6}$		$10^{-2.5}$	

D/I ---- Deaths due to rabies/number inoculated

M.I.T. - Mean Incubation Time (days)

TABLE 3

THE EFFECT OF β-PHENYLSERINE ON RABIES INFECTION IN RATS

Experiment Number	Dosage mg/day	LD_{50} β-phenylserine treated Pre-infection	LD_{50} β-phenylserine treated Post-infection	LD_{50} Control
2	10	1.6	1.7	2.20
3	10	1.08	1.52	2.70
4	10	0.91*	1.00	2.70
5	15	0.85*	0.93*	2.63
6	15	0.79*	0.94*	2.67

* Calculated by probit analysis

Experiment 2 - 54 animals were used.

Experiments 3 to 6 - 90 animals were used in each experiment.

EFFECT OF β-PHENYLSERINE ON RABIES INFECTION IN RATS

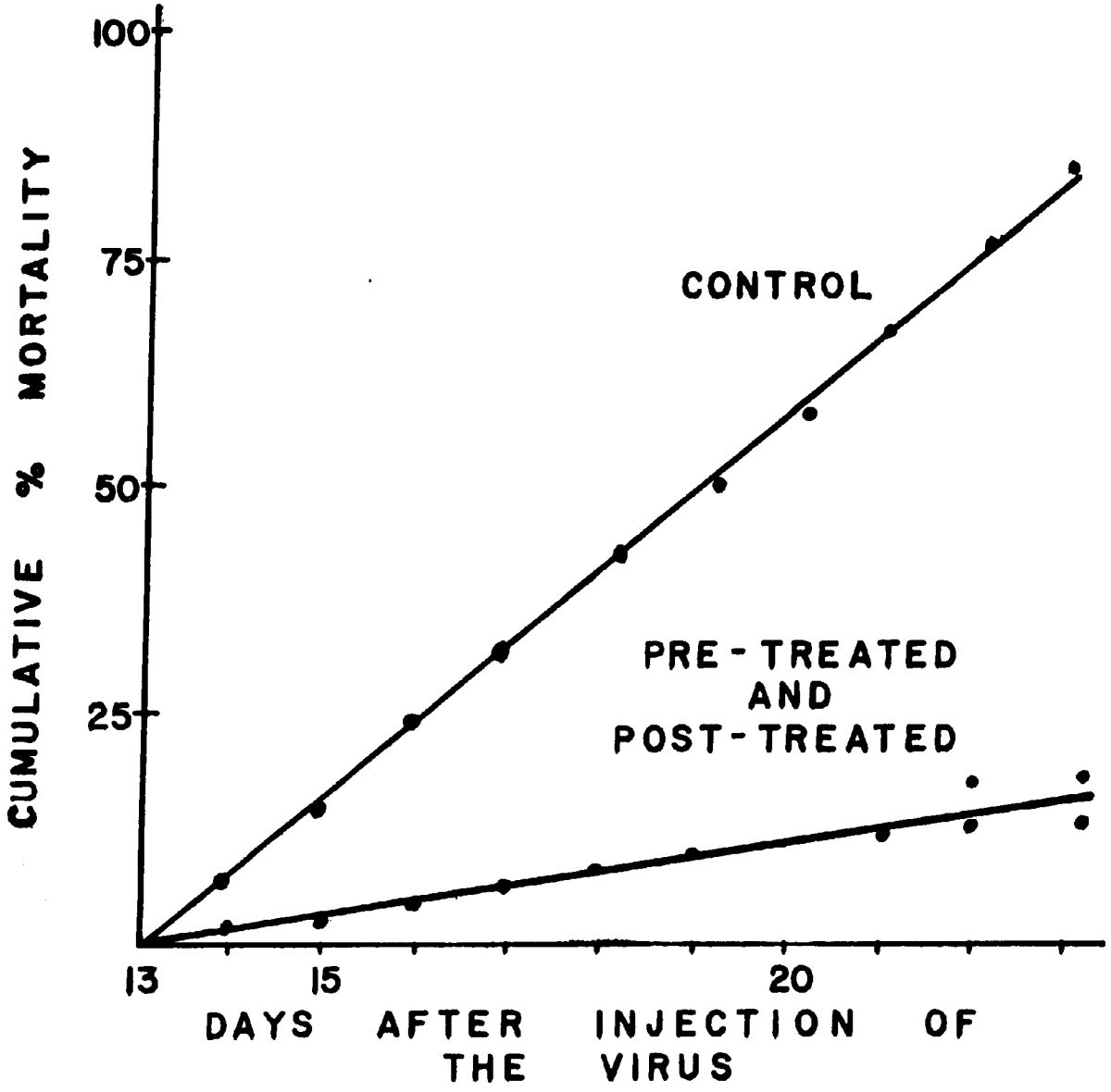

FIGURE 4

exceeded 20%. There was no significant difference between the LD_{50}'s of the pre- and post-treated groups, both of which showed that the animals were protected against the disease. It was of considerable interest that treatment could be delayed for 24 hours and still demonstrate a protective effect.

When the β-phenylserine dosage was increased to 15 mg/day a greater degree of protection was obtained (experiments 5 and 6), however, 20 mg/day proved toxic when administered for more than eight days.

The conditions of the experiments were varied to demonstrate the protective effect in a different way. Sixty rats were injected with 90 LD_{50}'s (in 0.03 ml). Twenty of the animals began receiving β-phenylserine (15 mg/day) 24 hours before challenging with the virus, twenty received the compound 24 hours after injection of the virus, and the remaining twenty served as controls. The mortality rates of the pre- and post-treated groups were 20% and 30% respectively, as opposed to a mortality of 100% in the control group.

Effect of Varying Time of Initiation and Termination of β-Phenylserine Treatment in Rats Injected with Rabies Virus.

It was of interest to know how soon after injection of the virus it was necessary to begin treatment, and conversely, when treatment could be stopped and still obtain protection. The experiment involved 120 animals (150 grams ± 10 grams) which were divided into two groups of 60. Each

of these groups was subdivided into 6 subgroups of ten animals. Each of the animals was injected with 90 LD_{50}'s of rabies virus and ten animals in group 1 and the 60 animals in group 2 received 15 mg of β-phenylserine. Each day an additional ten animals in group 1 began to receive the compound and continued to be treated until the first animal in the experiment died of rabies. In group 2, treatment was stopped on subgroups of ten animals each day. An additional thirty animals served as controls. The results are summarized in Table 4. From the data obtained it appeared that β-phenylserine protected the animals when the daily administration of the compound was begun within 48 hours after injection of the virus. There was slight indication that one dose of 15 mg of β-phenylserine could protect the animal when this was administered at the time the virus was injected.

Effect of Phenylalanine and β-Phenylserine as Diluents of Rabies Virus.

