THE EFFECT OF RIBOFLAVIN DEFICIENCY
ON PHAGOCYTOSIS, SUSCEPTIBILITY,
AND SERUM PROTEINS IN THE RAT.

by

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STATEMENT BY AUTHOR

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APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

[Kenneth F. Wertman] [July 10, 1960]

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Professor of Bacteriology
In sincere appreciation to Dr. Kenneth Wertman for his supervision, encouragement, and assistance during this investigation.
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INTRODUCTION

The influence of diet on the incidence and outcome of infection has been studied since early 1900. Research endeavors have been prompted by observations of higher infection rates in malnourished populations. The pestilence that accompanies war and other types of disaster is often associated with inadequate nourishment. Lusk (1921), observing higher frequency of tuberculosis among the Germans during World War I, stated that undernourishment diminishes physical and mental powers, and reduces resistance to infection. Cannon (1942) drew a close parallel between malnourishment and the increase in infection during wartime. This parallel was partially explained by the decrease in protein intake, which subsequently reduced antibody protein matrix. Werhman, et al. (1952) stated that there was increased susceptibility and mortality to typhus in malnourished human and animal populations.

However, observations made during war and disaster yield inconclusive evidence of a direct relationship between malnourishment and increased infection rates. Howie (1949) has suggested that these areas are deficient in other respects. Hospitals and health organizations are not available to isolate and control potential infectious agents; personal hygiene is either poor or lacking; living quarters are crowded and often infested; and members of the
community suffer from low morale, anxiety and fatigue.

Because of the existence of these other factors in poorly fed communities and disaster areas, the observer must proceed cautiously in drawing a direct relationship between poor diet and alteration in animal resistance to disease. Fatigue and anxiety alone have been shown to alter host susceptibility sufficiently to cause an increase in infection rate (Rasmussen, 1957; McQuire and Floyd, 1958). Therefore, it has become necessary to look to laboratory investigation for more conclusive results concerning the influence of diet on host susceptibility.

The experimentation of Schneider and Webster (1945) and Schneider (1946, 1958, 1960) has led them to attribute to heredity the resistance of animals to disease. However, after extensive animal studies, Schutz, et al. (1936) and Church (1939) have concluded that nutrition must be considered along with heredity in the study of animal resistance.

Various nutritional requirements and their effects on host resistance have been studied. Webster and Pritchett (1924) observed the effects of different synthetic diets on the susceptibility of mice to infection. Schneider and Webster (1945) found that inanition alone could account for differences in resistance that occurred among groups of mice fed on varied basal grains. Jackson and Smith (1931) reported evidence of increased susceptibility in animals restricted in their water intake for several months.
Seeler and Ott (1944) showed that feeding chicks 50% of the normal food consumption decreased survival to Plasmodium lophurae by the same percentage. Varying degrees of protein deficiency have been shown to lower the resistance of experimental animals (Fitzpatrick, 1948; Miles, 1951; Dubos and Schaedler, 1958; Hill and Garren, 1958; Ruebner and Bramhall, 1959).

The present emphasis in nutritional investigations is that of the effect of vitamin deficiencies on host susceptibility to infection. Extensive reviews have appeared which deal with all the known vitamins (Clausen, 1934; Robertson, 1934; Aycock and Lutman, 1944; Schneider, 1946; Clark, et al., 1949).

The effect of deficiency in members of the B-complex group of vitamins has been reported upon by numerous investigators. Werkman (1923b) found that "B vitamin" deficient rats were more susceptible to infection by anthrax bacilli and pneumococci than were non-deficient rats.

Rose (1928) maintained dogs on restricted "B vitamin" diets and noted that these animals gave positive blood cultures after injection with Bacillus welchii, while normal dogs gave negative cultures. Similarly, Barlow (1930) demonstrated a bacteremia only in the "B vitamin" deficient

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1 Throughout this paper, the nomenclature of organisms is that of the original authors.
Ross and Robertson (1932) were able to show a decrease in the resistance of rats to infection by Salmonella muriotidis after being deprived of "B vitamin". Rose and Rose (1936), aware of the importance of paired-weight control animals, found that the increased fatality in "B vitamin" deficient rats was not due to the state of inanition resulting from the deficiency. However, Rose, et al. (1936) attributed to inanition the decrease in resistance to intoxication by B. welchii toxin.

The diet-susceptibility relationship was studied further by Watson (1937), who maintained mice on diets deficient in "B vitamin". The deficient animals showed a definite decrease in resistance to per os infection with Salmonella typhimurium. These studies were extended by Watson, Wilson and Topley (1938), observing the effects of "B vitamin" deficiency on mouse typhoid epidemics. Utilizing ad libitum, inanition and deficient groups, several mice from each group were inoculated with S. typhimurium and placed in cages with 100 or more un inoculated mice of the same group. Animals in the deficient group were clearly more susceptible to an ensuing typhoid epidemic than were mice in the control groups.

Various members of the B-complex vitamin group were isolated and purified during the late 1930's and early 1940's. The availability of these intrinsic factors
in pure form made it desirable to study single-vitamin deficiencies.

Animals deficient in thiamin have been shown to be less resistant to bacterial and rickettsial infection than normal animals (Natvig, 1941; Wooley and Sebrell, 1942; Badger, et al., 1940; Badger, 1942; Robinson and Seigel, 1944; Guggenheim and Beuchler, 1946; Fitzpatrick, 1947; Wertman and Groh, 1959). Parasitic infections apparently are reduced in severity in the thiamin deficient host (Reiner and Paton, 1932; Watt, 1944; Brooke, 1945), although an exception to this has been noted in experimental rat coccidial infection (Becker and Dilworth, 1941).

