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"Effects of High and Low Barometric Pressures on Susceptibility and Resistance to Infection"

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Summary. During the period of this report two additional experiments on the effect of prolonged increased oxygen tension at one atmosphere (Fig. 1) on enteric flora of mice were completed and one additional and perhaps final experiment in this series was initiated. Tentative conclusions have been drawn. The completion of chambers for holding mice under increased pressure, up to 95 psig (Figs 2 and 3), have permitted initiation of a new series of experiments at this pressure. Final items of equipment have been obtained to allow experiments at simulated altitudes. During this period, two additional independent investigators have been added to the staff. Dr. Kun-yen Huang has begun a program of observations on the production and effect of interferon in vivo and in cell cultures in vitro under parabarosis. CAPT Charles E. Meyers, DC, USN, is planning observations on the normal and/or altered flora of personnel engaged in experimental diving tests preliminary to Sea Lab III or in other confined spaces.

Normal enteric bacterial flora of mice.

Experiment 4.-The preliminary report of this experiment was made in the Fourth Quarterly Status Report, 1 April - 30 June 1966, and the final results are now presented in Table 1, and in Figure 4. In this experiment a large group of mice were placed together in a single large mouse box under normal conditions for three weeks. They were then divided into three groups of 10 mice each, one of which was kept on the shelf in the animal room (Room Air Group), a second group was placed in a chamber through which air from a compressed air tank was allowed to flow (Tank Air Group), and a third group (O_2 Group) was placed in a similar chamber through which a mixture of oxygen, 60% total, in air was allowed to flow. All three groups were at one atmosphere of pressure. Cultures of weighed fecal pellets of each mouse were made at intervals from one week previous to placing in the 3 environments to 6 weeks after, as indicated in Table 1. Although there were changes in the level of several species of bacteria during the course of the experiment, the trend was for the levels in all three of the groups of mice to be similar. Standard errors were calculated in all cases.

Comparison of the levels of slow lactose fermenters in the 3 groups indicates a significant departure of the O_2 group of mice from the two control groups. This is indicated by underlined figures in Table 1 and by the charted levels in Fig. 4. At the 2nd and 4th weeks the numbers of slow lactose fermenters in the stools of the O_2 mice were considerably less from those of the 2 control groups which were not significantly different. By the 6th week this difference had become much less. Similar differences were seen with an atypical (aberrant) E. coli, which appears only on plates where slow lactose fermenter colonies are present and in proportion to them, and for this reason would be expected to follow the curve of the latter in incidence.

(Figures underlined in Table 1). The incidence of a Klebsiella sp. was also different in the O_2 mice and controls at weeks 4 and 6, the O_2 group having a higher incidence (Figures underlined in Table 1).

Experiment 5.-The plan of this experiment was similar to that of Experiment 4, except that an oxygen level of 77% total in air was used instead of the 60% previously employed. The control group of mice received air from a tank. Also included was a group of mice exposed to 90% total oxygen in air, but this level was found to be incompatible with a long term experiment. All mice of this last group died between the 1st and 4th week after being placed in this atmosphere. In contrast to Exp. 4, no significant differences were observed between oxygen exposed mice and controls, with respect to slow lactose fermenters and the aberrant coli type. The Klebsiella, however, behaved similarly as in Exp. 4, and the results are depicted in Figure 5. All but one of the control mice became negative for Klebsiella after being placed in the chamber (no recovery from 0.1 ml of a 10^{-3} dilution). In contrast, three and four out of ten of the oxygen group continued to show levels of Klebsiella. The curve for the controls, Figure 5, is not significantly different than that for the oxygen mice, but the former includes the negatives (no growth from 0.1 ml of a 10^{-3} dilution, i.e., negative at 1.0 ml x 10^{-4}) which are calculated as positive at the next lower log dilution. They are given a positive value of $10^{3.4}$, i.e., 1 of 4 plates possibly positive at the lower dilution. Since this confirms the trend seen in Exp. 4, the difference is probably significant. A reasonably significant difference was also seen between the 77% oxygen group and the controls at six weeks with respect to colony type 2 of an anaerobic lactobacillus (Fig. 6). The P value for the difference between the 77% oxygen and control groups (t test) was found to be less than 0.001. A similar difference with respect to colony type 2 of this lactobacillus was seen in Exp. 4 at 2 weeks, although the difference was not statistically significant.

