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ABSTRACT

Experiments are reported concerning the influence of low ambient temperatures on mice which have been challenged with K. pneumoniae. The metabolic behavior and virulence of the organisms isolated from mice maintained at 2^{o} or 21^{o} C were investigated. The oxygen uptake of the organisms appeared to be directly proportional to the temperature of incubation. The growth ability of K. pneumoniae isolated from mice maintained at 2^{o} C appeared to be temperature independent between 37^{o} and 32^{o} C, whereas the K. pneumoniae isolated from mice maintained at 21^{o} C exhibited growth curves that were temperature dependent. The virulence of the K. pneumoniae isolated from mice maintained at 2^{o} or 21^{o} C did not appear altered.

STUDIES ON KLEBSIELLA PNEUMONIAE PASSED THROUGH MICE MAINTAINED AT LOW AMBIENT TEMPERATURES*

Numerous studies on the effect of temperature on the disease course of various infectious agents have been reported. Sulkin (1945) reported that environmental temperatures had little effect on the mortality of mice challenged with type A influenza virus. Similar results have been reported by Sarracino and Soule (1941), who concluded that the appearance of influenza disease in an individual is a function of the amount and virulence of the virus rather than related to the general resistance of the host. Muschenheim, et al. (1943) reported that reduced temperatures caused a decrease in local inflammatory reactions but were without influence on the bacteremia and death of rabbits challenged with pneumococcus type I. Furthermore, Castaneda (1937) reported that lowering of the core temperature of guinea pigs, rabbits and sheep as little as 1^{o} to 4^{o} C resulted in good growth of rickettsia in the peritoneal cavities of these animals. Conversely, temperatures greater than 39^{o} C definitely interfered with growth of the rickettsia. It is of interest to note that phagocytic activities of animals are reported to be more influenced by the general health of the host than by temperature changes (Muschenheim, et al., 1943; Cottingham and Mills, 1943).

In the above reports it was consistently noted that the investigators failed to characterize the metabolic behavior and virulence of the challenge agent at the temperatures employed in their experiments. The experiments to be described were undertaken to determine if any changes in metabolic behavior and virulence of K. pneumoniae occurred when the organism was passed in mice chronically exposed to low ambient temperatures.

MATERIALS AND METHODS

Klebsiella pneumoniae obtained from departmental stock cultures was maintained on heart infusion blood agar slants (Difco). Adult albino mice (Mus musculus) obtained

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from local sources were employed in the experiments. The low ambient temperatures were obtained by use of walk-in refrigerators. The temperature of the refrigerator used in these experiments was 2^{o} C and did not vary more than $\pm 1^{o}$ C during the time the mice were kept in the box. Mice were also maintained at room temperature ($21^{o} \pm 1^{o}$ C) as controls. The temperature of the refrigerators and the animal room was constantly monitored with a calibrated temperature recording instrument (Tempscribe, Bacharach Industrial Instrument Company, Pittsburg).

Mouse temperature measurements. Mouse temperatures were obtained from the core, skin and upper respiratory cavity by calibrated probes and were recorded by an Electric Universal Thermometer type TE3 (Chemical and Pharmaceutical Industry Company, Inc., New York). Specifically, the core temperatures were obtained with model R.M. 4 probes inserted 2 cm into the rectum. The skin temperatures were obtained with model H. 1 probes placed on the center of the abdominal cavity. Upper respiratory cavity measurements were obtained with a model K. 8 probe suitably bent to be inserted to the region of the upper respiratory cavity. Anatomical considerations prevented the probe from being inserted into the trachea; to avoid trauma in the mice, the probes were inserted the same distance into each mouse (approximately to the region of the lower pharynx). For purposes of discussion the temperatures obtained in this region are taken to be indicative of the upper respiratory cavity.

Passage of organism. A single isolated colony of K. pneumoniae was inoculated into tryptose phosphate broth (Difco) and incubated for 18 hours at 37^{o} C. The passage inoculum was 0.1 ml of this broth suspension of organisms per mouse intraperitoneally. At the end of 24 hours the surviving mice were sacrificed by decapitation. The peritoneal cavities were opened aseptically and the peritoneal exudate was obtained. This exudate was reinjected into a number of mice after a sample had been seeded to heart infusion blood agar plates (Difco) for isolation and identification procedures. This procedure was repeated each day for 7 days. The mice that were inoculated were either kept at $21^{o} \pm 1^{o}$ C or $2^{o} \pm 1^{o}$ C during this time of organism passage. The mice had been kept at these respective temperatures for 30 days prior to injection with the organisms. The K. pneumoniae isolated from the animals kept at 2^{o} C was incubated at room temperature (21^{o} C), while the organisms isolated from the animals kept at 21^{o} C were incubated at 37^{o} C.

LD_{50} determinations. The LD_{50} of the K. pneumoniae isolated from the mice kept

at the two ambient temperatures was determined by employing the procedure of Litchfield and Wilcoxon (1949).

Oxygen uptake. Oxygen uptake studies were conducted by employing the Warburg constant volume respirometer technique (Umbreit, et al., 1957). The temperatures chosen for the studies were 37° C and 32° C, and variation from these temperatures was no greater than ± 0.5° C during the time of the experiments. The reaction flask contained 2.5 ml of tryptose phosphate broth (Difco) with 10% normal mouse serum as substrate. The bacteria were added in 0.5 ml volumes taken from 18-hour broth cultures which were standardized to contain approximately the same number of organisms per ml. The center well contained 0.2 ml of 20% KOH plus a 1 cm^2 fluted filter paper to increase the CO_2 absorbing area. Duplicate flasks were set up, and an equilibration period of 15 minutes was allowed before the system was closed.

