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# UNIVERSITY OF CINCINNATI

May 24 1944

*I hereby recommend that the thesis prepared under my supervision by* Isaac Ruchman

*entitled* The effect of fatigue, dehydration, pregnancy and nutritional deficiencies on the development of neutralizing antibodies and associated changes in cerebral resistance against the virus of Western equine encephalomyelitis.

*be accepted as fulfilling this part of the requirements for the degree of* Doctor of Philosophy

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# THE EFFECT OF FATIGUE, DEHYDRATION, PREGNANCY AND NUTRITIONAL DEFICIENCIES ON THE DEVELOPMENT OF NEUTRALIZING ANTIBODIES AND ASSOCIATED CHANGES IN CEREBRAL RESISTANCE AGAINST THE VIRUS OF WESTERN EQUINE ENCEPHALOMYELITIS

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requirements for the degree of

DOCTOR OF PHILOSOPHY

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I.R.

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## Introduction

Resistance to infection may be either natural or acquired, the principal difference being that in the acquired type the body produces specific antibodies. They are formed in response to antigenic stimulation and manifest themselves as complement fixing, agglutinating, precipitating or neutralizing antibodies. The presence of neutralizing antibodies are detected by neutralization tests. Up to the present time these tests have remained the only method of determining past or present exposure to many of the diseases caused by filterable viruses as well as the presence or absence of past infection. Since neutralizing antibodies are used to a large extent as indicators of immunity in virus diseases, it becomes of importance to investigate those conditions which could conceivably affect the production of neutralizing substances. Therefore, the influence of the following factors on immunity has been selected for investigation: 1. Fatigue, 2. dehydration, 3. pregnancy, and, 4. undernourishment, either partial or complete. Furthermore, according to many investigators the presence of neutralizing antibodies and immunity to virus infection do not necessarily parallel each other and an attempt has been made in certain instances to correlate the level of antibody attained with the degree of cerebral resistance.

The virus of Western equine encephalomyelitis (W.E.E.) was selected for these investigations for the following reasons: 1. Mice vaccinated with W.E.E. respond by producing a high level of antibody: 2. These antibodies are formed in a comparatively short period of time, reaching their maximum titre in approximately two weeks: 3. Such

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vaccinated mice develop a high degree of active immunity: and 4. the relationship between antibodies and cerebral resistance in mice after W.E.E. has been fairly well studied.

Western equine encephalomyelitis virus has all of the characteristics of those obligatory intracellular parasites known as filterable viruses. These requirements are: 1. the ability to multiply only in the presence of living susceptible cells; 2. the ability to traverse filters which will retain most bacteria; 3. the invisibility under the ordinary microscope; and, 4. the failure to respond to sulfonamide therapy.

Although W.E.E. has probably been present in this country for many years, it was first recognized as a seperate entity in 1931 (1), when it was found to be the cause of an epizootic among horses and mules in California. Since then cases have been reported from various parts of the country as well as abroad. More recently W.E.E. has been found to attack human beings with subsequent invasion of the central nervous system. Recovery is followed by the production of specific substances in the blood stream which inhibit the activity of the virus. This virus is one of a small group which causes encephalitis in man, the others being Eastern equine, St. Louis, Japanese B and Spring-Summer. All five have been isolated and they have several features in common: 1. They can cause epidemics in man: 2. They occur chiefly during the summer months: 3. The virus is found in the blood stream early in the disease; and 4. there is good evidence that they are transmitted by insects.

The virus of W.E.E. is strictly neurotropic and when experimentally injected into mice tends to localize in the central nervous

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system causing encephalitis and death. After peripheral inoculation of sufficient virus, if death does not ensue, the animal responds with a rapid production of antibodies. These protective substances are likewise produced after the peripheral injection of sufficient amounts of formalin-inactivated virus. By the use of such vaccines, it was found that the protective capacity of the serum and the active immunity of the animals were equal in degree to that induced by active virus (2). It was also observed that as the micegrew older they showed not only an increased resistance to peripheral inoculation of active virus (3), but also an increased ability to develop an immune response after the injection of formalin-inactivated virus (4). Antibodies appear as early as the fourth day, attain their peak a few days later and maintain a slightly diminishing level for several months (2, 5). The resistance curve roughly parallels the antibody curve.

A search through the literature has disclosed the fact that some work had been done on the problem of establishing a relationship between resistance to virus infection and certain specific factors, such as undernutrition, dehydration, pregnancy and fatigue. The effect on antibody formation after virus antigen stimulation, however, has been almost completely neglected. In the studies to be presented only those factors have been selected which from the literature appeared most likely to exert an influence. Fatigue has recently been implicated in poliomyelitis and has been chosen for that reason. The effects of dehydration have been offered as a cause for increased susceptibility to infection-- also decreased susceptibility. Pregnancy seems to influence antibody production, but its effect on active immunity is still obscure.

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The close relationship between resistance to infection and undernourishment has long been observed, but the reasons are still not evident. It has been postulated that some of the members of the "B" complex function in the enzyme-coenzyme systems which catalyze oxidations in the tissues. In some manner the "B" complex vitamins are involved in the intermediary metabolism of the body and thus maintain the health of the tissues generally. Thiamin specifically is concerned with many functions which influence the general health of the individual. Loss of appetite, polyneuritis, decreased gastric motility, faulty carbohydrate metabolism are some of the conditions ascribed to thiamin deficiency. Riboflavin is essential for maintaining the health of the cells and it, too, has been accused of exerting an effect on immunity to infection. Some attention has been given to protein deprivation on resistance to infection. It is an established fact that antibodies, regardless of their method of formation, are protein in nature, and it is, therefore, quite possible that the protein intake may influence the antibody production and determine the immunity of the host.

Finally, carbohydrate metabolism is carried on to a large extent in the liver. Since the liver is one of the chief sites of antibody formation, it may not be beyond the realm of possibility to influence antibody production by a decreased carbohydrate intake.

In this study only those antibodies have been considered which are produced as a result of antigenic stimulation. This would omit such natural antibodies as complement, bactericidins, opsonins and phagocytes, but would include agglutinating, precipitating, com-

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plement fixing and neutralizing antibodies. As far as neutralizing antibodies are concerned no distinction will be made between those formed after antigenic stimulation and those formed otherwise.

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## Methods and Materials

Virus.-- The strain of Western equine encephalomyelitis (W.E.E.) virus used in these experiments was obtained from Doctor P. K. Olitsky and has been maintained by numerous mouse brain passages. At first, infected mouse brains were stored in 50 per cent glycerol, but more recently the source of supply has been either material kept frozen in dry ice or material lyophilized from the frozen state. Virus maintained in the frozen state was found to be better suited to our purpose and was prepared as follows. The brains of mice succumbing to intracerebral injection of W.E.E. virus were removed under aseptic conditions, ground up with sand and enough undiluted, inactivated normal rabbit serum was added to make a 10 per cent suspension. After centrifugation at 1500 r.p.m. for 5 minutes, the supernate was drawn off and distributed in 1 cc. amounts in glass sealed ampules. These were quickly frozen in a dry ice-alcohol mixture and then stored in a dry ice box. Only those suspensions were used which were negative on culture and which titred about 8.5 before freezing. The keeping quality of the virus was found to be much enhanced when stored in undiluted serum. For use, an ampule was thawed rapidly and the suspension designated $10^{-1}$ or 1:10. Dilutions were prepared in physiological saline to which had been added enough undiluted, inactivated normal rabbit serum to make a final concentration of 10 per cent. This was found to be a more satisfactory diluent than broth.

When a 20 per cent virus suspension was required, only half the amount of diluent was used; otherwise the procedure was the same as above.

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In the studies on the factors influencing immunity one ampule of 10 per cent virus was sufficient for the two types of intracerebral neutralization tests as well as the cerebral immunity test performed on the same day. Thus, for convenience, the following general procedure was used. The ampule was thawed and dilutions of 1:50, 1:500, etc., were prepared for the regular neutralization test. These were distributed, the sera added and the mixtures set in the 37° C. water bath. Next a dilution of 1:2,000,000 was made which was added to the series of serum dilutions and these mixtures were incubated at 37° C. During the two hour incubation period serial tenfold dilutions of the virus were injected intracerebrally into the vaccinated mice as well as the controls.

Mice.-- All of the mice employed in these investigations were of the same strain of albinos obtained locally, and most of the animals were bred and reared in our own laboratory. Unless otherwise specified the mice were given adequate amounts of purina dog chow checkers, which were kept suspended from the side of the cage by a wire basket. Water in liberal amounts was supplied from a bottle. Mice used for the intraperitoneal neutralization tests were between 14 and 15 days old and of either sex. Such mice weighed in the neighborhood of 7 grams. For the preparation of virus or vaccines, mice approximately 3 weeks old were used, also without regard to sex. For the intracerebral neutralization tests the mice ranged in age from 3 to 5 weeks. The mice to be vaccinated and their controls were those selected from among healthy, non-pregnant virgin females. This was accomplished by segregating the females at an early age. For most experiments the females were used when they were 8 weeks old or a little over and weighed between 20 and 23 grams.

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Immunization.-- The procedure adopted for the vaccination of mice was reached after preliminary work to be described later. W.E.E. mouse brain suspension inactivated with 0.4 per cent formalin was selected as the immunizing agent for the tests to be described under the heading, experimental. With one exception the dosage in these same tests was 0.3 cc. intra-abdominally on 3 separate days with a 48 hour interval between injections (0.3 x 3). In one series of tests a single dose of 0.3 cc. (0.3 x 1) was administered intra-abdominally. The reason for the choice of vaccine and dosage will be discussed under the heading orientation. In all cases only one day's supply of formalinized vaccine was removed from the stock bottle at a time. Four mg. of powdered $NaHSO_3$ was added to each cc. of vaccine to neutralize the effects of the free formalin still present in the suspension.

Sera for Neutralization Tests.-- Two weeks after the initial dose of vaccine, the mice were bled from the heart under light ether anesthesia. Only sufficient blood was obtained from each group of mice to perform the tests. In this way as many animals as possible were saved. However, some animals did die after the cardiac puncture and in some instances the number of animals remaining was small. The bleedings from each group were pooled in a long, narrow centrifuge tube, allowed to clot at room temperature and the serum drawn off after centrifugation. Sera were tested for neutralizing capacity on the following day and the remainder was frozen in the dry ice box. With the exception of a few intraperitoneal neutralization tests, all sera were tested the day after bleeding.

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Neutralization Tests.— For the conventional neutralization test with undiluted serum the following procedure was adopted. An ampule of virus was thawed rapidly. From this 1:10 suspension dilutions of 1:50, 1:500, etc., up to 1:5,000,000,000 were prepared in 10 per cent rabbit serum saline, a separate pipette was used for each dilution. Next 0.15 cc. amounts of the proper virus dilution was added to 0.15 cc. amounts of undiluted serum and the mixtures thoroughly shaken. These mixtures were kept in an ice bath until all were ready and then incubated for 2 hours, in a 37° C. water bath. At the end of this time the mixtures were returned to the ice bath and then injected intracerebrally into mice under light ether anesthesia. To facilitate delivery of 0.03 cc. intracerebrally, 0.25 cc. tuberculin syringes fitted with 27 gauge needles were used. With some exceptions 4 mice were employed for each dilution. Highest dilutions were injected first and the controls were done last. The mice were observed for 10 days and then discarded. Those dying within 24 hours after inoculation were regarded as non-specific deaths and were not included in the results.

As a further test for antibody in the sera of vaccinated mice the procedure of Morgan and Olitsky (2) was utilized. Dilutions of serum were tested against constant amounts of virus. To this end serial two-fold or four-fold dilutions of the serum to be tested were prepared in undiluted normal rabbit serum which was inactivated at 56° C. for one-half hour. To each of the serum dilutions was added an equal amount (0.15 cc.) of a virus suspension containing about 50 $LD_{50}$ doses of active virus. This was prepared in the following manner. From the same frozen ampule of virus, which was used for the conventional neutralization test described above enough suspension was withdrawn to make a 1:2,000,000

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dilution in 10 per cent rabbit serum saline. This when added to the serum dilution became 1:4,000,000 or the log of 6.6. Since the frozen virus suspension titred 8.3, then 8.3 minus 6.6 give 1.7, whose anti-log equals 50, the number of $LD_{50}$ doses required. Sometimes the control titre was above or below 8.3 so that the number of $LD_{50}$ doses ranged between 25 and 80. From this point on the procedure was the same as for the neutralization test. The mixture containing the 50 $LD_{50}$ doses and the proper serum dilution was shaken, incubated at 37° C. for 2 hours and then injected intracerebrally into 4 mice.

For the intraperitoneal neutralization tests (8) virus dilutions of 1:50, 1:500, etc., were prepared in 10 per cent rabbit serum saline as before. Later in the investigations a 20 per cent suspension of virus was prepared so that dilutions of 1:5, 1:50, etc., were obtained. The virus dilutions were added to equal amounts of undiluted serum, the mixtures thoroughly shaken and 0.03 cc. immediately injected intra-abdominally into 14 to 15 day old mice. The mice were observed for 2 weeks and then discarded.

Cerebral Resistance Tests.— On the same day the neutralization tests were performed, the mice that had supplied the sera for the tests, vaccinated as well as controls, were subjected to an intracerebral resistance test. Serial ten-fold dilutions of virus prepared in 10 per cent rabbit serum saline were injected intracerebrally. Ether anesthesia was used and 0.25 cc. tuberculin syringes were used to inject 0.03 cc. The highest dilutions were injected first and controls for a dilution were injected last. Animals were observed for 21 days and those dying within 24 hours were regarded as non-specific. Passage was resorted to

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in order to establish the cause of death among some vaccinated animals. In such cases the brain was removed aseptically and, if a Gram's stain revealed no contaminating organisms, the brain suspension was cultured on a blood agar plate and then injected intracerebrally into 3 mice. A mouse brain which was sterile on culture and which caused central nervous system involvement and death in two days after passage, was regarded as positive and, therefore, specific for W.E.E. In some cases where the brain had been cannibalized the spinal cord was used for passage.

Calculations According to Reed and Muench.-- The results of both neutralization and cerebral resistance tests were calculated according to the Reed and Muench method (6). In the case of a simple titration the $LD_{50}$ titre was expressed as the logarithm of the dilution where 50 per cent of the animals succumbed. Where several titrations were done to compare different groups of mice, such as cerebral resistance tests, the immunity index was obtained. This was expressed as the ratio between the $LD_{50}$ titre of the controls and the $LD_{50}$ titre of the test animals. Thus the immunity index of the controls was always 1. For neutralization tests in which undiluted serum was used, the method was the same. The $LD_{50}$ titre was figured for each serum and the ratio between the controls and test animals computed. This is called the neutralization index and shows how many $LD_{50}$ doses were neutralized. Again the index of the controls was always 1.

For estimating the fifty per cent end point of a serum when tested against a constant amount of virus, the method is that described by Reed and Muench for a protective serum. The final value obtained

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represents the highest calculated serum dilution which could still protect at least half the mice.

Vaccine.-- In the studies on the factors influencing the production of immunity the vaccine employed was a formulinized mouse brain suspension. The preparation has been described for two other types of encephalitis virus and is essentially the same (7). Mice 18 to 21 days old are injected intracerebrally with a $10^{-6}$ dilution of frozen W.E.E. virus. Two days later the mice showing nervous symptoms are chloroformed and then exsanguinated by cutting the heart. The brains are removed aseptically and dropped into cold saline solution to remove any adherent blood. The brains are weighed after the saline has been drained off and are then ground in a mortar. Sand is used as an abrasive. Cold physiological saline is added in the proportion of 9 cc. for each gram of mouse brain. The crude suspension is next filtered through enough layers of gauze to remove gross particles and sand. Some of the filtrate is removed for culture and intracerebral titration in mice. To the remainder 0.4 per cent by volume of the concentrated forty per cent formaldehyde is added and the mixture is thoroughly shaken. This vaccine is stored in the refrigerator and shaken daily. When first used for vaccination it is tested intracerebrally in mice in order to determine whether or not all the virus has been inactivated. Four mg. of powdered $NaHSO_3$ is added to each cc. of vaccine. This amount is not sufficient to neutralize the formaldehyde still present as tested by the Schryver method, but, nevertheless, is sufficiently effective for inoculation.

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Three different batches of formalinized W.E.E. mouse brain vaccine were prepared in the manner described. An infectivity test to determine whether the virus had been completely inactivated was performed on each batch two days prior to its first use. In all cases these tests were negative. To be effective, a vaccine before the addition of formalin should have titred at least 8.0. Reference to Table I reveals that the three lots of virus used to make the vaccines titred 8.6, 8.8 and 8.5 respectively.

Fatigue.-- Non-pregnant virgin female mice weighing between 21 and 23 grams were placed in a revolving drum which had a diameter of 15 inches and made 13 revolutions per minute. The animals were kept in the rotating drums for three hours in the morning and an additional three hours in the afternoon. There was a rest period of about one hour between the three hour runs during which time the mice were allowed food and water. It was calculated that the animals ran a little over three and a half miles each day. Animals in these experiments were exercised either one or two weeks. The one week animals were fatigued for seven consecutive days and then allowed to rest for one week. At the end of this time they were bled and tested for immunity. The two week group was fatigued for fourteen consecutive days and then bled and tested in the same manner. The three doses of vaccine were made to coincide with the first, third and fifth days of fatigue. Vaccinations were made after the animals had received about two hours of exercise. In one series of tests only one dose of vaccine was employed. When this occurred it was injected on the first day of fatigue, and also after the animals had been two hours in the drum.

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Table I

Formalinized W.E.E. Mouse Brain Vaccines

| Vaccine batch | Date Prepared | Amount Prepared cc. | Titre before formalin-ization | Test for infectivity |
|---|---|---|---|---|
| I | 6/23/43 | 75 | 8.6 | 5 days - negative |
| II | 7/23/43 | 147 | 8.8 | 24 days - negative |
| III | 9/20/43 | 271 | 8.5 | 9 days - negative |

The negative tests for infectivity indicate when vaccine was first used and do not necessarily mean when infectivity was first lost.

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Dehydration.-- Mice to be dehydrated were placed in individual compartments with wire mesh bottoms sufficiently large to allow feces and urine to pass through. Liberal amounts of purina were allowed each mouse, but the water supply was restricted. Preliminary work had demonstrated that adult mice could go for 3 to 4 days without water, but would die shortly thereafter. Consequently the following procedure was adopted. The mice were numbered and their individual weights were recorded daily. Water was withheld for three days until the average weight of the mice was between 14 and 15 grams. Then a measured amount of water was given each mouse. To do this, graduated 10 cc. pipettes whose tips were broken were utilized and the required amount of fluid drawn up by means of a rubber bulb which was left on the pipette. It was then inserted into the mouse compartment and usually the thirsty animal drank all of the liquid within a few minutes. Between 0.5 cc. and 1.5 cc. was given to each animal daily depending on the weight recorded for that day. In general the mice maintained their weight around 14 grams when they were allowed to drink 1 cc. of water each day and as a rule survived if they managed to stay above 13 grams.

