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The work under this contract was directed toward detection and evaluation of evidence for either the maintenance or the breakdown of natural body defenses or mechanisms of acquired resistance of small animals exposed to simulated spacecraft environments. Efforts were also directed toward determination of alterations in invasiveness, antigenic specificity and biochemical characteristics of selected bacteria exposed in vivo and in vitro to such environments. The following is a description of methods and materials used, and of results obtained in the period of this contract.

The work was begun in September 1968 with the selection of bacterial strains to be used and the determination of cultural, biochemical, and behavioral characteristics of those strains. Included in these determinations were repeated tests to establish the degree of stability which could be expected in characteristics of these bacteria.

Organisms chosen for in vivo studies were: Salmonella california (Texas State Department of Health isolate); Streptococcus pyogenes, ATCC 12962; Pseudomonas aeruginosa, ATCC 7700; and Streptococcus sp., ATCC 12394. The characteristics of these organism are shown in Table 1. Antigens were prepared from the S. california, P. aeruginosa and S. pyogenes (ATCC 12062), cultures and injected into rabbits for the production of antisera to be used in serological testing of experimental animals.

In addition to the above cultures, other bacterial strains were selected for in vitro studies. These included: Staphylococcus aureus, ATCC 12600; Psuedomonas aeruginosa, ATCC 15442; P. aeruginosa, ATCC 17933; Escherichia coli, ATCC 128; S. aureus, ATCC 6020; S. epidermidis, ATCC 155; and S. pyogenes, ATCC 8668.

Most experimental tests involving animal exposure to simulated space environments involved the use of chamber environments modified to a barometric pressure of 380 mm Hg (equivalent to 18,000 feet altitude) and an atmosphere of 100% oxygen. In those tests where noted the modifications were changed to 250 mm Hg (27,000 feet) and/or an atmosphere of 43% oxygen. In all cases CO_2 was continuously removed by exhaust and/or absorption. During the period of this work a total of 17 tests of one week chamber exposure were run in which animals were exposed to simulated space conditions. Of these, seven tests used S. california, three tests used S. pyogenes, one test used Staphylococcus aureus and three tests used P. aeruginosa as the experimental bacterial strain in guinea pigs. Three tests were

TABLE I
Characteristics of Test Organisms

Test	Salmonella California(1) TSDH	Pseudomonas aeruginosa (2) ATCC 7700	Streptococcus pyogenes (3) ATCC 12962	Streptococcus sp. (Group G)(4) ATCC 12394
FERMENTATIONS				
arabinose	+	-	-	-
dextrose	+	-	+	+
dulcitol	+	-	-	-
lactose	-	-	-	-
maltose	+	-	+	+
mannitol	+	-	-	-
raffinose	-	-	-	-
salicin	-	-	+	+
sucrose	-	-	+	+
xylose	+	-	-	-
OTHERS				
MethylRed	+	-	ND	ND
V - P	+	-	ND	ND
Gelatin	-	+	-	-
Citrate	+	+	-	-
Nitrate	+	-	ND	ND
Indole	-	-	ND	ND
H_2S	+	-	ND	ND

(1) Rough colony (2) Soluble green pigment produced
(3) Beta hemolytic (4) No hemolysis

done using S. california as the bacterial strain in rabbits exposed to simulated space environment.

Two tests were done in which animals were exposed to simulated space conditions for a two week period. No essential differences could be observed in these tests as compared to one week exposures, and therefore results of these tests are not reported separately. One test was done in which animals were exposed for a period of six weeks. All of these tests involved Salmonella california as the bacterial strain.

One test of one week duration was done at a simulated altitude of 27,000 feet. In this test, the animals appeared to be placed under such stress as to produce distortions in the responses, and therefore other tests were not done under these conditions. The six week test was done at 43% oxygen in order to gain more accurate information on the immune responses of the animals as well as information on bacteriologic flora.

Some test exposures of in vitro bacterial cultures were done in the same chamber with animals, others were done separately. A total of 10 chamber exposures of in vitro cultures were done for one week periods only. Four tests were done for one week intervals with re-exposure to a total of three times. Cultures were exposed to chamber environments on semi-solid slants of trypticase soy agar, and in liquid trypticase soy broth.

PROCEDURES AND RESULTS

1. One week exposures using test organism Salmonella california.

The strain of S. california isolated by the Texas State Department of Health was chosen for this work because of its apparent low capacity to produce progressive disease in guinea pigs. This characteristic was desirable because of the aim of establishment of the carrier state in the test animals. This bacterium, as other test organisms was capable of producing disease, but simply was not highly virulent. In several repeat tests, it was established that 6×10^{10} cells in a one ml. amount was a suitable dosage for establishment of a carrier state. This dosage was given orally by dropper just before the animals were placed in chambers for exposure.

For each experimental run, it was necessary to establish the normal throat and rectal flora of the test and control animals. This was accomplished by taking swabs from these sites for one to three times, at intervals of two days, prior to application of the test organism and chamber exposure. The swabs were immediately placed in tubes of measured amounts of trypticase soy broth and thoroughly agitated to remove the adhering bacteria. This broth was then diluted and used to inoculate the necessary culture media for identification and enumeration of bacterial colonies. Media used were trypticase agar, EMB agar and mannitol-salt agar for rectal swabs, and the same media plus blood enriched agar and PEA agar for throat swabs. Following incubation, colonies were identified by differential reaction, colonial morphology or subculture methods, and counts were made for each bacterial variety.

The organisms most frequently found as normal flora populations from rectal swabs are shown in Table 2. Of these, Staphylococcus sp. (mannitol + and mannitol -), and diphtheroids were most frequently predominant. In general, very few Gram negative bacteria were recovered

TABLE 2

MOST COMMON NORMAL FLORA BACTERIA
FOUND IN RECTAL SWAB CULTURES
FROM HARTLEY STRAIN GUINEA PIGS

Staphylococcus species, mannitol positive
Staphylococcus species, mannitol negative
Diphtheroid bacilli (5 colonial types)
Micrococcus species
Gaffkya species
Bacillus species
Enterococci
Escherichia coli (rare)
Proteus sp (rare)

from rectal swabs of these guinea pigs as normal flora, and this number was greatly reduced following chamber exposure. The diphtheroids isolated were of obviously different types and were therefore separately counted on the basis of colonial morphology and size. The designation of diphtheroid types is shown in Table 3.

The total presence of aerobic bacterial cells recovered from rectal swab cultures varied from approximately 1×10^4 to 1×10^8 in different animals.

The work with throat cultures from guinea pigs proved to provide little information regarding alterations in flora due to the almost constant intake of food by these animals. Even though sterile food could be offered to the animals, the animals would rapidly reinoculate any residual, and the microbial flora changes due to food intake could not be eliminated. As one indication of the effect of food intake on bacterial flora, Gram negative organisms were routinely found in relatively large numbers in these cultures, while such bacteria were essentially lacking in rectal swab cultures. Organism found most commonly in normal flora throat cultures were: Staphylococcus species (mannitol + and -), alpha hemolytic Streptococcus sp., beta hemolytic Streptococcus sp., Sarcina lutea, Proteus sp., Escherichia coli, and yeasts. The total numbers in these throat swab cultures varied greatly, (again apparently due to differences in time and frequency of eating) but were usually between 1×10^6 and 8×10^9. It is felt that little information was gained from these or post exposure throat cultures with the exception that test organisms were occasionally isolated from throat swab cultures but not from rectal swab cultures. Alterations in total numbers or predominant organisms were not detectable in post chamber studies and throat cultures were dropped from some of the later test runs.

Following determination of normal flora and oral inoculation of the test organism dose, animals were placed in chamber environment. In all experiments of one or two weeks animals were divided into four groups as follows:

TABLE 3

COLONY CHARACTERISTICS OF
DIPHTHEROIDS ISOLATED

Type I. Colonies 3-4 mm. diameter, flat granular, irregular edge, ivory, dry.

Type II. Colonies 3-4 mm. diameter, umbonate, granular, irregular edge, orange, dry.

Type III. Colonies 2-3 mm. diameter, convex, smooth, entire edge, white, butyraceous.

Type IV. Colonies 2-3 mm. diameter, convex, smooth, entire edge, yellow to orange, butyraceous.

Type V. Colonies 0.8-1 mm. diameter, effuse, finely granular, fimbriate edge, white, adheres to agar.

A. Infected in altitude chamber

B. Normal in altitude chamber

C. Infected at ground level isolation

D. Normal at ground level isolation

Infected and normal animals were housed together both in chamber and in ground isolation in most experiments in order to determine the transfer of the test organism under each set of conditions.

In two tests, animals (total of 16 guinea pigs) were exposed to Salmonella california 35 to 42 days prior to chamber exposure. In all cases these animals had ceased to shed the test organism before being placed in the chamber. In post chamber cultures the eight animals exposed to chamber conditions again were shedding the organisms, while those infected and remaining at ground level did not shed the organism again.

In one week chamber exposure test using Salmonella california as the test organism (seven test runs) a total of 56 guinea pigs were exposed to the test organisms and 40 normal controls were tested at the same times and intervals. The two, two week chamber exposure tests comprised a total of 40 guinea pigs exposed to the test organism and 40 normal controls. Representative results only are shown in Tables 4-7 showing total rectal flora prior to chamber exposure and that following chamber exposure.