Growth characteristics. Growth of the bacteria was determined by employing turbidity as a measure of growth. The change in turbidity of the growing cultures was quantitated in a Klett-Summerson photoelectric colorimeter with a blue filter. The tryptose phosphate broth (Difco) containing 10% normal mouse serum was seeded with a standard inoculum. The number of organisms present for any given turbidity measurement was determined by reference to a standard curve relating organisms per ml as function of optical density. The temperatures of incubation chosen for these studies were 37° C and 32° C, and variation in temperature during the time of the experiments was not greater than ± 0.5° C.

RESULTS

Mouse temperature measurements. The results of well-designed experiments of other workers were obscure or made equivocal by failure to observe precautions such as whether the animals were allowed to huddle (Sulkin, 1945; Sarracino and Soule, 1941; Muschenheim, et al., 1943; Castaneda, 1937; Cottingham and Mills, 1943). In order to determine the effect huddling had on mouse temperature measurements, the following experiment was conducted. Mice were placed at 2° C or 21° C in cages containing either a single mouse or a group of five mice. Core, skin and upper respiratory cavity temp-

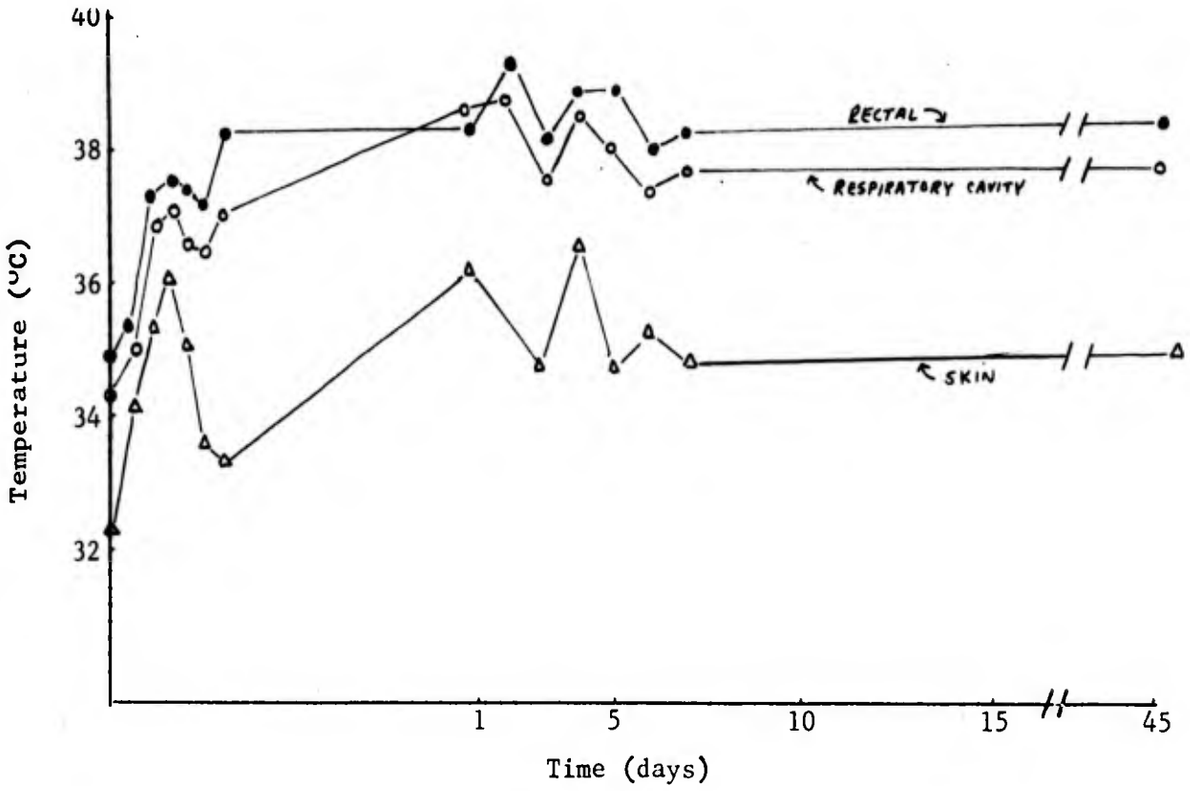

Figure 1. Effect of grouping (5 mice/group) on average temperatures of mice maintained at $2^{o} \pm 1^{o}C$.

