milk secreted in 24-hour periods and 129 collections from 56 women of milk secreted in periods of 4-8 hours. Mean values for concentrations of the amino acids in the 2 groups of samples agreed well. 2. Wide individual variations were found among successfully nursing women and at different intervals postpartum. Mean concentrations of nitrogen and the essential amino acids in 24-hour samples of colostrum, transitional, and mature milk showed a general lowering to the levels in mature milk as lactation was established and application of the Analysis of Variance to the data for 11 women, each of whom provided a sample during the same postpartum interval of mature milk production also indicated a gradual reduction in amino acid concentrations.

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Action of *Bacillus cereus* Toxins on Chicken Erythrocytes.*† (18690)

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A preliminary survey of the action of bacterial toxins on cells indicated that the toxins of *Bacillus cereus* had little or no effect on the rate of respiration of chicken erythrocytes(1), dogfish erythrocytes, *Arbacia* or *Asterias* eggs(2). These toxins did, however, have a marked effect on the osmotic behavior of dogfish and chicken erythrocytes(3). The present investigation was undertaken to analyze these changes in more detail. Such a study might help one to understand more fully the way in which these toxins exert their *in vivo* action and might contribute to an understanding of the relationship between the structure of the cell membrane and the passage of non-electrolytes through it.

Materials and methods. General. An isolation of *B. cereus* was made from the laboratory air. The organisms were grown on agar slants and then transferred to proteose peptone broth for incubation at 37.5°C for 36-60 hr. Following filtration, the toxins were stored at 4°C, 95% humidity. Since some of the material was stored for approximately 2 years before it was used, there was considerable variation in the strength of the toxins in different experiments. Consequently, an assay value would have no meaning. It was noted, however, that the qualitative changes in the erythrocytes were the same regardless of the age of the toxins. The older toxins merely had to act on the cells for a longer time before comparable changes were observed.

Chicken and rabbit bloods were obtained by cardiac puncture and heparinized. Sterile technics were employed except during the final 5-10 minutes when measurements were being made and only data obtained from sterile stocks were considered.

Permeability. Using the apparatus previously described(4), rates of hemolysis, shrinking and swelling were measured. The experimental stock solution contained a mixture of erythrocytes and toxins while the control stocks contained cells and broth or

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cells and Ringer Locke. These stocks were incubated at 37°C and after various time intervals aliquots (0.05-0.2 cc) were added to 10 cc of the non-electrolyte or salt solution in the chamber of the apparatus. To measure the hemolysis which occurred during swelling or shrinking, the cell suspension was removed after the measurements were made with the photoelectric apparatus, transferred to a centrifuge tube and the cells were thrown down. The amount of hemoglobin in the supernatant fluid was measured at 545 mμ using a Coleman Universal Spectrophotometer. The volumes of the cells were determined by hematocrit measurements obtained using an air turbine and by making cell counts in the manner usually employed in this laboratory.

Lipid analysis. The stock suspensions were centrifuged, the supernatant fluid discarded and the cells hemolyzed. Two methods of determining the lipid content of the cell membranes were used. One was a weight-loss method(5), which consisted essentially of extracting with alcohol-ether a known weight of dried ghosts and determining the loss in weight. The other was a gasometric technic (6) as used by Dziemian(7).

Results. Permeability. In Fig. 1 are presented typical records. Both sets of hemolysis curves show that the cells which have been exposed to the toxins hemolyze more rapidly than do the controls. This might suggest a difference in permeability but the swelling and shrinking curves show no change in permeability. The fact that the deflection of curve 6 is greater than that of curve 5 indicates a slight increase in "fragility". Spectrophotometric measurements showed that 4%§ of the control and 32% of the experimental cells hemolyzed during swelling in 0.3 M glycerol in Ringer Locke. Similar measurements of cells which had been equilibrated in 0.6 M glycerol in Ringer Locke and then shrank in 1.25x Ringer Locke or 2x Ringer Locke showed 5% and 3% hemolysis for controls and 41% and 22% for experimentals respectively. These data as well as hematocrit measurements indicate that the experimental cells swell. In 2 experiments, for example, after 9 hours exposure to the toxins, the control volumes were 73 μ^3 and 74 μ^3 while the experimental volumes were 118 μ^3 and 116 μ^3. After 15 hours the control volumes were essentially the same, 68 μ^3 and 70 μ^3, while it was impossible to obtain hematocrit readings on the experimental cells since about two-thirds of them had swelled to their hemolytic volumes.

