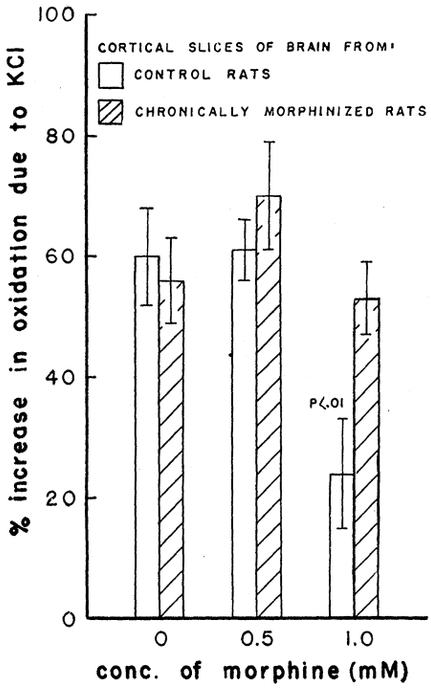

Fig. 2. Effect of morphine on the stimulated oxidation of cortical slices. Each bar represents an average of 15 rats. The vertical lines at the top of each bar show the standard error. Only one group, as indicated ($p < 0.01$), is significantly different from the control group (no morphine).

for 3 weeks until the rats were receiving 45 mg/kg twice daily during the third week. The rats became tolerant to the depressant and analgesic effects of morphine during this treatment. Control animals received isotonic saline injections throughout the course of the experiment. Eighteen hours after the last dose, the rats were decapitated and cortical slices of their brains were prepared for analysis.

Respiration was measured manometrically by the conventional Warburg technique at 37°C with oxygen as the gas phase. The incubating medium contained 0.135*M* NaCl, 0.005*M* KCl, 0.0013*M* $MgSO_4$, 0.0005*M* $CaCl_2$, 0.012*M* glucose, and 0.01*M* KH_2PO_4–Na_2HPO_4 buffer, *p*H 7.4. After readings were taken every 10 minutes for 40 minutes, KCl (0.1*M*) or various concentrations of morphine or both were tipped from the side arm into the main compartment, and readings were taken for an additional 50 minutes at 10-minute intervals. There was a 10-minute lag period before the effect of KCl became fully apparent and before the rate again became linear.

At concentrations of 5×10^{-4} and 1×10^{-3} *M* morphine does not affect the oxidation of unstimulated cortical slices from control or chronically morphinized rats (Fig. 1). This agrees with other reports (*5*) that morphine is without effect at concentrations as high as $3.2 \times 10^{-3}M$ and 0.01*M*. Potassium chloride stimulates the oxidation of slices from both control and chronically morphinized rats to about 60 percent above the unstimulated rate in the absence of morphine (Fig. 2). The respiratory stimulation of slices from control rats is only about 24 percent in the presence of $1 \times 10^{-3}M$ morphine, while the respiratory stimulation of slices from morphinized rats remains unaffected. At a concentration of $5 \times 10^{-4}M$ morphine appears to have no significant effect on the stimulated slices from either control or chronically morphinized rats. The results show an adaptation to morphine at the cellular level. This adaptation occurs at a time when the whole animal is pharmacologically adapted to the effects of morphine.

Eddy (*6*) pointed out that among the numerous explanations offered for the phenomenon of tolerance to narcotic analgesics, increased biotransformation of the drug and cellular adaptation have the greatest likelihood for clarifying this problem. The metabolism of morphine in tolerant animals appears to be unrelated to the development of tolerance (*7*). In fact, enzymic studies show less *N*-demethylating capacity (*8*) and less transferase activity (*9*) in livers obtained from tolerant rats than in livers obtained from nontolerant rats. Cellular adaptation is an attractive explanation for the formation of tolerance, but supporting evidence for this hypothesis is meager.

Although the in vitro concentration of morphine employed in this study is still quite high compared to the theoretical in vivo concentration of morphine in the central nervous system, the demonstration of a definite difference between the tissues from control and chronically morphinized rats in resisting the depressive effect of morphine offers a promising tool for studying cellular adaptation in vitro (*10*).