In order to examine the possibility that β-phenylserine might be inactivating the virus directly or preventing the adsorption of the virus on host cells, rabies virus was diluted in solutions of phenylalanine or β-phenylserine. After incubating the mixtures at 37C for 1 hour, 0.03 ml of the mixture was injected intracerebrally into mice. When a solution composed of 13.5 mg β-phenylserine and 13.5 mg of phenylalanine was used as the diluent the LD_{50} was considerably below that of the control. However, when the experiment was repeated using 6.75 mg each of

TABLE 4

EFFECT OF VARYING TIME OF INITIATION AND TERMINATION OF β-PHENYLSERINE TREATMENT ON RABIES INFECTION IN RATS

Compound begun on day	D/I	Compound stopped on day	D/I
1.	5/10	1.	7/10
2.	6/10	2.	8/10
3.	9/10	3.	9/10
4.	9/10	4.	9/10
5.	10/10	5.	8/10
6.	9/10	6.	8/10

D/I --- Deaths due to rabies virus/number inoculated

Experimental animals received a 1/50 dilution of the virus (LD_{50} $10^{-2.8}$)

Treatment - 15 mg β-phenylserine/day

β-phenylserine and phenylalanine there was no difference from the control. Table 5 shows that under these circumstances β-phenylserine and phenylalanine apparently did not affect the adsorption of the virus nor inactivate it.

Reversal of β-Phenylserine Inhibition.

β-phenylserine inhibition of bacteria can be reversed by the addition of excess phenylalanine or tyrosine. Morgan (1954) and Heggie and Morgan (1956) found that phenylalanine reversed β-2 thienylalanine inhibition of psitticosis, poliomyelitis, vaccinia, and mouse encephalomyelitis viruses cultured in vitro. However, Hosley (1955) found that tyrosine did not reverse the inhibition of rabies virus cultured in minced mouse-brains.

The effects of phenylalanine and tyrosine were tested in rats injected intramuscularly with rabies virus. These compounds, both alone and in combination with β-phenylserine, were injected each day intraperitoneally in the amounts indicated in Table 6. β-phenylserine protected rats injected with the virus but tyrosine or phenylalanine reversed this inhibition. Treatment with β-phenylserine and tyrosine (8 mg/day of each) only partially reversed the inhibition. Thus, it appears that the inhibitory action of β-phenylserine involves phenylalanine and tyrosine metabolism in the intact animal. The reversal of β-phenylserine inhibition using other amino acids was not attempted. The possibility that other amino acids could reverse the inhibition is unlikely.

TABLE 5

EFFECT OF PHENYLALANINE AND β-PHENYLSERINE AS DILUENTS OF RABIES VIRUS

DILUENT COMPOSITION	LD50*
13.5 mg β-phenylserine	3.63
13.5 mg phenylalanine	3.38
13.5 mg each of β-phenylserine and phenylalanine	2.83
6.75 mg each of β-phenylserine and phenylalanine	3.72
Gelatine Saline	4.00

* 40 animals in each group.

TABLE 6

REVERSAL OF β-PHENYLSERINE INHIBITION WITH TYROSINE AND PHENYLALANINE

COMPOUND	DOSAGE	LD_{50}*
Tyrosine	6 mg/day	3.00
β-Phenylserine	6 mg/day	1.58
Tyrosine plus β-phenylserine	6 mg/day of each	3.15
Tyrosine	8 mg/day	2.62
β-phenylserine	8 mg/day	1.39
Tyrosine plus β-phenylserine	8 mg/day of each	2.52
Phenylalanine	10 mg/day	2.83
β-phenylserine	10 mg/day	1.00
Phenylalanine plus β-phenylserine	10 mg/day of each	2.83
Control	--	3.35

Animals began to receive compound 24 hours after infection

* 30 animals in each group

Effect of Compounds Structurally Related to β-Phenylserine on Rats Injected with Rabies Virus.

Previous studies using antimetabolites have indicated that certain molecular groups on the inhibitor are responsible for its activity. In an attempt to determine the group or groups in the β-phenylserine molecule responsible for its protective effect, five compounds, structurally related to β-phenylserine, were tested for their ability to protect animals inoculated with rabies virus. Treatment with the various compounds was begun on the day virus was inoculated and was continued until the first animal died of rabies on the tenth day. The animals treated with β-hydroxypropionic acid became increasingly difficult to handle when they were injected. Treatment of these animals was discontinued on the eighth day when it became impossible to hold them without being bitten. The results summarized in Table 7 indicate that only β-phenylserine protected the animals.

Immunity to Rabies Virus After β-Phenylserine Treatment.

It was of interest to know whether there had been enough multiplication of the virus in β-phenylserine treated animals to elicate an antibody response. If the action of β-phenylserine occurred at an early stage in the development of the disease, and this prevented the growth of the virus, there would be little or no antibody response to the virus. Since the original challenge dose (0.03 ml of a 10^{-1} or 10^{-2} dilution) was so small, it should account for very little antibody production.

TABLE 7

EFFECT OF β-PHENYLSERINE AND STRUCTURALLY RELATED COMPOUNDS ON RATS INJECTED WITH RABIES VIRUS

COMPOUND	LD_{50}*
Phenylpyruvate	>2.5
Serine	>2.4
Hydrocinnamic acid	>2.5
Phenylglycine	>2.4
β-hydroxyproprionic acid	>2.4
β-phenylserine	1.32
Control	3.28

Dosage of β-phenylserine and other compounds 10 mg/day

* 20 animals in each group

Twenty rats which had survived injection of rabies virus after treatment with β-phenylserine were divided into two groups; the members of each group consisted of animals which had survived the injection of 10^{-1} and 10^{-2} dilutions of virus. Group 1 and Group 2 were challenged intramuscularly with 0.03 ml of a 10^{-2} dilution of rabies virus 6 and 7 weeks, respectively, after the first virus inoculation. All of the animals succumbed to the infection. The mean incubation time and the manifestations of the disease were no different from those of animals which had been injected with this amount of virus for the first time.

On the basis of this evidence it can be stated that the treatment of animals with β-phenylserine did not allow sufficient multiplication of the virus to induce an immunity capable of protecting the animals against subsequent challenge doses of virus. Furthermore, the protective mechanisms through which β-phenylserine operates were not in effect 30 days and 37 days after the treatment was discontinued.

Effect of β-Phenylserine on the Inflammatory Response to the Injection of Rabies Virus.

Since there did not appear to be any multiplication of the virus in the animals as evidenced by the apparent lack of an immunological response in β-phenylserine treated rats, it was felt that the action of the compound might involve host defense mechanisms at the site of injection of the virus. To examine this possibility, six rats were injected into the masseter with 0.03 ml of a 10^{-2}

dilution of pool virus. Three of the animals had been treated with 15 mg of β-phenylserine per day for three days prior to the injection of the virus. Treatment was continued for two more days at which time the animals were killed and the masseter muscles excised. Sections were prepared and examined in the Department of Pathology.

