EXPERIMENTAL TULAREMIA IN WILD ANIMALS

by

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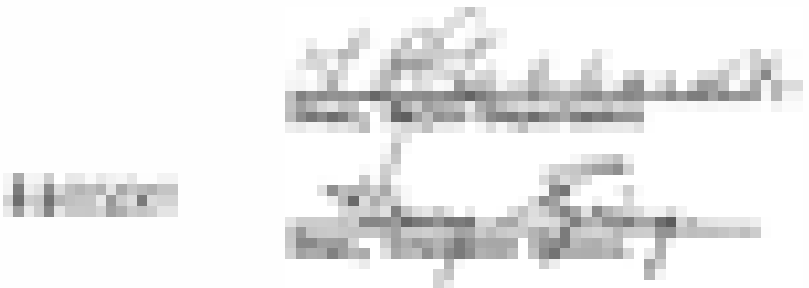

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INTRODUCTION

Recent studies on the ecology of disease agents enzootic in the Great Salt Lake Desert of Utah have demonstrated the presence of Pasteurella tularensis in the mammalian fauna. The first isolation of P. tularensis in this region was made in 1952 from ectoparasites of the jack rabbit, Lepus californicus*, the antelope ground squirrel, Citellus leucurus, and the deer mouse, Peromyscus maniculatus, (Woodbury and Parker, 1954). During the period of June, 1954 to the present time, isolations were made from a number of jack rabbits and jack rabbit ticks, Dermacentor parumapertus, (Stoenner, et al., 1959; and unpublished data). However, since the 1952 isolation of P. tularensis from ectoparasites, no further isolations have been made from rodent ectoparasites or rodent tissues, although thousands of specimens have been examined in the past eight years.

Under certain conditions, tularemia in North America can and does become epizootic in rodent populations (Kartman, et al., 1958; Jellison, Bell and Owen, 1959). The nature of the predisposing factors contributing to the initiation of an epizootic and the maintenance of the disease agent between epizootics is far from clear. One of the major objectives of this study is the elucidation of some of these factors, at least in the particular desert habitat under study.

The presence of enzootic tularemia in the rabbit - rabbit tick population in this region and the almost complete absence of the disease

* Scientific mammalian nomenclature follows Miller and Kellogg (1955).

from the extensively studied rodent fauna presents a puzzling situation. In studying the problem, it became desirable to define as nearly as possible the host-parasite relationship between P. tularensis and the native mammalian fauna of this region. Part of this report is the results of studies on the susceptibility of native rodents, rabbits and carnivores to experimental tularemia, and conversely, the virulence of strains of P. tularensis isolated from this and other areas.

There has been a great deal of work on the ecology of many of the zoonoses (diseases of wild animals transmissible to man) along the lines of classical ecology. The relationships between host and parasite, host and vector, and vector and parasite have been investigated for many diseases, exhaustively so for some. However, there have been few attempts to study the physiology of zoonotic agents and to use such information to better describe and understand their distribution and their relationship to vertebrate hosts and invertebrate vectors.

Part of the present study utilizes this latter approach in the study of the ecology of P. tularensis. From the data obtained by classical ecological studies and that obtained from physiological studies, an attempt is made to formulate a logical picture of the geographical and host distribution of P. tularensis.

LITERATURE REVIEW

I. Historical

Tularemia was first recognized as a specific disease entity by McCoy (1911) in California ground squirrels, Citellus beecheyi. It was described as a plague-like disease of rodents differing from classical plague primarily by the fact that the etiological agent could not be cultured on any of the ordinary culture media used at that time. A year later, however, McCoy and Chapin (1912) did isolate, on Dorset egg medium, an organism which they called Bacterium tularense, and subsequently proved it to be the etiological agent of the new disease. In the following two decades, tularemia was discovered in wild rodents throughout the United States.

In 1919, Francis (1919) showed that the disease known locally in Utah, Wyoming, Idaho, and Colorado as deer fly fever was actually tularemia. This disease has been endemic and epizootic in these four states at least since 1908 (Francis, 1922). The disease agent was apparently maintained in jack rabbits from which it was transmitted to humans by the blood-sucking fly, Chrysops discalis.

The first human case of tularemia in Canada was diagnosed from a varying hare, Lepus americanus columbiensis, and from ticks, Haemaphysalis leporis-palustris, infesting it (Parker, Hearle and Bruce, 1931).

Tovar (1944) reported the first bacteriologically confirmed case of tularemia in Mexico in 1944. In Alaska the first reported human case occurred in 1946 (Williams, 1946), although Philip and Parker (1938), much earlier, had isolated P. tularensis from H. leporis-palustris collected from a snowshoe hare.

According to Schmidt (1951), tularemia probably was first observed in Europe in 1917 in an Austrian. However, the first documented case was described in Russia in 1928. Thereafter, it has been reported from most of the countries of Europe and northern Africa. The earliest and/or most complete accounts were published from Belgium by Nelis (1950), from Finland by Gonroos (1939), from Sweden by Olin (1934), from Norway by Thjotta (1930), from Turkey by Huseyn (1937), and from Tunis by Anderson (1934).

The literature on tularemia in Russia is vast and difficult to obtain. No attempt will be made specifically to review it here other than to mention that the first isolation of P. tularensis in that country was from a human a human in 1926 (Sil'chenko, 1957).

Yato Byo (Ohara's Disease), as tularemia is known in Japan, was recognized as a distinct clinical entity in rabbits and humans in 1925 (Ohara, 1925; Francis and Moore, 1926).

Interestingly enough, tularemia has not been reported from tropical regions, nor from the southern hemisphere.

In the study area comprising a portion of the Great Salt Lake Basin of Utah, an epizootic among jack rabbits, Lepus californicus deserticola, was first reported by Francis (1921) to have occurred during July and August, 1920, in Millard County. Between 1952 and 1959, P. tularensis was isolated repeatedly from jack rabbits and their ectoparasites in Tooele County (Woodbury and Parker, 1954; Stoenner, et al., 1959). A tularemia epizootic among muskrats, Ondatra zibethica, occurred in the spring of 1950 along the shores of Utah Lake (Jellison, Kohls and Philip, 1951).

II. Natural Infection in Wild Animals

In 1945, Burroughs, et al., (1945) listed for North America, Asia, Europe and North Africa 48 vertebrates in which natural tularemia infection had been demonstrated. Today the list has grown to include 57 vertebrates in North America alone (Table 1), and it is growing longer all the time.

III. Wild Animal Susceptibility

Morgan (1947) reported six species of fresh water fish (black bullhead, black crappie, large-mouth bass, northern pike, yellow perch, and rainbow trout) to be completely refractory to experimental infection with P. tularensis. On the other hand, certain amphibians can be experimentally infected. The infection in Rana esculenta (frog) and Bufo viridis (toad) tends to become chronic or latent (Novikova and Lalazarov, 1940), whereas in R. ridibunda it is acute and results in death without formation of macroscopic lesions (Bilal, 1939). It was shown also that infected R. ridibunda shed P. tularensis into the water through their excretions and other frogs could thus become infected in this way. This may also explain some instances of P. tularensis-contaminated water.

Among the Reptilia, only the turtle has been tested for susceptibility to tularemia. Intraperitoneal inoculation of virulent P. tularensis was fatal to young animals, but caused a chronic or latent infection in adults (Bilal, 1939a). In this particular study, viable P. tularensis was isolated from the liver of an adult turtle 45 days after inoculation. In addition, it was found that the turtle tick, Hyaloma aegyptium, could transmit P. tularensis from infected to healthy turtles.

TABLE 1

Vertebrates of North America which have been shown to be naturally infected with tularemia. Where more than one published record exists for a given species, the earliest and/or most complete description only is cited.

Species	Common name	Published record	Diagnosis
AVES			
Bonasa umbellus togata	Ruffed grouse	Green and Shillinger, 1932	culture
Pedioecetes phasianellus	Sharp-tailed grouse	Green and Shillinger, 1932	culture
Centrocercus urophasianus	Sage hen	Parker, Philip & Davis, 1932	culture
Colinus virginianus	Bob-white quail	Green and Wade, 1929	culture
Bubo virginianus	Horned owl	Green and Evans, 1938*	culture
Buteo jamaicensis calurus	Red-tailed hawk	Nakamura, 1950	culture
Larus pipixican	Franklin gull	Ozburn, 1944	culture **
MARSUPALIA			
Didelphis marsupialis	Opossum	McKeever, et al., 1958	serology
MAMMALIA			
Sorex vagrans monticola	Wandering shrew	Kohls and Steinhaus, 1943	culture
Lepus americanus	Varying hare	Green and Shillinger, 1934*	culture
L. californicus	Black-tailed jack rabbit	Francis, 1921	culture
L. townsendii	White-tailed jack rabbit	Bow and Brown, 1943	culture
Sylvilagus floridanus	Cottontail rabbit	Wherry, 1914	culture
S. idahoensis	Pigmy rabbit	Bacon and Drake, 1958	serology
Marmota flaviventris	Marmot	Menges and Galton, 1959	serology
Citellus townsendii (mollis)	Piute ground squirrel	Francis, 1921	culture
C. richardsonia elegans	Wyoming ground squirrel	Burroughs, et al., 1945	culture
C. columbianus columbianus	Columbian ground squirrel	Burroughs, et al., 1945	culture
C. beecheyi	Beechey ground squirrel	McCoy and Chapin, 1912	culture
C. armatus	Uinta ground squirrel	Nakamura, 1950	culture
C. fisheri		Simons, et al., 1953	culture
C. douglasii		Simons, et al., 1953	culture
Sciurus niger	Fox squirrel	McKeever, et al., 1958	serology
Eutamias sp.	Chipmunk	Parker, 1945	culture
E. minimus	Least chipmunk	Nakamura, 1950a	culture
Tamiasciurus hudsonius ventorum	White River pine squirrel	Nakamura, 1950a	culture
Castor canadensis	Beaver	Hammersland & Joneschild, 1940	culture

TABLE 1 (continued)

Species	Common name	Published record	Diagnosis
MAMMALIA (cont'd)			
Peromyscus maniculatus rubidus	Redwoods white-footed mouse	Burroughs, et al., 1945	culture
P. m. artemesiae	White-footed mouse	Ozburn, 1944	culture**
P. m. osgoodi	Osgood white-footed mouse	Ozburn, 1944	culture**
P. m. borealis	White-footed mouse	Humphreys and Campbell, 1947	culture
Zapus princeps	Jumping mouse	Nakamura, 1950a	culture
Mus musculus	House mouse	Humphreys and Campbell, 1947	culture
Neotoma fuscipes	Wood rat	Burroughs, et al., 1945	culture
N. albigula	Wood rat	Ecke and Holdenried, 1952	culture
Microtus pennsylvanicus	Meadow mouse	Jellison, et al., 1942	culture
M. p. modestus	Sawatch meadow mouse	Kohls and Steinhaus, 1943	culture
M. californicus aestuarinus	Tule meadow mouse	Perry, 1928	culture
M. montanus	Montane meadow mouse	Kartman, et al., 1958	culture
M. p. drummondi	Drummond meadow mouse	Ozburn, 1944	culture**
Dipodomys sp.	Kangaroo rat	Kartman, et al., 1958	culture
Ondatra zibethica	Muskrat	Green and Shillinger, 1934	culture
Rattus norvegicus	Norway rat	Dieter and Rhodes, 1926	culture
Erethizon dorsatum epizanthum	Porcupine	Nakamura, 1950a	culture
Urocyon cenereoargenteus	Gray fox	Schlotthauer, et al., 1955	culture
Vulpes fulva	Red fox	McKeever, et al., 1958	serology
Mustela vison	Mink	Nakamura, 1950a	culture
Taxidea taxus taxus	Badger	Nakamura, 1950a	culture
Procyon lotor	Raccoon	McKeever, et al., 1958	serology
Mephistis mephistis	Striped skunk	McKeever, et al., 1958	serology
Spilogale putorius	Spotted skunk	McKeever, et al., 1958	serology
Felis rufa	Wild cat	McKeever, et al., 1958	serology
F. domestica	Feral house cat	McKeever, et al., 1958	serology
Canis latrans lestes	Coyote	Philip, et al., 1955	serology
C. familiaris	Domestic dog	Ey and Daniels, 1941	serology
Ovies aries	Domestic sheep	Parker and Dade, 1929	culture
Bos taurus	Domestic cow	Burroughs, et al., 1945	culture
Equus caballos	Domestic horse	Claus, et al., 1959	culture

* Cited by Burroughs, et al., 1945

** See footnote in Burroughs, et al., 1945, p. 118

Among birds the Hungarian partridge, Perdix perdix perdix; ruffed grouse, Bonasa umbellus togata; and sharp-tailed grouse, Pedioecetes phasianellus, have been reported to be susceptible (Green and Wade, 1928, 1928a; Green and Shillinger, 1932), although Theiler (1928) has questioned the validity and reliability of some of these experimental results. Bob white quail, Colinus virginianus, apparently are highly susceptible (Green and Wade, 1929; Parker, 1929), whereas the ring-necked pheasant Phasianus colchicus tarquatus, and pigeons, are relatively resistant (Green, Wade and Kelly, 1928; Green and Wade, 1928). Chickens appear to be completely resistant (Green and Wade, 1928; Theiler, 1928).

With the exception of wild and laboratory strains of Rattus norvegicus, which are relatively resistant (McCoy, 1911; Dieter and Rhodes, 1926; Downs, et al., 1946), all North American rodents tested have proved to be very susceptible to experimental tularemia. McCoy's (1911) initial experiments showed that gophers, Thomomys bottae, were lethally susceptible to parenteral inoculation of P. tularensis. McCoy and Chapin (1912), a year later, demonstrated the susceptibility of desert ground squirrels, Ammospermophilus citellus leucurus, to high doses of organisms administered via the subcutaneous and the oral routes. In rather limited experiments, Stagg, Tanner and Lavender (1956) showed that deer mice, P. maniculatus sonoriensis, and kangaroo rats, D. ordii and D. microps, were very susceptible to tularemia infection administered by both the subcutaneous and the respiratory routes. In addition, antelope ground squirrels, C. leucurus leucurus, and long-tailed pocket mice, Perognathus formosus incolatus, were shown to be very susceptible to respiratory infection. Rapidly fatal infections were produced by parenteral inoculation of P. tularensis into

muskrats, O. zibethicus (Green, Wade and Dewey, 1929), white-tailed prairie dogs, Cynomys leucurus (Davis, 1935), beavers, Castor canadensis (Green, 1937), and montane meadow mice, M. montanus (Kartman, Prince and Quan, 1958).

Although rodents are undoubtedly lethally susceptible to parenterally administered virulent P. tularensis, they seem to be less susceptible to infection by the oral route (Quan, 1954; Dunaeva and Olsuf'ev, 1951; Tereschenko, 1954). The potential role that carnivorism and cannibalism may play in the transmission of tularemia among rodents has been demonstrated by Vest and Marchette (1958).

Cottontail rabbits, Sylvilagus spp., are extremely susceptible to natural and experimental tularemia infection (Bell and Green, 1939; Bell and Chalgren, 1943; Jellison and Parker, 1945; Yeatter and Thompson, 1952; McGinnes, 1958).

On the basis of extensive epizoological evidence, Bell and Green (1939) considered the snowshoe hare, L. americanus, to be "usually highly resistant" compared to cottontails. They reported tularemia in the snowshoe hare to be a symptomless infection characterized by chronic focal lesions. Also, a high proportion of hares trapped in highly enzootic and epizootic areas possessed agglutinins against P. tularensis, indicating recovery from infection (Green, Larson and Bell, 1938; Bell and Green, 1939). The natural occurrence of tularemia agglutinins in cottontails is extremely rare. The only published reports are those of McKeever, et al., (1958), and Bacon and Drake (1958).

Stagg, Tanner and Lavender (1956) induced fatal tularemia infection in jack rabbits, L. californicus, by exposure to an aerosol of virulent

P. tularensis. Philip, Bell and Larson (1955) killed four jack rabbits with subcutaneous inoculations of approximately 10^6 viable organisms, but two rabbits inoculated with approximately 10^2 organisms survived. However, these same authors suggest that the general lack of agglutinins against P. tularensis in jack rabbit sera collected in enzootic and epizootic areas indicates that no rabbits survive natural infection with tularemia. This thesis is supported by Lechleitner (1959) who could find no tularemia agglutinins in jack rabbits in California. However, Bacon and Drake (1958) reported finding tularemia agglutinins at a titer of 1/320 in two jack rabbits from which no organisms could be isolated.

Investigating an epizootic in gray foxes, Urocyon cinereoargentatus, Schlotthauer, Thompson and Olson (1955) found a number dead or dying of tularemia, and suggested that this proved them to be susceptible. Lillie and Francis (1936) reported red foxes, Vulpes fulvus, to be at least partially susceptible to per os and subcutaneous infection, and Olsuf'ev and Dunaeva (1951) reported the Russian species to be completely refractory, as is the skunk, and to a lesser extent the weasel. Mink, Mustela vison, were shown to contract fatal infections by feeding on infective rabbit carcasses (Gorham, 1949: Shmuter and Abramova, 1956).

Coyote pups were shown by Parker and Francis (1926) to be lethally susceptible to massive oral infection. However, Stagg, Tanner and Lavender (1956) found coyote pups to develop non-lethal, subacute infections after exposure to aerosols; and Lundgren, Marchette and Smart (1957) found a similar situation in adults exposed to massive doses of P. tularensis by both oral and subcutaneous routes.

Dogs appear to be almost entirely immune (Green, 1942; Ey and Daniels, 1941; Johnson, 1944; McCoy, 1911; McCoy and Chapin, 1912); although Downs, et al., (1946) were able to infect them by inoculation of a highly virulent strain, and in general a good immunological response was elicited. Cats possess a high degree of resistance (McCoy, 1911; McCoy and Chapin, 1912), but it is possible to induce fatal infections by feeding them infected meat (Downs, et al., 1946; Green and Wade, 1928).

Calhoun, Mohr and Alford (1956) demonstrated the presence of tularemia agglutinins in the sera of raccoons and opossums, indicating recovery of these animals from natural infections.

In farm animals, tularemia has been demonstrated most frequently in sheep (Gwatkin, Painter and Moynihan, 1942; Jellison and Kohls, 1955; McArthur and Brown, 1950; Parker and Butler, 1929; Parker and Dade, 1929, 1929a; Philip, Jellison and Wilkins, 1935). In all cases the infection in bands of sheep is associated with infected ticks. Rodents and/or rabbits usually are found dead of tularemia in the regions through which the sheep have grazed. In general, the younger sheep, lambs and yearlings, are much more susceptible to natural tularemia infection than are the older members of a band (Jellison and Kohls, 1955).

Cattle appear to be not as often involved in tularemia epizootics as do sheep, and they are, in general, much less susceptible to natural infection, (Geiger, 1931; Vail and McKenny, 1943; Calhoun, 1954).

While nothing is known about tularemia infection in hogs, Bell (1944) has reported a case of tularemia in man which was definitely associated with these animals.

Finally, Kamil and Golem (1938) have reported that the water buffalo develops an acute infection after inoculation with virulent P. tularensis.

