THE EFFECTS OF FOLIC ACID DEFICIENCY ON
PHAGOCYTOSIS AND SUSCEPTIBILITY TO INFECTION

by

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STATEMENT BY AUTHOR

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APPROVAL BY THESIS DIRECTOR

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Professor of Bacteriology
Head of Department
With acknowledgement to Dr. Kenneth Wertman, Mr. Paul Sypherd, and the staff of the Department of Bacteriology and Medical Technology for advice and guidance during this investigation.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. STATEMENT OF PROBLEM</td>
<td>9</td>
</tr>
<tr>
<td>III. MATERIALS AND METHODS</td>
<td>10</td>
</tr>
<tr>
<td>A. Animals and Housing</td>
<td>10</td>
</tr>
<tr>
<td>B. Experimental diets and feeding</td>
<td>10</td>
</tr>
<tr>
<td>C. Bacterial cultures</td>
<td>13</td>
</tr>
<tr>
<td>D. Phagocytic study</td>
<td>15</td>
</tr>
<tr>
<td>E. Susceptibility study</td>
<td>16</td>
</tr>
<tr>
<td>F. Bacteremia study</td>
<td>16</td>
</tr>
<tr>
<td>IV. RESULTS</td>
<td>19</td>
</tr>
<tr>
<td>V. DISCUSSION</td>
<td>24</td>
</tr>
<tr>
<td>VI. SUMMARY</td>
<td>27</td>
</tr>
<tr>
<td>VII. REFERENCES</td>
<td>29</td>
</tr>
</tbody>
</table>
INTRODUCTION

Early in the civilization of man, many philosopher-physicians of India remarked in the scriptures of Ayurved (Charak Saṁhitā, Susruta Saṁhitā) that there existed a definite relationship between diet and disease.

Unaware of the existence of microorganisms in the days of Hypocrates, the belief was strengthened that malnutrition, as well as polluted water and bad air, was a cause of disease.

Since 1800 evidence has been accumulating that diet may have some influence upon the incidence, course, and final outcome of infection. The supposition that diet might exert an important influence on susceptibility to infection was well worth making. Reviews of the literature revealed many suggestive correlations between the food of men and animals and the incidence of infection in the community or herd. (Clausen, 1934; Robertson, 1934; Watson, 1934; Wilson and Topley, 1938; Perla and Marmorston, 1941; Aycock and Lutmann, 1944; Barry et al., 1945; Leitel, 1945; Schneider, 1946; Wilson and Miles, 1946; Gell, Perry, Leitner, Howie and Hartley, 1948; Clark et al., 1949).

The historical association of famine and war to susceptibility to infection was observed. (Lusk, 1921, Cannon, 1942). The cause attributed to increased susceptibility was undernourishment. Indeed the problem of host-parasite relationship is so complicated that to arrive at a
definite pattern is extremely difficult.

Much evidence exists demonstrating the role of nutrition in the susceptibility to tuberculosis. It is now well established that both the incidence and severity of pulmonary tuberculosis increases during periods of severe restriction and inadequacy of food. For example, during World War I the mortality rate in Denmark between 1914 to 1917 rose from 138 to 176 per 100,000 due to low level of food consumption, independent of over-crowding, unemployment and the like. (Faber, 1938). The incidence of other infectious diseases also increased to a considerable extent during the period i.e., enteritis, broncho-pneumonia, otitis media (Kolmer, 1955). The data from World War II has also indicated that tuberculosis morbidity and mortality were increased by malnutrition (Keys, 1948). Leyton (1946) reported that the incidence of the disease among slowly starved Russian prisoners at Tost was 15 times that of the British prisoners whose rations were substantially supplemented by food supplied by the Red Cross. (Leyton, 1946).

Clinical observation gives a general impression that optimum nutrition commonly aids resistance, but our knowledge of nutrition is still too fragmentary to give an adequate statement of "optimum nutrition" for persons living under markedly diverse conditions throughout the world.

