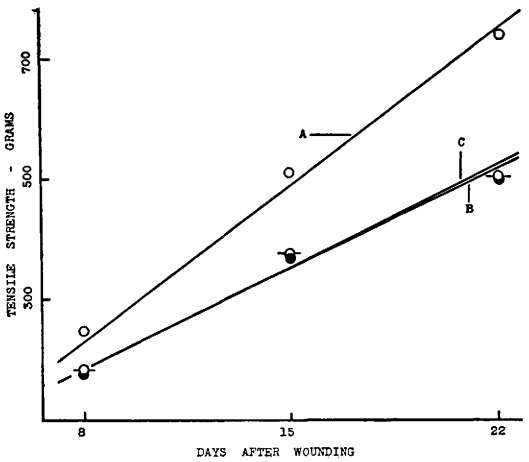

FIG. 3. Tensile strength of healing wounds in rats on a 5% casein diet plotted against time. Curve A (Group I), alanine supplement, *healing index (K)* = 31; Curve B (Group II), ethionine and methionine supplement, $K = 21$; Curve C (Group III), ethionine and cystine supplement, $K = 21$. Significance between mean values of tensile strength for Groups I and II is "p" = .01.

relatively small as compared to the cystine requirement.

Summary. The effect of methionine and cystine on the *healing index* of standard experimental wounds in rats was determined. Since both amino acids have the same effect, per equivalent of sulfur, it is concluded that methionine is converted to cystine before being used in the healing process. When the utilization of methionine is blocked by ethionine, cystine is ineffective, indicating that some methionine, *per se,* is required for the healing of wounds. There appears to be a correlation between the *healing index* and the retention of amino acid sulfur in excess of that expected on the basis of nitrogen retention.

1. Williamson, M. B., McCarthy, T. H., and Fromm, H. J., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 302.

2. ———, *Fed. Proc.,* 1951, v10, 270.

3. Localio, S. A., Morgan, M. E., and Hinton, J. W., *Surg. Gyn. Obst.,* 1948, v86, 582.

4. Croft, P. B., and Peters, R. A., *Nature,* 1945, v155, 175; *Lancet,* 1945, 266.

5. Peters, R. A., *Brit. Med. Bull.,* 1945, v3, 81.

6. Morris, H. P., Dubnik, C. S., and Dunn, T. B., *J. Nat. Cancer Inst.,* 1945, v5, 271.

7. Williamson, M. B., and McCarthy, T. H., unpublished data.

8. Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutr.,* 1937, v14, 273.

9. Charney, J., Williamson, M. B., and Bernhart, F. W., *Science,* 1947, v105, 396.

10. Masters, M., *Biochem. J.,* 1939, v33, 1313.

11. Treon, J. F., and Crutchfield, W. E., *Ind. Eng. Chem.,* 1942, v14, 119.

12. DuVigneaud, V., Kilmer, G. W., Rachele, J. R., and Cohn, M., *J. Biol. Chem.,* 1944, v155, 645.

13. Binkley, F., *J. Biol. Chem.,* 1944, v155, 39.

14. Simpson, M. V., Farber, E., and Tarver, H., *J. Biol. Chem.,* 1950, v182, 81.

15. Goldberg, R. C., Chaikoff, I. L., and Dodge, A. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 869.

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Effects of Auxotrophic Mutations on the Adaptation to Inositol Degradation in *Aerobacter Aerogenes.** (19713)

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Enzymatic adaptation and cell division in microorganisms are closely allied. Both processes are inhibited by the same agents, and in many instances only dividing cells are capable of adaptation. When adaptation can occur in the absence of a source of exogenous nitrogen, lower levels of adaptive enzymes are attained than in media capable of supporting growth(1). It has been postulated that in resting cells an interconversion of enzymes is responsible for adaptation(1). On the other hand, very recently, evidence has been presented that resting yeast cells contain

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a pool of free amino acids and that adaptation can be prevented by amino acid analogues, suggesting synthesis of adaptive enzymes from a reserve of simple nitrogen compounds(2). In addition, the ribonucleic acids of the cell may be involved in adaptation.

The present paper reports preliminary results of an attempt to elucidate some of the phases of the mechanism of enzymatic adaptation with the use of amino acid, purine and pyrimidine-deficient mutants.