IV. Virulence and Variation

The virulence of wild and laboratory strains of P. tularensis has been investigated by a number of workers. Strains of lowered virulence have been isolated in nature from ticks (Davis, Philip and Parker 1934; Philip and Davis, 1935), snowshoe hares (Bell and Green, 1939), mice (Humphreys and Campbell, 1947), meadow mice (Jellison, Bell and Owen, 1959), and from a number of other rodents, rabbits and carnivores (Nakamura, 1950a)*. Green (1943) observed that natural passage of P. tularensis through birds tended to lower its virulence. Ransmeier (1943) obtained similar results by serial passage of a virulent strain through chick embryos. However, Davis (1940) demonstrated that mere persistence of the organism in ticks for as long as 701 days did not decrease its virulence.

The results of the recent comparison of virulent American and Russian strains of P. tularensis by Olsuf'ev (1959) are of considerable interest. Tularemia in Russia appears to be primarily a disease of rodents, with rabbits seldom, if ever, involved. The Russian strains of P. tularensis are avirulent for white rabbits. The American strains, of course, are extremely virulent for white rabbits. In North America, tularemia is "primarily" a disease of wild rabbits, although rodents are often implicated. Another interesting observation (originally suggested by Francis, 1942) is that the Russian strains do not ferment glycerin, whereas the vast majority of American strains do. However, there seems to be no correlation between the ability to ferment glycerin and virulence.

* There is some question that the organism isolated from these animals was actually P. tularensis. The published description of the organism, its isolation, and method of identification leave a great deal to be desired.

This apparently is an inherent difference between the Old World and the New World strains of P. tularensis. This is reminiscent of the classical metabolic differences in strains of P. pestis from different geographical areas (Pollitzer, 1954). Olsuf'ev (1959) suggested changing the name of P. tularensis to Francissella tularensis tularensis for the New World strain and to F. t. palearctica for the Old World strain. There is a great deal of merit in this suggestion.

Using laboratory strains of P. tularensis, Foshay (1932) was able to alter the virulence of a highly virulent strain by passage on coagulated egg-yolk media. The virulence of various other strains has been modified by serial passage through animals and subculture on various artificial media (Bell, Owen and Larson, 1955; Owen, Bell, Larson and Ormsbee, 1955; Shertinsky, 1951; Yaniv and Avi-Dor, 1953; Tomaszunas, 1959; Mashkov, 1958). The tendency of antibiotics to lower the virulence of P. tularensis was demonstrated by Yaniv, Avi-Dor and Olitski (1953). The development of resistance to streptomycin in vitro tended to decrease the virulence of the strain. However, it is possible that avirulent cells in the population were naturally more resistant to streptomycin and were thus selected. That the latter explanation might be the more correct one is suggested by the experiments of Chapman, et al., (1949), in which they were able to isolate resistant strains from mice treated with subcurative doses of streptomycin. Since the avirulent cells do not multiply in vivo, they could not be recovered. Only fully virulent, non-streptomycin resistant cells were recovered.

Eigelsbach, Braun and Herring (1951), Avi-Dor and Yaniv (1953), and Moody and Downs (1955) were able to correlate virulence with colonial morphology; electrokinetics of cell walls, growth rates, inhibition of growth of

virulent cells by avirulent cells, immunogenic potency and/or sensitivity to antibiotics. However, Owen, Larson and Ormsbee (1955) were unable to verify the above work in every detail in their laboratory.

Using visible light microscopy, Hesselbrock and Foshay (1945) could find no morphological features differentiating virulent from avirulent forms of P. tularensis. They discount the experiments of Ohara, Kobashi and Kudo (1935) which indicated a positive correlation between pleomorphism, "motility", and virulence. On the other hand, Ribbi (personal communication) has been able to correlate virulence with cell wall structure as shown by the electron microscope. This difference between the cell wall structure of virulent and avirulent cells is definitely not determined by the age of the culture when it is sampled, although there is a change in cell wall characteristics with age (Ribbi and Shepard, 1955).

The ability of P. tularensis to grow and multiply in vivo has been investigated by Downs, Buchele and Edgar (1949), and Yudenich (1956). They showed that the invasiveness and distribution of the organism in the infected body did not depend upon the virulence of the culture. The behavior of the attenuated strains varied in degree, but not in kind, from virulent strains.

Fleming and Foshay (1955) have demonstrated that the highly virulent Schu, Scherm, and Holt strains of P. tularensis possess a citrulline ureidase enzyme system capable of degrading citrulline to ornithine, CO_2 and NH_3. This system is lacking in the low virulent Jap_4 and SMR-1 strains and the avirulent 38 and 176 strains. This is the only qualitative metabolic difference between the strains of different virulence that

has been found to date. However, the relationship of this enzyme system to virulence is completely unknown. Indeed, its relationship to the overall metabolism of the organism is obscure. Fleming and Foshay (1955) were unable to demonstrate degradation of ornithine; and no arginine desimidase system was demonstrable, although most other bacteria capable of degrading citrulline to ornithine, CO_2 and NH_3 contain this enzyme (Knivett, 1952; Korzenovsky and Werkman, 1952; Oginsky and Gehrig, 1952; Schmidt, Logan and Tytell, 1952; Slade, 1953). However, arginine is required for the growth of P. tularensis in defined media (Traub, Mager and Grossowicz, 1955).

On a quantitative basis, virulent strains of P. tularensis were shown to possess more glutaminase activity than low or avirulent strains (Fleming and Foshay, 1955). Conversely, avirulent strain 38 possessed higher aspartic acid alanine transaminase activity than did the virulent Schu strain (Fleming and Foshay, 1956).

MATERIALS AND METHODS

I. Cultures

The 18 strains of P. tularensis* used in this study are listed in Table 2. All stock cultures were grown on glucose cysteine blood agar (GCBA) and stored at 4°C with routine monthly transfers to fresh media. Suspensions for the inoculation of animals were prepared either from 48-hour aerated cultures in modified casein partial hydrolysate (MCPH) broth (Mills, et al., 1949; Mills, 1954), or from a 24-48-hour GCBA culture. The 48-hour MCPH cultures regularly contained $1x10^9$ to $5x10^9$ viable organisms per ml, as determined by viable counts, using the method recommended by Snyder (1947).

The GCBA cultures were washed down with normal saline (0.85% NaCl) and adjusted to 24% light transmittance on a Bausch and Lomb Spectronic 20 spectrophotometer set at 525 mu. These suspensions regularly contained $1x10^9$ to $5x10^9$ viable organisms per ml.

Serial 10-fold dilutions were routinely made in normal saline containing 0.2% gelatine. Viable counts were always made immediately prior to animal inoculation.

In certain cases, described in a later section, broth cultures were also prepared in Snyder's peptone media (Snyder, et al., 1946).

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* The Schu A, Jap_4, 38, and NIIEG strains were obtained originally from Dr. H. T. Eigelsbach, Fort Detrick, Md.; the 425 F_4G and Kf-473 strains were supplied by Dr. Cora Owen of the Rocky Mountain Laboratory, Hamilton, Montana; H-8859 was obtained from Dr. K. D. Claus, Veterinary Research Laboratory, Bozeman, Mont.; DPG-1, 2, 3, 4, 5, and 6 were obtained from Dr. H. G. Stoenner of the Rocky Mountain Laboratory, Hamilton, Mont., who originally isolated them from rabbits and rabbit ticks collected in Utah; SKV-1, 2 and 3 were isolated by the author; 9K-161 was furnished by the Epizoology Laboratory, University of Utah.

TABLE 2

Strains of *Pasteurella tularensis* used in this study

Strain	Source	Location	Isolated by	Date of Isolation	Pertinent Reference
Schu A	Human	U. S.	Foshay	1941	Bell, Owen and Larson, 1955
Jap_4	Human	Japan	Ohara	1926	Moody and Downs, 1955
38	Human	U. S.	Francis	1920	Hesselbrock and Foshay, 1945
425 F_4G	*Dermacentor andersoni*	U. S.	RML *		Bell, Owen and Larson, 1955
Kf-473	*Microtus montanus*	Oregon	RML	1957	Jellison, Bell and Owen, 1959
H-8859	Horse	Montana	Claus	1958	Claus, Newhall and Mee, 1959
NIIEG-BLUE	?	Russia	?	?	Anon. 1944-46; Fairbich and Rumarina, 1946
NIIEG-GRAY	?	Russia	?	?	Ibid.
DPG-1	*D. parumapertus*	Utah	Stoenner	1956	Stoenner, et al., 1959
DPG-2	*L. californicus*	Utah	Stoenner	1956	Ibid.
DPG-3	*D. parumapertus*	Utah	Stoenner	1956	Ibid.
DPG-4	*L. californicus*	Utah	Stoenner	1956	Ibid.
DPG-5	*D. parumapertus*	Utah	Stoenner	1956	Ibid.
DPG-6	*D. parumapertus*	Utah	Stoenner	1956	Ibid.
SKV-1	*Sylvilagus audubonii*	Utah	Ecol. Res. Laboratory	1958	Ecol. Res. Ann. Rept. 1958-59
SKV-2	*L. californicus*	Utah	Ecol. Res.	1958	Ibid.
SKV-3	*L. californicus*	Utah	Ecol. Res.	1959	Ecol. Research Ann. Rept., 1959-60
9K-161	Jack rabbit ticks	Utah	Ecol. Res.	1959	Ibid.

* Rocky Mountain Laboratory, Hamilton, Montana

II. Experimental Animals

The following five rodent species* used in this study were reared by Mr. Harold J. Egoscue in the Ecological Research Faunal Colony (Egoscue, 1956); Peromyscus maniculatus sonoriensis (Le Conte), deer mouse; P. crinitus pergracilis Goldman, canyon mouse; Microtus montanus nexus Hall and Hayward, Montane meadow mouse; Neotoma lepida lepida Thomas, desert wood rat (Egoscue, 1957); Onychomys leucogaster utahensis Goldman, Northern grasshopper mouse (Egoscue, 1960). The animals used were 2-4 generations removed from the wild parent stock.

Six additional rodent species ** were live-trapped by Mr. John B. Bushman and associates, of Ecological Research Laboratories; Citellus leucurus leucurus Merriam, antelope ground squirrel; Eutamias minimus pictus (Allen), least chipmunk; Perognathus formosus incolatus Hall, long-tailed pocket mouse; Dipodomys ordii utahensis (Merriam), Ord kangaroo rat; D. microps bonnevillei Goldman, chisel-toothed kangaroo rat; Reithrodontomys megalotis megalotis (Baird, Western harvest mouse.

Audubon cottontails, Sylvilagus audubonii arizonae (Allen), and black-tailed jack rabbits, Lepus californicus deserticola Mearns, were also live-trapped by Mr. Bushman and associates.

Jack rabbits of the subspecies L. c. texianus (Waterhouse) were obtained from the Conrad D. Durant Animal Co., Ft. Sumner, New Mexico.

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* All mammal identifications are based upon descriptions of Durrant, (1952).

** The subspecific identifications may not be accurate in all cases, for they are often based primarily on the published range of each (Durrant, 1952; Shippee and Egoscue, 1958).

Coyotes, Canis latrans lestes Merriam, were supplied as pups by the U. S. Fish and Wildlife Service, Predator Control Unit. They were used when 6-12 weeks old.

All animals obtained by live-trapping were held in quarantine for at least two weeks before being used.

III. Techniques

A. Wild Animal Susceptibility

Experimental Infection: The standard laboratory strain of P. tularensis, Schu A, was used to determine the susceptibility of the 15 species of wild mammals listed above.

Some of the coyotes were exposed to tularemia by allowing them to feed on infective rodent carcasses. All other animals, and the remainder of the coyote pups, were inoculated subcutaneously in the flank or in the cervical region, with 0.2 or 0.5 ml of an appropriate suspension.

After inoculation, deer mice and canyon mice were caged in groups of 2-10 animals per cage. Animals of all other species were caged individually. The exposed rodents and rabbits were observed daily for 14-21 days depending upon the estimated susceptibility as determined by preliminary experiments. Carnivores were observed for periods up to thirteen weeks following exposure to experimental tularemia infection. Deaths

were recorded daily. All carcasses were autopsied and spleens cultured on GCBA. Routinely, all animals surviving the observation period were bled for serological study, autopsied, and the spleen of each cultured on GCBA.

All bacterial growth obtained from spleen cultures on GCBA were examined and the identification of P. tularensis made on the basis of colonial morphology, reaction of the organisms with the Gram stain, and with specific high titer tularemia antiserum. The antiserum was produced against the homologous strain in goats. The antiserum with an agglutinin titer of 1/1280 was diluted 1/3 with normal saline when used to identify cultures of suspect organisms.

Animals dying within 24 hours after inoculation were discarded and are not included in the results. Likewise, only animals dying of proven tularemia infection are included.

In addition to the above procedures, the carnivores were bled at intervals and aliquots inoculated intraperitoneally into deer mice and onto GCBA plates for the detection of a bacteremic condition. Serum was also collected for serological examination.

At autopsy of all carnivores, portions of the maxillary gland, spleen, liver, kidney, mesenteric lymph gland, lung, heart muscle, bone marrow and brain were homogenized in a mortar with sterile sand, suspended in normal saline, and inoculated intraperitoneally into normal deer mice and onto GCBA plates.

Standard tube agglutination tests were carried out on all sera, using formalin-killed P. tularensis strain Schu A cells suspended in normal saline with 0.5% phenol as a preservative. The suspension was

adjusted to 40% light transmittance at 525 mu in a Bausch and Lomb Spectronic 20 spectrophotometer.

Complement fixation tests were carried out on some serum samples, using a polysaccharide extract of Schu A cells (Nicholes, 1946; Alexander, Wright and Baldwin, 1950) supplied by Dr. Nicholes.

B. Virulence of enzootic and laboratory strains of P. tularensis

The virulence of various P. tularensis strains was tested by subcutaneous inoculation of aliquots of serial dilutions of fresh GCBA, MCPH, or peptone broth culture into one or more species of laboratory or wild animal. The ability to kill or to produce a detectable infection as determined by serological or skin-testing techniques were the criteria used to assess the virulence of each strain. Whenever possible, the 50% lethal dose or organisms for one or more species was calculated, using the method of probit analysis (Finney, 1952).

C. Assay of Citrulline Ureidase Enzyme Activity

Cultures of P. tularensis were prepared in MCPH or Snyder's peptone broth at pH 6.8. After 48 hours incubation at 35-37°C on a reciprocating shaker, the cultures were checked for purity by microscopic examination, washed twice in normal saline and suspended in minimal amounts of de-ionized water. The cell suspensions were then lysed by sonic vibration in a Ratheon Sonic Oscillator, Model S-102 (50 watt, 9 Kc/s). The cell lysates were kept at deep freeze temperature (-20°C) until used.

Bacterial nitrogen determinations were made on each lysate according to the method of Lang (1958).

Citrulline ureidase enzyme activity of sonic lysates was assayed by measuring the amount or ornithine produced from the breakdown of citrulline. The reaction mixture containing an aliquot of sonic lysate containing 1.50 mg bacterial nitorgen and DL-Citrulline was buffered at pH 6.5 with phosphate buffer and incubated at 30°C according to the procedure described by Fleming and Foshay (1956). Aliquots were removed at various intervals, applied to washed Whatman No. 1 filter paper and chromatogrammed at room temperature in n-butanol-pyridine-water solvent system for 3-4 hours, followed by drying for 20 minutes at 65°C. The chromatograms were sprayed with 0.5% alkaline solution of ninhydrin in 71% ethyl alcohol and the optical density of the solution determined at 575 mu in a Spectronic 20 spectrophotometer according to the method of Kay, et al., (1956).

The amount of ornithine produced was calculated by referring to a previously determined standard curve relating uM of ornithine to optical density of the eluates.

EXPERIMENTAL RESULTS

I. Susceptibility of Wild Animals to Experimental Tularemia

A. Contact Transmission of P. tularensis

Natural tularemia is generally transmitted by arthropod vectors, primarily ticks, although other biting or blood sucking arthropods may at times be implicated. Occasionally other modes of transmission assume local importance, e. g., ingesting of infective flesh or contaminated water. Although wild animals are susceptible to experimental respiratory infection with P. tularensis (Stagg, et al., 1956), it is very doubtful if this mode of infection is of any significance in nature. Nonetheless, it has to be considered when non-infected animals are held in contact with, or near, experimentally infected animals in the laboratory.

The early attempts of McCoy (1911) to transmit tularemia to guinea pigs by placing them in contact with infected squirrels were not successful. However, in a more recent paper, Owen and Buker (1956) demonstrated contact transmission of tularemia from experimentally infected white mice to healthy cage mates.

In order to investigate the possibility that contact tularemia transmission might occur among caged deer mouse populations, in the present study varying proportions of infected and non-infected mice (total of eight per cage) were confined in standard mouse cages. The non-infected mice were marked with dyes to distinguish them from their infected cage mates. Although the laboratory-reared deer mice used were almost completely free of ectoparasite infestation, they were at first dusted with rotenone powder to rule out transmission by vectors. This was

not done in later experiments. In no case was it possible to demonstrate transmission of tularemia by contact of infected mice with healthy cage mates, even in experiments in which respiratory infections were induced in the "infected mice" by intranasal instillation of P. tularensis broth cultures. To investigate this problem further, the experiments outlined below were conducted.

A cylindrical wire cage 8 x 10 inches, containing six healthy deer mice, was placed inside a larger wire and metal cage (24 x 24 x 24 inches), containing 12 experimentally infected mice. Thus, there could be no cannibalism of infected mice by non-infected mice. The inoculated mice were removed as soon as possible after death, which occurred by the 4th or 5th day. The healthy mice were checked daily for 18 days and dead animals removed and autopsied. On the third day, 7 of the 12 inoculated mice, and one of the six uninoculated mice were dead. The latter had been almost completely devoured by its cage mates and no attempt was made to diagnose the dause of death. However, on the 8th day, three more uninoculated mice were dead and a positive diagnosis of death by tularemia was made in each case. These presumably had contracted infection by ingestion of the first dead mouse, thus indicating that the first mouse had contracted tularemia from the inoculated mice in the outer cage. However, the possibility exists that the last three mice actually contracted tularemia from the inoculated mice.

This experiment was repeated three times but no additional uninoculated mice contracted tularemia from their infected cage mates.

In the second experiment, 16 healthy deer mice were placed in a wire and metal cage (24 x 24 x 24 inches) and allowed a week to establish themselves. Then, every day for 60 days, one healthy deer mouse and one marked mouse, inoculated intraperitoneally with a lethal dose of P. tularensis, strain Schu A, were added to the cage. Food and water were provided ad libidum. The inoculated deer mice died 2-4 days after being placed in the cage. Their carcasses were removed as soon after death as possible. On the 20th day the cage contained 34 uninoculated mice, and one dead and three live inoculated mice. The dead mouse had been almost completely devoured. Four days later, uninoculated mice began dying of tularemia infection and deaths were recorded over a period of 18 days. Evidence of cannibalism of the carcasses was observed throughout this period. From the 42nd day through the 60th day no cannibalism was noted, and no uninoculated mide died after the 46th day. On the 61st day, cannibalism was again noted and on the 66th day one uninoculated mouse was found dead of tularemia.

This experiment was repeated, adding three healthy and three inoculated deer mice per day, five days per week, with essentially the same results. In both cases, death of uninoculated mice from tularemia occurred only after evidence of cannibalism of infected mouse carcasses was noted. Although the cage population in the second experiment reached 80 mice on the 65th day, it was not possible to demonstrate tularemia transmission by any means other than by cannibalism.

