LYSINE DEFICIENCY AND HOST RESISTANCE TO ANTHRAX

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PLATE 19

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The interaction between the state of nutrition and the resistance of a host to infectious disease is well established. Recent reviews by Dubos and Schaedler (1), Scrimshaw *et al.* (2), and Schneider (3, 4) describe very adequately the state of our present knowledge. Although many data have been accumulated demonstrating the decreased resistance to several bacterial infections brought about by selected nutritional deficiencies, the mechanisms responsible for such changes are still obscure. The purpose of the studies reported here was to follow the host response to an organism and to determine how specific modification of the host will affect this response. The host selected was the rat and the parasite, *Bacillus anthracis*. In 1879, Feser (5) reported that rats on a meat diet were more resistant to anthrax than those on a bread diet. Although little has been done to study this change, it is now accepted that herbivorous animals are more sensitive to anthrax than are carnivores (6, 7). Despite this knowledge, no specific approach to the cause of this difference has been pursued. It is with this end in view that the series of studies to be reported here was undertaken.

Material and Methods

Female white rats, Sprague-Dawley strain, were obtained from a commercial source.[1] The animals were weaned, shipped by the supplier and received at our laboratory at 22 days of age. The rats, littermates, were allocated at random into appropriate sized groups and placed on a predetermined diet. The rats were caged in groups of five, weighed as a group, and the weight per rat calculated. The animals were weighed on day of arrival and at weekly intervals. Rats on experimental diets were kept on screened cage floors, whereas those on control diets were kept on wood shavings. The diets were obtained commercially.[2] The composition of the diets is as listed in Table I.

The experimental diet (G) was one designed to produce a lysine deficiency in the rats. In this diet, the casein of diet C was replaced by gluten and the caloric balance was maintained by increasing the cornstarch as necessary. In the experiments in which the effect of dietary supplements was studied, the desired amino acid, L-lysine (GL), L-methionine (GM), or both (GLM) was added to diet G in an amount to make the specific amino acid concentration equal to that in control diet C. The animals were allowed both food and water *ad libitum*. The diets were prepared by mixing 2000 gm of the dry powder with 2000 ml water and baking at approximately 350°F until set. The resulting cake was cut into squares and given to the animals. The rats were maintained on the diet for at least 30 days.

[1] Charles River Breeding Laboratories, Brookline, Massachusetts.

[2] Nutritional Biochemical Company, Cleveland.

The activity of the fixed macrophages of the reticuloendothelial system (RES) as reviewed by Benacerraf *et al.* (8) and Dobson (9) was studied using colloidal chromic phosphate–P^{32}. 0.5 ml $CrP^{32}O_4$, obtained from a commercial source,[3] was injected into the tail vein of the rat. The animals were stunned by a blow to the head and sacrificed by exsanguination at 1, 3, 5, 7, 9, 11, 13, 15, 20, 25, and 30 minutes following inoculation. The disappearance rates were calculated by the method of least squares as summarized by Batson (10). The liver and spleen were removed, weighed, and assayed for radioactivity. In those experiments in which 24 hour excretion of $CrP^{32}O_4$ was followed, the rats were placed in appropriate metabolic cages in which the urine and feces were separated and collected each 24 hour period following the injection.

In all experiments using $CrP^{32}O_4$, approximately 30 μc of radioactivity were injected.

To follow the fate of injected spores, the organisms were labeled with radioactive I^{131}. Washed spores were suspended in phosphate buffer, pH 7.4, to which was added 4.2 ml of a

TABLE I

Composition of the Control (C) and Experimental (G) Diets

Nutrient	Composition	
	Control diet (C)	Experimental diet (G)
	per cent	*per cent*
Vitamin-free casein	27	0
Gluten	0	20
Cornstarch	59	66
Corn oil	10	10
Salt mixture U.S.P. XIV	4	4
Vitamin diet fortification mixture, *gm/100 pounds diet*	152	152

mixture containing 0.7 ml of a solution of 126 mg I_2 in 100 ml 0.1 M KI and 3.5 of stock carrier-free I^{131} (containing 1 to 2 mc) as received from the supplier.[4] The spores remained in this mixture for 15 minutes. In the first experiments, the spores were dialyzed against distilled water to remove unbound I^{131} and finally made up to the desired concentration. However, it was found that the unbound I^{131} could be removed by centrifugation and washing 3 times with distilled water. The final preparation contained less than 5 per cent unbound I^{131}. The urine was collected from individual animals housed in appropriate metabolic cages.