There appeared to be no difference in the histological character of the response to the virus in the treated and untreated rats. In all animals there was a focal interstitial myositis with an infiltration of histiocytes and the beginning of a fibroblastic proliferation. When 0.03 ml of a 10^{-2} suspension of normal mouse brain was injected into the masseter muscle of 2 additional rats the inflammatory response 48 hours later was very mild and lymphocytic in character.

If the original supposition was correct, that is, if β-phenylserine were altering the character of the inflammatory response, one would expect that this alteration would be reflected in the type and quantity of cells infiltrating into the region where the virus had been injected. Since there were no histological differences in the lesions produced by the virus in the treated and untreated rats, it would appear that β-phenylserine was not altering the cellular response elicited by the injection of the virus.

The Effect of β-Phenylserine on Other Virus-Host Systems.

The effect of β-phenylserine on other virus-host systems was examined in a number of pilot experiments.

1. Neurotropic Viruses

a. Eastern Equine Encephalitis (EEE) - EEE virus was titrated in two groups of mice, a control and an experimental group, the latter receiving 1 mg of β-phenylserine intraperitoneally daily. Serial ten-fold dilutions (10^{-8} to 10^{-11}) of the virus were injected intracerebrally into 6 mice (weight 10-14 grams) at each dilution level. There was no significant difference between the control and experimental groups which had titers of $10^{-9.74}$ and $10^{-10.0}$, respectively.

Since infection via the intracerebral route was considered too drastic to allow the effects of the compound to be evaluated, mice weighing 8-10 grams were injected intramuscularly with the virus. Five mice were tested at 10^{-1}, 10^{-2}, and 10^{-3} dilutions of virus. The LD_{50}'s of the control and treated group (1 mg of β-phenylserine daily) were $10^{-2.2}$ and $10^{-2.1}$.

b. Poliovirus, Type 2, Lansing strain - Forty mice (weight 10-14 grams) were injected intracerebrally with 100 LD_{50}'s of poliovirus (LD_{50} $10^{-3.9}$). Half of the mice were treated with 1.0 mg of β-phenylserine intraperitoneally daily. There was no apparent difference in the course of the disease in either group.

c. Mouse Encephalomyelitis Virus (FA strain) - Two routes of injection of the virus were used, intracerebral and intramuscular. Duplicate titrations in treated and untreated mice (weight 10-14 grams) were made using 6 mice at each dilution level. The treated mice received 1 mg of

β-phenylserine intraperitoneally daily. The intracerebral LD_{50}'s in the treated and control groups were $10^{-6.0}$ and $10^{-6.2}$, respectively, intramuscularly the titers were $10^{-1.3}$, control, and $10^{-0.9}$ for the treated group. In both cases the differences are insignificant.

2. Non-Neurotropic Viruses

a. Vaccinia Virus - Vaccinia virus propagated in eggs was used. Three separate intracutaneous titrations were done on the dorsum of two rabbits. One rabbit received virus diluted in saline, the other virus diluted in β-phenylserine (15 mg/ml). A titration was performed by injecting 0.2 ml of serial ten-fold dilutions of virus (10^{-2} to 10^{-6}) intradermally. The titers obtained in the control rabbit were 10^{-4} while the endpoints of the titrations using virus diluted in β-phenylserine showed 1 titer at 10^{-2} and no lesions in the other two titrations.

Since β-phenylserine seemed to inhibit the propagation of this virus, the question was pursued further in two experiments. In each of these experiments three groups of rabbits weighing 5 to 6 1/4 pounds were used. In group 1, virus was diluted in saline. In group 2, the virus was diluted in 15 mg/ml of β-phenylserine. In group 3, virus was diluted in saline and 75 mg of β-phenylserine were injected intraperitoneally daily. There were three rabbits in each group and, as before, 3 titrations were done in each rabbit.

The first of these two experiments was conducted using discard rabbits and the results were: Group 1,

(control) 7 endpoints at 10^{-4} and two at 10^{-3}; Group 2, no lesions at any dilution level of all 9 titrations; Group 3, 1 titration had an endpoint at 10^{-2}, but there were no lesions in the other 8 titrations. Repeating the experiment with unused rabbits the following data were obtained: Group 1, (control) 5 endpoints at 10^{-4} and 4 endpoints at 10^{-5}; Group 2, two rabbits (or 6 titrations) showed no lesions while on the third rabbit the endpoints for the three titrations were 10^{-4}, 10^{-2}, no lesions; Group 3, Rabbit 1: 3 endpoints 10^{-4}, Rabbit 2: 2 endpoints 10^{-4} and one 10^{-2}, Rabbit 3: 2 endpoints 10^{-2} and one with no lesions.

The observed differences when submitted to probit analysis are statistically significant at the 5% level; however, variations in groups 2 and 3 do not inspire confidence in this interpretation. It is obvious that further investigation with this virus should be considered as a future possibility.

b. Myxoma Virus - Two rabbits were injected intradermally with 0.01 ml of a 10% suspension of tissue containing myxoma virus. One of the rabbits was treated with 75 mg of β-phenylserine intraperitoneally daily. In the treated animal the lesion was slower in developing, compared to that of the control, and the animal lived 48 hours longer. These results suggested that β-phenylserine might have an effect on the propagation of myxoma virus.

Using two rabbits for each dilution, 0.1 ml of serial ten-fold dilutions of myxoma virus were injected intradermally. The small number of animals used permitted

only an approximation of the LD_{50} as $10^{-7.0}$. On this basis six rabbits were injected with 10,000 LD_{50}'s. Three of the rabbits received 75 mg β-phenylserine intraperitoneally daily. The lesions approximated hemispheres, and so their volumes were measured daily; the average volume of the lesions of the three control rabbits and the three treated rabbits were plotted as shown in Figure 5. The deaths of the treated animals were delayed by 24 to 48 hours.

Another experiment was conducted using 12 rabbits. Three rabbits served as controls and were injected with 100 LD_{50}'s of the virus while the remaining rabbits were injected with 100 LD_{50}'s, 1000 LD_{50}'s, and 10,000 LD_{50}'s using three rabbits at each dilution. The nine latter animals received 75 mg β-phenylserine intraperitoneally daily. The results are shown in Figure 6. Again the control animals died 24 to 48 hours sooner than the treated animals which received the same amount of virus.

The slopes of the lines indicate that the development of the lesions in the treated animals receiving 100 LD_{50}'s was 20% slower than in the control group which received the same amount of virus. The same data, plotted semi-logarithmically (Figure 7), indicated that β-phenylserine caused a three day lag in the development of the lesions. It is also of interest that only the control curve can be extrapolated back to the origin and that only this group and the 10,000 LD_{50} group show truncated curves, indicating an immunological response to the virus.