Several experiments were also conducted in which infected and non-infected deer mice were placed together in varying proportions in arti-

cial burrows constructed in damp sand. Again, in no case was it possible to demonstrate transmission by contact.

All mice surviving these esperiments were challenged with low doses of virulent P. tularensis (Schu A) to determine if they may have acquired a non-fatal infection and recovered. In no case was the survival rate of these mice different from that of normal mice controls inoculated with the same doses of P. tularensis.

The results of these experiments indicate that under laboratory conditions, contact transmission of tularemia from infected deer mice to healthy cage mates does not readily occur. The single case of death of an uninoculated mouse in contact with infected cage mates, described in the first experiment above, was the only instance of apparent transmission by contact.

B. Susceptibility of Wild Rodents to Experimental Tularemia.

Eleven species of wild rodents were tested for susceptibility to experimental infection with the highly virulent Schu A strain of P. tularensis. The organisms were administered subcutaneously as described previously. All rodents tested were extremely susceptible to infection with this strain and readily succumbed with massive invasion of all tissues by the organism, Table 3. Only one animal out of 145 survived inoculation of 500 organisms; 18 out of 169 survived inoculation of 50 organisms. There is no indication that any of the species tested is more or less susceptible than any other. There was no indication of difference in susceptibility due to sex.

TABLE 3

Susceptibility of wild rodents to subcutaneous tularemia infection (*Pasteurella tularensis* strain Schu A). The data are presented as the ratio of the number of deaths in a 14-day period to the total number of animals used.

Species	Number of Organisms Inoculated (+10%)				
	5000	500	50	5	0.5
*Citellus leucurus**					
Antelope ground squirrel	8/8	8/8	8/16	8/16	0/8
Eutamias minimus					
Least chipmunk		8/8	7/7	3/8	
*Perognathus formosus**					
Long-tailed pocket mouse	8/8	8/8	14/15	15/16	8/8
Dipodomys ordii					
Ord kangaroo rat		16/16	16/16	11/11	9/16
D. microps					
Chisel-toothed kangaroo rat		8/8	8/8	2/8	
Reithrodontomys megalotis					
Western harvest mouse			8/8	7/8	0/8
Peromyscus crinitus					
Canyon mouse		8/8	8/8	4/8	
P. maniculatus *					
Deer mouse	10/10	50/50	52/56	47/62	10/38
*Onychomys leucogaster**					
Northern grasshopper mouse		8/8	8/8	13/16	5/16
Neotoma lepida *					
Desert wood rat	10/10	22/23	14/19	20/27	0/16
Microtus montanus					
Montane meadow mouse	8/8	8/8	8/8	5/8	

* Combined results of two or more experiments

Fifty-four per cent of the rodents dying from acute tularemia infection were dead by the 4th day after inoculation; 97% were dead by the 7th day. The size of the dose (in the range given) appeared to have little effect upon the course of infection and the time of death. However, in other experiments in which much larger doses were administered, most of the animals died between the second and fourth day after inoculation.

In no case was there any pathological, cultural, or serological evidence that any of the 14-day survivors had become infected and recovered, or continued to harbor the organism in a latent state. In the rodents tested, infection with the highly virulent Schu A strain appeared invariably to result in death. The animals surviving these experiments represent those receiving no viable organisms.

Five species of wild rodents were tested for susceptibility to the low virulent Jap_4 strain of P. tularensis, Table 4. The grasshopper mouse was found to be fatally susceptible to infection with this strain (LD_{50} 12.3 organisms), whereas both species of wood rats tested survived doses as high as 2×10^8 viable cells. The LD_{50} in kangaroo rats and deer mice was between these two extremes (1.4×10^5 and 7.4×10^6, respectively).

In contrast to infection with strain Schu A, rodents exposed to Jap_4 strain reacted much more slowly. Only nine per cent of those dying over a 21-day period were dead by the 5th day, 61% by the 9th day, 88% by the 13th day, and 97% by the 17th day after inoculation.

While none of the rodents tested survived proven infection with the Schu A strain, many recovered from acute or subacute infection with Jap_4 strain. This was demonstrated by positive skin reactions elicited by survivors tested with specific antigen (Ecological Research Annual Report 1957-58), and/or by the presence of strong agglutinating and complement fixing antibody titers (Tables 5 and 6).

TABLE 4

Susceptibility of five species of wild rodents to subcutaneous tularemia infection (*Pasteurella tularensis* strain Jap_4). Data are presented as the ratio of the number of deaths at 21 days to the total number of animals used. LD_{50} and 95% confidence limits were calculated by the method of probit analysis (Finney, 1952).

	Number of Organisms (± 10%)									LD_{50} and 95% Confidence limits
	$5x10^8$	$5x10^7$	$5x10^6$	$5x10^5$	$5x10^4$	$5x10^3$	$5x10^2$	$5x10^1$	$5x10^0$	
Dipodomys ordii Ord kangaroo rat			9/10	5/9	2/10	3/8	1/8			$1.4x10^5$ ($2.0x10^4$ to $3.4x10^6$)
*Peromyscus maniculatus** Deer mouse	7/16	10/14	29/53	28/54	20/69	8/54	7/42			$7.4x10^6$ ($1.6x10^6$ to $1.7x10^8$)
*Onychomys leucogaster** Grasshopper mouse			18/18	15/18	19/28	22/29	16/25	8/10	3/9	12.3 (1.0 to $3.2x10^3$)
Neotoma lepida Desert wood rat	0/16	0/16	0/16	0/8	0/8					$5x10^8$
N. cinerea Bushy-tailed wood rat	0/3			0/3						$5x10^8$

* Combined results of two or more experiments.

TABLE 5

Agglutinating antibody titers of pooled serum samples from rodents surviving infection with the Jap_4 strain of Pasteurella tularensis. Titers shown as the reciprocal of the highest serum dilution agglutinating standard P. tularensis antigen to +2 or greater.

	Number of organisms originally inoculated							
Rodent	$5x10^8$	$5x10^7$	$5x10^6$	$5x10^5$	$5x10^4$	$5x10^3$	$5x10^2$	$5x10^1$
Dipodomys ordii			(1)*	(4)	(8)	(5)	(7)	
Kangaroo rat			320	1280	640	640	320	
Peromyscus maniculatus**			(6)	(6)	(8)	(9)	(10)	
Deer mouse			1280	320	1280	2560	640	
	(8)		(4)	(7)	(8)			
	640		160	640	1280			
Onychomys leucogaster			(3)	(3)	(3)	(5)		
Grasshopper mouse			320	neg	320	160		
				(6)	(1)	(4)	(2)	(6)
				160	80	320	160	320
Neotoma lepida	(8)	(8)	(8)	(8)	(8)			
Desert wood rat	320	160	160	160	160			
	(8)	(8)	(8)					
	320	160	160					

* Numbers in parentheses indicate number of serum samples in pool tested.
** Serology done in two experiments only.

TABLE 6

Complement fixing antibody titers of pooled serum samples from rodents surviving infection with the Jap_4 strain of Pasteurella tularensis. Titers shown as the reciprocal of the highest serum dilution showing 50% lysis of sensitized sheep red blood cell suspension.

	Number of organisms originally inoculated							
Rodent	$5x10^8$	$5x10^7$	$5x10^6$	$5x10^5$	$5x10^4$	$5x10^3$	$5x10^2$	$5x10^1$
Dipodomys ordii			(1)*	(4)	(8)	(5)	(7)	
Kangaroo rat			640	40	160	320	320	
Peromyscus maniculatus**			(6)	(6)	(8)	(9)	(10)	
Deer mouse			640	320	320	1280	1280	
Onychomys leucogaster			(3)	(3)	(6)	(5)		
Grasshopper mouse			640	160	320	40		
				(6)	(1)	(4)	(2)	(6)
				70	640	640	640	320
Neotoma lepida	(8)	(8)	(8)	(8)	(8)			
Desert wood rat	20	20	40	neg	40			
	(8)	(8)	(8)					
	160	20	20					

* Numbers in parentheses indicate number of serum samples in pool tested.
** Serology done in one experiment only.

Table 7 indicates the susceptibility of grasshopper mice, wood rats, and/or deer mice to an additional 13 wild strains and 3 laboratory strains of P. tularensis. These results were established using 30-60 deer mice and 6-20 wood rats or grasshopper mice per strain. Inoculation was by the subcutaneous route. It is clear that these species are lethally susceptible to all the naturally isolated rodent, rabbit, rabbit tick, and horse strains. Strain 38, which is typically avirulent for all animals in which it has been tested, and NIIEG (gray variant) failed to kill any of the rodents tested, even when given in massive doses. The NIIEG (blue variant) resembled the Jap_4 strain in its virulence for deer mice.

TABLE 7

Susceptibility of deer mice, wood rats and grasshopper mice to sixteen laboratory and wild strains of Pasteurella tularensis.

Strain of Pasteurella tularensis	Number of organisms necessary to kill at least 50% of the animals tested		
	Deer mouse	Wood rat	Grasshopper mouse
38	$>10^9$	$>10^9$	$>10^9$
425 F_4G	1	1	1
Kf-473	1	1	1
SKV-1	1	1	
SKV-2	1	1	1
SKV-3	1		
H-8859	1		
DPG-1	1		
DPG-2	1		
DPG-3	1		
DPG-4	1		
DPG-5	1		
DPG-6	1		
9K-161	1		
NIIEG (Blue variant)	1.2×10^3 (1.0 to 4.2×10)*	10^5	
NIIEG (Gray variant)	$>10^8$	$>10^5$	

* LD_{50} and 95% confidence limits calculated by method of probit analysis (Finney 1952).

C. Susceptibility of Wild Rabbits to Experimental Tularemia

Cottontails, Sylvilagus audubonii, and jack rabbits of the subspecies Lepus californicus deserticola, that were tested were lethally susceptible to low doses of P. tularensis strain Schu A, Table 8. Deaths occurred over a 4-12 day period after inoculation. On the other hand, in one experiment, jack rabbits of the subspecies L. c. texianus (from New Mexico) resisted tularemia infection. All six inoculated with $5x10^4$ organisms died of tularemia infection, but only four out of 20 animals inoculated with doses of P. tularensis ranging from 1 to $1x10^4$ viable cells died of tularemia. All of the 16 surviving rabbits had negative agglutinin titers 21 days after inoculation. However, the two rabbits surviving inoculation of 10,000 cells each had a complement fixing antibody titer of 1/160; the one surviving the 1,000 cell inoculum had a titer of 1/640; the one surviving the 100 cell inoculum had a titer of 1/160; one of the five surviving the 50 cell inoculum had a titer of 1/80; the other four had no demonstrable titer. The rabbits that had received less than 50 viable cells had no demonstrable complement fixing antibody titer. No P. tularensis was isolated from any of these rabbits at autopsy 21 days after inoculation.

TABLE 8

Susceptibility of wild rabbits to subcutaneous tularemia infection. Data are presented as the ratio of the number of deaths at 21 days to the total number of animals used

Species	50,000	10,000	5,000	1,000	500	100	50	5
Sylvilagus audubonii								
Audubon cottontail					4/4		6/6	5/6
Lepus californicus deserticola								
Black-tailed jack rabbit			5/5				3/3	
L. c. texianus								
Black-tailed jack rabbit	6/6	1/3		1/2	2/3		0/5	0/7

D. Susceptibility of Coyote Pups to Experimental Tularemia Infection.

Three groups of coyote pups were used in this series of experiments:

Group 1 consisted of 4 pups, 2-4 months old
Group 2 consisted of 8 pups, 2-5 months old
Group 3 consisted of 7 pups, 1-2 months old

All of these animals were given 2-3 ml of canine distemper antiserum (Fort Dodge Laboratory, Inc., Ft. Dodge, Iowa; or Pitman and Moore, Indianapolis, Ind.), within a day or two after capture. Groups 1 and 2 were obtained and used in the spring of 1957; Group 3 in the spring of 1958. They were all held for 1-2 weeks before exposure to experimental infection. P. tularensis, Schu A, was used exclusively in these experiments.

Group 1: Two of the four pups were inoculated subcutaneously with 1.3×10^6 fully virulent organisms; and two were fed a total of 2,200 grams of infective rodent carcasses over a 14-day period. All the carcasses were of rodents that had been inoculated with lethal doses of P. tularensis and had died within a short time before being fed to the pups.

Approximately 2.0 ml of blood were taken from each pup at 0, 1, 2, 3 and 4 weeks after exposure, and for an additional two weeks from the two which were fed infective rodent carcasses. Approximately 0.2 ml of each blood sample was inoculated intraperitoneally into each of the three deer mice, 0.2 plated on GCBA, and the remainder centrifuged to obtain serum for agglutinating antibody determinations. At the end of

four weeks after their last exposure to tularemia infection, the pups were anesthetized by intravenous injection of 200 mg of nembutal (sodium pentobarbital), exsanguinated by severance of the carotid artery, and autopsied.

Significant agglutination antibody titers appeared one week after subcutaneous infection and two weeks after oral infection, Table 9. Titers of 1/80 and 1/160 persisted until all the animals were sacrificed at 4-6 weeks. No P. tularensis was isolated from the blood of any of these animals. All the tissues were sterile when tested 4-6 weeks after initial exposure to infection.

Group 2: In an attempt to repeat the above experiment, and verify the results, eight more coyote pups were obtained and tested. One pup was inoculated subcutaneously with 1.5×10^6 organisms, and one fed 100 grams of infective rodent carcasses. Blood was drawn at 0, 9, 10, 11, 12 and 13 weeks for determination of agglutinating antibody titers.

Antibody persisted in low titer for 11-13 weeks, Table 9. No bacteremia could be demonstrated. The pup receiving subcutaneous inoculation presumably had been exposed previously to tularemia, as evidenced by its pre-inoculation agglutinating antibody titer of 1/80. At autopsy, 13 weeks after exposure to experimental tularemia, all tissues examined were sterile.

Of the six remaining pups in this group, two were inoculated subcutaneously with 1.5×10^3 viable organisms; one with 1.5×10^6 organisms;

TABLE 9

Agglutinating antibody response of six coyote pups (Groups 1 and 2) exposed to tularemia by the oral or subcutaneous route. Titers are shown as the reciprocal of the highest serum dilution having a +2 or higher agglutination

Weeks after Exposure	Group No. 1				Group No. 2*	
	Inoc. with 1.3×10^6 organisms	Inoc. with 1.3×10^6 organisms	Fed 2,200 gms infected carcasses	Fed 2,200 gms infected carcasses	Inoc. with 1.5×10^6 organisms	Fed 100 gms infected carcasses
0	neg	neg	neg	neg	80	neg
1	40	40	neg	neg		neg
2	160	160	40	320		
3	320	160	160	640		
4	80	80	160	160		
5			80	160		
6					20	80
9					20	40
10					neg	80
11					neg	40
12					neg	neg
13						

* Six pups from this group contracted distemper and died. The two that did not contract distemper are presented here.

one was fed 100 grams of infective rodent carcasses; and two were fed 330 grams of infective rodent carcasses, over a four-day period. Within 4-5 days after exposure, all six pups appeared to be suffering from an acute infection. However, the symptoms did not resemble those commonly exhibited by other wild animals suffering from acute tularemia. There was a general lassitude, refusal of food, excessive lachrymation and nasal discharge, followed by apparent central nervous system impairment expressed as a partial paralysis of the hind limbs. The animals died within 2-3 days after these symptoms were noticed. At autopsy, the internal organs appeared normal and there was no gross pathological picture of tularemia.

Since the clinical symptoms suggested canine distemper, samples of brain, bladder, kidney, liver, and spleen were preserved in 10% formalin and sent to Dr. A. F. Alexander at Colorado A and M College, Boulder, Colo., for examination. Based on the clinical symptoms and the finding of inclusion bodies in the brain, the cause of death was diagnosed as canine distemper. It was also possible to culture P. tularensis from the spleen, liver, kidney, mesenteric lymph gland, and brain of the pups infected orally; and from the spleen, liver, and brain of the subcutaneously infected pups.

Group 3: The seven coyote pups in this group were 1-2 months younger than those in the other two groups previously used. Three were inoculated subcutaneously with 1.7×10^9 organisms. One of these died on the third day after inoculation; one died on the 7th day; and the third lived until sacrificed 33 days after inoculation. P. tularensis was

readily isolated from the heart blood and various organs of each of the pups that died. The tissues of the surviving pup were all sterile at autopsy 33 days after inoculation.

Attempts to detect P. tularensis in the blood stream at 3, 5, 6, 10, 12 and 14 days were successful in only one case. On the third day after inoculation, P. tularensis was isolated from the pup which subsequently died on the 7th day.

The antibody response of the pup surviving the infection is shown in Table 10. Measurable agglutination antibody appeared by the sixth day and developed to a peak between the 10th and 17th day, after which it diminished slightly. The complement-fixing antibody was nor measurable until the 10th day, after which it was at a maximum titer on the 17th day (1/1024) and persisted at a high level until the 33rd day, when the animal was killed.

A fourth pup in this group was inoculated subcutaneously with 1.5×10^7 organisms. It failed to develop a bacteremic condition at 2, 3, or 6 days after inoculation. All tissues were sterile at autopsy 28 days after inoculation. The antibody response, Table 10, was similar to that elicited by the previous pup inoculated with 1.7×10^9 organisms.

The final three pups (age approximately 2 months) in this group were each fed tularemia-infected deer mice (approximately 60 grams of infective flesh). No bacteremic condition was detectable at 3, 5, 6, 10, 12 or 14 days after exposure to oral tularemia infection. At autospy on the 28th-33rd day, all tissues were sterile. The antibody response, Table 10, was similar to that in the previously described pups.

TABLE 10

Agglutinating and complement fixing antibody titers of five coyote pups (Group 3 exposed to subcutaneous or oral infection with Pasteurella tularensis (Schu A). Titers shown as reciprocal of highest serum dilution giving a positive reaction (+2 agglutination or 50% hemolysis).

Days after Exposure	Dose and route of infection: Subcutaneous 1.7x10 Agg.	Subcutaneous 1.7x10 CF	Subcutaneous 1.5x10 Agg.	Subcutaneous 1.5x10 CF	Per Os 60 grams* Agg.	Per Os 60 grams* CF	Per Os 60 grams* Agg.	Per Os 60 grams* CF	Per Os 60 grams* Agg.	Per Os 60 grams* CF
0	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
3	neg	neg	neg	neg	neg	neg	20		neg	neg
6	80	neg	160	16	neg	neg	160	neg	20	neg
8			160	64						
10	1280	256	320	128		256		128	320	64
12	1280	512	640		160	512	320	128	640	128
14	1280	512			160	256	1280	512	2560	512
17	1280	1024	320	512	320	256	640	1024	1280	256
20	320	512	640	512	320	256	320	512	640	128
24			320	512	80	256	640	256	640	128
28	320	512	320	1024	160	128	640	256	640	256
33					160	64			640	256

* Infective rodent carcasses

II. Virulence and Citrulline Ureidase Enzyme Activity of Labora- and Wild Strains of Pasteurella tularensis.

The presence of citrulline ureidase enzyme activity in P. tularensis strains of high virulence, and its absence in strains of low or no virulence was reported by Fleming and Foshay (1955). This was the first reported qualitative physiological difference between strains of high and low virulence. However, the relationship between this enzyme system and virulence is still obscure.