Paul et al (1949) suggested that there seems to be genetic control of host and parasite as far as infectious disease is concerned. They concluded, "In the field of in
infectious disease, nutrition of the host operates in a
genetic frame work and the area of its operation is defin­
able as the area in which genetically heterogeneous hosts
meet with genetically heterogeneous pathogen population."
It is evident that true relations exist for infectious dis­
eases of such diverse etiology as leprosy (Rogers and Muir,
1925), rheumatic fever (Read et al, 1938, Gaulb et al, 1939),
poliomyelitis (Aycok, 1942), and tuberculosis (Puffer, 1944,
Kallman, 1943). Schneider and Webster (1945), and Schneider
(1946, 1958, 1960) reported on the hereditary influence on
animal susceptibility. Apart from genetic control, various
nutritional requirements have been reported to have an effect
on host resistance. Schneider and Webster (1945) reported
a difference in resistance in groups of mice maintained on
a restricted diet.

Jackson and Smith, (1931), observed that a reduced
consumption of water intake increased susceptibility of
animals to infection. Various dietary protein deficiencies
have shown increased susceptibility to infection. (Fitzpat­
rick, 1948, Miles, 1951, Dubos and Schaedler, 1958, Hill and
Green, 1958, Ruebner and Bramhall, 1959.)

Effects of vitamin deficiency in general on animals
have shown increased susceptibility to infection. (McCollum
and Davis, 1913; Osborne Mendal, 1913; Wolbach, 1925; Mellenby,
1918, 1921; Evans and Bishop, 1922; Evans and Burr, 1928;
Dam, H., 1935; Woolley, D.W. and L.O. Krampitz, 1943; Robert­
son, 1934; Wertman and Groh, 1958; and Wertman and Sypherd, 1960).
The effect of a deficiency of B complex group of vitamins has been studied and reported upon by numerous investigators. Rats deficient in vitamin B complex were reported more susceptible to infection by *antrax bacilli* and *Diplococcus pneumococci* (Werkman, 1923). Barlow (1930) demonstrated bacteremia in chicks due to *Plasmodium luphorae* infection in "B complex" deficiency. Decreased resistance to *Salmonella typhimurium* in vitamin B deficiency was shown by Watson (1937). Similar results were reported by Ross and Robertson (1932) when worked with *Salmonella murititis*.

Extensive studies of various single constituents of B complex vitamins were undertaken in recent years. These factors were isolated and were made available in pure form so that individual study of each separate member of the group was possible. Evidence shows that laboratory animals maintained on specific vitamin deficiencies show a marked increase in susceptibility to certain bacterial and rickettsial infections. Riboflavin deficient rats were found to be more susceptible to murine typhus rickettsiae and *D. pneumoniae* infections in rats. (Pinkerton and Bessey, 1939; Fitzpatrick, 1948; Wooley and Sebrell, 1942; Wertman and Sypherd, 1960).

Guggenheim and Buchhler (1946) observed that thiamine deficiency in rats lowered their resistance to infection. Badger et al (1940) and Lamb (1935) found that thiamine deficient rats were more susceptible to rat leprosy. Pinkerton and Swank (1940) observed increased susceptibility to psittacosis in thiamine deficient animals. The resistance of rats
to the blood flagellate *Trypanosoma lewisi* was markedly lowered by deficiency either in panthothenic acid or biotin (Caldwell and Gyoroy, 1943). Decreased resistance to bacterial and rickettsial infection in panthothenic acid deficiency was reported by West et al., (1944); Fitzpatrick, (1948); and Zucker and Zucker, (1954). Biotin deficiency, (Kligler et al., 1946; Trager, 1943) nicotinic acid deficiency, (Krahl et al., 1945) and pyridoxine deficiency, (Robinson and Seigal, 1944) have also shown decreases in resistance to infection.

Workers have also reported increased resistance to infection. Bacon et al. (1951) reported that multiplication of *Plasmodium berghei* in the rat occurs only if an adequate amount of paramino-benzoic acid was available in the diet of the infested animals. Thiamine deficient rats were found more resistant to poliomyelitis virus. (Foster et al., 1942). Rasmussen et al., (1944) and Linchstein et al., (1945) reported that Swiss mice fed on a diet deficient in pyridoxine, biotin, inositol, and thiamine had increased resistance to infection. Others workers found no alteration in resistance to infection. Fitzpatrick (1948) found no alteration in resistance of pyridoxine deficient rats challenged with murine typhus rickettsiae. Day and McClung (1945) found no appreciable change in resistance in panthothenic acid deficiency. Higgins and Fledman (1943) found that diets low in thiamine and riboflavin did not influence the resistance of the white rat to avian tuberculosis.