Previous studies in this laboratory have shown that the ability of capsulated strains of *Aerobacter aerogenes* to grow on *myo*-inositol as the only source of carbon is due to the formation of adaptive enzymes. Resting cell suspensions of this organism can adapt to the oxidation of inositol in 30-60 minutes(3). The adaptation seems to involve the synthesis of several enzymes, acting on a series of compounds intermediate in the degradation of *myo*-inositol(3,4). The inositol system was used in the present study, in the hope that the results would contribute to the understanding of enzymatic adaptation in general, as well as to the specific problem of inositol metabolism.

Materials and methods. Myo-inositol (formerly called *meso*-inositol or i-inositol) was obtained from the Corn Products Refining Co. *D,L-tryptophane,* and *L-histidine* were Merck preparations. *D,L-leucine* was a product of Hoffmann-LaRoche, and *guanine* and *uracil,* products of the Nutritional Biochemicals Corporation. The purity of these compounds was checked by paper chromatography and ultraviolet spectroscopy.

The salt base used in the growth experiments and as suspending medium in the manometric experiments consisted of Na_2HPO_4 0.54%, KH_2PO_4 1.26%, $MgSO_4 \cdot 7H_2O$ 0.02%, $CaCl_2$ 0.001%, pH 6.5.

Aerobacter aerogenes 1033, a capsulated strain, giving the characteristic biochemical reactions of this group was originally isolated from a patient at Boston City Hospital. It has been used in biochemical studies of inositol and glycerol metabolism in this laboratory(4,5). The *amino acid-deficient mutants* were obtained after ultraviolet irradiation by the penicillin selection method of Davis(6).‡

TABLE I. Growth Requirements of Mutants.

Strain	Requirement	Level for maximal growth on glucose (μg/ml)
44	Tryptophane	20
50	Histidine	20
54	Leucine	90
P-12	Uracil	20
P-14	Guanine	20

The *purine* and *pyrimidine-deficient mutants* were prepared by an analogous procedure: a suspension of cells of strain 1033 was irradiated by an ultraviolet lamp, adjusted to kill 99.9% of the cells. The survivors were cultured in a medium enriched with beef heart infusion at 37°C overnight. Several dilutions of the centrifuged cells were then inoculated into minimal broth containing 1,500 units of penicillin per ml, and incubated at 37°C for 5 hours.

Two series of minimal agar plates containing mixtures of purines and pyrimidines and their ribonucleosides and nucleotides at levels of 8 μg and 0.8 μg per ml were inoculated with 0.1 ml volumes of the penicillin broths. After 48 hours of incubation small colonies were picked and their requirements determined on agar plates and in liquid cultures. Five mutants were isolated in one irradiation experiment.

The minimal level of growth factor required for maximal growth was determined by shaking the cultures consisting of 50 ml of salt base, containing 0.2% glucose, 0.2% ammonium sulfate, and graded amounts of the growth factors, on a Camp type shaker for 16-18 hours at 37°C. The turbidity of the cultures was measured, after appropriate dilution, at 590 mμ in a Coleman spectrophotometer, Model 11. The same maximal turbidity was obtained by mutants and wild strain.

The growth requirements of the mutants and the amount of enrichment needed for maximal growth under these conditions are summarized in Table I. The amino acid-deficient mutants had requirements that could not be replaced by other amino acids; the

‡ We are indebted to Marcus S. Brooke for the preparation of these mutants.

purine and the pyrimidine mutants were less exacting: Strain P-12 which was found to excrete orotic acid, could utilize uracil, cytosine, their nucleosides and nucleotides, and thymine, although growth on the latter was very slow. Strain P-14 could grow on guanine, guanosine and guanylic acid, but not on adenine and its derivatives. All strains were maintained by weekly transfers on agar enriched with tryptic digest (amino acid-deficient mutants) or with beef heart infusion (purine-pyrimidine-deficient mutants).

The rates of growth of the mutant strains on glucose and on *myo*-inositol were determined in cultures consisting of 50 ml of salt base containing 0.2% of the carbon source, 0.2% $(NH_4)_2SO_4$, and the minimal level of growth factor listed in Table I; 3 ml of a similar culture, with glucose as carbon source, shaken for 16-18 hours at 37°C, served as inoculum. The cultures contained in 125 ml Erlenmeyer flasks, were shaken at 37°C; samples were withdrawn at half-hourly intervals and their turbidity measured.

