problem of atherosclerosis. Therefore the aortas of the pigs fed the diets deficient in essential fatty acids were inspected for lesions. Of the 8 pigs that survived longer than the normal weanling age of pigs, 56 days, 5 were found to have gross lesions of the aorta. None of the 4 pigs supplemented with ethyl linoleate were found to have gross lesions. Microscopic examination of the lesions by Dr. Jesse Edwards of the Mayo Foundation confirmed the macroscopic observations. The lesions were found to be in the inner third of the media, and consisted of areas of calcification. No lipid deposits were demonstrable by Sudan IV staining. Alizarin red and von Kossa stains for calcium were positive, and Prussian blue stain for iron was positive. Ceroid stain was negative.

Summary. Rigid exclusion of essential fatty acids from the diet of young swine leads to a deficiency disease not characterized by consistent or early skin lesions. The severity of the dietary regime imposed caused high mortality, but of the 8 pigs that survived beyond the normal weanling age, skin lesions were observed in one and aortic lesions in 5, at or before 98 days on experiment. The data suggest that essential fatty acids are required by swine and that deficiency of essential fatty acids may lead to impairment of the arterial wall.

1. Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, 1929, v82, 345.

2. Holman, R. T., *The Vitamins*. Ed.: Sebrell and Harris, v2, p. 268, Academic Press, N. Y., 1954.

3. Witz, W. M., and Beeson, W. M., *J. Animal Sci.*, 1951, v10, 112.

4. Young, G. A., Underdahl, N. R., and Hinz, R. W., *Am. J. Vet. Res.*, 1955, v16, 123.

5. England, D. C., Winters, L. M., and Carpenter, L. E., *Growth*, 1954, v18, 207.

6. Peifer, J. J., and Holman, R. T., *Arch. Biochem. Biophys.*, 1955, v57, 520.

7. See Holman, R. T., *Methods of Biochemical Analysis*. Ed.: Glick, vIV, 1957, p. 126. Interscience Publishers, N. Y.

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Effect of Time and Temperature upon Survival of *Escherichia coli* in Sodium Chloride.* (23193)

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When cells of *Escherichia coli* are suspended in 4.6 M NaCl and held at −22°C, the decline in number of viable cells does not proceed at a constant rate but at a continuously decreasing rate(1). To gain an insight into the kinetics of this decline, the effect of time and temperature upon survival has been investigated. The present paper summarizes some of the results of this investigation.

Materials and methods. The test organism was *Escherichia coli* strain 69L-15. It was cultivated in broth of the following percentage composition: yeast extract (Balt. Biol. Lab., dehydrated), 1.0; K_2HPO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.01; and "Tween 80" (Atlas Powder Co.), 0.1. The plating medium was of the same composition as the broth except 1.6% agar was an additional ingredient. Incubation was at 37°C. Cells of the test organism were transferred with a wire loop from a refrigerated stock agar culture to 5 ml of broth in a test tube and incubated for 24 hours. After 2 or more serial transfers through such tubes, 0.1 ml of the culture was used to inoculate 100 ml of broth in a 250 ml Erlenmeyer flask. After incubation for 24 hours, 30 ml of culture were centrifuged at 2,000 G for 30 minutes; the cells were washed twice with distilled water and finally resuspended to a volume of 30 ml with distilled water. The resulting aqueous suspension was

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immediately placed in an ice-water bath at +2°C.

One part of the aqueous bacterial suspension was mixed with 9 parts of 5.1 M NaCl at +2°C in the ice-water bath. This resulted in a 10-fold dilution of the cells and a reduction in concentration of the NaCl to 4.6 M. The pH of the mixture was 6. The bacteria-saline mixture was then dispensed in 5 ml aliquots into test tubes (15 mm x 150 mm) fitted loosely with aluminum caps. These manipulations at +2°C were completed in less than 3 min; then the tubes were placed at various temperatures for storage. The viable cell count at the moment of admixture of the bacteria with the saline was determined by a plate count on the original aqueous bacterial suspension and dividing this count by 10. This will be called the initial count. Four storage temperatures were employed: −22°, −9°, +2°, and +15°C. The tubed suspensions were maintained at −22° and −9°C in glycerol baths and at +2° and +15°C in water baths. When, during the course of an experiment, it was desired to alter the storage temperature the suspension was simply transferred from one bath to another. Only a few minutes were required for the suspension to attain the new temperature. Temperature was monitored by means of toluene thermometers and also by means of a "TRI-R" electronic thermometer (TRI-R Instruments, Long Island City, N. Y.), the sterile thermistor probe being placed within one of the tubed suspensions. The 4.6 M NaCl prevents solidification at −9° or −22°C, hence the effect of temperature may be studied in the absence of ice formation. To determine the number of survivors after storage in 4.6 M NaCl, 0.1 ml aliquots were withdrawn periodically and diluted serially in bottles containing 100 ml of diluent. Pour plates were prepared by plating 0.1 or 1. ml aliquots from these bottles. The plates were incubated for 24 hours. The diluent employed in the dilution bottles consisted of 0.05% trypticase (Balt. Biol. Lab., dehydrated) adjusted to pH 7.4 with phosphate buffer.