In the present study the citrulline ureidase system was used initially as a tool, along with virulence titrations in rodents and rabbits, to further characterize and compare strains of P. tularensis isolated from nature with laboratory strains of known virulence and pathogenicity. Thirteen wild strains of P. tularensis were compared with five laboratory strains on the basis of virulence for deer mice, rabbits and/or guinea pigs, and the presence of citrulline ureidase activity, Table 11. The virulence titrations were done, using 30-60 deer mice, 2-6 rabbits, and/or 4-40 guinea pigs for each strain tested. The number of deaths occurring over a 21-day period was recorded, and where possible LD_{50} determinations were made according to the method of Finney (1952).

According to the studies of Bell, Owen and Larson (1955), the virulence of tularemia strains can be determined most accurately by estimation of the number of organisms administered subcutaneously which are necessary to cause 50-100% mortality of mice, guinea pigs, and rabbits. On this basis, it is evident from Table 11 that the Schu A strain and all the wild isolates except strains Kf-473 and 425 F_4G, are fully virulent --

very low numbers kill all three species. Strains Kf-473 and 425 F_4G are moderately virulent -- very few organisms are required to kill mice, but 10^4 or higher are required to kill guinea pigs and rabbits. Strains Jap_4 and NIIEG (blue variant) are of low virulence. They are avirulent for rabbits, but can kill guinea pigs with high doses, and mice with somewhat lower doses. Strains 38 and NIIEG (gray variant) are completely avirulent for all three species.

Only those strains fully virulent for all three species possessed any citrulline ureidase enzyme activity, Table 11.

During the qualitative analysis of citrulline ureidase activity in these 18 strains of P. tularensis, it became evident that there were further quantitative differences among them. Consequently the enzyme activity in lysates of each strain was quantitated as previously described by determining the amount of ornithine produced from a standard amount of citrulline per hour per mg of bacterial nitrogen in the lysate. The results of these determinations are shown graphically in Figs. 1, 2, and 3. In each figure, strains Schu A and H-8859 are plotted for comparative purposes. It is quite evident from these results that two distinct populations of P. tularensis exist on the basis of citrulline ureidase enzyme activity. Strains Schu A and H 8859 showed the greatest activity, reaching equilibrium after approximately 2-4 hours incubation at 30°C. The activity of all the DPG, SKV, and 9K-161 strains was very low. Equilibrium was not reached until 48 hours or longer. The activity in the latter 10 strains was nearly identical.

TABLE 11

Virulence and citrulline ureidase activity of five laboratory and thirteen wild strains of *Pasteurella tularensis*

Strain	Virulence*			Citrulline ureidase activity**
	Deer mice	Guinea pigs	Rabbits	
Laboratory Strains				
Schu A	1	1	1	+
Jap_4	10^6	10^8	$>10^9$	-
38	10^9	$>10^9$	$>10^9$	-
NIIEG (blue variant)	10^3		$>10^9$	-
NIIEG (gray variant)	$>10^8$	$>10^9$	$>10^9$	-
Wild Strains				
425 F_4G	1	10^5	10^9	-
Kf-473	1	1	10^4	-
H-8859	1	1	1	+
DPG-1	1	1	1	+
DPG-2	1			+
DPG-3	1			+
DPG-4	1	1	1	+
DPG-5	1	1	1	+
DPG-6	1	1	1	+
SKV-1	1	1	1	+
SKV-2	1			+
SKV-3	1			+
9K-161	1	1	1	+

* Approximate numbers of subcutaneously inoculated organisms necessary to kill 50-100% of the animals indicated.

** Activity assayed qualitatively by demonstration of the enzymatic breakdown of citrulline to ornithine by sonic lysates during 2-4 hours incubation at 30°C. (-) indicates no sonic activity.

When citrulline ureidase activity of different concentrations of Schu A lysate (in terms of bacterial nitrogen) was determined, it was found that Schu A lysate containing 0.24 mg N were comparable to lysates of the less active strains containing 1.50 mg N, Fig. 4. In other words, the difference in activity of these strains can be explained by the lower concentrations of enzyme in the less active strains. The Schu A lysates were approximately 6-fold more active in the breakdown of cit-

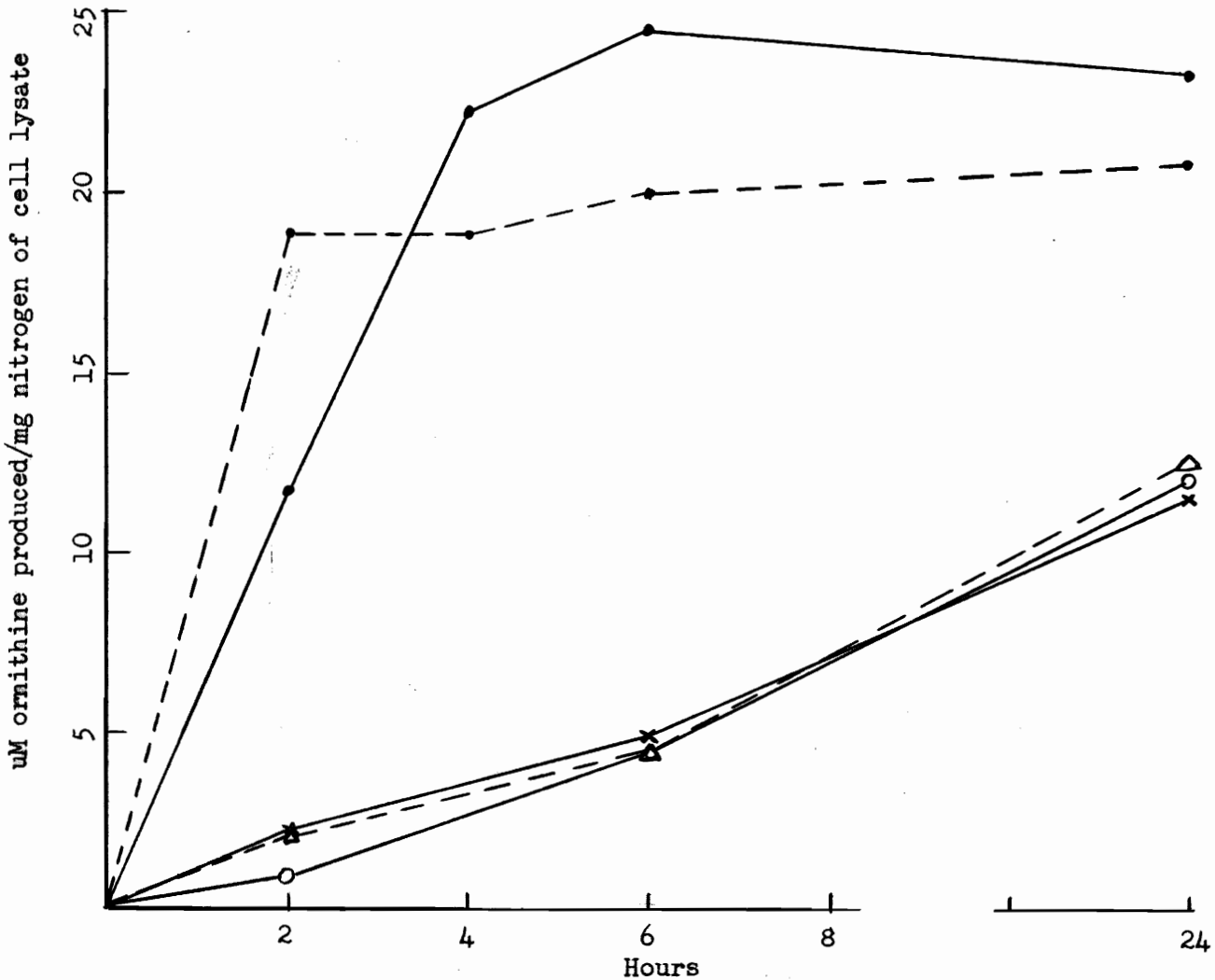

Figure 1. Citrulline ureidase enzyme activity of five strains of P. tularensis. Schu A•—•; H-8859•- - -•; DPG-1×—×; DPG-2△—△; DPG-3○—○.

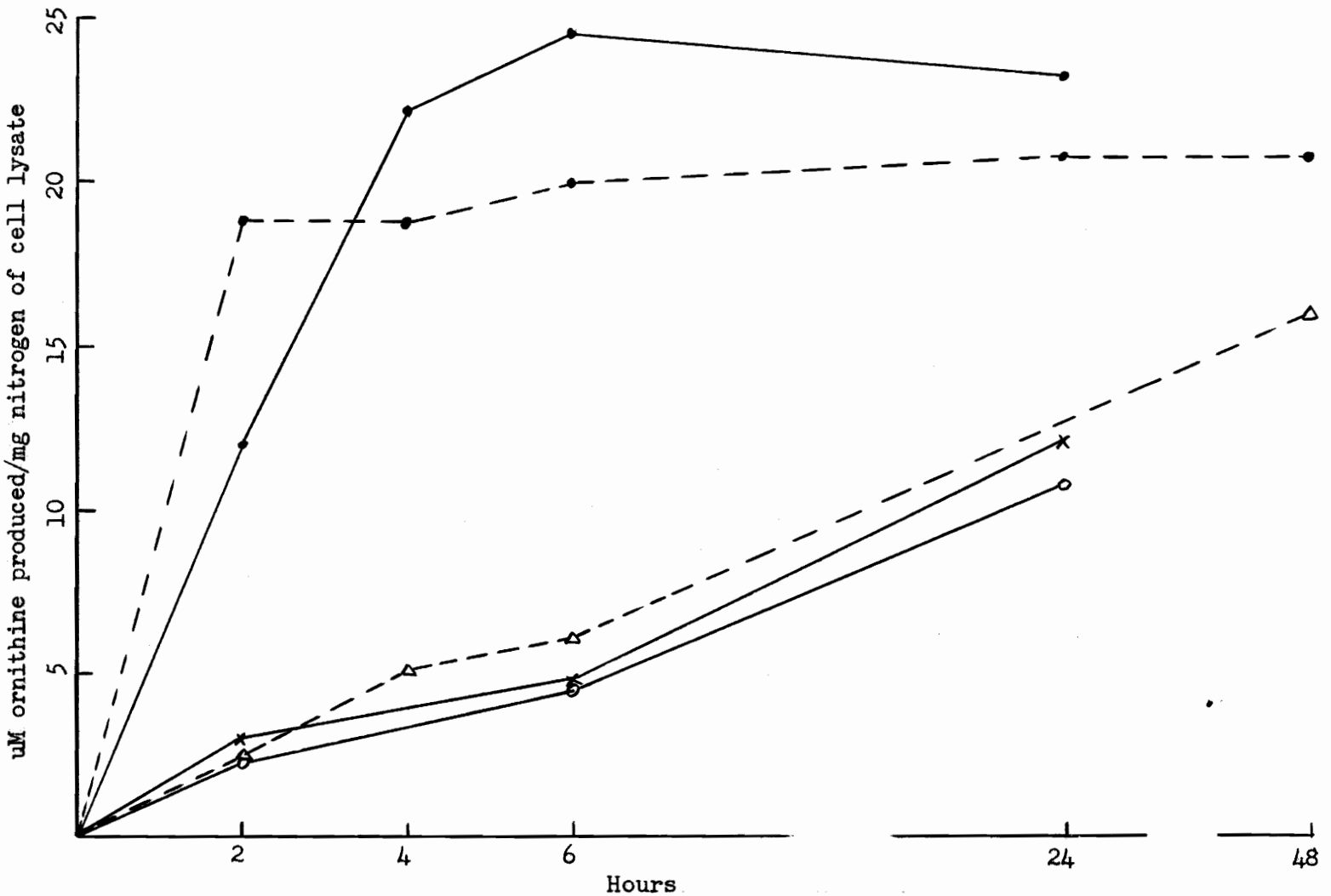

Figure 2. Citrulline ureidase enzyme activity of five strains of Pasteurella tularensis. Schu A●——●; H-8859●- - -●; DPG-4×——×; DPG-5△- -△; DPG-6○——○.

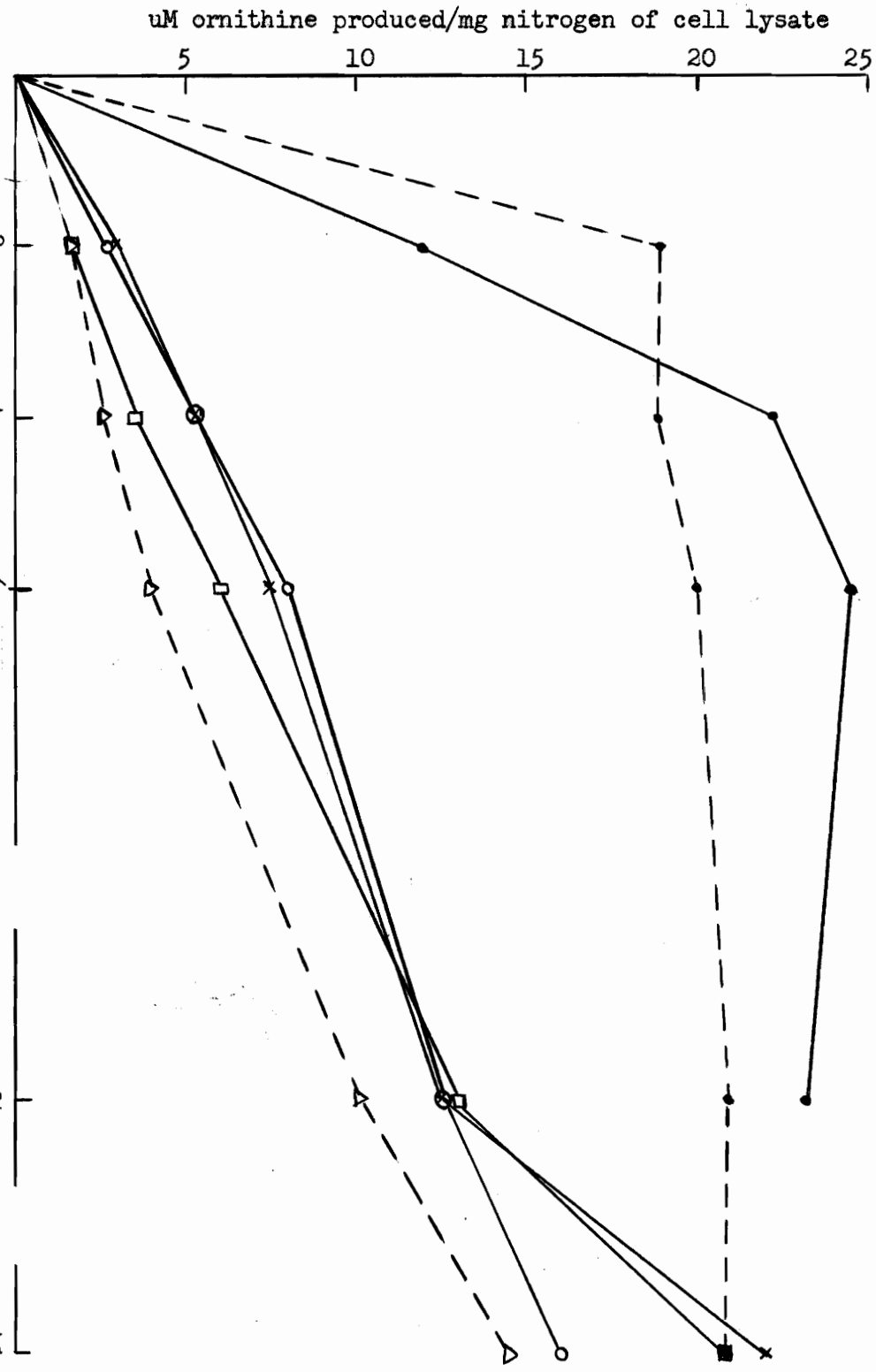

Figure 3. Citrulline ureidase enzyme activity of six strains of P. tularensis. Schu A●——●, H-8859●- - -●, SKV-1 ×——×, SKV-2 ◭-◭, SKV-3 ○——○, 9K-161□——□.

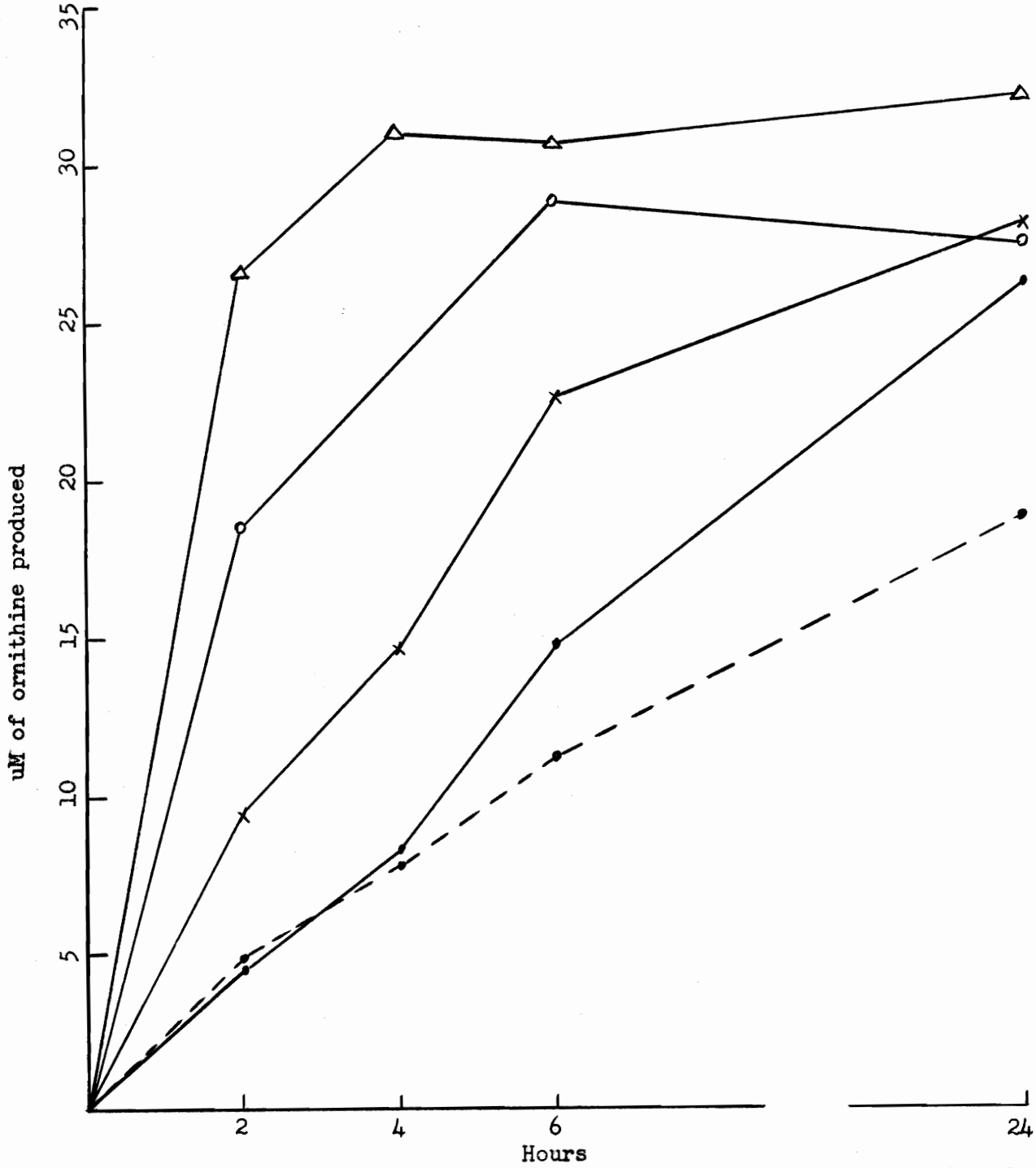

Figure 4. Ornithine production from a standard amount of citrulline (52uM) as a function nitrogen concentration of Schu A cell lysate compared to strain SKV-1 with a nitrogen concentration of 150 mg. This strain is taken as representative of all the SKV, DPG, and 9K-161 strains. Schu 1.92 mg N△—△, Schu 0.96 mg N○—○. Schu 0.48 mg N×—×, Schu 0.24 mg N•—•, SKV-1•- -•.

rulline to ornithine than were the lysates of the other strains under the standard set of conditions used.