Oxygen uptake was measured in a conventional Warburg apparatus. The cells were grown as described above, on glucose, and with the minimal level of growth factor required for maximal growth. It was found that most consistent results were obtained in the manometric experiments, when 0.5 ml of a 10% solution of glucose was added to 50 ml cultures after overnight growth, and the shaking continued for an additional period of 30 minutes. This treatment apparently restored the metabolic activities of the cells which had suffered from the earlier exhaustion of glucose in the growth medium. All cell suspensions used in the experiments reported here were treated in this manner.

The cells were collected by centrifugation at 5000 r.p.m. in the cold, and washed twice with 0.9% NaCl solution. They were suspended in the salt base containing, when indicated, 0.2% $(NH_4)_2SO_4$, and the growth factor at the levels listed in Table I, or at one tenth of that level. The concentration of the cells was adjusted to an optical density of 1.2 at 590 mμ; 2.5 ml portions of the cell suspension were placed in the main compartment of the Warburg vessels and 20 μM of *myo*-inositol in 0.5 ml of H_2O in the side arm. The center well contained 0.2 ml of 20% KOH.

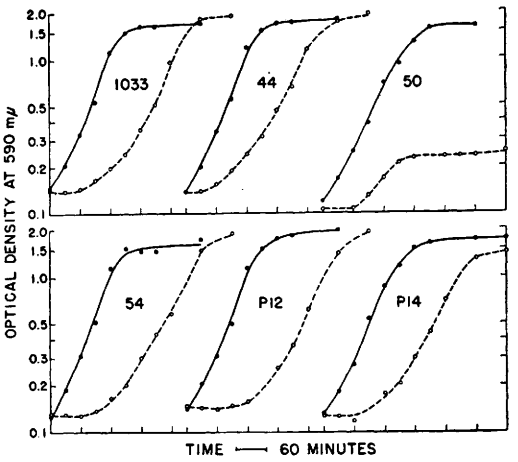

FIG. 1. Growth curves of the wild strain and of the mutants on glucose (full line), and on inositol (dotted line), in minimal medium with the enrichments shown in Table I.

In all the experiments reported here the mutant and the parent strain 1033 were tested simultaneously. All Warburg vessels contained salt base and cells. Vessels of each set varied by containing the following additional materials: 1, none; 2, 0.2% $(NH_4)_2SO_4$; 3, growth factor (0.1 of minimal level); 4, 0.2% $(NH_4)_2SO_4$ + growth factor (0.1 of min. level); 5, growth factor (min. level); 6, 0.2% $(NH_4)_2SO_4$ + growth factor (min. level). The vessels were shaken at 35°C. Readings were taken at 10-minute intervals. The oxygen uptake was measured for 30 minutes, then the *myo*-inositol was tipped in from the side arms, and readings taken for a period of 100 minutes. The cell density was determined at the end of the experiment.

Results and discussion. The growth curves of the parent and the mutant strains on glucose and on *myo*-inositol are shown in Fig. 1. At the level of growth factor required for maximal growth on glucose, the mutants grow on glucose, adapt to *myo*-inositol, and grow on *myo*-inositol at the same rate as the parent strain. The same level of growth was obtained on glucose as on inositol in all except the histidine requiring mutant, strain 50; in order to obtain full growth on inositol it was necessary to add 600 μg which is 30 times as much

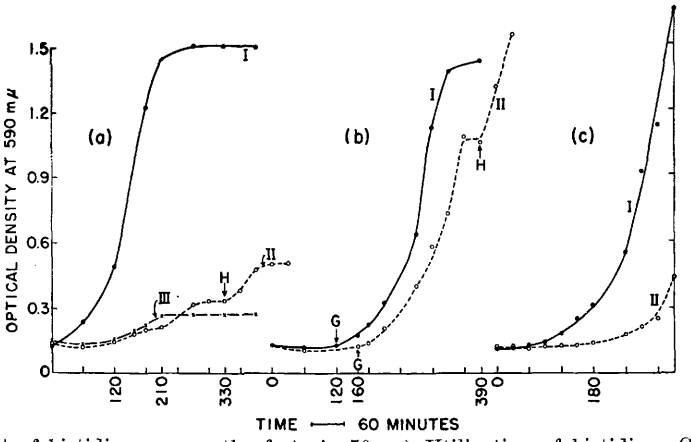

FIG. 2. Effect of histidine on growth of strain 50. a) Utilization of histidine. Curve I—Glucose and 20 μg/ml of histidine; Curve II—Inositol and 20 μg/ml of histidine; Curve III—Inositol and 40 μg/ml of histidine. Arrows mark addition of 20 μg/ml of histidine. b) Degradation of histidine. 20 μg/ml of histidine. Glucose (G), and histidine (H) added where indicated by arrows. c) Growth on 600 μg/ml of histidine. Curve I—with inositol; Curve II—without inositol.