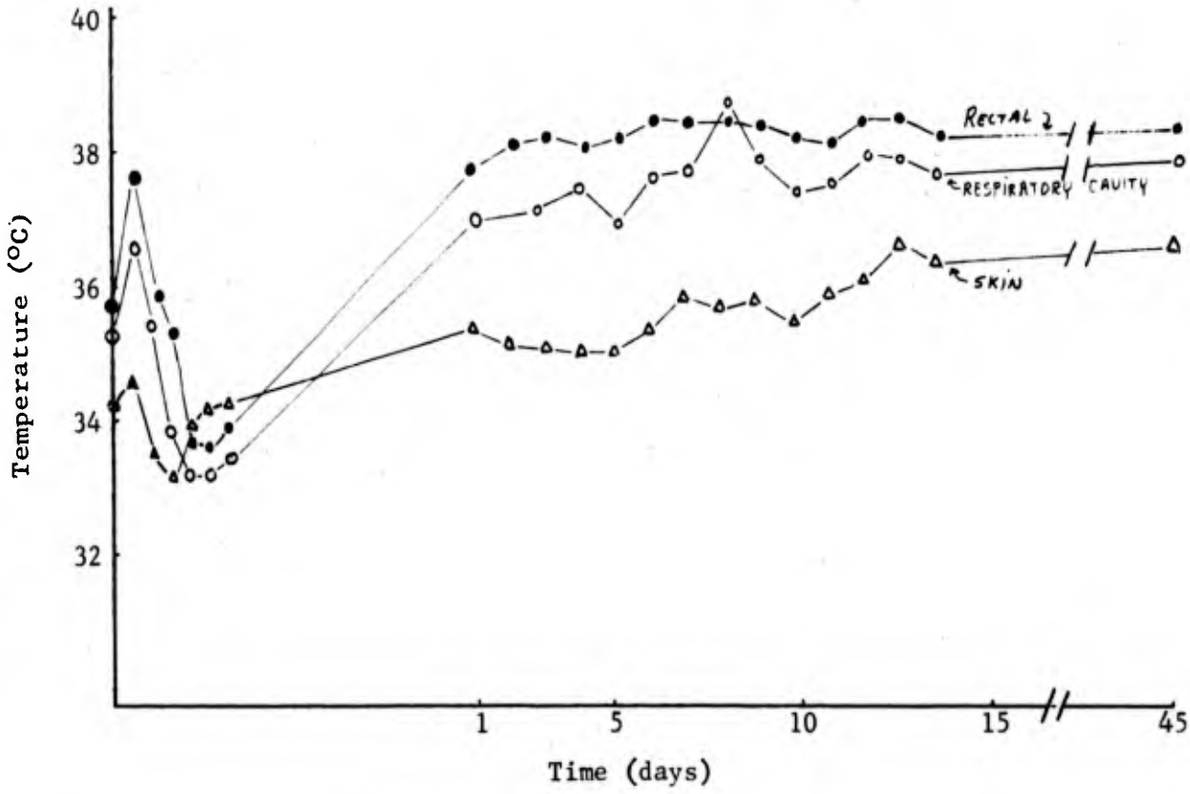

Figure 2. Effect of single caging on average temperatures of mice maintained at $2^{o} \pm 1^{o}C$ (average of 10 mice).

eratures were taken hourly for the first 4 to 5 hours on the first day, then once a day for 8 to 14 days, and finally on the 45th day of exposure. It can be seen in Figure 1 that the placing of five mice in one cage at 2^{o} C results in temperature measurements that increase gradually, reaching a maximum in 2 to 4 hours after exposure. The rectal temperature generally is consistently higher than the upper respiratory cavity, but it does not appear to be significantly greater. The skin temperatures are considerably less than the rectal or upper respiratory cavity temperatures, and in general the three temperature curves appear to parallel each other.

In Figure 2 are presented the results of temperature measurements obtained on singly-caged mice maintained at 2^{o} C. Again it is seen that the three curves parallel each other and that the magnitude of the temperatures is of the order rectal > upper respiratory cavity > skin. The rectal temperatures are not significantly greater than the upper respiratory cavity temperatures, but they do show consistently higher values. Similar to the results obtained with the grouped mice, the skin temperatures of the singly-caged mice are considerably lower than the rectal and upper respiratory cavity temperatures. It is of interest to note that an initial rise in temperature occurs within one hour after exposure to the low ambient environment, followed by a sharp drop in temperatures which reaches a maximal fall by four hours post-exposure. From this point the temperatures gradually rise to reach a stabilizing point by 24 hours post-exposure. The time of stabilization appears to be the same as required for the grouped mice.

When temperatures are measured on mice grouped five per cage and maintained at 21^{o} C, the results shown in Figure 3 are obtained. The rectal and upper respiratory cavity temperatures are about the same in magnitude, while the skin shows temperatures ranging about 2^{o} to 2.5^{o} C less. The temperature curves again parallel each other, but they do not exhibit any marked fluctuations as seen with mice maintained singly or grouped at 2^{o} C. Essentially, the temperatures of the mice remained quite constant throughout the experiment.

The data charted in Figure 4 demonstrate that mice singly caged and kept at 21o C exhibited temperature curves that were almost identical with those obtained with grouped mice maintained at the same ambient temperature.