Deficiency in other members of the B-complex group has also been shown to lower host resistance to bacterial and rickettsial infection. These include deficiency in pantothenic acid (West, et al., 1944; Fitzpatrick, 1948; Zucker and Zucker, 1954; Seronde, 1954), biotin (Kligler et al., 1946; Trager, 1943) and pyridoxine (Robinson and Seigel, 1944). Some investigations have apparently refuted the findings of increased susceptibility in these deficiency states. For example, Day and McClung (1945) found no change in the resistance of pantothenic acid deficient rats when challenged with pneumococci. Similar observations were made by Robinson and Seigel (1944), who induced lobar pneumonia in pantothenic acid deficient rats. Fitzpatrick (1948) found no alteration in the resistance of pyridoxine deficient
It can be seen that no unequivocal conclusions may be drawn concerning the susceptibility of animals deficient in various members of the B-complex vitamin group. The literature reveals the same degree of confusion with respect to alteration in the susceptibility of riboflavin deficient animals.

Pinkerton and Bessey (1939) reported that all riboflavin deficient rats died when challenged with murine typhus rickettsiae, while all animals on the complete ration survived. This work was later confirmed by Fitzpatrick (1948), who employed the same infecting organism. Wooley and Sebrell (1942) demonstrated that riboflavin deficient mice exhibited higher fatality rates to pneumococcal pneumonia than normal mice.

In contradiction to these results, Robinson and Seigel (1944) concluded that riboflavin deficient rats were susceptible to lebar pneumonia to the same degree as ad libitum animals. In retrospect, some shortcomings in the experimental technique of these workers might be noted. The authors stated that the pneumococci were "...maintained at a high peak of virulence..." by passage through rats. It is now established that rats are not normally susceptible to pneumococcal infection, and that rat passage would actually attenuate the virulence of the organisms (Burrows, 1958). The experimental infection was induced by slitting the rats'
tracheae and injecting the bacterial culture, suspended in hog mucin, through the surgical opening. There can be little doubt that the surgical manipulation introduced the factor of trauma. Moreover, the bactericidal properties of the serum were altered by employing hog mucin. Dewitt (1958) has shown that very small quantities of hog mucin lower the serum properdin level of rats to zero. The importance of properdin in host resistance has been demonstrated (Pillemer, et al., 1954; Wedgewood and Pillemer, 1958; Nelson, 1958; Todd, et al., 1959; Wardlaw and Pillemer, 1959).

Kligler, et al. (1944) have added support to the relationship between riboflavin and host susceptibility. These workers have shown that riboflavin deficiency was responsible for increased susceptibility of mice to spontaneous infection with Salmonella typhimurium. It was also demonstrated that inanition animals, paired-weight with the animals in the deficient group, were susceptible to a degree intermediate between the deficient and ad libitum groups.

Altered susceptibility of animals deficient in B-complex vitamins to virus infection has been studied (Rivers, 1939; Sprunt, 1941, 1942; Foster, et al., 1942, 1944; Bloomfield, 1943; Rasmussen, et al., 1944a, 1944b; Kearny, et al., 1948). These investigations have shown a general increase in resistance to virus infection during vitamin deficiency and starvation.

As evidence for lowered host resistance during avit-
aminosis increased, many workers turned their attention to certain specific and non-specific immunological factors which might be responsible for alteration in resistance. It also became apparent that the vitamin deficient animal provided an excellent tool for studying the qualitative and quantitative importance of host defense mechanisms. Of the immunological processes studied, the following will be discussed: 1) the production of circulating antibodies and serum proteins, 2) the production of opsonins and activity of phagocytic blood cells, 3) complement activity, and 4) blood and bone marrow cellular composition.

The ability of the vitamin deficient animal to produce circulating antibodies has been the most studied single factor important in host resistance. Zilva (1919), who was the first to investigate immune responses in deficient animals, found no alteration in the antibody production of animals deficient in various nutritional requirements. The observations with respect to "B vitamin" were verified by Werkman (1923a).

In an extensive review of the research concerned with the diet-susceptibility relationship, Clausen (1934) stated: "The majority of writers agree that both the normal antibodies of the serum and the power to form antibodies are not affected by dietary deficiencies; a few exceptions are noted." Furthermore, this view was supported experimentally prior to the use of purified vitamins and the preparation of
synthetic diets.

With the isolation of constituent members of the B-complex group, and improvements in the preparation of synthetic diets, subsequent investigators yielded more reliable data. Stoerk and Eisen (1946) and Stoerk, et al. (1947) demonstrated a severe impairment of the antibody response in pyridoxine deficient rats, but did not show a similar impairment in riboflavin and pantothenic acid deficient rats. Axelrod, et al. (1947) reported that antibody titers in the latter two deficiencies were significantly lowered. Although Stoerk (1948) explained this discrepancy by the difference in severity of the deficiency, Wertman and Sarandria (1951a, 1951b) showed that differing amounts of antigen and the different serological tests employed by these two groups accounted for the lack of agreement.

The decrease in antibody production by the pyridoxine deficient rat has been supported by Agnew and Cook (1949), Wertman and Sarandria (1951b), and Pruzansky and Axelrod (1955a), each group employing a different antigen. Stoerk (1950) has also demonstrated the absence of anamnestic response in pyridoxine deficient rats.

Impairment of antibody production has been shown in riboflavin, thiamin, biotin, niacin, pantothenic acid and folic acid deficiency states (Carter and Axelrod, 1948; Little, et al., 1950; Ludovici and Axelrod, 1951; Wertman and Sarandria, 1951a, 1951b; Wertman, et al., 1952; Axelrod
and Pruzansky, 1955a, 1955b; Pruzansky and Axelrod, 1955a; Zucker, et al., 1966). Axelrod (1952) states that in each of these deficiencies, inanition alone cannot account for loss of the ability to produce antibodies.