All radioactivity was measured to an error of 5 per cent or less in a well type scintillation counter.

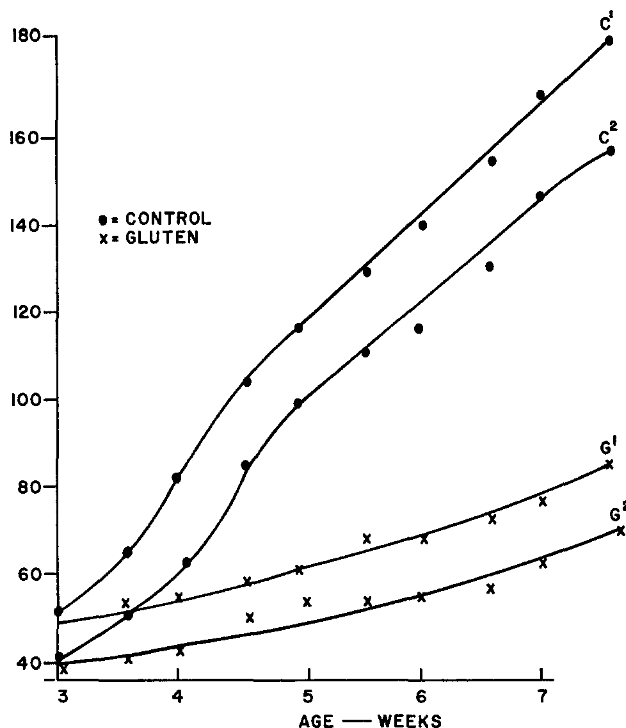
The challenge organism was the Vollum-1-b (lot 189) strain of *B. anthracis*. Spores were heat-shocked 48 hours prior to challenge which was by the subcutaneous route. Except for titrations of mortality studies, the challenge dose was 10^7 spores. After inoculation, daily mortality was recorded.[5] Inasmuch as it was determined in preliminary experiments that deaths rarely occurred after 6 days, all experiments were discontinued at that time.

[3] Abbott Laboratories, North Chicago. Colloidal chromic phosphate–P^{32}, 0.4 mg/ml, particle size 0.2μ to 2.0μ in 25 per cent glucose containing 2.0 per cent benzyl alcohol.

[4] Abbott Laboratories, North Chicago. Oriodide, carrier-free NaI^{131} in isotonic NaCl containing 0.9 per cent benzyl alcohol and 0.2 per cent cysteine HCl.

[5] Inoculation of the virulent organism and recording of deaths were carried out by the Animal Assessment Division of this unit.

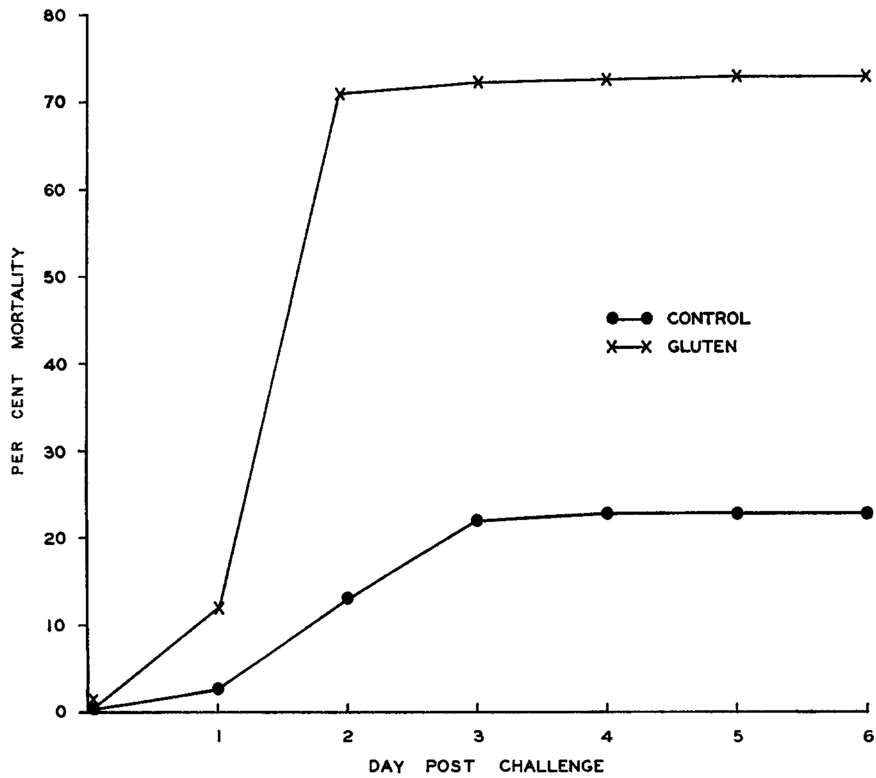
The data contained in Table V were obtained by standard clinical laboratory methods except for the lysozyme determinations. The latter were carried out as reported by Ribble (11) with the following details. Rat plasma was used at a 1/40 dilution and the standard was 5 μg/ml. For spleen concentration, the whole organ was homogenized in 10 ml of physiological saline, centrifuged at 1500 RPM for 30 minutes and 4 ml of the supernatant fluid taken for analysis. For these studies the rats were stunned by a blow to the head and sacrificed by exsanguination.



