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The Effect of Infection with *Pasteurella tularensis* on the Metabolism of White Rats

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To the Graduate Council:

I am submitting herewith a dissertation written by Gennaro John Miraglia entitled "The Effect of Infection with *Pasteurella tularensis* on the Metabolism of White Rats." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry and Cellular and Molecular Biology.

John M. Woodward, Major Professor

We have read this dissertation and recommend its acceptance:

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December 1, 1960

To the Graduate Council:

I am submitting herewith a thesis written by Gennaro John Miraglia entitled "The Effect of Infection with Pasteurella tularensis on the Metabolism of White Rats." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Bacteriology.

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THE EFFECT OF INFECTION WITH PASTEURELLA TULARENSIS ON THE METABOLISM OF WHITE RATS

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Gennaro John Miraglia

December 1960

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To Jean

whose devotion and understanding
was an inspiration

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INTRODUCTION

Although tularemia is generally considered to be a disease of rodents and of small ground animals, man is an occasional host. The early workers in this field succeeded in isolating and describing the causative organism, and in a relatively short time its nutritional requirements and cultural characteristics were fairly well understood. In slightly more than a decade after Pasteurella tularensis was first isolated (McCoy and Chapin, 1912), both American and Japanese workers had reported on its symptomatology, pathology, and epidemiology.

In more recent years, efforts have been directed to studies of the bacterium itself in an attempt to understand its virulence (Fleming and Foshay, 1955; Fleming and Foshay, 1956; Rendina and Mills, 1957). The literature, however, is meager concerning a systematic host-parasite study with tularemia.

Since the rat and P. tularensis afford a host-parasite system suitable for investigation in the laboratory, studies employing this model could elucidate the interaction of these two agents, namely, the invading organism on the one hand and the host on the other. It is conceivable that any knowledge so obtained could be pertinent to the understanding of other infections in mammals by gram-negative bacteria.

Previous studies in this laboratory have shown

disturbances in the nitrogen metabolism and in enzymes concerned with amino acid utilization. Liver dysfunction and the reduction in the activity of certain tricarboxylic acid cycle enzymes in the liver of the host have also been reported.

An analysis of these outward manifestations of the disease is of worth not only for its intrinsic value but also because this understanding may serve as a basis for the exploration of more fundamental disturbances of the host from which many other abnormalities follow, possibly through a series of complex and intricately related reactions.

The purpose of this thesis has been to follow the course of tularemia in rats with the aid of biochemical methods. An attempt was made to alleviate disease symptoms by administering the appropriate chemical substances, and to study the disease anew from this vantage point.

REVIEW OF THE LITERATURE

According to Ohara (1955a), Martin called attention to cases of tularemia in human beings in this country in 1907. McCoy and Chapin (1912) succeeded in isolating the causative organism from ground squirrels in San Francisco. However, Ohara (1955a) indicates that the earliest recorded account of this disease dates back to 1837. He reports that at that time Homma Soken, a Japanese physician, accurately reported in a text entitled A Memoir on Tumor Pathology the first clinical and epidemiological description of the disease in man resulting from eating infected hares.

The disease was rediscovered in Japan by Ohara in 1925 and called Yato-Byo (wild rabbit disease). Francis and Moore (1926) reported that both Japanese and American workers had been independently studying the same disease, i.e., tularemia.

Ohara (1926) showed that the disease was transmitted from infected rabbits to man by handling the infected animal tissue. This was unquestionably demonstrated by rubbing infected rabbit blood on the arm of his wife who subsequently contracted the disease. An accurate medical record of her progress was kept, and the incubation period established. The organism was isolated from the axillary lymph node on the 18th post-infection day and was found to be the same as the organism isolated from the infected rabbit. It was observed that on the 6th day the temperature was at a peak and the

symptoms were most pronounced. Ohara also described the autopsy of an infected rabbit.

He demonstrated its communicability and named the organism isolated Ohara-Haga coccus. Ohara's studies were the first truly scientific studies of this disease in Japan. Before this time tularemia was believed to exist only in the United States (Francis, 1925).

In Japan tularemia is transmitted mainly by ectoparasites of wild rabbits which form the principal animal reservoir for this disease. In the United States tularemia is more widely distributed among lower animals. The disease in Japan appears to be a milder form than that in the United States. Up to 1955 no mortalities had been reported in Japan, but in this country fatal infections are not uncommon (Ohara, 1955b).

Tularemia is a disease caused by Pasteurella tularensis and is primarily an infection of wild rodents and rabbits and some of their ectoparasites. Man is occasionally infected by his contact with animal tissues containing the organism or by the bite of certain insects.

Since tularemia was first described, it has been known by a variety of names. McCoy referred to it as a "plague-like disease of rodents"; in Utah it was known as "deer-fly fever", in Washington, D. C. as "rabbit fever", and in Japan as "Ohara's disease".

In 1911 McCoy described a "plague-like disease of rodents" in Tulare County, California, the county after which the disease was later named. The following year McCoy and Chapin (1912) isolated the causative agent of tularemia and named it Bacterium tularense.

Wherry and Lamb (1914) were the first to diagnose a case of tularemia in man by isolating the organism from an eye infection. They also reported on the relationship between the natural reservoir of infection in wild rabbits and human disease.

Pasteurella tularensis is a pleomorphic, nonmotile, gram-negative, nonsporulating rod which may or may not exhibit bipolar staining. Equal numbers of cocci and rod forms occur ranging in size from 0.2 to 0.7 microns. No growth occurs on ordinary culture media, the standard solid medium being glucose-cysteine-blood agar.

In human infections the incubation period is about three days. The onset is sudden and is characterized by headache, vomiting, and chills and fever. An ulcer frequently develops at the site where the organism entered the body, and the regional lymph nodes become enlarged and tender. The course of the disease is from three to four weeks. Convalescence is slow, lasting from two to three months. Recovery is attended by a solid and lasting immunity to secondary attack (Hull, 1955).

Six clinical types of tularemia are recognized--ulceroglandular, oculoglandular, glandular, typhoidal, pneumonic, and meningitic. In Japan (Ohara, 1955b) two additional types are recognized--tonsilloglandular and rhinoglandular.

Francis (1928) summarized the results of studies concerned with the symptoms, diagnosis, and pathology of tularemia in both man and animals.

Kavanaugh (1935), Amoss and Sprunt (1936), and Pullen and Stuart (1945) reported on blood dyscrasias in patients with tularemia. Clinical reviews have also been written by Foshay (1940), and Blackford and Casey (1941).

Earlier workers (Haga and Ohara, 1926) described methods for culturing P. tularensis. Later Downs et al. (1947) reported on a method for growing the organism in embryonated eggs. Ohara (1926) described the clinical symptoms of the disease in man and suggested treatment with salvarsan. The morphology was studied by Ohara et al. (1935), and they suggested that virulence is enhanced by cultures showing excessive pleomorphism. Hatchome and Sato (1954) described skin eruptions in tularemia, management of the disease, and an agglutination test for the detection of the organism. More recently, skin tests for diagnosis of tularemia have been studied by Ohara and Hoshishima (1957), and by Takizawa (1959a, 1959b).

With the completion of these basic studies, the

isolation and cultivation of the organism became a task more easily accomplished, and the disease it caused could be diagnosed both from the clinical symptoms and the appropriate laboratory tests.

Attention was then focused on the organism itself. Fleming and Foshay (1955) attempted to relate the virulence of certain strains of P. tularensis with the activity of several enzymes and found that citrulline ureidase activity was low in strains of low virulence. The following year Fleming and Foshay (1956) reported that a much greater aspartic-alanine transaminase activity was found in avirulent strains in comparison to virulent strains of this organism. Rendina and Mills (1957) characterized the glutamic acid dehydrogenase of P. tularensis, strain Sm. In Japan Saito (1959) found that virulence and the succinic dehydrogenase of P. tularensis increase in direct proportion following a series of animal passages.

Foshay (1950) wrote a review of most of the papers concerned with tularemia which had been published from 1939 to 1949. It is interesting to note that even this extensive paper gives no indication of any metabolic studies conducted on a host during infection.