Studies on K. Pneumoniae

Growth studies. The K. pneumoniae passed through the mice maintained at 2^{o} C was compared with the K. pneumoniae passed through mice maintained at 21^{o} C with

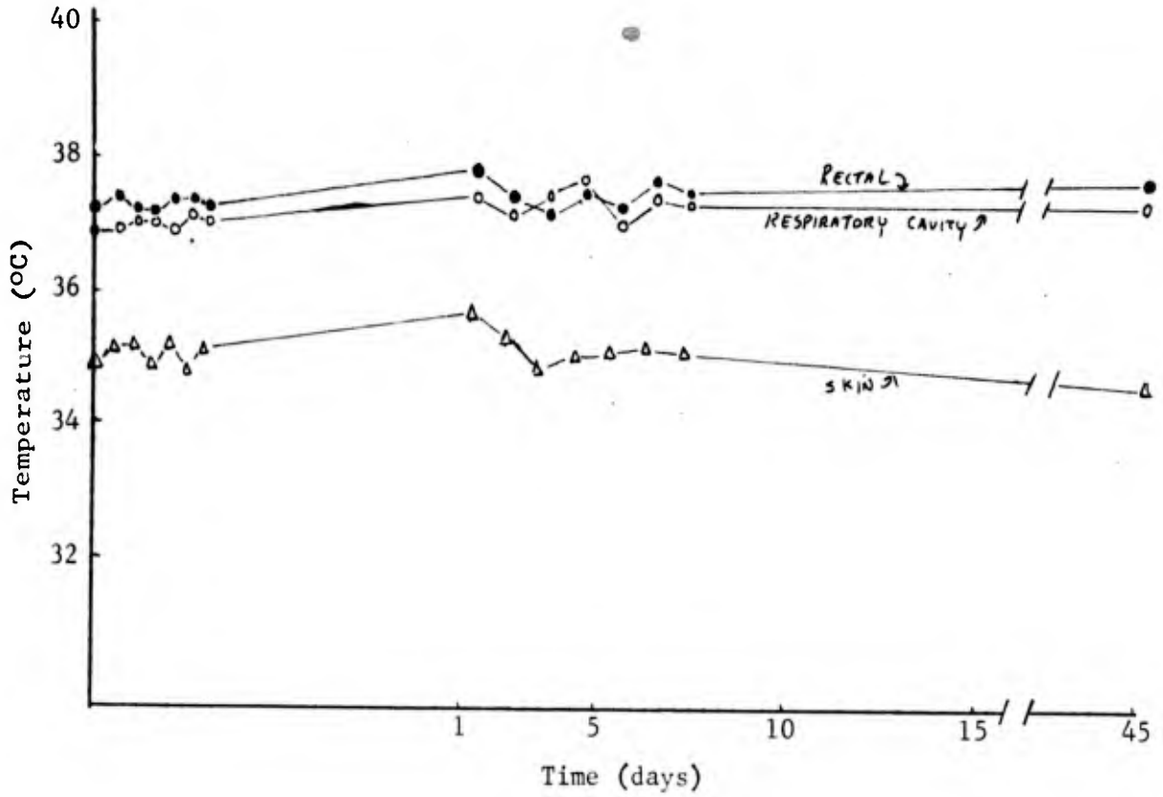

Figure 3. Effect of grouping (5 mice/group) on average temperatures of mice maintained at 21° ± 1°C.

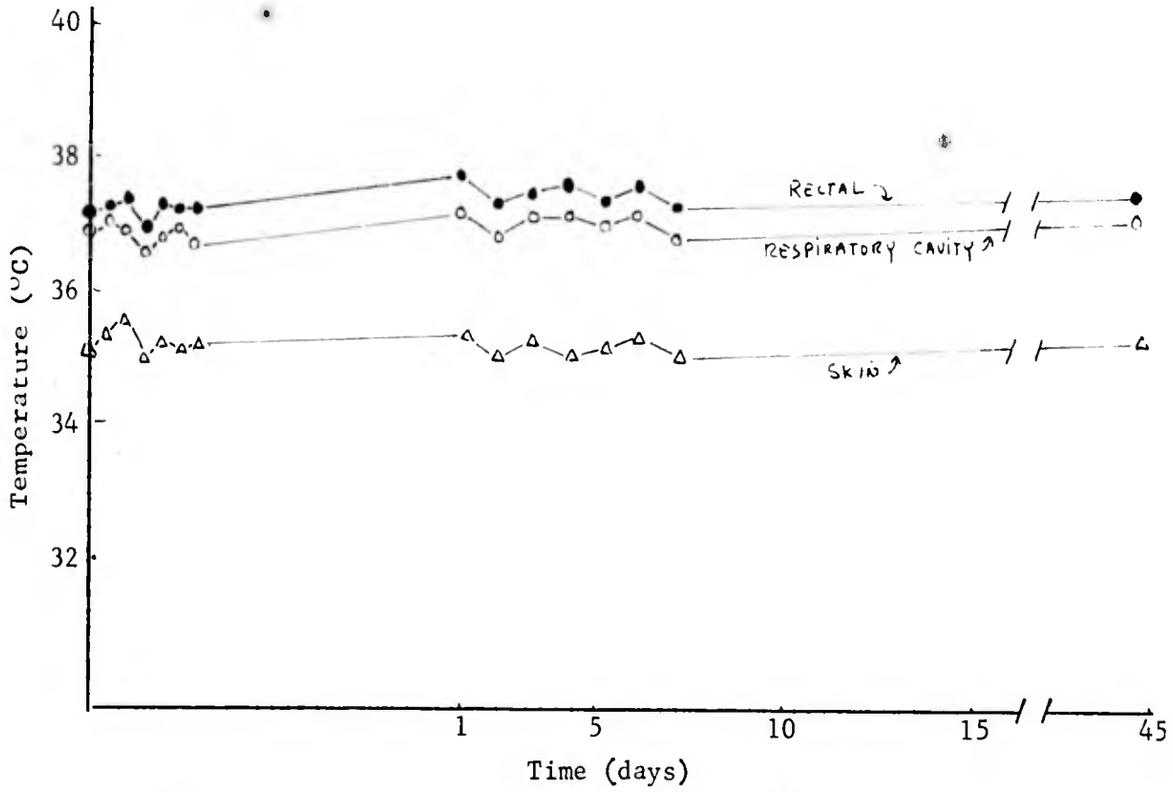

Figure 4. Effect of single caging on average temperatures of mice maintained at 21° ± 1°C (average of 10 mice).