TEXT-FIG. 1. The rate of growth of Sprague-Dawley female, littermate, rats maintained on the control (C), 27 per cent casein diet or the experimental (G), 20 per cent gluten diet. Superscripts indicate code of two separate experiments.

RESULTS

Text-figs. 1 and 2, Fig. 1, and Tables II and III illustrate the response of the rats to the experimental diet and the concomitant changes in resistance. When these animals are challenged with differing concentrations of *B. anthracis* spores, it is apparent that the lysine-deficient animals are less resistant to the infection at all levels of challenge and that this change in resistance is an acute phenomenon. It is seen that except for an occasional animal, all deaths in the control group occur by the 3rd day post inoculation. This suggests that the decreased resistance lies in the immediate host reaction to the challenge.



TEXT-FIG. 2. The effect of diet of rats on the mortality rate due to anthrax. The rats were challenged with 10^7 spores of *B. anthracis* after being on the selected diet for 30 days. Each point is the mean of at least 40 animals.

TABLE II

Relation between Challenge Dose, Mortality, and Diet

Dose	Cumulative mortality			
	Control		Gluten	
	Dead/Total	Per cent	Dead/Total	Per cent
10^{10}	22/40	55		
10^9	16/40	40	39/40	98
10^8	16/40	40	39/40	98
10^7	14/40	35	36/40	90
10^6	19/50	38	38/50	76
10^5			25/50	50

TABLE III

The Effect of Diet on Cumulative Mortality of Rats Challenged with 10^7 B. Anthracis Spores (Vollum)

Exp. No.	Control	Gluten
1	7/15	13/15
2	1/15	14/15
3	6/10	9/10
4	5/20	16/20
5	3/20	15/20
6	7/20	15/20
Total....................	29/100	82/100
Per cent.................	29	82

TABLE IV

The Effect of Specific Amino Acid Supplementation of the Diet on Mortality Due to Challenge of Rats by 10^7 B. anthracis Spores

Exp. No.	Diet				
	C	G	GL	GM	GLM
4	5/20*	16/20	8/20	16/20	9/20
5	3/20	15/20	6/10	9/10	7/10
8	3/15	10/15	5/15	9/15	8/15
Total........	11/55	41/55	19/45	33/45	24/45
Per cent......	20	75	42	73	53
Wt. per rat, *gm*.........	165	83	159	84	146

p Values by χ^2 test of significance

Diet	G	GL	GM	GLM
C	<0.001	<0.001	<0.001	<0.001
G	—	<0.001	Not sig.	<0.05
GL	—	—	<0.01	Not sig.
GM	—	—	—	<0.01

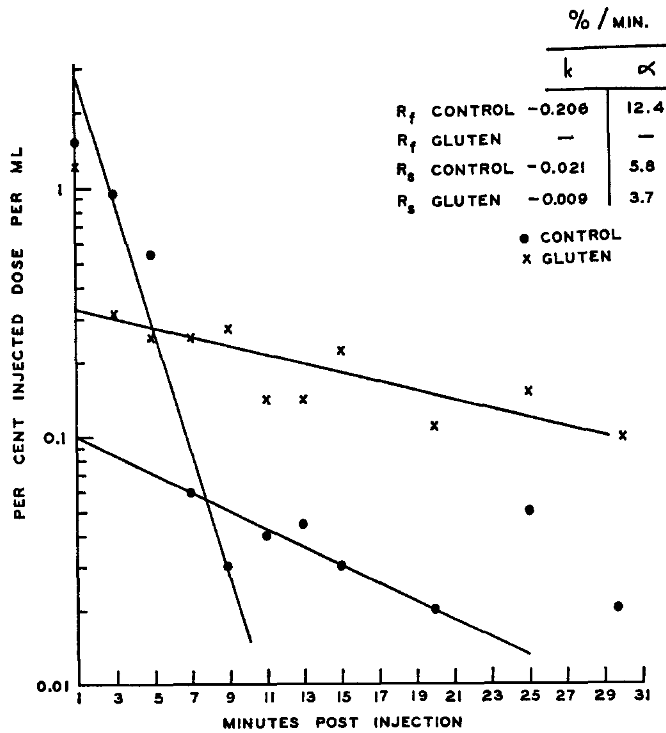
C, control diet; G, gluten diet; GL, G + Lysine; GM, G + Methionine; GLM, G + Lysine + Methionine.