In order to further characterize and compare the citrulline ureidase enzyme system in the different strains, the Michaelis Menten equilibrium constant (Km) was determined for 3 strains, Schu A, H-8859, and 9K-161, from the Lineweaver-Burk plots (Lineweaver and Burk, 1934) according to the following equation:

$$1/v = (K/V)(1/P) + (1/V)$$

where, v is the rate of the reaction
V is the maximum velocity
P is the amount of product formed at maximum velocity
K is the equilibrium constant

The production of ornithine from varying concentrations of citrulline was determined at 15-minute intervals under the standard conditions already described, except that the concentration of lysate used was 2.50 mg of bacterial nitrogen instead of 1.50 mg N. The rate of reaction was measured for each citrulline concentration by plotting the production of ornithine against time and calculating the velocity of the reaction along the steepest part of the curve (maximum velocity). Thus:

$$v = \frac{P_1 - P_2}{t_2 - t_1}$$

where, P_1 is the amount of ornithine produced at time$_1$; and
P_2 is the amount produced at t_2

When the reciprocal of the velocity is plotted against the reciprocal of the product formed, the intercept of the line on the 1/v axis is 1/V, and the slope of the line is K/V. Thus, from the above equation: K = (slope) (V).

The reciprocal plots for Strains Schu A, H-8859, and 9K-161 are shown in Figs. 5, 6, and 7 respectively. From these data:

$$K_{\text{Schu A}} = 1.55 \times 10^{-6} M$$
$$K_{\text{H-8859}} = 2.00 \times 10^{-6} M$$
$$K_{\text{9K-161}} = 1.60 \times 10^{-4} M$$

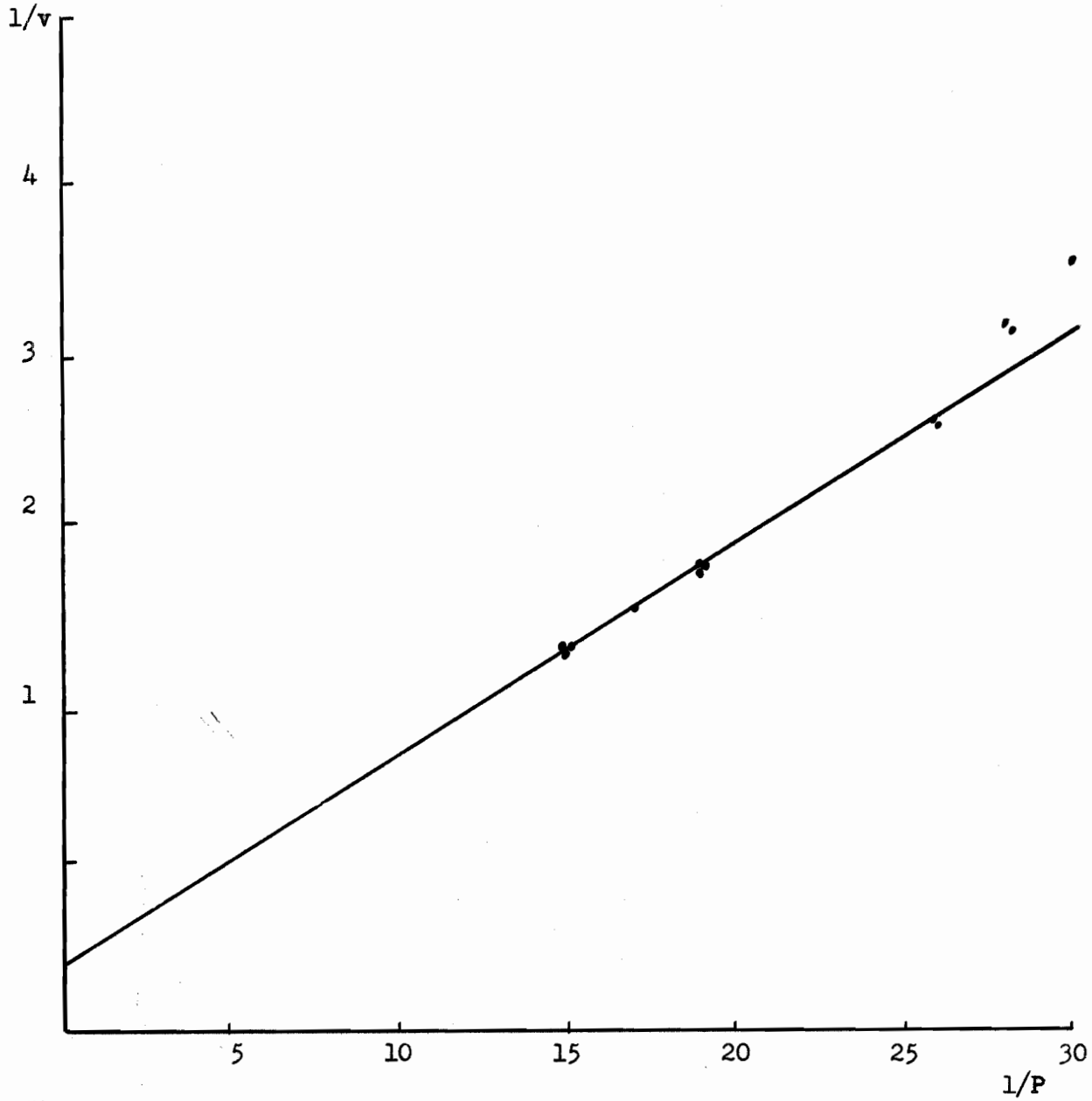

Figure 5. Reciprocal plot for the calculation of the equilibrium constant (K_m) for the citrulline ureidase enzyme system of P. tularensis strain Schu A.

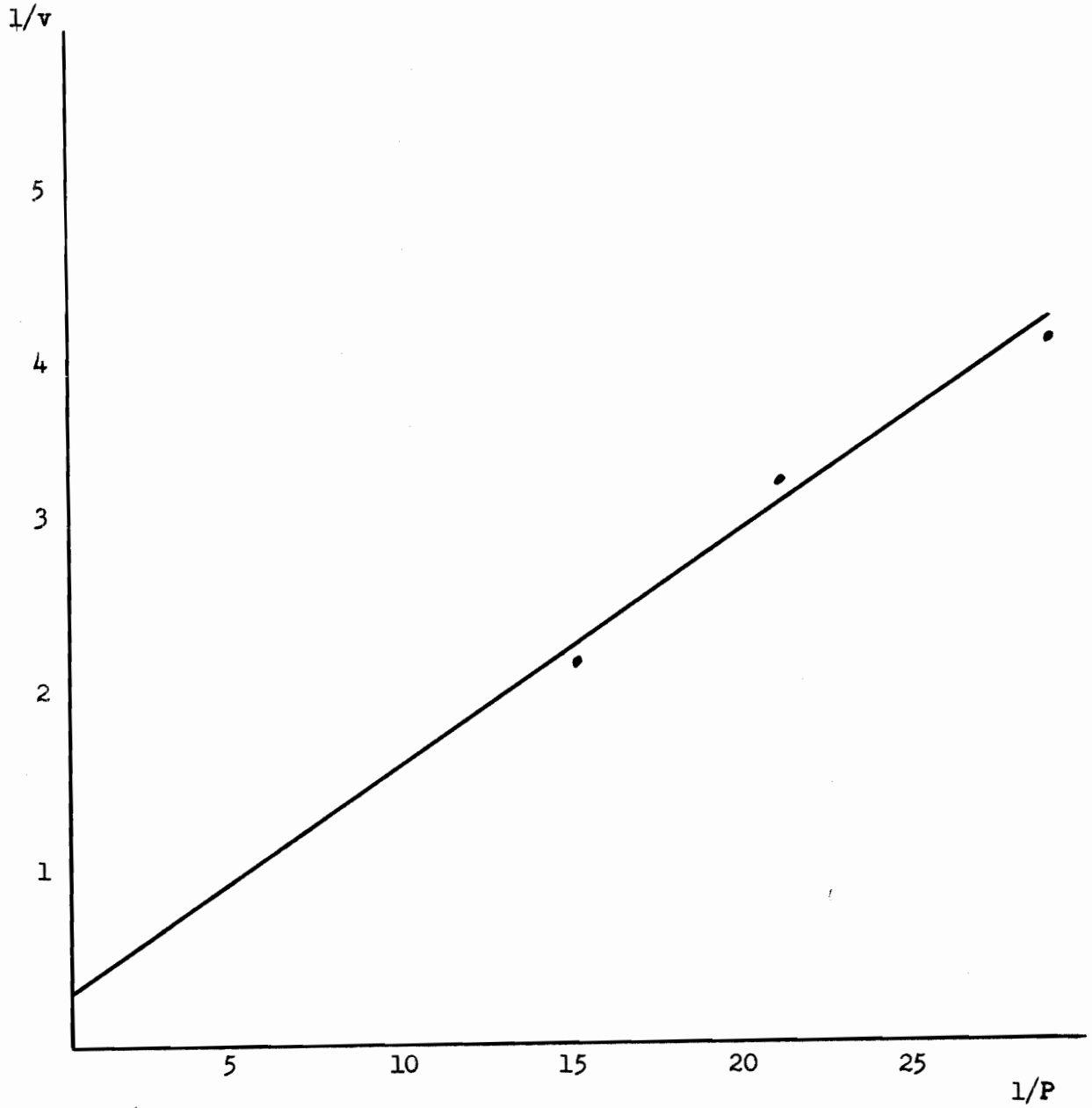

Figure 6. Reciprocal plot for the calculation of the equilibrium constant (K_m) for the citrulline ureidase enzyme system of P. tularensis strain H-8859.

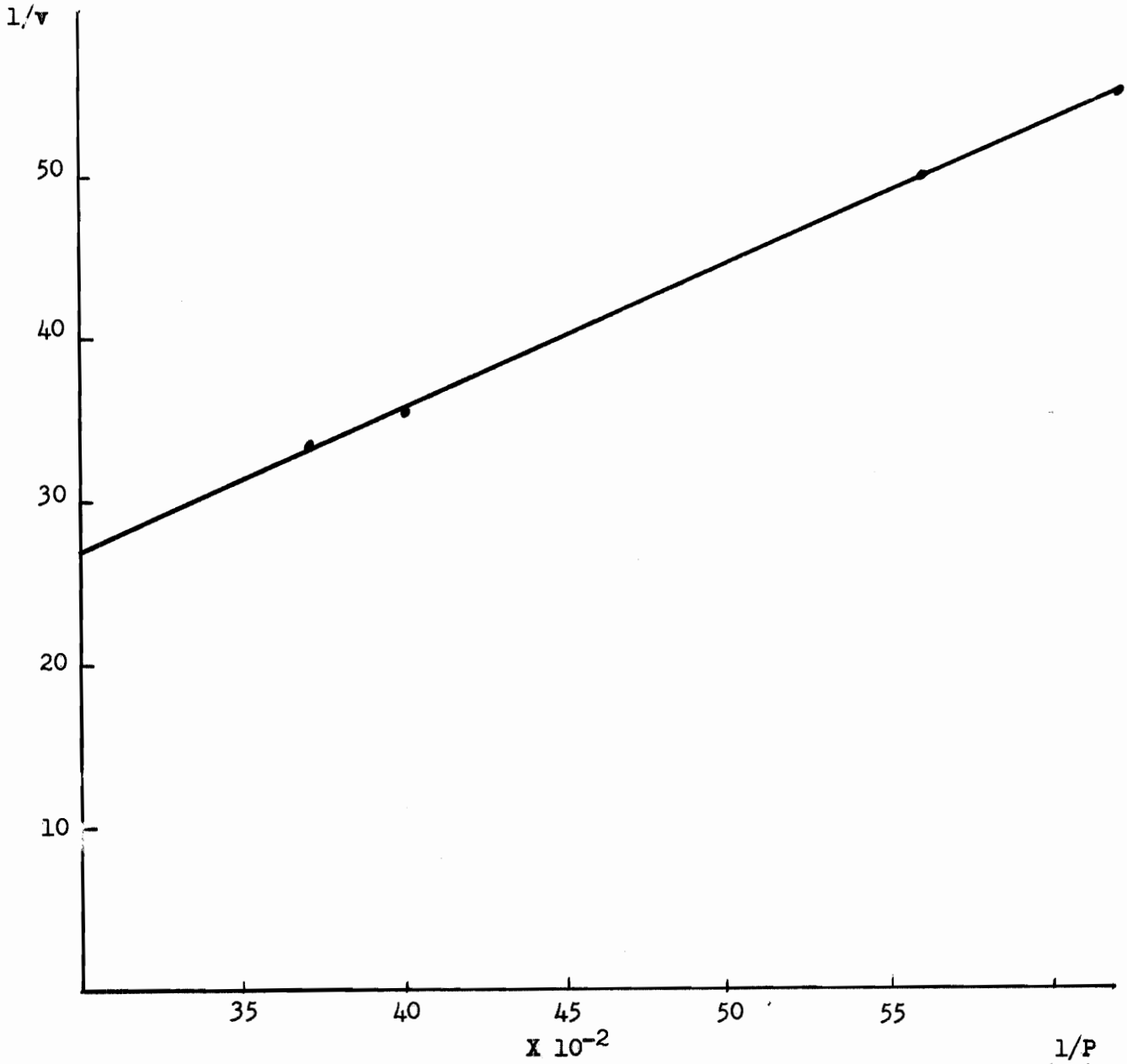

Figure 7. Reciprocal plot for the calculation of the equilibrium constant (K_m) for the citrulline ureidase enzyme system of P. tularensis strain 9K-161

III. Relationship between Citrulline Ureidase Enzyme Activity and Virulence

It has been demonstrated that only those strains of P. tularensis which are fully virulent (kill mice, guinea pigs and rabbits after low doses administered subcutaneously) possess an enzyme system capable of degrading citrulline to ornithine, NH_3 and CO_2. The question arises whether there is any causal relationship between this enzyme system and virulence. One approach towards testing this hypothesis is to attempt to alter the virulence of a strain which possesses this system and of a strain which does not, and then determine the effect of virulence change on citrulline ureidase enzyme activity.

In one series of experiments, attempts were made to enhance the virulence of the low virulent Jap_4 strain by serial passage through resistant deer mice, and through susceptible grasshopper mice. Heart blood from deer mice or grasshopper mice dead or dying of tularemia infection was passed intraperitoneally into healthy deer mice or grasshopper mice, respectively. At the end of six such serial passages in grasshopper mice, the organism was isolated from heart blood and tested for virulence for deer mice. Doses as high as 1.2×10^4 viable organisms inoculated subcutaneously failed to kill any deer mice.

After 15 serial passages, a culture was isolated from each species and its virulence for deer mice via the subcutaneous route tested. All deer mice receiving one or more viable organisms died of tularemia within 12 days after inoculation. Thus the virulence for deer mice had increased approximately a million-fold. (The LD_{50} of the parent strain in deer mice by the subcutaneous route is approximately 10^6).

Neither of these strains had developed the capacity to metabolize citrulline.

Each strain was then serially passed an additional 15 times through each species of mouse via the subcutaneous route. Then the virulence of each was titrated in deer mice and wood rats. The latter species is completely resistant to the parent strain. One organism of each strain was capable of killing deer mice. Most wood rats survived doses as high as 10^7 viable organisms inoculated subcutaneously, but a few died at doses as low as 10^5, Table 12. This indicates that the virulence for wood rats had increased somewhat over that of the parent strain.

Neither of these strains had developed the ability to metabolize citrulline.

The effect of suboptimal growth media on virulence and citrulline ureidase activity was next tested by serially subculturing strains Schu A, DPG-5, SKV-1, and Kf-473 on regular blood agar without cysteine or glucose. Each strain was subcultured at 3-4 day intervals for 60 serial transfers. These cultures were then maintained at 4°C in the stock culture collection and subcultured on blood agar slants every 4-6 weeks.

Only the Schu A blood agar passage strain was tested for virulence in deer mice and white rabbits. In each case one viable organism inoculated subcutaneously was sufficient to cause death within 10 days.

All four strains were tested for citrulline ureidase activity after 60 blood agar passages. There was no detectable change in enzyme activity of any of the four strains, Fig. 8. Strain Kf-473 still possessed no enzyme activity.