Experiment 6.-A group of mice was kept for a period of 7 weeks in room air and stool cultures were made at 0, 2 and 7 weeks. This was for the purpose of determining whether variations in bacterial count such as have been seen in control mice in chambers would be observed in the shelf mice. Expecially between the 2 and 7 week culturings there was little change in level of the different species. This suggests that unrecognized factors associated with maintenance of mice in exposure chambers even under normal air at one atmosphere influence the estimation of levels of enteric bacteria.

Experiment 7.-This experiment, now underway, is designed to test the hypothesis mentioned above (Exp. 6). Mice at 77% O_2, mice in air from a compressed air tank, and mice in normal room air (shelf mice) are being observed in the usual manner. It is anticipated that this

experiment will be the last in this particular series to determine the effect of increased oxygen tension at one atmosphere on enteric flora of mice.

Effect of hyperbaric environments on susceptibility of mice to aerosol challenge with mouse pneumonitis agent (Chlamydia). Three steel and plastic chambers that will hold approximately 15 mice each and allow the use of positive pressures up to 95 psig, and simulated altitude to nearly 0 mm Hg, pressure, were first used during the period of this report. Suitable pumps for handling oxygen in the hypobaric environment have recently been acquired. After some practice and training of personnel in maintaining mice under pressure, one experiment was completed.

Experiment Mopn 15.-Ten mice (Group A) were held in a pressure chamber for 3 weeks at a simulated depth of 207 ft. sea water (95 psig) in a mixture of O_2 (2.8%) and N_2. This provides a pO_2 equal to standard conditions at sea level. A control group (B) were held in the same type of chamber in air at 1 atmosphere. Six test and 9 control mice, available for challenge at 3 wks, were exposed to an aerosol of mouse pneumonitis agent (Fig. 7), and returned to their respective chambers. Two days later the test mice were suddenly decompressed due to a leak in the chamber, but survived and were returned to pressure within 90 minutes. On the 9th day all 6 of the test mice (under pressure) died, one only minutes before scheduled sacrifice. All of the control group survived but were sacrificed on the 9th day. Data on this experiment are summarized in Table 3. The results point to a greater susceptibility of the pressurized mice, and additional experiments are planned to determine what factor actually is responsible. One such experiment now underway will compare 3 groups of mice: at 95 psig with 2.8% O_2; at simulated 37,000 ft. altitude with 100% O_2; and in air at 1 atmosphere (0 psig).

Effect of increased oxygen on immunizability of mice with Vi antigen as determined by a challenge with Salmonella typhi (Ty 2). A group of 20 mice were placed in a chamber with a constant flow of oxygen (total 77%) in air at two liters per minute. A 2nd group of 20 mice were placed in a chamber with air from a compressed air tank flowing at the same rate, and a 3rd group of equal size were kept on a shelf in the room. After 7 days in these environments the mice of each group were arranged into 4 subgroups of 5 mice each, and injected subcutaneously into the neck region with a single dose of Vi antigen in tenfold varying amounts as indicated in Table 4. They were then returned to their respective environments. Six days later the mice were challenged intraperitoneally with a saline suspension of 6×10^7 cells per milliliter (0.25 ml) of Salmonella typhi (Ty 2). This represents approximately 5 ip LD_{50}'s. Three days after inoculation when the effect of the challenge could be expected to be complete the number of dead and

survivors were tabulated (Table 4). It may be seen that the mice in the 2 control environments varied considerably in their apparent response to immunization, supporting our conclusion from other observations that mice in a chamber in a normal atmosphere of air from a tank cannot be considered to be equal to mice held out of the chamber in room air. If the results in the "tank air" group represent a valid base for comparison with the O_2 group, the indication is that exposure to O_2 modified the ability of mice to respond to the Vi antigen. In a previous experiment, reported in the 4th Quarterly Report, evidence was presented that the 3 environments do not greatly affect the results of challenge with this S. typhi strain. Increased mouse holding facilities will now permit this experiment to be repeated on a larger scale.