EFFECT OF β-PHENYLSERINE ON RABBITS INJECTED WITH MYXOMA VIRUS

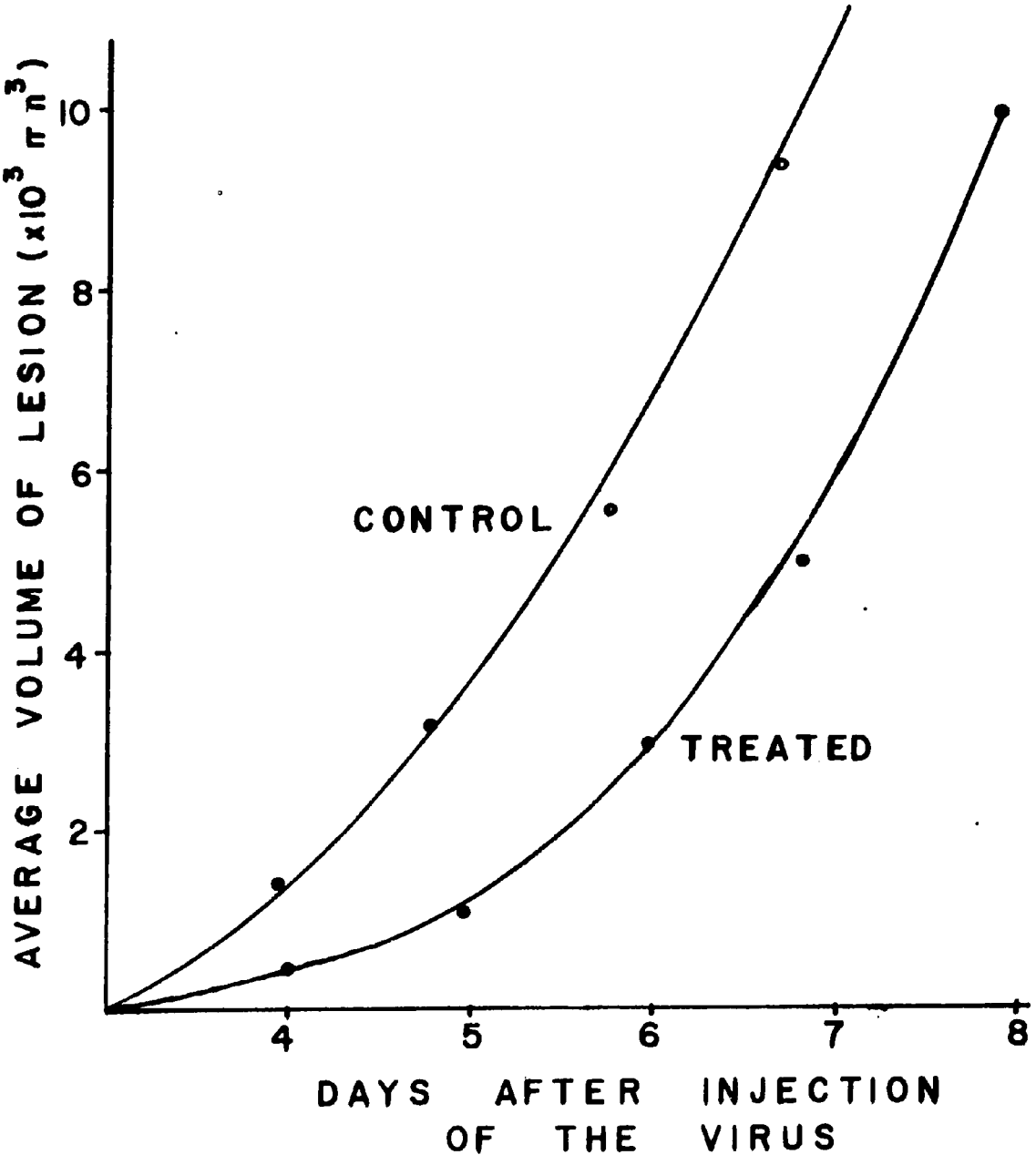

FIGURE 5

EFFECT OF β-PHENYLSERINE ON RABBITS INJECTED WITH MYXOMA VIRUS

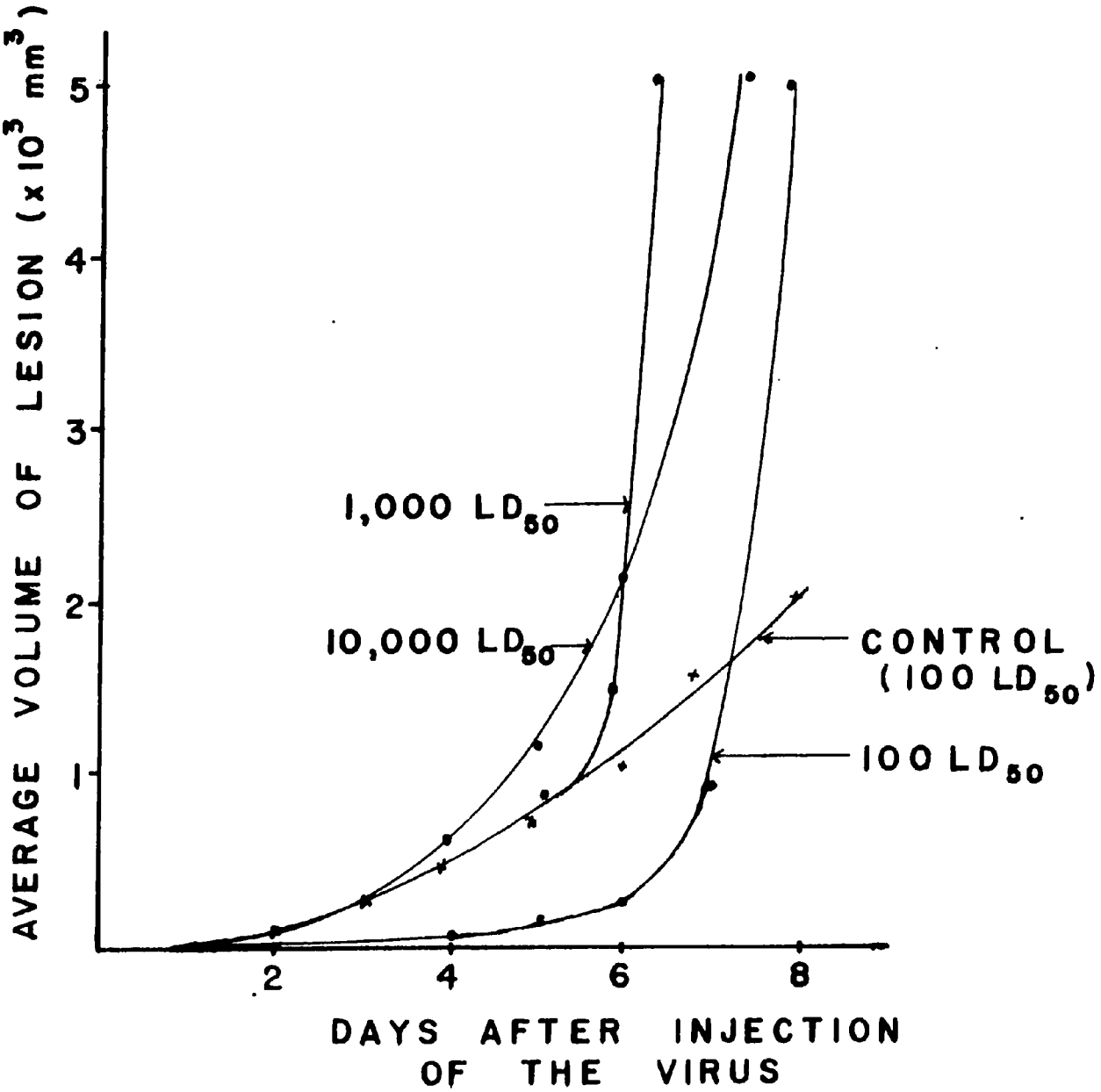

FIGURE 6

EFFECT OF β-PHENYLSERINE ON RABBITS INJECTED WITH MYXOMA VIRUS

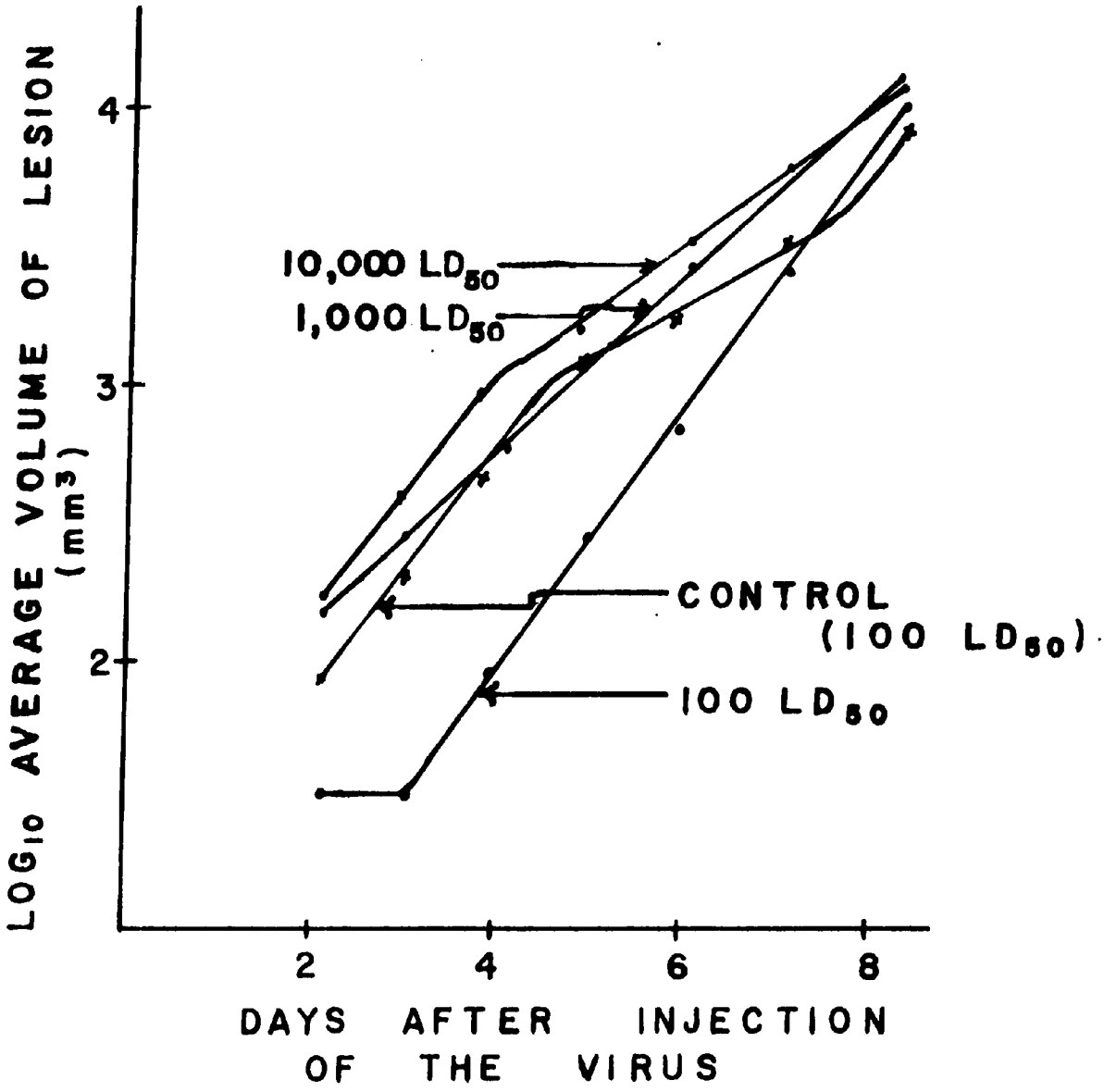

FIGURE 7

c. Influenza Virus - The effect of β-phenylserine on the propagation of Influenza A virus in eggs and in mice was examined. Serial ten-fold dilutions of the PR8 strain diluted in saline and in β-phenylserine (15 mg/ml) were injected into the allantoic cavities of 10 day old embryonated eggs. Three eggs were used at each dilution level. After an incubation period of 48 hours the allantoic fluids were harvested and the hemagglutinating titers determined. The titers varied from 1/640 to 1/1280 in both control and treated groups with no demonstrable difference between the two.

Mice were injected intranasally with serial ten-fold dilutions (10^{-4} to 10^{-7}) of influenza virus. Each dilution of virus was administered to 20 mice, 10 of which served as controls and the other ten received 1.0 mg of β-phenylserine intraperitoneally daily. On the fourteenth day all surviving mice were killed; the lungs of all dead mice were examined for evidence of consolidation. The LD_{50}'s of the control and treated groups were $10^{-6.4}$ and $10^{-6.2}$, respectively. This small difference was not significant.

Alterations in the Xanthine Oxidase Activity of the Brains of Rats Injected with Rabies Virus.