Results. Fig. 1 compares survival of *Escherichia coli* in 4.6 M NaCl at 4 temperatures (curves 1, 2, 3, and 4). It will be noted that

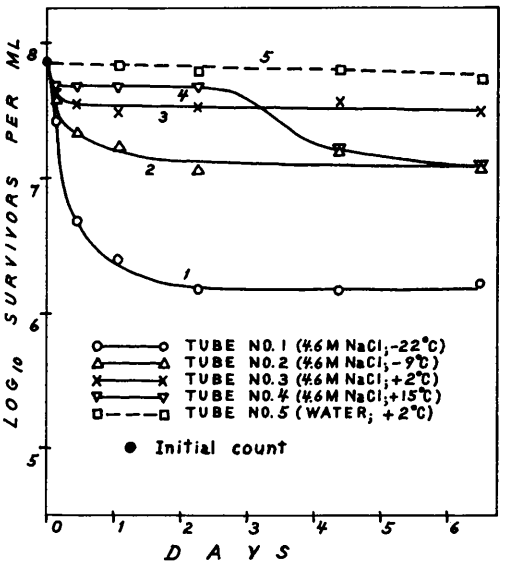

FIG. 1. Effect of temperature upon survival of *Escherichia coli.*

survival at −9°C is better than at −22°C; similarly, survival at +2°C is better than −9°C. The relationship between survival at these 3 temperatures (curves 1, 2, and 3) is maintained throughout the 6½ day period. For the first 2 days, survival at +15°C is as might be predicted from the results at −22°, −9°, and +2°C. However, sometime after 2 days the death rate increases, then diminishes, and the curve "levels off" once again (curve 4). Between −22°C and +2°C survival is better the higher the storage temperature. Above +2°C, however, the configuration of the survival curve may vary from one experiment to another. In some experiments, the viable count remains higher at +15°C than at any of the other temperatures over the entire test period (1 week and longer). In other experiments, the curve is sigmoidal (Fig. 1). Since this behavior above +2°C occurs despite our uniform experimental technic, we have assumed that it is due to inherent differences between different cultures.

Fig. 2 illustrates that holding *E. coli* at +2°C for various periods prior to storage at −22°C will improve subsequent survival at the latter temperature. Holding the cells at +2°C for as short an interval as 3 hours improves survival at −22°C considerably (compare curve 2 with curve 1). Even after the

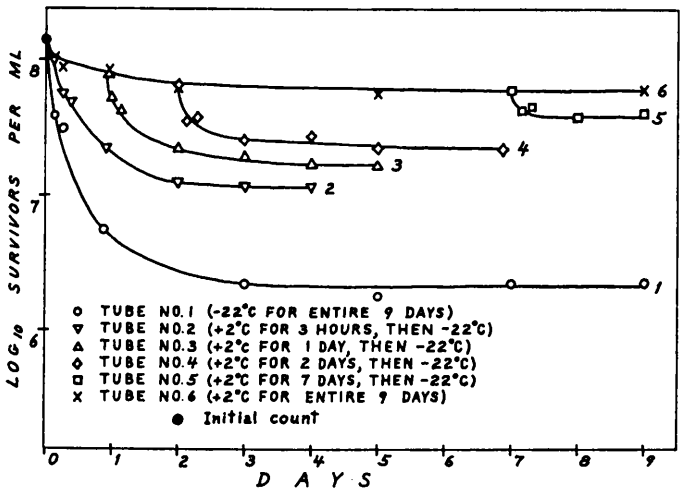

FIG. 2. Effect of length of holding at +2°C upon subsequent survival of *Escherichia coli* at -22°C.

suspensions at +2°C have become static, that is, after the death rate has become negligible (2 days, curve 6), the cells are undergoing some change, because continued holding at +2°C improves subsequent survival at -22°C even more (compare curve 4 with curve 5). In some experiments prior storage at +2°C for as little as 4 days conferred to the suspension complete resistance to -22°C. Improvement in survival at -22°C may also be acquired by prior storage at -9°C.