The findings from all animals in these tests are summarized in Tables 8-12. In changes in bacterial flora, the summaries are calculated to show increases and decreases in numbers of types of bacteria isolated. Results of this study indicate that these changes are greater and perhaps more meaningful than changes in total numbers of organisms.

Following re-isolation of the test organism after chamber exposure of the infected animals, the organism was again studied to determine specific characteristics. In the case of S. california the only characteristic changed in these tests was that the recovered

TABLE 4
RECTAL SWAB CULTURES FROM GUINEA PIGS
EXPOSED TO 380 mm. MERCURY PRESSURE
AND 100% OXYGEN IN CHAMBER

ANIMAL #1

ORGANISM	PRE-CHAMBER	INFECT	IMMEDIATE POST-CHAMBER	ONE WEEK POST-CHAMBER
Staphylococcus Mannitol +	4.8%	No	6%	2.6%
Staphylococcus Mannitol -	7.0%		0.1%	6.6%
Enterococcus	11.2%		3.8%	1.0%
Diphtheroid I	----		14.5%	----
Diphtheroid II	----		----	----
Diphtheroid III	76.7%		----	----
Diphtheroid IV	0.4%		----	1.8%
Diphtheroid V	----		75.6%	88.0%
ANIMAL #2				
Staphylococcus Mannitol +	92.0%	Yes	86%	----
Staphylococcus Mannitol -	2%		----	----
Diphtheroid I	4%		13%	66%
Diphtheroid II	----		----	----
Diphtheroid III	----		----	----
Diphtheroid IV	1%		0.5%	----
Diphtheroid V	----		----	----
Enterococcus	0.5%		----	33%
Proteus	0.5%		0.5%	----
Salmonella	----		----	1%

TABLE 4 (continued)

ANIMAL #3

ORGANISM	PRE-CHAMBER	INFECT	IMMEDIATE POST-CHAMBER	ONE WEEK POST-CHAMBER
Staphylococcus Mannitol +	88%	Yes	99%	----
Staphylococcus Mannitol -	8.0%		----	----
Enterococcus	1.0%		0.5%	----
Diphtheroid IV	3.0%		----	----
Salmonella	----		0.5%	Died, Salmonella Pneumonia

ANIMAL #4

Staphylococcus Mannitol +	0.1%	No	1.2%	----
Staphylococcus Mannitol -	0.2%		0.7%	----
Enterococcus	0.1%		----	----
Diphtheroid I	99.5%		98.0%	100%
Diphtheroid II	0.1%		----	----
Coliform	----		0.1%	----

TABLE 5

RECTAL SWAB CULTURES FROM GUINEA PIGS
EXPOSED TO 380 mm. MERCURY PRESSURE AND
ATMOSPHERIC OXYGEN IN CHAMBER
NOT INFECTED

ANIMAL #1

ORGANISM	PRE-CHAMBER	IMMEDIATE POST-CHAMBER	ONE WEEK POST-CHAMBER
Staphylococcus Mannitol +	12.0%	2.0%	3.5%
Staphylococcus Mannitol -	----	----	----
Enterococcus	76.0%	2.0%	64.2%
Diphtheroid III	----	96.0%	10.9%
Diphtheroid V	----	----	21.4%
Bacillus	12.0%	----	----

ANIMAL #2

Staphylococcus Mannitol +	7.0%	----	----
Enterococcus	56.0%	50.0%	19.0%
Diphtheroid V	37.0%	50.0%	76.0%
Bacillus	----	----	5.0%

TABLE 6

RECTAL SWAB CULTURES FROM GUINEA PIGS
EXPOSED TO ATMOSPHERIC PRESSURE AND
OXYGEN CONCENTRATION IN CHAMBER

ANIMAL #1

ORGANISM	PRE-CHAMBER	INFECT	IMMEDIATE POST-CHAMBER	ONE WEEK POST-CHAMBER
Staphylococcus Mannitol +	0.3%	No	11.3%	0.6%
Staphylococcus Mannitol -	1.2%		1.0%	1.1%
Diphtheroid III	0.8%		2.1%	0.8%
Diphtheroid V	97.5%		85.5%	97.0%
Coliform	----		----	0.5%
Bacillus	0.2%		0.1%	----
ANIMAL #2*				
Staphylococcus Mannitol +	94%	Yes	----	----
Staphylococcus Mannitol -	4%		----	----
Enterococci	1%		----	----
Diphtheroid I	----		98%	98%
Proteus	0.5%		1%	1%
Coliform	0.5%		----	----
Salmonella	----		1%	1%

*Dead Salmonella enteritis after post test. No Salmonella cultured from lungs.

TABLE 6 (continued)

ORGANISM	PRE-CHAMBER	INFECT	IMMEDIATE POST-CHAMBER	ONE WEEK POST-CHAMBER
ANIMAL #3				
Staphylococcus Mannitol +	95.8%	Yes	86.5%	89.0%
Staphylococcus Mannitol -	1.2%		----	1.5%
Enterococci	1.0%		0.5%	0.5%
Diphtheroid I	0.8%		4.0%	3.0%
Diphtheroid IV	0.8%		----	----
Coliform	0.1%		8.5%	4.5%
Salmonella	----		0.5%	1.5%
ANIMAL #4				
Staphylococcus Mannitol +	0.7%	No	0.6%	----
Enterococcus	30.8%		3.2%	7.0%
Diphtheroid III	15.1%		34.2%	57.0%
Diphtheroid V	53.1%		62.0%	36.0%
Mirc rococcus	0.3%		----	----

TABLE 7

RECTAL SWAB CULTURES FROM GUINEA PIGS
EXPOSED TO ATMOSPHERIC PRESSURE AND
OXYGEN CONCENTRATION IN NORMAL HOUSING

ANIMAL #1

ORGANISM	PRE-CHAMBER	INFECT	IMMEDIATE POST-CHAMBER*	ONE WEEK POST-CHAMBER
Staphylococcus Mannitol +	1.9%	No	0.8%	21.8%
Staphylococcus Mannitol -	0.7%		0.7%	8.2%
Enterococcus	0.1%		0.1%	0.1%
Diphtheroid I	97.1%		98.0%	47.2%
Diphtheroid III	----		0.2%	7.3%
Diphtheroid IV	0.2%		0.2%	0.9%
Coliform	----		----	14.5%

*These animals never in chamber. Tested at same time and intervals as chamber animals.

ANIMAL #2

Staphylococcus Mannitol +	11.7%	Yes	51.7%	56.2%
Staphylococcus Mannitol -	1.6%		52.1%	36.5%
Enterococcus	7.8%		10.3%	4.6%
Diphtheroid III	77.3%		----	----
Diphtheroid IV	1.6%		4.9%	0.7%
Salmonella	----		1.0%	1.0%

TABLE 7 (continued)

ANIMAL #3

ORGANISM	PRE-CHAMBER	INFECT	IMMEDIATE POST-CHAMBER	ONE WEEK POST-CHAMBER
Staphylococcus Mannitol +	61.1%	No	69.5%	21.2%
Staphylococcus Mannitol -	23.8%		15.3%	25.0%
Enterococcus	0.4%		10.9%	28.8%
Diphtheroid I	----		----	21.1%
Diphtheroid III	9.6%		1.9%	----
Diphtheroid IV	5.1%		2.4%	3.8%
ANIMAL #4				
Staphylococcus Mannitol +	1.0%	Yes	14.0%	32.6%
Staphylococcus Mannitol -	----		2.0%	----
Diphtheroid I	61.8%		84.0%	31.4%
Diphtheroid II	37.2%		----	----
Diphtheroid III	----		----	32.6%
Diphtheroid IV	----		----	2.2%
Salmonella	----		----	1.2%

TABLE 8

CHANGES IN NUMBER OF TYPES OF BACTERIA
IN RECTAL FLORA OF NORMAL ANIMALS
AT 380 mm MERCURY PRESSURE AND
100% OXYGEN CONCENTRATION
(Tests # 3, 6, 7, 8)

TEST CONDITIONS	NUMBER OF TYPES		
	INCREASED	SAME	DECREASED
Animals in Chamber	0	27.8%	72.2%
Control Animals in Normal Housing	25.0%	37.5%	37.5%

TABLE 9

CHANGES IN NUMBER OF TYPES OF BACTERIA
IN RECTAL FLORA OF NORMAL ANIMALS ONE
WEEK AFTER EXPOSURE TO 380 mm MERCURY
PRESSURE AND 100% OXYGEN CONCENTRATION
(Tests # 3, 6, 7, 8)

TEST CONDITIONS	NUMBER OF TYPES		
	INCREASED	SAME	DECREASED
Test Groups in Chamber	73.6%	21.0%	5.4%
Control Groups in Normal Housing	11.2%	60.8%	28.0%