Pregnancy.-- Virgin females weighing between 22 and 24 grams were selected and distributed 3 to a cage. A healthy male of the same stock was added to each cage. The females were numbered and vaginal smears were taken and examined every morning. The presence of sperm in the vaginal tract was regarded as the first day of insemination. Subsequently daily smears were made and individual weights recorded as a check on the continued pregnancy. The date of delivery was recorded and in nearly every case the period of gestation was 19 days. The young were allowed

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to remain with their mothers throughout the experiment. Pregnancy was established in those mice that eventually gave birth. However, in the first experiment three females that did not give birth were included among the pregnant mice. They were animals that had become inseminated as evidenced by the presence of sperm and subsequent cessation of the estrus cycle as well as a mounting weight curve. As a result of the vaccination these three mice resorbed their young 3 to 5 days after the third dose of vaccine. Evidence of resorption of young was borne out by the resumption of the estrus cycle as well as a sharp drop in the weight curve.

Underfeeding.-- Purina dog chow was the diet chosen for the experiments on underfeeding. In these tests mice were placed in individual compartments containing wire mesh bottoms to allow passage of feces. The solid dog chow was selected because it could be weighed out in one piece and would not fall through the wire floor. The analysis of the dog chow on the label is given below:

Crude protein not less than. . . . . . . . . .21.0%
Crude fat not less than. . . . . . . . . . . . 4.0%
Crude fibre not more than. . . . . . . . . . . 6.0%
Nitrogen free extract not less than. . . . .46.0%

It is said to contain the following ingredients: Meat, meal, dried skimmed milk, wheat germ, barley malt, dried beet pulp, corn grits, cereal feed (from corn and wheat), dried raisins, soybean oil meal, molasses, riboflavin supplement, brewer's dried yeast, Vitamin A and D feeding oils, 1% steamed bone meal, 1% iodized salt.

The animals were numbered and placed in their individual cubicles. Individual weights were taken on the mice daily and a weighed amount of food was given each mouse. Preliminary work had demonstrated

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that mice could go for 2 to 3 days without food. After that time there was slight chance of survival. Therefore, the food was withheld for the first 2 days until the weight had dropped to around 14 grams. Then the measured amounts of food were given to maintain the weights of the animals at this level. On the third day of starvation about one gram of food was given to each mouse. This was increased on subsequent days until about 3 grams of food were consumed daily by each mouse, which is slightly less than the required minimum of 4 grams daily per mouse to maintain a body weight of a little over 20 grams. Of course, these are all average figures so that more or less food was given to each individual mouse, depending on the body weight for the day. Water was given *ad libitum*.

Synthetic diets.-- The synthetic diet employed in these experiments was that used by Woolley (9). It was found to be adequate for mice.

| | |
|---|---|
| Sucrose | 76 gm. |
| Vitamin-free casein | 18 gm. |
| Salts | 5 gm. |
| Fortified corn oil | 1 gm. |
| Thiamin | 200 Y |
| Riboflavin | 500 Y |
| Nicotinic Acid | 10 mg. |
| Pyridoxine | 200 Y |
| Calcium pantothenate | 2 mg. |
| Choline | 10 mg. |
| Inositol | 100 mg. |

Salts (10)

| | |
|---|---|
| NaCl | 335 |
| $K_2HPO_4 \cdot 3H_2O$ | 645 |
| $Ca_2H_2(PO_4)_2 \cdot 4H_2O$ | 190 |
| $MgSO_4 \cdot 7H_2O$ | 204 |
| $CaCO_3$ | 600 |
| $Fe(C_6H_5O_7)_2 \cdot 6H_2O$ | 55 |
| KI | 1.6 |
| $MnSO_4 \cdot 4H_2O$ | 0.7 |
| $ZnCl_2$ | 0.5 |
| $CuSO_4 \cdot 5H_2O$ | 0.6 |
| | 2232.4 gms. |

Fortified Corn Oil (11)

| | |
|---|---|
| Corn oil | 100 gm. |
| Vitamin A concentrate[1] (200,000 USP units) | 1 gm. |
| Viosterol (10,000 USP units) | 1 gm. |
| Concentrate of Natural mixed tocopherols[1] | 1 gm. |
| 2 Methyl 1-4 Naphthoquinone | 10 mg. |

[1] Kindly supplied by Dr. P. L. Harris, Distillation Products, Inc.

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Mice were placed in boxes equipped with screen bottoms and the prescribed diet given in liberal amounts. Group weights were taken daily and the average weights recorded. For optimum results fresh diets were prepared each week and kept in the refrigerator when not in use. The ration utilized was Woolley's diet slightly modified to meet the needs of certain experiments in which deficiencies were required. The diets are listed below.

1. Synthetic or adequate diet.-- This diet was prepared according to the formula with no modifications. Mice on this diet did as well as animals on the stock purina diet.

2. "B" deficient diet.-- This was the adequate diet from which the "B" components (thiamin, riboflavin, nicotinic acid, pyridoxine, calcium pantothenate, choline and inositol) had been omitted. On this diet mice lost weight rapidly and died in 3 to 4 weeks.

3. Thiamin deficient diet.-- This was the adequate ration with the exception that $B_1$ was completely lacking until near the end of the experimental period. Then in order to keep the mice alive they were put on a diet containing 40 Y thiamin per 100 gm. of ration. The adequate diet contained 200 Y although mice can get along with 80 Y (12). On this formula the animals did not increase in weight during the first weeks at a time when the control mice were gaining in weight. Then they began to lose weight and at 3 weeks were showing such obvious symptoms as hunching, weakness, tremors and spasticity. It was at this point that 40 Y thiamin was included in the diet to reduce the mortality.

4. Riboflavin deficient diet.-- On this diet which contained no riboflavin, mice showed no gain in weight for 2 weeks and then very

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slowly began to lose weight. They were still losing weight at the end of six weeks when the experiment was terminated. No obvious signs were noted other than those which accompany weight loss.

5. Carbohydrate deficient diet.-- In this experiment the sucrose, which was the source of the carbohydrate was replaced by casein, otherwise the formula remained unchanged. The additional casein was equal in weight to that of the replaced sucrose and consequently the diet was light and fluffy. This last may be a possible explanation for the failure of the mice to put on weight on this diet. It is known that removal of carbohydrate from a diet leads to no weight reduction. Toward the end of the experiment, however, the weight of the mice rose almost to their former level.

6. Protein deficient diet.-- Since casein was the source of protein, it was replaced by a weight of sucrose equal to that of the replaced casein. Mice on such a deficient diet lost weight steadily from the very first day and began to die at the end of three weeks. They showed symptoms related to the loss of weight. The animals were thin, somewhat hunched and scraggly, but active and spry at all times.

Finally, it should be mentioned that in some cases in order to conserve mice, one set of control animals acted as controls for more than one factor at a time.

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## Orientation

The following experiments were performed before the choice of a vaccine or the proper dosage had been made. Not until after these tests had been completed, was the decision made to use the mouse brain vaccine administered in 3 separate doses for optimum results. For convenience in the early experiments a commercial W.E.E. vaccine prepared by Lederle Laboratories[2] was used. This vaccine, stated to contain chick embryo preserved with 0.35 per cent phenol and 0.1 per cent formalin, was found to be effective in protecting against W.E.E. In the experiments to be reported it will be referred to as the Lederle vaccine. The mouse brain vaccine used for comparison in the latter half of these experiments is the one used in studying the effect of the various physiological factors on immunity in the main body of the thesis. Only the more important experiments have been selected and these will be described briefly.

The first experiment was set up in order to determine the neutralization range over varying periods of time. A large group of mice was given one dose of the Lederle vaccine, 0.3 cc. intra-abdominally. They were bled 3, 4, 7, 10 and 14 days after vaccination and the sera stored in dry ice. All of the sera were tested simultaneously and the final results are set forth in Table II. Antibodies appeared as early as the 3rd day and remained at a rather low titre until the 14th day when an index of 1,600 was obtained. From this response it was apparent that a minimum of 2 weeks was necessary for a good antibody response.

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[2] Kindly supplied by Dr. H. R. Cox, Lederle Laboratories, Inc.

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Table II.

Development of Neutralizing Antibodies after Intraperitoneal Vaccination with Lederle W.E.E. Vaccine.

Neutralizing Antibodies (intracerebral method)
Mixtures at 37° C. for 2 hours

| Serum in Mixture | Days after vaccination bled | Dilution of virus in mixture | | | | | | | $LD_{50}$ titre | Neutralization index* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | $10^{-10}$ | | |
| Control rabbit | -- | | | | 3/3# | 2/3 | 1/3 | 0/3 | 8.5 | -- |
| Vaccinated mouse 0.3 cc. intra-abdominally | 3 | | | 3/3 | 2/3 | 1/3 | 0/3 | | 7.5 | 10 |
| | 4 | | | 3/3 | 1/3 | 0/3 | 0/3 | | 6.7 | 60 |
| | 7 | | 3/3 | 3/3 | 0/2 | 0/3 | | | 6.5 | 100 |
| | 10 | 3/3 | 3/3 | 3/3 | 1/3 | | | | 6.7 | 60 |
| | 14 | 3/3 | 2/3 | 0/3 | 0/3 | | | | 5.3 | 1600 |

$$* \text{ Neutralization Index} - \frac{LD_{50} \text{ titre of controls}}{LD_{50} \text{ titre of vaccinated mice}}$$

# 3/3 denotes 3 mice out of 3 inoculated died with the disease.

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It then became necessary to determine the potency of serum in another way, that is, by testing dilutions of serum against dilutions of virus. A large group of mice received 0.3 cc. of Lederle vaccine intra-abdominally. They were bled at 1, 2 and 3 weeks and the sera drawn off. Ten-fold dilutions of these sera were prepared in normal undiluted rabbit serum. Both the undiluted and diluted vaccinated mouse sera were tested for neutralization in the usual manner. The summarized results are presented in Table III and they show first, a low neutralization index for the undiluted serum, and second, a complete disappearance of the antibody when the serum is diluted at least ten times. In the 3 week test serum diluted 1:10 still afforded some protection, but in general the conclusion may be drawn that the low titre of antibody present in undiluted serum is almost or completely lost on dilution.

After the inability of one dose of vaccine to elicit a strong antibody response had been demonstrated, it seemed reasonable to test the effect of varying doses on the immune response. Groups of animals were given vaccine according to the schedule in Table IV and then bled and tested at 1 week, 2 weeks and 4 weeks. The results shown in Table IV again demonstrate that in general neutralization indexes were low for the undiluted serum and that diluted serum became ineffective in neutralizing W.E.E. virus except in low dilution. Examination of the serum $LD_{50}$ column shows that low serum dilutions protect against 50 $LD_{50}$ doses of virus in the one week test and against 32 $LD_{50}$ doses of virus in the 2 week test. The 4 week test revealed somewhat higher values, but results were not consistently high and more important this period of time was impractical. The broad conclusion to be drawn from

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Table III

Development of Neutralizing Antibodies after Intraperitoneal Vaccination with Lederle Vaccine

Vaccination: one dose 0.3 cc. i.p.
Neutralizing Antibodies (i.cer. method)

| Serum in mixture | Dilution of serum in mixture | Neutralization Index of Serum | | |
|---|---|---|---|---|
| | | One week test | Two weeks test | Three weeks test |
| Vaccinated mice | Undiluted | 150 | 60 | 150-* |
| 0.3 cc. intra-abdominally | 1:10 | -4# | -2 | 10 |
| | 1:100 | -4 | -2 | 4 |
| | 1:1000 | 2- | -2 | -2 |

* 150- signifies no end point

\# -4 signifies $LD_{50}$ titre higher than that of control

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Table IV

Development of Neutralizing Antibodies after Intraperitoneal Vaccination with Varying Doses of Lederle Vaccine

Vaccination Schedule:
- 0.5 cc. I.P. one dose....................0.5 x 1
- 0.3 cc. I.P. one dose....................0.3 x 1
- 2 doses 0.5 cc. I.P. 2 days apart.........0.5 x 2
- 2 doses 0.3 cc. I.P. 2 days apart.........0.3 x 2
- 2 doses 0.15 cc. I.P. 2 days apart........0.15 x 2
- 3 doses 0.2 cc. I.P. 3 successive days....0.2 x 3
- 3 doses 0.1 cc. I.P. 3 successive days....0.1 x 3

Neutralizing Antibodies (i.cer. method)

| Interval mice bled | Vaccination I.P. | Neutralization index of serum | Neutralizing antibodies--using serum dilutions | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | $LD_{50}$ doses used | Serum diluted | | | | Serum $LD_{50}$ |
| | | | | Und. | 1:5 | 1:10 | 1:20 | |
| One week | 0.5 x 1 | 60 | | 1/5 | --- | 5/5 | 5/5 | 1:2 |
| | 0.3 x 1 | 250 | 50 | 3/5 | --- | 5/5 | 5/5 | <1:1 |
| | 0.5 x 2 | 400 | | 0/5 | --- | 3/5 | 5/5 | 1:7 |
| | 0.3 x 2 | 650 | $\left(\frac{8.2}{6.5}\right)$ | 0/5 | --- | 5/5 | 4/5 | 1:4 |
| | 0.15 x 2 | 500 | | 0/5 | --- | 5/5 | 4/5 | 1:4 |
| | 0.2 x 3 | 60 | | 1/5 | --- | 5/5 | 5/5 | 1:2 |
| | 0.1 x 3 | 60 | | 2/5 | --- | 4/5 | --- | 1:2 |
| Two weeks | 0.5 x 1 | 650 | | 1/4 | 3/4 | 4/4 | --- | 1:2 |
| | 0.3 x 1 | 300 | 32 | 3/4 | 3/4 | 4/4 | --- | <1:1 |
| | 0.5 x 2 | 100 | | 0/4 | 1/4 | 4/4 | --- | 1:6 |
| | 0.3 x 2 | 100 | $\left(\frac{8.0}{6.5}\right)$ | 1/4 | 2/4 | 2/4 | --- | 1:6 |
| | 0.15 x 2 | 300 | | 1/4 | 1/4 | 3/4 | --- | 1:6 |
| | 0.2 x 3 | 1000 | | 2/4 | 3/4 | 4/4 | --- | 1:1 |
| | 0.1 x 3 | 16- | | --- | --- | --- | --- | |
| Four weeks | 0.5 x 1 | 150 | 32* | 2/4 | --- | 3/4 | 3/4 | 1:2 |
| | | | 100* | 1/4 | 2/4 | 3/4 | --- | 1:5 |
| | 0.3 x 1 | 300 | 32 | 0/4 | --- | 1/4 | 1/4 | 1:20 |
| | | | 100 | 0/4 | 2/4 | 2/4 | --- | 1:7 |
| | 0.5 x 2 | 600 | 32 | 0/4 | --- | 0/4 | 2/4 | 1:20 |
| | | | 100 | 0/4 | 1/4 | 1/4 | --- | 1:10 |

* 32 $LD_{50}$ doses - $\frac{8.5}{7.0}$

* 100 " " - $\frac{8.5}{6.5}$

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these experiments was that the Lederle vaccine was ineffective in inducing the formation of strong neutralizing antibodies. Further evidence to substantiate this claim was found in the next experiment.

Since Morgan and Olitsky (2) had demonstrated that sufficient dosage of formalinized virus could evoke an immune response in mice equal to that obtained after active virus, it now appeared desirable to repeat their work using the Lederle vaccine as the source of formalin inactivated virus. Consequently one group of mice received 6 intra-abdominal injections of 0.25 cc. (0.25 x 6). Injections were made on the first 3 days of the first week and first 3 days of the second week. The animals were bled 2 weeks after the first dose was given. For comparison a second group of mice was vaccinated with living virus. They received 2 intra-abdominal injections of 0.25 cc. of $10^{-3}$ dilution one week apart (0.25 x 2) and were bled 2 weeks after the first dose.

The results, shown in Table V, demonstrate the inability of mice vaccinated with the Lederle vaccine to produce an immune response at all comparable to that of animals vaccinated with active virus. In fact, under comparable circumstances the values after the formalinized virus are much lower than those reported by Morgan and Olitsky after vaccination with formalinized virus. The results of the intracerebral immunity test showed a distribution of deaths among the Lederle vaccinated mice over a wide range of virus dilutions and no sharp end point. This is strongly indicative of a low grade immunity. Finally, in the test for neutralizing antibodies by the intraperitoneal method in young mice no difference was apparent between both groups of vaccinated mice.

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Table V

Immunogenic Capacity of W.E.E. Virus
Comparison of Formalinized (Lederle) Virus and Living Virus

1. Neutralizing Antibodies--I.Cer. method

| Vaccine | Dosage | Neutralization Index * | Serum $LD_{50}$ Protecting | |
|---|---|---|---|---|
| | | | against 10 $LD_{50}$ doses | against 50 $LD_{50}$ doses |
| Living virus | 0.25 x 2 | 1000 | 1:790 | 1:40 |
| Form. virus | 0.25 x 6 | 400 | 1:60 | 1:10 |

* Control titre 8.3

2. Intracerebral Immunity

| Group of Mice | 0.03 cc. intracerebrally | | | | | | | | | $LD_{50}$ titre | Immunity Index |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | | |
| Unvac. Controls | - | - | - | - | - | - | 3/3 | 3/3 | 1/3 | 8.7 | --- |
| Vac. Living virus 0.25 x 2 | 3/4 | 0/3 | 0/3 | - | - | - | - | - | - | 1.3 | 25,000,000 |
| Vac. Form. virus 0.25 x 6 | 2/3 | 1/3 | 2/3 | - | - | - | - | - | - | $2.0^{+}$ | $5,000,000^{-?}$ |

3. Neutralizing Antibodies--I. Peritoneal method
0.03 cc. I.P. -- 14-15 day mice

| Serum in Mixture | Dilution of virus in mixture | | | | | | | | | $LD_{50}$ titre | Neutralization Index |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1:20 | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | | |
| Unvac. Controls | - | - | - | - | - | 2/3 | 3/3 | 2/3 | 0/3 | 8.0 | --- |
| Vac. Living virus | 2/3 | 1/3 | 0/3 | 0/3 | 0/3 | - | - | - | - | 1.7 | 2,000,000 |
| Vac. Form. virus | 2/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | - | - | - | 1.5 | 3,000,000 |

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However, this method is not sufficiently sensitive to pick up quantitative differences in antibody content as will be seen from this test as well as others.

Since the Lederle vaccine was ineffective in producing a strong immunity against the strain of virus used throughout all these experiments, it next seemed logical to test another vaccine. Therefore, vaccine was prepared from W.E.E. infected mouse brains according to the directions in methods and materials and its immunizing potentialities compared with that of the Lederle vaccine. One group of mice received the Lederle vaccine while a second received the mouse brain vaccine. Both groups received 6 intra-abdominal injections (0.25 x 6) as before and were then tested 2 weeks after the first dose.