Thus conflicting evidence is available concerning the
susceptibility of animals deficient in various components of the B complex group. It is an established fact that variations in dietary factors and introduction of infectious agents in the animal will give varying observations.

Folic acid deficiency was reported by many workers under the name Lactobacilli casei factor, vitamin Bc, vitamin M, vitamin U, and pteroylglutamic acid (P.G.A.). Littel et al. (1950) reported that folic acid deficiency interfered with the resistance of chicks to the experimental infection with P. multocida. Folic acid deficient chicks had lower resistance to P. lophorea. (Seeler and Ott, 1945). The experimentally induced Streptococcus hemolyticus infection and influenza virus infection in folic acid deficient monkeys resulted in high mortality (Saslaw et al., 1946).

Apart from the role of folic acid in relation to susceptibility, it has been reported as a hemopoetic factor (Hundell, 1959), and growth factor. (Snell and Peterson, 1940; Edwardson and Elvehjem, 1942; Simon Black et al., 1942). Day et al. (1935) induced a syndrome of anaemia, leukopenia, diarrhoea, and stomatitis in monkeys with folic acid deficient diets. Later they called this vitamin M deficiency and recognized that it was similar to sprue. (Day et al., 1938). Stockstad and Manning (1938) described a growth factor for chicks which they name vitamin U. Hogan and Parrot (1939) reported vitamin Bc as an anti-anaemic factor for chicks. Snell and Peterson (1940) described a growth factor for Lactobacilli and named it the Lactobacilli factor. Mitchell et al., (1945)
obtained the same substance from deep green leaves and suggested the name folic acid. Day et al., (1945) successfully treated vitamin M deficiency in monkeys with folic acid. Pfiffner et al., (1943) showed that chick growth factor vitamin Bc is related to folic acid. Soon it became apparent that all these factors were one and of the same substance.

In animal nutrition, folic acid is considered important for the activity of certain hormones (Hartz, 1948; Nelson et al., 1952). Thus the important role of this vitamin in normal physiology of animal and man is increasingly recognized.

Lowered host resistance in vitamin deficiency created an interest for the research worker in the study of specific and non-specific physiological factors which might be responsible for this altered resistance. Animals deficient in vitamins provided the opportunity for a study of the complex host defense mechanism.

The decrease in antibody production by pyridoxine deficient rats was reported by Wertman and Sarandria (1951). Inhibition of antibody production was also reported in folic acid deficient rats (Wertman et al., 1952). Folic acid deficient rats also exhibited decreased number of granulocytes and leukocytes (Wertman et al., 1956).

With the impairment of antibody formation during vitamin deficiency demonstration, it was expected that study of phagocytosis and susceptibility to infection would yield useful information. The present study was undertaken with the view to study the role of folic acid deficiency in relation
to phagocytosis and infection to virulent Type I Diplococcus pneumococci.
STATEMENT OF PROBLEM

The purpose of this investigation was to study the defense mechanisms of the folic acid deficient rat with special reference to (1) the phagocytic activity of leukocytes, (2) the susceptibility of the vitamin deficient rat to infection when challenged intraperitoneally with virulent Type I Diplococcus pneumoniae, and (3) to determine the degree of bacteremia developing at various time intervals during the disease.
MATERIALS & METHODS

Animals & housing. The experiment was conducted on male, weanling albino rats of the Sprague-Dawley strain. Twenty to twenty-five day old rats initially weighing approximately 40 grams each were employed for the purpose of this study. All animals were placed in individual wide-mesh screened-bottom metal cages and were provided water and food in sufficient quantities.

The animals for this study were divided into three groups: (1) ad libitum (15 rats); (2) inanition group (25 rats); and (3) folic acid deficient group (57 rats). All animals were weighed weekly during the time of the experiment.