The first reports dealing with the host-parasite relationship in tularemia which encompassed studies of host metabolism during the disease were those of Sbarra et al.

(1952), Sbarra and Woodward (1954), Woodward et al. (1954), Sbarra and Woodward (1955), and Woodward and Mayhew (1956).

Miraglia and Woodward (1959) extended this work and conducted a series of studies which clearly demonstrated a depression in energy metabolism in tularemic rats and the amelioration of this condition by the administration of phosphates. Woodward and Miraglia (1960) observed that the administration of cortisone acetate to infected animals alleviated disease symptoms, supported fumarase and aconitase activity, but did not alter the mortality rate. This observation was in accord with similar studies by Pinchot et al. (1949) using E. coli and P. tularensis, and with reports by Berry et al. (1959) using mice and Salmonella typhimurium endotoxin. In still another host-parasite system, Gilfillan et al. (1956) showed that the activity of several enzymes, including fumarase and aconitase, was reduced in the livers of baby chicks infected with Salmonella pullorum.

Miraglia and Woodward (1959) showed that the administration of adenosine triphosphate (ATP) and KH_2PO_4 to tularemic rats reduced markedly the mortality rate and is in agreement with the report of Takeda et al. (1955) who observed the protective effect of ATP in rabbits intoxicated with Salmonella typhosa, Salmonella paratyphi B, and Shigella flexneri endotoxins. Similarly, Dooley et al. (1958) demonstrated the protective effect of ATP when administered

to chicks treated with the endotoxin of *Salmonella pullorum*.

MATERIALS AND METHODS

Animals. Adult male rats of the Wistar strain, weighing 200-250 g, were used throughout this investigation.

Rat ration. Animals were sustained on Purina Laboratory Chow manufactured by the Ralston Purina Co., St. Louis, Missouri. Water was available ad libitum at all times.

Stock culture. The highly virulent Sm strain of P. tularensis, having a rat LD_{50} of approximately 350 organisms, was used for all infectivity experiments.

Medium. Stock cultures were cultivated on glucose-cysteine-blood agar (Downs et al., 1947).

Reagents and chemicals. Chemicals designated as chemically pure were used throughout.

ATP, creatine phosphate (sodium salt), and adenosine employed in these studies were from the Nutritional Biochemicals Corp., Cleveland, Ohio.

Cortisone acetate (11-dehydro-17-hydroxycorticosterone-21-acetate) was obtained from the Upjohn Co., Kalamazoo, Michigan.

The source of 2,4 dinitrophenol (DNP) was the Fisher Scientific Co.

Preparation of inoculum for animal inoculation. Growth from a 24 hour culture of P. tularensis was emulsified in physiological saline. The suspension was then adjusted to 27 per cent transmittance on the Spectronic 20 Colorimeter

(Bausch and Lomb Optical Co.) using a 600 mu filter.

From this standard suspension containing approximately 3.5×10^9 organisms, the desired number of cells were obtained by making appropriate ten-fold serial dilutions. The inoculation was always made by the intraperitoneal route.

Determination of fumarase activity. This was conducted according to the method of Racker (1950). The rats to be used for this enzyme study were fasted for approximately 12 hours prior to use. The animals were killed by decapitation in a mechanical guillotine, and 0.5 g of tissue from the median lobe of the liver was removed without delay, blotted dry on a filter paper, and weighed on a torsion balance.

The tissue was homogenized at 4 C in 10 ml of 0.1 M phosphate buffer at pH 7.4. The homogenate was centrifuged at 3000 rpm for 10 minutes in an International Centrifuge, Model SBV. The supernatant was then filtered through Whatman no. 1 filter paper.

One ml of the filtrate was dried to constant weight at 90-100 C in a tared 10 ml beaker. An aliquot of 0.1 ml was used to determine the fumarase activity as measured in a Beckman Model DU Spectrophotometer at a wave length of 240 mu.

The enzyme test system contained the following:

a) 0.1 M phosphate buffer	1.0 ml
b) 0.05 M sodium L-malate	1.0 "
c) glass distilled water	0.9 "
d) homogenate	0.1 "
Total volume in absorption cell	3.0 ml

Activity was recorded at intervals of 15 seconds following the addition of the substrate.

A control was employed which contained all of the above materials except malate.

Administration of test materials. The initial injection of the test substances was always by the subcutaneous route to avoid immediate contact with the infecting organisms. After the first post-infection day, these compounds were administered by intraperitoneal injection to normal controls and also to infected animals.

Blood samples. Blood was obtained by cardiac puncture from rats which were under ether anesthesia. Serum was always removed within 1 hour after clotting had occurred.

Urine samples. Urine was collected by placing two rats matched by weight in a circular cage over a Buchner funnel for approximately 12 hours. A crystal of thymol was placed in a flask below the funnel to inhibit bacterial growth during the collection period.

Urine albumin. The determination for albumin in the urine was conducted according to the method described by Exton (1923).

Determination of inorganic phosphorus. For the determination of inorganic phosphorus in both the urine and the blood, the method of Fiske and Subbarow (1925) was used. Samples were obtained from animals which were in the post-

absorptive state.

Serum alkaline phosphatase. The method described by Bodansky (1932, 1933) for the determination of serum alkaline phosphatase was followed.

Serum acid phosphatase. For this determination, the method of Shinowara et al. (1942) was used.

Electrophoresis. The distribution of the various serum proteins was determined electrophoretically by the paper strip method (Kunkel and Tiselius, 1951). A 0.01 ml aliquot of each sample of serum was placed at a reference point in the center of the paper strip, and electrophoresis was conducted at 7.5 milliamperes for 18 hours. This procedure was carried out in a Spinco Model R cell containing barbital buffer, ionic strength 0.1 at pH 8.6. Each serum sample was analyzed in duplicate. The strips were then dried and stained according to the method described by Block et al. (1955). Analysis of the paper strips was accomplished in a Spinco Analytrol Scanner, Model R, which automatically records dye intensities by plotting a curve.

Inanition studies. Measured amounts of food and water were given to normal, infected, and infected rats receiving treatment to determine the time of onset and the severity of anorexia during infection and/or treatment, in comparison to normal controls.

Statistical methods. The standard error of the mean

was calculated employing standard formulae.

When means were compared, their significance was determined by Student's *t* test.

The Chi Square test was employed to determine significance in mortality studies.

RESULTS

General macroscopic observations of tularemic rats. Rats infected with approximately 1 LD_{50} of P. tularensis were found to be asymptomatic for the first two days following infection. On the third to fourth day, most of the animals showed a ruffled, dull fur coat and bleeding about the nose. From post-infection day 4 to 6, the disease symptoms were most pronounced. This period was characterized by periorbital hemorrhage and conjunctivitis, diarrhea, abdominal distention, labored breathing, and sluggishness.

Necropsy during this period invariably revealed a spleen enlarged 5-6 times its normal size. This organ appeared normal in color, but was very hard in consistency. Necrotic areas were very seldom noted. The liver was twice the normal size and was frequently covered with necrotic foci. The entire alimentary canal was consistently free from hemorrhagic areas or ulcers.

Studies of fumarase activity in livers of the rat. Fumarase activity was studied in the livers of normal, infected, and infected rats treated with cortisone (Table I). The data demonstrate that fumarase activity rapidly decreases in tularemic rats. This activity is supported by the administration of cortisone for the first 48 hours post-infection.

TABLE I

FUMARASE ACTIVITY IN THE LIVER OF NORMAL AND INFECTED RATS, AND IN INFECTED RATS TREATED WITH CORTISONE

Post Infection Day	Number of Animals	Condition of Animals	Fumarase Units*
1	6	Inf.**	78.1 ± 4.1
2	6	"	72.2 ± 1.0
3	5	"	62.8 ± 4.5
4	4	"	62.8 ± 0.1
1	4	Inf. + T***	81.2 ± 8.5
2	4	"	85.9 ± 9.5
3	4	"	42.8 ± 3.6
4	3	"	59.6 ± 0.5
	40	Normal	99.5 ± 4.1

* Each value is the mean ± the standard error of the mean.