TABLE 8A

ANIMAL EXPOSURE
CHAMBER EXPERIMENTS

Test No.	Begun	Duration	Organism	Exposed Animal on	Animals	Conditions
1	9/23/68	7 days	S. california	8/10/68	GP	A
2	10/17/68	7 days	S. california	10/10/68	GP	A
3	11/14/68	6 days	S. california	11/9/68	GP	A
4	11/26/68	7 days	S. pyogenes	11/22/68	GP	A
5	12/20/68	7 days	S. pyogenes	12/16/68	GP	A
6	12/13/68	7 days	S. california	12/12/68	GP	A
7	1/31/69	14 days	S. california	2/7/69	GP	B
8	2/7/69	14 days	S. california	2/6/69	GP	A
9	2/21/69	7 days	S. california	2/27/69	GP	C
10	3/5/69	6 weeks	S. california	4/16/69	GP	B
11	3/19/69	7 days	None	3/26/69	GP	D
12	3/19/69	7 days	None	3/26/69	GP	E
13	5/10/69	7 days	P.aeruginosa	5/17/69	GP	A
14	6/2/69	7 days	S. california	6/9/69	Rab	A
15	6/23/69	7 days	S. califomia	6/30/69	Rab	A
16	7/7/69	7 days	P. aeruginosa	7/14/69	GP	A
17	7/17/69	7 days	P. aeruginosa	7/24/69	GP	A
18	8/1/69	7 days	S. pyogenes	8/8/69	GP	A

TABLE 8A (continued)

Test No.	Begun	Duration	Organism	Exposed Animal on	Animals	Conditions
19	8/10/69	7 days	S. aureus	8/17/69	GP	A
20	8/10/69	7 days	S. california	8/17/69	Rab	A

A. 18,000 feet equivalent, 100% oxygen.
B. 18,000 feet equivalent, 43% oxygen.
C. 27,000 feet equivalent, 100% oxygen.
D. 18,000 feet equivalent, normal air.
E. Ground, normal air.

TABLE 10

CHANGES IN NUMBER OF TYPES OF BACTERIA IN RECTAL FLORA OF NORMAL ANIMALS PRODUCED BY CHAMBER EXPOSURE TO 380 mm MERCURY PRESSURE AND ROOM AIR AND BY CHAMBER EXPOSURE WITH NORMAL ATMOSPHERIC PRESSURE AND AIR
(Test # 11)

TEST CONDITIONS	NUMBER OF TYPES		
	INCREASED	SAME	DECREASED
380 mm Mercury Pressure, Room Air	20.0%	40.0%	40.0%
Normal Pressure, Room Air	0%	60.0%	40.0%

TABLE 11

CHANGES IN NUMBER OF TYPES OF BACTERIA IN RECTAL FLORA OF NORMAL ANIMALS ONE WEEK AFTER CHAMBER EXPOSURE TO 380 mm MERCURY PRESSURE AND ROOM AIR AND BY CHAMBER EXPOSURE WITH NORMAL ATMOSPHERIC PRESSURE AND AIR
(Test # 12)

TEST CONDITIONS	NUMBER OF TYPES		
	INCREASED	SAME	DECREASED
380 mm Mercury Pressure, Room Air	80.0%	20.0%	0%
Normal Pressure, Room Air	20.0%	40.0%	40.0%

TABLE 12

CHANGES OF NUMBERS OF TYPES OF BACTERIA
IN RECTAL CULTURES OF GUINEA PIGS
FOLLOWING EXPOSURE TO
SALMONELLA CALIFORNIA
(Tests # 1-3, 6, 7)

Test Conditions	Number of Types			Salmonella Isolated		Animal Survived	
	Increased	Same	Decreased	1	2	No	Yes
Test Group in Chamber	10.2%	27.2%	63.6%	45.4%	36.3%	18.1%	81.9%
Control Group in Chamber	22.4%	11.0%	66.7%	0%	5.8%	5.8%	94.2%
Test Group Ground Level	11.1%	22%	66.9%	48%	52%	27.2%	72.8%
Control Group Ground Level	36.1%	37.0%	26.7%	0%	0%	0%	100%

1. Isolated prior to chamber exposure or test period.
2. Isolated after chamber exposure or test period.

strain demonstrated a smooth colony form, while the inoculated strain was a rough form. Time prohibited complete testing of this alteration, however, for the guinea pigs used as test animals there appeared to be little or no change in invasiveness or pathogenicity associated with this variation. Conversion occurred only in chamber test animals.

Animals exposed to Salmonella california cultures and to chamber environments were tested for the production of specific antibodies for up to two weeks following removal from the chamber. In these tests, of fourteen guinea pigs tested following chamber exposure only three had developed specific antibodies by two weeks post chamber. Of eleven tested following exposure to the bacterium and remaining at ground level for the same time period, no animal developed demonstrable antibody.

At first glance, these results would appear to indicate that chamber environment exposure stimulated antibody production. However, results of the long term study are not in agreement with this interpretation, and it must be assumed that another interpretation is necessary in this case.

If one considers antibody production as correlated with infections and deaths produced in exposed animals, some conclusions may be drawn. In those two tests in which animals were inoculated with S. california seven to eight weeks before chamber exposure, no animal in the test group succumbed to the infection. In all these (16) animals, the test organism was shed for four to twelve days after which apparent shedding stopped, although there was evidence that the salmonella was spread to normal guinea pigs in the chamber from these carrier animals. At ground level then, it would appear that some resistance was developed, although not demonstrable as antibody titers, and the organism was not able to proliferate to an extent that it was shed in the feces.

When animals were inoculated with S. california and immediately placed in the chamber environment, 18% of the test group died from apparent salmonella infection. The majority of these

infections occurred as lung disease rather than the more usual enteritis produced by salmonella. Uninoculated animals in the chamber also showed 5.8% deaths. Ground controls showed 27.2% deaths among the inoculated group (mostly in the form of enteritis disease) and those uninoculated ground controls housed with them showed no deaths and no salmonella recovered from cultures of these animals.

From these results, it may be concluded that exposure to this organism just prior to chamber environment, or in the simulated altitude, 100% oxygen environment, may change the type of infection produced in the guinea pig. The differences in numbers of deaths produced at altitude or at ground do not permit conclusions as to a likelihood of one environment enhancing the infection.

II. One week exposures using test organisms Streptococcus pyogenes and/or Streptococcus species (Group G).

The inoculation dosage of Streptococcus cells used in these three experiments was 1×10^{10} cells in one ml. volume. The living, 24 hour culture was dropped into the pharynx of the guinea pig, and the animal was held until the dosage was swallowed.

Due to the normal flora presence of both beta and alpha hemolytic streptococci in throat cultures of the guinea pigs, it was impossible to be assured of re-isolation of the inoculated strain. No infections of any type were produced and no development of specific antibody was detected. The trend toward reduction in numbers of types of bacteria present in both throat and fecal swabs was seen in these tests to follow that seen in salmonella tests after chamber exposure. In these three runs, of 32 animals exposed to chamber conditions, both Streptococcus sp. inoculated and controls, 69% showed a decrease in number of bacterial types present in rectal swab cultures. Throat cultures were more difficult to analyze as mentioned previously. Ground controls were similar to those in the salmonella tests.

Because of difficulties in establishing the carrier state and isolation of the inoculated organism, further studies using this test organism were not attempted.

III. One week exposures using test organism Pseudomonas aeruginosa.

The inoculation dosage of P. aeruginosa used was 1×10^{10} cells of a 24 hour culture, inoculated as in other tests. Twenty-seven guinea pigs were exposed to the culture, and nineteen uninoculated controls were used.

Two animals possessed a Pseudomonas sp. in the normal flora which could be distinguished from the test species by pigmentation type. Animals exposed to chamber showed fecal flora type increases of 35%, decreases of 43% and no change 22%. However, of those animals showing increases in number of bacterial types in the chamber, 90% of them showed an increase by the addition of the test pseudomonas strain. Apparently the inoculation of P. aeruginosa in this dosage does not produce an alteration in fecal flora similar to that produced by salmonella inoculation. In ground controls 75% showed an increase in types.

Of inoculated test, chamber exposed animals, the organism was isolated from nine of eighteen inoculated, and three deaths were produced by the organism infecting the lungs. Inoculated animals remaining at ground showed two positive P. aeruginosa recoveries, and one death of nine animals tested.

The test organism was passed to six of ten normal controls in chamber and to four of nine normal controls at ground level. The normals were housed with the inoculated animals in both environments. These results indicate that this organism is spread with equal ease in either environment.

These tests indicate that P. aeruginosa is more easily established in the intestinal tract of guinea pigs in chamber environment than at normal ground level environment, and it would appear that fatal infections are somewhat more likely to be produced.

IV. One week exposures using test organism Staphylococcus aureus, ATCC 12600.

Since a major portion of the intestinal flora of guinea pigs used in this project has been found to be Staphylococcus species strains, one test was run in which nine animals were orally inoculated with 1×10^{10} living cells of the above strain, and were compared to seven uninoculated controls. Results as to flora types were similar to those reported for other tests.

No infections or deaths were produced in any animal. The net result of the experiment was simply to increase the predominance of mannitol positive staphylococci in the flora detected by rectal swab cultures. Uninoculated controls did not show this increase in level of predominance.

