of the females is the X (since the Y chromosome here is passed from father to daughter). We do not yet have information on the map location of the responsible genetic unit.

The length of the human X chromosome is, at metaphase, about 0.06 of the total haploid set (*9*); it is the only chromosome on which there are markers (*10*). Any marker would be expected to have, on the basis of chromosome length alone, about one chance in 16 of being found on the X chromosome. Actually, of course, the recovery is much higher, because of the ease of analysis of X-linked traits. In *D. melanogaster*, the X chromosome represents about 0.19 of the total haploid complement, and hence there is about one chance in five of finding that a given marker is X-linked. The locus controlling the electrophoretic mobility of this enzyme in both man and *Drosophila* would be expected to be found on the sex-associated chromosome about once in 80 samples, by chance alone. Since this particular sample contains such disparate organisms, we wonder whether the association is purely coincidental, or whether selection pressure has maintained the relationship of this structural locus with the sex-determining apparatus for functional reasons which are not evident to us at this time.

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28 October 1963

Heat Adaptation and Ion Exchange in Bacillus megaterium Spores

Abstract. *A new spore phenomenon is described in which induction of increased heat resistance occurs during the process of heating. In buffered environments the acid form of dormant mature bacterial spores shows a temperature dependent adaptation to heat which is correlated with the rate of uptake of $Ca(OH)_2$ by titration at constant* p*H in neutral salt solution.*

In a recent investigation (*1*) relating base exchange and heat resistance in bacterial spores, heat-survival curves similar to curve *A*, Fig. 1 were obtained for the acid form of *Bacillus megaterium* spores when heated in calcium buffer solutions. Such curves frequently have been interpreted to indicate innate heterogeneity in the population with respect to heat resistance. An alternate explanation of such decreasing death-rate curves would be the hypothesis that heating slowly evokes a protective response in the spore. This view, that greatly increased heat resistance gradually developed during the heating, is supported by our data. By manipulation of the duration and severity of heating, the spores could be protected substantially against subsequent heat treatments which, otherwise, would have been quickly lethal. Rates of the later stages of calcium hydroxide consumption at constant *p*H by suspensions of the spores in neutral salt solutions were correlated with heat adaptation.

The *B. megaterium* spores, from the preparation previously described (*1*), were converted to the hydrogen form (stripped) at 25°C by holding them at *p*H 4 with nitric acid for 4 to 5 hours in a *p*H-stat. These stripped spores were washed with water and dried from the frozen state. Heating for the resistance tests was carried out in sealed 7-mm glass tubes containing 0.1 to 1.0 ml of spore suspension (2.5 mg of spores/ml). Survivors were measured as previously described (*1*). According to common practice, heat death was defined as failure to grow to visible colonies under these conditions.

Curve *A* of Fig. 1 with decelerating death rate was obtained when a freshly made suspension of stripped spores in 0.02*M* calcium acetate buffer at *p*H 5.7 was subjected to lethal temperatures in the usual way, that is with almost instant equilibration to the heating temperature. That the decelerating death rate was due to an adaptive reaction was indicated by a curve of a type like *B*, which shows high resistance after either of the following treatments of similar spore suspensions: (i) bringing the spore suspension slowly up to the lethal temperature over a 2- to 3-hour period, or (ii) holding the spore suspension at a moderately elevated, but sublethal, temperature such as 50°C overnight. Either pretreatment changed the initial slope of the curve of the log of the number of survivors plotted against time more than 30-fold.

The rate of this adaptation to heat was followed at several sublethal temperatures in calcium acetate buffer (0.02*M* Ca) at *p*H 5.7 and in calcium diethyl barbiturate (0.012*M* Ca) at *p*H 7.9. For assay the spores were brought to 83°C instantly and the number surviving for 10 minutes was used as a measure of the reaction's progress. This number would have been influenced somewhat by any adaptation occurring during the 10-minute test at 83°C. In Fig. 2 the log of survivors per gram of spores is plotted against time. In addition to the moderate p*H* effect the results showed a marked temperature dependence. Days were required at moderately low temperatures such as 18°C to accomplish the protection afforded by 1 or 2 hours at 50° to 65°C. The rate data of Fig. 2 roughly approxi-

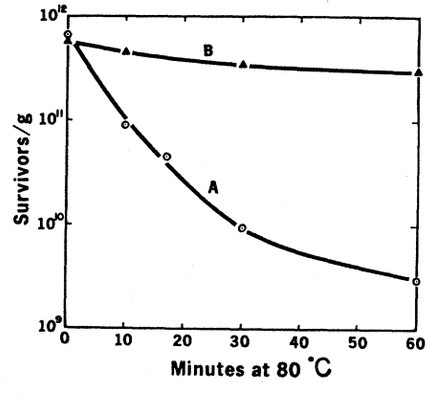

Fig. 1. Heat resistance of stripped *B. megaterium* spores in *p*H 5.7 calcium acetate buffer (0.02*M* Ca). Curve *A*, tubes of freshly made suspension plunged directly into 80°C water bath. Curve *B*, spore suspension conditioned prior to lethal heating (80°C) by either (i) slow ascent (2 to 3 hr) to lethal temperature, or (ii) holding at 50°C overnight.