Experimental diet and feeding. The basal vitamin-free diet which was employed successfully by Wertman et al. (1952, 1956) was used for all groups of rats in this folic acid study. The ingredients (1 & 2) used for preparation of basal diet were of the highest purity available. The basal diet consisted of the following: Sucrose, 57.47%; casein, (vitamin free) 25%; (3) vegetable oil, (Hydrogenated) 10%; salt mixture no. 2, (U.S.P.) 4%; corn oil, 2.00%; (4)

(1) General Biochemical Company Inc., Chagrin Falls, Ohio.
(2) The Nutritional Biochemical Company, Cleveland, Ohio.
(3) Wesson oil.
(4) Mazola oil.
sulfa-suxidine, (succinyl sulfathiazole) 1.00%; choline chloride, 0.20%; D-inositol, 0.03%; dl-alpha-tocopherol acetate, 0.01%; and 2-methyl-1, 4-naphthoquinone, 0.001%.

Vitamin B complex tablets were prepared using lactose as a binder. For the control groups of rats, *ad libitum* and inanition, complete B complex vitamin tablets were prepared. The following ingredients, expressed as micrograms, were incorporated in the preparation of the tablets: thiamine hydrochloride, 40; riboflavin, 60; pyridoxine, 50; calcium pantothenate, 300; nicotinic acid, 150; folic acid, 20; and biotin, 3. The rats in the deficient group were fed vitamin B complex tablets from which folic acid was omitted. All animals were given the appropriate vitamin tablet daily prior to being fed. Sulfa-suxidine was added in the diet to inhibit the growth of the bacterial intestinal flora, which forms folic acid in the intestine. Thus folic acid deficiency would be possible in the experimental animals. The addition of sulfa-suxidine to a diet has been reported to have an adverse affect on the bacterial synthesis of biotin in the rat intestine (Martin 1942, Welch 1942). A biotin deficiency is produced and pantothenic acid utilization is interfered with. (Wright and Welch 1944). It was for this reason that a supplement of biotin, 2 ug., and calcium pantothenic, 150 ug., was added beyond the accepted normal requirement of the rat. Hence it was anticipated that folic acid deficiency alone would result at the end of the experimental feeding diet.
Each rat in all three groups received two drops of cod-liver oil\(^{(5)}\) once a week to provide them with 300 U.S.P. of vitamin A and 30 U.S.P. of vitamin D. Vitamin supplements in the above mentioned dose were successfully employed by Wertman et al. (1952-1956), and was employed for this study.

All the animals were fed ad\ libitum for one week so that they could adjust to the new environment and to the synthetic diet. All rats (without any group difference) during this period received the full basal diet and the complete vitamin B complex tablet. After the week period of stabilization, ad\ libitum animals and folic acid deficient animals received the basal diet ad\ libitum. The inanition control animals were paired with folic acid deficient animals, and hence they received basal diet in quantities sufficient to maintain their weights equal to that of the vitamin deficient animals.

When typical deficiency symptoms were established in the rats of the deficient group, the phagocytic and susceptibility studies were performed. This occurred at the end of the 5th week of experimental feeding in phagocytic study and at the end of the 7th week of experimental feeding in susceptibility study. At this time, the ad\ libitum and inanition control groups were subdivided into two groups in

\(^{(5)}\) Squibb.
which one group was studied at the end of the 5th week for
the phagocytic activity and at the end of the 7th week for
the susceptibility study. The second group was challenged
a week later.

The folic acid group was divided into three sub-groups.
The first sub-group was studied at the end of the 5th week
for phagocytic activity and at the end of the 7th week for
the susceptibility determination. Sub-group #2 received 20
μg. of folic acid for one week following the establishment of
the folic acid deficiency and then challenged. Sub-group #3
was included to determine if any deaths occurred due to
folic acid deficiency during the entire experimental period.
Sub-group #3 received the basal diet and folic acid deficient
vitamin B complex tablets for a period of eight weeks.

Initial and final mean weight for each group of
rats appears in Table I.