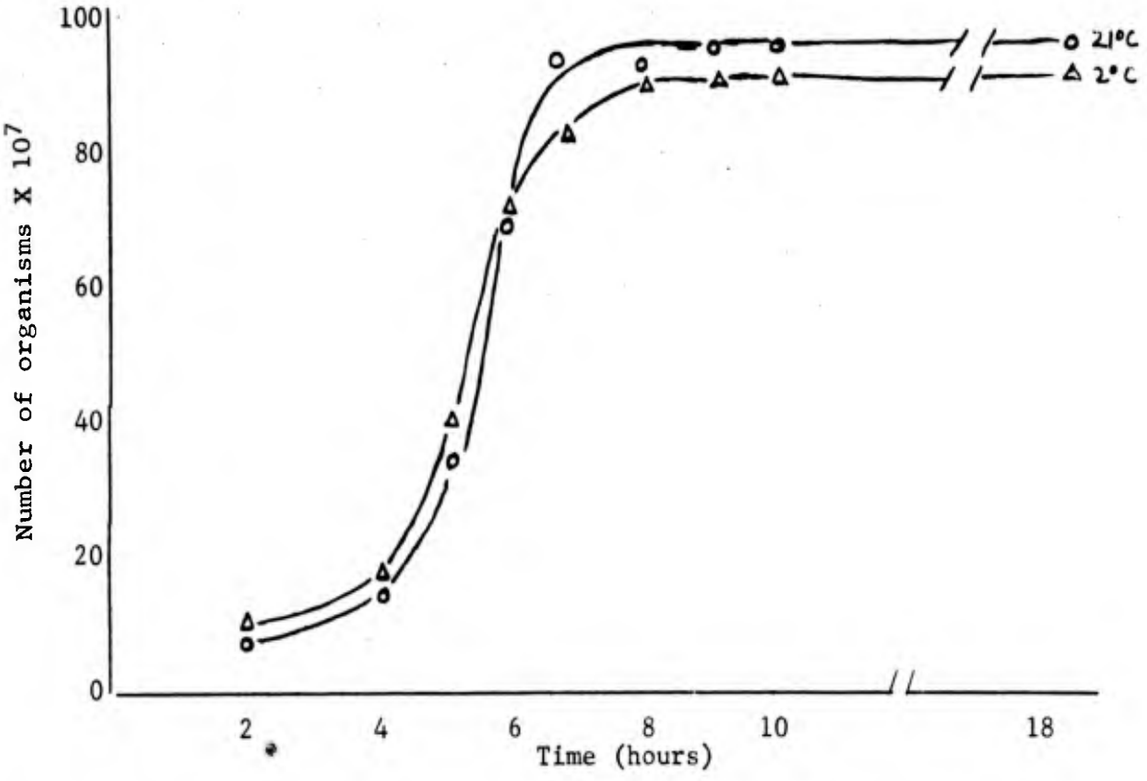

Figure 5. Effect of 37°C incubation temperature on growth behavior of K. pneumoniae isolated from mice maintained at 2°C and 21°C.