In consideration of the obvious reduction in antibody titers during certain vitamin deficiencies, it might be expected that a study of the serum proteins, globulins in particular, would yield useful information. However, only fragmentary information is available concerning the effect of vitamin deficiency on serum protein composition. Stoerk, et al. (1947) reported a lowering of the alpha and gamma globulin percentages in pyridoxine deficient rats. This observation was partially substantiated by Bäshing (1950), who demonstrated a lowering of the total globulin nitrogen in pyridoxine deficient rats. However, Axelrod and Pruzansky (1955) have not been able to detect any change in the gamma globulin fraction of deficient rats which are unable to form demonstrable antibody titers to human erythrocytes.

Mudd, et al. (1934), in reviewing the factors which influence phagocytosis, suggested that the influence of nutrition on phagocytic activity offered a promising field for study. Although circulating and fixed phagocytic cells account for the first line of defense against bacterial invaders, little has been reported concerning the effect of vitamin deficiency on their activity.
Werkman (1923c) investigated the effect of B-complex avitaminosis on the ability of the host to elicit opsonins and maintain normal phagocytic activity. In vivo determinations of phagocytic activity were accomplished by injecting a suspension of *Salmonella typhosa* intraperitoneally in rats and recovering the peritoneal washings. By counting the number of bacteria engulfed in active leukocytes, it was shown that rats in the deficient group had a lower phagocytic rate than normal animals. However, results from in vitro studies demonstrated no difference in the activity of deficient and ad libitum leukocytes. These observations led to the conclusion that the impairment of phagocytic activity was not due to the inability of the deficient animals to elicit opsonins. These conclusions confirmed the results of Findlay and McKinzie (1922), who found no change in the opsonic index of B-complex deficient rats.

It was not until vitamins of the B-complex group became available in purified form that studies of this nature were continued. Cottingham and Mills (1943) investigated the influence of various vitamins on phagocytic activity of rat leukocytes. It was shown that rats deficient in thiamin, riboflavin, pyridoxine, pantothenic acid, choline, and ascorbic acid exhibited depressed phagocytic activity in vitro against *Micrococcus albus*. In order to verify the results obtained in vitro, duplicate experiments were performed with thiamin and choline deficient mice, in which
intraperitoneal phagocytosis was determined. Values for this and the previous study corresponded. Unfortunately, these workers did not include inanition control animals, hence it was not possible to rule out underfeeding as being responsible for the lowered activity of phagocytic blood cells. However, Gellhorn and Dunn (1937) reported that starvation did not affect the phagocytic rate until there was a loss of over 35% of the body weight.

Guggenheim and Buechler (1946) reported that rats deficient in thiamin and riboflavin showed no alteration of the in vivo phagocytic rate after being injected intraperitoneally with Salmonella typhimurium. Similarly, Wertzman and Groh could not demonstrate a reduction in phagocytic activity during thiamin deficiency in rats, employing the in vitro method of Cottingham and Mills (1943) and Diplococcus pneumoniae as the test organism.

Berry, et al. (1945) gave support to the findings of Cottingham and Mills (1943) by reporting a reduction in phagocytosis in B-complex deficient rats to 60–65% of the normal activity.

The influence of B-complex deficiency on the activity of complement has received little attention in the voluminous literature concerning the relationship of diet to disease susceptibility. In pioneer work, Zilva (1919) found no change in the ability of "B vitamin" deficient rats to form complement. In agreement, Rose and
Kolmer (1936) failed to show any decrease in the complement activity of "B vitamin" deficient dogs.

Wertman and his coworkers (Wertman, et al., 1954, 1955, 1956, 1957; Wertman and Groh, 1959) have studied the effects of various B-complex deficiencies on the complement activity of the rat. These studies were carried out under carefully controlled, identical experimental conditions, employing well defined synthetic diets. This group has reported a lowering of complement activity, not accounted for by inanition, during deficiencies in thiamin, pyridoxine, and folic acid. No activity could be demonstrated in the sera of rats deficient in niacin-tryptophane, while no change in activity was attributed to riboflavin deficiency. Pruzansky and Axelrod (1955b) claimed that inanition was responsible for lowering the complement activity of pyridoxine deficient rats.

Shukers and Day (1943) had early access to reasonably good diets, and studied the effects of vitamin deficiency on the distribution and quantitation of blood cells. They noted that the changes taking place during riboflavin deficiency could be attributed to the state of inanition. This work was verified by Carpenter and Kodicek (1948, 1952) and Wertman, et al. (1957). Wertman and his group of investigators (Wertman, et al., 1954, 1955, 1956, 1957; Wertman and Groh, 1959) found that the blood and bone marrow composition was altered to some degree during deficiencies in
various other members of the B-complex group of vitamins.

It is evident that conflicting results have been obtained in the study of the diet-susceptibility relationship. As Schneider (1958) would have it, no such relationship exists, but attention should be paid to the evolutionary and hereditary factors which govern species resistance. Evidence from the literature prior to 1940 demonstrated the lack of a real relationship between diet and infection. However, research accomplished more recently supports the concept that the nutrition of an animal species may directly influence its ability to resist infectious disease.