Studies on altered gaseous environment at the cellular level. (This portion of the report prepared by Doctors Neptune and Weiss). As noted in the last report, alterations occur in microbial enzymatic activity as a function of the gas environment. This work has been extended and specific mechanisms have been delineated.

As reported before, the pyruvic oxidase of meningopneumonitis agent (Chlamydia) is stimulated by oxygen. This finding is contrary to our hypothesis that inhibition of pyruvic oxidase is one of the specific mechanisms for the toxic effects of oxygen. It has been reported that hydrogen peroxide reacts spontaneously with pyruvate and the pyruvate is decarboxylated. This reaction has been studied in our laboratory in considerable detail. The results indicate that without any doubt H_2O_2 and pyruvate do form an highly unstable mixed anhydride which decomposes to yield CO_2, water and acetate. As reported before, this microbe has a pentose phosphate pathway which we have portrayed in Fig. 8. Note that the terminal sequence on the right involves a peroxidation reaction with the generation of H_2O_2. If we assume that endogenous traffic over this pathway is continuous and that endogenous H_2O_2 is decarboxylating pyruvate, then destroying part or all H_2O_2 should decrease the amount of CO_2 released from pyruvate. The enzyme catalase destroys H_2O_2. When the pyruvic oxidase of meningopneumonitis was studied in the presence of added catalase there was definitely less stimulation by oxygen as shown in Table 5.

Since the endogenous metabolism that produces H_2O_2 is presumably not maximal, it seemed important to attempt maximal stimulation and observe the decarboxylation of pyruvate. The obvious experiment is to add excess glucose-6-PO_4, NADP and GSSG. Glucose-6-PO_4, however, enters not only the pentose phosphate pathway but is also degraded to pyruvate. This latter reaction permits the mixing of metabolically formed pyruvate with added radioactive pyruvate and thus the yield of $C^{14}O_2$ from pyruvate appears to be diminished. Therefore, it was necessary to add an inhibitor that would impede the breakdown of

glucose-6-PO_4 to pyruvate. We chose iodoacetate which inhibits glyceraldehyde-3-PO_4 dehydrogenase.

As shown in Table 6 the pentose pathway (carbon 1) is only modestly inhibited by iodoacetate, whereas the pathway to pyruvate (carbon 3 and 4) is severely depressed by iodoacetate. Therefore, it appeared that experiments with iodoacetate were feasible.

The data in Table 7 clearly indicate that in the presence of glucose-6-PO_4 there is a marked stimulation of the decarboxylation of pyruvate and that this stimulation is abolished by catalase.

Effect of parabaric conditions on induction and effect of interferon. A facility has been set up, including a laboratory, equipment and reagents for performing quantitative tests for interferon. Standard interferon inducers and interferon preparations have been prepared and titrated.

Table 1. (Exp. 4) Stool cultures of mice in 3 types of gaseous environment. Numbers of bacteria per gram. (Figures are logarithms of the geometric means of counts from 10 mice).

Type of bacterium	Interval (weeks)	Environment in which mice were kept. (1 atmosphere) Room air	Tank air	O_2(60% total) in air
E. coli (aberrant)	-1	7.04	7.46	7.10
	0	7.76	7.22	7.55
	1	6.92	7.50	6.93
	2	7.67	7.39	5.56
	4	6.76	6.34	4.94
	6	5.23	5.76	4.80
Slow lactose fermenters	-1	8.98	8.64	8.08
	0	8.52	8.02	8.49
	1	7.57	8.13	7.36
	2	8.11	7.73	6.25
	4	7.28	6.86	5.75
	6	6.19	6.75	5.80
Klebsiella sp.	-1	5.28	5.35	5.44
	0	4.49	4.76	5.05
	1	3.71	3.95	4.30
	2	3.80	4.02	4.38
	4	3.65	<3.40	4.63
	6	3.56	3.55	4.12
Lactobacillus anaerobic, colony type 1	-1	7.80	7.66	8.24
	0	7.75	8.64	8.78
	1	8.34	8.29	8.03
	2	8.41	8.33	7.80
	4	8.01	7.96	8.13
	6	7.06	8.16	7.61
colony type 2	-1	7.71	7.53	7.91
	0	8.61	8.62	8.46
	1	8.27	8.10	7.89
	2	7.82	7.03	5.88
	4	8.07	7.93	7.28
	6	7.86	8.85	7.81

(Table continued on next page)

Figures discussed in text are underlined.