**Bacterial cultures.** A virulent *Diploccoccus pneumoniae*,
Type I, was used for the study of phagocytosis and suscepti-
bility to infection. *D. pneumoniae* Type I was obtained from
the Department of Medical Microbiology, University of Wis-
consin. The organism was maintained in beef "hormone" broth
(beef heart infusion broth with 2% glucose and 0.5% gelatine).
The virulence was developed by daily injecting 0.5 ml. of
an 18 hr. broth culture intraperitoneally into the mouse. At
the end of six hours, the mouse was sacrificed and the thoracic
cavity was swabbed and the exudate inoculated into blood-heart


<table>
<thead>
<tr>
<th>group</th>
<th>total</th>
<th>mean weight in grams</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>no. rats</td>
<td></td>
</tr>
<tr>
<td>A. Phagocytic studies</td>
<td></td>
<td></td>
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<tr>
<td>Ad libitum control</td>
<td>15</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>163 (11) 195 (4)</td>
</tr>
<tr>
<td>Inanition control</td>
<td>25</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>129.5 (25)</td>
</tr>
<tr>
<td>Folic acid deficient</td>
<td>57</td>
<td>67</td>
</tr>
<tr>
<td>Sub-group #1*</td>
<td></td>
<td>130 (47)</td>
</tr>
<tr>
<td>Sub-group #2**</td>
<td></td>
<td>130 (10) 162 (10)</td>
</tr>
<tr>
<td>B. Susceptibility and bacteremia study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum control</td>
<td>18</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>263 (10) 281 (6)</td>
</tr>
<tr>
<td>Inanition control</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>202 (16) 229 (14)</td>
</tr>
<tr>
<td>Folic acid deficient</td>
<td>58</td>
<td>55</td>
</tr>
<tr>
<td>Sub-group #I*</td>
<td></td>
<td>200 (25)</td>
</tr>
<tr>
<td>Sub-group #II**</td>
<td></td>
<td>200 (25) 232 (25)</td>
</tr>
<tr>
<td>Sub-group #III***</td>
<td></td>
<td>211 (8) 210 (8)</td>
</tr>
</tbody>
</table>

* Rats maintained 5 wks on deficient diet.
** Rats maintained 5 wks on deficient, followed by 1 wk on complete B-complex diet.
*I Rats maintained 7 wks on deficient diet.
**II Rats maintained on 7 wks deficient diet followed by 1 wk on complete B-complex diet.
***III Rats maintained on 8 wks deficient diet.

/ Number of animals in experiment at the end of indicated time period in parenthesis.
infusion glucose broth. After six passages, the virulence was raised to a degree that it killed mice in 6-8 hours.
The virulent state of the organism then was maintained by passing through mice once a week. The bacterial suspension was prepared by suspending washed cells from an 18-hour broth culture in sterile broth. It was adjusted to a reading of 0.08 O.D. in a Lumatron on 650 mu wave length and was checked repeatedly for adjustment if necessary. The bacterial count of 0.08 O.D. was determined to be $2 \times 10^5$ organisms/ cu mm.

**Phagocytosis.** This experiment was conducted to determine the activity of phagocytic white cells, particularly neutrophils, of rats deficient in folic acid as compared to ad libitum and inanition controls.

The phagocytic studies were performed according to the method of Cottingham and Mills and modified by Wertman and Groh (1959) and Wertman and Sypherd (1960). The phagocytic studies were performed at a time when typical folic acid deficiency was established in the rats. At the end of the 5th week of experimental feeding, the animals were bled from the heart by cardiac puncture, using syringes previously rinsed in heparin. Approximately 0.6 ml. to 0.7 ml. of blood was withdrawn and 0.5 ml. was placed in the sidearm of a Warburg flask. To this blood sample, 0.2 ml. of the appropriate bacterial suspension was added. ($2 \times 10^5$ organisms/ cu mm.) The blood-bacterial suspension mixture was then placed immediately in a constant temperature water bath at 38°C and gently agitated by
lateral motion for four minutes. The flasks were then removed from the water bath and five smears were made of each sample. The slides were air dried and stained with Wright's stain. The cells were counted and the percentage of active phagocytizing neutrophiles recorded. In addition, the number of organisms phagocytized by neutrophiles in 100 unclumped and unruptured cells were counted. Average number of organisms per cell was determined.

At the end of the 6th week of experimental feeding, the second phagocytic study was undertaken. During the period of the 6th week, folic acid deficient rats were given the daily requirements of 20 μg. of folic acid. Ad libitum and inanition control rats were continued on the same experimental diet as before.