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References and Notes

1. C. A. Ashford and K. C. Dixon, *Biochem. J.* **29**, 157 (1935).
2. H. McIlwain, *ibid.* **53**, 403 (1953).
3. J. J. Ghosh and J. H. Quastel, *Nature* **174**, 28 (1954).
4. V. C. Sutherland, C. H. Hine, T. N. Burbridge, *J. Pharmacol. Exptl. Therap.* **116**, 469 (1956); D. W. Clarke and R. L. Evans, *Can. J. Biochem. and Physiol.* **37**, 1525 (1959); H. Wallgren and E. Kulonen, *Biochem. J.* **75**, 150 (1960).
5. M. H. Seevers and F. E. Shideman, *J. Pharmacol. Exptl. Therap.* **71**, 373 (1941); H. W. Elliott, A. E. Warrens, H. P. James, *ibid.* **91**, 98 (1947).
6. N. B. Eddy, in *Origins of Resistance to Toxic Agents*, M. G. Sevag, R. D. Reid, O. E. Reynolds, Eds. (Academic Press, New York, 1955), p. 223.
7. H. L. Zauder, *J. Pharmacol. Exptl. Therap.* **104**, 11 (1952); L. A. Woods, *ibid.* **112**, 158 (1954); E. L. Way, C. Y. Sung, J. M. Fujimoto, *ibid.* **110**, 51 (1954).
8. J. Axelrod, *Science* **124**, 263 (1956); G. J. Mannering and A. E. Takemori, *J. Pharmacol. Exptl. Therap.* **127**, 187 (1959).
9. A. E. Takemori, *J. Pharmacol. Exptl. Therap.* **130**, 370 (1960).
10. This work was supported by grant No. M-3897 from the National Institute of Mental Health, U.S. Public Health Service.

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Stability of Protein in Intestinal Epithelial Cells

Abstract. In vivo measurements have revealed a high degree of stability of synthesized protein in rapidly proliferating intestinal epithelial cells. A slow loss of protein has been found during migration of mature cells to the villus tip.

Although previous studies have provided support for the concept of "dynamic equilibrium" of proteins in mammalian cells (*1*), the occurrence of rapid intracellular protein degradation and turnover has recently been questioned (*2*). In bacteria, several studies (*2, 3*) have indicated that protein, once synthesized, is stable. The rates of protein degradation here were so low that dynamic intracellular protein turnover was not believed to play a role in protein synthesis in these cells. Recently, similar evidence was found in mammalian cells of the L strain grown in tissue culture (*4*). In addition, although protein breakdown was found in resting yeast cells, a much smaller rate of degradation was present in dividing cells (*5*). The results of the present studies, performed in vivo, suggest that visceral parenchymal cells which are renewed rapidly maintain a high degree of stability of synthesized proteins with little degradation, while protein breakdown or loss is greater in the older nonproliferating cells derived therefrom.

The incorporation of labeled leucine into proteins of the jejunum was measured. Adult C57 Brown mice were maintained on a standard nutritional diet. Microautoradiographic measurements were made of the cells of the proximal jejunum removed after intraperitoneal injection of 100 μc of tritiated leucine (specific activity, 370 mc/mmole). Chromatographic analysis of injected material revealed no free label. In addition, the tissues were washed with solutions containing trichloracetic acid, and chemical analysis revealed no free amino acid present, indicating that most grains activated

were due to tritium incorporated into protein.

For the microautoradiographic studies, slides carrying sections of labeled material were dipped into liquid emulsion (NTB), exposed for 1 to 2 weeks at 4°C, developed, stained, and examined. Since the electrons produced in the decay of tritium have a range in tissue of about one μ, activated silver grains tend to be closely apposed to the structures labeled. It was therefore possible to identify sites of incorporation of tritiated leucine with a resolution of a fraction of a cell size.

Quantitative estimates of relative rates of leucine incorporation were based on counts of grain density over various kinds of cells, and various portions of cells. Since the sections were cut 5 μ thick, that is, infinitely thick compared to the range of tritium beta rays, these counts are proportional to the amount of tritium incorporated. Exposure time was identical in all experiments.