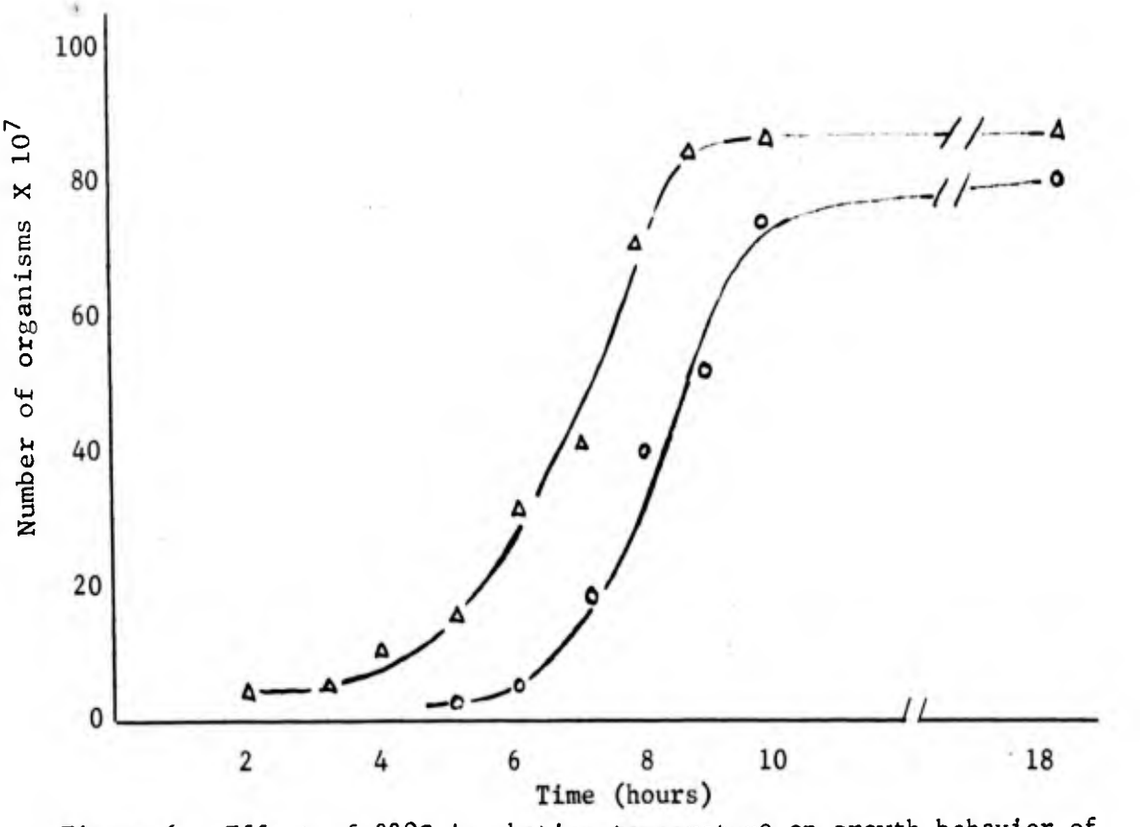

Figure 6. Effect of 32°C incubation temperature on growth behavior of K. pneumoniae isolated from mice maintained at 2°C and 21°C.

respect to their growth behavior at different temperatures. Previous studies (Marcus, et al., 1960a) indicated that temperature (25°, 32°, and 37° C) had no significant effect on the generation time of the organism, but only delayed the lag phase as the temperature of incubation was lowered to 25° C. The hypothesis was offered that an organism conditioned or adapted to lower temperatures would exhibit the same lag phase as an organism incubated under temperature conditions optimal for its shortened lag phase (i. e., at 37° C). Since rectal temperatures obtained from mice maintained singly in cages at 2° C drop to as low as 33.5° C early in the exposure period, it was felt that the organism to be used as the challenge agent in acute and chronic exposure experiments would necessarily need to grow optimally at all temperatures of fluctuations in order to evaluate the results. The growth curves of the isolated K. pneumoniae organisms when incubated at 37° and 32° C are shown in Figures 5 and 6. It is seen in Figure 5 that the bacteria isolated from mice maintained at 2° and 21° C exhibit very similar growth curves when these organisms are incubated at 37° C. However, a different picture is seen when the same organisms are incubated at 32° C (Fig. 6). It is apparent that the K. pneumoniae isolated from mice maintained at 2° C begin their logarithmic phase earlier than the organisms isolated from mice maintained at 21° C. It is of interest to note that the effect of a 5° C decrease in incubation temperature does not significantly affect the growth behavior of the K. pneumoniae isolated from mice maintained at 2° C (compare Figure 6 with Figure 5).

Oxygen uptake studies. Oxygen uptake studies of the organisms isolated from mice maintained at 2° and 21° C were conducted at 37° and 32° C. The flasks contained approximately the same number of organisms initially since care was taken to standardize the different broth suspensions used as inoculum. The results of the experiment conducted at 37° C are shown in Figure 7. It is seen that the K. pneumoniae isolated from mice maintained at 2° and 21° C have similar oxygen uptake curves when incubated at 37° C. However, the K. pneumoniae isolated from mice maintained at 2° C has greater oxygen uptake activity than do the organisms isolated from mice maintained at 21° C for any given increment of time (Fig. 8). The oxygen consumption is less than that obtained at 37° C, since comparison of Figure 8 with Figure 7 shows that for any given time interval there is greater consumption (twofold increase) at 37° C.

Virulence studies. The virulence of the K. pneumoniae isolated from mice maintained at 2° C was compared to the K. pneumoniae isolated from mice maintained at 21° C. The results are summarized in Table I. It is seen that there is no significant difference or changes in virulence in the organisms. The results are in agreement with those previously obtained (Marcus, et al., 1955 and 1960b).