Table VI shows the decided superiority of the mouse brain vaccine over the Lederle vaccine when tested for neutralization by the intracerebral method. The neutralization index induced by the mouse brain vaccine was much higher than that induced by the Lederle vaccine being 3,200 against 800. The serum $LD_{50}$ of 1:500 against 10 $LD_{50}$ doses and the serum $LD_{50}$ of 1:44 against 50 $LD_{50}$ doses for the mouse brain vaccine was significantly higher than the respective values of 1:50 and 1:11 for the Lederle vaccine. The neutralization index by the intraperitoneal method revealed no difference between the 2 vaccines, thus confirming the previous observation of the inadequacy of this method of testing for quantitative differences in neutralizing antibodies. The cerebral resistance test also revealed the superiority of the mouse brain vaccine. Whereas this vaccine produced an immunity index of at least 2,000,000 with good end points, the Lederle vaccine showed an index of 200,000 with no end point at the higher dilution. This is

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Table VI

Immunogenic Capacity of Formalinized W.E.E. Virus
Comparison of Lederle and Mouse Brain Vaccines

0.25 cc. x 6

1. Neutralizing Antibodies

| Serum in Mixture | Intracerebral Method | | | Intraperitoneal Method |
|---|---|---|---|---|
| | Neutralization index | Serum $LD_{50}$ | | Neutralization index |
| | | 10 $LD_{50}$ doses | 50 $LD_{50}$ doses | |
| Lederle vaccine | 800 | 1:50 | 1:11 | 1,000,000 |
| Mouse brain vaccine | 3200 | 1:500 | 1:44 | 1,000,000 |

2. Intracerebral Immunity

| Group of Mice | | 0.03 cc. intracerebrally | | | | | | | | | $LD_{50}$ titre | Immunity Index |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | $10^{-10}$ | | |
| Unvaccinated controls | | | | | | | 5/5 | 3/5 | 1/5 | 0/5 | 8.3 | --- |
| Vaccinated | Lederle vaccine | 3/3 | 1/3 | 0/3 | 1/2 | | | | | | 3.0 | 200,000 |
| Vaccinated | Mouse brain vac. | 1/2 | 0/2 | 0/2 | 0/2 | | | | | | 2.0 | 2,000,000+ |

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more than a ten-fold drop in immunity. Again, the distribution of deaths over many dilutions is indicative of the low grade immunity induced by the Lederle vaccine.

In summary it would appear that the Lederle vaccine itself was inadequate, or some other factor was responsible for the failure to produce an adequate immune response in mice. In a personal communication Major A. B. Sabin has suggested that differences in the strains of W.E.E. virus may be the underlying cause for the weak immunogenic ability of the vaccine. Examination of the last few tests tend to bear out this possibility. Mice injected with the mouse brain vaccine were protected against its homologous strain of virus, but animals vaccinated with a heterologous preparation (Lederle vaccine) were protected to a lesser degree against the same strain of virus. Nevertheless, reports indicate that the Lederle vaccine has produced good immunity when tested against its homologous strain of virus. As a result the mouse brain vaccine was employed in all the tests in which physiological factors were studied.

Finally, a comparison was made between two doses and 3 doses of mouse brain vaccine on their respective immunogenic capacities and the results are set forth in Table VII. Three doses of vaccine appeared distinctly superior on the basis of the neutralization index and the 2 serum $LD_{50}$ tests. The intraperitoneal neutralization index was about the same for both dosages, but no end points were obtained and again it can be pointed out that this method does not give sufficiently accurate quantitative results. Finally, the immunity index was apparently the same for both groups, but as no end points were obtained, we can only say that considerable cerebral resistance resulted from either 2 or 3 doses of vaccine.

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Table VII

Immunogenic Capacity of Formalinized W.E.E.
Mouse Brain Vaccine

Comparison between 2 and 3 doses

| Serum in Mixture | Neutralization Tests | | | | I.Cer. Immunity |
|---|---|---|---|---|---|
| | Intracerebral | | | Intraperitoneal | |
| | Neutralization index | Serum $LD_{50}$ Protecting against | | Neutralization index | Immunity index |
| | | 5 $LD_{50}$ doses | 25 $LD_{50}$ doses | | |
| Vaccinated 0.3 x 2 | 300 | 1:58 | 1:5 | 1,000,000+ | 16,000+ |
| Vaccinated 0.3 x 3 | 1600 | 1:330 | 1:7 | 1,000,000+ | 16,000+ |

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There finally remained the possibility that the intraperitoneal neutralization test in young mice is a more suitable method for detecting differences in antibody titre. If it could be shown that the use of undiluted serum, over many dilutions, could obviate the necessity of using serum dilutions, then the intraperitoneal test would be the neutralization test of choice. An experiment was set up to test the effect of dilution on the serum antibody by the intraperitoneal method and the results are summarized in Table VIII.

W.E.E. hyperimmune rabbit serum, which was on hand, was utilized and serial ten-fold dilutions were prepared in normal rabbit serum. The serum dilutions were distributed in tubes and appropriate virus dilutions added to each tube. The mixtures were thoroughly shaken and 0.03 cc. immediately injected intra-abdominally into 14 to 15 day old mice. Two tests were done and the results were combined to form Table VIII. The results demonstrate that serum dilutions are still necessary for a quantitative antibody determination when the intraperitoneal method in young mice is employed. By using the hyperimmune serum definite neutralization was obtained with a serum dilution of 1:1000 and slight neutralization, with a dilution of 1:10,000. The fact that serum dilutions were necessary, and the test did not permit a quantitative differentiation between sera, as well as that the maintaining of a constant supply of 14 to 15 day old mice was an additional inconvenience, ruled out the intraperitoneal neutralization test as a practical method for detecting quantitative differences in antibody content.

From the results it was finally decided to do the following:

1. use the mouse brain vaccine

2. inject 3 doses of 0.3 cc. each intra-abominally into 21 gram female mice

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Table VIII

Effect of Dilution on the Neutralizing Antibody of W.E.E.
When Tested Intraperitoneally
in 14-15 Day Mice

| Rabbit Serum | Serum Diluted | 0.03 cc. of mixture intraperitoneally | | | | | | | | | $LD_{50}$ titre | Neutralization Index |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1:20 | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | | |
| Normal | undiluted | - | - | - | - | 2/2 | 2/2 | 5/5 | 2/5 | 0/5 | 7.8 | --- |
| | undiluted | 0/5 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | - | - | - | $1.1^-$ | $5,000,000^+$ |
| | 1:10 | 2/3 | 0/5 | 0/1 | 0/1 | 0/2 | 0/2 | - | - | - | 1.5 | 2,000,000 |
| W.E.E. | 1:100 | - | 0/3 | 2/5 | 0/5 | 0/2 | 0/1 | 0/2 | - | - | $1.8^-$ | $1,000,000^+$ |
| Hyper- | 1:1,000 | - | - | 2/3 | 3/5 | 0/5 | 0/2 | 0/2 | 0/2 | - | 4.0 | 6,000 |
| immune | 1:10,000 | - | - | - | - | 4/5 | 5/5 | 2/5 | 0/2 | 0/2 | 6.7 | 13 |
| | 1:100,000 | - | - | - | - | - | - | 2/3 | 0/3 | 0/3 | 7.3 | 3 |
| | 1:1,000,000 | - | - | - | - | - | - | 3/3 | 1/3 | 0/3 | 7.7 | 1 |

Note: The results of two tests are combined to make up this table.

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3. test for immunity at 2 weeks

4. titrate the antibody by means of the intracerebral neutralization test using:

(a) undiluted serum against decimal dilutions of virus

(b) dilutions of serum against 50 $LD_{50}$ doses of virus.

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## Experimental

Effect of Fatigue. Some work has been reported on the relation of fatigue to immunity, but the results are conflicting and a general statement on the subject is impossible. Some investigators find a decreased resistance to bacterial infection after exercise or fatigue, while others find exactly the opposite, that is, an increased resistance. According to recent reports the same state of affairs holds true for filterable viruses. Fatigue can reduce the resistance of monkeys to poliomyelitis (13), whereas no definite effect has been obtained in mice infected with influenza virus (14). A survey of the literature on the relationship between fatigue and the production of antibodies after antigenic stimulation reveals that here again no definite conclusions can be drawn. When typhoid agglutinins were used as a measure of antibody production, negative results were usually obtained, but decreases in titre were also reported. The same survey disclosed the important fact that absolutely no work had been attempted on the relationship between fatigue and the antibody produced by a filterable virus. It was, therefore, decided to include in these investigations a study of the effect of fatigue on the neutralizing antibodies of W.E.E.

Experiment 1. Mice weighing between 21 and 23 grams were selected from the stock of nonpregnant virgin females and divided into 3 groups of 10 mice each. One group was exercised in the fatigue machine for 1 week, another for 2 weeks and a third group was set aside to be used as controls. All three groups were vaccinated simultaneously and received 0.3 cc. intra-abdominally on 3 separate days. There was a 48 hour interval between injections. A group of normal mice was kept in reserve as unvaccinated controls. The weight curves of the vaccinated

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mice are shown in Fig. 1. Among the 1 week group, 3 animals died during the first week of exercise, one died during the second week and two died after being bled from the heart. In the 2 week group, one mouse died the first week, none the second week, but two died after bleeding. None of the controls died before or after cardiac puncture. After exercise mice lost weight at the average rate of approximately two grams a day. They managed to regain some of the lost weight at night, but did not achieve a return to the normal level. This loss in weight was not in any way due to the withholding of food from the mice, because the food baskets were removed from the vaccinated controls as well, and they demonstrated no such weight loss.

Two weeks after vaccination the animals were bled from the heart under light ether anesthesia, the blood from several mice pooled and the sera tested for neutralizing capacity. At the same time, the animals whose sera were being tested were subjected to a test for cerebral immunity. Reference to Table IX. reveals no significant difference in antibody titre as measured by the conventional method of mixing undiluted serum with decimal dilutions of virus. The one week and two week exercised groups of mice showed neutralization indexes of 650 and 2,000 respectively, which compares favorably with the index of 1,000 attained by the vaccinated controls. These values do not differ much from one another. There was no difference in antibody titre when the same sera were tested in another way. Two-fold dilutions of sera were added to a constant amount of virus dilution, in this case 32 $LD_{50}$ doses. The $LD_{50}$ dilutions of sera were 1:5 for the one week group, 1:11 for the two week group and 1:10 for the vaccinated controls (Table X). Here again, there was no difference between fatigued and nonfatigued

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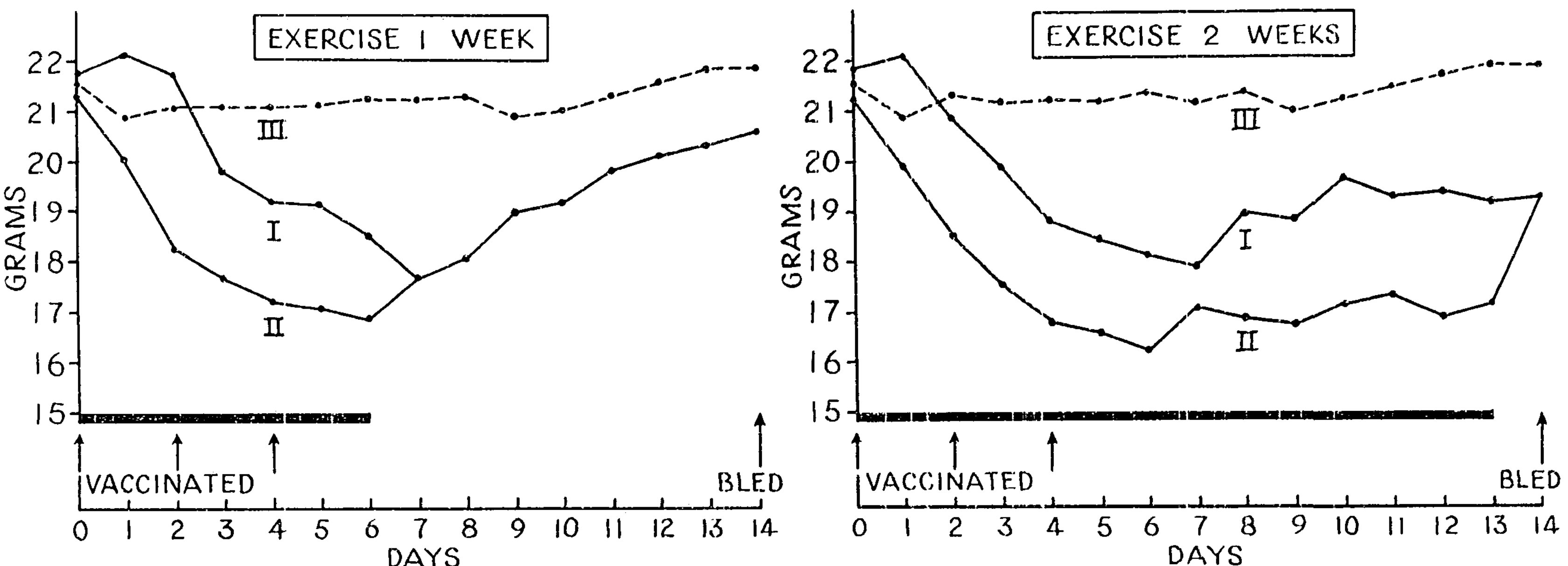


FIG. I WEIGHT CURVES OF MICE

I-BEFORE EXERCISE, II-AFTER EXERCISE, III-NON EXERCISED CONTROLS

▬▬ PERIOD OF EXERCISE 3 HOURS IN A.M. + 3 HOURS IN P.M.

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Table IX

Development of Neutralizing Antibodies in W.E.E.
Vaccinated Mice after Exercise

Neutralizing Antibodies (i.cer. method)
Serum virus mixtures at 37° C. for two hours

| Serum in Mixture | | Dilution of virus in mixture | | | | | | $LD_{50}$ titre | Neutralization Index* |
|---|---|---|---|---|---|---|---|---|---|
| | | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | | |
| Unvaccinated Control | | - | - | - | 4/4+ | 3/4 | 0/4 | 8.3 | --- |
| Vaccinated 0.3 x 3 | Control | 4/4 | 3/4 | 0/4 | 0/4 | - | - | 5.3 | 1000 |
| | Exercised one week | - | 3/4 | 1/4 | 0/4 | 0/3 | - | 5.5 | 650 |
| | Exercised two weeks | - | 2/4 | 0/4 | 0/4 | 0/4 | - | 5.0 | 2000 |

* Neutralization Index - $\frac{LD_{50} \text{ titre of controls}}{LD_{50} \text{ titre of vaccinated mice}}$

\+ 4/4 denotes 4 mice out of 4 inoculated died.

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Table X

Development of Neutralizing Antibodies in W.E.E. Vaccinated Mice after Exercise

Neutralizing Antibodies (intracerebral method) using dilutions of serum

| Serum in Mixture | | $LD_{50}$ doses used | Dilution of serum added | | | | | Serum $LD_{50}$ |
|---|---|---|---|---|---|---|---|---|
| | | | Und. | 1:2 | 1:4 | 1:8 | 1:16 | |
| Unvaccinated Control | | 32 | 4/4 | - | - | - | - | -- |
| Vaccinated 0.3 x 3 | Control | | 0/4 | 0/4 | 1/4 | 1/4 | 3/4 | 1:10 |
| | Exercised one week | (8.3 control / 6.8) | 0/4 | 0/4 | 2/4 | 3/4 | 4/4 | 1:5 |
| | Exercised two weeks | | 0/4 | 0/4 | 1/4 | 0/4 | 3/4 | 1:11 |

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Table XI

Development of Neutralizing Antibodies in W.E.E.
Vaccinated Mice after Exercise

Neutralizing Antibodies (intraperitoneal method)
Serum virus mixtures injected immediately into
14-15 day mice. 0.03 cc. i.perit.

| Serum in Mixture | | Dilution of virus in mixture | | | | | | | | $LD_{50}$ titre | Neutralization Index |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | | |
| Unvaccinated Control | | - | - | - | - | - | 2/2 | 0/2 | 1/2 | 6.7 | --- |
| Vaccinated 0.3 x 3 | Control | 0/2 | 0/2 | 0/2 | - | - | - | - | - | $0.5^-$ | $1,600,000^+$ |
| | Exercised one week | 1/2 | 1/2 | 0/2 | - | - | - | - | - | 1.5 | 160,000 |
| | Exercised two weeks | 1/2 | 0/2 | 0/2 | - | - | - | - | - | $1.0^-$ | $500,000^+$ |

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mice as regards antibody production. A comparison of Table IX with Table X shows that the results obtained parallel one another.

Table XI reveals no striking differences between these same sera when they were tested for neutralization by means of the intraperitoneal method in 14 to 15 day mice.

The animals tested for intracerebral immunity showed considerable resistance, but since no end points were obtained on the few survivors and since we were primarily concerned with the antibody, this aspect of the work was not repeated.

Experiment 2. This test was set up to determine whether or not a smaller amount of antigen could bring out any differences in antibody response between exercised and nonexercised mice. Therefore, three groups of 10 mice each were vaccinated with one dose of 0.3 cc. intra-abdominally on the first day of exercise. As before, one group was exercised one week, another for two weeks and still another set aside as nonexercised controls. A fourth group received neither exercise nor vaccination and acted as controls for those receiving antigen. The weight curves of this series of animals were similar to those shown in Fig. 1. This time only three mice in the one week group died and none in any of the others. Preliminary tests had demonstrated that the neutralization range after one dose of vaccine was the same as that after three doses, when the test was done with undiluted serum. However, when the test was performed by using dilutions of serum, some drop in titre was observed. There was also a marked drop in the resistance of mice tested for cerebral immunity after only one dose of vaccine.

Two weeks after vaccination the mice were tested for neutralizing antibodies as well as active immunity. Neutralization was determined

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by the two methods previously outlined. From the data in Table XII no significant difference could be observed, when the sera were tested either undiluted or diluted. The results further corroborate the findings of other investigators that, although the neutralization index is the same whether one or three doses of vaccine is given, differences in the antibody content can be discerned by using dilutions of serum. When the mice that had been bled were tested for cerebral resistance a slight difference was noted between exercised animals and controls as seen in Table XIII. It is true that fewer exercised than control mice succumbed at the lower dilutions, but, nevertheless, over a wider range of dilutions more fatigued animals succumbed. Furthermore, no end point was reached for either of the two fatigued groups. This lack of complete protection over a wide range of dilutions contrasts somewhat with the control group of vaccinated mice which completely resisted the dilutions above $10^{-4}$.

From these experiments one may conclude that fatigue exerts no influence on the formation of neutralizing antibodies under the conditions set forth in these tests. However, there is a possibility that after slight antigenic stimulation there is a decreased cerebral resistance after exercise.

Effect of Dehydration. From a review of the literature on the effect of dehydration one may conclude that dehydration of the body tissues leads to an increased susceptibility to virus infection. However, it has been pointed out by Sprunt (27) that an increased resistance is possible under certain conditions of dehydration. No attempt has been made to assay the level of antibody present at a time when the resistance of the host to invasion had been decreased. Therefore, dehydration as it affects immunity was included among these studies.

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Table XII.

Development of Neutralizing Antibodies in W.E.E. Vaccinated Mice after Exercise.