V. One week chamber exposure experiments using S. california as test organism in rabbits as experimental animals.

In these experiments, random bred white rabbits weighing two to four pounds were used as experimental animals. Chamber conditions, culture specimens and media, testing schedules and inoculated dosage were the same. Three separate tests were run using a total of ten infected animals and nine uninoculated controls.

Normal flora determination in both throat and rectal swab cultures showed considerable differences in the aerobic bacteria present in these animals as compared to guinea pig cultures. The major general difference was seen in the consistent presence of Gram negative organisms in both types of cultures from rabbits.

In one test involving four infected test and four uninfected control rabbits, the test was abandoned after two days chamber exposure due to the death of the inoculated animals in the chamber. Inoculated animals at ground level survived and in the test period did not shed Salmonella organisms as determined by rectal swab cultures. The inoculated chamber animals were autopsied and initially were thought to have died from widely disseminated blood clots. On culture, however, S. california was isolated from every tissue sampled and it must be assumed that the cause of death was this generalized infection.

In the two additional experiments, all animals survived the test period, although evidence of disease did appear in three of the four animals inoculated with Salmonella and exposed to chamber environment. In these animals, an abnormality in the clotting of blood was noted as an increased bleeding time. Although inconclusive, this observation was of interest in view of the autopsy on the infected animals which did not survive chamber exposure.

The normal flora and post-chamber cultures for rabbits in these two experiments are shown in Table 12R. Animals were inoculated and placed in the chamber for one week immediately after taking pre-exposure culture #2. Post-exposure culture #1 was taken one week later, immediately after animals were removed from chamber, and #2 was taken one week later. Inoculated and normal were housed together in both environments.

In these experiments, the flora demonstrated by rectal swab cultures was reduced in variety following one week chamber environment exposure. In practically all cases, this flora had recovered or was in process of recovery one week later. Animals at ground level did not show this consistent drop in numbers of types of organisms.

Throat cultures did not change as consistently, nor to such an extent, however, the trend toward reduction appears to be present here as well.

In animals inoculated and placed in chamber, S. california was recovered from two of four. In both cases, the organism was recovered from throat swabs, not rectal swabs. The test organism was recovered from three of three inoculated and remaining at ground level. No evidence of passage of Salmonella from inoculated to normal animals was observed in the chamber environment. S. california was recovered from a rectal swab culture of one normal animal which remained at ground level.

Six rabbits in this group were tested for antibody titer one week after inoculation and after chamber exposure for that group. No animal exposed to chamber environment produced an antibody titer above 1:40. Both inoculated and normal animals in chamber did, however, show at

TABLE 12 R

ORGANISMS ISOLATED FROM RABBITS BEFORE AND AFTER EXPOSURE TO ALTITUDE CHAMBER
(Rabbits were housed either in pairs or threes in chamber according to chamber size.)

ORGANISMS	PRE-EXPOSURE #1 CULTURES	#2	POST-EXPOSURE #1 CULTURES	#2
Rabbit #1 - Inoculated, Chamber Environment				
Throat				
Staph., mannitol pos.	5,000	20,000	---	13,000
Staph., mannitol neg.	2,000	10,000	2,000	---
Alkaligenes	1,300	700	100	1,100
E. coli	2,400	600	---	---
Diphtheroids	250,000	---	10,000	---
Alpha Strep	400,000	17,000	2,000	---
Bacillus, rough	---	6,000	2,000	---
Bacillus, vesicular	---	---	---	100
Unident. Gram neg. rod	---	---	---	40,300
Rectum				
E. coli	1,300,000	500	---	---
Alpha Strep.	9,000	---	---	---
Staph, mannitol pos.	1,000	---	---	---
Staph., mannitol neg.	1,000	---	---	---
Bacillus, rough	30,000	---	---	5,000
Bacillus, vesicular	---	---	---	2,000
Sarcina	---	---	---	200
Rabbit #2 - Inoculated, Ground Environment				
Throat				
Sal. calif ornia	---	---	1,000	---
E. coli, typical	2,000	1,000	---	600
E. coli, atypical*	---	---	1,000	---
E. coli, atypical**	---	---	---	100
Alpha Strep	2,100,000	50,000	8,000	8,000
Staph., mannitol pos.	30,000	---	2,000	---
Staph., mannitol neg.	20,000	---	9,000	30,000
Bacillus	---	20,000	---	---
Diphtheroids	---	30,000	16,000	---
Alkaligenes, atypical	---	---	22,000	---
Sarcina	---	---	---	30,000

*E. coli., indole negative
**E. coli., non-lactose fermenting

TABLE 12 R
(Continued)

ORGANISMS	PRE-EXPOSURE #1 CULTURES	PRE-EXPOSURE #2	POST-EXPOSURE #1 CULTURES	POST-EXPOSURE #2
Rabbit #2				
Rectum				
Bacillus, rough	200	5,000	---	---
Bacillus, moist	---	9,000	---	---
Fecal Strep	40,000	8,000	13,000	16,000
Alpha Strep	---	---	---	100,000
Staph., mannitol pos.	10,000	---	---	6,000
Staph., mannitol neg.	3, 000	1,000	---	2,000
Proteus mirabilis	---	---	6,000	40,000
Micrococcus	---	---	40,000	---
Sarcina	---	---	---	200,000
Rabbit #3 - Inoculated, Chamber Environment				
Throat				
Alpha Strep	200,000	300,000	11,000	
Staph., mannitol pos.	10,000	10,000	1,000	
Staph., mannitol neg.	100,000	20,000	3,000	
Sarcina	1,000	---	---	
Gaffkya	40,000	---	---	
Diphtheroids	10,000	500,000	---	
E. coli	---	200	1,500	
Bacillus	---	---	400	
Fecal Strep	---	---	300	
Sal. californica*				
Rectum				
Bacillus	500	10,000	---	
Staph., mannitol pos.	2,000	1, 000	1,000	
Staph., mannitol neg.	6,000	1, 000	---	
E. coli	---	85,000	---	
Alpha Strep	---	210,000	---	
Fecal Strep	---	---	7,000	

*Sal. californica present on isolation plates, but not on dilution plates.

TABLE 12 R
(Continued)

ORGANISMS	PRE-EXPOSURE #1 CULTURES	#2	POST-EXPOSURE #1 CULTURES	#2
Rabbit #4 - Normal, Chamber Environment				
Throat				
Alkaligenes	100	---	---	100
Staph., mannitol pos.	1,000	20,000	20,000	---
Staph., mannitol neg	1,000	---	30,000	---
Pleo. Gram neg rod	100,000	15,000	---	200
Bacillus	---	9,000	---	100
Alpha Strep	---	---	---	10,000
Rectum				
Bacillus, rough	100	18,000	---	9,000
Bacillus, moist	---	1,000	---	1,000
Bacillus, vesicular	---	---	---	1,000
Staph., mannitol pos.	---	1,000	20,000	1,000
Staph., mannitol neg.	---	---	40,000	7,000
E. coli, typical	---	---	10,000	---
E. coli, atypical**	---	---	---	1,500
Gram neg. coccobacillus	---	---	4,000	---
Sarcina	---	---	---	1,000
Rabbit #5 - Normal, Chamber Environment				
Throat				
Alkaligenes	200	---	1,100	14,000
Staph., mannitol pos.	2,000	---	---	2,000
Staph., mannitol neg.	---	---	---	2,000
Alpha Strep	150,000	---	---	5,000
E. coli, typical	---	200	---	3,000
E. coli, atypical*	---	---	---	1,000
Klebsiella	---	100	---	---
Bacillus	---	17,000	---	1,200
Diphtheroids	---	23,000	---	---
Unident. Gram neg. rod	---	---	8,900	2,000
Gram neg. coccobacillus	800	---	---	---

**E. coli, indole negative
*E. coli, citrate positive

TABLE 12 R
(Continued)

ORGANISMS	PRE-EXPOSURE #1	PRE-EXPOSURE #2	POST-EXPOSURE #1	POST-EXPOSURE #2
Rabbit #5				
Rectum				
Bacillus, spreading	500	300	---	---
Bacillus, discrete	800	300	---	---
Staph., mannitol pos.	1,000	---	---	---
Staph., mannitol neg.	---	---	---	6,000
Sarcina	---	---	---	8,000
Rabbit #6 - Normal, Ground Environment				
Throat				
Alkaligenes, typical	---	---	---	200
Alkaligenes, atypical	---	---	42,000	---
Staph., mannitol pos.	1,000	3,000	---	2,000
Staph., mannitol neg.	100,000	---	2,000	---
Alpha Strep	320,000	50,000	7,000	9,000
Bacillus	---	50,000	---	100
Diphtheroids	---	30,000	---	---
E. coli**	---	---	300	---
E. coli***	---	---	100	---
Fecal Strep	---	---	400	---
Unident. Gram neg rod	---	---	---	800
Rectum				
Staph., mannitol pos.	5,000	30,000	22,000	19,000
Staph., mannitol neg.	2,000	90,000	44,000	6,000
Sal. california	---	---	2,000	---
E. coli	---	---	1,000	---
Fecal Strep	---	---	31,000	---
Sarcina	---	---	15,000	---
Bacillus, spreading	200	---	---	---
Bacillus, discrete	500	---	---	---
Bacillus, rough	---	---	23,000	1,000
Bacillus, vesicular	---	---	2,000	---
Bacillus, moist	---	---	1,000	---