** Animals were infected with approximately 350 cells of P. tularensis.

*** Animals were infected and treated with 5 mg cortisone acetate/rat/day.

To determine what effect the administration of a high energy phosphorus compound had on fumarase activity, ATP was given to a group of normal and infected rats as shown in Tables II, III, and IV. Fumarase activity was not altered in normal animals treated with ATP, but enzyme activity was supported temporarily in infected animals treated with 30 mg of ATP.

Survival studies using organic and inorganic phosphate treatments. The influence of ATP on tularemic rats was studied employing reduction of mortality rate as a criterion. The results of this phase of the study are illustrated in Tables V, VI, and VII. This series of experiments demonstrated the protective effect of ATP. It may be noted that 30 mg of ATP was found to be the most effective treatment only against 1 LD_{50} of P. tularensis. By using only 10 mg of ATP, a significant protective effect could be noted on post-infection day 4; however, protection diminished as the disease progressed.

Creatine phosphate, another source of high energy phosphate, was found to be very effective in preventing death in tularemic animals if used daily (Table VIII).

Tables IX, X, and XI show the results of a series of experiments conducted in an effort to determine which portion of the ATP molecule afforded the protective effect. It was also the purpose of these experiments to study the mortality ratio of tularemic animals when the dosages of the testing

TABLE II

FUMARASE ACTIVITY IN THE LIVER OF NORMAL RATS, AND IN NORMAL RATS TREATED WITH 30 MG ATP

Day	Number of Animals	Condition of Animals	Fumarase Units*
1	3	T**	99.8 ± 3.2
2	3	"	94.5 ± 3.3
3	3	"	99.1 ± 2.0
4	3	"	101.5 ± 5.1
	10	Normal	97.05 ± 2.1

* Each value is the mean ± the standard error of the mean.

** Animals were treated with 30 mg ATP/rat/day.

TABLE III

FUMARASE ACTIVITY IN THE LIVER OF NORMAL AND INFECTED RATS, AND IN INFECTED RATS TREATED WITH 10 MG ATP

Post Infection Day	Number of Animals	Condition of Animals	Fumarase Units*
1	3	Inf.**	76.5 ± 1.8
2	3	"	57.1 ± 7.9
3	3	"	57.1 ± 6.4
4	3	"	58.6 ± 3.4
5	3	"	54.3 ± 1.0
1	3	Inf. + T***	71.4 ± 1.4
2	3	"	64.3 ± 1.4
3	3	"	44.4 ± 1.3
4	3	"	48.2 ± 3.6
5	3	"	51.1 ± 0.7
	10	Normal	97.05 ± 2.1

* Each value is the mean ± the standard error of the mean.

** Animals were infected with approximately 350 cells of P. tularensis.

*** Animals were infected and treated with 10 mg ATP/rat/day.

TABLE IV

FUMARASE ACTIVITY IN THE LIVER OF NORMAL RATS, AND IN INFECTED RATS TREATED WITH 30 MG ATP

Post Infection Day	Number of Animals	Condition of Animals	Fumarase Units*
1	2	Inf. + T**	56.4 ± 6.1
2	2	"	75.1 ± 7.2
3	2	"	106.1 ± 0.9
4	2	"	63.8 ± 9.1
5	2	"	65.1 ± 8.8
6	2	"	72.2 ± 3.2
	7	Normal	93.3 ± 6.9

* Each value is the mean ± the standard error of the mean.

** Animals were infected with approximately 350 cells of P. tularensis and treated with 30 mg ATP/rat/day.

TABLE V

THE EFFECT OF ATP ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 10 mg ATP/day	Rats Receiving No Treatment
1	0	0
2	0	0
3	0	0
4	1	5
5	2	1
6	1	1
7	0	0
8	2	2
9	0	0
10	0	0
Mortality Ratio:	6/15	9/15
Per cent Dead:	40.0%	60.0%

* Animals were infected with approximately 350 cells of P. tularensis.

TABLE VI

THE EFFECT OF ATP ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 30 mg ATP/day	Rats Receiving No Treatment
1	0	0
2	0	0
3	0	0
4	0	1
5	1	5
6	0	0
7	1	2
8	0	0
9	0	0
10	0	0
Mortality Ratio:	2/30	8/30
Per cent Dead:	6.7%	26.7%

* Animals were infected with approximately 350 cells of P. tularensis.

TABLE VII

THE EFFECT OF ATP ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 30 mg ATP/day	Rats Receiving No Treatment
1	0	0
2	0	0
3	5	7
4	8	7
5	2	0
6	0	1
7	0	0
8	0	0
9	0	0
10	0	0
Mortality Ratio:	15/15	15/15
Per cent Dead:	100%	100%

* Animals were infected with approximately 3500 cells of P. tularensis.

TABLE VIII

THE EFFECT OF CREATINE PHOSPHATE ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 15 mg Creatine Phosphate/ Day	Rats Receiving No Treatment
1	0	0
2	0	0
3	0	0
4	0	1
5	1	7
6	0	3
**		
7	5	5
8	4	1
9	3	2
10	1	0
11	1	1
12	1	0
13	1	1
14	1	0
15	0	0
16	0	0
17	1	0
18	0	0
19	0	0
20	0	0
Mortality Ratio:	19/40	21/40
Per cent Dead:	47.5%	52.5%

* Animals were infected with approximately 35 cells of P. tularensis.

** Treatment terminated.

TABLE IX

THE EFFECT OF ADENOSINE AND INORGANIC PHOSPHATES ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 30 mg Adenosine/day	Rats Receiving 20 mg KH_2PO_4/day	Rats Receiving 20 mg K_2HPO_4/day	Rats Receiving No Treatment
1	0	0	0	0
2	0	0	0	0
3	10	4	6	11
4	2	6	6	2
5	2	1	2	1
6	0	1	0	0
7	1	0	0	1
8	0	0	0	0
9	0	0	0	0
10	0	0	0	0
Mortality Ratio:	15/15	12/15	14/15	15/15
Per cent Dead:	100%	80.0%	93.3%	100%

* Animals were infected with approximately 350 cells of P. tularensis.

TABLE X

THE EFFECT OF ADENOSINE AND KH_2PO_4 ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 30 mg Adenosine/day	Rats Receiving 20 mg KH_2PO_4/day	Rats Receiving No Treatment
1	0	0	0
2	0	0	0
3	1	0	0
4	8	0	6
5	0	3	1
6	0	0	1
7	1	2	1
8	0	0	0
9	0	0	0
10	0	0	0
Mortality Ratio:	10/15	5/15	9/15
Per cent Dead:	66.7%	33.3%	60.0%

* Animals were infected with approximately 35 cells of P. tularensis.

TABLE XI

THE EFFECT OF INORGANIC PHOSPHATES ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 20 mg KH_2PO_4/day	Rats Receiving 20 mg K_2HPO_4/day	Rats Receiving No Treatment
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	0	0	2
6	0	1	3
7	1	2	1
8	1	2	1
9	1	2	0
10	0	1	1
Mortality Ratio:	3/15	8/15	8/15
Per cent Dead:	20.0%	53.3%	53.3%

* Animals were infected with approximately 35 cells of P. tularensis.

substances and the numbers of infecting organisms were altered. The data presented in Table IX reveals, for the first time during this investigation, that the virulence of the infecting organism had increased; henceforth, the LD_{50} could be reached with fewer organisms.

Table X shows the ineffectiveness of treatment with adenosine and suggests a possible protective effect by administration of KH_2PO_4, which was subsequently proved by the data in Table IX. Table XI also indicates the lack of protective effect with the use of K_2HPO_4. This is further substantiated by the data in Table IX.

Tables XI, XII, XIII, and XIV indicate the unpredictability of treatment with KH_2PO_4. It can be noted that in Table XII this treatment significantly protected the host (above the 95 per cent level of significance on the basis of Student's t test), but failed to protect animals infected with one-tenth this number of organisms, as shown in the following experiment (Table XIII). When the number of infecting organisms was decreased, no benefecial effect was detected following treatment with KH_2PO_4 (Table XIV).