Neutralizing Antibodies (intracerebral method)
Serum virus mixtures at 37°C. for 2 hours.

| Serum in mixture | | Undiluted serum | | Serum dilutions | |
|---|---|---|---|---|---|
| | | $LD_{50}$ titre | Neut. Index | $LD_{50}$ doses used | Serum $LD_{50}$ |
| Unvaccinated control | | 8.5 | - | 80<br>(8.5 Control / 6.6) | - |
| Vaccinated 0.3 x 1 | Control | 5.0 | 3200 | | 1:2 |
| | Exercise 1 wk. | 5.5 | 1000 | | 1:4 |
| | Exercise 2 wks. | 5.3 | 1600 | | 1:2 |

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Table XIII

Development of Cerebral Resistance in W.E.E. Vaccinated Mice after Exercise.

| Group of mice | | 0.03 cc. intracerebrally | | | | | | LD50 titre | Immunity Index |
|---|---|---|---|---|---|---|---|---|---|
| | | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | | |
| Unvaccinated controls | | - | - | - | 3/3 | 2/3 | 0/3 | 8.3 | - |
| Vaccinated 0.3 x 1 | Controls | 3/3 | 0/3 | 0/2 | 0/2 | - | - | 4.5 | 6,300 |
| | Exercise 1 wk. | 1/2 | 1/3 | 1/2 | - | - | - | 4.7+ | 4,000- |
| | Exercise 2 wks. | 1/3 | 2/3 | 1/2 | 1/2 | - | - | 5.3+ | 1,000- |

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Female mice averaging 21 grams in weight were placed in individual compartments and were subjected to the regimen of restricted water intake. On the fourth day when the weights of the animals had become stabilized between 14 and 15 grams, they were given their initial dose of vaccine followed by the other two doses on the sixth and eighth days. Two weeks after the first dose of vaccine, or on the eighteenth day of dehydration, the animals were tested. As shown in Fig. 2, the animals maintained a uniform weight of about 14 grams. Fig. 2 also shows the average water consumption per mouse and the resultant weight curve. Eight mice comprising little more than half the total survived the dehydration program and 3 of these died after being bled. Next day the serum was tested for neutralization and the vaccinated mice were tested for immunity. Since we were primarily interested in the antibody the mice were returned to an adequate water supply 24 hours after the intracerebral injection. This was also to make certain that the injected mice died of virus infection and not of dehydration. It will be noted that the mice almost returned to their previous normal weight 24 hours after they were put on an adequate water intake and then put on weight more slowly. Eventually they returned to their normal weight.

The summarized data in Table XIV show that no significant differences were present. The neutralization index of the dehydrated animals was almost as high as that of the vaccinated controls. However, since no complete end point was reached on the serum of the dehydrated mice, even this slight difference would tend to disappear. When tested by means of the serum dilution method the sera obtained from the thirsted animals revealed a slight drop in the protective

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Table XIV

Effect of Dehydration on Development of Neutralizing Antibodies (intracerebral method)

| Serum in mixture | Undiluted serum | | Serum dilutions | |
|---|---|---|---|---|
| | $LD_{50}$ titre | Neut. Index | $LD_{50}$ doses used | Serum $LD_{50}$ |
| Unvaccinated control | 8.3 | - | 50 | - |
| Vaccinated 0.3 x 3 — Control | 4.5 | 6300 | | 1:20 |
| Vaccinated 0.3 x 3 — Dehydrated | 5.0 | 2000+ | $\frac{8.3}{6.6}$ | 1:8 |

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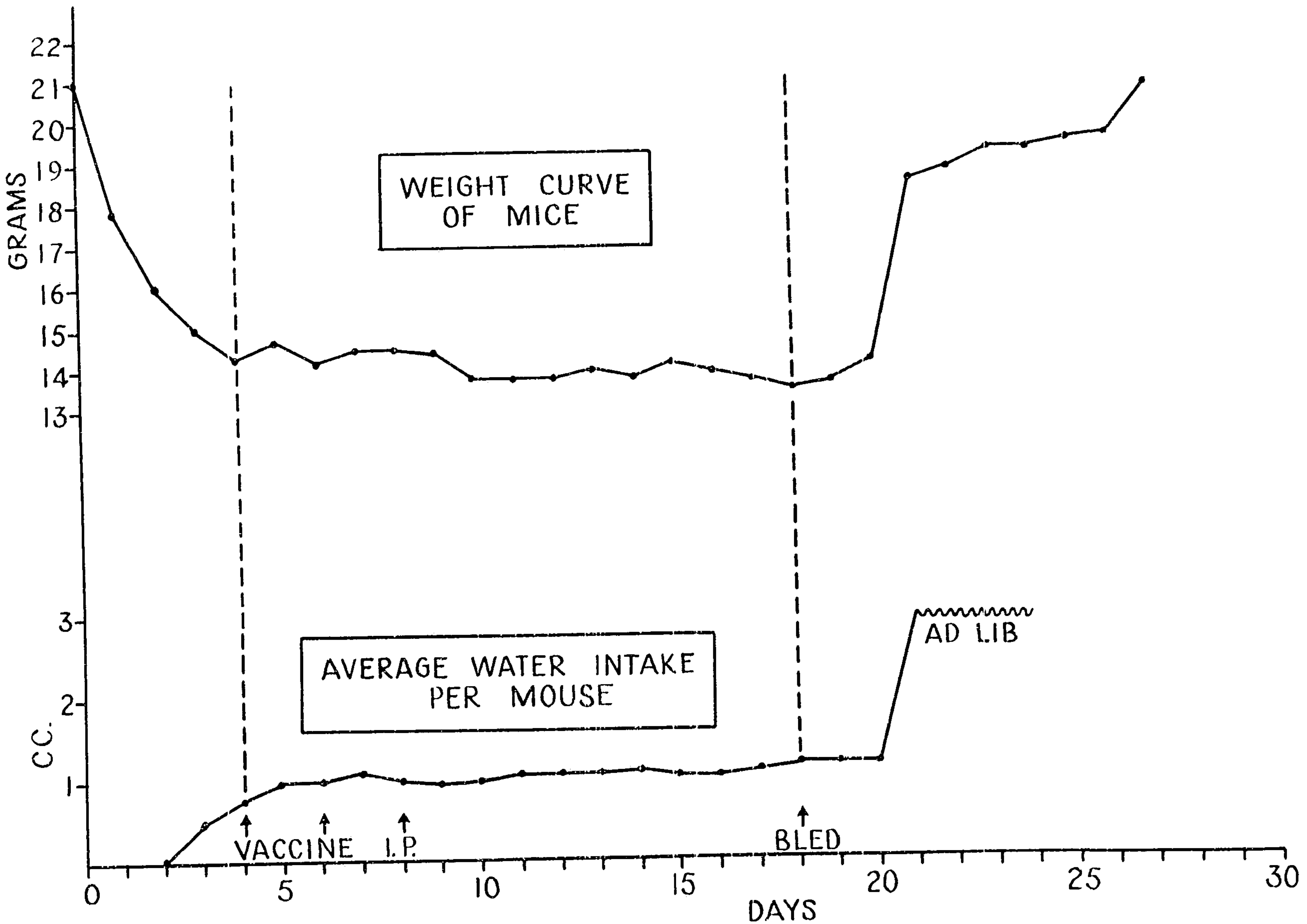


FIG. 2 WEIGHT CURVE OF MICE ON RESTRICTED WATER INTAKE

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power . Against 50 $LD_{50}$ doses of virus the dehydrated mouse serum diluted 1:8 protected half the mice as compared with a dilution of 1:20 for the vaccinated controls. To become significant in these tests a drop in protective power much greater than 1:8 would be necessary. Since no end points were obtained in the cerebral immunity test, these results are not tabulated. It may be of interest to mention that none of the five surviving dehydrated mice tested intracerebrally succumbed after the $10^{-4}$ dilution and one of two died after an injection of the $10^{-5}$ dilution of virus. The cause of death was verified by passage and culture. A larger number of animals should be used to determine whether there is a decreased resistance, but, since our primary concern was with antibodies, no further work was done on this problem.

These results indicate that dehydration induced by a restricted water intake does not influence antibody formation.

Effect of Pregnancy. The clinical observation has been made that pregnancy modifies the response of women to certain diseases. In the laboratory, on the other hand, it has been amply demonstrated that pregnancy can interfere with the development of an acquired immunity. Hence, no generalization about the relationships of pregnancy to immunity is possible. Consequently, experiments were designed to determine whether pregnancy could influence the production of immunity. The level of antibody as well as the degree of cerebral resistance was, therefore, selected as the means of measuring the immunity produced.

In the first experiment 14 mice which had been inseminated were given three doses of vaccine intraperitoneally. Of these 6 were

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eventually discarded for various reasons, while the remaining eight mice were bled for serum 2 weeks after the first dose of vaccine. The mice were vaccinated during the period of insemination so that of the mice that delivered one gave birth before and 4 after being bled. Probably as a result of the intraperitoneal vaccination the remaining three mice resorbed their young 3 to 5 days after the third dose of vaccine. They were animals well advanced in pregnancy since they had been inseminated some time before the first dose of vaccine. The time of vaccination in relation to the period of pregnancy is shown in Fig. 3. The important thing is that the mice bled for serum had been vaccinated early in pregnancy so that any influence present could exert its effect over the maximum period of time.

In the results obtained (Table XV, Expt. 1) there are no striking differences between nonpregnant vaccinated controls and pregnant vaccinated mice. The slight decrease in antibody shown by the pregnant mice is demonstrated in the three types of neutralization tests. They are a neutralization index of 650 compared with 1,000 in the controls, a serum $LD_{50}$ of 1:6 compared with 1:10 and an intraperitoneal neutralization index of 160,000 compared with at least 1,600,000 for the controls. The last comparison would appear to be significant except for the fact that too few animals were employed in this test. However, of interest is the fact that this serum tested by 3 different methods showed proportionate drops in titre of doubtful significance in all 3 of the methods when compared with the control vaccinated mouse serum in each case. An intracerebral immunity test was performed on the 7 vaccinated pregnant mice as well as the vaccinated controls. In summing up the results all that can be said is that the animals resisted at least 100 $LD_{50}$ doses of vaccine since no lower end point was reached.

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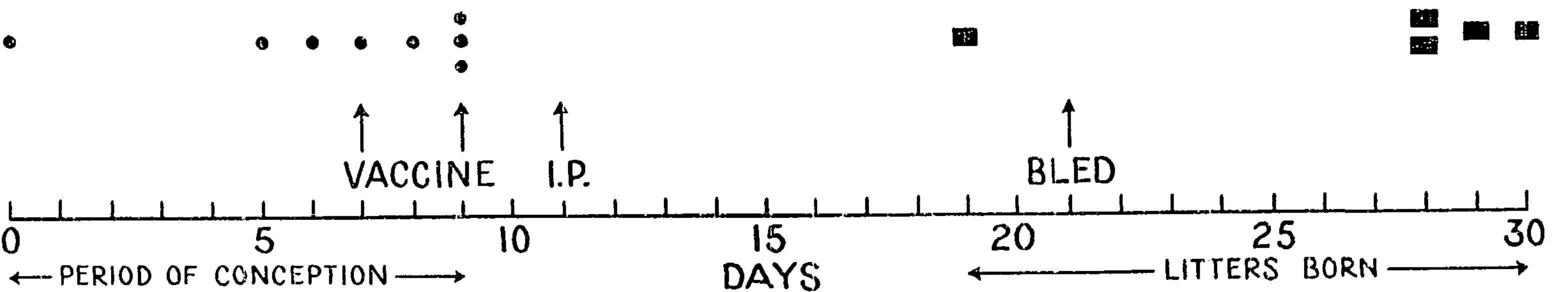


FIG. 3 PERIOD OF PREGNANCY AND VACCINATION (3 DOSES)

● INDIVIDUAL MOUSE INSEMINATED
■ INDIVIDUAL MOUSE LITTER BORN

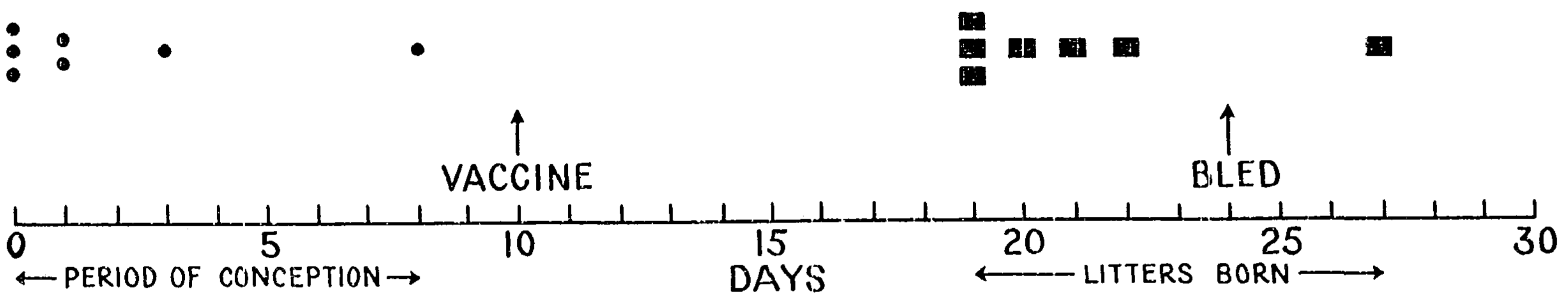


FIG. 4 PERIOD OF PREGNANCY AND VACCINATION (1 DOSE)

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In the second experiment only one dose of vaccine was administered intraperitoneally. It was given about themiddle of pregnancy. This time only 7 pregnant vaccinated mice were chosen and Fig. 4 shows at what time during the pregnancy the vaccine was given. All 7 mice were impregnated before vaccination and with one exception all gave birth before tests for immunity were performed. The one exception produced a litter 3 days after bleeding. The mice as usual were bled 2 weeks after the initial dose of vaccine.

Examination of Table XV, Experiment 2, reveals two things. First, there is no difference between nonpregnant mice and pregnant mice as far as antibody content of serum is concerned when they are compared on the basis of the serum $LD_{50}$. The dilution of serum protecting 50% of the mice against 80 $LD_{50}$ doses of virus was identical in both groups of mice, 1:2 being the calculated dilution. Second, there is an almost ten-fold drop in the neutralization index of the serum obtained from the pregnant mice as compared with the nonpregnant. This drop in protective power assumes more significance when welook at Table XVI. In this table are recorded the results of the test for cerebral immunity on those mice whose sera were tested for neutralization in Experiment 2. The vaccinated controls gave complete end points and an immunity index of 6,300, which is contrasted with the results of the vaccinated pregnant mice. They showed no such complete end points and had an immunity index of less than 500, a drop of more than 12 $LD_{50}$ doses. The distribution of deaths among the pregnant mice over many dilutions also indicates a low grade type of protection. There is then a correlation present which is borne out by the lowered neutralization index of the serum on the one hand and the lowered immunity index of the mice on the other.

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Table XV

Effect of Pregnancy on Development of Neutralizing Antibodies.

| Expt. | Serum in mixture | Undiluted serum i. cer. test | | Serum dilutions i. cer. test | | Undiluted serum i. per. test | |
|---|---|---|---|---|---|---|---|
| | | LD50 titre | Neut. Index | LD50 doses used | Serum LD50 | LD50 titre | Neut. Index |
| 1. | Unvaccinated control | 8.3 | - | 32 | - | 6.7 | - |
| | Vaccinated 0.3 x 3 — Control | 5.3 | 1000 | (8.3/6.8) | 1:10 | 0.5- | 1,600,000+ |
| | Vaccinated 0.3 x 3 — Pregnant | 5.5 | 650 | | 1:6 | 1.5 | 160,000 |
| 2. | Unvaccinated control | 8.5 | - | 80 | - | - | - |
| | Vaccinated 0.3 x 1 — Control | 5.0 | 3200 | (8.5/6.6) | 1:2 | - | - |
| | Vaccinated 0.3 x 1 — Pregnant | 5.8 | 500 | | 1:2 | - | - |

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Table XVI

Effect of Pregnancy on Intracerebral Resistance.

| Group of mice | | 0.03 cc. intracerebrally | | | | | | LD50 titre | Immunity Index |
|---|---|---|---|---|---|---|---|---|---|
| | | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | | |
| Unvaccinated controls | | - | - | - | 3/3 | 2/3 | 0/3 | 8.3 | - |
| Vaccinated 0.3 x 1 | Controls | 3/3 | 0/3 | 0/2 | 0/2 | - | - | 4.5 | 6,300 |
| | Pregnant | 2/3 | 2/2 | 1/2 | - | - | - | $5.6^{+}$ | $500^{-}$ |

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Thus a slight decrease in immunity is produced during pregnancy. This decrease is shown by a drop in neutralizing capacity as well as a fall in cerebral resistance and becomes manifest after slight antigenic stimulation, but not after larger amounts of antigen are administered.

Effect of Underfeeding. In the articles on the effect of underfeeding on resistance or the production of immune bodies, the results reported are irregular and, therefore, of doubtful significance or they varied with the organism used. In general the conclusion is that a lowered intake of food is not a factor in diminished resistance to infection. In fact, from recent work, the impression gained is that with holding food leads to increased resistance to infection (15).

Up to the present time no experiments have been conducted to determine whether the neutralizing substances present in the blood stream after virus stimulation can be influenced by simple inanition, although some work has been done with bacterial antibodies. Consequently, experiments were planned to test the effect of underfeeding on the immune response in mice after inoculation with W.E.E. The results to be reported demonstrate a drop in the neutralizing capacity of the blood which is correlated with a lessened ability to resist cerebral infection.

In the first experiment 12 virgin female mice averaging 21 grams in weight were placed on a diet of purina dog chow insufficient in amount to meet their needs. They lost weight rapidly and weighed about 14 grams by the fourth day, when they received their first dose of vaccine. They received 3 doses of vaccine in all and were bled 2 weeks after the first dose. During this 2 week period their weight

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averaged between 13 and 14 grams which was a little too low. As a result only two mice survived the ordeal. These were bled to death and their serum tested for neutralization.

Although there was insufficient serum for a complete test, the results (Table XVII, Expt. 1) were striking enough to warrant a second trial. The neutralization index of the undiluted serum dropped to less than 60 compared to 6,300 for the vaccinated controls. This was a drop of more than 100 $LD_{50}$ doses. The drop in protective power also became evident, when the blood was tested by the serum dilution method. Against 50 $LD_{50}$ doses of virus the serum from the underfed mice showed a serum $LD_{50}$ of 1:3 as compared with $1:20^{+}$ for the vaccinated controls. This was the first evidence of a marked drop in the production of neutralizing antibodies.

The test was repeated in Experiment 2 with substantially the same results. This time, however, 10 mice survived the underfeeding, 2 of which died after bleeding and one the following day, probably as a result of trauma. The neutralization index of the underfed vaccinated animals was less than 150 compared with 1,300 for the vaccinated controls, a drop of at least 10 $LD_{50}$ doses. The serum dilution showed a drop, too, being 1:1 as compared to 1:8 for the controls. In the test for cerebral immunity no end points were obtained, but a drop in active immunity appeared possible.