The lack of agreement which exists between the early nutrition experiments and those recently performed may be attributed to several discernible factors. The recognition and isolation of certain dietary constituents, specifically the vitamins, has given to modern-day researchers well defined experimental diets. The use of starch in food rations has ceased since Guerrant, et al. (1936, 1937) have shown that starch, when employed as the carbohydrate source in synthetic diets, increases the rate of intestinal synthesis of various members of the B-complex group. Refined immunological procedures and modifications in our concepts of virulence and disease have resulted in better experimental design.

Taken together, these innovations have increased the proficiency and validity of present-day nutrition studies.
Repetition of much of the work done prior to 1945 has provided new answers to traditional problems.
STATEMENT OF PROBLEM

The purpose of this investigation was to study the defense mechanisms of the riboflavin deficient rat, with special reference to: (1) the phagocytic activity of leukocytes as compared with those of normal rats, (2) the susceptibility to infection of the deficient rat when challenged intraperitoneally with virulent Type I *Diplococcus pneumoniae*, and (3) the relative distribution of serum albumin and globulin proteins.
MATERIALS AND METHODS

Animals and housing. Male weanling albino rats of the Sprague-Dawley strain were employed throughout this investigation. All animals were housed individually in metal cages with mesh bottoms. The animals were maintained in an air-conditioned room with a constant temperature of 25°C. Water bottles and food dishes provided for each cage were replenished daily. The rats for each study were divided into three groups for feeding purposes: ad libitum control, inanition control, and riboflavin deficient. The animals weighed between 30 and 45 grams when received in the laboratory.

Experimental diets and feeding. The basal diet was that employed by Wertman, et al. (1957). The ingredients used in the preparation of the diet were obtained from commercial sources and were of the highest purity available. The basal diet had the following percentage composition: vitamin-free casein, 25.00; C.P. sucrose, 58.75; hydrogenated vegetable oil, 10.00; corn oil, 2.00; U.S.P. salt mix #2, 4.00; choline chloride, 0.20; β-inositol, 0.03; p-amino-

2 General Biochemicals Co., Inc., Chagrin Falls, Ohio. National Biochemicals Co., Cleveland, Ohio

3 Wesson Oil

4 Mazola Oil

17
benzocic acid, 0.01; d-alpha-tocopherol acetate, 0.01; and 2-methyl-1,4-naphthoquinone, 0.001.

Each animal was given one vitamin tablet daily prior to receiving the food ration for that day. Tablets were prepared with the following vitamin composition (in micrograms): thiamin, 40; pyridoxine, 50; calcium pantothenate, 150; niacin, 150; biotin, 1; folic acid, 1; and riboflavin, 60. Riboflavin was omitted from the tablets prepared for the deficient group.

Vitamin tablets were prepared employing lactose as a binder. The vitamin-lactose mixture was granulated by wetting with 50% ethanol and passing through a #40 sieve. When the granulation had thoroughly dried, it was sprayed lightly with mineral oil to provide for lubrication of the tablet machine punch (Remington, 1956).

In addition to the basal diet and vitamin tablet supplement, each animal was given 300 U.S.P. units of vitamin A and 30 U.S.P. units of vitamin D once weekly by adding 5 drops of cod liver oil5 to the diet for that day.

All animals were maintained on the basal diet and complete vitamin supplement for one week after arrival in the laboratory. This was intended to stabilize the nutritional intake and allow the animals to adapt to the new diet and living quarters (Wertman, et al., 1954). Thereafter, the

5 Squibb's
animals in the deficient and ad libitum groups received the basal diet ad libitum while the inanition group received only enough to maintain their weights equal to those of the deficient group. The rats were maintained on the experimental rations for seven weeks. After this time, the rats in the deficient group showed the typical deficiency symptoms.

At the end of the seventh week of experimental feeding, the ad libitum and inanition control groups were divided into two sub-groups. One sub-group was challenged at the end of the seventh week, and the second sub-group was challenged at the end of the eighth week. The riboflavin deficient group of animals was arranged into three sub-groups. Sub-group #1 was challenged at the end of the seventh week of experimental feeding, sub-group #2 was administered 60 μg of riboflavin daily for one week following the seven weeks of deficient diet and then challenged, and sub-group #3 was maintained on deficient diet for the entire eight week experimental period. This last group was included to determine the number of animals, if any, that would die from the vitamin deficiency during the total experimental feeding period. Initial and final mean weights for each group appear in Table I.

**Bacterial cultures.** All bacterial cultures employed in this investigation were derived from a strain of Type I *Diplococcus pneumoniae* provided by Dr. C.V. Seastone, University of Wisconsin. The bacteria were transferred daily.
TABLE I

Distribution and initial and final mean weights of rats.

<table>
<thead>
<tr>
<th>group</th>
<th>total no. rats</th>
<th>mean weight in grams</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>initial 7th week 8th week</td>
<td></td>
</tr>
<tr>
<td>Ad libitum control</td>
<td>19 35</td>
<td>220 (19) 248 (5)</td>
</tr>
<tr>
<td>Inanition control</td>
<td>18 34</td>
<td>89 (13) 126 (5)</td>
</tr>
<tr>
<td>Riboflavin deficient</td>
<td>59 35</td>
<td></td>
</tr>
<tr>
<td>Sub-group #1*</td>
<td></td>
<td>80 (39) - - -</td>
</tr>
<tr>
<td>Sub-group #2**</td>
<td></td>
<td>83 (20) 149 (20)</td>
</tr>
</tbody>
</table>

B. Susceptibility study.

| Ad libitum control          | 24 57          | 232 (20) 240 (4)     |
| Inanition control           | 40 54          | 88 (30) 127 (10)     |
| Riboflavin deficient        | 60 57          |                      |
| Sub-group #1                |               | 84 (30) - - -        |
| Sub-group #2                |               | 88 (22) 125 (22)     |
| Sub-group #3***             |               | 90 (8) 92 (8)        |