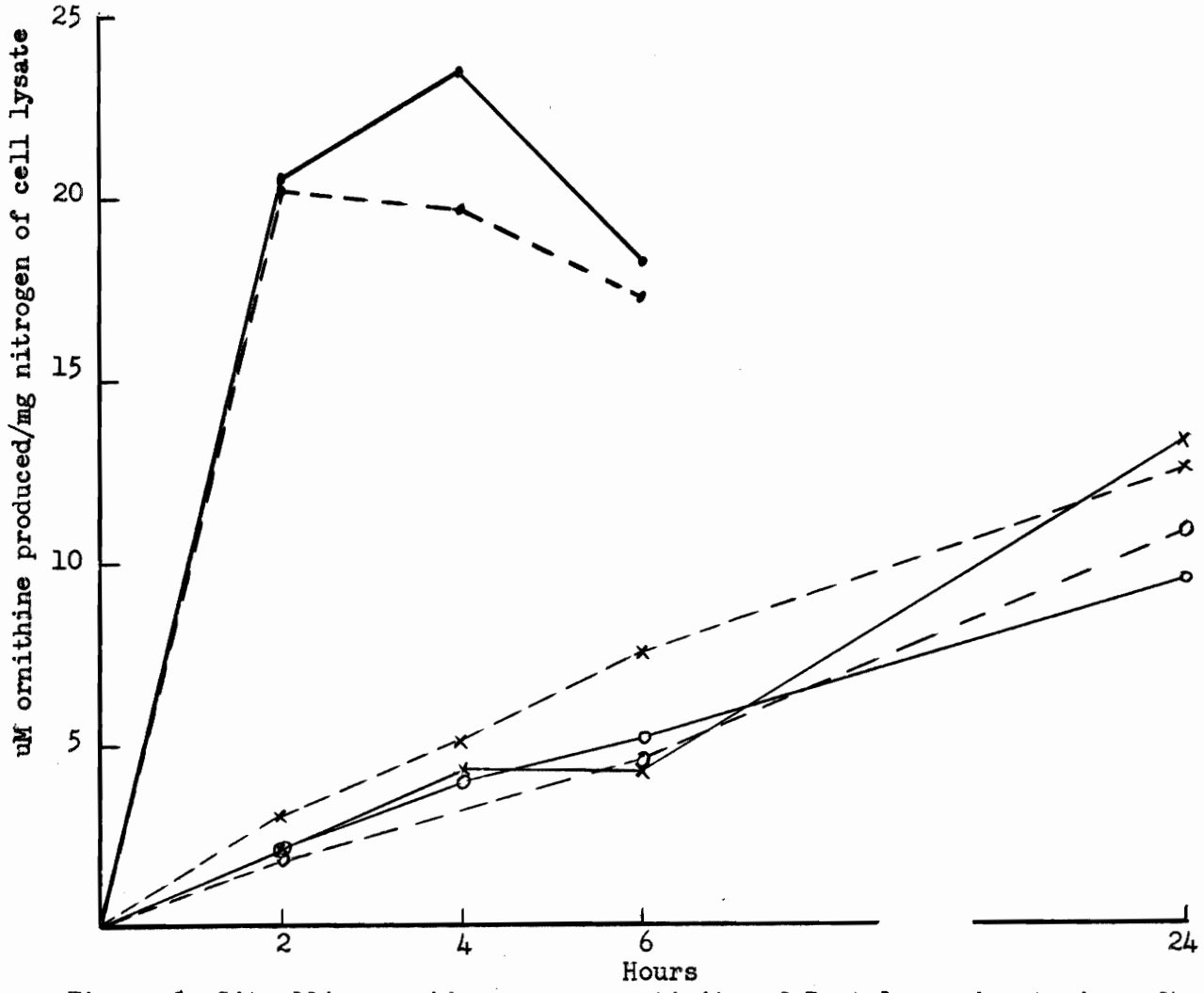

Figure 8. Citrulline ureidase enzyme activity of P. tularensis strains after 60 serial passages on blood agar compared with the enzyme activity of strains maintained on GCBA. Schu-blood agar●- - -●, Schu-GCBA ●—●, SKV-1-blood agar ×- - -×, SKV-1-GCBA ×——×, DPG-5-blood agar ●- - -●, DPG-5-GCBA ●——● .

TABLE 12

Virulence for wood rats of Pasteurella tularensis strain Jap_4 (parent), Jap_4-Pm-30 (30 serial passages in deer mice), and Jap_4-Ol-30 (30 serial passages in grasshopper mice). Data shown as the ratio of the number of animals dying of tularemia over a 21-day period to the total number used.

Jap_4 sub-strain	Number of Organisms					
	10^8	10^7	10^6	10^5	10^4	10^3
Parent	0/8	0/8	0/8	0/8	0/8	0/8
Pm-30		2/4	1/4	1/4		
Ol-30		2/8	0/8	0/5	0/7	0/8

Having failed either to alter the virulence appreciably or change the enzyme activity of these strains by animal passage or by subculture on artificial media, it was decided to attempt to induce variation more directly. Eigelsbach, Braun and Herring (1951) were able to produce a high percentage of variants of the Schu A strain by utilizing the method of Stakman, et al., (1948). Peptone broth (pH 6.8) containing 0.01% uranium acetate was inoculated with a heavy suspension of P. tularensis Schu A cells and incubated at 37°C without aeration for 15 days. Aliquots were then subcultured on peptone agar plates (pH 7.2). The isolated colonies obtained were observed under oblique light (Henry, 1933), and after staining with 1/1000 solution of crystal violet (White and Wilson, 1951). Approximately 99.9% of the colonies were translucent, "bluish" colored under oblique light illumination, stained a deep violet-red with 1/1000 crystal violet, and were nonsmooth in appearance. The remainder of the colonies were smooth, opaque and "whitish" colored under oblique light, and stained blue with crystal violet. Each colony form

was isolated in pure culture and subcultured for several passages on peptone agar or GCBA without either reverting to the parent type.

Each variant was cultured in Snyder's peptone broth (pH 6.8) for 48 hours at 35-37°C on a reciprocal shaker. An aliquot of each was removed for virulence titrations in deer mice and rabbits. The remainder was washed and lysed according to the standard procedure and tested quantitatively for citrulline ureidase enzyme activity.

The nonsmooth (NS) variant failed to kill any deer mice in doses up to $4x10^5$ viable organisms. Rabbits survived doses as high as $1x10^8$ organisms, Table 13. None of the deer mice or rabbits developed agglutinin antibody titers. The rabbits surviving the $1x10^8$ inoculum of the NS strain survived challenge with a normally lethal dose of the virulent Schu A parent strain. The other two rabbits died within 10 days after challenge.

The smooth variant (S) was partially virulent for deer mice, but avirulent for rabbits, Table 13. The agglutinin titers in deer mice surviving 21 days after inoculation ranged from 1/80 to 1/640. The three rabbits had agglutinin titers of negative, 1/80, and 1/640, respectively, depending upon the number of organisms inoculated. The one with agglutinin titer of 1/640 survived challenge with the virulent Schu A strain which readily kills normal rabbits in 5-7 days. The rabbit which had developed no agglutinating antibody succumbed to challenge with Schu A.

The citrulline ureidase enzyme activity of both smooth and nonsmooth variants was the same as that of the parent strain, Fig. 9.

TABLE 13

Virulence of smooth (S) and nonsmooth (NS) variants of Pasteurella tularensis strain Schu A for deer mice and rabbits. Data shown as the ratio of the number of animals dying of tularemia in a 21-day period after subcutaneous inoculation to the total number used.

	Number of organisms*			
Variant of Schu A	10^7	10^5	10^3	10^1
Smooth				
Deer mice		4/8	3/8	2/8
Rabbits	0/1	0/1	0/1	
Non-smooth				
Deer mice		0/8	0/8	0/8
Rabbits	0/1	0/1	0/1	

* Number of organisms x 3 for the smooth variant
Number of organisms x 10 for the non-smooth variant.

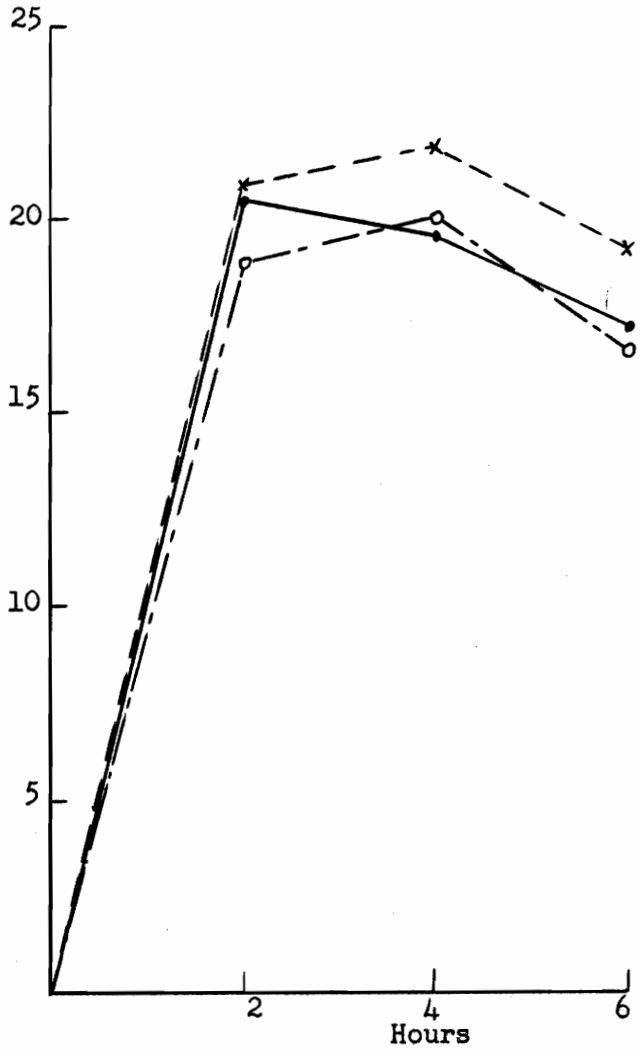

Figure 9. Citrulline ureidase activity of smooth and non-smooth variants of P. tularensis strain Schu A compared to that of the parent strain. Schu (parent) •—•, Schu (S) x-- x. Schu (NS) o--o.

DISCUSSION

Although no investigators have actually tested the susceptibility of wild rodents to tularemia on a strictly quantitative basis, the general consensus of opinion is that North American rodents are generally very susceptible to infection with P. tularensis. The wild rat, R. norvegicus, seems to be the only exception (McCoy, 1911; Dieter and Rhodes, 1926). In the present study, none of the 11 species of wild rodents tested survived infection with the virulent Schu A strain of P. tularensis. Subcutaneous inoculation of as few as one viable organism invariably resulted in death of the animal in a few days. The lethality for deer mice of 13 strains of P. tularensis variously isolated from ticks, rabbits, rodents, and a horse was also demonstrated. Wood rats and/or grasshopper mice were shown to be lethally susceptible to experimental infection with very low doses of four strains.

No chronic or latent infections were detected in any of the animals tested, although every effort was made to demonstrate such infections. This is in contrast to the situation in Russia where ground squirrels, Citellus spp., (Gorokov and Kozantseva, 1940) and water rats, Arvicola amphibius, (Dunaeva and Olsuf'ev, 1958) are reported to have a chronic form of tularemia as well as showing an acute form. However, this is most probably due to a difference in strains of P. tularensis enzootic in Russia and North America (Olsuf'ev, 1959), rather than to a difference in susceptibility of the rodents.

That rodents can survive infection with some strains of tularemia organisms has been demonstrated in this study in the case of deer mice, kangaroo rats, and wood rats inoculated with infective doses of the Jap_4 and NIIEG (blue variant) strains. There are also at least two cases in which apparently healthy rodents (fox squirrel and Marmot), with significant agglutinin titers have been collected, indicating recovery from tularemia infection (McKeever, et al., 1958; Menges and Galton, 1959). It is possible that in reports of rodents surviving experimental infection, less virulent strains comparable to the Jap_4 or Russian strains may have been involved.

As expected, the cottontails tested were highly susceptible to virulent tularemia. Tularemia epizootics occur frequently in these rabbits in many areas. However, there is very little evidence that they develop a chronic form of infection, or that they recover from natural infections (Jellison and Parker, 1945; Bell and Chalgren, 1943; Bell and Green, 1939). McKeever, et al., (1958) report an agglutinin titer of 1/320 from an apparently healthy cottontail, S. floridanus, indicating that even these highly susceptible animals may on occasion survive tularemia infection in nature.

In contrast to cottontails, jack rabbits and other rabbits of the genus Lepus are suspected to be resistant or only moderately susceptible to tularemia. Various field studies have reported the presence of P. tularensis agglutinins in rabbits collected for one purpose or another (MacLulich, 1937; Larson and Bell, 1938; Bell and Green, 1939; Jellison and Parker, 1945; Bacon and Drake, 1958; Menges and Galton, 1959).

Philip, Bell and Larson (1955) report the survival of two black-tailed jack rabbits, L. c. deserticola, after injection of approximately 100 P. tularensis cells, without any detectable agglutinating antibodies appearing after 34 days. Four rabbits inoculated with 10^6 organisms all died on the sixth day after injection.

Other investigators have reported the absence of tularemia agglutinins in jack rabbits collected from several tularemia epizootic areas (Lechleitner, 1939; Philip, Bell and Larson, 1955). In addition, a total of approximately 1500 serum samples from jack rabbits collected in Tooele County, Utah, have been examined serologically over the past six years without finding a single positive sample (Stoenner, et al., 1959; and unpublished data), even though P. tularensis has been isolated repeatedly from these rabbit populations and their ectoparasitic ticks.

In the present study, jack rabbits survived experimental tularemia infection under some conditions, and yet the survivors had no detectable agglutinating antibody when tested 21 days after inoculation with virulent P. tularensis. However, they did possess high complement fixing antibody titers. Unfortunately, these animals are very difficult to obtain and maintain in captivity. Consequently, it has not been possible to conduct further satisfactory experiments to confirm or clarify this rather puzzling situation. Certainly some strains of jack rabbits can and do survive tularemia infection at least under certain conditions in nature and in the laboratory. The probable intimate connection of jack rabbits with the maintenance and transmission of tularemia justifies further work on this species, difficult as it may be.

Carnivores, in general, appear to be resistant to natural tularemia infection. However, there are reports that, under certain conditions, some carnivores can and do contract fatal tularemia. An actual "epizootic" has been reported among gray foxes in Minnesota in which ten animals were involved (Schlotthauer, Thompson and Olson,1935). Lillie and Francis (1936) state that red foxes are susceptible to both per os and subcutaneous infection. Commercially important fur-bearing foxes and mink raised on fur farms have been reported to be lethally susceptible to tularemia contracted by eating infective rabbit carcasses (Gorham, 1949; Shmuter and Abramova, 1956). On the other hand, Olsuf'ev and Dunaeva (1951) were unable to kill Russian foxes or skunks with injections of 10^8 organisms, although a weasel did die after such an injection. Here again the difference between Russian and North American strains of P. tularensis must be considered.

Other evidence of the resistance of carnivores to tularemia is presented by McKeever, et al., (1958) who showed that tularemia agglutinins are not uncommon among Georgia and Florida carnivores -- red foxes, gray foxes, raccoons, striped and spotted skunks, wildcats, and feral house cats.

Parker and Francis (1926) showed coyote pups to be lethally susceptible to oral tularemia infection. However, Stagg, Tanner and Lavender (1956) found them to be resistant to aerosol exposure. Lundgren, Marchette and Smart (1957) were unable to kill full grown adults by either oral or subcutaneous infection.

The present experiments indicate that coyote pups are easily infected by both oral and subcutaneous exposure to virulent P. tularensis as determined by a four-fold or greater rise in tularemia antibodies. The course of the disease is usually mild and recovery spontaneous and uneventful. However, fatal infections were induced in very young pups with massive doses of P. tularensis inoculated subcutaneously. Animals surviving infections are probably not carriers since they readily clear their tissues of the infecting organisms.

The carnivores investigated in this study responded to experimental tularemia infection with the rapid production of both agglutinating and complement fixing antibodies. Although no attempts were made to determine the persistence of these antibodies, significant agglutinin titers did not persist beyond the 10th to 12th week in the two pups tested. If this is generally the case, then wild carnivores which possess tularemia antibodies can be assumed to have been recently exposed to tularemia infection.

Tularemia in the Great Salt Lake Desert of Utah appears to be principally a disease of the desert jack rabbit, L. c. deserticola. These animals and their ectoparasitic ticks, D. parumapertus, apparently are able to maintain the disease in restricted foci over long periods of time. On the other hand, the associated rodent fauna have not been shown to be involved in the maintenance or transmission of tularemia.

The existence of P. tularensis in the rabbits, and its absence from the rodent population of this region of the Great Basin is difficult to explain from an ecological standpoint. The main arthropod vector of rabbit tularemia, D. parumapertus, is commonly found on many

species of rodent. It has been shown that this tick can carry P. tularensis throughout its life and transmit it after each molt to susceptible hosts, including rodents (unpublished data). At least some and probably all of the indigenous rodents are lethally susceptible to the native strains of P. tularensis that have been tested. In addition, under laboratory conditions, rodents are able to transmit the disease to other rodents through ectoparasites or by carnivorism. Everything seems to be present for the involvement of rodents in natural tularemia. However under ordinary circumstances, the natural populations of rodents in this region are not very dense(Ecol. Res. Annual Reports), and there is probably not a sufficient amount of contact between rodents to support an epizootic or maintain the agent in the population. Among colonial rodents such as the muskrat, Ondatra zibethicus, there is sufficiently close contact among the animals to support epizootic outbreaks, and such outbreaks have been recorded (Jellison, Kohls and Philip, 1951).

On the other hand, one cannot rule out the possibility that the particular strain or strains of P. tularensis enzootic in this region are peculiarly adapted to the wild rabbit populations. The organisms may not be able to maintain themselves in the rodent populations because of their extreme virulence for the native rodents, or they may not be transmitted to these animals because of some factor(s) in the ecology of the rabbits, rabbit ticks and/or rodents which has not yet been elucidated.

There is very little evidence to support the above hypothesis, but there is no doubt that strains of varying virulence do exist in nature (Davis, Philip and Parker, 1934; Philip and Davis, 1935; Bell and Green,

1939; Humphreys and Campbell, 1947; Jellison, Bell and Owen, 1959). Unfortunately, virulence is the only characteristic that has been systematically checked. There is no reason to believe that these and/or other strains may not differ in any number of less obvious, but no less important, characteristics. More complete characterization of P. tularensis strains isolated from different sources as well as more detailed ecological studies are needed to clarify this complex relationship between host and parasite.

Recently, studies have been made on the comparative physiology of a few laboratory strains of P. tularensis. A survey of transaminase activity of two virulent and one avirulent strain revealed no qualitative strain differences (Fleming and Foshay, 1956). However, quantitatively the avirulent strain showed a markedly higher reaction velocity in the aspartic acid-alanine system compared to that of the two virulent strains. The same workers also showed earlier that the same avirulent strain possesses less glutiaminase activity than the virulent Schu strain (Fleming and Foshay, 1955). However, they made no attempt to assess the significance of these probably minor differences in metabolic activity.

Of considerably more interest was the demonstration by Fleming and Foshay (1955) of a citrulline ureidase enzyme system in three virulent strains of P. tularensis, and its complete absence in two low virulent and two avirulent laboratory strains. The correlation of this enzyme system with high virulence was striking, but the relationship was not elucidated.

The results of the present study indicate that the relationship between citrulline ureidase activity and virulence is merely coincidental and is probably not of a causal nature. It is true that, on a qualitative basis, the only naturally occurring strains tested which possessed the ability to metabolize citrulline were those which are fully virulent. That is, small numbers of organisms inoculated subcutaneously are lethal for mice, guinea pigs and rabbits.

None of the strains which are avirulent or of attentuated virulence for rabbits possessed any demonstrable citrulline ureidase activity. Thus, strain Kf-473 and 425 F_4G, although highly virulent for mice, do not kill rabbits except when large numbers of organisms are injected. Neither of these strains possessed any citrulline ureidase activity.

These data would lead one to postulate the correlation of ability to metabolize citrulline with virulence for rabbits. This hypothesis was tested by inducing and selecting variants of the virulent-citrulline ureidase positive Schu A strain. It was relatively easy to obtain pure cultures of a smooth and a non-smooth variant of the parent strain. The virulence of each of these variants, Table 13, was considerably less than that of the parent strain. Neither variant was virulent for rabbits in doses as high as 10^7 organisms. The smooth variant was avirulent also for deer mice. However, the ability to metabolize citrulline had not diminshed in either strain, Fig. 9.

Thus, it appears that the possession of a citrulline ureidase enzyme system <u>per se</u> is not related to virulence.

Other evidence supporting this conclusion is the quantitative difference in the citrulline ureidase enzyme activity demonstrated in the local rabbit and rabbit tick strains of P. tularensis, compared to human (Schu) and the horse (H-8859) strains. All these strains are fully virulent for mice, guinea pigs, and rabbits, yet the local strains possessed only about one sixth the citrulline ureidase activity of the Schu and H-8859 strains.