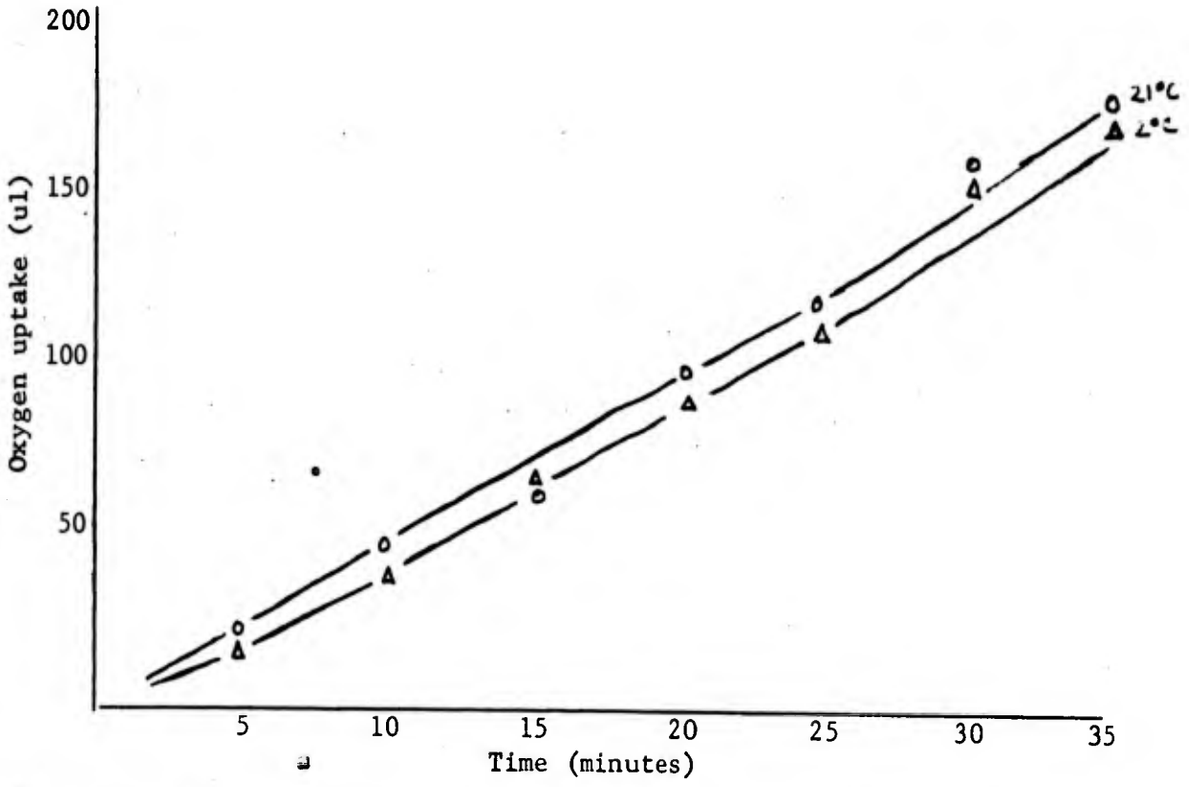

Figure 7. Effect of 37°C incubation temperature on oxygen uptake of K. pneumoniae isolated from mice maintained at 2°C and 21°C.

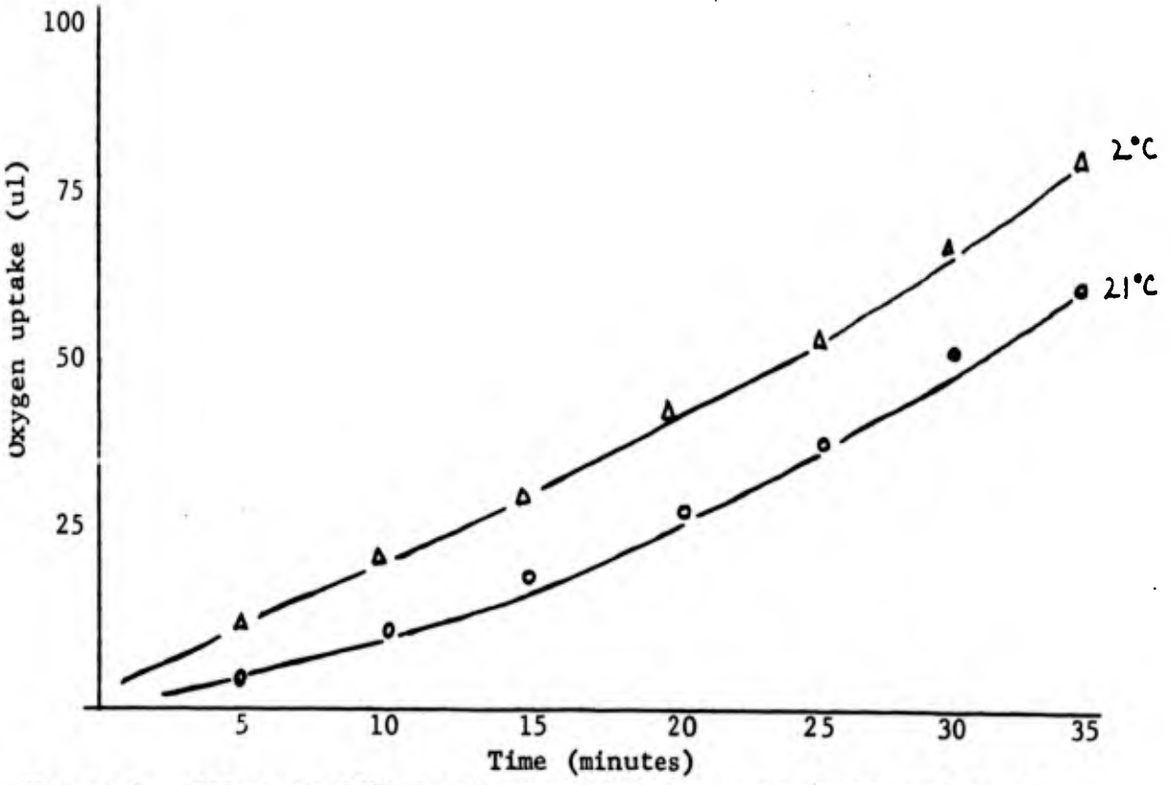

Figure 8. Effect of 32°C incubation temperature on oxygen uptake of K. pneumoniae isolated from mice maintained at 2°C and 21°C.