as was necessary for full growth on glucose. This unexpected consequence of the inability of a mutant to synthesize histidine was one of the most striking observations made in the present study. The increased level of histidine was required by adapting as well as by previously adapted cells as shown by the experiment in Fig. 2. In this experiment the addition of histidine to the medium in which growth on inositol had ceased, resulted in an immediate resumption of growth which, however, came to a halt after an increase in cell density similar to that attained in the first period of growth. This cessation of growth was due to the exhaustion of histidine, because addition of fresh histidine resulted again in immediate growth. In the absence of a source of carbon, histidine was not broken down during the first 150 minutes. When glucose was added during this time interval as much growth was obtained as in the case when both histidine and glucose had been present from the beginning (Fig. 2). Exposure of histidine to the cells beyond 150 minutes apparently caused adaptive degradation of histidine. Lower levels of growth were reached when glucose was added after this time interval, and growth occurred with histidine at 600 μg per ml as the only source of carbon (Fig. 2).

The enormous increase in the histidine requirement in cells growing on inositol may be due to the production of inositol degrading enzymes excessively rich in histidine. In that case, inositol grown cells should contain many times as much histidine as glucose grown cells. However, histidine does not seem to be stored to an appreciable extent in either glucose or inositol grown cells, as shown by their inability to grow on glucose in the absence of histidine. Yet, glucose grown cells are able to form adaptive enzyme for inositol degradation in the absence of histidine (Fig. 4). The increased requirement for histidine by cells growing on inositol thus does not seem to be due to a utilization of histidine in large amounts for the production of inositol degrading enzymes, but rather to be connected with some aspect of inositol metabolism. *Myo*-inositol which is degraded by a pathway distinct from that by which glucose is broken down(4), may fail to provide a building block which can be formed from histidine. This interesting relationship between inositol and histidine metabolism will be further investigated.

The results of the manometric experiments are illustrated in Fig. 3 and 4. Fig. 3 compares the kinetics of adaptation of the wild strain and the tryptophane requiring mutant, strain 44, and Fig. 4 summarizes the results obtained with all the strains. The results ex-

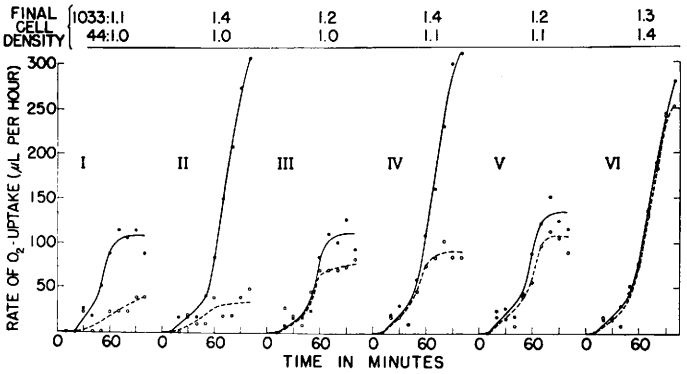

FIG. 3. Rate of oxygen uptake on inositol by strains 1033 (full line) and 44 (dotted line). Additions: I—None, II—$(NH_4)_2SO_4$, III—2 μg/ml tryptophane, IV—$(NH_4)_2SO_4$ + 2 μg/ml tryptophane, V—20 μg/ml tryptophane, VI—$(NH_4)_2SO_4$ + 20 μg/ml tryptophane. Original optical density of the cell suspensions was 1.