* No. Dead/No. Challenged

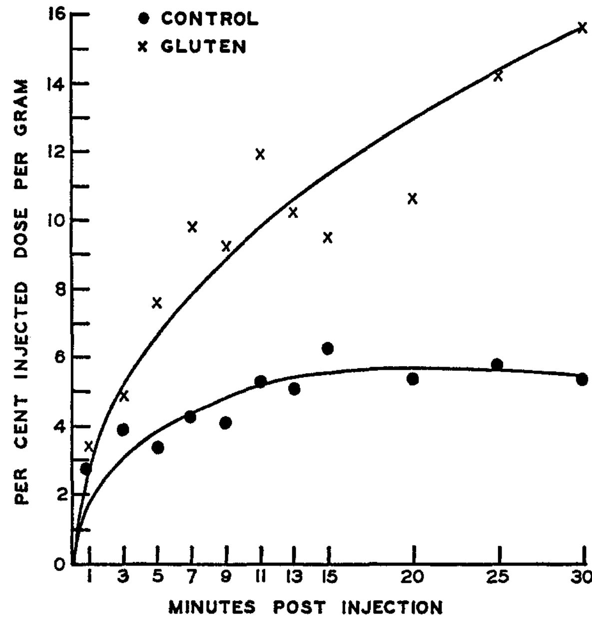
TABLE V

In vivo Changes Associated with Lysine Deficiency in Rats

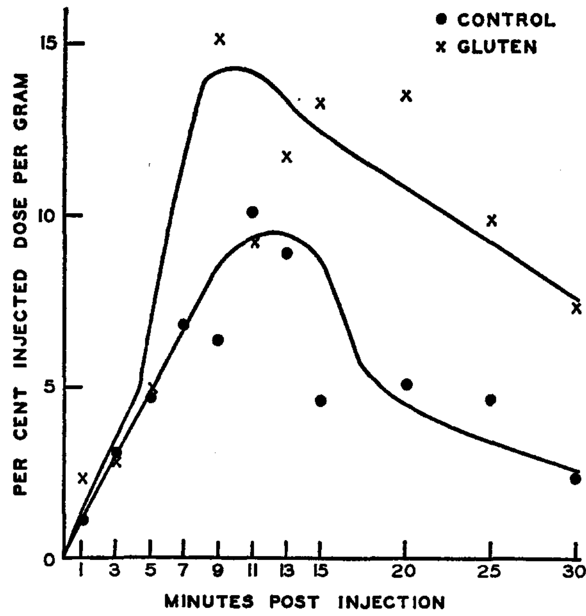
Substance	Control	Gluten	Significance
Total blood leukocytes (*/mm³*)	12,700	9,900	$p<0.01$
Total blood platelets (*/mm³*)	478,000	354,000	$p<0.05$
Serum complement (50 per cent units/ml)	148	61	$p<0.001$
Serum lysozyme (*μg/ml*)	15	11	$p<0.001$
Spleen lysozyme (*μg/gm*)	27	56	$p<0.001$
Serum total protein (gm per cent)	6.3	5.1	$p<0.05$
Serum Na^+ (*meq/liter*)	143	138	Not sig.
Serum K^+ (*meq/liter*)	4.8	6.7	$p<0.01$
Urine pH	5.8	7.3	$p<0.05$
Urine Na^+ (*meq/24 hrs*)	0.66	0.3	—
Urine K^+ (*meq/24 hrs*)	1.12	0.55	—
Urine Cl^- (*meq/24 hrs*)	3.1	1.4	—
Blood urea N (*mg per cent*)	18	31	$p<0.01$
Urea clearance (*ml/min.*)	0.87	0.12	$p<0.05$



TEXT-FIG. 3. Rate of clearance of colloidal $CrP^{32}O_4$ from the blood of control and lysine-deficient (gluten diet) rats. k is the slope of the line calculated by least squares. $\alpha = \frac{W}{W_{ls}} \sqrt[3]{k}$. W is the weight of rat; W_{ls} is the weight of the liver and spleen (5).