**E. coli, indole negative
***E. coli, non-lactose fermenting

TABLE 12 R
(Continued)

ORGANISMS	PRE-EXPOSURE #1 CULTURES	#2	POST-EXPOSURE #1 CULTURES
Rabbit #9 - Inoculated, Chamber Environment			
Throat			
Alpha Strep	200,000	300,000	11,000
Staph., mannitol pos.	10,000	10,000	1,000
Staph., mannitol neg	100,000	20,000	3,000
Sarcina	1,000	---	---
Gaffkya	40,000	---	---
Diphtheroids	10,000	500,000	---
E. coli	---	200	1,500
Bacillus	---	---	400
Fecal Strep	---	---	300
Sal. califomia *			
Rectum			
Bacillus	500	10,000	---
Staph., mannitol pos.	2,000	1,000	1,000
Staph., mannitol neg.	6,000	11,000	---
E. coli	---	85,000	---
Alpha Strep	---	210,000	---
Fecal Strep	---	---	7,000
Rabbit #10 - Normal, Ground Environment			
Throat			
Alkaligenes	100	---	---
Staph., mannitol pos.	1,000	20,000	20,000
Staph., mannitol neg.	1,000	---	30,000
Pleo. Gram neg rod	100,000	15,000	---
Bacillus	---	9,000	---
Rectum			
Bacillus, rough	100	18,000	---
Bacillus, moist	---	1,000	---
Staph., mannitol pos.	---	1,000	20,000
Staph., mannitol neg.	---	---	40,000
E. coli	---	---	10,000
Gram neg. coccobacillus	---	---	4,000

*Sal.californio present on isolation plates, but not on dilution plates.

TABLE 12 R
(Continued)

ORGANISMS	PRE-EXPOSURE #1 CULTURES	#2	POST-EXPOSURE #1 CULTURES
Rabbit #11 - Normal, Chamber Environment			
Throat			
Alkaligenes	200	---	1,100
Staph., mannitol pos.	2,200	---	---
Alpha Strep	150,000	---	---
E. coli	---	200	---
Klebsiella	---	100	---
Bacillus	---	17,000	---
Diphtheroid	---	23,000	---
Unident. Gram neg rod	---	---	8,900
Gram neg coccobacillus	800	---	---
Rectum			
Bacillus, spreading	500	300	---
Bacillus, discrete	800	300	---
Staph., mannitol pos.	1,000	---	---
Rabbit #12 - Inoculated, Ground Environment			
Throat			
Alkaligenes, atypical	---	---	42,000
Staph., mannitol pos.	1,000	3,000	---
Staph., mannitol neg.	100,000	---	2,000
Alpha Strep	320,000	50,000	7,000
Bacillus	---	50,000	---
Diphtheroids	---	30,000	---
E. coli	---	---	400
Fecal Strep	---	---	400
Rectum			
Staph., mannitol pos.	5,000	30,000	22,000
Staph., mannitol neg.	2,000	90,000	44,000
Sal. california	---	---	2,000
E. coli	---	----	1,000
Fecal Strep	---	---	31,000
Sarcina	---	---	15,000
Bacillus, spreading	200	---	---
Bacillus, discrete	500	---	---
Bacillus, rough	---	---	23,000
Bacillus, vesicular	---	---	2,000
Bacillus, moist	---	---	1,000

#1 Pre-Exposure: Taken one week prior to chamber exposure.
#2 Pre-Exposure: Taken immediately prior to chamber exposure.

least a minimum titer, indicating that the organism was spread from inoculated to normal, although it was not recovered by culture. The two ground level animals produced titers of 1:160 and 1:320 respectively. These results compare favorably with results obtained from the long term guinea pig experiment. Electrophoretic analysis of sera from these animals did not indicate any changes in protein concentrations or patterns regardless of treatment or environment exposure.

VI. Long term study using Salmonella califomia as test organism.

One study was performed at the USAF School of Aerospace Medicine, Brooks Air Force Base, Texas in which animals were maintained in chamber for a period between five and six weeks. This study included rectal swab cultures done at the North Texas laboratory, but was primarily concentrated on immune responses of the animals subjected to various treatments. The immune responses are described in detail below.

The biologic simulator consisted of two connecting compartments, the main chamber and air lock, each about 6 x 6 x 6 feet (215 cubic feet). Animals were placed in the environment of the main chamber which was maintained at a barometric pressure of 380 mm Hg., equivalent to a simulated altitude of 18,000 feet. Personnel working with the animals wore protective clothing and oxygen masks and were placed in contact with the animals by being brought to a matching pressure in the air lock and then opening the connecting door to allow access to the chamber. The simulator was specifically designed to handle animals infected with pathogenic organisms and is described in a recent publication by Wynne, et al. (1968).

Conditions during the test period were controlled so that a constant oxygen level of 43% was maintained. Carbon dioxide concentration was held to levels below 1% by use of an absorbent hydroxide bed within the chamber (Baralyme, National Gas Co.). Relative humidity, although not controlled, was monitored and found to remain at 60 to 70%. Temperature was 20 to 23 degrees Centigrade throughout the experiment.

Animal Techniques

English strain male guinea pigs, purchased from Camm Research Laboratories, Wayne, N. J., were randomly picked from a group of 100 and arbitrarily assigned to cages in groups of five. They were individually tagged and identified by means of tatoos on the ear margins.

Adequate food and water was always available and routine changes of cage bedding were accomplished without disturbing the animals. Animals in the chamber remained at altitude during routine bedding changes. Control animals were housed in identical cages in an adjacent r

Individual animals were bled on a routine weekly basis. This was accomplished by anaesthetizing with methoxyflurane (Penthrane, Abbott Laboratories) according to procedures established by Pindak and Kendrick (1969) and subsequently withdrawing three to five milliliters of blood from the heart, using glass syringes and twenty-one gauge needles.

Eighty animals were used for the experiment and were assigned in four groups of twenty animals each, as shown in Table 13.

Experimental Design

Each of the four groups of twenty guinea pigs was subdivided into four additional groups of five each. These sub-groups were subjected to the various environmental conditions outlined in Table 14.

Hematological Studies

Blood Sampling and Processing

A bleeding schedule was established so that individual animals were bled once per week, but no more than 25 were bled on a sampling day. Blood was processed in the following manner. Approximately 1.0 ml of blood was added to a 13 x 100 mm plastic tube to which 2-3 mg of dry EDTA was added as an anticoagulant. This tube was immediately swirled and agitated until the EDTA was in solution. The remainder of the sample was transferred to an identical tube and

TABLE 13

ANIMAL ASSIGNMENTS

Group Designation	Number in Group	Purpose
I	20	Immunization with Salmonella "O" antigen
II	20	Immunization with Bovine Serum Albumin
III	20	Oral inoculation with Salmonella and flora studies
IV	20	Control group

TABLE 14

ENVIRONMENTAL CONDITIONS

Sub-group Designation	Environment
A-A	Altitude acclimation for two weeks, immunized or infected and remained at altitude for three more weeks, then two weeks at normal ground level conditions.
A-G	Altitude acclimation for two weeks, treatment given, brought to ground for remainder of experiment (5 additional weeks).
G-A	Ground conditions for two weeks, treatment given and then brought to altitude for three weeks, followed by two weeks at ground level.
G-G	Ground level during entire experiment.

allowed to clot at room temperature. The tube containing the clotted blood was placed in a 37 degree incubator for 30-60 minutes. The clot was rimmed with a wooden stick, allowed to contract at room temperature, and placed in a cold room for an additional one to three hours. The tube was centrifuged at 5,000 x g for ten minutes and the clear serum withdrawn and stored in the frozen state (-70 degrees Centigrade) in small vials for future testing. Serum samples were frozen within eight to ten hours after blood sampling.

Hematocrit

Hematocrit values were obtained by use of the micro-hematocrit method. Capillary tubes of the blood samples were centrifuged for five minutes at 11,000-13,000 rpm and the packed cell volume read with a circular micro-hematocrit reader (International Equipment Co.).

Hemoglobin

Hemoglobin was determined by the cyanmethemoglobin method using commercially prepared reagents (Acculute, Ortho Diagnostics, Raritan, N. J.). A hemoglobin standard (Accuglobin, Ortho Diagnostics) was used each time determinations were made and was consistent throughout the experimental period.

Red Blood Cell Counts

Red blood cell counts were performed on each blood sample using a Coulter Counter, Model A (Coulter Electronics, Hialeah, Florida). Initial dilutions of 1:500 were made in a 18 x 150 mm tube with Isoton dilution fluid (Coulter Electronics). The above tube was swirled and the contents mixed thoroughly before a final dilution of 1:50,000 was made for counting. The counts obtained were periodically checked against counts with a hemocytometer and microscope. Settings of the counter were standardized and consistent throughout the experimental period.

White Blood Cell Counts

White blood cell counts were performed with a blood sample (1:500 dilution) which was stromatalized with three drops of a three per cent saponin solution. Window settings of the Coulter Counter were established so that comparable counts of white blood cells were obtained with the counter and with a hemocytometer.