The findings were as follows: Grain counts were highest about 2 to 4 hours after injection. This corresponded to the maximum height of specific activity curves of labeled leucine incorporated into mucosal protein precipitate, measured in a separate series of radiochemical studies. In the microautoradiographs, incorporation at 2 to 4 hours was higher in the epithelial cells than in smooth muscle cells and fibroblasts. The epithelial cells in the crypts were more heavily labeled than those on the villi. On the villus a definite gradient was also observed, with the cells near the base being more heavily labeled than those near the tip. Within the epithelial cells label was most heavy near the surface; this was more pronounced in villus cells than in crypt cells. Nuclei were less heavily labeled than cytoplasm, particularly on the villi.

In the jejunum, epithelial cell production is restricted to the crypts. The majority of epithelial cells in the crypts are proliferating, a smaller fraction are in process of differentiation, and still fewer are fully mature. Villus cells are in steady migration from the mouths of the crypts to the tips of the villi, from where they are sloughed off into the lumen. During this phase of migration, they do not proliferate; however, they differentiate further, and microvilli grow rapidly as cells migrate from the villus base to the tips.

The concentration of label near the surface of villus epithelial cells appears to be due to protein synthesis or accumulation of protein in or near rapidly growing microvilli. The heavy concentration of label in crypt epithelial cells indicates rapid protein synthesis in the proliferating cells. The microvilli do not grow rapidly in crypt cells and those cells do not show heavy labeling near the surface.

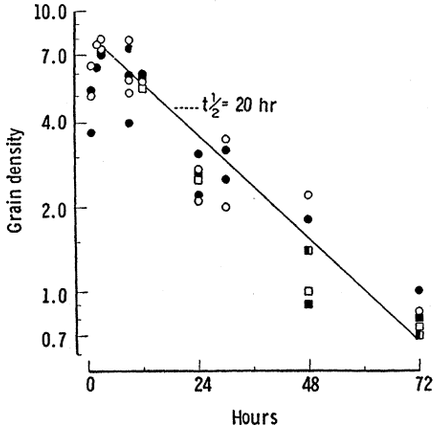

Fig. 1. Grain densities (grains per 64 μ^2 per 100 μc per week exposure) over jejunal crypt epithelial cells, plotted against time. Each point represents one animal. The circles and squares indicate separate experiments. Empty symbols, cytoplasm; full symbols, nuclei.

Studies of microautoradiographs of animals sacrificed up to 3 days after labeling yielded the data shown in Figs. 1 and 2. In the *crypts* (Fig. 1) the grain density is about the same over nuclei and cytoplasm; it reaches a peak shortly after labeling and then declines roughly exponentially with a half-life of about 20 hours. This is precisely what should result if there is no intracellular degradation and loss of protein in these proliferating cells. They have been shown to divide approximately every 19 hours (*6*). At each division, the labeled protein is diluted to one-half by production of new unlabeled protein, each daughter cell receiving roughly equal amounts of labeled and unlabeled protein. The data, however, are not precise enough to exclude a minor loss of labeled protein.

Separate studies have shown that, within about 1¾ days after labeling, the cells which occupied the *base of the villi* at the time of injection have been replaced by cells which have come up from the crypts (*6*). Some of these have divided before leaving the crypts; others, in the process of differentiation at the time of labeling, have left without previous division.

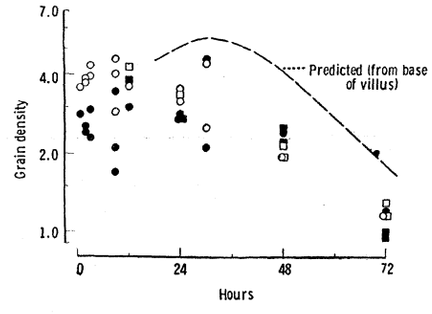

Fig. 2. Grain densities over jejunal epithelial cells at tip of villus, plotted against time.

The results of counts taken over the base of the villi up to 12 hours after labeling showed heavier label in the cytoplasm (about 5.6 units) than in the nuclei (about 4.2). In preparations taken one or more days after labeling, this difference disappeared. The peak grain count, reached between 12 and 24 hours, was near 5.5. From 1 to 3 days, the grain count declined roughly exponentially with a half-life of about 20 hours. These changes again can be accounted for by cell movement.