The data recorded in Tables XV and XVI were obtained from two experiments in which identical infective doses were employed. No protection was noted in the former, but a significant protective effect (above the 95 per cent level) was evident in the latter. Additional protective effect

TABLE XII

THE EFFECT OF KH_2PO_4 ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 20 mg KH_2PO_4/day	Rats Receiving No Treatment
1	0	0
2	0	0
3	0	0
4	1	7
5	2	8
6	3	0
7	2	0
8	0	0
9	0	0
10	2	0
Mortality Ratio:	10/15	15/15
Per cent Dead:	66.7%	100%

* Animals were infected with approximately 350 cells of P. tularensis.

TABLE XIII

THE EFFECT OF KH_2PO_4 ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 20 mg KH_2PO_4/day	Rats Receiving No Treatment
1	0	0
2	0	0
3	0	0
4	1	1
5	3	4
6	3	6
7	4	4
8	4	3
9	0	2
10	0	0
Mortality Ratio:	15/30	20/30
Per cent Dead:	50.0%	66.7%

* Animals were infected with approximately 35 cells of P. tularensis.

TABLE XIV

THE EFFECT OF KH_2PO_4 ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 20 mg KH_2PO_4/day	Rats Receiving No Treatment
1	0	0
2	0	0
3	0	0
4	0	0
5	0	0
6	0	1
7	2	0
8	1	0
9	1	1
10	1	3
Mortality Ratio:	5/15	5/15
Per cent Dead:	33.3%	33.3%

* Animals were infected with approximately 3.5 cells of P. tularensis.

TABLE XV

THE EFFECT OF INORGANIC PHOSPHATES
ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 20 mg KH_2PO_4/day	Rats Receiving 60 mg KH_2PO_4/day	Rats Receiving No Treatment
1	0	0	0
2	0	0	0
3	0	0	0
4	1	0	0
5	3	4	3
6	3	4	2
7	1	2	2
8	1	0	0
9	0	1	0
10	0	0	0
Mortality Ratio:	9/15	11/15	7/15
Per cent Dead:	60.0%	73.3%	46.7%

* Animals were infected with approximately 350 cells of P. tularensis.

TABLE XVI

THE EFFECT OF INORGANIC PHOSPHATES ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 20 mg KH_2PO_4/day	Rats Receiving 20 mg K_2HPO_4/day	Rats Receiving No Treatment
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	2
5	1	0	3
6	1	4	2
7	0	1	0
8	0	2	0
9	1	2	0
10	2	1	1
Mortality Ratio:	5/15	10/15	8/15
Per cent Dead:	33.3%	66.7%	53.3%

* Animals were infected with approximately 350 cells of P. tularensis.

could not be detected by increasing the dose of KH_2PO_4 (Table XV). In infections approaching the LD_{100}, KH_2PO_4 was without protective effect (Table XVII).

In an experiment employing a larger number of animals (Table XVIII), treatment with KH_2PO_4 showed a very high degree of protection (above the 99 per cent significance level).

The influence of delayed treatment on tularemic rats. A delay in treatment nullified the beneficial effect afforded by KH_2PO_4 (Table XIX) even when the dosage was tripled.

By postponing ATP administration for 48 hours, the final mortality ratio of the treated and untreated groups was nearly identical, but some protection is observed briefly immediately after treatment (Table XX).

The requirement by the host for a readily available source of energy seems to be preferred to that of a potential energy source. Table XXI presents data which indicates that ATP was significantly more beneficial (above the 99 per cent level) than glucose under the conditions of this experiment.

Studies of the distribution of serum proteins. In an attempt to explain the protective effect of treatment, the distribution of proteins in the serum was determined electrophoretically. These results are shown in Table XXII. The finding that serum albumin was at a low level at the time when disease symptoms were most pronounced prompted a determination of the albumin content of the urine which is

TABLE XVII

THE EFFECT OF KH_2PO_4 ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 20 mg KH_2PO_4/day	Rats Receiving No Treatment
1	0	0
2	0	0
3	2	2
4	5	6
5	3	0
6	1	2
7	0	0
8	0	0
9	0	1
10	0	0
Mortality Ratio:	11/15	11/15
Per cent Dead:	73.3%	73.3%

* Animals were infected with approximately 3500 cells of P. tularensis.

TABLE XVIII

THE EFFECT OF KH_2PO_4 ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 20 mg KH_2PO_4/day	Rats Receiving No Treatment
1	0	0
2	0	0
3	0	0
4	2	13
5	3	8
6	6	9
7	6	2
8	4	3
9	1	2
10	3	0
Mortality Ratio:	25/45	37/45
Per cent Dead:	55.6%	82.2%

* Animals were infected with approximately 350 cells of P. tularensis.

TABLE XIX

THE EFFECT OF DELAYED INORGANIC PHOSPHATE TREATMENT ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 20 mg KH_2PO_4/day	Rats Receiving 60 mg KH_2PO_4/day	Rats Receiving No Treatment
1	0	0	0
2**	0	0	0
3	0	0	0
4	1	0	0
5	0	0	1
6	2	2	2
7	1	3	2
8	1	1	2
9	1	1	1
10	0	0	0
Mortality Ratio:	6/15	7/15	8/15
Per cent Dead:	40.0%	46.7%	53.3%

* Animals were infected with approximately 35 cells of P. tularensis.

** Treatment begun.

TABLE XX

THE EFFECT OF DELAYED ATP TREATMENT ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 30 mg ATP/day	Rats Receiving No Treatment
1	0	0
2	0	0
3**	6	11
4	4	2
5	2	1
6	0	0
7	1	1
8	0	0
9	1	0
10	0	0
Mortality Ratio:	14/15	15/15
Per cent Dead:	93.3%	100%

* Animals were infected with approximately 350 cells of P. tularensis.

** Treatment begun.

TABLE XXI

THE EFFECT OF ATP AND OF 2% GLUCOSE ON TULAREMIC RATS*

Post Infection Day	Rats Receiving 30 mg ATP/day	Rats Receiving 2% glucose/day	Rats Receiving No Treatment
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	0	2	2
6	0	2	5
7	0	2	5
8	0	0	2
9	1	1	0
10	0	1	0
11	0	2	0
12	1	1	0
13	0	0	0
14	0	1	0
15	0	0	0
16	0	0	0
Mortality Ratio:	2/30	12/30	14/30
Per cent Dead:	6.7%	40.0%	46.7%

* Animals were infected with approximately 35 cells of P. tularensis.

TABLE XXII

THE DISTRIBUTION OF SERUM PROTEINS AND THE ALBUMIN/GLOBULIN RATIO IN NORMAL, INFECTED, AND IN RECOVERED RATS AS DETERMINED ELECTROPHORETICALLY

Condition of Animals	Albumin	Alpha 1 Globulin	Alpha 2 Globulin	Beta Globulin	Gamma Globulin	Albumin/ Globulin Ratio
Normal (8)*	35.7 ± 4.9	20.0 ± 2.4	12.4 ± 1.8	19.2 ± 1.9	12.7 ± 1.9	0.56 ± 0.14
Infected (6)*	12.6 ± 2.5	23.9 ± 2.1	21.0 ± 1.7	30.7 ± 2.0	11.8 ± 2.4	0.15 ± 0.32
Recovered (6)*	26.2 ± 6.1	17.7 ± 2.0	14.8 ± 3.3	22.7 ± 5.2	18.4 ± 4.1	0.37 ± 0.11

* Number of animals tested.

Note: The measurements reported are the mean per cents of each fraction ± the standard error of the mean.

recorded in Table XXIII.

Serum phosphatase studies. Studies of serum alkaline phosphatase and serum acid phosphatase determinations were conducted to ascertain whether or not tularemic animals and/or tularemic animals treated with phosphate caused any changes in these enzyme systems which could result in the protective effect noted with some therapeutic compounds.