At the end of the 6th week, all animals were bled by cardiac puncture and phagocytic determinations were performed. Results of the phagocytic study are shown in Table II.

Susceptibility and Bacteremia studies. This experiment was conducted to determine the degree of susceptibility of the folic acid deficient rats to infection. The results were compared with Ad libitum and inanition control animals.

The normal albino rats are susceptible with a high mortality to lobar pneumonia (Loughlin et al 1945), but Wertman and Groh (1959) and Wertman and Sypherd (1960) reported that normal rats are not susceptible by the intraperitoneal route. Therefore, the intraperitoneal route of injection was selected
for the challenge study.

At the end of the 7th week of experimental feeding, typical deficiency symptoms were observed and 2 ml. of virulent \textit{D. pneumoniae} Type I were introduced intraperitoneally into each rat in all three groups. No death occurred in any group within the first 96 hours.

It was observed in an earlier pilot challenge study that none of the deficient rats died due to challenge dose of \textit{D. pneumoniae}. Therefore, it was desirable to study bacteremia at intervals of 12, 24, and 48 hours. At the end of each selected time period, every third animal from each group was bled by cardiac puncture. Approximately 0.6 ml. to 0.7 ml. of blood was drawn from each animal and 0.5 ml. was inoculated in 25.0 ml. of hormone broth. Each tube was then shaken vigorously. Five-tenths ml. and 1 ml. of each mixture was placed on a petri dish. Following 36 hours of incubation at 37°C the colonies of \textit{D. pneumoniae} occurring on each plate were counted. Results on bacteremia study are shown in Table III.

During the period of the 8th week, (Sub-group #2) folic acid deficient rats received 20 \textmu g. of folic acid per day while \textit{ad libitum} and inanition control rats were continued on their same experimental diets. Likewise, at the end of the 8th week of experimental feeding, a second susceptibility study was performed.

At the end of the 8th week, the remaining animals were
challenged and the degree of bacteremia was determined by the plate culture technique.

Sub-group #3 of the folic acid deficient group was continued on the deficient diet for a period of eight weeks to determine the number of deaths occurring due to the vitamin deficiency. This group was not challenged with *P. pneumonieae* infection. No deaths occurred in this group through the entire experimental period.
RESULTS

Evidence of typical folic acid deficiency symptoms developed during the 5th week of experimental feeding in phagocytic study and during the 7th week in susceptibility and bacteremia study. Delay in establishing deficiency in rats in susceptibility and bacteremia study may be due to developed resistance to sulpha-suxidine of intestinal flora. The ad libitum control rats gained weight, possessed smooth, even coats, and appeared very active and healthy. The inanition animals, although given limited quantities of food, had healthy growth and possessed smooth, even coats. The rats in the folic acid deficient group gained very little weight, particularly in the last two weeks of experimental feeding. The skin was rough, uneven, and alopecia was becoming apparent. Ulceration on the mouth and lymphocytopenia was observed. There was no evidence of anemia.

The distribution and initial and final mean weights of each group appear in Table I.

Two separate but related studies were completed.

(1) The in vitro phagocytosis of virulent Type I D. pneumoniae by neutrophiles of rats, (2) susceptibility of the groups to infection by D. pneumoniae, and (3) the degree of bacteremia produced by this organism in vitamin deficient and control animals.

The phagocytic experiment demonstrated a significant
TABLE II

Phagocytic activity of leukocytes from folic acid deficient and control rats.

5th week study

<table>
<thead>
<tr>
<th>group</th>
<th>average percent active neutrophils</th>
<th>no. bacteria/ cell average</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad libitum control</td>
<td>72.1 (11)*</td>
<td>14</td>
<td>11.8 - 15.9</td>
</tr>
<tr>
<td>Inanition control</td>
<td>53.8 (25)</td>
<td>5.9</td>
<td>2.4 - 6.6</td>
</tr>
<tr>
<td>Folic acid deficient</td>
<td>22.6 (47)</td>
<td>2.9</td>
<td>1.6 - 4.7</td>
</tr>
</tbody>
</table>

6th week study**

<table>
<thead>
<tr>
<th>group</th>
<th>average percent active neutrophils</th>
<th>no. bacteria/ cell average</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad libitum control</td>
<td>80.0 (4)</td>
<td>15.9</td>
<td>15.5 - 16.6</td>
</tr>
<tr>
<td>Folic acid deficient</td>
<td>53.5 (10)</td>
<td>4.2</td>
<td>3.5 - 4.7</td>
</tr>
</tbody>
</table>

* Number of animals in experiment.