Differential Counts

Each sample of blood was examined further by making a blood smear on a glass slide. After air drying, the cells were stained with a standard Wright's stain. Cells were counted and enumerated by types by standard counting methods. Maturation lines of the neutrophil series were not specifically followed unless a marked shift in types was observed.

Serological Studies

Antigen Preparation

Salmonella O Antigen. An overnight broth culture of Salmonella california was seeded to three two-liter flasks each containing 250 ml Trypticase Soy Agar (Difco). After fifteen hours growth, the cells were harvested, washed three times with phosphate buffered saline, and suspended in a minimal amount of saline. The thick suspension was placed in a boiling water bath for one hour and then centrifuged at 10,000 x g. The supernatant was discarded and the precipitate washed once with saline, centrifuged and resuspended in saline. After an additional hour of heating in the boiling water bath the cells were centrifuged and suspended in saline as a stock preparation.

For injection, the sterile cell suspension was diluted in saline to 10^9 cells per ml as measured by comparison with a series of standards prepared according to Campbell, et al. (1964).

For use as cells for agglutination studies, the stock preparation was diluted with saline so that the concentration had an optical density of 0.1 at 600 mu with a 1 cm. cuvette in a Beckman DU spectrophotometer.

Bovine Serum Albumin. Bovine Serum Albumin (BSA) purchased from Nutritional Biochemicals Co., Cleveland, Ohio, was prepared for injection in the following manner. Crystalline BSA was suspended in saline and emulsified with a modified Freunds adjuvant (Difco) composed of a mixture of incomplete and complete adjuvant. The final preparation contained 7.5 mg BSA plus 0.12 ug mycobacterium in a total volume of 0.10 ml.

Immunization Procedures

Group I. Each of the twenty guinea pigs of Group I was immunized with a single injection of 10^9 cells of the Salmonella O antigen preparation. The injection was given subcutaneously in the suprascapular region.

Group II. Each of the twenty guinea pigs of Group II was immunized with a single injection of BSA--Freund's adjuvant mixture at a level of 15 mg BSA per kilogram body weight. The dose was divided and given in four portions of 0.025 ml to each footpad. The mean weight of the guinea pigs at time of injection was 500 grams and each animal received a total of 7.5 mg BSA.

Serological Testing

Agglutination Titers. Individual serum samples were diluted two-fold in saline within the cups of a plastic tray (Linbro Chemical Co., New Haven, Conn.) in 0.5 ml amounts. Following dilution, Salmonella O antigen preparation (0.5 ml) was added and the trays incubated for one hour at 55 degrees Centigrade, refrigerated overnight, and the agglutination read under oblique illumination in a darkened room.

Gel Double Diffusion. Antibody response to the injected BSA in Group II was measured in a double gel diffusion system similar to that described by Ouchterlony (1962).

Gel diffusion plates were prepared at North Texas State University in the following manner. Oxoid Ionagar number two (Oxoid Ltd., London) at a one per cent concentration was prepared in pH 7.4 phosphage buffer with a phenol concentration of 0.5 per cent. This agar preparation was

autoclaved for fifteen minutes at 121 degrees Centigrade and then held at 50 degrees Centigrade for pouring. Ten milliliter aliquots were distributed in 60 x 15 mm. plastic perti dishes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.).

After solidifying, the agar was punched with a Grafar Gel Punch Assembly (Grafar Corp., Detroit, Mich.). The pattern consisted of a central well surrounded by six other wells equidistant from the center well. The plates were kept moist with a fitted piece of wet filter paper and were shipped by bus to Brooks Air Force Base for use. Prior to use, the wells were formed by pulling the agar plugs with a suction apparatus. The test sera were placed in the center well and ten-fold dilutions of BSA in saline were placed in successive wells surrounding the sera, beginning with 100 mg per ml and ending with .001 mg per ml. Precipitin bands were observed after 24 hours incubation at 37 degrees Centigrade.

Electrophoresis

All serum samples were subjected to electrophoresis on Sepraphore III cellulose acetate strips (Gelman Instrument Co., Ann Arbor, Mich.). A Shandon electrophoresis chamber (Shandon Scientific Co., London) and a standard D. C. power source were used for the separation of the serum components on the 1 inch by 6 inch strips. The strips were subjected to a current of 1.5 milliamps per strip for 40 minutes utilizing Gelman High Resolution Buffer, pH 8.8 and .025 ionic strength. Immediately following separation the strips were stained with Ponceau S stain (Consolidated Laboratories, Inc., Chicago Heights, Ill.) for 5-10 minutes and then washed of excess stain with three successive washings of 2-5 minutes each in five per cent acetic acid. After initial air drying on plexiglass plates, the strips were dehydrated further in absolute methanol, placed in a solution of 10 per cent acetic acid in methanol for one minute, removed and smoothed on a plexiglass sheet, and dried at 60 degrees Centigrade for 15 minutes. The cleared strips were then flattened between the pages of a heavy book and subsequently removed to storage packets for future study. The clear strips were analyzed by scanning on a Densicord

Recording Electrophoresis Densitometer equipped with a Model 49 Automatic Integrator (Photovolt Corp., New York).

Protein Determinations

Protein of each serum sample was measured by the biuret method of Gornall, Bardawill, and David (1949). Protein standards were either bovine serum albumin (Nutritional Biochemicals, Inc.) or Versatol (General Diagnostics Div., Warner-Chilcott Laboratories, Morris Plains, N. J.) serum standard.

Organisms

Salmonella californiа was chosen from the stock culture collection of North Texas State University by virtue of its ability to es tablish itself as a potential pathogen in guinea pigs and its ease of detection with differential media. Organisms were maintained on Trypticase Soy Agar slants (Difco) and transferred monthiy. Guinea pigs in Group III were infected by oral administration of one ml of an overnight broth culture containing 6×10^{10} organisms.

Flora Studies

Group III was used to study the ecological aspects of the spread of a specific pathogen under chamber conditions. Two samples were taken at each sampling date: one throat swab and one rectal swab. Each animal was swabbed on a weekly basis throughout the seven week experiment. The cotton swabs (Culturette, Scientific Products) were saturated with modified Stuart's transport medium contained in the plastic unit, shipped by bus to North Texas State University, and were available for testing within 24 hours after sampling.

More intensive determinations of the presence of Salmonella californiа were made at the Infectious Diseases Branch. Cotton swabs of the sampling sites were placed in tubes of SBG-Sulfa Enrichment Broth (Difco) and allowed to incubate for 12-18 hours. The swabs were

then streaked on XLD Media (BBL) and the plates were allowed to incubate for 24 hours. Appearance of typical black colonies on the XLD agar surface was indicative of the presence of Salmonella california. Confirmation of Salmonella california colonies was done with various biochemical tests and serological testing.

RESULTS

Survival

Out of the original eighty guinea pigs chosen for the study, sixty-five survived the fifty day experiment. Deaths of the animals were observed to occur randomly throughout the period although more occurred during the first two weeks. This was likely due to inexperience in bleeding the animals. Most deaths were observed to occur on the day following a blood sampling date. The results are tabulated in Table 15.

The results tabulated in Table 16 show the numbers of surviving guinea pigs in each experimental environment.

Weight Changes

Progressive weight changes of individual animals were recorded and tabulated according to six periods during the experiment. The first two periods correspond to the acclimation period, the second three to the period following injection of the antigen or oral inoculation of the infecting organism, and the last time period corresponds to the first week post-chamber. Mean weights for each group (I, II, III, or IV) were tabulated. Table 17 shows the weight changes of the animals during the three periods of the experiment. Mean weights of each group were calculated and recorded as positive if the mean increased by fifty grams or more during the period. A minus sign indicated the group lost a mean weight of fifty grams per animal.

TABLE 15

SURVIVORS OF FIFTY-DAY STUDY

Group	Original Number	Surviving Number
I	20	17
II	20	16
III	20	18
IV	20	14

TABLE 16

SURVIVORS ACCORDING TO ENVIRONMENT

Environment	Original Number of Animals	Number of Survivors
A-A	20	17
A-G	20	14
G-A	20	18
G-G	20	16

TABLE 17

CHANGES IN MEAN WEIGHTS OF
50 GM OR GREATER

Environment	Group	Acclimation Period	Post-Inoculation	Post-Chamber
A-A	I	0	+	0
	II	0	0	0
	III	-	+	0
	IV	0	0	0
A-G	I	+	+	0
	II	+	0	0
	III	0	+	0
	IV	+	+	0
G-A	I	-	0	+
	II	-	+	+
	III	0	0	+
	IV	-	0	+
G-G	I	0	0	0
	II	0	0	0
	III	-	+	+
	IV	0	+	0

Hematological Studies

All hematological values were placed on punch cards and averaged by computer for each of the six time periods. Changes of ten per cent or greater in the red blood cell count, hematocrit, and hemoglobin were consistent within groups. Although overall trends towards loss of red cell mass were not observed, fluctuations during the three main phases of the experiment were observed and are tabulated in Table 18. Table 19 lists changes in leukocytes for each group showing increases or decreases of 2,000 cells per cubic millimeter.