An initial decrease in serum alkaline phosphatase followed by a subsequent rise and another decrease were observed in tularemic rats. The same pattern was observed for infected animals receiving treatment (Table XXIV).

A definite decrease was observed in serum acid phosphatase (Table XXV) in the infected controls. The treated group consistently showed activity that could not be distinguished from the acid phosphatase activity of normal controls.

Studies of inorganic phosphorus in blood and urine. Serum inorganic phosphorus analyses were conducted on infected animals receiving inorganic phosphate, ATP, and creatine phosphate in an effort to detect any variation from the phosphate values in normal rats which might explain the beneficial effect of treatment. These results are shown in Tables XXVI, XXVII, XXVIII, and XXIX.

It was noted that serum inorganic phosphorus was increased in normal animals receiving ATP but not in those which were infected. Treatment with creatine phosphate tended

TABLE XXIII

ALBUMIN LEVELS IN URINE IN NORMAL AND IN TULAREMIC RATS

Post Infection Day	Number of Animals	Condition of Animals	Urine Albumin (mg/rat/ 12 hr)*
1	4	Inf.**	7.8 ± 1.96
2	2	"	8.7 ± 0.42
3	3	"	8.6 ± 0.10
4	3	"	8.9 ± 1.98
5	3	"	9.2 ± 0.10
6	3	"	11.2 ± 0.08
7	3	"	15.6 ± 0.69
8	3	"	10.2 ± 0.87
9	3	"	2.5 ± 0.19
10	3	"	1.8 ± 0.90
	12	Normal	1.10 ± 0.05

* Each value is the mean ± the standard error of the mean.

** Animals were infected with approximately 35 cells of P. tularensis.

TABLE XXIV

SERUM ALKALINE PHOSPHATASE IN NORMAL, INFECTED, AND IN INFECTED RATS RECEIVING ATP

Post Infection Day	Number of Animals	Condition of Animals	Serum Alkaline Phosphatase (Bodansky units/ 100 ml serum)*
1	3	Inf.**	19.3 ± 0.90
3	4	"	20.2 ± 1.45
5	4	"	37.7 ± 1.40
7	4	"	41.3 ± 9.85
9	4	"	26.6 ± 4.35
12	4	"	29.2 ± 0.01
14	4	"	30.2 ± 2.10
1	3	Inf. + T***	20.2 ± 1.00
3	4	"	28.9 ± 2.10
5	4	"	28.9 ± 1.95
7	4	"	35.5 ± 4.50
9	4	"	23.0 ± 4.65
12	4	"	39.3 ± 0.12
14	4	"	18.8 ± 1.30
	8	Normal	37.02 ± 1.60

* Each value is the mean ± the standard error of the mean.

** Animals were infected with approximately 35 cells of P. tularensis.

*** Animals were infected and treated with 30 mg ATP/rat/day.

TABLE XXV

SERUM ACID PHOSPHATASE IN NORMAL, INFECTED, AND IN INFECTED RATS RECEIVING ATP

Post Infection Day	Number of Animals	Condition of Animals	Serum Acid Phosphatase (Bodansky units/ 100 ml serum)*
1	4	Inf.**	2.9 ± 0.32
3	4	"	0.9 ± 0.44
5	4	"	1.3 ± 0.45
7	4	"	2.1 ± 0.57
9	4	"	1.1 ± 0.19
10	4	"	1.5 ± 0.03
1	4	Inf. + T***	2.1 ± 0.10
3	4	"	2.0 ± 0.34
5	4	"	1.9 ± 0.24
7	4	"	1.9 ± 0.43
9	4	"	2.0 ± 0.09
10	4	"	1.9 ± 0.11
	8	Normal	2.0 ± 0.57

* Each value is the mean ± the standard error of the mean.

** Animals were infected with approximately 35 cells of P. tularensis.

*** Animals were infected and treated with 30 mg ATP/rat/day.

TABLE XXVI

SERUM INORGANIC PHOSPHORUS IN INFECTED RATS TREATED WITH KH_2PO_4, AND IN INFECTED RATS TREATED WITH ATP

Post Infection Day	Number of Animals	Condition of Animals	Serum Inorganic Phosphorus (mg %)*
1	3	Inf. + KH_2PO_4**	5.8 ± 0.10
2	4	"	3.4 ± 1.20
3	4	"	4.9 ± 0.42
4	4	"	6.1 ± 0.30
5	3	"	4.2 ± 0.72
6	4	"	5.4 ± 0.52
7	4	"	4.2 ± 0.12
8	4	"	4.3 ± 0.85
9	4	"	4.8 ± 0.01
10	4	"	5.6 ± 0.65
2	4	Inf. + ATP***	4.7 ± 0.13
3	4	"	4.7 ± 0.01
4	3	"	5.6 ± 0.29
5	4	"	4.6 ± 0.52
6	4	"	6.4 ± 0.83
7	3	"	3.7 ± 1.30
8	4	"	4.7 ± 0.12
9	3	"	5.6 ± 1.00
10	4	"	4.0 ± 0.01
	9	Normal	5.52 ± 0.18

* Each value is the mean ± the standard error of the mean.

** Animals were infected with approximately 35 cells of P. tularensis and treated with 20 mg KH_2PO_4/rat/day.

*** Animals were infected with approximately 35 cells of P. tularensis and treated with 30 mg ATP/rat/day.

TABLE XXVII

SERUM INORGANIC PHOSPHORUS IN NORMAL RATS TREATED WITH KH_2PO_4, AND IN NORMAL RATS TREATED WITH ATP

Day	Number of Animals	Condition of Animals	Serum Inorganic Phosphorus (mg %)*
1	4	N - KH_2PO_4**	5.5 ± 0.15
2	4	"	5.4 ± 0.01
3	4	"	4.6 ± 0.06
4	3	"	7.7 ± 1.25
5	4	"	5.7 ± 0.32
6	4	"	4.8 ± 0.61
7	3	"	5.7 ± 0.13
8	3	"	4.8 ± 0.90
9	4	"	5.8 ± 0.25
10	3	"	5.4 ± 0.13
1	3	N - ATP***	4.7 ± 0.04
2	4	"	5.0 ± 0.14
3	4	"	4.6 ± 0.01
4	4	"	7.0 ± 0.01
5	3	"	5.3 ± 0.10
6	3	"	6.4 ± 0.11
7	4	"	5.1 ± 0.52
8	4	"	5.5 ± 0.01
9	4	"	5.5 ± 0.50
10	4	"	5.0 ± 0.14
	12	Normal	6.63 ± 0.14

* Each value is the mean ± the standard error of the mean.

** Normal rats treated with 20 mg KH_2PO_4/rat/day.

*** Normal rats treated with 30 mg ATP/rat/day.

TABLE XXVIII

SERUM INORGANIC PHOSPHORUS IN INFECTED RATS, AND IN INFECTED RATS TREATED WITH CREATINE PHOSPHATE

Post Infection Day	Number of Animals	Condition of Animals	Serum Inorganic Phosphorus (mg %)*
3	2	Inf.**	9.2 ± 4.80
5	2	"	8.8 ± 2.60
8	2	"	8.4 ± 3.40
12	2	"	9.1 ± 3.10
14	2	"	10.1 ± 0.34
16	2	"	8.6 ± 0.68
19	2	"	9.4 ± 0.34
1	2	Inf. + T***	9.1 ± 1.80
3	2	"	8.1 ± 0.34
5	2	"	8.1 ± 0.34
8	2	"	7.8 ± 2.10
12	2	"	8.8 ± 0.68
14	2	"	8.0 ± 0.68
16	2	"	8.8 ± 0.68
19	2	"	9.1 ± 0.34
	8	Normal	7.0 ± 0.35

* Each value is the mean ± the standard error of the mean.