Table 1 (continued)

Bacteroides	-1	6.49	6.76	6.52
	0	8.07	7.97	8.28
colony type 1	1	8.48	8.30	8.04
	2	6.92	7.92	7.39
	4	7.66	7.24	7.33
	6	7.82	8.22	7.64
colony type 2	-1	8.99	8.94	8.93
	0	8.81	8.67	8.78
	1	8.63	8.99	8.68
	2	6.19	6.79	6.76
	4	7.93	7.39	7.72
	6	7.62	7.91	7.40
colony type 3	-1	9.01	8.82	8.79
	0	8.45	8.56	8.34
	1	8.35	8.33	8.18
	2	7.89	8.14	7.56
	4	7.72	7.69	7.53
	6	7.27	8.59	7.56

Table 2. (Exp. 5) Stool cultures of mice in 3 types of gaseous environment. Numbers of bacteria per gram. (Figures are logarithms of the geometric means of counts from 10 mice).

Type of bacterium	Interval (weeks)	Environment in which mice were kept. (1 atmosphere) Tank air	O_2 (90% total in air)	O_2 (77% total in air)
E. coli (aberrant)	-2	8.91	8.57	8.92
	0	3.66	3.76	3.81
	+2	7.74	6.02	7.31
	+4	5.67	--*	5.68
	+6	5.79	--	5.05
Slow lactose fermenters	-2	9.93	9.99	9.77
	0	9.61	9.50	9.56
	+2	8.42	9.01	7.81
	+4	6.33	--	7.37
	+6	6.46	--	5.70
Klebsiella sp.	-2	7.96	6.84	6.78
	0	8.75	8.58	8.94
	+2	3.51	4.15	3.93
	+4	3.58	--	4.16
	+6	3.53	--	4.21
Lactobacillus anaerobic, colony type 1	-2	8.35	9.14	7.98
	0	8.80	8.92	8.74
	+2	6.68	6.59	6.91
	+4	3.76	--	4.56
	+6	6.19	--	7.25
colony type 2	-2	7.95	8.77	8.38
	0	8.85	8.92	8.74
	+2	8.18	8.75	7.96
	+4	6.95	--	7.04
	+6	7.53	--	6.49
Bacteroides colony type 1	-2	8.82	8.44	8.64
	0	9.42	9.30	9.45
	+2	7.26	8.28	7.26
	+4	8.76	--	8.58
	+6	4.18	--	5.46

(Table continued on next page)

Figures discussed in text are underlined.

*All animals died between 2nd and 4th week under a 90% oxygen atmosphere.

Table 2 (continued)

colony type 2	-2	7.66	7.55	7.51
	0	9.56	9.37	9.52
	+2	7.57	9.11	7.55
	+4	7.94	--	8.13
	+6	6.95	--	6.00
colony type 3	-2	8.43	7.12	7.67
	0	9.07	8.71	9.03
	+2	7.38	8.91	6.55
	+4	8.48	--	8.24
	+6	6.10	--	5.45

Table 3.-- Effect of pressure on susceptibility of mice to mouse pneumonitis agent.

	A. Mice held at 95 psig in 2.8% O_2 in N_2.	B. Mice held in air at 0 psig.
D/T	5/6	0/9
Gross changes in lung, hemorrhage	2.8*	1.9
consolidation	2.1	1.3
Degree of infection (smear)	2.0	1.4
Titer of agent in lungs	8.6×10^5 (1 sacrif'd mouse)	4.1×10^5 (9 sacrif'd mice)
	4.2×10^5 (5 mice found dead)	

*Average score for each group.

Table 4.-- Resistance to challenge of mice immunized with Vi antigen after being held in 77% O_2 and in air.