A differential leukocyte count was performed on 150 slides representing approximately 30 animals in each of four time periods during the experiment. Lack of a sufficient number of counts for each sub-group precluded statistical analysis for these designations. However, when a group of approximately thirty guinea pigs representing animals from all sub-groups was tabulated in each of three time periods, mean values of lymphocytes and eosinophils were shown to be significantly different in the first and last weeks. A slight increase in neutrophils was also observed. Differential counts are indicated in Table 20.

Serological Studies

Group I

Animals in Group I, injected with the somatic O antigen of Salmonella california, were checked for antibody production by agglutination tests. The highest titers reached for individual animals in the group are tabulated in Table 21.

Group II

All serum samples for Group II were run with gel diffusion plates made on the same day. In all cases, serum which was positive in a precipitin reaction in the gel reacted optimally with the well containing 0.1 mg/ml BSA. Table 22 indicated the date of first antibody appearance and mean date of appearance for the environmental group.

TABLE 18

COMBINED CHANGES IN
HEMATOLOGICAL PARAMETERS

Environment	Group	Acclimation Period*	Post-Inoculation	Post-Chamber
A-A	I	0	-	0
	II	0	0	-
	III	0	0	0
	IV	0	-	0
A-G	I	-	0	0
	II	0	0	0
	III	-	0	0
	IV	0	0	0
G-A	I	0	-	0
	II	+	-	0
	III	0	0	0
	IV	0	0	0
G-G	I	0	0	0
	II	0	0	0
	III	-	+	0
	IV	0	0	0

*The (+) or (-) symbols indicate ten per cent changes during each period in at least two of the three parameters of the red blood cell counts, hematocrit, and hemoglobin.

TABLE 19

CHANGES IN MEAN WHITE BLOOD CELL COUNTS
OF 2,000 PER MM^3 OR GREATER

Environment	Group	Acclimation Period	Post-Inoculation	Post-Chamber
A-A	I	0	+	0
	II	0	0	0
	III	0	+	-
	IV	0	+	0
A-G	I	0	+	0
	II	0	0	0
	III	0	0	-
	IV	+	-	0
G-A	I	0	+	0
	II	0	+	+
	III	0	+	0
	IV	0	+	0
G-G	I	0	+	0
	II	0	0	0
	III	0	+	0
	IV	0	0	0

TABLE 20

DIFFERENTIAL LEUKOCYTE COUNT
(PER CENT OF TOTAL CELLS)

Type	Range	Mean Value of Counts from 30 Animals		
		Week 1	Week 4]	Week 7
Neutrophils	20-77	46.3 ± 10.1	50.4 ± 10.7	52.9 ± 9.1
Lymphocytes	22-79	51.8* ± 10.8	47.5 ± 10.2	45.5 ± 9.1
Monocytes	0-5	0.7 ± .7	1.96 ± 1.4	0.9 ± 0.9
Eosinophils	0-5	1.3* ± .9	0.6 ± 1.0	0.6 ± 0.9
Basophils	0-2	. . .	. . .	. . .

*Significantly different from week 7 (P less than .05).

TABLE 21

HIGHEST TITERS REACHED FOR GROUP I

Environment	Number of Animals	Highest Titers	Mean Titer
A-A	5	40 320 1,280 80 40	352
A-G	3	40 80 20	47
G-A	4	40 10 40 40	33
G-G	5	320 80 80 5,120 5,120	2,144

TABLE 22

DAY OF FIRST APPEARANCE OF ANTIBODY
IN GROUP II

Environment	Number of Animals	Day of First Appearance of Antibody	Mean Day of Appearance*
A-A	3	22 22 **	27
A-G	4	15 20 34 **	26
G-A	5	20 22 23 ** **	28
G-G	4	20 20 22 31	23

*Assuming production on the thirty-sixth day by those not detected before that time.
**Animals not producing antibody by the thirty-sixth day.

Group III

Animals in Group III, infected orally with live Salmonella california, were checked for presence of the organism on the thirty-second day post-inoculation and for two subsequent days. All animals were bled on the thirty-sixth day post-inoculation and the serum was analyzed for presence of antibody specific for the organism. Table 23 shows the antibody titer on the thirty-sixth day post-inoculation as well as the results of the check for the presence of Salmonella california on throat and rectal swabs. Table 24 shows the number of animals having antibody titers specific for the organism according to whether they are carriers or non-carriers of the disease at time of sampling.

Electrophoretic Components

All scans of the electrophoretic strips were divided into six regions and the data transferred to punch cards. Computer programs calculating relative concentrations of components were designed ro indicate differences between groups, environments, and time periods. Significant increases in alpha-I globulin and decreases in beta-globulin fractions were found to occur during the experiment.

Protein

Protein levels of 250 serum samples from Groups I and IV were tabulated and found to fluctuate over a range of 49-62 mg/ml with a mean value of 53.8 ± 6.2. Variations were random throughout the samples. No major trends towards increased protein levels were seen with time or with environmental treatments. However, the final serum samples appeared to have lower protein levels.

TABLE 23

ANTIBODY TITER OF GROUP III AND
INCIDENCE OF INFECTION

Environment	Animal Number	Antibody Titer on 36th Day	Salmonella Recovery on 32nd Day
A-A	1	10	+
	2	40	+
	3	10	+
	4	20	+
	5	5	+
A-G	1	640	-
	2	640	-
	3	80	-
	4	20	+
	5	40	-
G-A	1	10	-
	2	10	-
	3	5	+
	4	10	+
	5		
G-G	1	320	+
	2	160	+
	3	5	+
	4	80	-

TABLE 24

ANTIBODY TITERS OF GUINEA PIGS ON
36TH DAY POST INFECTION AND
ASSOCIATED CARRIER STATE

Titer	Number of Animals	
	Carriers*	Non-Carriers
1:5	3	0
1:10	3	2
1:20	2	0
1:40	1	1
1:80	0	2
1:160	1	0
1:320	0	0
1:640	0	2

*Salmonella recovered on thirty-second day.

DISCUSSION

Environmental Effects on Weight

The guinea pigs in this study were young males approximately three months old. An increase in weight was expected and found to occur in all groups. However, the group held at altitude for acclimation and then brought to ground level after treatment by antigen injection or oral inoculation, group A-G, unexpectedly showed higher weight gains. The wide range in mean weights may be explained by the fewer numbers in the group, giving a statistically wider spread. The only consistent rise in all four groups at any time period occurred with the post-chamber period and the G-A environment. It appears that this group was stressed more inasmuch as the altitude stress coincided with the antigenic stress and that recovery in weight came only after the altitude stress was removed.

Hematological Changes

Although erythropoiesis is seen to occur with hypoxic, hypobaric conditions, the normoxic environment of simulated altitude in this study had no apparent effect on the hematological parameters.

A consistent leukocytosis was observed in all groups which were at altitude following antigenic treatment. This increase in white blood cell count was of unknown etiology, although it may be speculated that the increase was due either to the effects of hypobaric pressure per se, or to the slowly increasing levels of trace contaminants such as ammonia present in the chamber during the last three weeks of the experiment. Transient increases in white blood cell counts were observed in individual animals which were brought to ground level after acclimation at altitude. This transient increase has been observed in humans following orbital flights (Kaplan, 1967).

The ranges of cell types found in the present study compare very favorably with the reported

distributions of leukocytes in guinea pigs (Schermer, 1967). As the experiment progressed, a shift in relative numbers of leukocyte types was observed. This eosinopenia, lymphocytopenia, and neutrophilia is possibly explained by recalling that these effects are seen when adrenocortical hormones act on leukocyte production (Nelson, et al., 1952). Adrenocortical hormone liberation as a result of stress is well documented (Di Raimondo, 1961).

Antibody Production

Antibody response in guinea pigs injected with the Salmonella antigen appeared to be different according to the experimental environment. Statistical analysis of the data concerning animals producing antibody titers of 1:80 or greater revealed a significant (P less than .05) difference between the ground controls and the group shifted to altitude at the time of antigen injection. Although the size of the group precluded this evaluation for the A-G environment, the same effect appeared to have occurred. That is, those animals subjected to a change in environment at the time of antigen stimulation failed to produce high titers of antibody. This is indicated in the combined results of Table 25.

When the antibody titers for Group I are combined into groups which were not changed and groups which were changed at the time of antigen stimulation, the differences are even more dramatically revealed. Table 26 shows eight of ten animals of the combined A-A and G-G groups having an antibody titer of 1:80 or greater while the mean titer for the ten animals reached 1:1,248. However, only one of seven animals in the groups subjected to a change in environment showed a titer of 1:80 or greater and the mean titer for the seven animals only was 1:40.

The differences in antibody response of Group I is reminiscent of the work of Giron, Pindak, and Schmidt (1967) who reported that resistance to mengovirus infection was related to changes in the barometric pressure, regardless of the nature of the preinfection treatment.

TABLE 25

ANTIBODY PRODUCTION IN GROUP I

Environment	Number of Animals Having Highest Titer	
	1:80 or Greater	1:80 or Less
A-A	2	3
A-G	0	3
G-A	0	4
G-G	5	0

TABLE 26

COMBINED ANTIBODY TITERS OF GROUP I

Environment	Number of Animals	Mean* Titer	Number Producing Titer of 1:80 or Greater	Number Producing Titers of Less Than 1:80
A-A + G-G	10	1,248	8	2
G-A + A-G	7	1:40	1	6

*Obtained from highest titer measured for each animal.