TEXT-FIG. 4. Rate of accumulation of colloidal $CrP^{32}O_4$ in the liver of control and lysine-deficient (gluten diet) rats.



TEXT-FIG. 5. Rate of accumulation of colloidal $CrP^{32}O_4$ in the spleen of control and lysine-deficient (gluten diet) rats.

In Table IV the effect of supplementation with the amino acids that are limiting in the gluten diet is reported. When lysine is added to the diet, with or without methionine, the resistance increases over that of totally deficient animals but does not return to that of the control animals. It should be noted that the weight of the experimental animals is similar to that of the controls. This indicates a separation of lack of growth due to amino acid deficiency and resistance to the disease. These findings are similar to those of Schaedler and Dubos (12) who used mice challenged with several different bacterial species.

Some changes found to be associated with lysine deficiency have been listed in Table V. The possible importance of the decrease in the number of circulating leukocytes and platelets will be discussed below. From the relative serum

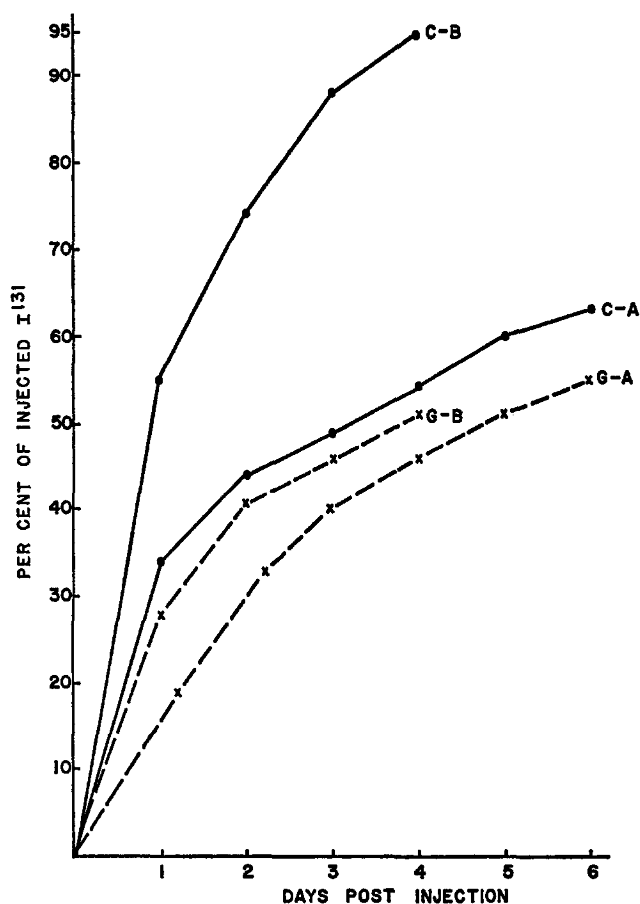
TABLE VI

Excretion of $CrP^{32}O_4$ Following Intravenous Injection of Colloid in Control (C) and Lysine-Deficient (G) Rats.

Day postinoculation	Per cent of injected dose			
	In urine/ml		In feces/gm	
	C	G	C	G
1	0.24	0.44	0.69	0.21
2	0.003	0.03	0.66	0.39
3	0.005	0.04	0.62	0.19
5	0.0026	0.0011	0.27	0.18
Total	0.063	0.128	0.56	0.24

and urine values of several components, there appears to be a decrease in renal function. However, unpublished data from our laboratory indicate that the endogenous creatinine clearance is unchanged. Since the latter is reported (13) to be not too accurate a measure of glomerular filtration rate in the rat, other studies are underway to investigate the importance of the renal function changes.

The effect of lysine deficiency on the phagocytic capability of fixed macrophages was studied using colloidal $CrP^{32}O_4$. From Text-figs. 3 to 5, it is apparent that the rate of clearance of colloid from the blood of lysine deficient rats is reduced (Text-fig. 3). Furthermore, when the direct rates of loss as measured by the slope, k, of disappearance are corrected for the weight of the liver and spleen as suggested by Benacerraf *et al.* (8), the differences between rates, α, are still evident. Although the loss of radioactive colloid from the blood proceeds at a slower rate, the concentration of colloid in the organs studied is greater in the lysine-deficient animals than in the controls (Text-Figs.