It is possible that different enzymes are involved in these two varieties of P. tularensis. The equilibrium constants for the Schu A and H-8859 strains are almost identical, being of the order of 10^{-6}M. The equilibrium constant for the one rabbit strain tested, 9K-161, is somewhat higher, being of the order of 10^{-4}M.

Attempts to increase the virulence of the low virulent Jap_4 strain by serial passage through a susceptible and a resistant host were only partially successful. Strains of full virulence for deer mice were obtained, but the virulence for wood rats, which are completely resistant to infection with the parent strain, was only slightly altered. No ability to metabolize citrulline had developed.

The significance of this enzyme system in terms of the overall metabolic activity of P. tularensis is still obscure. Citrulline is not an essential nutrilite in completely defined media (Fleming and Foshay, 1955; Mager, Traub and Grossowicz, 1954; Nagle, Anderson and Gray, 1960). Arginine is essential, but there is no direct evidence indicating that it is metabolized to citrulline by P. tularensis. However, no definitive work has been reported on this particular aspect of the problem.

An arginine desimidase enzyme system is present in all other organisms possessing a citrulline ureidase system that have been studied: Streptococcus faecalis (Oginsky and Gehrig, 1952; Knivett, 1952; Slade, 1953), S. lactis (Korzenovsky and Werkman, 1953), Clostridium perfringens (Schmidt, Logan and Tytell, 1952), and Pseudomonas sp. (Slade, Doughty and Slamp, 1954). Further work may demonstrate such a system in P. tularensis. However, the citrulline ureidase system of P. tularensis differs somewhat from that found in these other organisms and may not be the same enzyme at all (See Fleming and Foshay, 1955, for a discussion of these differences).

Likewise, P. tularensis has not been shown to be able to metabolize or utilize ornithine, nor is it necessary for growth and reproduction in defined media.

What then is the significance of the citrulline ureidase enzyme system in P. tularensis? It has been demonstrated that there exist in nature strains of P. tularensis which possess a citrulline ureidase system of a high order of activity, strains which possess activity of a low order, and strains which completely lack this enzyme system. It has also been shown that this system is not directly correlated with virulence. It is therefore suggested that the presence or absence of the citrulline ureidase system (and possibly its level of activity) are characteristics which define varieties of P. tularensis. Thus, one can distinguish three distinct varieties on the basis of this characteristic alone: 1) strains possessing citrulline ureidase enzyme activity of a high order (Schu A and H-8859); 2) strains with a low order of activity (DPG, SKV, and 9K-161); and 3) strains lacking this system entirely (Kf-473 and 425 F4G).

If additional study shows this scheme to be essentially correct, how can it be explained in terms of the ecology of P. tularensis? Or conversely, can it shed some light on the ecology of P. tularensis? It is possible that strict geographical isolation has led to the evolution of three distinct varieties. However, there is at present no evidence for or against this hypothesis.

What little evidence exists (and meager it is) suggests that each of these varieties may have become adapted to a particular mammalian host-ectoparasite reservoir complex. Thus, the two non-citrulline ureidase possessing strains, Kf-473 and 425 F_4G, were isolated from a rodent (M. montanus) and a tick which is commonly found on rodents (D. andersoni) respectively. All the strains possessing citrulline ureidase activity of a low order were isolated from rabbits or rabbit ticks (D. parumapertus). On the other hand, the only two strains possessing enzyme activity of a high order, H-8859 and Schu A were isolated from a horse and a human, respectively. Since these latter two creatures are probably only accidental hosts of P. tularensis (certainly they play no significant role in the ecology of this agent), it is possible that these two strains are actually derived from rabbit strains. Passage through these "unnatural" hosts may have served to induce citrulline ureidase enzyme activity of a higher than normal order. If this is the case, then only two wild varieties are probably present in nature -- rodent strains lacking this system and the rabbit strains possessing it.

The data presented here are certainly not sufficient to prove the existence of different natural varieties of P. tularensis. However,

they do suggest the presence of a variety of P. tularensis which has evolved in rodents and is distinct from the variety which is found in rabbits. Only extensive sampling and testing of natural isolates can prove or disprove this hypothesis. However, if this is the case, its significance in the study of the ecology of P. tularensis would be considerable.

SUMMARY

1. Eleven species of wild rodents and one species of cottontail were found to be extremely susceptible to subcutaneous infection with the virulent Schu A strain of P. tularensis. In addition, grasshopper mice, wood rats, and/or deer mice were very susceptible to subcutaneous infection with any of the 12 strains of P. tularensis isolated from rabbits, rodents or ticks, and one strain isolated from a horse. All three species were completely resistant to inoculation with the avirulent 38 strain. Deer mice and wood rats were completely resistant to the Russian NIIEG (gray variant) and partially resistant to NIIEG (blue variant).

2. Jack rabbits of the subspecies L. c. deserticola were highly susceptible to subcutaneous infection with the Schu A strain, whereas the subspecies L. c. texianus showed some resistance. Complement fixing, but not agglutinating, antibodies were demonstrated in the sera of L. c. texianus surviving experimental infection.

3. Of five species of rodents exposed to subcutaneous infection with the moderately virulent Jap_4 strain of P. tularensis, only the grasshopper mouse was very susceptible. Wood rats were completely resistant, while deer mice and kangaroo rats were lethally susceptible only to high doses.

4. Tularemia infection in young coyote pups caused death when massive doses were inoculated. However, subcutaneous and oral infection usually resulted only in production of antibody. The disease followed a mild, subacute course and was completely resolved within 2-3 weeks. No carrier state or chronic infection resulted.

5. The presence of citrulline ureidase enzyme system in P. tularensis strains of high virulence and its absence in avirulent strains and strains of low virulence has been confirmed.

6. The only wild strains of P. tularensis tested which lacked a citrulline ureidase system were the two isolated from rodents or rodent ticks. All the strains isolated from rabbits, rabbit ticks, a human, and a horse, that were tested, possessed this system.

7. The citrulline ureidase system has been shown to be not directly related to virulence.

8. The existence of two North American strains of P. tularensis has been postulated on the basis of the presence or absence of a citrulline ureidase enzyme system. It has been further postulated that the strains lacking this enzyme system may have evolved in rodents and the strains possessing the ability to metabolize citrulline may have evolved in rabbits.

REFERENCES CITED

Alexander, M. M., Wright, G. G., and A. C. Baldwin. 1950. Observations on the agglutination of polysaccharide treated erytrhocytes by tularemia antisera. J. Exp. Med. 91(6):561-566.

Anderson, C. 1934. Bacillus with characteristics of Bacterium tularense found in Tunis. Arch. Inst. Pasteur Tunis, 23:53-59.

Anon. 1944-46. Tularemia. Excerpts from the published literature on the development of NIIEG vaccines. Tico-5954 55-INT-397, 1936-1946. Trans. #41, 7 pp.

Avi-Dor, Y. and H. Yaniv. 1953. Relation between changes in the stability of P. tularensis suspensions and its bacterial population. I. The stability of suspensions of P. tularensis in the presence of electrolytes. J. Bact., 66:1-5.

Bacon, M. and C. H. Drake. 1958. Bacterial infections in wild rabbits of Eastern and Central Washington. Northwest Science. 32:124-131.

Bell, I. R. 1944. A case of tularemia of unusual origin. Can. Med. Assoc. J., 50:555-556.

Bell, J. F. and W. S. Chalgren. 1943. Some wildlife diseases in Eastern United States. J. Wildlife Management. 8:270-278.

Bell, J. F. and R. G. Green. 1939. Nonfatal infections with Pasteurella tularensis in the snowshoe hare. J. Bact., 38:114.

Bell, J. F., Owen, C. R. and C. L. Larson. 1955. Virulence of Bacterium tularense. I. A study of the virulence of B. tularense in mice, guinea pigs, and rabbits. J. Inf. Dis., 97:162-166.

Bilal, S. 1939. La sensibilite' a la tularemie de la grenouille (Rana ridibunda). Bull. Soc. Path. Exot., 32:869-871.

Bilal, S. 1939a. Essais de transmission de la tularemic aux tortues terrestres. Bull. Soc. Path. Exot. 32:141-143.

Bow, M. R. and J. H. Brown. 1943. Tularemia in the "Seven Persons Coulee", Alberta. Can. J. Pub. Health. 34:415-418.

Burroughs, A. L., Holdenried, R., Longanecker, D. S. and K. F. Meyer. 1945. A field study of latent tularemia in rodents with a list of all known naturally infected vertebrates. J. Inf. Dis. 76:115-119.

Calhoun, E. L. 1954. Natural occurrence of tularemia in lone star tick, anddogs in Arkansas. Amer. J. Trop. Med. Hyg. 3:360-366.

Calhoun, E. L., Mohr, C. O. and H. I. Alford, Jr. 1956. Dogs and other mammals as hosts of tularemia and of vector ticks in Arkansas. Amer. J. Hyg. 63:127-135.

Chapman, S. S., Downs, C. M., Coriell, L. L. and S. F. Kowal. 1949. Streptomycin studies in tularemia. I. Effect of streptothricin and streptomycin on Bacterium tularense in vitro and in vivo (mouse).

Claus, K. D., Newhall, J. H. and D. Mee. 1959. Isolation of Pasteurella tularensis from foals. J. Bact. 78:294-295.

Davis, G. E. 1935. Tularemia. The susceptibility of the white-tailed prairie dog to tularemia. Pub. Health Repts., 50:731-732.

Davis, G. E. 1940. Bacterium tularense: its persistence in the tissues of the argasid ticks Ornithodoros turicata and O. parkeri. Pub. Health Repts., 55:676-680.

Davis, G. E., Philip, C. B. and R. R. Parker. 1934. The isolation from the Rocky Mountain wood tick (Dermacentor andersoni) of strains of Bacterium tularense of low virulence for guinea pigs and domestic rabbits. Amer. J. Hyg. 19:449-456.

Dieter, L. V. and B. Rhodes. 1926. Tularemia in wild rats. J. Inf. Dis. 38:541-546.

Downs, C. M., Buchele, L. and E. P. Edgar. 1949. Studies on pathogenesis and immunity in tularemia. I. The pathogenesis of tularemia in the white rat. J. Immunol. 63:117-133.

Downs, C. M., Coriell, L. L., Pinchot, G. B., Maumee, E., Klauber, A., Chapman, S. S. and B. Owen. 1946. Comparative susceptibility of various laboratory animals. J. Immunol. 56:217-228.

Dunaeva, T. N. and N. G. Olsuf'ev. 1951. Quoted from Suraleva. 1959.

Dunaeva, T. N. and N. G. Olsuf'ev. 1958. The possibility of a latent or chronic course of tularemia in water voles and other animal species highly susceptible to that infection. Zool. Zhur. 37:430-440. In Russian with an English summary.

Durrant, S. D. 1952. Mammals of Utah. Univ. of Kans. Publ. Mus. Nat. Hist. 6:1-59.

Ecke, D. H. and R. Holdenried. 1952. Tularemia from a wood rat in New Mexico. Pub. Health Repts. 67:588-589.

Ecological Research. 1957. Annual Report of the Ecological Research Laboratories of the Univ. of Utah, Contract No. DA-18-064-CML-2639, 1956-57, to U. S. Army Chemical Corps, Dugway, Utah.

Ecological Research. 1958. Annual Report of the Ecological Research Laboratories of the Univ. of Utah, Contract No. DA-18-064-CML-2639, with the U. S. Army Chemical Corps, Dugway, Utah.

Ecological Research. 1960. Annual Report of the Ecological Research Laboratories of the Univ. of Utah, Contract No. DA-42-007-403-CML-327, with the U. S. Army Chemical Corps, Dugway, Utah.

Egoscue, H. J. 1956. The faunal colony as a reservoir of native animals. IN: Symposium on ecology of disease transmission in native animals. Dugway, Utah. pp. 50-54.

Egoscue, H. J. 1956a. Preliminary studies of the kit fox in Utah. J. Mammalogy. 38:472-481.

Egoscue, H. J. 1960. Laboratory and field studies of the Northern grasshopper mouse. J. Mammalogy. 41:99-110.

Eigelsbach, H. T., Braun, W. and R. Herring. 1951. Studies on the variation of Bacterium tularense. J. Bact. 61:557-570.

Ey, L. G. and R. E. Daniels. 1941. Tularemia in dogs. J. Amer. Med. Assoc. 117:2071-2072.

Fairbich, M. M., and T. S. Ramarina. 1946. Dry living tularemia vaccine of NIIEG of Red Army. Zhur. Mikrobiol., Epidemiol., Immunobiol. #7:59-63; #10:23-27.

Finney, D. J. 1952. Probit Analysis. Cambridge Univ. Press. London. 318 pp.

Fleming, D. E. and L. Foshay. 1955. Studies on the physiology of virulence of Pasteurella tularensis. I. Citrulline ureidase and deaminase activity. J. Bact. 70:345-349.

Fleming, D. E. and L. Foshay. 1956. Studies on the physiology of virulence of Pasteurella tularensis. II. Serine deaminase and transaminase activity. J. Bact. 71:324-327.

Foshay, L. 1942. Induction of avirulence in Pasteurella tularensis. J. Infect. Dis. 51:280-285.

Francis, E. 1919. Deer-fly fever: A disease of man of hitherto unknown etiology. Pub. Health Repts. Sept. 12, pp. 2061-2062.

Francis, E. 1921. The occurrence of tularemia in nature as a disease of man. Pub. Health Rept. 36:1731-1738.

Francis, E. 1922. Tularemia: A new disease of man. Intern. Clin. 33:73-78.

Francis, E. 1942. Fermentation of sugars by Bacterium tularense. J. Bact., 43:343-346.

Francis, E. and D. Moore. 1926. Identity of Ohara's disease and tularemia. J. Amer. Med. Assoc. 86:1329-1332.

Geiger, J. C. 1931. Tularemia in cattle and sheep. Calif. and West. Med. 34:1-3.

Gorham, J. R. 1949. Mink, fox, susceptible to tularemia. Amer. Nat. Fur. Market Jour. 28:21.

Gorokov, V. I. and A. L. Kazantseva. 1940. Chronic course of tularemia in ground squirrels, and their significance as carriers of virus. Vestnik., Mikrobiol., Epidemiol., Parazitol. 19:92-95. (Biol. Abstr. 15(7):15124).

Green, R. G. 1937. Susceptibility of beaver to tularemia. Minnesota Wildlife Dis. Invest. 3:110.

Green, R. G. 1942. Tularemia as a hunter's problem. Cons. Volunteer, 3:41-45.

Green, R. G. 1943. Virulence of tularemia related to animal and arthropod hosts. Am. J. Hyg. 38:282-292.

Green, R. G., Larson, C. L. and J. F. Bell. 1938. Shock disease as the cause of the periodic decimation of the snowshoe hare. Amer. J. Hyg. 30:83-102.

Green, R. G. and J. E. Shillinger. 1932. A natural infection of the sharp-tailed grouse and ruffed grouse by Pasteurella tularensis. Proc. Soc. Exp. Biol. Med. 30:284-287.

Green, R. G. and E. M. Wade. 1928. Experimental tularemia in birds. Proc. Soc. Exp. Biol. Med. 25:637.

Green, R. G. and E. M. Wade. 1928a. Ruffed grouse (Bonasa umbellus) are susceptible to tularemia. Ibid 25:515-517.

Green, R. G. and E. M. Wade. 1929. A natural infection of quail by Bacterium tularense. Proc. Soc. Exp. Biol. Med. 26.626-627.

Green, R. G., Wade, E. M. and E. T. Dewey. 1929. Experimental tularemia in muskrat. Proc. Soc. Exp. Biol. Med. 26:426-427.

Green, R. G., Wade, E. M. and W. Kelly. 1928. Experimental tularemia in ring-necked pheasant. Proc. Soc. Exp. Biol. Med. 26:260-263.

Gronroos, B. 1939. First case of tularemia in Finland. Duodecin. 55:740-751.

Gwatkin, R., Painter, R. H. and I. W. Moynihan. 1942. Tularemia in sheep. Can. J. Comp. Med. 6:163-168.

Hammersland, H. L. and C. M. Joneschild. 1940. Tularemia in beaver. J. Amer. Vet. Med. Assoc. 96:96.

Henry, B. S. 1933. Dissociation in the genus Brucella. J. Inf. Dis. 52:374-402.

Hesselbrock, W. and L. Foshay. 1945. The morphology of Bacterium tularense. J. Bact. 49:209-231.

Humphreys, F. A. and B. A. Campbell. 1947. Plague, Rocky Mountain spotted fever and tularemia surveys in Canada. Can. J. Pub. Health. 38:124-130.

Huseyn, K. 1937. Die Tularamie in der Turkei. Zeit. f. Hyg. und Infektionskrankheiten. 119:425-430.

Jellison, W. L., Bell. J. F. and C. R. Owen. 1959. Mouse disease studies. The Oregon Meadow Mouse Irruption of 1957-1958. Federal Coop. Extension Service, Oregon State College, Corvallis. pp. 71-80.

Jellison, W. L. and G. M. Kohls. 1955. Tularemia in sheep and in sheep industry workers in western United States. Pub. Health Serv. Publ. #421. Public Health Monograph #28.

Jellison, W. L., Kohls, G. M. Butler, W. J. and J. A. Weaver. 1942. Epizootic tularemia in a beaver, Castor canadensis, and the contamination of stream water with Pasteurella tularensis. Amer. J. Hyg. 26:168-182.

Jellison, W. L., Kohls, G. M. and C. B. Philip. 1951. Tularemia - - muskrats as a source of human infection in Utah. Rocky Mtn. Med. Jour. 48:594-597.

Jellison, W. L. and R. R. Parker. 1945. Rodents, rabbits and tularemia in North America; some zoological and epidemiological considerations. Amer. J. Trop. Med. 25:349-362.

Johnson, H. N. 1944. Natural occurrence of tularemia in dogs used as a source of canine distemper virus. J. Lab. and Clin. Med. 29:906-915.

Kamil, S. and S. Golem. 1938. Recherches experimentales sur l'etioligie de la tularemie en Turquie. Ann. de. Parasit. 16:530-542.

Kartman, K., Prince, F. M. and S. F. Quan. 1958. "Epizootiologic Aspects" in Oregon Meadow Mouse Irruption of 1957-1958. Federal Coop. Extension Service, Oregon State College, Corvallis. pp. 43-53.

Kay, R. E., Harris, D. C. and C. Entenman. 1956. Qyantification of the ninhydrin color reaction as applied to paper chromatography. Archives Biochem. and Biophys. 63:14-25.

Knivett, V. A. 1952. Citrulline as an intermediate in the breakdown of arginine by Streptococcus faecalis. Biochem. J. (Lond.) 50:xxx-xxxi.

Kohls, G. M. and E. A. Steinhaus. 1943. Tularemia: A spontaneous occurrence in shrews. Pub. Health Repts. 58:842.

Korzenovsky, M. and C. H. Werkman. 1953. Conversion of citrulline to ornithine by cell-free extracts of Streptococcus lactis. Arch. Biochem. and Biophys. 46:174-185.

Lang, C. A. 1958. Simple microdetermination of Kjedahl nitrogen in biological materials. Analytical Chemistry. 30:1692-1694.

Lechleitner, R. R. 1959. Some parasites and infectious diseases in a black-tailed jack rabbit population in the Sacramento Valley, California. Calif. Fish and Game. 45:83-91.