In Experiment 3 this test was repeated once more on a larger number of mice in order to have enough survivors for the intracerebral immunity test. Forty-two mice averaging slightly over 21 grams in weight were numbered and distributed in their individual cubicles. When their average weights had dropped to 15 grams the mice as well as

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Table XVII

Effect of Underfeeding on Neutralizing Antibodies.
(i. cer. method)

| Expt. | Serum in mixture | | Undiluted serum | | Serum dilutions | |
|---|---|---|---|---|---|---|
| | | | $LD_{50}$ titre | Neut. Index | $LD_{50}$ doses used | Serum $LD_{50}$ |
| 1. | Unvaccinated control | | 8.3 | - | 50 | - |
| | Vaccinated 0.3 x 3 | Control | 4.5 | 6,300 | | 1:20+ |
| | | Underfed | 6.5+ | 60- | $\left(\frac{8.3}{6.6}\right)$ | 1:3 |
| 2. | Unvaccinated control | | 8.3 | - | 50 | - |
| | Vaccinated 0.3 x 3 | Control | 5.2 | 1,300 | | 1:8 |
| | | Underfed | 6.2+ | 150- | $\left(\frac{8.3}{6.6}\right)$ | 1:1 |
| 3. | Unvaccinated control | | 8.5 | - | 80 | - |
| | Vaccinated 0.3 x 3 | Control | 4.8 | 5,000 | | 1:16 |
| | | Underfed | 6.5 | 100 | $\left(\frac{8.5}{6.6}\right)$ | 1:2 |
| 4. | Unvaccinated control | | 8.5 | - | 80 | - |
| | Vaccinated 0.3 x 3 | Control | 5.3 | 1,600 | | 1:6 |
| | | Prolonged under-feeding | 6.6 | 80 | $\left(\frac{8.5}{6.6}\right)$ | 1:2 |

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their adequately fed controls were vaccinated and 2 weeks later bled. The weight curves of both groups of mice are depicted in Fig. 5. The curve shows that slightly more than 3 grams of food was sufficient to maintain the weights of the mice at 14 to 15 grams while at a 4 gram level the mice slowly began to put on weight. The food allowance was increased to 4 grams the day after the intracerebral resistance test was done in order to minimize the possibility of mice dying of starvation. Initially, there was a slight increase in weight; then as one of the effects of the virus the weight curve began to drop. After this period the surviving mice slowly put on weight. The control mice on an adequate food supply also showed a drop in weight at a time when they had become cerebrally infected with virus.

Examination of the results (Table XVII, Expt. 3) reveals that again there is a decrease in the neutralization index and the serum $LD_{50}$ of blood obtained from underfed mice. The index of the underfed mice was 100, or a drop of 50 $LD_{50}$ doses as compared with 5,000 for the controls. The serum $LD_{50}$ fell from 1:16 for the controls, to 1:2 for the underfed mice. The serum was obtained from 25 surviving underfed mice who were subsequently tested for active immunity. Table XVIII presents the result of this test. The vaccinated controls gave a reasonably good end point of 1.3 and an immunity index of 25,000,000. In comparison the underfed animals were only partially resistant to all the dilutions up to and including $10^{-6}$ with a negative end point at $10^{-7}$. The calculated $LD_{50}$ titre was 3.6 with an immunity index of 126,000, or a drop of about 200 $LD_{50}$ doses. To make sure that the underfed mice did

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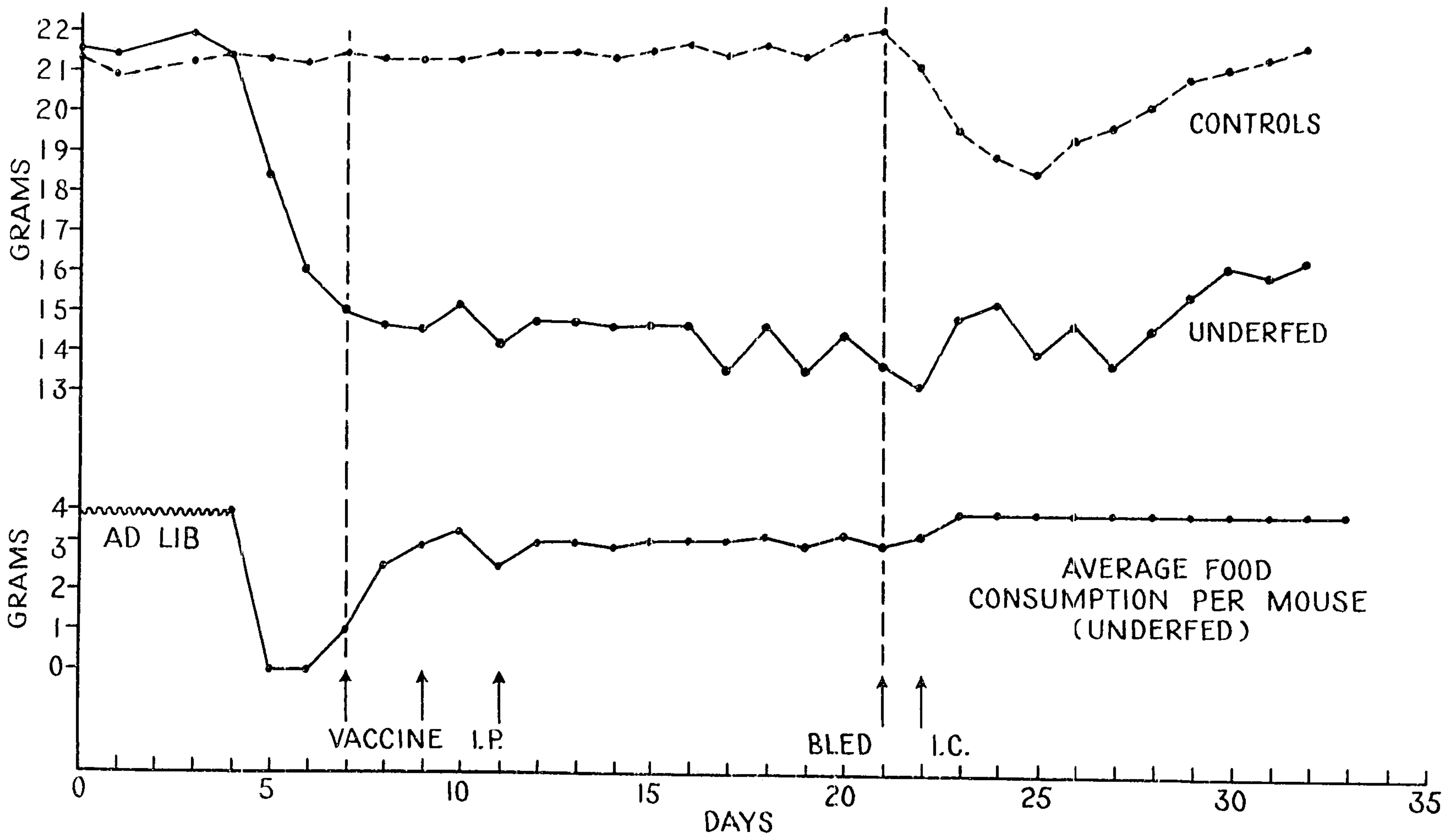


FIG. 5 WEIGHT CURVES OF UNDERFED MICE ON 'PURINA' DIET ALSO AVERAGE FOOD CONSUMPTION PER SINGLE MOUSE TO OBTAIN WEIGHT CURVE OF UNDERFED ANIMALS

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Table XVIII

Effect of Underfeeding on Cerebral Immunity

| Expt. | Group of mice | | 0.03 cc. intracerebrally | | | | | | | | | $LD_{50}$ titre | Immunity Index |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | | |
| 3. | Unvaccinated controls | | - | - | - | - | - | - | 4/4 | 3/3 | 1/4 | 8.7 | - |
| | Vaccinated 0.3 x 3 | Controls | 3/5 | 1/4 | 0/4 | - | - | - | - | - | - | 1.3 | 25,000,000 |
| | | Underfed | - | 2/4 | 2/4 | 1/4 | 2/4 | 2/4 | 0/3 | - | - | 3.6 | 126,000 |
| 4. | Unvaccinated controls | | - | - | - | - | - | - | 4/4 | 3/4 | 2/4 | 8.8 | - |
| | Vaccinated 0.3 x 3 | Controls | 4/4 | 2/4 | 0/4 | 0/2 | - | - | - | - | - | 2.0 | 6,300,000 |
| | | Prolonged under-feeding | 5/5 | 3/3 | 1/4 | 2/4 | 2/4 | 1/4 | 0/3 | - | - | 4.0 | 63,000 |

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not die from starvation, the brains of those found dead were cultered and tested by passage. Only those mice that were negative on culture and positive on passage were regarded as dying from specific causes.

After the depressing effect of underfeeding on the immune response had been established, an attempt was made in Experiment 4 to accentuate this effect by a more prolonged period of feed restriction. Fifty mice averaging 18.5 grams in weight, to allow for gain in weight, were put on a diminished food intake. They were kept on this regimen for 2 weeks before they were given their 3 doses of vaccine and then kept on this restricted foor intake for the duration of the experiment. Control mice of the same weight had been set aside and were vaccinated at the same time. The weight curves of the mice undergoing the prolonged underfeeding schedule as well as the controls are presented in Fig. 6. Time of vaccination and bleeding with respect to the underfeeding program is also shown. Twenty-nine surviving mice were bled 2 weeks after vaccination and tested for immunity.

From the results (Table XVII, Expr. 4) it can be seen that the neutralization index of the underfed mice fell from 1,600 to 80, a drop of 20 $LD_{50}$ doses. The serum $LD_{50}$ was 1:2 which was in keeping with the previous results. However, the vaccinated control serum was only 1:6 when tested against 80 $LD_{50}$ doses of virus.

The results of the intracerebral immunity test (Table XVIII, Expr. 4) were the same as those obtained in Experiment 3, that is, a partial protection of vaccinated underfed mice. The immunity index of the underfed mice was 63,000, or a drop of 100 $LD_{50}$ doses as compared with the value of 6,300,000 for the controls. There was a good end point for the vaccinated controls, but fatalities occurred over a wide range of dilutions for the underfed vaccinated animals.

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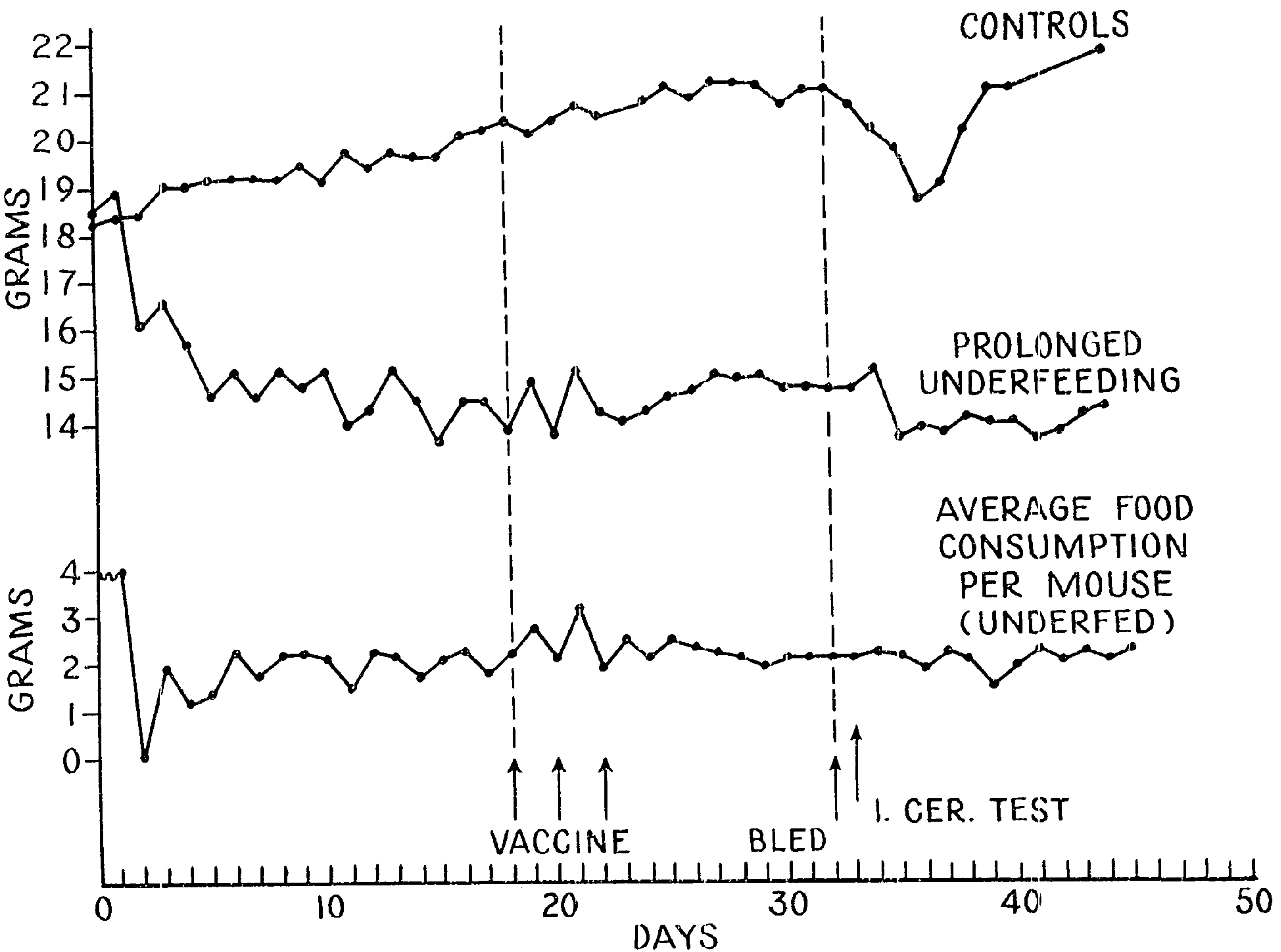


FIG. 6 WEIGHT CURVES OF MICE AFTER PROLONGED UNDERFEEDING ON 'PURINA' DIET. ALSO AVERAGE FOOD CONSUMPTION PER SINGLE MOUSE TO OBTAIN WEIGHT CURVE OF UNDERFED ANIMALS.

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The results obtained in the several underfeeding experiments were combined. Frozen ampules of virus prepared from one batch of material were used in the neutralization tests. Control titrations demonstrated $LD_{50}$ titres between 8.0 and 8.5 with a cumulative $LD_{50}$ titre of 8.3. These values are in all probability, basically the same so that for convenience we can regard all tests as having been done with the same ampule of virus. Therefore, the results of the four tests on underfeeding were combined and the cumulated results tabulated. Good end points were obtained in both types of calculated neutralization tests and the minor differences arising when smaller numbers of mice are employed were eliminated. The index in the experiments on underfed mice dropped from 2,500 for the controls to 80, a decrease of 32 $LD_{50}$ doses (Table XIX). In Table XX in which results on the serum dilution method of testing are brought together, a decided drop is demonstrated in the ability of the serum of underfed animals to neutralize W.E.E. virus. A serum dilution of 1:11 from vaccinated controls could protect 50% of the test animals against 50 $LD_{50}$ doses whereas the figure dropped to a dilution of 1:2 for the sera of the underfed mice when tested in the same manner.

In summary, these tests prove that underfeeding causes a marked drop in antibody titre as well as a lowered resistance to cerebral infection.

Effect of Nutritional Deficiencies. An extensive literature has been accumulated on the relationship between nutrition and resistance to infection and the excellent reviews by Robertson (16) and by Clausen (17) cover the field in an adequate manner. Although the consensus is that nutritional deficiencies lead to a lowered resistance to infection, no

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Table XIX

Effect of Underfeeding on Neutralizing Antibodies.

Cumulative Summary

| Serum in mixture | | Mixture | | | | | | | $LD_{50}$ titre | Neut. Index |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | | |
| Unvaccinated control | | - | - | - | - | 16/16 | 14/16 | 0/16 | 8.4 | - |
| Vaccinated 0.3 x 3 | Control | 12/12 | 12/16 | 10/16 | 0/16 | 0/4 | - | - | 5.0 | 2,500 |
| | Underfed | - | 12/12 | 15/16 | 11/15 | 4/12 | 0/8 | - | 6.5 | 80 |

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Table XX

Effect of Underfeeding on Neutralizing Antibodies
Using Serum Dilutions

50-80 $LD_{50}$ doses $\left(\frac{8.3 \text{ to } 8.5}{6.6}\right)$

Cumulative Summary

| Serum in mixture | | Serum dilution | | | | Serum $LD_{50}$ |
|---|---|---|---|---|---|---|
| | | Und. | 1:4 | 1:16 | 1:64 | |
| Unvaccinated control | | 16/16 | - | - | - | - |
| Vaccinated 0.3 x 3 | Control | 1/16 | 5/16 | 8/16 | 11/12 | 1:11 |
| | Underfed | 3/16 | 13/16 | 15/16 | 4/4 | 1:2 |

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such general statement is possible about the formation of antibodies. Decreases in agglutinin titres have been reported after certain nutritional deficiencies, but this is not a constant finding and the many reports of negative results testify to the controversial nature of the subject. Reports of the effect on neutralizing antibody formation after viral stimulation are negligible and for that reason a study has been made of deficiencies in the "B" complex, thiamin, riboflavin, carbohydrate and protein and their effect on antibody formation as well as cerebral immunity.

In order to study the effect of the removal of specific dietary factors from the synthetic diet, one must first compare the complete synthetic diet with the purina ration in its ability to elicit an immune response in mice. Mice averaging 22 grams in weight were divided into 2 groups, one group was kept on the stock purina diet and the other was put on the synthetic diet. They were given three doses of vaccine intraperitoneally and then bled 2 weeks after the first dose. The serum was tested for neutralizing substances and the mice for active immunity to decimal dilutions of virus given intracerebrally. Two such tests were done and the results of one are given below. The neutralization index of 1,300 was identical for both groups of mice (Table XXI). The serum $LD_{50}$ was slightly higher for the synthetic group being 1:12 as compared to 1:8 for those on the purina diet. The test for cerebral immunity showed a somewhat similar response. (Table XXII). The response in the mice on the synthetic diet was slightly higher than in those on the purina diet. At first glance the difference in the immunity index between 10,000,000+ and 2,500,000 appears quite large, but examination of the detailed account shows that only one more mouse died

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Table XXI

Comparison of Synthetic and Stock (Purina) Diets on Immunogenic Capacity of Mice

Neutralization Tests (intracerebral method)

| Serum in Mixture | | $LD_{50}$ Titre | Neut. Index | $LD_{50}$ doses used | Serum $LD_{50}$ |
|---|---|---|---|---|---|
| Unvaccinated Control | | 8.3 | | 50 | ---- |
| Vaccin. 0.3 x 3 | Stock diet | 5.2 | 1300 | $\left(\frac{8.3}{6.6}\right)$ | 1:8 |
| | Synthetic diet | 5.2 | 1300 | | 1:12 |

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Table XXII

Comparison of Synthetic and Stock (Purina) Diets

Test for cerebral immunity

| Group of Mice Injected | Virus in Mixture | | | | | | | | $LD_{50}$ titre | Immunity Index |
|---|---|---|---|---|---|---|---|---|---|---|
| | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | | |
| Unvaccinated Controls | - | - | - | - | - | 3/3 | 2/2 | 1/3 | 8.7 | ---- |
| Vaccin. 0.3 x 3 — Stock diet | 2/3 | 0/3 | 0/2 | 0/2 | - | - | - | - | 2.3 | 2,500,000 |
| Vaccin. 0.3 x 3 — Synthetic diet | 1/3 | 0/3 | 0/2 | 0/2 | - | - | - | - | $1.7^{-}$ | $10,000,000^{+}$ |

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on the purina diet over comparable dilutions. Therefore, we may safely conclude that mice on an adequate synthetic diet are capable of producing an immune response to W.E.E. equal to that of mice on a stock diet.