** Folic acid deficient group was fed 20 μg. folic acid daily during the 6th week.
difference in both the percentage of active neutrophiles and in the degree of activity. At the end of the 5th week, 72.1% of the leukocytes ad libitum; 53.5% of the leukocytes inanition; and 22.6% of the leukocytes from folic acid deficient rats were actively engaged in the phagocytosis of virulent *D. pneumoniae*. The average number of bacteria engulfed per neutrophile was: Ad libitum, 14, inanition, 5.9, and folic acid deficient, 2.9. The result of the present study made it apparent that the rat deficient in folic acid has less phagocytic activity than control animals.

A similar study was conducted with a second group of rats, maintained on folic acid deficient diet for 5 weeks and then placed on the complete diet that provided 20 ug. of folic acid daily for 1 week. The difference between this deficient group and control groups at the end of the 6th week was not as great as it was in the 5th week study. The deficient rats that had received folic acid for one week after deficiency was established showed greater percentage of phagocytizing neutrophiles, (53.5%), than those that did not receive this vitamin (22.6%). Also there was an increase in the number of bacteria engulfed per cell. At the end of the 5th week the average number of organisms per phagocyte was 2.9. After folic acid was administered for one week it was found to be 4.2.

The infectivity studies indicated that folic acid deficient animals did not die from infection with the inoculum of *D. pneumoniae*. All three groups of animals survived
<table>
<thead>
<tr>
<th>group</th>
<th>total no. rats</th>
<th>number of bacteria/ ml. of blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12 hrs.*</td>
</tr>
<tr>
<td>7th week challenge</td>
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</tr>
<tr>
<td>Ad libitum control</td>
<td>10</td>
<td>2,000</td>
</tr>
<tr>
<td>Inanition control</td>
<td>15</td>
<td>10,800</td>
</tr>
<tr>
<td>Folic acid deficient*</td>
<td>25</td>
<td>&gt;300,000</td>
</tr>
<tr>
<td>8th week challenge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum control</td>
<td>8</td>
<td>2,300</td>
</tr>
<tr>
<td>Inanition control</td>
<td>14</td>
<td>15,000</td>
</tr>
<tr>
<td>Folic acid deficient**</td>
<td>25</td>
<td>15,000</td>
</tr>
<tr>
<td>Folic acid deficient***</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

* Time after injection of virulent *D. pneumoniae*.

** Fed 20 μg. of folic acid daily for 1 wk following 7 wks of deficient diet.

*** Extended deficiency group---not challenged.
the challenge dose of *D. pneumoniae*. Studies by Wertman and Groh (1958), and Wertman and Sypherd (1960), indicated the thiamin and riboflavin deficiency had high mortality rate.

The bacteremia study indicated that folic acid deficient animals developed severe bacteremia. Inanition animals showed a greater degree of bacteremia than *ad libitum* rats but comparatively less than the deficient group. The number of bacteria per ml. of blood 12 hrs. after injection was: *ad libitum* 2,000, inanition 10,800, and deficient >300,000. After 24 hours, the numbers were: *ad libitum* 1,900, inanition 4,500, and deficient 12,500; and after 48 hours: *ad libitum* 200, inanition 300, and deficient 9,500.

The deficient group of animals that received 20 µg. of folic acid per day after the deficiency period showed a lesser degree of bacteremia than the deficient group. The results of the 6th week study at the end of 48 hours were: *ad libitum* 300 bacteria/ml., inanition 600 bacteria/ml., and deficient 3,300 bacteria/ml.

Eight rats were maintained on the folic acid deficient diet (Sub-group #3) and remained unchallenged for the entire term of the experiment. This group was maintained to indicate the number of rats that would die from deficiency alone. None died due to the folic acid deficiency.
DISCUSSION

It is apparent that variation in diet may alter the course of bacterial infection. The study of the mechanism of this altered susceptibility has only recently been undertaken. Many investigations have indicated that in order to fully understand the role of dietary factors in infection, it will be necessary to study more thoroughly certain defense mechanisms of the host; e.g., phagocytosis, inflammatory response, bacteremia, etc.