Bauer and Bradley (1956) reported that in mice injected with neurotropic viruses the xanthine oxidase activity of the brain increases as the infection progresses. Younathan (1956) has shown that β-2 thienylalanine can cause a decrease in the xanthine oxidase activity of rat

liver. Since the action of β-2 thienylalanine and β-phenylserine is similar in some systems we compared the effect of β-phenylserine on the xanthine oxidase activity of the brains of normal rats and rats infected with rabies.

Five groups of twenty rats weighing 190-195 grams were used. Group 1, β-phenylserine controls, received 15 mg of β-phenylserine daily. Group 2, virus controls, were injected intramuscularly with a 10^{-2} dilution of pool virus. Group 3, were normal animals. Group 4, test animals were injected intramuscularly with a 10^{-2} dilution of pool virus and received 15 mg of β-phenylserine intraperitoneally daily beginning 24 hours after the virus had been injected. Group 5, virus titration controls, were injected intramuscularly with a 10^{-2} dilution of pool virus. Since the toxicity of the borate buffer used in preparing the brei precluded the assay of the virus present in the brain suspension by mouse inoculation, it was necessary to use this fifth group of animals to determine time of appearance of virus in the brains of infected animals.

Two and a half hours after the virus had been injected, twenty-four hours later and then on alternate days, two animals from each of the first four groups were sacrificed and the xanthine oxidase activity of the brains determined. Each point on the graph (Figure 8) represents the mean of the activity (expressed as optical density/dry weight of brain tissue) in the two animals. Determinations for each animal were made in triplicate. On the ninth day the xanthine oxidase activity in the virus-control animals

XANTHINE OXIDASE ACTIVITY AND APPEARANCE OF RABIES VIRUS IN RAT BRAIN

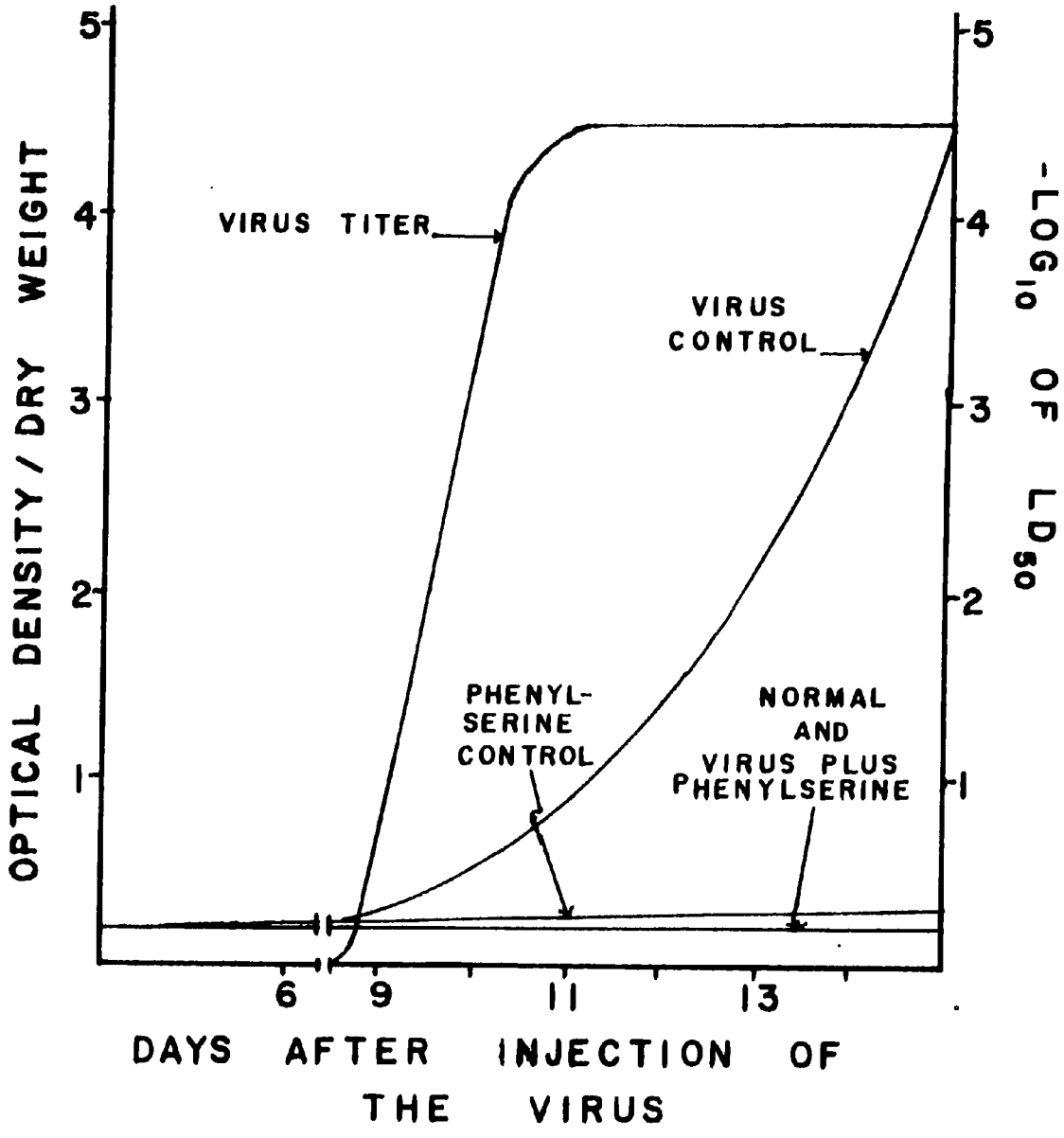

FIGURE 8

began to increase until it reach a maximum when the animals were close to death. The animals of the fifth group were sacrificed on alternate days and homogenates of their brains injected into mice to assay for virus.

It is obvious that the effect of rabies on the xanthine oxidase activity of rat brains is similar to that reported by Bauer and Bradley in mouse brains. However, in normal animals β-phenylserine did not have the same effect on the xanthine oxidase activity of rat brain as β-2 thienylalanine did on rat liver. Furthermore, the activity of the enzyme did not increase in the treated animals which had been injected with virus.

The role of xanthine oxidase in the propagation of neurotropic viruses is unknown; however it is known that xanthine oxidase is intimately involved in purine metabolism. Since this enzyme is capable of oxidizing a wide variety of substrates including aldehydes, xanthine, hypoxanthine, and reduced co-enzyme I, it is difficult to speculate on its possible function in the formation of new virus particles.

Alkaline Phosphatase Activity in the Brains of Rats Injected with Rabies Virus.