TEXT-FIG. 6. Rate of excretion of radioactive I^{131} following injection of 10^7 spore, *B. anthracis*, labeled with I^{131}. Figure illustrates two representative experiments, A, B. C and G refer to control (C) or deficient (G) rats.

4 and 5). Table VI summarizes the results obtained when the excretion of the $CrP^{32}O_4$ is followed in the urine and feces. It is apparent that there is a difference in both the rate of urinary and fecal excretion. There is almost twice the radioactivity per gram of feces in the control over that in the deficient animals. On the other hand, the excretion rate in the urine of the gluten animals is greater. This is compatible with the high blood level of P^{32} in this group.

Text-fig. 6 summarizes the results on the urinary excretion of breakdown products of the I^{131}-labeled spores. The excretion is more rapid and rises to greater values in the control animals. The significant differences occur in the first 3 days following inoculation. After this time, both groups excreted the radioactivity at the same rate.

DISCUSSION

As shown above, lysine deficiency, brought about by a gluten diet, reduced the resistance of the rat to anthrax. In the experimental and control groups, deaths occurred within 2 to 3 days post inoculation. This type of response, we postulate, could result primarily from an alteration of the ability of the host to overcome the invading parasite. The interrelation between resistance to infection and the phagocytic activity of the RES has been reviewed, and that such a relation exists is quite evident (14, 15). In this paper we have reported on the activity of the RES and the probable fate of anthrax spores following subcutaneous inoculation into the rat.

It has been demonstrated that there is a decreased rate of loss of colloidal material from the blood of the lysine-deficient animals. Salvidio and Crosby (16) have reported that RES activity could be dependent on the number and activity of the circulating platelets. We have shown that lysine deficiency was associated with a significantly decreased number of platelets. These two facts could contribute to the decreased colloidal clearance by the RES. At the same time, there is a greater concentration of colloid in the liver and spleen of the deficient animals. Individual reports cited above (9, 14) have shown that the colloid is removed by the fixed macrophages of the RES. At first glance, it would seem that the high tissue concentration with a low blood clearance rate was an anomalous situation. However, this could be explained by the inability of the deficient animals to clear from the organ the colloid that had been removed from the blood. If the difference does lie in the inability of an organ to get rid of the colloid, then considering that the liver secretes its products into the gastrointestinal tract, more of the colloid should be found in the feces of the control than of the deficient animal. Table VI illustrates that such is the case. If this mechanism applied to a material that could be metabolized in the RES of the animal, then it might be expected that the metabolic products would be excreted more rapidly in the control than in the deficient animals. This is seen in Text-fig. 6. When I^{131}-labeled spores were injected, dead or living, the greater amount of radioactivity was excreted in the urine by the control animals. Furthermore, for both the colloid $CrP^{32}O_4$ in the feces and the I^{131} in the urine, the significant difference occurred during the first 2 days after the inoculation of the labeled material, the same period during which the maximum mortality rate occurred.

SUMMARY

1. Lysine deficiency has been produced in rats by placing the weanling animals on an experimental diet in which gluten replaced the casein of the control diet. Both diets were complete in all other known requirements.

2. The resistance of the deficient animals to a subcutaneous challenge of

several concentrations of *B. anthracis* spores, was decreased. Deaths occurred within 2 days post inoculation.

3. When lysine was added to the gluten diet to bring the total concentration to that of the control diet, the growth rate of the animals was maintained but a decrease in resistance remained, although not as great as on the gluten diet.

4. Changes in the tissues associated with lysine deficiency are reported.

5. It is not unreasonable to state that within the time frame of our experiments, the decreased ability of the RES of the host to clear the invading organism from the tissues and subsequently to breakdown the organism is a major factor of the decreased resistance of the lysine-deficient rats to anthrax.

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EXPLANATION OF PLATE 19

Fig. 1. The appearance of Sprague-Dawley rats after being fed on a control, casein, diet (C) or an experimental, gluten, diet (G). Rats were weaned at 21 days of age and maintained on the diet for 30 days. C rats weighed 180 gm, G rats weighed 80 gm. United States Army photograph.

(Gray: Lysine deficiency and host resistance to anthrax)