** Animals were infected with approximately 35 cells of P. tularensis.

*** Animals were infected and treated with 15 mg of creatine phosphate/rat/day.

TABLE XXIX

SERUM INORGANIC PHOSPHORUS IN NORMAL, INFECTED, AND IN INFECTED RATS TREATED WITH KH_2PO_4

Post Infection Day	Number of Animals	Condition of Animals	Serum Inorganic Phosphorus (mg %)*
1	8	Inf.**	9.9 ± 0.93
2	6	"	7.6 ± 0.09
3	8	"	6.5 ± 0.55
4	4	"	4.9 ± 0.78
5	4	"	6.8 ± 0.01
6	4	"	9.2 ± 0.95
7	6	"	7.4 ± 0.35
8	6	"	8.0 ± 0.65
9	4	"	8.2 ± 1.23
10	2	"	6.8 ± 1.50
15	2	"	8.2 ± 0.98
1	6	Inf. + T***	8.5 ± 1.10
2	8	"	8.0 ± 0.85
3	6	"	7.6 ± 0.11
4	6	"	5.6 ± 0.45
5	4	"	7.5 ± 1.25
6	6	"	7.6 ± 0.60
7	4	"	7.2 ± 0.60
8	4	"	6.7 ± 2.40
9	4	"	8.1 ± 0.61
10	2	"	7.6 ± 0.21
15	2	"	7.8 ± 1.11
	12	Normal	6.63 ± 0.14

* Each value is the mean ± the standard error of the mean.

** Animals were infected with approximately 35 cells of P. tularensis.

*** Animals were infected and treated with 20 mg KH_2PO_4/rat/day.

to maintain serum phosphorus at levels approaching those of the normal controls. Results obtained with infected animals following treatment with KH_2PO_4 were more erratic but showed a significant increase in the serum inorganic phosphorus.

A similar study following the same rationale was conducted for urine phosphates. These results are shown in Tables XXX, XXXI, and XXXII.

The results presented in Table XXVI were obtained using a Coleman Junior Spectrophotometer, and although the amounts obtained are lower than those using the Spectronic 20 Colorimeter, the same pattern develops.

Inanition studies. Table XXXIII shows the results of a study on inanition which demonstrated that tularemia did not significantly alter the food and water intake of rats during a 10 day test period.

Studies concerning an in vivo uncoupling of phosphorylation. This phase of the investigation was concerned with experiments designed to learn whether any of the phosphates known to protect against tularemia in rats would also protect rats which had received DNP, a compound known to uncouple oxidative phosphorylation. The results of these experiments are recorded in Tables XXXIV, XXXV, and XXXVI. On the basis of the Chi Square test, results obtained following treatment with ATP were significant beyond the 99 per cent level, treatment with KH_2PO_4 at approximately the 80 per cent level, and

treatment with K_2HPO_4 showed no significant change.

TABLE XXX

INORGANIC PHOSPHORUS IN URINE EXCRETED BY NORMAL RATS RECEIVING KH_2PO_4

Day	Number of Animals	Condition of Animals	Urine Inorganic Phosphorus (mg/rat/12 hr)*
1	4	N - T**	3.80 ± 0.64
2	4	"	2.75 ± 0.58
3	4	"	5.75 ± 1.15
4	4	"	4.60 ± 1.13
5	4	"	4.84 ± 0.16
6	4	"	2.70 ± 0.08
7	4	"	2.30 ± 0.12
	7	Normal	3.95 ± 1.01

* Each value is the mean ± the standard error of the mean.

** Normal rats treated with 20 mg KH_2PO_4/rat/day

TABLE XXXI

INORGANIC PHOSPHORUS IN URINE EXCRETED BY NORMAL AND INFECTED RATS, AND BY INFECTED RATS RECEIVING ATP

Post Infection Day	Number of Animals	Condition of Animals	Urine Inorganic Phosphorus (mg/rat/12 hr)*
1	2	Inf.**	2.88 ± 0.51
2	2	"	3.45 ± 0.65
3	2	"	2.63 ± 0.12
4	8	"	4.35 ± 0.86
5	8	"	5.78 ± 0.76
6	8	"	9.84 ± 0.64
7	8	"	6.25 ± 0.95
8	2	"	3.15 ± 0.45
9	2	"	5.40 ± 1.10
10	2	"	9.60 ± 2.10
1	2	Inf. + T***	3.15 ± 0.64
2	2	"	3.45 ± 0.93
3	2	"	4.40 ± 1.24
4	8	"	3.25 ± 1.13
5	8	"	4.71 ± 0.43
6	8	"	5.20 ± 0.94
7	6	"	3.98 ± 0.81
8	2	"	4.85 ± 1.85
9	2	"	3.15 ± 0.11
10	2	"	6.35 ± 0.35
	17	Normal	4.36 ± 0.38

* Each value is the mean ± the standard error of the mean.

** Animals were infected with approximately 35 cells of P. tularensis.

*** Animals were infected and treated with 30 mg ATP/rat/day.

TABLE XXXII

INORGANIC PHOSPHORUS LEVELS IN BLOOD AND URINE IN NORMAL RATS, AND IN NORMAL RATS RECEIVING ATP

Day	Number of Animals	Condition of Animals	Urine Inorganic Phosphorus (mg/rat/12 hr)*	Blood Inorganic Phosphorus (mg %)*
1	4	N - T**	5.2 ± 0.45	7.0 ± 0.01
2	4	"	5.0 ± 1.25	8.0 ± 0.01
3	4	"	4.8 ± 1.85	6.3 ± 0.45
4	4	"	3.7 ± 0.70	7.3 ± 1.20
5	4	"	3.5 ± 0.32	6.0 ± 0.01
6	4	"	4.4 ± 1.96	9.5 ± 1.30
7	4	"	3.6 ± 0.94	9.6 ± 0.92
	12	Normal	5.15 ± 1.91	7.35 ± 0.24

* Each value is the mean ± the standard error of the mean.

** Normal rats treated with 30 mg ATP/rat/day.

TABLE XXXIII

FOOD AND WATER INTAKE FOR NORMAL AND TULAREMIC RATS

Post Infection Day	Number of Animals	Condition of Animals	Intake/rat/24 hr Food (g)	Intake/rat/24 hr Water (ml)
1	10	Inf.*	15.0	30.8
2	10	"	15.0	32.1
3	10	"	15.0	27.9
4	10	"	15.0	26.5
5	10	"	14.5	23.6
6	10	"	15.3	24.0
7	10	"	11.4	27.7
8	10	"	15.0	21.0
9	10	"	17.5	37.5
10	10	"	15.8	31.0
	10	Normal**	14.8	31.9

* Average daily food and water intake for animals infected with approximately 35 cells of P. tularensis.

** Average daily food and water intake for normal rats taken over a 10 day period.

TABLE XXXIV

THE EFFECT OF ATP ON NORMAL RATS
TREATED WITH 2,4 DINITROPHENOL

Day	Rats Receiving DNP*	Rats Receiving DNP + ATP**
1	8	2
2	1	0
3	0	0
4	0	0
5	0	0
6	0	0
7	0	0
8	0	0
9	0	0
10	0	0
Mortality Ratio:	9/10	2/20
Per cent Dead:	90.0%	10.0%

* 7 mg of DNP/rat on day one only.

** 30 mg of ATP/rat/day.

TABLE XXXV

THE EFFECT OF KH_2PO_4 ON NORMAL RATS TREATED WITH 2,4 DINITROPHENOL

Day	Rats Receiving DNP*	Rats Receiving DNP + KH_2PO_4**
1	7	6
2	1	1
3	0	0
4	0	0
5	0	0
6	0	0
7	0	0
8	0	0
9	0	0
10	0	0
Mortality Ratio:	8/12	7/20
Per cent Dead:	66.7%	35.0%

* 7 mg of DNP/rat on day one only.

** 20 mg of KH_2PO_4/rat/day.

TABLE XXXVI

THE EFFECT OF K_2HPO_4 ON NORMAL RATS TREATED WITH 2,4 DINITROPHENOL

Day	Rats Receiving DNP*	Rats Receiving DNP + K_2HPO_4**
1	6	3
2	0	0
3	0	0
4	0	0
5	0	0
6	0	0
7	0	0
8	0	0
9	0	0
10	0	0
Mortality Ratio:	6/10	3/10
Per cent Dead:	60.0%	30.0%

* 7 mg of DNP/rat on day one only.