Although phagocytosis is not the sole, protective mechanism against infection, it does assist the body in the removal of foreign particles such as pathogenic micro-organisms. In spite of its relatively important role, little has been reported on the effect of folic acid deficiency on the process of phagocytosis. The present investigation attributes the marked reduction in phagocytic activity to folic acid vitamin deficiency, and not to inanition resulting from the deficiency.

The exact mechanisms for reduced phagocytic activity of leukocytes from deficient rats have not been elucidated at this time. However, certain facts are available which may account for the reduction. Humoral influence on the phagocyte in the case of phagocytosis of pneumonias was demonstrated by Ward and Ender (1933). This investigation
demonstrated the requirement of specific antibody for the phagocytosis of pneumococci. Earlier work (Wertman et al. 1956) have shown that (1) a relative decrease of polymorphonuclear neutrophiles, (2) a relative increase in lymphocytes, (3) a reduction of complement activity in folic acid deficient albino rats, and (4) a relative decrease in granulocytes occurs in folic acid deficiency.

So reduced phagocytic activity, which was observed in this present study may be due to a decrease in polymorphonuclear neutrophiles or to a hampered mechanism of formation of granulocytes in the bone marrow.

Wertman et al. (1956) reported that there was a significant reduction of total leucocytes recovered from inflammatory exudate of deficient animals over the ad libitum and inanition animals. This confirms with the present result of reduced activity of phagocytosis in folic acid deficient rats.

Whatever the explanation for reduction and subsequent recovery of phagocytic activity after deficiency and vitamin therapy, it can be seen from Table II that activity approaches normal values after administration of the daily requirement of 20 μg. of folic acid for one week following established deficiency.

The organisms employed in the infectivity studies showed that vitamin deficient animals are not susceptible to death. All animals looked sick 6 hours after challenge
with *D. pneumoniae*. None died within 12 hours after challenge. Deficient animals appeared sick for about 4 days while the inanition animals were active in 2 to 3 days. *Ad libitum* fed rats returned to their normal activity earlier than inanition animals.

Although no deaths occurred in any group, there was a definite degree of bacteremia established in each group of animals, folic acid deficient group showing a greater degree of bacteremia than inanition and *ad libitum* groups. They remained sick for a longer period of time than inanition and *ad libitum* animals. This observation revealed that there is a definite impaired defense mechanism in the deficient group of rats. When 20 μg. of folic acid was administered for 7 days, after experimental feeding for 7 weeks, challenge study showed a lesser degree of bacteremia in this group than previous deficient animals. Evidently, replacement of folic acid played a definite role in the degree of decrease of bacteremia found.
SUMMARY

Male albino rats of the Sprague-Dawley strain were maintained on a well-defined diet in folic acid to study the effects of the deficiency on (1) the phagocytic activity of leukocytes, (2) the susceptibility to infection by virulent Type I Diplococcus pneumoniae, and (3) the determination of the degree of bacteremia developing. Adequate numbers of inanition and ad libitum control animals were included. The following observations were made: (1) folic acid deficient groups showed remarkably decreased activity of phagocytosis against D. pneumoniae. Neutrophiles actively engaged in phagocytosis was considerably less in the deficient animals than in control animals. Number of bacteria engulfed by deficient leukocytes was less than those engulfed by ad libitum leukocytes. Similarly, the phagocytic white cells of the inanition group rats engulfed more than folic acid deficient group animals but less than ad libitum animals. (2) Folic acid deficient animals were not susceptible to death by infection with D. pneumoniae. (3) The deficient group of animals showed a very high degree of bacteremia in comparison with controls. Inanition animals showed less degree of bacteremia and ad libitum animals showed still lesser degree of bacteremia to that of deficient animals. The group of deficient rats which received the daily require-
ment of 20 mg. folic acid for 7 days following the establishment of folic acid deficiency had less degree of bacteremia than the previous deficient group.

There were no fatalities due to the extension of the deficiency for the total period of the research project.
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