Since the role of xanthine oxidase in purine metabolism is usually considered to be catabolic, an examination of the effect of rabies virus on some anabolic enzymes was made. Alkaline phosphatase activity was chosen as it has been shown that many diseases cause alterations in the activity. Table 8 shows the alkaline phosphatase activity

TABLE 8

ALKALINE PHOSPHATASE ACTIVITY IN THE BRAINS OF NORMAL AND RABIES INFECTED RATS

Days after injection of virus	µM of phosphorus converted per hour per gram of brain (dry weight)	
	Infected	Normal
3	0.680	0.912
	0.694	0.794
		0.763
5	0.727	0.871
	0.700	0.855
		0.992
7	0.743	0.846
	0.748	0.816
9	0.702	0.887
	0.736	0.760
11	0.883	0.688
	0.766	0.785
		0.697
12	0.751	0.763
	0.800	0.763
	0.788	
Mean and Standard Deviation	0.747±0.054	0.813±0.081

determined in homogenates of the brains of normal and infected rats. After intramuscular injection of 0.03 ml of a 10^{-2} dilution of virus, the rats were sacrificed at periodic intervals and the activity of the enzyme determined. Each value represents the mean of two determinations on one brain. Statistical analysis (t test) of the data reveals that there was a significant decrease in the alkaline phosphatase activity at the 5% level but none at the 1% level.

Urinary Glucuronide Levels of Normal and β-phenylserine-Treated Rats.

In 1909, Dakin reported that cats injected with β-phenylserine excreted more hippuric acid in their urine than did normal animals. He postulated that the compound underwent beta-oxidation to benzoic acid which then was conjugated with glycine to form hippuric acid. If the rat metabolizes β-phenylserine in the same way, that is, if it removes the glycine molecule from the compound and oxidizes the aromatic residue to benzoic acid, this latter compound will unite with glycine to form hippuric acid or with glucuronic acid to form benzoylglucuronic acid. To test this assumption the amount of glucuronic acid excreted by normal and β-phenylserine-treated animals was determined by the method of Dische. Quantitative tests showed there was no hippuric acid being excreted.

Eight rats were placed in individual metabolism cages with food and water supplied ad lib. Every twenty-four

hours the urine was collected and the amount of glucuronic acid determined. After sufficient determinations were made on normal animals to establish the normal excretion pattern of glucuronides, the animals were injected intraperitoneally daily with 15 mg of β-phenylserine for 7 to 9 days, and the determinations continued. The results summarized in Table 9, show that the injection of β-phenylserine did not cause an increase in the excretion of glucuronides. However, if the animal were excreting the compound slowly, the small increase in glucuronides being excreted would not be detected if the system were not sufficiently sensitive. Furthermore, if the animal were metabolizing β-phenylserine in some other way the excretory pattern of glucuronides might not be affected.

TABLE 9

URINARY GLUCURONIDE LEVELS OF NORMAL AND β-PHENYLSERINE TREATED RATS

Animal No.	NORMAL No. of assays	NORMAL mg glucuronic acid/24 hours*	TREATED (15 mg β-phenylserine/day) No. of assays	TREATED (15 mg β-phenylserine/day) mg glucuronic acid/24 hours*
1.	6	9.52 (12.47 to 5.22)	9	7.23 (1.61 to 10.80)
2.	6	8.72 (11.00 to 4.94)	9	7.71 (15.37 to 4.89)
3.	6	9.18 (12.97 to 6.48)	9	12.07 (21.65 to 3.90)
4.	15	12.60 (21.20 to 4.91)	7	12.56 (16.56 to 9.19)
5.	15	6.08 (8.80 to 3.08)	7	4.94 (6.08 to 3.31)
6.	5	4.09 (2.71 to 6.56)	7	5.46 (11.94 to 1.61)
7.	5	7.19 (4.73 to 10.64)	7	7.64 (10.45 to 5.16)
8.	5	12.52 (6.25 to 19.21)	7	11.43 (15.70 to 7.18)

* mean (range)

DISCUSSION

Evidence has been presented to show that β-phenylserine inhibits the development of rabies in rats. Consistently, in experiments involving more than 700 animals, it was possible to demonstrate protection against 40 to 100 LD_{50}'s of virus, and in some cases as much as 230 LD_{50}'s. The prophylactic effect was greatest when administration of the compound was begun one to three days before challenge; however, some protection could be obtained when treatment was delayed 24 to 48 hours after injection of the virus. Clearly then, β-phenylserine inhibits the propagation of rabies virus not only in tissue culture but also in vivo.

The literature abounds with reports of inhibitors of virus multiplication in tissue culture systems. In most cases, there is uncertainty as to the mechanisms by which the inhibition is effected. When an in vivo inhibitor of virus multiplication is found, the problems of interpretation of the mechanism of action are greatly increased. Even when the pathogenesis of the disease is well understood it is difficult to evaluate this mechanism because it is necessary to be fully cognizant of the effect of the compound on the virus, the effect of the inhibitor on the host and the host's effect on the virus. This study has been concerned with the effect of β-phenylserine on the virus and on the normal and infected host. In the animal injected with rabies virus β-phenylserine may conceivably act in one or more of the following ways: 1. at the site of

inoculation, either to inactivate the virus directly or to prevent adsorption of the virus onto a susceptible cell; 2. on the normal defense mechanisms of the host; 3. intracellularly to prevent the formation of new virus particles. Each of these possibilities will be examined in turn.

If β-phenylserine were combining with the free virus to inactivate or prevent its adsorption onto a susceptible host cell, one would expect inhibition of the virus when it was mixed with the compound before injection. This was not found to be the case. There was no apparent difference in disease between animals injected with rabies virus diluted in gelatine saline or in β-phenylserine. Furthermore, protection was demonstrated when the compound was administered to the animals 24 hours after the virus had been injected. It is unlikely that after 24 hours enough free virus exists in the tissue so that its inactivation could seriously alter the course of the disease.

Although it is doubtful that β-phenylserine inactivates rabies virus or inhibits its adsorption to a host cell, its action on vaccinia virus may be different. A greater degree of protection was observed when vaccinia virus was mixed with β-phenylserine prior to intradermal injection into rabbits than when the virus was injected alone and the compound given intraperitoneally. This would indicate that the effect was due to a localized action, such as inhibition of adsorption; however, Dickinson and Thompson (1957) showed that β-phenylserine did not inhibit the adsorption of influenza A (PR8) and

vaccinia viruses in tissue culture. In the rabbit, the action of vaccinia virus is confined, initially, to the prickle-cells (Scott, et al., 1950) and the decreased protective effect of intraperitoneally administered β-phenylserine may be a reflection of the inability of the compound to reach the avascular prickle-cell layer of the epidermis.

Until more is known of the role phagocytic cells play in the pathogenesis of rabies and other viral diseases it is difficult to speculate how a prophylactic effect of a chemical compound could be mediated through these cells. Undoubtedly some phagocytosis of virus particles by the cells of the reticulo-endothelial system must occur. One can assume that if an inhibitor of virus multiplication were increasing the phagocytic abilities of these cells this alteration in their activity might be reflected in the type and quantity of cells present at the site of the inflammatory reaction to the virus. Although the character of the inflammatory response to normal and infected mouse-brain suspensions injected intramusculalry into rats was different, there were no differences in the response of β-phenylserine treated and control rats to injected material.