Immunizing dose, Vi antigen, µg	Result of challenge with Sal. typhosa; dead/total mice		
	77% O_2	Tank air	Room air
5.0	0/5	0/5	0/5
0.5	0/5	0/5	0/5
0.05	1/5	0/5	1/5
0.005	3/5	1/5	4/5
0.0 (shelf mice): (4/5)			

Table 5.-- Pyruvic Oxidase of meningopneumonitis agent; response to catalase

Addition	Gas Phase		
	N_2	Air	O_2
None	36*	41	48
Catalase	37	43	41

*mμmoles CO_2

Each flask contained a high K^+, low Na^+, phosphate buffered solution and 0.4 μc and 4 μmoles of pyruvate. 300 units of sterile catalase was added to appropriate flasks. The final volume was 1.9 ml and the pH 7.0. The flasks were incubated for 2 hours at 34 C.

Table 6.-- Dehydrogenases of meningopneumonitis agent

Micromoles added			mμmoles CO_2 per mg agent protein from C of glucose	
NADP	GSSG	IAc	1	3,4
-	-	-		46
-	-	2		3
3	10	-	145	56
3	10	2	103	3.5

Conditions were similar to those given for Table 5. The special additions were added as given. The gas phase was air.

Table 7.-- Pyruvic oxidase of MN agent

Addition	mμmoles CO_2/mg protein
None	66
Iodoacetate	54
G-6-P, NADP, GSSG	42
G-6-P, NADP, GSSG, Iodoacetate	120
G-6-P, NADP, GSSG, Iodoacetate, Catalase	32

As indicated, flasks contained 4 μmoles of pyruvate (1-C^{14}), 5 μmoles of glucose-6-PO_4, 3 μmoles of NADP, 10 μmoles of NADP. Iodoacetate was 10^{-3} M and catalase was 300 μmolar units. The gas phase was air. Other conditions were as noted in Table 6.

Figure 1. One of a battery of 4 chambers used for exposing mice to altered O_2 tensions at one atmosphere (normal) pressure.

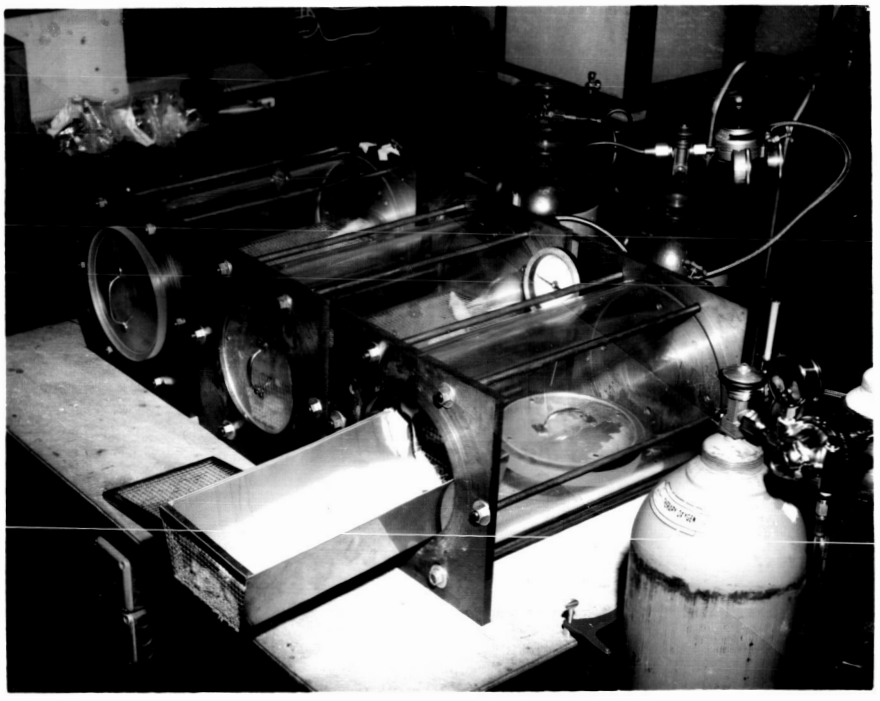

Figure 2. Chambers for holding mice at various atmospheric pressures from 0 (simulated altitude) to 95 psig (simulated depth of 207 ft in the sea) and varying pressures of O_2, N_2 and air.