It was found that cells which had been in the presence of the toxins a sufficient length of time to make them quite "fragile", as indicated by a large increase in galvanometer deflection during swelling measurements, still were selectively permeable. The relative rates of swelling in ethylene glycol, glycerol, urea, thiourea and erythritol (all in Ringer Locke) were unchanged by this treatment.

Another series of observations showed that rabbit erythrocytes were much less sensitive to the toxins than were the chicken erythrocytes. Whereas a marked change in "fragility" was observed after chicken cells had been exposed to the toxins for 2½ hours, no change in rabbit cells was observed after 4½ hours exposure. Seven hours exposure did make the rabbit erythrocytes quite "fragile" as indicated by the swelling measurments.

Lipid analyses. In Table I the total lipid contents of control and experimental cells are compared. These data indicate that there is no consistent difference between controls and experimentals. In Table II are presented the data on total and free cholesterol contents. Here again, no consistent difference is noted between the controls and experimentals.

Discussion. Apparently these toxins can alter the structural integrity of the cell membrane without causing a loss of lipid (*cf.* Ballentine and Parpart) (8). It seems reason-

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§ These values are all high for they include hemolysis which had already occurred in the stock suspensions before these measurements were made. The problem of measuring this hemolysis in the stock suspension will be discussed in another publication.

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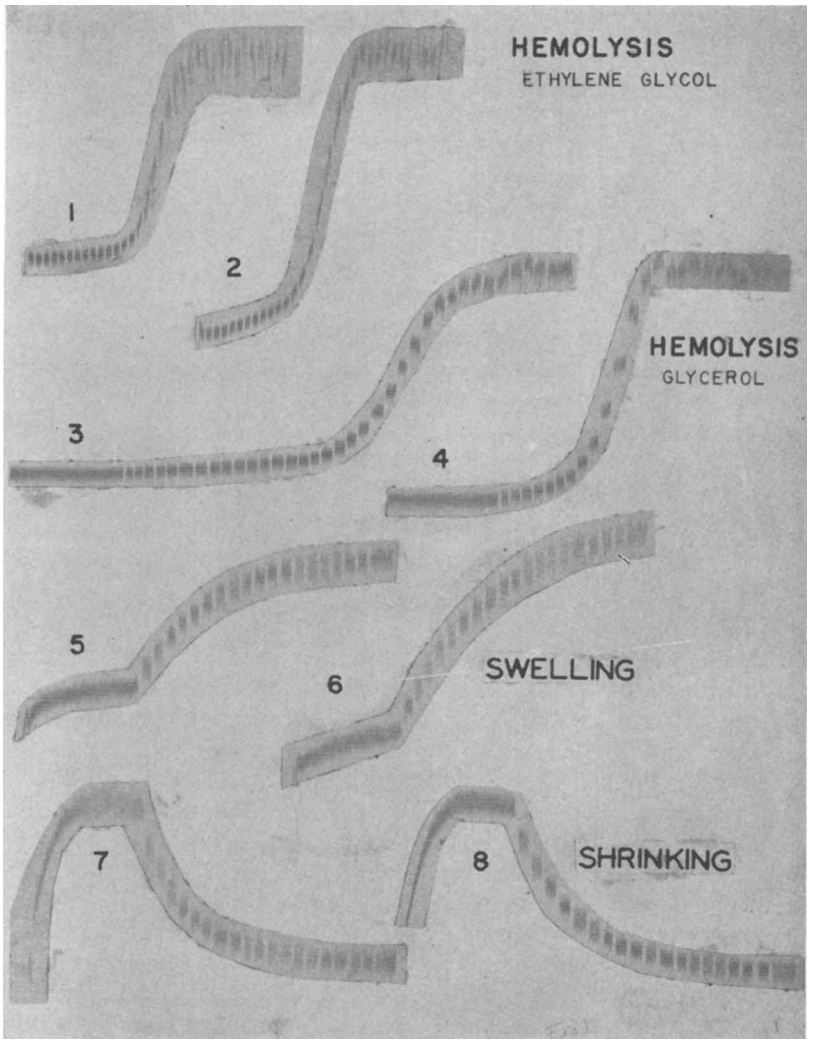