Fig. 3 summarizes data obtained from fluctuating *E. coli* suspensions between -22° and +2°C. If, during the period of rapid decline at -22°C, the suspension is transferred to +2°C, death will be arrested. In other words, during the period of rapid decline at -22°C, degree of death varies in proportion to length of exposure to this low temperature (compare curves 2, 3, and 4). Once the death rate of the suspension has diminished to a negligible value (after 1 day) warming to +2°C brings about no demonstrable change in viable cell count. In fact, once a suspension has attained this relatively static condition, fluctuations between -22° and +2°C have no effect whatsoever upon survival (compare curves 4 and 5).

The cells which remain viable during the period of rapid decline are able to maintain themselves for long periods. This resistance,

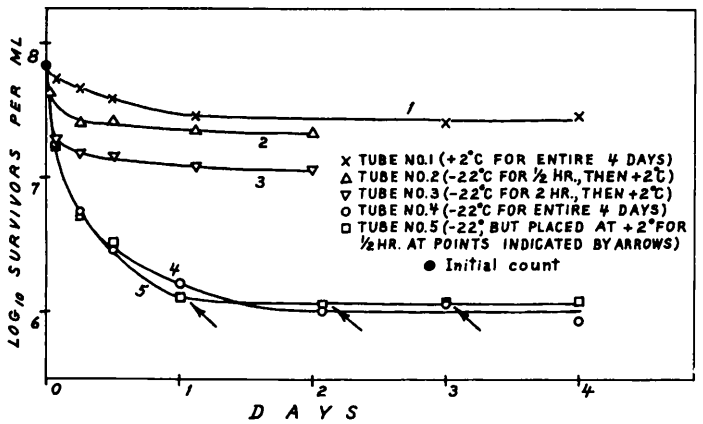

FIG. 3. Effect of temperature fluctuations between -22°C and +2°C upon survival of *Escherichia coli*.

however, is not heritable. If an aliquot of a stored (−22°C for 1 week) suspension is plated and the resulting colonies emulsified with water and subjected to a second exposure to 4.6 M NaCl, they are found to be no more resistant than an emulsion of colonies prepared from the original aqueous suspension.

The test organism will not grow at 37°C in broth containing 1.1 M NaCl. Therefore, it would hardly be expected to proliferate in a concentration of salt over 4 times as great in the absence of nutriments and at temperatures between +2° and −22°C. Even in the absence of salt there is no increase in the viable cell count at +2°C (curve 5; Fig. 1). In fact, there is no increase in viable count after 2 weeks in broth at this temperature. Nevertheless, it was considered prudent to make microscopic counts on the cell suspensions. No change in the number of cells per unit volume was detected from zero time to 1 week at either +2° or −22°C, nor was the number different at these 2 temperatures after any given storage period. Suspensions which had been fluctuated between +2° and −22°C showed no change in cell numbers. (The microscopic counts were made with dark field illumination, since the similarity in refractive index between the 4.6 M NaCl and the cells rendered detection of the latter difficult otherwise.)

Discussion. Suspensions of *E. coli* in 4.6 M NaCl stored at low temperatures undergo at first a rapid decline in viable cells, but as the storage interval is extended the death rate continuously decreases until eventually it becomes negligible. Cells that endure the period of rapid decline are able to maintain themselves in the saline for long periods. Most important, however, is the fact that once these cells are removed from the saline their resistance to it is lost. One may demonstrate this by centrifuging the cells from the NaCl and resuspending them in diluent. Upon a second exposure to 4.6 M NaCl, the suspension will undergo a decline proportionally as great as that obtained upon the first exposure. This same result is obtained with *Lactobacillus fermenti*(1). These data suggest that the resistance is acquired while the cells reside in the saline and does not exist preformed in a segment of the original population. Additional support for this idea is the adjustment which occurs when a suspension is held at +2°C prior to subsequent storage at the more lethal condition of −22°C. Here all cells (that is, all those which have not already succumbed at +2°C) may acquire resistance to salt at −22°C.