TABLE I

LD_{50} VALUES OF KLEBSIELLA PNEUMONIAE ISOLATED FROM MICE MAINTAINED AT 2° C (P) and 21° C (NP) FOLLOWING INTRAPERITONEAL INJECTION.

Temperature of Experiment	Dose	Organism	Mortality Ratio	LD_{50} (95% Confidence Limits)
2° C	1040		10/10	
	104	P	9/10	20
	10.4		4/10	(14.1 - 28.4)
	1.04		0/10	
2° C	1040		10/10	
	104	NP	8/10	35
	10.4		3/10	(14.6 - 84.0)
	1.04		0/10	
21° C	1040		10/10	
	104	P	7/10	35
	10.4		4/10	(15.9 - 77.0)
	1.04		0/10	
21° C	1040		10/10	
	104	NP	4/10	35
	10.4		5/10	(3.5 - 350)
	1.04		0/10	

DISCUSSION

Mouse temperature measurements taken from the core, skin or upper respiratory cavity parallel each other during the course of chronic exposure to low ambient temperatures. The rectal temperature generally showed the highest temperature values, with the upper respiratory cavity and skin following in that order. The low skin temperatures are not unusual since there is a greater surface area for increased heat loss. In addition, vasoconstriction occurs as a compensatory mechanism in animals exposed to low temperatures, thus leading to a decreased blood flow to the surfaces exposed to the cold. The temperature of the upper respiratory cavity was intermediate between the rectal and the skin, but tended to approach that of the rectum rather than the skin. This is not unexpected since the upper respiratory cavity is somewhat shielded from direct exposure to cold air, and the incoming air is warmed by countercurrent exchange as it progresses to the lungs. It is our opinion that in this study the rectal temperature more accurately reflected the temperature of the mouse than did the skin or upper respiratory cavity.

It is of interest to note that mice kept singly in cages exhibited fluctuations in temperature early in the chronic exposure period, in contrast to mice caged in groups of five. The initial increase in temperature seen with the singly-caged mice is ascribed to the immediate increase in body heat due to reflex heat regulation. This initial effect of body heat increase is either transient or inadequate since a sharp drop in temperature occurs which reaches a maximum in four hours and then gradually rises due to initiation of compensating heat mechanisms (shivering, increased metabolic rate). The temperature stabilizes by 24 hours but at final temperatures that are 2^{o} and 3^{o} C higher than the temperatures recorded at the beginning of the exposure to cold. This same increase in temperature is seen with grouped mice kept in the cold; the only difference is that no initial fluctuations occurred. The lack of fluctuating temperatures is probably due to huddling of the mice in an effort to conserve or minimize body heat loss.

Regardless of whether the mice were caged singly or in groups, the temperatures remained quite constant from the second day until the 45th day of exposure to cold. However, the level of temperature stabilization was 2^{o} to 3^{o} higher than the baseline

temperature. In contrast, when mice were kept at 21° C no fluctuations of baseline changes in temperatures occurred whether the mice were caged singly or in groups. The measured temperatures were not significantly altered from the baseline values as the experiment progressed. We believe that a likely explanation is that the degree of temperature stress imposed on mice kept at 21° C is not nearly so severe as that on mice exposed to 2° C. No huddling occurred at the higher temperature, and normal movements were the rule rather than the exception.

The metabolic behavior of the K. pneumoniae isolated from mice exposed to 2° C or 21° C was investigated as to growth and oxygen uptake activity. The bacteria isolated from the mice exposed to 2° C appeared to grow equally well at 37° or 32° C, whereas the organisms isolated from mice kept at 21° C showed a definite decrease in growth ability when incubated at 32° C, as compared to growth at 37° C. This is what one would expect and is in good agreement with results of others (Marcus, et al., 1960a; Barber, 1908; Graham-Smith, 1920). However, it might be expected that adaptation of an organism to a certain temperature would endow this organism with minimal lag phase when cultured at the temperature of adaptation. In the experiments reported herein, it is apparent that the organisms grown in mice whose rectal temperatures were 38° to 39° C exhibited completely unexpected results. Normally the mouse has a rectal temperature of about 37° C (Hart, 1951) when kept at 21° C, yet K. pneumoniae isolated from mice kept at 21° C exhibit growth behavior that is temperature dependent. In contrast, the K. pneumoniae isolated from mice kept at 2° C (rectal temperatures of 38° to 39° C) exhibit growth curves that appear to be temperature independent, at least between 37° and 32° C.

The oxygen uptake studies correlate well with known effects of temperature on enzymatic reactions, regardless of whether the K. pneumoniae tested is isolated from mice kept at 2° or 21° C. It is of interest to note that the K. pneumoniae isolated from mice maintained at 2° C exhibited consistently higher oxygen uptake activity than organisms isolated from mice kept at 21° C when both isolates were incubated at 32° C. The K. pneumoniae appears to grow well under conditions that cause a decrease in oxygen uptake. The explanation for this observation is not readily apparent.

The virulence of the organism does not appear to be influenced by passage into mice kept at 2° or 21° C. The LD_{50} of the K. pneumoniae isolates was found to be within the range of virulence reported by others (Marcus, et al., 1955 and 1960b). Questionable suggestion toward enhanced virulence of both types of isolates appeared to occur if the ambient temperature of the mice was low. This aspect of the experiment is being further pursued in an effort to gather more definitive evidence.

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