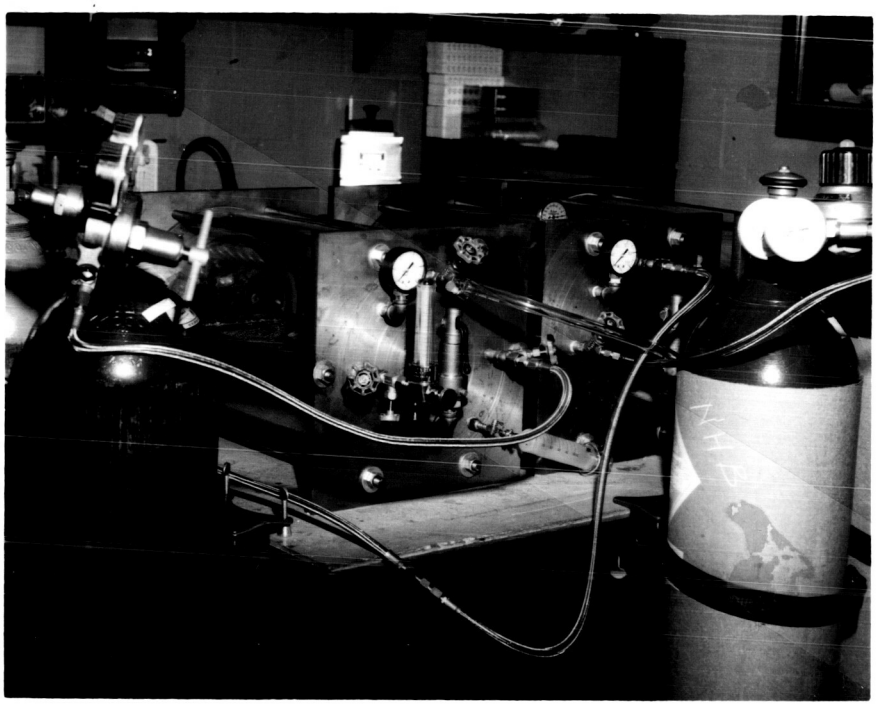

Figure 3. Opposite end of chambers illustrated in Figure 2, showing controls.

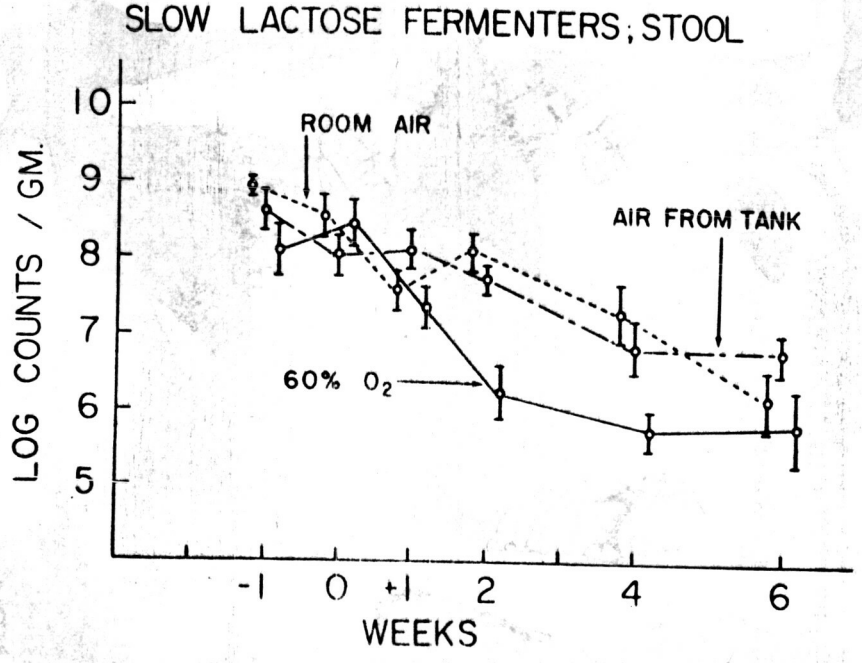

Figure 4. Chart of levels of slow lactose fermenters in the 3 groups of mice in Experiment 4. Levels of the atypical E. coli were of the same pattern.