* Rats maintained 7 weeks on deficient diet.
** Rats maintained 7 weeks on deficient diet, followed by 1 week on complete diet, including 60 µg riboflavin daily.
*** Rats maintained 8 weeks on deficient diet.
† Number of animals in experiment at end of indicated time period in parentheses.
in beef "hormone" broth (beef infusion broth with 2% glucose and 0.5% gelatine). Virulence was maintained by weekly mouse passage and daily mouse passage for two weeks prior to use in the phagocytic determinations and challenge studies. Mouse passage was accomplished by injecting 0.5 ml of a 24 hour broth culture intraperitoneally and recovering the exudate from the thoracic cavity 5 to 6 hours after injection. Suspensions for the phagocytic and challenge studies were prepared by suspending washed cells from a 24 hour broth culture in sterile broth and adjusting to $2 \times 10^5$ bacteria/ cu mm (O.D. 0.8 at 650 mu on a Lumatron). The organisms were centrifuged and washed to remove any soluble, antiphagocytic substances that might be present in the growth medium (Ward and Enders, 1933).

**Phagocytic study.** The phagocytic studies were performed using the method described by Cottingham and Mills (1943) for in vitro determinations. The animals were ether-ized and bled by the cardiac puncture technique (Burhoe, 1940), withdrawing at least 1 ml of blood. Five-tenths ml of blood were placed in the side arm of a paraffin coated Warburg flask, to which had been added 1 drop of heparin. The remainder of the blood specimen was pooled for electrophoretic analysis.

Flasks containing the blood were held at 38°C for no longer than 1 hour. When 10 rats had been bled and the blood placed in flasks, 0.2 ml of a bacterial suspension con-
taining $2 \times 10^5$ bacteria/ cu mm were added. The flasks were agitated laterally in a constant temperature water bath (38° C) at 240 reversals/min for 4 minutes. This allowed thorough mixing of the blood and pneumococci. When the time period was completed, the flasks were promptly removed and 5 thin smears made of each specimen.

Slides prepared from the blood specimens were stained by Wright's method and examined for: 1) the percentage of neutrophils that were active in phagocytosis, and 2) the average number of bacteria engulfed in each active neutrophil. In the first determination, 200 unclumped and unruptured leukocytes in each sample were counted, and the number containing bacteria recorded. The degree of phagocytosis was determined by counting the pneumococci in 100 unclumped and unruptured phagocytically active neutrophils. The results of the phagocytic determinations appear in Table II.

A consideration was made of the time involved in bleeding and making smears. Cottingham and Mills (1943) reported that there was no alteration in the phagocytic activity of leukocytes held for as long as 5 hours at 37° C. This corresponded with the observations made during this investigation. Furthermore, it was here noted that the time required for adding the bacterial suspension to each flask corresponded to the time for making smears. As a result of these observations, no difference in the activity of leuko-
cytes was attributed to the time difference in bleeding and preparing smears.

**Susceptibility study.** The rats in the susceptibility study were challenged after 7 weeks of experimental feeding by intraperitoneal injection of 2 ml of a suspension of virulent Type I *Streptococcus pneumoniae*. The bacterial suspensions were prepared as described previously. The intraperitoneal route of injection was selected to test the defense mechanisms of tissue and blood. After injection, the animals were observed periodically for the first 6 hours so that death due to trauma might not be included in the final results. All deaths were recorded by noting the approximate time at which it occurred.

**Serum protein study.** The evaluation of serum proteins was done by paper electrophoresis, employing a Spinco Model R apparatus for hanging strip electrophoresis (Durrum, 1950). A modification of the procedure recommended by the manufacturer was utilized in the analysis.

Specimens were obtained from the serum pools collected during the phagocytic determinations, and were held at -10°C until used. Each pool contained the sera of 4 to 5 rats. For analysis, 0.006 ml of each pool were placed in duplicate on Schleicher and Schuell 2043-A mg1 filter paper strips. Veronal buffer, pH 8.6 and 0.10 M was employed, and the runs made at 3.0 ma/cell for 24 hours at 20°C. The staining procedure was that described by Block, et al. (1958)
in which the strips were fixed by heating for 30 minutes at 130° C followed by a 6 minute methanol rinse. After staining, the strips were scanned and recorded on a self-recording densitometer (Spinco Analytrol Model RB).

An additional group of rats was employed for the serum protein studies and were identified as the "normal" group. These animals were maintained on stock feed (Purina Laboratory Chow) from the time of weaning. This group was included to serve as control on changes produced in the serum protein distribution due to feeding rats the synthetic experimental diets.
RESULTS

Evidence of typical riboflavin deficiency symptoms became apparent before the end of the sixth week of experimental feeding. The ad libitum control rats gained weight, appeared healthy, and possessed smooth even coats. The inanition control animals, although severely restricted in their food intake, appeared healthy and possessed smooth even coats. The rats in the riboflavin deficient group were stunted and gained relatively little weight during the sixth and seventh weeks of feeding. Their coats were ruffled, uneven, and alopecia was becoming apparent. The distribution and initial and final mean weights of each group appear in Table I.

Three separate but related studies were completed: (1) the in vitro phagocytosis of virulent Type I pneumococci by neutrophils of rats in each group, (2) the susceptibility of the various groups to infection by this organism, and (3) the electrophoretic analysis of sera from each group in the investigation.