On the other hand, the antibody response to oral challenge with Salmonella california appeared to be related to the environment after infection. The animals maintained at altitude after challenge showed the lowest antibody titers with seven of the nine animals remaining carriers of the organism after thirty-two days. Animals kept at ground level after infection showed the highest antibody titers with only four of nine animals showing persistance of the organism after thirty-two days. These results are indicated in Table 27.

Groups showing the fewest number of carriers per cage were those which were shifted in test environment after challenge. A possible explanation of this lowered carrier state could be that the cage change brought about a state where less chance of re-infection from the surrounding environment was possible.

Antibody response to the injected antigens and the orally administered antigen may be summarized in the following statements.

1. The greatest amount of antibody depression occurred in those animals which were placed in the chamber at the time the antigen was administered. This environment (G-A) had the lowest number of animals producing antibody regardless of challenge.

2. Antibody depression in Group I was greatest in those animals which were shifted in environment at the time of antigenic stimulation.

3. Antibody depression in Groups II and III was greatest in those groups which were at simulated altitude following administration of the antigen.

Electrophoresis

The albumin-globulin (A/G) ratios in Groups I and III which were challenged with bacterial antigens, appeared to increase with time. Since the albumin and protein levels were essentially constant during this time, a decrease in relative concentrations of beta-globulins may be hypothesized. Although no specific function for beta-globulins has been established, these

TABLE 27

ANTIBODY PRODUCTION IN GROUP III

Environment	Number of Animals	Mean Titer at 36 Days	Number Infected* at 32 Days
A-A	5	1:30	5
A-G	5	1:280	1
G-A	4	1:10	2
G-G	4	1:140	3

* Salmonella california detected in either throat or rectal swabs taken daily for three days.

proteins may be involved in the ratio changes since alpha-1 levels increased while gamma and alpha-2 levels remained constant. An increase in alpha-1 concentration may be involved in the adaptive changes required to phagocytize the invading bacterial cells. Alpha-1 globulin seems to have some role in the enhancement of phagocytosis (Downey, 1964).

Conclusions

These data support the concept that nonspecific stress such as that found in hypobaric pressures and pressure changes may produce variations in the immune response of animals challenged with diversified antigens. In this study, hematological shifts due to this hypobaric stress were not apparent in the erythroid line of cells. However, the stress produced effects on the leukocytes which resembled those of adrenocortical hormone interraction. The depression of antibody titers in guinea pigs which were shifted in environment at the time of antigenic challenge can be considered to be due to one or more of the following. 1.) Proliferation of essential germinal centers of antibody production may be reduced by the increased hormonal levels which were produced by the nonspecific stress. 2.) Relative shifts occurred in mobility of leukocytes and/or monocytes necessary for initial ingestion and processing of the antigen. This may be concomitant with shifts of body water towards increases or decreases in intracellular fluids. Or, 3.) An initial inflammatory response, often involved in reaction to antigenic stimulus, was inhibited by the hormonal production which came when the atmospheric pressure abruptly changed.

The immune response of animals is a complex mechanism involving many physiological parameters. As knowledge concerning individual responses to single or related environmental factors is increased, a more complete understanding of the mechanism of antibody response may be revealed. This knowledge may be useful as man adapts to environments devised for outer space.

Rectal Swab Cultures

The animals included in the long term chamber exposure were also followed by rectal swab cultures at bi-weekly intervals, as well as for the continued presence of Salmonella california in the intestinal tract.

The general flora studies indicated the same trend as reported for short term experiments of reduction in numbers of types of bacteria present. This reduction occurred early in the period of chamber exposure and was not progressive. This result was expected since the animals were not isolated in the chamber and were re-exposed during feeding, handling, and cleaning at frequent intervals throughout the test period. These results do not then, indicate what may be expected when isolation experiments may be carried out for this period of time.

VII. In vitro tests of bacterial cultures exposed to chamber environment.

All tests were performed at the equivalent of 18,000 feet altitude and 100% oxygen environment. Cultures were placed in the chamber immediately after inoculation into broth and onto agar slants, and were exposed to culture environments for a one week period. After removal from the chamber the cultures were transferred to broth for 18-24 hours and were then inoculated into the battery of fermentation and biological test media as listed in Table I for the experimental organisms. Bacteria tested have been listed previously (page 1). There was some change in results following a second week of chamber exposure as compared to the one week exposure results, however, few altered characteristics reverted to the original organisms which were observed to show altered fermentative characteristics, and the characteristics changed are shown in Tables 28 and 29.

TABLE 28

IN VITRO TESTS WITH BACTERIA

Test	Organisms: Pseudomonas (1) Before	Pseudomonas (1) After* 1	Pseudomonas (1) After* 2	Pseudomonas (2) Before	Pseudomonas (2) After 1	Pseudomonas (2) After 2	Pseudomonas (3) Before	Pseudomonas (3) After 1	Pseudomonas (3) After 2	Salmonella (4) Before	Salmonella (4) After 1	Salmonella (4) After 2
Dextrose	-	+	+	-	+	-	-	+	+	+	-	+
Maltrose	-	+	+	-	-	+	-	+	+	+	+	+
Mannitol	-	-	-	-	-	-	-	-	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	+	-	-	-
Rhamnose	-	-	-	-	-	+	-	-	-	+	+	+
Salicin	-	+	+	-	-	-	-	-	-	-	-	+
Trehalose	-	+	+	-	-	-	-	-	-	+	+	+
Xylose	-	-	-	-	-	+	-	-	-	+	+	+
Sorbitol	-	-	-	-	-	-	-	-	-	+	+	+
Citrate	+	+	+	+	+	+	+	+	+	-	+	+
Gelatin	+	+	+	+	+	+	+	+	-	+	+	+

(1) P. aeruginosa, ATCC 7700
(2) P. aeruginosa, ATCC 15442
(3) P. aeruginosa, ATCC 17933
(4) S. califomia (experimental strain)
*1 = one week exposure, 2 = two weeks exposure.

TABLE 29

IN VITRO TESTS WITH BACTERIA

Test	Organisms														
	Staph (1)			Staph (2)			Staph (3)			Strep (4)			Strep (5)		
	Before	After*		Before	After		Before	After		Before	After		Before	After	
		1	2		1	2		1	2		1	2		1	2
Inulin	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Salicin	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-
Sorbitol	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Litmus Milk	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-
Gelatin	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-

(1) Staphylococcus epidermidis, A CC 155
(2) S. aureus, ATCC 12600
(3) S. aureus, ATCC 6020
(4) Streptococcus pyogenes, ATCC 12962
(5) Streptococcus sp. (Group G), ATCC 12394
*1 = one week exposure, 2 = two weeks exposure

VIII. Summary and Conclusion.

Based on results shown in this report, the following conclusions may be drawn.

1. There is a general overall reduction in numbers of types of aerobic bacteria present in rectal swab cultures in either guinea pigs or rabbits following exposure to simulated space-craft environment for a one week period. Some results indicate a similar trend toward reduction in bacterial types in throat cultures, although results are less conclusive.

2. Exposure to Salmonella califomia or Pseudomonas aeruginosa immediately prior to chamber environment exposure appears to increase the possibility of obvious infection being produced.

3. Exposure of S. california, rough strain, in vivo, in guinea pigs resulted in consistent conversion to a smooth strain.

4. Exposure of guinea pigs to S. california by the digestive route or to S. pyogenes by the respiratory route results in little or no immune response after two weeks, either following chamber exposure for one week or retention at ground level.

5. In guinea pigs, there is a tendency toward increases in Gram positive organisms in rectal swab flora concurrent with decreases in numbers of types following one week chamber exposure.

6. In longer term exposure to chamber environment (a) there was little difference in chamber vs. ground level immune response If the antigenic stimulus was applied after acclimatization to the environment and the environment was not altered for three to four weeks. (b) Little or no immune response was produced if the antigen was applied just prior to a change from altitude to ground or from ground to altitude indicating that the stress of the change alters the immune mechanism. (c) No measurable effects of chamber environment were observed in weight, leukocyte count or differential, erythrocyte count, hemoglobin, hematocrit, or electrophoretic patterns.

7. Serological tests on rabbits exposed to S. california confirmed the effect of change from ground to altitude on the immune response as was observed in guinea pigs.

8. Confirmation is seen in these results of the ability to transfer potential pathogens from carrier to normal animals in a simulated space environment. Some evidence is seen that a dormant carrier may become active when changed from ground to chamber environments.

9. Exposure of guinea pigs to S. california as much as five weeks prior to chamber environment appears to confer some degree of resistance on the animal as compared to exposure at the time of change to the chamber environment.

10. In vitro exposure of a number of bacterial cultures to the simulated spacecraft environment results in a number of changes in biochemical and/or fermentative characteristics.

XIX. Based on the results contained in this report, it is requested that the subject contract be re-negotiated with Clemson University, Clemson, South Carolina in order that this work may be continued as previously proposed by the investigator.

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