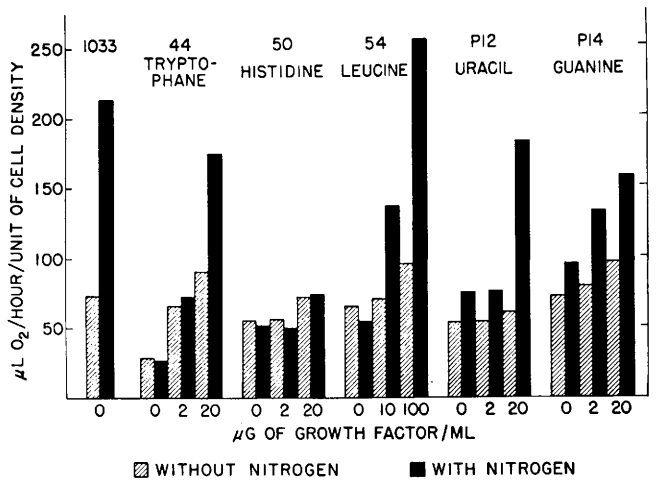

FIG. 4. Effect of addition of growth factor and of exogenous nitrogen on production of adaptive enzyme. Amount of adaptive enzyme formed by 3 ml of a cell suspension with an optical density of 1 is expressed in μL of oxygen taken up in one hr, and was calculated from: R_{100}/D_{100}—R_o/D_o, where R stands for rate of oxygen uptake and D for cell density, at the time of addition of inositol and at 100 min.

pected by analogy with other adaptive processes in bacteria and yeasts were observed with the parent strain 1033. Adaptation took place in the absence of added $(NH_4)_2SO_4$, but considerably higher levels of adaptive enzyme were attained in its presence. Under the latter condition an increase in cell density was observed, but was insufficient to account for the increase in the concentration of adaptive enzyme, as shown in Fig. 4. The addition of the growth factors to the wild strain, either in the absence or presence of $(NH_4)_2SO_4$, had no appreciable effect on the adaptation.

The auxotrophic mutants, on the other hand, could achieve similarly high levels of adaptive enzyme in an $(NH_4)_2SO_4$ containing medium only when the individual growth factor was also supplied, that is, the highest levels of adaptive enzyme were attained only by dividing cells. This relation between cell division and adaptation is well illustrated by the behavior of the histidineless mutant, strain 50. The level of histidine supplied was insufficient for growth as discussed previously, and similarly inadequate for the production of large amounts of adaptive enzyme.

A more specific role of some of the growth factors in adaptation could be demonstrated

by experiments in which cell suspensions of the mutants were exposed to inositol in media incapable of supporting growth. It was found (Fig. 3) that the loss of the ability to synthesize tryptophane in strain 44 reduced the amount of adaptive enzyme formed by this strain to about one-third of that formed by the wild strain. The ability of strain 44 to produce adaptive enzyme in the absence of growth could be restored by the addition of tryptophane, but not by the addition of $(NH_4)_2SO_4$.

Other experiments provided evidence that lack of added tryptophane interfered neither with the oxidation of glucose nor, in adapted cells, with the oxidation of inositol; regardless of the absence or presence of tryptophane the oxygen uptake on either glucose or inositol amounted to the theoretical one (6 M/M) in 0.0001 M 2,4-dinitrophenol, but was lower (3 M/M) without dinitrophenol. This latter finding shows that the normally occurring oxidative assimilation, which accounts for the low oxygen uptake(7), does not depend on the presence of the growth factor. The demonstration that assimilation, an energy requiring process, can take place in the absence of added tryptophane, makes it unlikely that the inability of mutant 44 to adapt under these conditions is due to an impairment of the energy metabolism of the cell.

The ability of the leucineless and the histidineless mutant to adapt in the absence of added growth factor was only slightly less than that of the wild strain. Still, addition of leucine and of histidine, respectively, stimulated adaptive enzyme formation, while addition of $(NH_4)_2SO_4$ alone was without effect.

In contrast to these findings are the observations made on the adaptation of the pyrimidineless and the guanineless strains. Ammonium sulfate alone caused a small increase in the production of adaptive enzyme; addition of uracil alone to strain P-12 was without significant effect, and addition of guanine alone to strain P-14 was less effective than addition of ammonium sulfate; the improvement in adaptation caused by the addition of guanine might be explained by the ability of guanine to act as an amino donor. As was observed with the amino acid requiring mutants, the highest levels of adaptive enzyme were attained only when cell division could occur, *i.e.*, in the presence of both ammonium sulfate and growth factor.

The observations on the adaptation of auxotrophs in media not capable of supporting growth may be summarized as follows: while the synthesis of adaptive enzyme is limited by the lack of the specific growth factor in the amino acidless mutants, it is limited by the lack of an exogenous source of nitrogen in the guanine- and the pyrimidineless mutants.