Wertman, et al. (1957) reported a significant reduction in the total leukocyte count of riboflavin deficient and inanition control animals. The ad libitum controls had a median of 18.2 X 10^3 cells/cu mm, compared with 2.6 X 10^3 cells/cu mm for the deficient group. In addition, the co-
plement activity of the sera from the inanition and deficient groups was reduced approximately 50%. The phagocytosis experiment reported here was to determine possible differences in the activity of phagocytic white cells of animals deficient in riboflavin compared to ad libitum and paired-weight animals. This was accomplished by observing (1) the percent of neutrophils that were active in phagocytosis and (2) the degree of activity exhibited by these cells as measured by the average number of organisms engulfed per phagocyte. A significant difference was found in both the percent active neutrophils and in the degree of activity.

In the experiments performed at the end of the seventh week (Table II), 72.5% of the ad libitum leukocytes, 54.0% of the inanition leukocytes, and 15.5% of the leukocytes from riboflavin deficient rats were active in the phagocytosis of virulent D. pneumoniae. The average number of bacteria engulfed per cell was: ad libitum neutrophils, 11.5; inanition neutrophils, 5.3; and riboflavin deficient neutrophils, 2.5. The results of Wertman, et al. (1957) and of the present study make it apparent that the rat deficient in riboflavin had an impaired mechanism for bacterial clearance.

A similar study was conducted with a second group of rats maintained on a riboflavin deficient diet for 7 weeks and then placed on a complete diet, including 60 μg of riboflavin daily, for one week. The differences between deficient and control groups at the end of the eighth week
TABLE II

Phagocytic activity of leukocytes from riboflavin deficient and control rats.

<table>
<thead>
<tr>
<th>group</th>
<th>average percent active neutrophils in each group</th>
<th>no. bacteria/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>average</td>
</tr>
<tr>
<td>7th week study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>72.5 (19)†</td>
<td>11.5</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inanition</td>
<td>54.0 (13)</td>
<td>5.3</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Riboflavin deficient</td>
<td>15.5 (39)</td>
<td>2.5</td>
</tr>
<tr>
<td>8th week study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>61.0 (5)</td>
<td>6.8</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inanition</td>
<td>49.4 (5)</td>
<td>4.0</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Riboflavin deficient</td>
<td>24.5 (20)</td>
<td>3.4</td>
</tr>
</tbody>
</table>

† Number of animals in experiment at end of indicated time period.

* Riboflavin deficient group was fed 60 µg of riboflavin daily during the 8th week.
were not as great as those found at the end of the seventh week. The deficient rats that had received riboflavin for one week showed a greater percentage of active neutrophils (24.5%) than those that did not receive the vitamin (15.5%). In addition to a greater number of active phagocytes, there was an increase in the number of bacteria engulfed per cell in those animals that were given the daily requirements of riboflavin for one week. At the end of the seventh week, the average number of organisms per phagocyte was 2.5. When riboflavin was administered for one week this figure rose to 3.4.

It was difficult to compare the results of the seventh and eighth week experiments. The overall activity of all three groups was less in the eighth week study. The difference between experiments may have been due to the difficulties involved in producing bacterial suspensions of equal virulence at different times. By comparing the results within each weekly experiment, it can be seen that the number of active leukocytes from deficient rats was 22% that of the active number from ad libitum rats at the end of the seventh week. However, this percentage was elevated to 40% of the active ad libitum cells when deficient animals received 60 µg of riboflavin daily for one week. Similarly, the average number of bacteria engulfed per deficient phagocyte increased from 22% of the average for ad libitum cells at the end of the seventh week to 50% at the end of the eighth
The infectivity studies indicated that riboflavin deficient rats were susceptible to infection with virulent D. pneumoniae while ad libitum animals were not (Table III). All the ad libitum control rats survived the challenge dose of pneumococci, 10 of the 30 inanition control rats died, and 25 of the 30 deficient rats died from the infection. The fatality rates for the three groups were as follows: ad libitum, 0%; inanition, 33.3%; and deficient, 83.3%.

The group of animals that received riboflavin for one week after establishment of the deficiency were not as susceptible to the infection as the first group challenged. Twenty of the initial 22 rats in this group survived the challenge dose of pneumococci. Ten animals that were inanition for seven weeks then fed ad libitum for one week were challenged with an identical inoculum of D. pneumoniae. Eight of this group survived the infection. There were no fatalities in the ad libitum group in this second challenge study.

Fatality rates for each group in the eighth week challenge were: ad libitum, 0%; inanition, 20%; and deficient, 9%.

Eight rats were maintained on the deficient diet for the entire term of the experiment, and remained unchallenged. This group was included to indicate the number of animals that would die from deficiency. None of these rats died from extended deficiency.

The data obtained from the serum protein study are
TABLE III
Susceptibility of riboflavin deficient and control animals to *D. pneumoniae*.

<table>
<thead>
<tr>
<th>group</th>
<th>no. of rats</th>
<th>no. survived</th>
<th>fatality %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>7th week challenge</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum control</td>
<td>20</td>
<td>20</td>
<td>0.0</td>
</tr>
<tr>
<td>Inanition control</td>
<td>30</td>
<td>20</td>
<td>33.3</td>
</tr>
<tr>
<td>Riboflavin deficient</td>
<td>30</td>
<td>5</td>
<td>83.3</td>
</tr>
<tr>
<td><strong>8th week challenge</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum control</td>
<td>4</td>
<td>4</td>
<td>0.0</td>
</tr>
<tr>
<td>Inanition control</td>
<td>10</td>
<td>8</td>
<td>20.0</td>
</tr>
<tr>
<td>Riboflavin deficient*</td>
<td>22</td>
<td>20</td>
<td>9.1</td>
</tr>
<tr>
<td>Riboflavin deficient**</td>
<td>8</td>
<td>8</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Rats in this group were fed 60 μg of riboflavin daily for 1 week following 7 weeks of deficient diet.