Effect of Removal of the "B" Complex. After it was established that the synthetic diet did not affect the immune response in mice, the effect of the removal of specific dietary factors on the immune response was tested. In the first experiment the entire "B" complex was omitted from the diet and its effect studied. Ten mice whose average weight was 22 grams were vaccinated in the usual manner and tested at the end of 2 weeks. Vaccination was instituted after the mice had been on the deficient diet for 17 days. Because on the 21st day of deficiency, some of the mice died, they were fed the complete synthetic diet which included all the "B" components on the 22nd day and the 27th day. Otherwise the mice were kept on the "B" deficient regimen for the experimental period.

At the end of 2 weeks, the 5 surviving mice were bled for serum. Three of these died immediately after cardiac puncture. The two remaining mice were tested intracerebrally with 300 $LD_{50}$ doses of virus; one of these subsequently succumbed to the disease as was demonstrated by mouse passage. The serum of the vaccinated mice tested for neutralization are shown in Table XXIII. The neutralization index of the "B" deficient mice dropped from 6,300 for the controls to 400, which was more than a ten-fold loss in potency. The same serum tested against 50 $LD_{50}$ doses of virus demonstrated a drop which, although not as marked, was somewhat lower. The dilution of 1:8 for the deficient animals was only slightly lower than the more than 1:16 for the controls.

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Table XXIII

Effect of "B" Deficient Diet on the Production of Neutralizing Antibodies

| | Serum in Mixture | | Serum Undiluted | | Serum Diluted | |
|---|---|---|---|---|---|---|
| | | | $LD_{50}$ titre | Neutralization index | $LD_{50}$ doses used | Serum $LD_{50}$ |
| Exp. 1 | Unvaccinated Control | | 8.3 | --- | 50 | --- |
| | Vaccin. 0.3 x 3 | Synthetic diet | 4.5 | 6300 | $\left(\frac{8.3}{6.6}\right)$ | 1:16+ |
| | | "B" Defic. diet | 5.7 | 400 | | 1:8 |
| Exp. 2 | Unvaccinated Control | | 8.3 | --- | 50 | --- |
| | Vaccin. 0.3 x 3 | Synthetic diet | 5.2 | 1300 | $\left(\frac{8.3}{6.6}\right)$ | 1:12 |
| | | "B" Defic. diet | 5.5 | 650 | | 1:2 |

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As no end point was reached for the controls, 1:8 may represent a more significant drop than appears at first.

In the test on the "B" deficient diet, the mice were allowed the synthetic diet containing the "B" complex on 2 occasions. In the second experiment the "B" complex was entirely eliminated from the ration and the mice were vaccinated earlier. Ten mice were placed on the "B" deficient diet and were vaccinated one week later. They were maintained on the diet throughout the experiment. The weight curves of the mice on the deficient and adequate diets are presented in Fig. 7.

The results of experiment 2 are shown in Table XXIII. There was a drop in neutralizing capacity of the deficient serum when tested by both methods. Now, however, the serum $LD_{50}$ drop was striking whereas the neutralization index showed only a minor fall in potency. A comparison of the "B" deficient neutralization index in experiment 1 with that in experiment 2 shows that both have approximately the same value which might lead one to think that the control synthetic diet was at fault. However, the low index was not due to the diet, since the same neutralization index of 1,300 was obtained on the purina diet controls done at the same time.

Seven vaccinated deficient mice survived the cardiac puncture and were subsequently tested for cerebral resistance. No end points were reached and we can only say they resisted more than 160,000 $LD_{50}$ doses of virus. Again since we were primarily interested in the antibody this work was not repeated.

Thus mice show somewhat of a drop in antibody titre after removal of the "B" complex from the diet. No conclusions about the cerebral resistance can be drawn.

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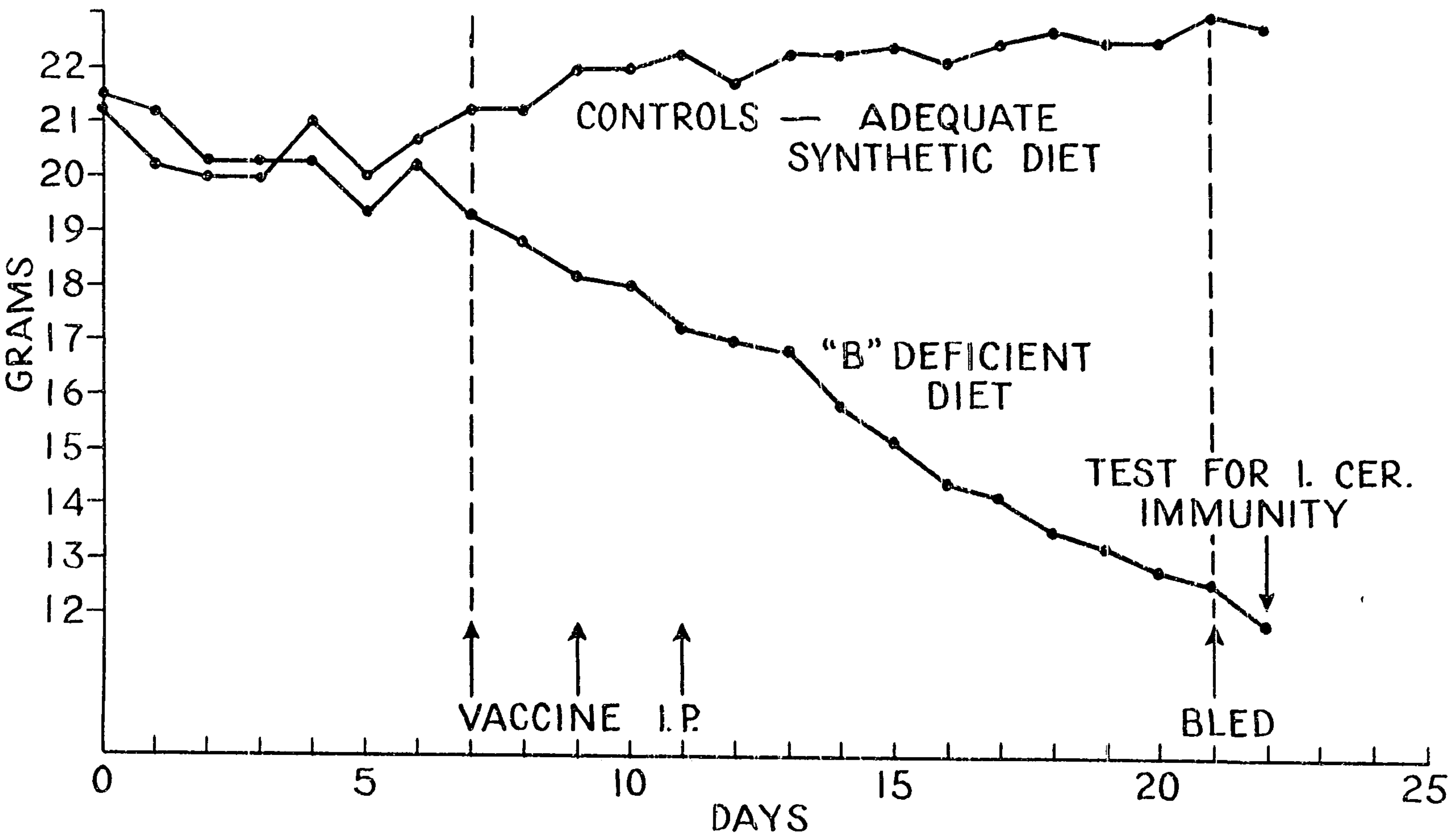


FIG. 7 WEIGHT CURVE OF MICE ON SYNTHETIC DIET LACKING ALL "B" COMPONENTS.
CONTROLS — SAME DIET + ADDED "B" COMPLEX.

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<u>Effect of Removal of Thiamin and Riboflavin.</u> These experiments were planned to include deficiencies of thiamin and riboflavin over long periods of time, but it soon became evident that mature female mice are more rapidly depleted of thiamin than are weanlings. As a result mice were vaccinated after they had been on their respective diets for only two weeks. There were 14 mice on the thiamin deficient diet, 14 on the riboflavin deficient diet and 12 on the adequate diet. They were kept on their diets throughout with only a slight modification in the thiamin diet, at one point. At two weeks the mice were vaccinated and then bled two weeks later. Two mice on the adequate diet died after cardiac puncture, none died on the riboflavin diet, but a few did so on the thiamin deficient diet ostensibly from lack of $B_1$ as early as the 16th day of deficiency. By the 22nd day all mice on the $B_1$ deficient diet were showing such obvious signs of deficiency as tremors and weakness. It was at this point that sub-obtimal amounts of thiamin were included in the diet to keep the mice alive. Ten mice finally survived for the immunity tests.

As seen in Fig. 8 the mice averaged 18 to 19 grams in weight at the start. Those on the adequate diet gained continuously until their test for cerebral resistance. They lost weight at a time when the virus was present in the body. However, this weight was slowly restored after the effect of the virus had worn off. Mice on the riboflavin deficient diet showed a constant decline in weight and were still losing weight when the experiment was terminated. The thiamin deficiency casued a sharp loss which would have ended fatally for all of the mice, but for the minimal allowance of thiamin to maintain their weights.

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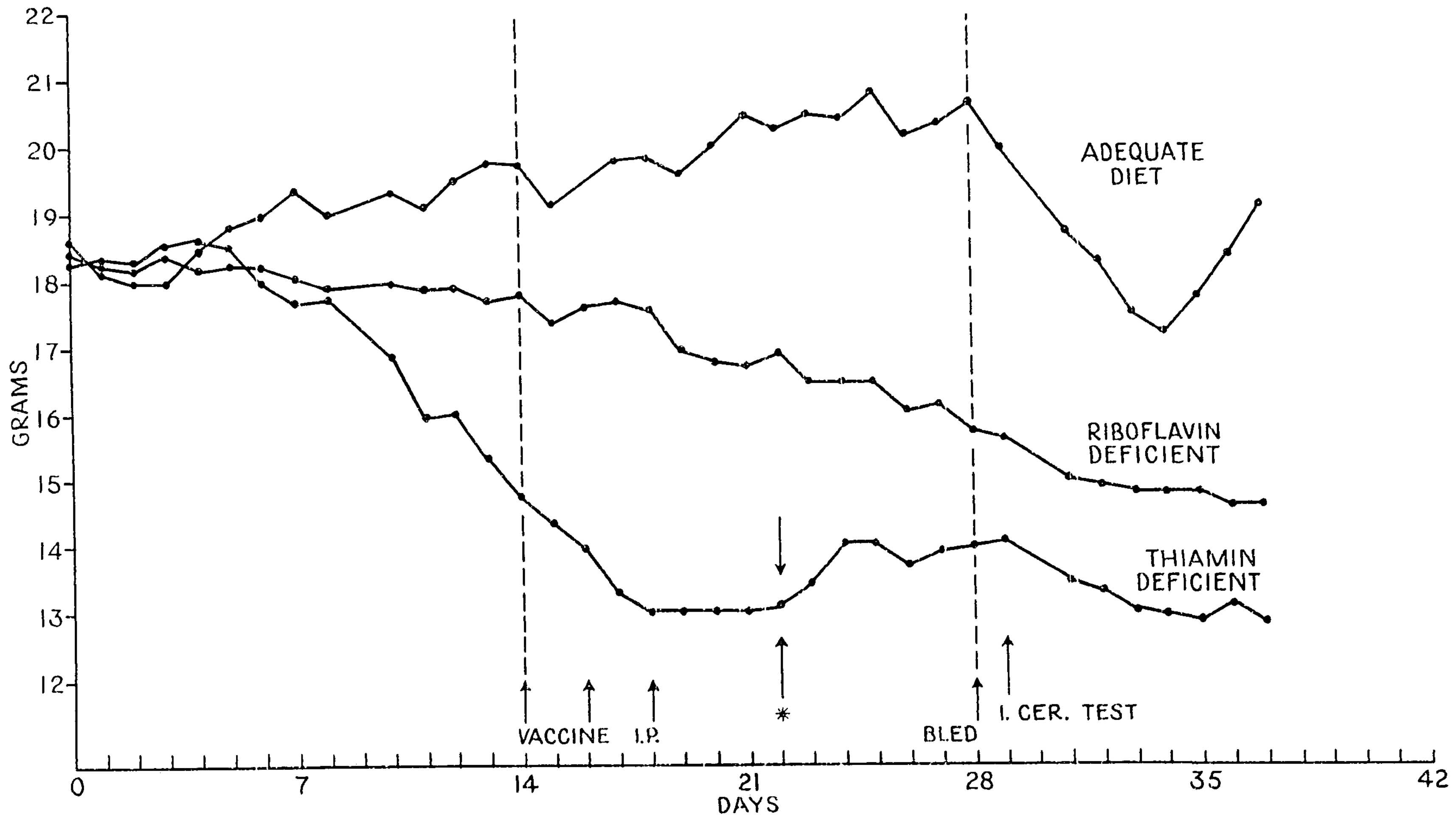


FIG. 8 WEIGHT CURVES OF MICE ON SYNTHETIC DIETS.
* STARTED ON DIET MADE UP WITH 0.5 mgm. THIAMIN (PREVIOUSLY 2.5 mgm.)

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As usual the serum obtained from the vaccinated animals was examined for neutralizing antibodies and the mice themselves tested against decimal dilutions of active virus. Passage and culture was used on the vaccinated mice in order to establish the cause of death. The results are summarized in Table XXIV and no significant deviations can be observed. The neutralization indexes of 300 for the two deficient diets compare favorably with the index of 650 for the control group. The same can be said for the serum $LD_{50}$ of 1:3 for both deficient diets when compared with 1:5 for the controls. The immunity index shows at best only a slight decrease in cerebral resistance for the deficient diets. Two things should be pointed out here. First, the figures obtained for the two deficient groups are noticeably identical for the three tests and in all cases are lower than the controls by about the same amount, thus showing remarkable parallelism. Second, despite the rather low concentration of antibody present in the serum, the vaccinated mice demonstrated a strong active immunity as evidenced by the high immunity indexes.

Thus, removal of either thiamin or riboflavin from the diet leads to no significant changes in the immune response of the vaccinated mice as compared with adequately fed controls. However, one important item to be noted is the presence of the high immunity indexes associated with neutralizing antibodies of low titre.

Effect of Carbohydrate and Protein Deficiencies. The deficiency tests were concluded with a study of the influence of carbohydrate and protein deficient diets on the immunity engendered in mice after W.E.E. Since preliminary work had established that mice begin to die after three weeks

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Table XXIV

Effect of Thiamin and Riboflavin Deficiencies on Immunity

| Groups Tested | | Neutralizing Antibodies (i.cer. meth.) | | | | I.Cerebral Immunity | |
|---|---|---|---|---|---|---|---|
| | | Serum Undiluted | | Serum Diluted | | | |
| | | $LD_{50}$ titre | Neutralization index | $LD_{50}$ doses used | 50% end point of serum | $LD_{50}$ titre | Immunity index |
| Unvaccinated--Adequate diet | | 8.3 | ---- | 50 | --- | 8.5 | --- |
| Vaccinated 0.3 x 3 | Adequate diet | 5.5 | 650 | | 1:5 | 1.5 | 10,000,000 |
| | Thiamin deficient | 5.8 | 300 | $\left(\frac{8.3}{6.6}\right)$ | 1:3 | 2.0 | 3,200,000 |
| | Riboflavin deficient | 5.8 | 300 | | 1:3 | 2.0 | 3,200,000 |

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on the protein (casein) deficient diet, it was decided to vaccinate the mice after they had been on the diet for one week. This would allow a period of two weeks for the bleeding experiments. Therefore, a group of 16 mice was started on the carbohydrate deficient diet, 16 were placed on the protein deficient diet and a control group of 12 were given the adequate synthetic diet. One week later the animals were vaccinated and subsequently during the next two weeks were bled for serum. In the test for cerebral immunity 13 mice on the carbohydrate free diet, 12 on the protein deficient diet and all 12 on the adequate diet survived the cardiac puncture. The weight curves of these mice are plotted in Fig. 9 and it can be seen that the mice on the adequate diet showed constant weight increments until the cerebral test for immunity. Mice on the carbohydrate free regime slowly lost weight for some unexplained reason and then slowly gained until a subnormal level was obtained. The protein deficient animals at first lost precipitously and then more slowly. Two days after being bled from the heart the mice were put on a diet containing 2% casein in order to minimize deaths from protein insufficiency. They were kept on this diet for six days and then the casein was omitted entirely from the diet for the remainder of the experiment.

The results of this neutralization test are summarized in Table XXV. The vaccinated mice on the adequate diet exhibited a neutralization index of 2,000 and a serum $LD_{50}$ of 1:8. In contrast the index of the carbohydrate free and the protein deficient diet dropped to 100 which was more than a ten-fold decrease. The serum $LD_{50}$ fell to a 1:3 dilution for the carbohydrate deficient and to 1:2 for the protein deficient diets. Both of these are significant. In Table XXVI is set forth the results

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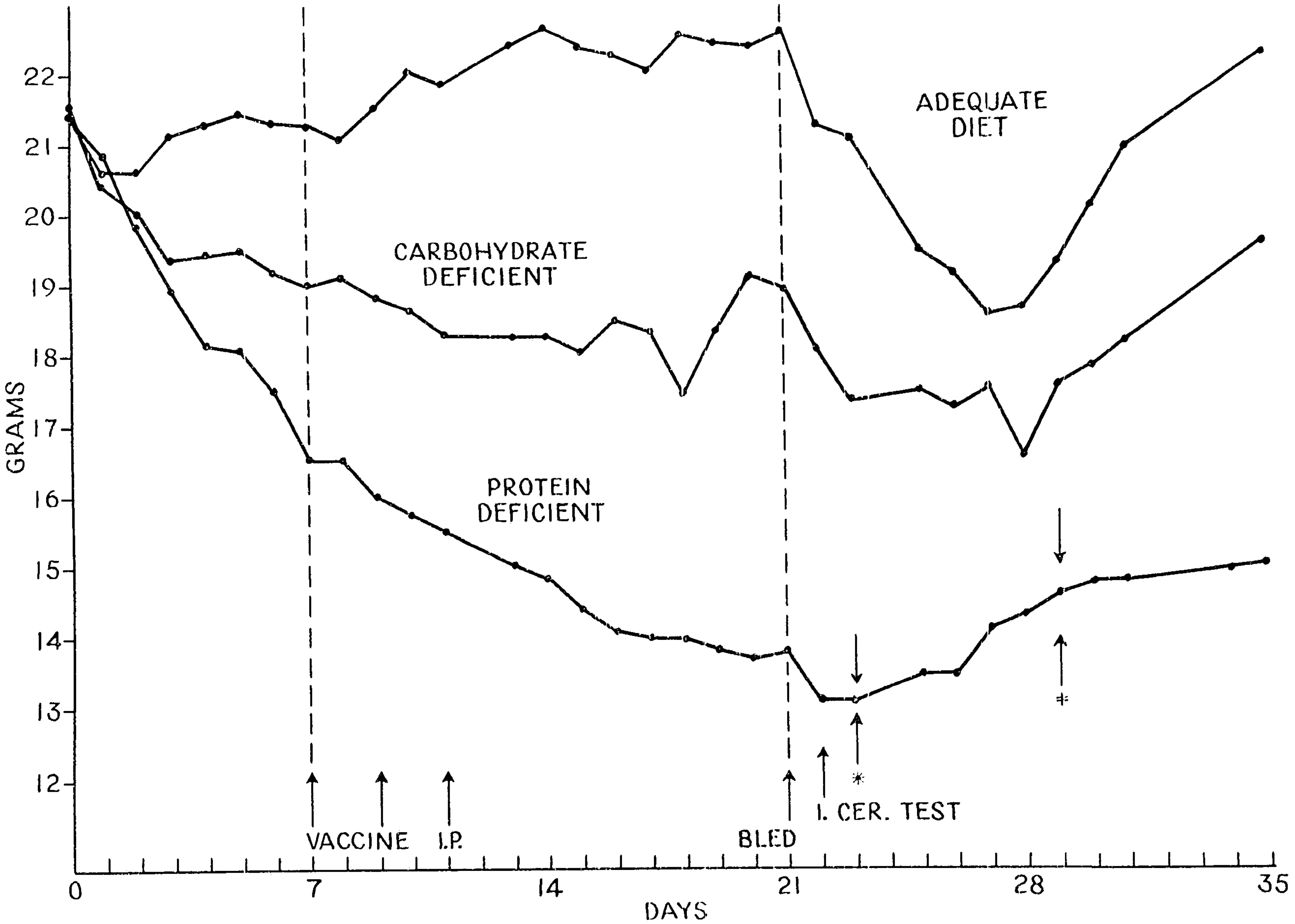