FIG. 1

The osmotic behavior of chicken erythrocytes which have been exposed to the toxins of *Bacillus cereus*. 1—hemolysis of control cells in 0.3 M ethylene glycol; 2—hemolysis of experimental cells in 0.3 M ethylene glycol; 3—hemolysis of control cells in 0.3 M glycerol; 4—hemolysis of experimental cells in 0.3 M glycerol; 5—swelling of control cells in 0.3 M glycerol in Ringer Locke; 6—swelling of experimental cells in 0.3 M glycerol in Ringer Locke; 7—shrinking of control cells in 1.25x Ringer Locke after previous equilibration in 0.6 M glycerol in Ringer Locke; 8—shrinking of experimental cells in 1.25x Ringer Locke after previous equilibration in 0.6 M glycerol in Ringer Locke. Vertical breaks = 1 second, curves 1 and 2; 15 seconds in 3-8, first 15 seconds recorded continuously.

able to suggest that the lecithinase(9) in the toxins is responsible for the changes in the cell surface. As might be expected, the toxins affect the erythrocytes of one species more rapidly than others, the action being most marked with chicken erythrocytes, less with rabbit and least with dogfish.

Apparently the initial action of the toxins can best be described as a "weakening" of the membrane without any marked alteration in the size of the aqueous channels through which small, lipid insoluble non-electrolytes

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TABLE I. Lipid Content of Chicken Erythrocytes Exposed to the Toxins of *Bacillus cereus*.

	Control			Experimental		
Method	% lipid by wt	mg lipid/ 10^9 cells	mg lipid/ cc cells	% lipid by wt	mg lipid/ 10^9 cells	mg lipid/ cc cells
Weight loss	4.1			4.4		
	3.8			3.8		
	3.3			3.3		
	3.9			3.9		
Combustion		.23	2.7		.39	3.1
		.33	3.4		.22	2.7

TABLE II. Cholesterol Content of Chicken Erythrocytes Exposed to the Toxins of *Bacillus cereus*.

	Control		Experimental	
	mg/10^9 cells	mg/cc cells	mg/10^9 cells	mg/cc cells
Total cholesterol	.055	.65	.062	.65
	.060	.61	.052	.62
	.047	.51	.078	.50
	.069	.74	.095	.61
Free "	.038	.45	.037	.35
	.043	.44	.038	.46

penetrate. Also, there is an increase in the size of the toxin treated cells (*cf.* Wilbrandt) (10). As a consequence of one or both of these changes, the time for hemolysis in isosmotic solutions of lipid insoluble nonelectrolytes is considerably decreased. The increase in "fragility" as indicated by the hemolysis when the cells shrink and then swell might well be a consequence of the structural "weakening" of the membrane. Thus, when the cells go through the mechanical process of shrinking followed by swelling, additional disorientation of the molecules in the membrane superimposed on that due to the action of the toxins occurs and some of the cells hemolyze (*cf.* Hunter) (11). The hemolysis observed when the cells swell first and then shrink is probably a consequence of the increased size of the experimental cells.

Conclusions. 1. Chicken erythrocytes exposed to the toxins of *Bacillus cereus* undergo a change in the surface of the cells which eventually leads to hemolysis. 2. Rabbit erythrocytes similarly treated are much more slowly altered by the toxins, but eventually do show a "fragility" change. 3. There is no significant change in the total lipid, total cholesterol or free cholesterol content of the experimental cells. 4. The experimental cells hemolyze more rapidly in solutions of nonelectrolytes. 5. There is an increase in "fragility" and in the size of the experimental cells. 6. The toxins have no effect on the rate of shrinking of cells placed in 1.25x Ringer Locke following previous equilibration in 0.6 M glycerol in Ringer Locke.

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