** 20 mg of K_2HPO_4/rat/day.

DISCUSSION

The depression of fumarase activity in the livers of tularemic animals indicates that P. tularensis interferes with energy metabolism. This may be demonstrated within the first 24 hours after infection at a time when the animals are otherwise asymptomatic. The difference in the activity of fumarase between normal and infected rats after the first 24 hours differs beyond the 95 per cent level of significance. This observation is compatible with the report of Gilfillan et al. (1956) who demonstrated that the activity of several enzymes of the citric acid cycle is reduced in baby chicks infected with Salmonella pullorum. It is reasonable to assume that this effect is the result, at least in part, of the influence of bacterial endotoxin which both Salmonella pullorum and P. tularensis are known to possess.

Fumarase activity in livers of infected rats and in infected rats receiving cortisone treatment indicated that treatment with this hormone exerted a beneficial effect. This was suggested by evidence that this treatment tended to support fumarase activity at nearly normal values at least for the first 48 hours following infection. In addition, cortisone treatment alleviated almost all macroscopic disease symptoms but did not alter the death rate.

Pinchot et al. (1940) reported that a significant

adrenal involvement occurs in tularemic rats resulting in adrenal hypertrophy and subsequent exhaustion of these glands.

From the data presented in Table I, it appears that diminished adrenal output of cortisone was compensated for by administering this hormone to the host, thus maintaining normal energy metabolism for a limited period of time following infection.

The ability of cortisone to protect the host against the toxic effects of bacterial endotoxins has been reported by Duffy and Morgan (1951), Thomas and Smith (1954), and by Berry et al. (1959). Although the mechanism by which cortisone accomplishes this has not been made clear, the implication has been that the anti-inflammatory property of cortisone is responsible.

Although the daily administration of 30 mg of ATP to normal rats appeared to increase fumarase activity, this was not found to be statistically significant. Infected rats showed a continuous decrease in fumarase activity from post-infection day 1 through day 5. In the group treated with ATP, there was a definite support of enzyme activity for the first 48 hours even though the level attained failed to reach that of the normal controls. In this respect this observation was similar to that noted with cortisone which is further evidence supporting the contention that energy metabolism is disturbed in tularemia. The administration of 30 mg of ATP supported

fumarase activity at post-infection day 3, the approximate time of peak infection.

The fumarase activity reported for day 3 was not significantly different from that of the normal controls. This suggested that ATP therapy was effective at a critical time during the infection. Further support for this was indicated by the data which showed that this dosage of ATP not only delayed the time of death but significantly reduced the mortality rate of infected rats. It was observed that 30 mg of ATP was the optimal dosage and that this dosage was most effective only when the host received 1 LD_{50} of infecting organisms. Larger amounts of ATP failed to demonstrate an increase in beneficial effect.

The data obtained indicates that the beneficial effect of ATP does not reside in the adenosine portion of the molecule but may possibly be related to inorganic phosphate. A parallel study showed that adenine was likewise without protective effect to the host.

Treatment with adenosine, KH_2PO_4, and K_2HPO_4 was also limited in effectiveness. Greater numbers of infecting organisms appeared to overwhelm both the host and any beneficial effect of the treatment, although significant reduction in the numbers of deaths could be noted on post-infection day 3 for both groups of rats treated with inorganic phosphates. This observation is consistent with the one noted when using

ATP and which is discussed above.

When 1 LD_{50} of P. tularensis was injected into rats, it was apparent that KH_2PO_4 was the more effective therapeutic agent in comparison to K_2HPO_4.

The administration of KH_2PO_4 occasionally produced unpredictable results. In one experiment it significantly protected the host against an inoculation of 350 organisms but failed to protect a group of rats against one-tenth this number of organisms. With still fewer numbers of P. tularensis, the effect of treatment with KH_2PO_4 could not be distinguished from the untreated controls.

It is interesting to note that the administration of creatine phosphate showed a highly significant reduction in mortality rate for infected rats, but this effect was nullified rapidly when treatment was withdrawn. This lends further credence to the belief that tularemia interferes with energy metabolism in the host and that administration of an energy source in the form of ATP or creatine phosphate benefits the host both by reducing the death rate and by alleviating macroscopic symptoms of tularemia. Infected animals treated with creatine phosphate exhibited no external appearance of infection until several hours before death. At this time, inactivity was the only noticeable symptom.

The administration of an energy source appears to be a requirement needed immediately after infection, since a 48

hour delay does not alter the death rate over a 10 day observation period. The favorable effect of treatment in this manner was evident immediately, but this was overcome as the disease progressed. Alleviation of tularemic symptoms was not observed. Similar results were obtained with infected animals when treatment with KH_2PO_4 was delayed only 24 hours.

The dosage of KH_2PO_4 used was 20 mg/rat/day which is the approximate amount of phosphate found in 30 mg of ATP. Higher amounts of KH_2PO_4 were without beneficial effect; likewise, it was found that amounts below 20 mg were without protective effect. Further evidence supporting the unpredictability of treatment with KH_2PO_4 was evident from an experiment in which no significant difference could be detected between infected controls and treated groups even though the LD_{50} was closely approached.

In other experiments the protective effect of KH_2PO_4 treatment was highly significant when compared to untreated controls. In Table XX, for example, a very highly significant difference is noted when KH_2PO_4 treatment is applied to rats employing fewer organisms. Attention is called to post-infection day 4 in which a definite reduction in death rate can be observed in the treated group in comparison to the untreated controls.

When greater numbers of organisms were used for infection, treatment with KH_2PO_4 failed to show any alteration

in the death ratio at the end of a ten day period or during any earlier period. In this regard it differed from ATP which at least delayed the time of death even when it failed to alter the final mortality ratio. Administration of K_2HPO_4 was lacking in protective effect for the host.

The effect of ATP was consistently more predictable than that of the inorganic phosphates tested. Evidence is presented, especially in experiments employing larger numbers of animals, which demonstrated the high degree of protection afforded by ATP. The data suggest that the energy offered must be in a readily available form, such as found in ATP, and not a potential source, such as glucose.

In order to determine whether or not treatment with phosphates altered the distribution of serum proteins in any manner which could explain protection of the host, a study was conducted employing serum electrophoretic techniques. It was found that treatment failed to alter serum protein fractions either in the distances in which they migrated or in their quantitative amounts per fraction. A significant decrease in serum albumin in tularemic animals was observed during the height of the infection. If this is compared with the increased amount of albumin excreted in the urine during the same period, it becomes evident that albuminuria can explain the decrease in serum albumin. In addition, energy metabolism is altered during this disease, and protein

synthesis is expected to be affected.

The significant increase in the beta globulin fraction during infection may be explained by the incorporation of immature antibodies (C. M. Downs, personal communication) into this portion of the serum. Attention is called to this fraction in the recovered animals. It may be noted that beta globulin in recovered animals returned to normal while there was a corresponding increase in gamma globulin. This is interpreted to mean that antibody now mature due to aging migrates with gamma globulin. The gamma globulin fraction remains high in recovered animals, and this may account for their immunity to subsequent attack of P. tularensis. This protection to re-infection may be due to the presence of antibodies which are known to migrate with this fraction.

Studies of serum alkaline phosphatase in infected rats revealed a highly significant decrease in this enzyme for the first two days. At a period corresponding to peak infection, this activity was at a normal level but again showed a decrease as the disease progressed.

Zinner and Ehrlich (1959) reported that a decrease in the alkaline serum phosphatase could be caused by damage on the cells of the tubules of the kidney and on its epithelial linings, both of which are carriers of alkaline renal phosphatase. An increase in alkaline serum phosphatase is observed in diseases associated with liver damage.

It would appear from the data presented that the kidneys are affected before the liver. In the infected group receiving treatment, liver damage, as indicated by an elevated serum alkaline phosphatase activity, occurred 24 hours later than in the infected controls. This may help to explain the protective effect of ATP. Also, it was observed that by post-infection day 12 the animals in the treated group had normal values; whereas, those not receiving treatment had values significantly below normal.