** Rats in this group were on extended deficiency and were not challenged.
presented in Table IV. There was a decrease in the percentage composition of each globulin component and in total globulin of the sera from riboflavin deficient rats. Analysis of the sera from inanition control rats revealed percentage values that were intermediate between those for the deficient and ad libitum groups. Values obtained for the ad libitum group were: albumin, 39.7%; alpha\(_1\) globulin, 17.0%; alpha\(_2\) globulin, 14.6%; beta globulin, 22.8%; and gamma globulin, 7.0%. Percentage composition of the serum proteins for ad libitum rats varied from that obtained from "normal" rats, which were fed Purina Laboratory Chow from the time of weaning. The values for protein components of "normal" sera were as follows: albumin, 48.8%; alpha\(_1\) globulin, 16.7%; alpha\(_2\) globulin, 8.6%; beta globulin, 17.0%; and gamma globulin, 14.2%. The riboflavin deficient rats that had received vitamin supplement for one week after deficiency showed a relative rise in the total globulin fraction after the eighth week. Total albumin prior to the administration of riboflavin was 56.1%, and total globulin was 43.9%; At the termination of the week of vitamin therapy, these values became: albumin, 49.5% and total globulin, 49.5%.

Values for albumin and globulins were obtained by the method of Tiselius and Kabat (1939), dropping perpendicular lines for demarcation of the various fractions. Longsworth (1942) reported good agreement by this method.
TABLE IV

Distribution of serum protein components of riboflavin deficient and control rats.

<table>
<thead>
<tr>
<th>group</th>
<th>no. of pools</th>
<th>percent albumin</th>
<th>percentage globulins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>alpha&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>Normal control*</td>
<td>5</td>
<td>48.8</td>
<td>16.7</td>
</tr>
<tr>
<td>Ad libitum control</td>
<td>5</td>
<td>39.7</td>
<td>17.0</td>
</tr>
<tr>
<td>Inanition control</td>
<td>3</td>
<td>47.6</td>
<td>16.5</td>
</tr>
<tr>
<td>Riboflavin deficient</td>
<td>7</td>
<td>56.1</td>
<td>13.5</td>
</tr>
<tr>
<td>Riboflavin deficient**</td>
<td>2</td>
<td>49.5</td>
<td>16.4</td>
</tr>
</tbody>
</table>

* Control animals maintained on stock feed (Purina Laboratory Chow) for the term of the experiment.

** Rats maintained on deficient diet for 7 weeks, then fed 60 mg riboflavin daily for 1 week.
when the fractions are well separated.
DISCUSSION

The results obtained in the phagocytic study closely parallel those of Cottingham and Mills (1943). However, these investigators did not include paired-weight animals in their study, and thus did not rule out the possibility of inanition accounting for the reduction in phagocytic rate. The present investigation attributes the marked reduction in phagocytic activity to the deficiency state and not to inanition resulting from the deficiency. Guggenheim and Buechner (1946) reported that there was no change in the phagocytic index of animals that had been maintained on riboflavin-free diets for seven weeks. These workers employed Salmonella typhimurium as the test organism and measured the degree of leukocyte activity by injecting the bacterial suspension into the peritoneum of rats and recovering the peritoneal washings for microscopic observations. Differences between the results of Guggenheim and Buechner and those obtained in this study must be attributed to the dissimilarity in experimental procedure.

The exact mechanisms for reduced phagocytic activity during riboflavin deficiency have not been elucidated at this time. However, certain facts are available which may account for the reduction. Ward and Enders (1935) have shown the humoral influence on the phagocyte in the case of
phagocytosis of pneumococci. These investigators demonstrated the requirement of specific antibody for the phagocytosis of these bacteria. Depletion of the humoral influence on phagocytosis is possible, since Wertman, et al. (1952) and Pruzansky and Axelrod (1954) have shown that the antibody response is inhibited in the riboflavin deficient rat.

Another humoral factor that might effect alteration of the leukocyte activity is the level of various serum proteins. Mudd, et al. (1954) have pointed out that phagocytosis occurs at a lesser rate in albumin than in globulin. Analysis of serum protein from riboflavin deficient rats has demonstrated a relative increase in the total albumin (Table IV). This resulted in lowering the albumin/globulin (Table IV). This resulted in lowering the albumin/globulin ratio, and could have decreased the rate at which phagocytes

It is more probable that alteration in phagocytic activity is due to a change in the leukocyte during vitamin deficiency (Gottimgham and Mills, 1945), since the changes in humoral factors apparently are not of sufficient magnitude to account for the gross lowering of activity.

Cellular composition might be modified by alteration in the metabolic processes in which riboflavin plays a role. The metabolic functions of riboflavin in lipid synthesis are especially significant in the light of recent evidence.

Sbarra and Karnovsky (1959) have shown that there is
increased lipid synthesis in the leukocyte during active phagocytosis, which the authors indicated was required for maintenance of the cell membrane. Kaunits, et al. (1954) have shown that there is a relationship between fat synthesis and dietary riboflavin. This relationship appears to be more direct than simply the role of flavin compounds in ATP synthesis. In support of this direct relationship, McHenry and Gavin (1958) observed an increase in fat synthesis from carbohydrate when riboflavin was included in the diet. Snell (1953) has presented evidence showing that fatty acid dehydrogenases, enzymes important in fatty acid synthesis and degradation, are flavoproteins.