The above discussion leads to the consideration that the cells may adapt to the adverse environment presented by 4.6 M NaCl. Those cells which, by chance, miss the lethal event for a sufficient period have time to make the adjustments necessary to enable them to resist the event when it does occur. Such activity by the cells would account for the effect of temperature (Fig. 1), the improvement acquired by holding at a relatively high temperature prior to subsequent storage at a low temperature (Fig. 2), and also the continuously decreasing slope of the survival curve at a given temperature. Both death and adjustment could presumably result from the same stimulus. Which occurs would depend upon the conditions within the cell that influence the rates of the 2 processes. This hypothesis, therefore, assigns to the cell an active role in adjustment. One alternate hypothesis must be considered. The adjustment to NaCl tolerance may be the result of an effect brought about by the NaCl alone, for example, some diffusion phenomenon—possibly dehydration. According to this hypothesis the cell would have no active role in adjustment. The first of the 2 hypotheses given above was formulated by Eddy and Hinshelwood(2,3,4) to account for the behavior of declining populations of *Aerobacter aerogenes* at +25°C in proflavine, *m*-cresol, and low pH.

E. coli strain 69L-15 attains the "maximum stationary phase" in 8 hours or less. The cells used in our experiments are from cultures 24 hours old. Therefore, "cold shock," which occurs only with cells in the "logarithmic phase"(5), would not be expected to be a factor here. This is substantiated in our experiments. When a suspension is transferred from +2° to −22°C it cools to the latter temperature within a very few minutes. In spite of this, death continues for hours, unless it is

arrested by placing the suspension back at +2°C (compare curves 2, 3, and 4; Fig. 3). Furthermore, relatively little death results upon exposure to −22°C in distilled water. In one experiment the initial viable cell count was 1.4 x 10^8 per ml; after 1 day the viable count was 1.2 x 10^8 per ml.

Since the NaCl in the suspensions prevents freezing, we are not concerned with extracellular ice. However, the possible formation and role of intracellular ice must be considered. Although our experiments do not permit a definite conclusion as to the presence or absence of intracellular ice, they do demonstrate that, if this ice is formed, it is not an important lethal factor. Firstly, periodic fluctuations from −22° to +2°C and back to −22°C bring about no greater death than uninterrupted storage at −22°C (curves 4 and 5; Fig. 3). Secondly, we have never detected between +2° and −22°C a crucial temperature on each side of which survival differs sharply.

Summary. Suspensions of *E. coli* in 4.6 M NaCl stored under conditions which preclude cell division undergo at first a rapid decline in viable cells, but as the storage interval is extended the death rate continuously decreases until eventually it becomes negligible. Cells that endure the period of rapid decline are able to maintain themselves in the saline for long periods. This resistance is not heritable. In fact, once the cells are removed from the saline their resistance to it is lost. Generally, the higher the storage temperature the better is survival. Also, holding suspensions at a relatively high temperature (+2° or −9°C) prior to subsequent storage at a low temperature (−22°C) improves survival at the low temperature. These data suggest that the tolerance to 4.6 M NaCl is acquired while the cells reside in the saline and does not exist preformed in a segment of the original population. Two hypotheses have been offered to account for the adjustment to NaCl tolerance.

1. Harrison, A. P., Jr., *Antonie van Leeuwenhoek*, 1956, v22, 407.

2. Eddy, A. A., and Hinshelwood, C. N., *Proc. Roy. Soc. London*, Series B, 1953, v141, 118.

3. Eddy, A. A., *ibid.*, 1953, v141, 126.

4. ———, *ibid.*, 1953, v141, 137.

5. Sherman, J. M., and Naylor, H. B., *J. Bact.*, 1942, v43, 749.

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Swine and Experimental Atherosclerosis. (23194)

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Gottlieb and Lalich(1) have reported that the aortas of swine obtained from slaughterhouses occasionally show atheroma-like lesions, and that incidence of these lesions increases with age. Lewis and Page(2) have reported rather high serum cholesterol levels and rather high concentrations of low-density lipoproteins in at least one strain of pig. It was therefore hoped that this species might prove a suitable one for studies on effects of diet on serum lipoproteins and on the atherosclerotic process.

Materials and methods. The experimental animals were 8 2-year-old boars of a cross-bred strain, bred and maintained at Beltsville. They had previously been used in breeding experiments. Their basal diet consisted of a mash made of 60 parts yellow corn meal, 20 parts soybean oil meal, and 2 parts of mineral mixture. This diet contains approximately 2.9% crude fiber, 58.3% nitrogen-free extract, 17.6% protein, 3.2% fat, 4.8% ash and 13.4% moisture. The experimental diets were made by adding 2.2 parts of corn oil or 2.6 parts of butter to 10 parts of the basal diet. Approximately 40% of the caloric value of these diets was thereby derived from fat. The diets were made up weekly and