Studies were conducted to determine if phosphate treatment altered the amount of inorganic phosphorus found in the serum of treated animals. These results revealed that the inorganic phosphate found in the serum of normal rats was not significantly altered by a daily injection of 20 mg of KH_2PO_4 per animal. Treatment with ATP, on the other hand, showed a retention of phosphate as indicated by a slightly elevated serum inorganic phosphorus content when compared to the group treated with inorganic phosphate but not higher than normal values obtained from untreated rats. This implies that either the amount of test material was administered in amounts too small to cause a change or that a renal threshold for phosphate was operative.

When this analysis was conducted on infected animals treated with ATP and with KH_2PO_4, it was noted that the group treated with ATP failed to show an increase in serum

phosphate. This may be explained by assuming that ATP is rapidly removed from the serum and migrates to ATP-deficient centers, possibly the mitochondria of the liver. The group treated with KH_2PO_4, however, showed a cumulative effect of phosphorus in the serum, since an increase in inorganic phosphate was noted by post-infection day 4.

In a more recent experiment conducted with a carefully controlled temperature in the animal room, the serum inorganic phosphate content in infected rats and in infected rats receiving KH_2PO_4 revealed a significant increase 24 hours post-infection in both groups. The group treated with KH_2PO_4 did not show values as widely scattered from the normal values as did the infected controls. Nevertheless, the protective effect of KH_2PO_4 could still not be understood from an evaluation of serum inorganic phosphorus levels.

It is suggested that treatment with creatine phosphate may exert its protective effect not only by providing high energy bonds but also by stabilizing the inorganic phosphate content of the serum and maintaining these values close to those of normal controls. Inorganic phosphorus determinations in the infected group showed somewhat higher readings, but due to the limited number of animals tested and the high standard error, the significance of this observation was not clear. In the infected group receiving treatment, the standard error was much less, and this is evidence favoring

a stabilization of serum phosphorus due to treatment with a compound possessing high energy bonds. Survival studies revealed that creatine phosphate significantly reduced the mortality rate during the treatment period, but this was immediately followed by a collapse of the host after treatment was discontinued.

Since it has been well established that elevated serum acid phosphatase levels are obtained in neoplastic diseases, an attempt was made to study tularemia in rats using this approach. The activity of serum acid phosphatase in infected animals treated with ATP was not significantly different from those of normal controls. The activity of this enzyme in the infected controls showed a subnormal value on post-infection day 2, followed by a slight tendency to return to normal over a 10 day period. This may be explained by considering that while neoplasms are concomitant with proliferation of tissue, tularemia is attended by extensive tissue degeneration. Nevertheless, the suggestion is made that ATP supports acid phosphatase activity during infection.

In a further attempt to ascertain the fate of the phosphates injected, a study was conducted to determine the inorganic phosphate content of the urine. It appeared that in the infected animals receiving ATP, the urine inorganic phosphate content was quite stable and did not differ significantly from the normal controls. This further supports

the theory that ATP is bound in infected animals. The ATP injected was apparently not excreted in the urine since the phosphate content failed to show a steady increase following daily ATP injections. There is a possibility that the ATP administered was balanced by a similar quantity of ATP which was not synthesized due to the interference of energy metabolism by the microorganisms. In contrast, the infected group showed phosphorus levels with a wide range and with considerable fluctuations. Post-infection days 6 and 10, in particular, showed a significant increase in urine phosphate. This was not observed in the treated group and may be evidence indicating that ATP indirectly protects the integrity of kidney membranes.

There is also the possibility that the ATP administered is rapidly bound or utilized by the host which may have deficient amounts of this material during infection. This theory is supported by evidence that following ATP administration in infected animals, no cumulative effect could be noted in either the serum or urine reflected as an increase in inorganic phosphate, as previously discussed.

To determine if membrane damage in the kidneys actually existed which could account for phosphate leakage into the urine and also to explain the fate of albumin lost from the serum, an examination for albumin in the urine was conducted. Urine albumin reached a maximum at the height of

peak infection and was well correlated with the sharp decrease in serum albumin during the same period. This strongly suggests that the albumin lost from the serum was spilled into the urine. For this to occur, considerable kidney damage must first exist.

A study was made comparing the inorganic phosphorus content in the urine and the blood of normal rats, and normal rats receiving treatment. Serum inorganic phosphorus in normal rats treated daily with 30 mg of ATP remained at near normal values for the first 5 days, but showed a significant increase on days 6 and 7.

Several investigators including Berry *et al.* (1959) have reported that endotoxins produce an initial hyperglycemia followed by a fall in blood sugars to hypoglycemic levels.

It has been definitely established that phosphate clearance consistently parallels the blood glucose level whether the latter is elevated by glucose administration or is depressed by hypoglycemic agents (Huffman *et al.*, 1958).

These reports may explain the observed increase in the phosphorus content of the blood on the 6th and 7th days. Urine inorganic phosphorus analysis during the same period showed no apparent change, but attention is called to the fact that while serum inorganic phosphorus levels between animals treated on the same day are relatively constant and

usually have small standard errors, the opposite was frequently true for studies with urine.

When normal animals were treated with KH_2PO_4, on the other hand, the treatment was immediately evident by an increase in urine inorganic phosphorus excreted from day 1 through day 5.

To evaluate the possibility that any increase in serum inorganic phosphate during infection could be the result of an uncoupling of phosphorylation, a study was conducted using a known uncoupling agent. In addition, some of the treatments used for infected animals were used here for a comparison. It was found that rats treated daily with KH_2PO_4 following an initial injection of DNP were protected from death. Similar results were obtained with K_2HPO_4. The most dramatic protection occurred with the use of ATP; this is also true in rats infected with P. tularensis. The results showed the highest levels of significance for ATP, followed by KH_2PO_4, and then K_2HPO_4. This is also the order in which these compounds protect against tularemia in rats. This is further evidence that energy metabolism is disrupted and that an uncoupling of phosphorylation probably occurs. Furthermore, it shows that ATP is effective in protecting the host during this period. This theory is supported by the results of Takeda et al. (1955) who showed that ATP alleviated the effects of Shigella and Salmonella endotoxins administered

to rabbits. Further support is derived from the work of Dooley *et al.* (1958) who demonstrated similar effects in chicks treated with *Salmonella pullorum* endotoxin. In an *in vitro* investigation, Mager and Theodor (1957) found that endotoxin uncouples oxidative phosphorylation in mitochondria isolated from rat livers.

The host requires a readily available high energy source during the early stages of infection, and this suggests that one of the primary targets of tularemia in rats is an energy synthesizing center. That ATP protected animals treated with DNP further supports this contention.

SUMMARY

A depression in the energy metabolism of rats was demonstrated by the reduction in the fumarase activity in livers of infected animals.

Cortisone administered to infected animals alleviated disease symptoms and temporarily supported fumarase activity, but failed to alter the death rate.

The administration of ATP and creatine phosphate alleviated the disease symptoms of tularemic animals markedly and significantly reduced the mortality rate.

The administration of both the adenine and the adenosine portion of the ATP molecule was without beneficial effect to tularemic rats.

Inorganic phosphorus in the form of KH_2PO_4 was effective in protecting tularemic animals to a lesser degree than ATP or creatine phosphate. Furthermore, it was less reliable in exerting a protective effect.

All compounds used for treatment were without beneficial effect to the host if not given on the day of infection. Treatment was beneficial only when animals were infected with 1 LD_{50} of P. tularensis.

Treatment failed to alter the distribution of serum proteins as determined by paper strip electrophoresis, but the low level of serum albumin observed during the height of

the infection coincided with the marked albuminuria noted during the same period.

Rats were protected from the uncoupling effect of DNP by administration of ATP, KH_2PO_4, and K_2HPO_4. The effectiveness of these compounds was in the order named. This identical order of effectiveness was noted in infectivity experiments. It is suggested that tularemia in rats is caused, at least in part, by an uncoupling of phosphorylation. This is also consistent with the observation that there is an elevation of serum inorganic phosphorus in tularemia.

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