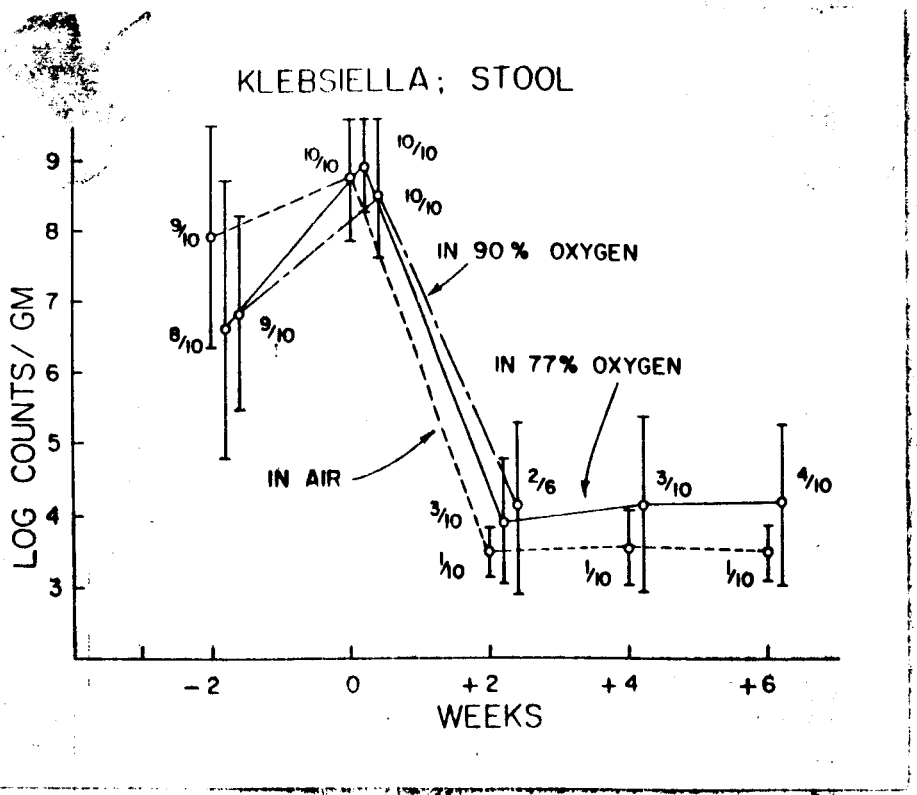

Figure 5. **Chart of levels of Klebsiella sp. in the mice of Experiment 5. Numbers of mice, of the total of 10, in which this bacterium was detected at each culturing are also indicated.**

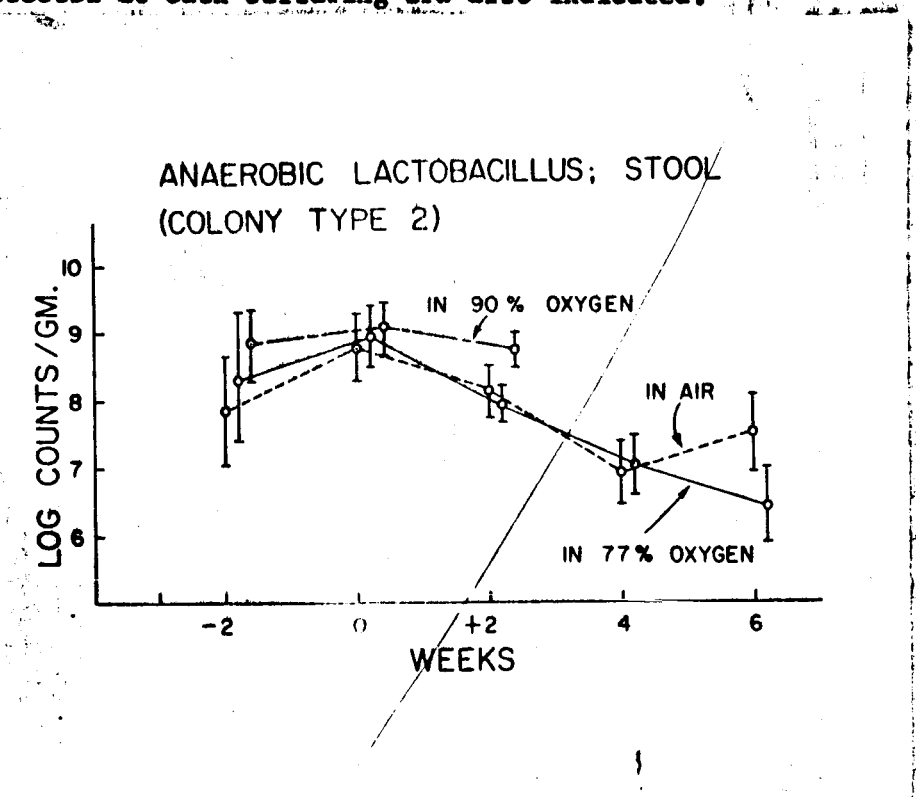

Figure 6. **Chart of levels of colony type 2 of an anaerobic lactobacillus in mice of Experiment 5.**