The cells which pass through the base of the villi reach the region of the *tip* 1 day later. If there were no loss of label during this migration, the grain counts over the tip of the villi should follow the dashed line in Fig. 2. Actually, the values obtained 30 hours and later after labeling are about one-half less. This indicates that during the migration about one-half the label was lost.

In these experiments there was, therefore, no evidence of major intracellular loss of protein, or turnover, in proliferating intestinal epithelial cells. There was, however, evidence of a moderate degree of protein loss in the mature cells derived from them. The same experiments revealed rapid loss of label over Paneth and pancreatic acinar cells, presumably due to secretory processes. It is not known how much of the protein lost from mature intestinal epithelial cells is accounted for by secretion of intact protein from the cells, or from loss of protein molecules that have undergone intracellular degradation. It may well be that, in the mature cell, protein contained in the vesicular endoplasmic reticulum of the proliferative cell undergoes degradation and possibly is partly reutilized in forming the tubular endoplasmic reticulum of the mature cell as well as enzymes and microvilli. Leblond *et al.*, in following S^{35}-labeled methionine incorporated into the epithelial cells of the duodenum of the rat, found that intensely labeled cells at the base of the villi moved to the tip (*7*).

The findings reported here indicate that protein in the proliferating epithelial cells of the jejunum is either highly stable or, if broken down, is reutilized not only in the same cell but even in the same fraction of the cell. The latter hypothesis appears less reasonable. It is, therefore, proposed that, in the rapidly growing cells of the intestinal epithelium, synthesized protein is suf-

ficiently stable to last through the proliferative cycle of the cells, and intracellular degradation or protein turnover does not have a significant role in fixing the net rate of protein synthesis or in contributing appreciably to the composition and protein structure of the proliferating cells (*8*).

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References and Notes

1. R. Schoenheimer, *The Dynamic State of Body Constituents* (Harvard Univ. Press, Cambridge, Mass., 1942).
2. D. S. Hogness, M. Cohn, J. Monod, *Biochim. et Biophys. Acta* **16**, 99 (1955).
3. B. Rotman and S. Spiegelman, *J. Bacteriol.* **68**, 419 (1954).
4. D. W. King, K. G. Bensch, R. B. Hill, Jr., *Science* **131**, 106 (1960).
5. H. Halvarson, *Biochim. et Biophys. Acta* **27**, 255 (1958).
6. H. Quastler and F. G. Sherman, *Exptl. Cell Research* **17**, 420 (1959).
7. C. P. Leblond, B. Everett, B. Simmons, *Am. J. Anat.* **101**, 225 (1957).
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Immunization as a Factor Affecting the Course of Septicemic Anthrax

Abstract. Immunization of guinea pigs with alum-precipitated antigen increased resistance approximately 1600 times. The growth rate of *Bacillus anthracis*, in the septicemic phase, was the same in both normal and immunized animals; however, the number of bacilli per milliliter of blood at death was decreased by 75 percent in immunized animals. Neither the enhancement of virulence by treatment with egg yolk nor the size of the challenge dose affected the growth rate or the number of bacilli per milliliter of blood at death. Mean time to death for the treatments tested varied from approximately 20 to 100 hours. Nonimmunized animals challenged with a strain of low virulence had a terminal number of bacilli per milliliter of blood and a growth rate in the blood the same as that for the highly virulent strain.

Anthrax is typified by an extremely rapid progress of disease, with symptoms and septicemia occurring some few hours before death of the animal. Bloom *et al.* (*1*) and Keppie *et al.* (*2*) state that the number of organisms per milliliter of blood appeared to be lower in partially immunized guinea pigs than in nonimmunized animals. This paper presents quantitative data regarding the in vivo rate of growth and the level of bacilli in the blood of immunized and normal guinea pigs during the septicemic stage of anthrax and notes the constancy of these observations regardless of certain treatment variations of the initial challenge dose.