The bearing of these findings on the problem of adaptive enzyme synthesis may now be considered briefly. The stimulation of adaptation by the amino acids required for growth seems to suggest that even in the absence of cell division adaptive enzymes are synthesized *de novo* from amino acids. The ability of the amino acid auxotrophs to form adaptive enzyme, though in smaller amount, in the absence of the required growth factor, may be due either to the storage of the amino acid or perhaps to the liberation of the amino acid from proteins by hydrolysis. Such an indirect utilization of proteins for formation of new enzymes might explain the competitive interactions observed in non-proliferating cells(1).

Uracil and guanine do not seem to be directly involved in the synthesis of adaptive enzyme. The stimulation of adaptation in these auxotrophs by ammonium sulfate in the absence of growth constitutes further evidence for the biosynthesis of new enzyme from simple nitrogen compounds.

The observation that dividing cells can attain a level of adaptive enzyme several times as high as that attained by resting cells (Fig. 4) suggests that still other factors in addition to the inducing agent, the building blocks, and energy are involved in the control of adaptation.

Finally, the preliminary nature of the results reported here must be emphasized: only one substance, *myo*-inositol, has been used to induce adaptation and the responses of only 5 auxotrophs have been recorded. We hope to test the usefulness of this approach and the general validity of the conclusions drawn by extending this study to other un-

related systems and to additional mutants of different types.

Summary. The ability of amino acid and of purine and pyrimidine auxotrophs of *Aerobacter aerogenes* to form adaptive enzymes for the degradation of *myo*-inositol has been investigated. It was found that adaptive enzyme synthesis by the amino acid auxotrophs could be stimulated in the absence of growth by addition of the required nutrilite, but not by addition of $(NH_4)_2SO_4$. In contrast, adaptation in the guanine and pyramidine auxotrophs was enhanced by addition of $(NH_4)_2SO_4$ alone. In all cases, dividing cells attained higher levels of adaptive enzyme than resting cells. The bearing of these results on the mechanism of adaptation is discussed. The poor utilization of histidine by strain 50 for growth on inositol does not seem to be due to the effect of histidine on adaptation, but rather seems to suggest a metabolic relationship between inositol and histidine.

1. Spiegelman, S., and Dunn, R., *J. Gen. Physiol.*, 1947, v31, 153.
2. Halvorson, H. O., and Spiegelman, S., *Bact. Proc.*, 1952, 151.
3. Magasanik, B., *Am. Chem. Soc. Abst. of Papers*, 119th Meeting, 20C, 1951.
4. ———, *J. Am. Chem. Soc.*, 1951, v73, 5919.
5. Magasanik, B., Brooke, M. S., and Karibian, D, *Bact. Proc.*, 1952, 164.
6. Davis, B. D., *Proc. Nat. Acad. Sc.*, 1949, v35, 1.
7. Clifton, C. E., *Enzymologia*, 1937, v4, 246.

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Response of X-irradiated Mice to Intravenous Inoculation of Intestinal Bacteria.* (19714)

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The possibility that infection by bacteria of intestinal origin plays a significant role in death following exposure to ionizing radiation has been suggested by a number of observations. Bacteria common to the lower intestinal tract have been found in blood and organs of animals after total body X-irradiation(1-4). Miller *et al.*(5,6) showed a high incidence of bacteremia identified with normal intestinal inhabitants in mice during the second week after exposure to 450 or 600 r total body X-irradiation. This was the period of greatest mortality. Furthermore, it has been possible to reduce mortality among irradiated animals by the administration of various antibiotics(7,8). The protective effect was considerably reduced in mice if there was a prevalence of invading intestinal bacteria which were relatively insensitive to the antibiotics(7). Increased susceptibility of mice to inoculation with *Escherichia coli* following sublethal total body X-irradiation was shown by Shechmeister *et al.*(9,10). Subcutaneous or intraperitoneal injection of *E. coli* caused much higher mortality among irradiated mice than among normal animals receiving the same inoculum.

The present experiments were undertaken to study the effect in mice of intravenous inoculation of another normal intestinal organism, a member of the genus *Proteus*, after moderate total body X-irradiation.

Experimental procedure. C_3H mice, 60-90 days of age, were subjected to total body X-irradiation in groups of 3 in plastic (Styron) containers. The radiation was delivered from a 200 kv Picker-Waite X-ray therapy machine operating at 20 ma and a distance of 20 cm. The filters consisted of 0.5 mm Cu and 1.0 mm Al. Doses ranged from 400 to 550 r, given at a rate of 230 r per minute. Non-irradiated controls were kept in the plastic containers for the same length of time as the irradiated animals. The mice were housed in plastic cages in groups of 6 in an air-condi-

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