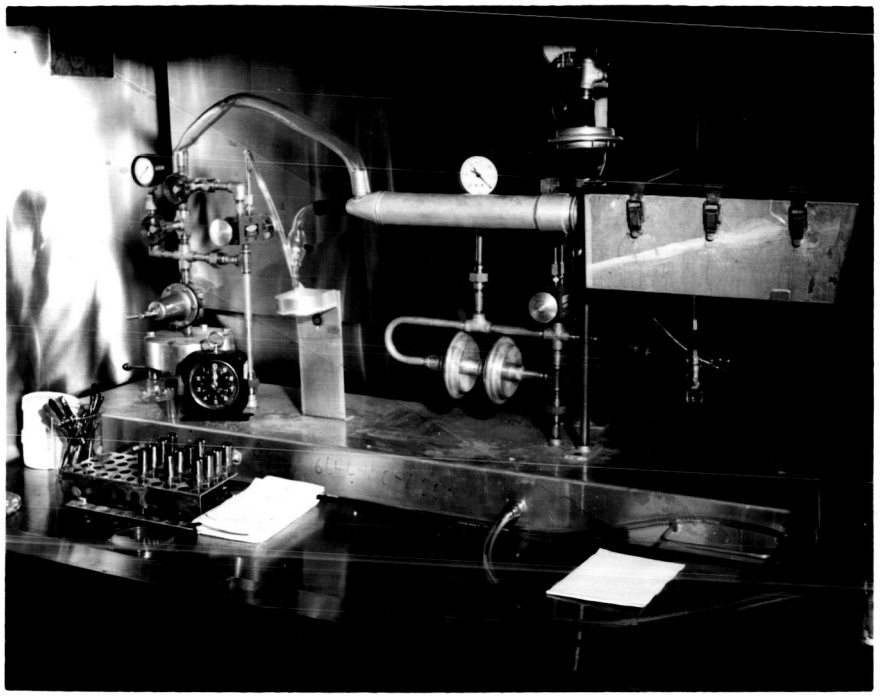

Figure 7. Henderson apparatus used for exposing small animals to an aerosol of infectious agents.

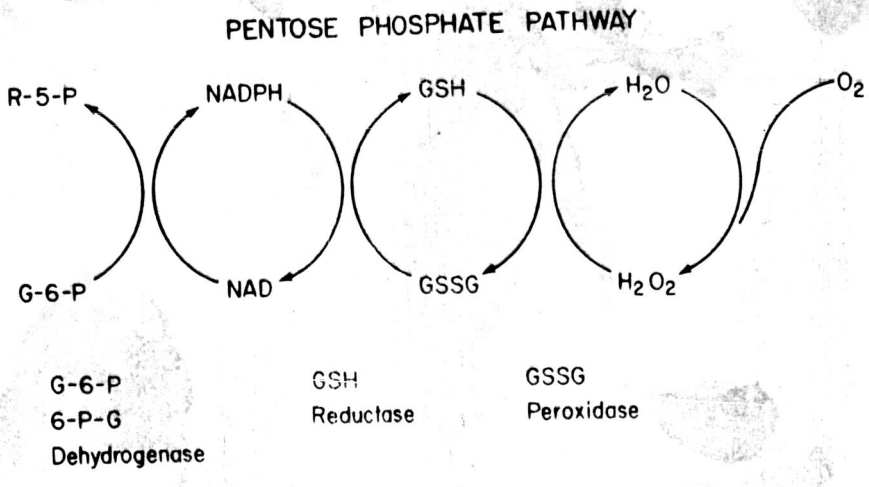

Figure 8. Pentose phosphate pathway used by meningopneumonitis agent.