FIG. 9 WEIGHT CURVES OF MICE ON SYNTHETIC DIETS.
* 2% CASEIN DIET STARTED, + BACK TO CASEIN FREE DIET

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Table XXV

Effect of Protein and Carbohydrate Deficiency on Immunity

Neutralization test (i.cer. method)

| Serum in Mixture | Serum Undiluted | | Serum Diluted | |
|---|---|---|---|---|
| | $LD_{50}$ titre | Neutralization index | $LD_{50}$ doses used | Serum |
| Unvaccinated Control | 8.0 | --- | 25 | --- |
| Vaccin. 0.3 x 3: Adequate diet | 4.7 | 2000 | | 1:8 |
| Vaccin. 0.3 x 3: Carbohydrate deficient | 6.0 | 100 | $\left(\frac{8.0}{6.6}\right)$ | 1:3 |
| Vaccin. 0.3 x 3: Protein deficient | 6.0 | 100 | | 1:2 |

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Table XXVI

Effect of Protein and Carbohydrate Deficiency on Immunity

(Test for Cerebral Resistance)

| Group of Mice | | Virus Dilution in Mixture | | | | | | | | | $LD_{50}$ titre | Immunity Index |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | | |
| Unvaccinated Control | | - | - | - | - | - | - | 4/4 | 4/4 | 1/4 | 8.7 | --- |
| Vaccin. 0.3 x 3 | Adequate | 2/3 | 1/3 | 1/3 | 0/3 | - | - | - | - | - | 1.7 | 10,000,000 |
| | Carbohydrate deficient | - | 2/4 | 1/3 | 0/2 | 1/2 | - | - | - | - | 2.5 | 1,600,000 |
| | Protein deficient | - | 2/3 | 1/3 | 0/3 | 1/3 | - | - | - | - | 2.7 | 1,000,000 |

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of the resistance test. The vaccinated controls showed the usual high immunity index of 10,000,000. The vaccinated mice on the deficient diets also show rather high indexes, but to a lesser degree than the controls. Examination of the detailed account in this last table shows a distribution of mortality over a wider range of dilutions in the deficient animals than in the controls. This distribution somewhat resembles that seen in other experiments, such as in underfeeding in which a lower grade of immunity prevails. Since not enough of the higher dilutions were employed the only thing to be said at the present time is that the cerebral resistance of deficient animals is not quite as high as that of control animals after vaccination.

In summary, therefore, removal of either carbohydrate or protein results in a decreased immune responce. This decrease manifests itself both by a drop in humoral antibodies and a somewhat decreased cerebral resistance. To be noted is the finding of low titre antibodies in the presence of strong cerebral resistance.

Summary of Control Tests. In Table XXVII are listed the vaccinated controls used in the various experiments and the immune results obtained with 3 doses of vaccine. It shows first that the neutralization index values range between 650 and 6,300 there being less than a ten-fold difference between the lower and upper limits. Therefore, as values drop below 650, they assume a greater significance. The same may be said of the serum $LD_{50}$ range between 1:5 and 1:20. Values of 1:2 and 1:3 are drops which can not be ignored. Finally, examination of the column under immunity index reveals that where end points were obtained the lowest index was 2,500,000. Usually, however, it was higher. Here

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Table XXVII

Immunogenic Capacity of W.E.E. Mouse Brain Vaccine
Summerization of Vaccinated Controls

Tested 2 weeks after 1st dose of vaccine.
0.3 cc. intra-abdominally--3 doses--2 days interval

| VACCINE | | Neutralization index | Serum Dilution | | Immunity index | Diet used by vaccinated mice |
|---|---|---|---|---|---|---|
| Batch used | Days kept at 4°C | | $LD_{50}$ doses used | Serum $LD_{50}$ | | |
| I | 87 | 1000 | 32 | 1:10 | 100,000+ | Purina |
| II | 67 | 6300 | 50 | 1:20+ | 1,000,000+ | Purina |
| | | 6300 | 50 | 1:16+ | 63,000+ | Synthetic |
| II | 100 | 1300 | 50 | 1:8 | 2,500,000 | Purina |
| | | 1300 | 50 | 1:12 | 10,000,000+ | Synthetic |
| II | 124 | 2000 | 25 | 1:8 | 10,000,000 | Synthetic |
| III | 68 | 5000 | 80 | 1:16 | 25,000,000 | Purina |
| III | 75 | 650 | 50 | 1:5 | 10,000,000 | Synthetic |
| III | 86 | 1600 | 80 | 1:6 | 6,300,000 | Purina |

+ signifies no end point reached.

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again a drop in the value of the immunity index below 2,500,000 becomes of consequence.

Additional evidence is brought forward when the same results are summarized in another way. As previously mentioned in the two types of neutralization tests as well as the cerebral immunity test the same ampule of frozen virus was used. However, all the ampules were prepared from one batch of virus so that to all intents and purposes we can regard the results obtained on all the vaccinated control mice as the results of one large single test. Consequently, the figures reported in Table XXVIII are the cumulative results. They are a neutralization index of 2,000, of serum $LD_{50}$ of 1:9 against 60 $LD_{50}$ doses and finally an immunity index of 6,000,000. These values on the control animal further emphasize the importance of the lower values obtained from vaccinated animals after underfeeding, protein deficiency, etc. In retrospect then a neutraliza-tion index less than 500, a serum $LD_{50}$ below 1:5 and an immunity index less than 1,000,000 are of importance and the drop can most certainly be regarded as due to the specific physiological influence studied and not to experimental error.

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Table XXVIII

Summarization of Vaccinated Controls

Accumulated Results

| Group of Mice | Neutralization Tests (i.cer. method) | | | | Active Immunity | |
|---|---|---|---|---|---|---|
| | | | Serum Dilutions | | Intracerebral Test | |
| | $LD_{50}$ titre | Neutralization index | $LD_{50}$ doses | Serum $LD_{50}$ | $LD_{50}$ titre | Immunity index |
| Unvaccinated | 8.4 | 1 | * | <1:0.5 | 8.6 | 1 |
| Vaccinated | 5.1 | 2000 | 60 | 1:9 | 1.7 | 8,000,000 |

* All controls injected with this number of $LD_{50}$ doses of virus succumbed.

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## Discussion

These experiments were designed primarily to establish a relationship between certain selected physiological factors and immunity to the virus of Western equine encephalomyelitis. It has already been found in many diseases that with increasing age there develops an increasing immune response manifested by either an increased antibody production or a greater resistance to infection. When maturity is reached many other factors pres nt themselves which can influence the state of immunity, and this report deals with a few of them. Fatigue, state of hydration, pregnancy and certain aspects of undernourishment were studied with regard to their effect on the production of immunity. The greater emphasis was placed on antibody production and wherever possible this was correlated with active immunity.

The relationship between fatigue and susceptibility to infection has been the subject of speculation and investigation for a long time. That fatigue decreases resistance to infection is a possibility that has been considered and attempts to establish a relationship have been based either on the production of antibodies or on the development of active immunity after vaccination. In the present report both aspects of immunity are considered and examination of the data fails to reveal any significant effect upon the immunity engendered in vaccinated mice. It is true that exercise after the lesser antigenic stimulation exerts a slight inhibitory influence on the development of active immunity, but until more work is done on this subject it can be generally stated that fatigue fails to exert an influence. This finding is in accord with those of others who studied vaccinated animals after periods of

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exercise. Thus Boycott and Price-Jones (18) found that fatigue had no influence on the mortality of rats inoculated with Gaertners bacillus. There was, however, increased illness and mortality when the organism was given orally. Friedberger, et al (19) observed no difference between fatigued and nonfatigued guinea pigs in respect to vibrio antibody production. No striking difference was noticed by Wedberg (20) in the ability of vaccinated rabbits to produce typhoid agglutinins. He concluded that fatigue does not materially affect the capacity to react to antigenic stimulation. More recently Sarracino and Soule (14) reported fatigue to be without effect on experimental influenza virus infection in mice. In fact, some investigators report a decreased susceptibility after exercise. Using rats, Oppenheimer and Speeth (21) stated that fatigue tended to increase resistance to tetanus toxin and definitely increased resistance to Type I pneumococcus. This was further corroborated by Nicholls and Spaeth (22) using Type I pneumococcus in guinea pigs. On the other hand questionable diminished agglutinins after typhoid vaccination was reported by Trommsdorff (23) in guinea pigs. A more definite redution in typhoid agglutinins was obtained by Lara and de Jesus (24) in fatigued guines pigs than in controls not exercised. Moreover, the effect was more pronounced in females than in males. Employing rats infected with <u>Pseudomonas aeruginosa</u>, Merrill and Howe (25) concluded that fatigue favors susceptibility to infection and that fatigue after infection was more disastrous than before. However, they noted that a training period before infection tended to increase the resistance of the rats. The close association of a history of physical overexertion just before attacks of poliomyelitis in human beings prompted

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Milzer, Lewin and Levinson to study the disease in monkeys (13). They observed that exercised animals developed a higher incidence and more serious paralysis than the controls.

The failure to obtain a definite altered immune response in exercised mice may be explained by the fact that mice normally lead an active existence and that vigorous exercise is not a radical departure from their normal activity. Also, if weight loss can be shown to be a factor in reduced immunity it may be that the drop in weight was not drastic enough. Nevertheless, the loss in weight after exercise was sufficient to slightly decrease the resistance of the mice to cerebral infection without altering their antibody response.

The results obtained with dehydrated mice show that no marked change in antibody production occurs. The literature failed to reveal any other attempts at establishing a relationship between dehydration and its influence on immune body production. However, the fact that dehydration or its attendant phenomena led to an increased susceptibility to virus infection was established. King (26) noted an enhanced activity of equine encephalomyelitis virus in mice dehydrated as a result of a previous inoculation of 50% glycerine. The same effect was produced by peripheral inoculation of concentrated salt solution. Moreover, a restricted water intake which gave a blood concentration equal to that produced by glycerine or salt led to no such increase of invasiveness of the central nervous system. The author concluded, therefore, that dehydration, suddenly produced, facilitated the ability of the virus to penetrate the nervous system. Gradual removal of water did not result in any significant dehydration of the brain with a consequent

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failure to facilitate entry into that organ. Quite another explanation was offered by Sprunt (27) who showed that dehydration of the interstitial spaces caused an increase in the number of vaccinial lesions. However, the end result is the same. Dehydration leads to an increased susceptibility to virus invasion. He postulated that hydration tends to localize the virus *in situ* because fewer cells are exposed to it, while dehydration has the opposite effect. It has been noted that poliomyelitis occurs after strenuous exercise thus suggesting that perhaps dehydration brought on by the exercise may have facilitated entry of the virus into the nerve cells causing infection.

From the above findings it would appear that the state of resistance of the dehydrated individual depends not so much on humoral antibodies as on the increased ability of the virus to enter susceptible cells. Therefore, it is not surprising that withholding of water from the mice has not led to antibody change. That the one dehydrated mouse succumbed to $10^{-5}$ dilution of virus is an indication of the decreased resistance to virus infection in the presence of an unaltered antibody level.

During pregnancy physiological changes occur which markedly affect the body tissues and in some way this change is reflected by an altered immune response. Observations have been made over many years that pregnancy modifies the course of some diseases in women. Experimentally this was borne out by the work of Rosahn and associates (28) who demonstrated that pregnancy increases resistance to vaccinia virus. Moreover, they found a lower incidence of both localized and generalized lesions in pregnant rabbits as compared to males. Using

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the neutralization test as an indicator, but not as a sure sign of immunity, Jüngeblut and Engle (29) tested the sera obtained from 12 pregnant women and found that they all neutralized the virus of poliomyelitis. Their conclusions were quite obvious. However, recent observations, although limited in number, lend no credence to this possibility. Peelen (30) and Harmon and Hoyne (31) reported the inability of pregnancy to modify the course of the disease in human poliomyelitis. In addition both agree that congenital poliomyelitis does not occur.

Far from substantiating any of the above findings Hodes (32) reported a decreased ability on the part of pregnant mice to elicit an immune response to St. Louis virus as measured by their cerebral resistance. Moreover, he also showed that pregnancy not only interfered with the development of immunity, but it also diminished a previously established immunity. The author finally concluded that since pregnancy did not increase the susceptibility of the mice it must in some manner affect the tissues which were rendered immune by vaccination. This last was corroborated to a certain extent in the experiments reported here. It will be recalled that where the smaller amount of antigen was given a slight drop in immunity was produced in pregnant mice. There was a decreased cerebral resistance which confirmed Hodes' work. In addition the decreased level of antibody was noted. The drop in immunity did not become manifest when the dose of vaccine was increased. No other work on the effect of pregnancy on neutralizing antibodies appeared in the literature.

The relationship between pregnancy and brucellosis experimentally induced is of considerable interest. According to Huddleson (33)

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calves were insusceptible to <u>Brucella abortus</u> infection up to the time of breeding, but beyond this period infection was quite common during pregnancy. Of interest too were the changes in agglutinin titre which were noted among goats experimentally infected with <u>Brucella melitensis</u>. Nonpregnant animals showed at best only a slight rise in agglutinating power whereas pregnant goats responded with a high titre antibody content in the blood. After pregnancy the titre reverted to its original low level.

The problem of underfeeding is important for many reasons, not the least of which is its relationship to infection. The long associated observation of starvation and increased susceptibility to disease has prompted investigation along this line, but no conclusive statement is possible at the present time. It seems very likely from the present experiments that a marked reduction in immunity takes place. There is a drop in cerebral resistance which is paralleled by a fall in neutralizing antibodies, and more important these declines can be obtained when the experiment is repeated. Obviously the action is one of a lowered ability on the part of the starved tissues to produce immune substances after antigenic stimulation. In the well fed animal vaccination leads to a strong immunity as evidenced by a high level of resistance as well as of neutralizing antibodies. A restricted food intake, on the other hand, affects the tissues in a manner which becomes manifest after vaccination by a reduced capacity on the part of these tissues to form the soluble immune substances which are found in the blood stream as well as those which are bound up in some manner with the cells and which cause tissue immunity. Thus whether the impaired

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immune response is due to fewer healthy cells remaining or whether it is due to a lack of some substance or substances in the cells remains to be seen. Regardless of the mechanism the diminished immune response is apparent. Examples of lowered immune response after vaccination appear in the literature. Müller (34) noted that underfed pigeons showed lower titre agglutinins against Vibrio metchnikovii and B. proteus. On the other hand, the titre was higher in the starved pigeons after vaccination with B. pyocyaneus, B. dysenteriae and B. typhosus. However, the agglutinins did not parallel the immunity since the starved pigeons were more susceptible to typhoid. Trommsdorff (23) not only reported the effect of fatigue, but also the effects of hunger on vaccinated guinea pigs. The results were irregular so that at best only questionable diminished agglutinin titres could be reported.

Although no other studies on the influence of underfeeding on immune response after vaccination were encountered, two reports dealing with increased resistance after the withholding of food are deemed worthy of mention. Deprivation of food results in either fewer or smaller vaccinial lesions in injected rabbits. As explained by Sprunt (27) this is presumably due to the reduction of available nutrients in the cell for the parasitizing virus. Therefore, according to this concept, increased resistance to virus infection can be brought about to a certain extent by depriving susceptible cells of materials required by the multiplying virus. Foster and associates (15) attempted to establish a correlation between thiamin deficiency in mice and their susceptibility to the Lansing strain of poliomyelitis. They concluded that $B_1$ deficiency increased the resistance of the animals to the Lansing strain. Since this vitamin deficiency leads to an impaired

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appetite, these investigators undertook a study of the effect of a restricted food intake on the ability of the mice to resist poliomyelitis. Here, again, the conclusion was reached that far from decreasing the susceptibility of mice to the Lansing strain underfeeding caused an increased resistance. These few reports tend to bear out the concept of an increased resistance to virus infection by keeping the tissue cells in an undernourished state. Further examples are cited by Sprunt. However, the same notion does not apply to starved animals that are vaccinated. Here there is a decreased resistance to infection which is brought about by an inhibition of the immune producing mechanism. Still another mechanism prevails among rapidly growing mice which can prevent the spread of disease. As pointed out by Sabin (35) this mechanism does not depend on the resistance or immunity of the animal, but rather on the insusceptibility of certain specific tissues which act as a barrier to further spread of the virus to the nervous system. He showed that the development of increasing resistance to intramuscular injection of vesicular stomatitis due to age was definitely retarded by restricting the amount of food consumed. This retardation was not the result of vitamin deficiencies since these were supplied in liberal amounts. Thus underfed mice even at the age of eight weeks were fully as susceptible to intramuscular injection of virus as two-week old mice at a time when well fed mice were developing resistance as early as the fourth week of life.

The effect of removal of the entire "B" complex from the diet on resistance to infection generally has been reported in the literature as a decreased immunity. The findings in the present report tend to

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substantiate this conclusion. In one case a definite drop in antibody titre was detected by the use of undiluted serum and only a suggestive fall by the serum dilution method while in the other case a slight decline was recorded with the use of undiluted serum and a definite decrease by the serum dilution method. The clinical observations by Riddle and associates (36), that "B" deficient patients showed a lowered immune response to Staphylococcus aureus and Streptococcus hemolyticus prompted Morey and Spies (37) to investigate the effect of varying types of "B" deficiency in human beings on their ability to produce agglutinins after B. tularense vaccination. Patients with a mild deficiency demonstrated fairly good agglutination titres, those with moderate symptoms a diminished titre and those with severe symptoms a more marked diminution, almost to the point of complete disappearance. The authors finally concluded that "B" deficient patients not only show a lowered immune response, but also a lessened ability to maintain whatever titre was present. It appears that the above results were due to the lessened ability on the part of the deficient tissues to produce the agglutinins. Breakdown of resistance after "B" deficiency in rats was demonstrated by Robertson and Tisdall (38). These authors found that rats rendered vitamin B deficient were more susceptible to Salmonella muriotitis (rat typhoid) than healthy controls. From the results it would appear that the "B" complex aids in maintaining cellular resistance to infection as well as in producing antibodies.