The activity of β-phenylserine is probably directed against the intracellular multiplication of the virus. Dickinson and Thompson (op. cit.) found that β-phenylserine was acting during the first half of the latent period of influenza A virus multiplication in chorioallantoic membrane fragments. Furthermore, the

addition of phenylalanine reversed the inhibition. Although it is impossible to perform a one-step growth curve in our system one can state that β-phenylserine is acting on one of the earlier stages of virus replication as evidenced by the fact that protection is obtained only when the administration of the compound is begun before or 24 to 48 hours after injection of the virus.

Hosley (1955) was unable to show reversal by tyrosine of β-phenylserine inhibition of rabies virus in tissue culture of minced mouse brains. However, it has been demonstrated that phenylalanine can reverse the inhibition of other viruses grown in tissue culture in the presence of β-2 thienylalanine. (Brown, 1952; Morgan, 1954; Heggie and Morgan, 1956). Eagle (1957) stated that Hela, normal liver, and normal conjunctiva cells grown in tissue culture could not hydroxylate phenylalanine to tyrosine. Therefore, it is obvious that the mechanism of β-2 thienylalanine and β-phenylserine inhibition of viruses propagated in tissue culture is not related to the inhibition of phenylalanine hydroxylase as it is in bacteria. It is more likely that these compounds inhibit the incorporation or utilization of phenylalanine in tissue culture.

Although the inhibition of rabies virus in rats may be the result of a direct interference with the incorporation of phenylalanine or tyrosine into new virus protein, the fact that both phenylalanine and tyrosine reversed the inhibition indicates that there is a relationship between the two with which β-phenylserine interferes. Since the

rat forms most of its tyrosine form phenylalanine, β-phenylserine may be inhibiting phenylalanine hydroxylase. Compounds structurally related to β-phenylserine but lacking either the amino, hydroxy, or phenyl groups were not active in preventing the development of the disease.

Armstrong and Lewis (1950) have shown that the rat does not convert β-phenylserine into phenylalanine or tyrosine. Therefore, if β-phenylserine were inhibiting phenylalanine hydroxylase it would be necessary for the animal to retain the compound as such in the body long enough to allow it to act. This would appear to be the case for there was no increase in the excretion of glucuronides in rats treated with β-phenylserine. It seems unlikely that the rat is capable of excreting the unaltered compound. An understanding of the fate of β-phenylserine in the rat may provide insight into the mechanism of its action.

The inhibition of myxoma and vaccinia viruses in the rabbit has also been demonstrated. The inhibition of the latter was greatest when the virus and compound were mixed, thereby indicating that the adsorption of the virus was being inhibited. However, as I have pointed out, the compound might be acting intracellularly, the close proximity of virus, compound, and cell increasing the efficiency of the inhibition. β-phenylserine did not completely prevent the development of myxomatosis but it did retard its course. Evidently, when the virus reached a certain concentration in the animal the compound was no

longer effective. As in the case of rabies, the myxoma virus was inhibited by intraperitoneally administered β-phenylserine, and the mechanisms involved could be similar.

The role of xanthine oxidase in the formation of new virus is unknown. Although it has not been possible to show an increased activity in the brains of infected rats, we cannot say whether this is related to the actual participation of the enzyme in the formation of new virus or if the increase is due to the catabolic activity of inflammatory cells acting on nucleoproteins released by damaged cells. However, Bauer (1947) and Bauer and Bradley (1956) have presented evidence against this possibility. They showed that the extent of the increase in activity was a characteristic of each particular virus tested, and so, unless one assumes a quantization of the inflammatory response to each virus disease, the catabolic activities of phagocytic cells cannot entirely account for the increase.

It was not possible to demonstrate any effect of β-phenylserine on the xanthine oxidase activity of rat brain. Following the administration of this compound to normal animals there was no measurable change, and treated rats which had been injected with virus also showed no deviation from the normal. Therefore, it is impossible to state whether the compound's inhibitory action is directed against the xanthine oxidase activity of the brain to prevent its increase, or if the failure to detect an increase in treated animals is due to the inhibition of some other

mechanism which precedes the enzyme's increased activity. Xanthine oxidase is a transfering enzyme. The increased activity may be due to the oxidation of reduced groups in the virus; the purines of the damaged cells supplying the oxygen. Therefore, since β-phenylserine inhibition of the virus would result in a decreased amount of tissue destruction by the virus, the activity of the enzyme would not be increased.

In conclusion, it has been shown that β-phenylserine inhibited the development of rabies in rats. The evidence which has been presented seems to indicate that the inhibition of mechanisms involved in the intracellular multiplication of the virus was responsible for the effects observed. If this is the case, β-phenylserine should prove a valuable tool in the study of the in vivo multiplication of animal viruses.

SUMMARY

An investigation has been made on the effect of β-phenylserine on normal rats and rats injected with rabies virus. The following conclusions were drawn.

1. The daily intraperitoneal administration of 10 or 15 mg of β-phenylserine protected rats injected intramuscularly with rabies virus. The protective effect was most marked when the animals were treated for three days prior to the injection of the virus, although initiation of treatment 24 hours after the virus was injected also gave protection.
2. The administration of phenylalanine or tyrosine in combination with β-phenylserine reversed the inhibition of rabies virus.
3. Ring substitution or removal of the -OH or $-NH_2$ groups from the side chain rendered the compounds inactive.
4. β-phenylserine had no in vitro antirabies virus effect.
5. β-phenylserine apparently did not alter the histological character of the inflammatory response to the virus.
6. Treated animals challenged with a second injection of virus 6 to 7 weeks after the first did not show any apparent immunity.
7. The xanthine oxidase activity of infected rat brain increased as the disease progressed. The increased activity paralleled the appearance of virus in the brain. There was no alteration in the xanthine

oxidase activity when normal animals and animals injected with the virus were treated with β-phenylserine.

8. The alkaline phosphatase activity of infected rat brain was no different from the normal.
9. Animals injected with β-phenylserine did not show an increased excretion of glucuronides.
10. β-phenylserine inhibited the propagation of vaccinia virus in rabbit skin when the compound and virus were mixed prior to injection. Equivocal results were obtained when the compound was administered intraperitoneally to rabbits injected intradermally with vaccinia virus.
11. β-phenylserine injected intraperitoneally daily delayed the development of myxomatosis in rabbits.
12. β-phenylserine had no apparent effect on the multiplication of influenza A (PR8), Eastern Equine Encephalitis, poliovirus (Lansing strain), or mouse encephalomyelitis (FA strain) viruses in mice. The compound was also inactive against influenza A virus in embryonated eggs.

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