Lillie, R. D. and E. Francis. 1936. The pathology of tularemia in other animals. Nat. Inst. Health Bull. 167:187-202.

Lineweaver, H. and D. Burk. 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56:658-666.

Lundgren, D. L., Marchette, N. J. and K. L. Smart. 1957. Tularemia in the coyote, Canis latrans lestes Merriam. J. Inf. Dis. 101:154-157.

MacArthur, F. Y. and S. B. Brown. 1950. Tularemia in sheep. J. Amer. Vet. Med. Assoc. 116:204-205.

McCoy, G. M. 1911. A plague-like disease of rodents. Pub. Health Bull. 43:53-71.

McCoy, G. W. and C. W. Chapin. 1912. Further observations on a plague-like disease of rodents, with a preliminary note on the causative agent, Bacterium tularense. J. Inf. Dis. 10:61-72.

McGinnes, B. S. 1958. Some factors influencing the cottontail rabbit in southwestern Virginia. Thesis Ph.D. Virginia Polytechnic Institute Biology.

McKeever, S., Schubert, J. H., Moody, M. D., Gorman, G. W. and J. F. Chapman. 1958. Natural occurrence of tularemia in marsupials, carnivores, lagomorphs and large rodents in southwestern Georgia and northwestern Florida. J. Inf. Dis. 103:120-126.

MacLulich, D. A. 1937. Fluctuations in numbers of the varying hare (Lepus americanus). Univ. of Toronto Studies, No. 43:136 pp.

McNabb, A. L. 1930. Tularemia: The first case reported in Canada. Can. Public Health Jour. 21:91-92.

Mager, J., Traub, A. and N. Grossowicz. 1954. Cultivation of Pasteurella tularensis in chemically defined media; effect of buffers and spermine. Nature (Lond.).174:747-748.

Mashkov, A. V. 1958. The immunogenic properties of two old strains of Pasteurella tularensis (virulent and vaccine). J. Microbiol., Epidemiol., Immunobiol. 29:1184-1186.

Menges, R. W. and M. M. Galton. 1959. Investigation of wildlife animals for leptospirosis. IN: The Oregon Meados Mouse Irruption of 1957-1958. Federal Coop. Extension Service, Oregon State College, Corvallis. pp. 81-88.

Miller, G. S., Jr. and R. Kellogg. 1955. List of North American recent mammals. U. S. National Mus. Bull. 205.

Mills, R. C. 1954. Research studies on the growth and metabolism of Bacterium tularensis. Univ. Kans. Quart. Prog. Rept. of Research to The U. S. Army Chemical Corps. 1 Jan.-31 Mar. 1954.

Mills, R. C., Berthelson, H., Donaldson, E., and P. L. Wilhelm. 1949. Nutritional requirements of Bacterium tularensis. Bact. Proc. 37.

Moody, M. D. and C. M. Downs. 1955. Studies on tularemia. I. The relation between certain pathogenic and immunogenic properties of variants of Pasteurella tularensis. J. Bact. 70:297-304.

Morgan, B. B. 1947. Experimental studies with Pasteurella tularensis in fish. Amer. J. Trop. Med. 27:399-401.

Nagle, S. C., Jr., Anderson, R. E. and N. D. Gray. 1960. Chemically defined medium for the growth of Pasteurella tularensis. J. Bact. 79: 566-571.

Nakamura, M. 1950. Tularemia in the red-tailed hawk. Auk, 67:383-384.

Nakamura, M. 1950a. The survey of Pasteurella tularensis infection in the animals of the Jackson Hole area. Zool. 35:129-131.

Nelis, P. 1950. First case of tularemia in Belgium. Ann. Inst. Pasteur. 79:749-756.

Nicholes, P. S. 1946. Thesis, Ph.D. University of Cincinnati.

Novikova, E. and G. Lalazarov. 1940. The part taken by amphibians in the epizootiology of tularemia. Vestnik. Mikrobiol. 19:271-294. (In Russian with English summary).

Oginsky, E. L. and R. F. Gehrig. 1952. The arginine dehydrolase system of Streptococcus faecalis. I. Identification of citrulline as an intermediate. J. Biol. Chem., 198: 791-797.

Ohara, H. 1926. On an acute febrile disease transmitted by wild rabbits. Jap. Med. World. 6:263-270.

Ohara, H., Kobayashi, T. and J. Kudo. 1935. A study on pleomorphism of Bacterium tularense (Yato-Byo bacteria, Ohara) and their special flagellum. Tohoku J. Exp. Med. 25:520-530.

Olin, G. 1934. New research on tularemia in Sweden. Bull. Off. Intern. Hyg. Publ. 26:890-892.

Olsuf'ev, N. G. 1959. Certain differences in the tularemia pathogen in the old and new world. Vestnik Akademii Nauk SSSR. 14:51-58. (OTS: 59-13, 938. JPRS:L-1015-N Translation).

Olsuf'ev, N. G. and T. Dunaeva. 1951. Sensitivity to tularemia of the weasel, skunk and fox and their possible epidemiological significance. Zoologicheskii Zhurnal. 30:78-83.

Owen, C. R., Bell, J. F., Larson, C. L. and R. A. Ormsbee. 1955. Virulence of Bacterium tularense. II. Evaluation of cirteria of virulence of Bacterium tularense. J. Inf. Dis. 97:167-176.

Owen, C. R. and E. O. Buker. 1956. Factors involved in the transmission of Pasteurella tularensis from inoculated animals to healthy cage mates. J. Inf. Dis. 99:227-233.

Ozburn, R. H. 1944. Probelms of medical entomology of military importance in Canada. J. Econ. Entom. 37:455-459.

Parker, R. R. 1929. Quail as a source of tularemia infection in man. Pub. Health Repts. 44:999-1000.

Parker, R. R. 1945. Tularemia: A spontaneous occurrence in the chipmunk. Pub. Health Repts. 60:17.

Parker, R. R. and W. J. Butler. 1929. Results of preliminary investigations in Montana of pathological conditions in sheep due to the wood tick, Dermacentor andersoni Stiles. 7th Biennial Report of the State Board of Entomology, 1927-28, Helena, Mont., State Board of Entomology, 1929, pp. 77-85.

Parker, R. R. and J. S. Dade. 1929. Tularemia in sheep in nature. Pub. Health Repts. 44:126-130.

Parker, R. R. and J. S. Dade. 1929a. Tularemia: Its transmission to sheep by the wood tick, Dermacentor andersoni Stiles. J. Am. Vet. Med.Assoc. 75:173-191.

Parker, R. R. and Francis, E. 1926. Susceptibility of the coyote, Canis lestes to tularemia. Pub. Health Repts. 41:1407-1410.

Parker, R. R., Hearle, E. and E. A. Bruce. 1931. The occurrence of tularemia in British Columbia. Pub. Health Repts., 45:45-46.

Parker, R. R., Philip, C. B. and G. E. Davis. 1932. Tularemia: occurrence in the sage hen, Centrocercus urophasianus. Pub. Health Repts. 47:479-487.

Perry, J. C. 1928. Tularemia among meadow mice in California. Pub. Health Repts. 43:260-263.

Philip, C. B., Bell, J. F. and D. L. Larson. 1955. Evidence of Infectious diseases and parasites in a peak population of black-tailed jack rabbits in Nevada. Jour. Wildlife Mngmt. 19:225-233.

Philip, C. B. and G. E. Davis. 1935. Tularemia: observations on a strain of low initial virulence from rabbit ticks. Pub. Health Repts. 50:909-911.

Philip, C. B., Jellison, W. L. and H. F. Wilkins. 1935. Epizootic tick-borne tularemia in sheep in Montana. J. Am. Vet. Med. Assoc. 86:726-744.

Philip, C. B. and R. R. Parker. 1938. Occurrence of tularemia in the rabbit tick (Haemaphysalis leporis-palustris) in Alaska. Pub. Health Repts. 53:574-575.

Pollitzer, R. 1954. Plague. World Health Organization Monograph, series No. 22.

Quan, S. F. 1954. Quantitative oral infectivity of tularemia for laboratory animals. Amer. J. Hyg. 59:282-291.

Ransmeier, J. C. 1943. The reaction of the chick embryo to virulent and non-virulent strains of Bacterium tularense. J. Inf. Dis. 72:86-90.

Ribbi, E. and C. Shepard. 1955. Morphology of Bacterium tularense during its growth cycle in liquid medium as revealed by the electron microscope. Expt. Cell Res. 8:474-487.

Schlotthauer, C. F., Thompson, L. and C. Olson. 1935. Epizootic of tularemia in gray foxes. J. Inf. Dis. 56:28-30.

Schmidt, H. W. 1951. Die Entwichlung der Tularemie in Europa. Zbl. Allg. Path. Anat. 87:180-184.

Schmidt, G. C., Logan, M. A. and A. Tytell. 1952. The degradation of arginine by Clostridium perfringens (BP6K). J. Biol. Chem. 198:771-783.

Sherstinsky, H. 1951. Variations of virulence in a strain of Pasteurella tularensis. Bull. Rec. Coun. Israel. 1:159-160.

Shippee, E. A. and H. J. Egoscue. 1958. Additional mammal records from the Bonneville Basin, Utah. J. Mammalogy. 39:275-277.

Shmuter, M. F. and S. G. Abramova. 1956. A case of the isolation of a culture of Bacterium tularense from an American mink. Trudy Rostov-skogona-Donu gosudarstvennogo nauchno-issledovatel'skogo protivo-chumnogo instituta, tom X, Astrakan. pp. 229-230.

Sil'chenko, V. S. 1957. Thirty years study of tularemia in the Soviet Union. J. Microbiol., Epidem., Immunobiol. 28:1428-1413.

Simons, S. A., Stevens, I. M. and W. C. Reeves. 1953. Some epidemiological observations on tularemia in California, 1927-1951. Amer. J. Trop. Med. 2:483-494.

Slade, H. D. 1953. Hydrolysis of arginine by soluble enzymes of Streptococcus faecalis. Arch. Biochem. and Biophys. 42:204.

Slade, H. D., Doughty, C. C. and W. C. Slamp. 1954. The synthesis of high-energy phosphate in the citrulline ureidase reaction of soluble enzymes of Pseudomonas. Arch. Biochem. and Biophys. 48:338-346.

Snyder, T. L. 1947. The relative errors of bacteriological plate counting methods. J. Bact. 54:641-654.

Snyder, T. L., Penfield, R. A., Engley, F. B., Jr. and J. C. Creasy.1946. Tularemia: cultivation of Bacterium tularense in peptone media. Proc. Soc. Expt. Biol. Med. 63:26-30.

Stagg, G., Tanner, W. S. and J. Lavender. 1956. Experimental infections of native animals with Pasteurella tularensis. J. Inf. Dis. 99:34-37.

Stakman, E. C., Daley, J. M., Gattani, M. L. and I. Wahl. 1948. Variation induced by uranium nitrate in corn smut and the cultivated mushroom. Science. 108:554.

Stoenner, H. G., Holdenried, R., Lackman, D. and J. S. Orsborn, Jr. 1959. The occurrence of Coxiella burneti, Brucella and other pathogens among the fauna of the Great Salt Lake Desert in Utah. Am. J. Trop. Med. and Hyg. 8:590-596.

Sural'eva, R. A. 1958. An investigation into the pathogenesis of experimental tularemia produced by alimentary infection. J. Microbiol., Epidemiol., and Immunobiol., 29:1187-1192.

Tereshchenko, M. P. 1954. Quoted from Sural'eva, 1958. An investigation into the pathogenesis of experimental tularemia produced by alimentary infection. J. Microbiol., Epidemiol., and Immunobiol., 29:1187-1192.

Theiler, H. 1928. Attempted experimental infection of chickens with tularemia. Cornell Vet. 18:348-352.

Thjotta, T. 1930. Three cases of tularemia, a disease not hitherto diagnosed in Norway. Norsk. Mag. Laegevidenskap. 91:224-236 (Bull. Hyg. 5:490, 1930).

Tomaszunas, S. 1959. Attempts at attenuation of Bacterium tularense strains. Bull. Inst. Marine Med. Gdansk. 10:73-82.

Tovar, R. M. 1944. Tularemia in Mexico. I. Primer caso confirmada Bacteriologicamente. Medicina (Mexico). 24-25 and 343-351.

Traub, A., Mager, J. and N. Grozzowicz. 1955. Studies on the nutrition of Pasteurella tularensis.

Vail, E. L. and F. D. McKenny. 1943. Diseases of domestic animals. U. S. Dept. of Interior, Fish and Wildlife Service. Conservation Bull. 31.

Vest, E. D. and N. J. Marchette. 1958. Transmission of Pasteurella tularensis among desert rodents through infective carcasses. Science. 128:363-364.

Wherry, W. B. 1914. A new bacterial disease of rodents transmissible to man. Pub. Health Repts. 29:3387-3390.

White, P. G. and J. B. Wilson. 1951. Differentiation of smooth and non-smooth colonies of Brucella. J. Bact. 61:239-240.

Williams, R. B. 1946. Tularemia. First case to be reported in Alaska. Pub. Health Repts. 61:875-876.

Woodbury, A. M. and D. D. Parker. 1954. Ecology of the Great Salt Lake Desert. Studies of tularemia, Pasteurella tularensis. Special Report No. 2 to the U. S. Army Chemical Corps, Dugway Proving Ground, Dugway, Utah. June, 1953.

Yaniv, H. and Y. Avi-Dor. 1953. Relation between changes in the stability of Pasteurella tularensis suspensions and its bacterial population. II. Mutual influence between salt-agglutinations and salt-nonagglutinable types. J. Bact. 66:6-9.

Yaniv, H., Avi-Dor, Y. and A. L. Olitzki. 1953. In vitro development of streptomycin resistance in Bacterium tularense. Experientia. 9:23-24.

Yudenich, V. A. 1956. Distribution of the tularemia organism in the body of immune and non-immune animals. Abst. of Soviet Medicine. 1:168. 1957.

EXPERIMENTAL TULAREMIA IN WILD ANIMALS

by

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The susceptibility to experimental tularemia infection of thirteen species of wild animals native to the Great Salt Lake Desert of Utah was tested. Eleven species of rodents, Citellus leucurus, antelope ground squirrel; Eutamias minimus, least chipmunk; Perognathus formosus, long-tailed pocket mouse; Dipodomys ordii, Ord kangaroo rat; D. microps, Chisel-toothed kangaroo rat; Reithrodontomys megalotis, western harvest mouse; Peromyscus crinitus, canyon mouse; P.maniculatus, deer mouse; Onychomys leucogaster, northern grasshopper mouse; Neotoma lepida, desert wood rat; and Microtus montanus, montane meadow mouse, were all lethally susceptible to subcutaneous inoculation of as few as one viable organism of the Schu A strain of Pasteurella tularensis. Cottontails, Sylvilagus auduboni, and jack rabbits, Lepus californicus deserticola,were as susceptible as the rodents tested. However, a subspecies, L. c. texianus from New Mexico survived subcutaneous inoculation of more than 10^3 times the number of P. tularensis organisms necessary to kill the Utah subspecies, L. c. deserticola.

Massive doses (10^9 viable organisms or greater) of P. tularensis strain Schu A inoculated subcutaneously into coyote pups, Canis latrans lestes, caused the death of two out of the three tested. Inoculation of lower doses or exposure to oral infection caused only a mild, subacute infection in other pups of this species. No chronic infection or carrier state was detectable in the survivors.

The Jap_4 strain of P. tularensis, which is moderately virulent for laboratory albino mice, was found to be highly virulent for grasshopper mice, moderately virulent for deer mice and kangaroo rats, and avirulent for wood rats.

Twelve strains of P. tularensis isolated from wild rabbits, rodents, or ticks, and one strain isolated from a horse were highly virulent for grasshopper mice, wood rats, and/or deer mice. The 38 strain and the Russian NIIEG (gray var.) strains were avirulent for these rodents. The blue variant of the NIIEG strain, however, was moderately virulent for deer mice and wood rats.

In order to further characterize strains of P. tularensis in terms of a physiological parameter, five laboratory and 13 wild strains of varying virulence were tested for the ability to metabolize the amino acid citrulline. Only those strains which were of high virulence for mice, guinea pigs, and white rabbits were found to possess a citrulline ureidase enzyme system which converts citrulline to ornithine, NH_3 and CO_2. This enzyme system was absent from all avirulent strains and strains of low virulence for rabbits that were tested. The only wild strains of P. tularensis tested that lacked a citrulline ureidase enzyme system were two that had been isolated from a rodent (M. montanus) and rodent ticks (Dermacentor andersoni) respectively. These strains were also of low virulence for rabbits.

However, it was possible to demonstrate that there is no direct cause and effect relationship between the presence of a citrulline ureidase enzyme system and virulence of P. tularensis for rabbits. Colonial variants of the virulent-citrulline ureidase enzyme possessing Schu A strain were produced by culture of the parent strain for two weeks or longer in a peptone broth containing 0.01% uranium acetate followed by plating onto peptone agar. Under oblique light illumination, two distinct colonial variants, one smooth (S) in appearance and the other non-smooth (NS) were detected and isolated in pure culture.

Neither of these variants were virulent for white rabbits in doses as high as 10^7 organisms inoculated subcutaneously. However, both variants possessed citrulline ureidase enzyme activity equal to that of the parent organism.

Attempts to increase the virulence of low virulent strains of P. tularensis by passage through susceptible and resistant hosts were only partially sucessful. In no case was their virulence for rabbits increased, nor had they developed the ability to metabolize citrulline.

On the basis of the presence or absence of a citrulline ureidase enzyme system, it is postulated that there are two distinct varieties of P. tularensis in North America. The present study suggests, although it does not prove, the presence of a variety of P. tularensis which has evolved and is being maintained in the rodent and associated arthropod fauna, and one which has evolved and is maintained in the rabbit and its associated arthropod fauna. The rodent variety lacks a citrulline ureidase enzyme system and is avirulent or of low virulence for rabbits. The rabbit variety possesses a citrulline ureidase enzyme system and is highly virulent for rabbits.

RESEARCH PROPOSALS

1. To determine if a citrulline ureidase enzyme system can be induced in strains of Pasteurella tularensis lacking this system, and if so, to determine the effect on virulence.

2. To determine the geographic and host distribution of the two varieties of P. tularensis postulated to exist on the basis of virulence and the presence or absence of a citrulline ureidase enzyme system.

3. To determine if the citrulline ureidase enzyme system present in some strains of P. tularensis functions in the metabolism of arginine.

4. To determine what metabolic differences besides ability to metabolize citrulline exist between the two postulated varieties of P. tularensis.

5. To test, by use of simulated natural wild populations, the hypothesis that there is a variety of P. tularensis peculiarly adapted to rodents and one peculiarly adapted to rabbits.

6. To compare the pathogenicity in wild rabbits of a rodent strain and a rabbit strain of P. tularensis.

7. To determine if there is a physiological basis (in the host) for the resistance of rabbits to some strains of P. tularensis and their susceptibility to others.

8. To determine the serological response of wild jack rabbits to sublethal infection with P. tularensis.

9. To determine what effect passage through natural and un-natural hosts has on the virulence and physiology of various strains of P. tularensis.

10. To determine how rodent tularemia is transmitted and maintained in nature.