Not only has the relationship between fat metabolism and dietary riboflavin been demonstrated, but the lack of riboflavin has been shown to impair lipid synthesis. Milbrandt (1930) and Monaghan (1932) reported a decrease in blood and tissue phospholipid during B-avitaminosis, and Snell (1953) pointed out a diminution in tissue flavoprotein during riboflavin deficiency. It is conceivable that the reduction in phospholipids and flavin-enzymes would reduce leukocyte lipid synthesis, especially since the phospholipids are believed to be active in transporting fatty acids for synthesis into fats (Cantarow and Schepartz, 1957). This concept is further demonstrated by the observation that blood phospholipid increases in quantity during high fever and infection (Sinclair, 1934), suggesting an increased
demand for fat metabolic intermediates during infection.

There is yet another mechanism by which the leukocytes of deficient animals may be altered. Dining, et al. (1950) have shown the importance of methyl groups in the formation of the rat leukocytes. In view of the role of choline in providing methyl groups, and the evidence for the participation of flavin-adenine-dinucleotide (FAD) in the choline oxidase system (Rothschild, et al., 1954), riboflavin deficiency could strike another link in the maintenance of rat phagocytes. More directly, Ebisusaki and Williams (1953) demonstrated a significant lowering of the choline oxidase activity in riboflavin deficient rats, thus decreasing the potential availability of methyl groups for white cell production.

Gottingham and Mills (1945) observed a lag in the recovery of normal phagocytic activity when deficient animals were fed the proper vitamins. This lag during riboflavin therapy may be made to correspond with the half-life of serum proteins (Borsook, 1950), the release of new phagocytes from bone marrow (Gottingham and Mills, 1945), or the regeneration of tissue flavoproteins and flavin-enzymes (Burch and Combs, 1956). Whatever the explanation for the reduction and subsequent recovery of phagocytic activity after deficiency and vitamin therapy, it can be seen from Table II that the activity approaches normal values after the administration of the daily requirement.
of riboflavin for one week.

The organism employed in this infectivity study allowed clear-cut results. The normal albino rat is sus-
ceptible to death from lobar pneumonia (Loughlin, et al., 1945), but not from bacteremia after intraperitoneal injec-
tion (Wertman, and Groh, 1959). The death rate among the
inanition rats might be expected due to the decrease of
phagocytic activity (Table II), and the reduction in com-
plement activity and circulating leukocytes (Wertman, et al.,
1957). The results of the infectivity study, showing the
riboflavin deficient rat more susceptible to infection,
agree with those obtained by Pinkerton and Bessey (1939),
Wooley and Sebrell (1942), and Fitzpatrick (1948). Each
of these groups used different infecting organisms from
those employed in this study.

The relative decrease in serum globulins has some
significance. Heidelberger (1938), Pauling (1940) and
Cannon (1942) have shown the relationship of serum glob-
ulins to antibody, although the relationship is apparently
an indirect one. Evidence indicates the formation of gamma
globulin and antibody globulin from the same precursor mol-
ecule (Green and Anker, 1954; Axelrod and Pruzansky, 1955).
The decrease in serum globulin during riboflavin deficiency
corresponds with the antibody titer decrease in the same
deficiency (Wertman, et al., 1952).

A review of the literature has yielded conflicting
opinions of the investigators as to the identification of
the various protein components of rat serum. Employing free electrophoresis, several workers have reported only one alpha globulin fraction (Deutsch and Goodloe, 1945; Li and Reinhardt, 1947; Moore, et al., 1944, 1945; Arboys, et al., 1954). The present study has clearly demonstrated the alpha1 and alpha2 globulin fractions, and corresponds to the data of Metcoff, et al. (1948), Gjessing and Chanutin (1947), Cohen and Thompson (1947), Enselme, et al. (1954) and Wostman and Gordon (1959), all obtained by free electrophoresis. Peterson and Beatty (1958) noted similar fractions employing paper electrophoresis of the Durrum type.

The relative percentage composition for each protein fraction obtained in this study is similar to the values reported by Gjessing and Chanutin (1947) and Enselme, et al. (1954). Values for albumin in the ad libitum group of rats are somewhat lower than those for the "normal" group. This discrepancy might be explained by the observation of McNaught et al. (1938) that certain plant and animal protein sources distinctly favor the production of albumins and others the production of globulins.
SUMMARY

Male albino rats of the Sprague-Dawley strain were maintained on well defined diets deficient in riboflavin to study the effect of the deficiency on (1) the phagocytic activity of leukocytes, (2) the susceptibility to infection by virulent Type I Diplococcus pneumoniae, and (3) the relative distribution of serum albumin and globulin protein. Adequate numbers of inanition and ad libitum control animals were included. The following observations were made:

1. Riboflavin deficiency had a marked effect on the capacity of leukocytes to phagocytize D. pneumoniae. The percentage of neutrophils actively engaged in phagocytosis and the number of bacteria each neutrophil had engulfed were considerably lower than values for ad libitum rats. Similarly, leukocytes from inanition animals, paired-weight with the deficient rats, showed a reduction in activity over that of ad libitum cells. However, inanition neutrophils were a good deal more active than deficient neutrophils.

2. Riboflavin deficient animals were highly susceptible to infection by D. pneumoniae, while ad libitum rats were not susceptible. Rats in the inanition group were less susceptible to infection than those in the deficient group. Fatality to infection by D. pneumoniae was lowered significantly in the deficient group by administering the
daily riboflavin requirement for one week.

3. There was a significant relative decrease in the serum globulin proteins of rats maintained on riboflavin free diets. This decrease was not accounted for by the inanition control animals. The analysis of rat serum by paper electrophoresis demonstrated the \( \alpha_1 \), \( \alpha_2 \), \( \beta \), and \( \gamma \) globulin fractions.
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