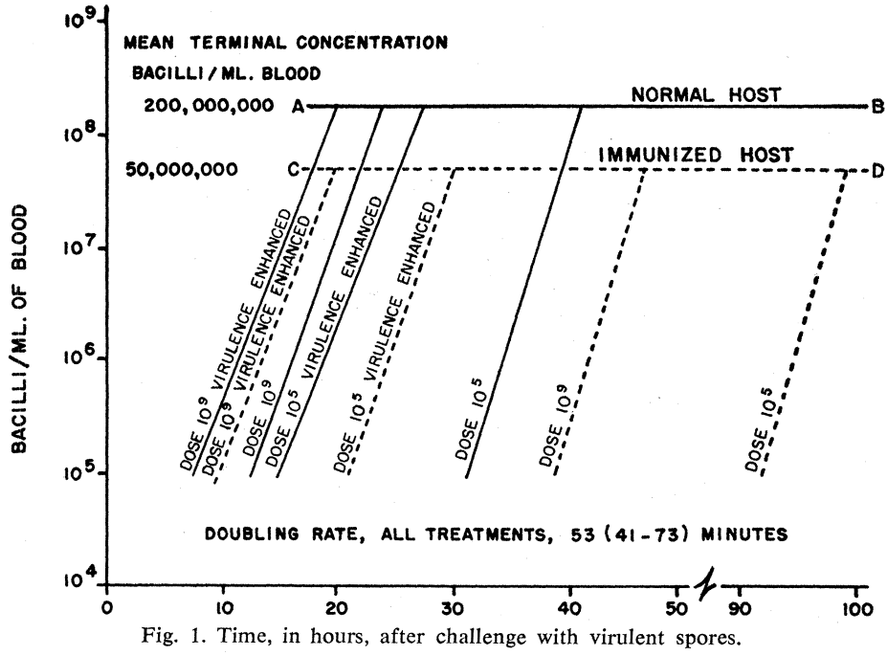

Fig. 1. Time, in hours, after challenge with virulent spores.

An experiment of four factors each at two levels was designed to investigate the septicemic phase of anthrax. These factors were host (immune or nonimmune), virulence (high or low), virulence-enhancement (enhanced or normal), and dose (10^9 or 10^5 spores); however, only the high dose level of the low virulent strain was used, because the low dose did not kill the host. Hartley strain guinea pigs, weighing 250 to 300 g, raised by the Fort Detrick animal farm were immunized by administration of 0.1 ml of a 1:10 dilution of antigen (*3*) given by intraperitoneal injection on days 1, 3, 5, 8, and 11. The animals were challenged on the 21st day with spores of either the virulent V1b strain or the low virulent 30R strain, a mutant of V1b. The immunity attained by this procedure, when the animals were challenged with spores of the virulent V1b strain, was approximately 1600-fold, that is, equal responses of immunized and control hosts were obtained by increasing the challenge dose of immunized animals 1600-fold. Virulence was enhanced by the addition of egg yolk medium to the inoculum (*4*). The measurement of virulence was based on a graded response method (*5*). After two initial experiments to establish techniques, groups of four immunized and two control animals were challenged with each of the treatment combinations, and tests were replicated on three occasions. The number of cells in the blood was determined quantitatively both by direct observation of stained slides, by the methods of Keppie *et al.* (*2*), and by a plate count assay method. The concentration of bacilli per milliliter of blood at the time of death is known as the terminal concentration. The factorial design was used to gain efficiency in regard to number of animals required for significant conclusions.

Data reported in a schematic graph (Fig. 1) are quantitative counts of bacilli observed on stained slides. For all treatment conditions the growth of bacilli in the blood, observed during approximately the 12 hours preceding death, was exponential, and all growth curves were parallel as shown in Fig. 1. The doubling rate of bacilli in the blood (apparent generation time) averaged 53 minutes (41 to 73 minutes for 95 percent confidence limit). The number of bacilli per milliliter of blood in the normal animals was constant for all treatment conditions (line *AB*, Fig. 1) and approximately four-fold higher than the average number found in the immunized host (line *CD*, Fig. 1). This difference is statistically significant and is based on 23 immunized and 15 control guinea pigs. Survival time in normal guinea pigs is considerably shorter than in immunized guinea pigs. Response of animals challenged with the 30R strain of low virulence was only partial; therefore, data on these