Attempts to implicate specific factors of the "B" complex in disease resistance have been many with emphasis on thiamin and riboflavin. Although decreased resistance has been postulated for vitamin deficient

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animals, no such conclusion can be drawn from the present experiments. Mice deficient in either $B_1$ or $B_2$ were just as responsive to immunization as were control mice. The deficient animals developed neutralizing antibodies and cerebral resistance to the same degree as did the controls. No other work appeared in the literature on vaccination against a virus disease under conditions of vitamin $B_1$ or vitamin $B_2$ deficiency. There were, however, several reports dealing with a change in resistance as a result of the above deficiencies. Cowdry, et al, (39) recorded that $B_1$ or $B_2$ deficient rats injected intracerebrally with herpes virus were slightly more susceptible than the controls. However, these results were not clear cut. According to Sabin and Duffy (40) depriving young mice of $B_1$ or $B_2$ during their period of growth retarded the normal development of the constitutional barriers to involvement of the nervous system to a neurotropic virus (vesicular stomatitis). Moreover, this type of resistance, once acquired, could not be broken down in full grown animals, even after they had been rendered vitamin deficient. On the other hand reports have appeared which indicate an increased resistance to inoculation of virus after thiamin deficiency. As previously mentioned, Foster and coworkers (15) pointed out that vitamin $B_1$ deficiency increased the resistance of mice to the Lansing strain of poliomyelitis. This was corroborated by Rasmussen and associates (41) who extended the work to include Theiler's virus.

The results obtained indicate that removal of either protein or carbohydrate leads to a lowered activity of the immunity producing mechanism in vaccinated mice. Both groups of mice showed some decrease in cerebral immunity and an appreciable decline in antibody as determined by the two methods of testing for neutralizing antibodies. Since carbohydrate

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deficiency *per se* does not exist in the presence of adequate fat or protein, it was more or less of a surprise to find that removal of carbohydrate from the diet resulted in a lowered state of immunity. Nevertheless that it did occur leads to the belief that in some way the metabolism was altered sufficiently to exert an influence on the mechanism of immunity. Sako (42) in a statistical analysis found that mice showed no change in resistance to pneumococcus injection when the diet was low in carbohydrate, but showed a lowered resistance when the diet was low in protein. A decreased resistance to rat typhoid in rats on a protein inadequate diet was reported by Robertson and Tisdall (38). Hotta (43) found a slightly decreased resistance to mouse typhoid on a low protein diet. The effect of a low protein diet on antibody formation was made the subject of investigation by Cannon, et al (44). They concluded that hypoproteinemic rabbits exhibited a definitely lessened capacity to produce agglutinins when compared with well fed rabbits after typhoid vaccination. Besides the article cited other reports indicated that deficiencies in vitamins A, B, C or D decreased agglutinin production. At the same time some investigations reported negative results. Consequently, at the present time this aspect of the work is still controversial. The whole subject is thoroughly reviewed in the article by Robertson (16). Noted also was the almost complete absence in the literature of reports about factors influencing neutralizing antibody production. The influence of age (4) has already been mentioned in which it was found that vaccinated mice showed increasing antibody titres with small increments of age. Feller and associates (45) recorded no change in influenza virus antibody formation in patients with marked vitamin A or vitamin C deficiency. However, these patients

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were not vaccinated with influenze. Wilson and others (46) using monkeys infected with influenza virus, also reported negative results. They found that some monkeys on the various deficient diets succumbed to intranasal instillation of influenza A virus, whereas none of the controls died. Studies of the humoral antibodies revealed no difference in titre between deficient animals and controls. To be noted is the fact that the diet which was deficient for the monkeys, appeared on analysis to be adequate for mice.

The relationship between level of antibody and degree of cerebral resistance has been studied in equine encephalomyelitis. A correlation was established by Morgan (4) who found that antibody in the serum of mice paralleled, though at a considerably lower level, their resistance to infection. Casals (5) reported maximum levels of neutralizing antibodies despite different titres of resistance. He found that antibodies appeared where there was no resistance and were present where the resistance was negligible. High indexes of resistance were also obtained at the same antibody level. In all of the above tests undiluted serum was used. Certain discrepancies seemed to clear up when antibody was titrated by the serum dilution method. Using this method Morgan and Olitsky (2) demonstrated that different antisera apparently were equal in neutralizing capacity when they were tested undiluted, but, when tests were made with dilutions of serum, different levels of protection were obtained. Thus a single dose of either killed vaccine or living vaccine produced in mice approximately the same titre of antibodies when undiluted serum was used. However, when serum dilutions were used, it was found that serum obtained from mice vaccinated with living virus could be diluted as much as one hundredfold

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and still protect the animal. The sera of mice vaccinated with killed virus was not protective to the same degree. Furthermore, the level of neutralizing antibodies in mice vaccinated with killed virus could be made to equal the level in mice vaccinated with living virus merely by increasing the amount of vaccine inoculated. From their results these authors concluded that since a change in cerebral resistance was reflected by a similar change in antibody level, a correlation was demonstrated between titre of antibody and the degree of cerebral resistance.

It is apparent from the results obtained by Morgan and Olitsky (2) that serum dilution is the method of choice for a quantitative determination of antibody titre. Their method, whereby different serum dilutions were mixed with approximately 10 units (8 to 15 $LD_{50}$ doses) of virus showed that antiserum could be diluted to as high as 1:10,000 and still protect animals. Since it was shown by Sabin and Ward (47) that a neutralization index of less than 10 is negative and an index between 10 and 49 equivocal, it can be seen that Morgan and Olitsky were using an amount of virus, which was too close to the borderline and, therefore, of doubtful significance. The use of such small amounts of virus may explain the distribution of survivors over a wide range of dilutions in their neutralization experiments. More clean cut results were obtained in our experiments by employing 50 $LD_{50}$ doses of virus in the serum dilution neutralization tests.

In general, when a group of vaccinated mice showed a decreased resistance, they also showed a decrease in neutralizing antibodies. However, the reverse did not obtain since in at least two of the tests low levels of antibody were accompanied by high levels of cerebral resistance

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(Tables XXIV and XXV). Furthermore, when a drop in cerebral immunity occurred there was left behind a marked immunity to infection as measured by immunity indexes of 63,000 to 1,600,000. Only by direct comparison with the vaccinated controls did the diminished resistance become perceptible. From this it would appear that an effect of some of the physiological conditions on vaccination is to depress the cellular immunity somewhat, but not to the extent that soluble immune substances in the blood stream are diminished (Table XXV).

The finding of low levels of antibody in the presence of high titres of resistance is not compatible with Morgan's concept of a parallel response and does not warrant the belief that antibody level parallels the degree of cerebral resistance in equine encephalomyelitis. If, as Margan believes, true titration of antibody can be obtained by serum dilution rather than virus dilution, it would be difficult to reconcile negligible serum $LD_{50}$ titres of 1:2 or 1:3 with immunity indexes of more than a million (Tables XXIV and XXV). On the other hand, it could be corroborated that quantitative differences in antibody content became apparent only when serum dilutions are used. As an example, mice vaccinated with three doses of vaccine responded with a thousandfold greater cerebral resistance than those with one dose (Tables XIII and XXII). When undiluted serum was used, both groups produced approximately the same neutralization index, but the difference in antibody content became apparent, when it was found that serum from the three-dose group of animals could be diluted further than that from the one-dose group and still retain its protective power (Tables XXI and XXI). In this way a relationship between antibody and resistance was demonstrated.

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The immune response of the vaccinated mice under the various physiological conditions studied was seldom as good as that of the vaccinated controls. The decrease in immunity, although often small and of no significance, was a decrease nevertheless. Analysis of the results showed that four of the conditions studied (underfeeding, "B" deficiency, carbohydrate deficiency, protein deficiency) led to a marked loss of weight and a definitely diminished immunity. However, loss of weight *per se* was not responsible, since no such decrease in immunity was noted for some other conditions (fatigue, dehydration, thiamin deficiency, riboflavin deficiency) where a definite loss in weight occurred. Although a reduced immunity was found in the presence of an increased weight during pregnancy, it should be borne in mind that the increased weight of pregnancy is more apparent than real.

Vaccination stimulates the healthy body cells to the production of tissue immunity and of circulating antibodies which neutralize the effects of the virus. An effect of a lowered weight is to decrease the number of available healthy cells for antibody formation as well as the number of healthy cells for dissipating the effects of the invading organisms. In pregnancy there is no weight loss, but profound changes take place in the body and the mechanism whereby it diminishes immunity is still unknown. The suggestion is made that it affects the tissues which are made immune by vaccination in some manner. The effects extend to antibody production both qualitatively and quantitatively. For example, a lowered neutralization index was reflected in a lowered serum $LD_{50}$ denoting a quantitative change. Occasionally a decreased neutralization index was encountered which did not accompany a decreased serum $LD_{50}$, thus denoting a qualitative change.

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An effect of certain biological conditions is to diminish the immune response of vaccinated animals. One of the manifestations is a lessened resistance to cerebral infection. This type of lowered resistance must not be confused with the lowered resistance that occurs in nonvaccinated animals under certain physiological states and is due in some way to a breakdown in tissue resistance or a breakdown in barriers to infection. No attempt has been made to determine the amount of lowered resistance which is carried over from nonvaccinated to vaccinated mice under the same physiological conditions.

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## Summary

Albino mice were tested for immunity two weeks after vaccination with the formalinized virus of Western equine encephalomyelitis. Neutralizing antibodies and cerebral resistance were determined. For the determination of antibodies serial dilutions of serum as well as undiluted serum was used. Antibody response did not always parallel the degree of resistance.

The immune response of mice subjected to various physiological conditions rarely equalled that of control animals. Specifically the following observations were made:

1. Fatigue did not cause any change in the level of neutralizing antibodies. A questionable decrease in resistance occurred after small amounts of vaccine, but not after larger doses.

2. Dehydration had no effect on antibody production.

3. In pregnancy, decreases in resistance as well as antibody titre were observed after small amounts of vaccine, but not after larger amounts.

4. Underfeeding resulted in a reduction of resistance as well as production of neutralizing antibodies.

5. Removal of specific dietary factors from the synthetic diet effected the production of immunity in the following manner:

a. Deficiency of the "B" complex resulted in a decreased level of antibody.

b. Thiamin or riboflavin deficiency had no effect either on the production of neutralizing antibodies or on the cerebral resistance.

c. In protein or carbohydrate deficiency a drop in cerebral resistance and in neutralizing antibodies was found.

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6. Mice on a synthetic diet exhibited the same immune response to vaccination as did mice on a stock diet.

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BIBLIOGRAPHY

1. Meyer, K. F., Haring, C. M., and Howitt, B.: Etiology of epizootic encephalomyelitis of horses in the San Joaquin Valley, 1930. Science, 1931, 74, 227.

2. Morgan, I. M., and Olitsky, P. K.: Immune response of mice to active virus and to formalin-inactivated virus of eastern equine encephalomyelitis. J. Immunol., 1941, 42, 445.

3. Olitsky, P. K., Sabin, A. B., and Cox, H. R.: Acquired resistance of growing animals to certain neurotropic viruses in the absence of humoral antibodies or previous exposure to infection. J. Exp. Med., 1936, 64, 723.

4. Morgan, I. M.: Influence of age on susceptibility and on immune response of mice to Eastern equine encephalomyelitis virus. J. Exp. Med., 1941, 74, 115.

5. Casals, J.: Neutralizing and complement-fixing antibody production and resistance following vaccination in experimental encephalitis infections. J. Exp. Med., 1943, 78, 447.

6. Reed, L. J., and Muench, H.: Simple method of estimating 50 per cent endpoints. Am. J. Hyg., 1938, 27, 493.

7. Sabin, A. B., assisted by Duffy, C. E., Warren, J., Ward, R., Peck, J. L., and Ruchman, I.: St. Louis and Japanese B types of epidemic encephalitis; development of noninfective vaccines; report of basic data. J. Am. Med. Assn., 1943, 122, 477.

8. Olitsky, P. K., and Harford, C. G.: Intraperitoneal and intracerebral routes in serum protection tests with virus of equine encephalomyelitis; comparison of antiviral serum constituents from guinea pigs immunized

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

with active or formolized inactive virus. J. Exp. Med., 1938, 68, 779.

9. Woolley, D. W., and Krampitz, L. O.: Production of a scurvy-like condition by feeding of a compound structurally related to ascorbic acid. J. Exp. Med., 1943, 78, 333.

10. Phillips, P. H., and Hart, E. B.: Effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., 1935, 109, 657.

11. Woolley, D. W.: Some new dietary essentials required by guinea pigs. J. Biol. Chem., 1942, 143, 679.

12. Woolley, D. W., and White, A. G. C.: Production of thiamine deficiency disease by feeding of pyridine analogue of thiamine. J. Biol. Chem., 1943, 149, 285.

13. Milzer, A., Lewin, P., and Levinson, S. O.: Effect of fatigue, chilling and trauma on resistance to experimental poliomyelitis. J. Bact., 1943, 45, 78.

14. Sarracino, J. B., and Soule, M. H.: Effect of heat, cold, fatigue and alcohol on resistance of mice to human influenza virus. Proc. Soc. Exp. Biol. and Med., 1941, 48, 183.

15. Foster, C., Jones, J. H., Henle, W., and Dorfman, F.: Effect of vitamin $B_1$ deficiency and of restricted food intake on the response of mice to the Lansing strain of poliomyelitis virus. J. Exp. Med., 1944, 79, 221.

16. Robertson, E. C.: Vitamins and resistance to infection. Medicine, 1934, 13, 123.

17. Clausen, S. W.: Influence of nutrition upon resistance to infection. Physiol. Rev., 1934, 14, 309.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

18. Boycott, A. E., and Price-Jones, C.: Experiments on the influence of fatigue on infection. J. Path. and Bact., 1926, 29, 87.

19. Friedberger, E., Andersen, O., Callerio, C., and Rutchko, I.: Ueber den Einfluss der Ermüdung auf die Antikörperbildung und die Infektion. Z. Immunitätsforsch., 1931, 72, 225.

20. Wedberg, S. E.: Effect of exercise on the production of typhoid agglutinins. Yale J. Biol. and Med., 1942, 15, 263.

21. Oppenheimer, E. H., and Spaeth, R. A.: Relation between fatigue and susceptibility of rats towards a toxin and an infection. Am. J. Hyg., 1922, 2, 51.

22. Nicholls, E. E., and Spaeth, R. A.: Relations between fatigue and susceptibility of guinea pigs to infections of type 1 pneumococcus. Am. J. Hyg., 1922, 2, 527.

23. Trommsdorff, R.: Experimentelle Studien über die Ursachen der durch verschiedene Schädlichkeiten bedingsten Herabsetzung der natürlichen Widerstandsfähigkeit gegen Infektionen (Resistenz); ein Beitrag zur Immunitätslehre. Arch. Hyg., 1906, 59, 1.

24. Lara, H., and de Jesus, P. I.: Effect of fatigue and excitement upon the formation of immune bodies. Philippine J. Sci., 1930, 42, 497.

25. Merrill, I. B., and Howe, E. C.: Effect of exercise and fatigue upon resistance to infection; experiment on the albino rat using Pseudomonas aeruginosa as infecting agent. Am. Phys. Ed. Rev., 1928, 33, 67.

26. King, L. S.: Studies on eastern equine encephalomyelitis; facilitation of infection in the mouse. J. Exp. Med., 1942, 76, 325.

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27. Sprunt, D. H.: Effect of undernourishment on susceptibility of rabbit to infection with vaccinia. J. Exp. Med., 1942, 75, 297.

28. Rosahn, P. D., Hu, C. K., and Pearce, L.: Reaction of normal and rabbit pox immune adult rabbits to vaccination with vaccine virus. J. Immunol., 1936, 31, 59.

29. Jungeblut, C. W., and Engle, E. T.: Influence of pregnancy and of menstruation on the power of normal human sera to inactivate poliomyelitis virus. J. Immunol., 1933, 24, 267.

30. Peelen, J. W.: Pregnancy complicated by acute anterior poliomyelitis. Mich. State Med. Soc., 1943, 42, 30.

31. Harmon, P. H., and Hoyne, A.: Poliomyelitis and pregnancy, with special reference to failure of fetal infection; two hitherto unreported cases in one of which human fetal spinal cord was examined for virus. J. Am. Med. Assn., 1943, 123, 185.

32. Hodes, H. L.: Effect of pregnancy upon immunity of mice vaccinated against St. Louis encephalitis virus. J. Exp. Med., 1939, 69, 533.

33. Huddleson, I. F.: Brucellosis in man and animals. New York, The Commonwealth Fund, Revised edition, 1943, p. 195.

34. Müller, P. T.: Ueber den Einfluss künstlicher Stoffwechselalterationen auf die Produktion der Antikörper. Arch. Hyg., 1904, 51, 365.

35. Sabin, A. B.: Constitutional barriers to involvement of the nervous system by certain viruses, with special reference to the role of nutrition. J. Pediatrics, 1941, 19, 596.

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36. Riddle, J. W., Spies, T. D., and Hudson, N.P.: Note on interrelationship of deficiency diseases and resistance to infection. Proc. Soc. Exp. Biol. and Med., 1940, 45, 361.

37. Morey, G. R., and Spies, T. D.: Antibody response of persons with pellagra, beriberi and riboflavin deficiency. Proc. Soc. Exp. Biol. and Med., 1942, 49, 519.

38. Robertson, E. C., and Tisdall, F. F.: Nutrition and resistance to disease. Canad. Med. Assn. J., 1939, 40, 282.

39. Cowdry, E. V., Lucas, A. M., and Neff, C. F.: Resistance of vitamin $B_1$ and $B_2$ deficient and normal rats to intracerebral injection of herpes virus. J. Infect. Dis., 1935, 57, 174.

40. Sabin, A. B., and Duffy, C. E.: Nutrition as a factor in the development of constitutional barriers to involvement of the nervous system by certain viruses. Science, 1940, 91, 552.

41. Rasmussen, A., Waisman, H., Elvehjem, C., and Clark, P.: Influence of the level of thiamine intake on the susceptibility of mice to poliomyelitis virus. J. Infect. Dis., 1944, 74, 41.

42. Sako, W. S.: Resistance to infection as affected by variations in proportions of protein, fat, and carbohydrate in diet; experimental study. J. Pediatrics, 1942, 20, 475.

43. Hotta, Y.: Der Einfluss der Ernährung auf die natürliche Resistenz. Centr. Bakt., 1. Abt., 1928, 108, 413.

44. Cannon, P. R., Chase, W. E., and Wissler, R. W.: Relationship of protein-reserves to antibody-production; effects of low-protein diet and of

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plasmapheresis upon formation of agglutinins. J. Immunol., 1943, 47, 133.

45. Feller, A. E., Roberts, L. B., Ralli, E. P., and Francis, T., Jr.: Studies on the influence of vitamin A and vitamin C on certain immunological reactions in man. J. Clin. Inv., 1942, 21, 121.

46. Wilson, H. E., Saslaw, S., Schwab, J. L., Woolpert, O. C., and Doan, C.A.: Nutritional deficiency and resistance to infection in monkeys. J. Am. Med. Assn., 1943, 121, 1411.

47. Sabin, A. B., and Ward, R., Unpublished observations.

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