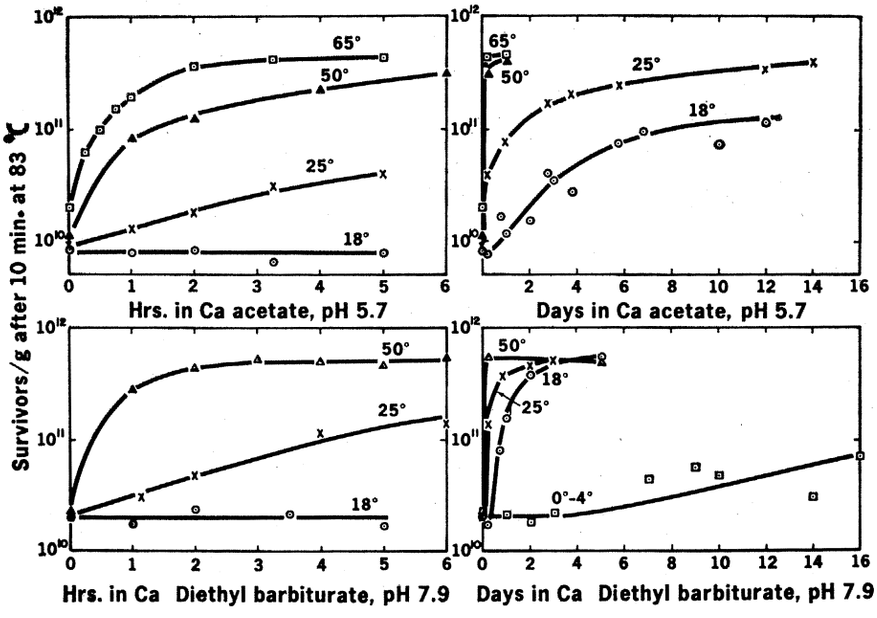

Fig. 2. Rates of heat adaptation for stripped *B. megaterium* spores in calcium buffers at various temperatures.

mated first-order kinetics. The calculated apparent rate constants approximately tripled for a rise of holding temperature of 10°C. A graph of the log of the rate constants plotted against the reciprocal temperature indicated an activation energy (E_a) of about 18,000 calories for the data at *p*H 5.7 and about 20,000 calories at *p*H 7.9, values within the expected range for ordinary chemical reactions. Heat killing of spores, on the other hand, has a much sharper temperature dependence (*2*, *3*) similar to that of protein denaturation reactions with E_a values around 100,000 calories and about a tenfold rate change for a rise of 10°C in the usual moist heat-killing range.

The difference in temperature dependence between these two reactions should be useful in minimizing the heat resistance of spores in environments where this type of heat adaptation is possible. Efficiency of spore heat killing in such environments should be promoted by: (i) low temperature storage prior to lethal heating, (ii) as nearly as possible instantaneous equilibration to the lethal temperature, (iii) the choice of a high lethal temperature (for a short time) so that the killing process with its sharper temperature dependence could outrun the adaptive reaction.

Recently we reported (*1*) that *B. megaterium* spores have a pronounced cation-exchange capacity and that their heat resistance is influenced by cations on the exchange system. The exchange is *p*H controlled, resembling somewhat that in the weak-acid cation exchange resins. In order to measure spore cation exchange under conditions simulating those of the heat adaptation biologically assayed (Fig. 2), the calcium hydroxide uptake of stripped *B. megaterium* spores was followed at constant temperature and *p*H in the presence of neutral salt ($CaCl_2$) in a *p*H-stat. The $Ca(OH)_2$ consumption occurred in two general stages, the first very rapid phase changing to a second slow stage. The rate of the slow stage was temperature and *p*H dependent. The rates (Fig. 3) of the final slow stage of calcium uptake

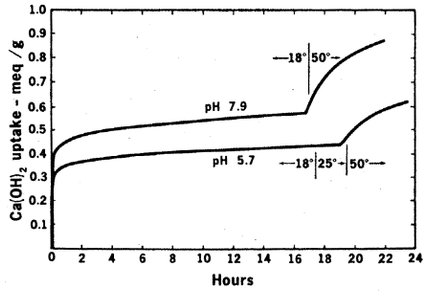

Fig. 3. Effect of temperature on the rate of titrimetric $Ca(OH)_2$ consumption of *B. megaterium* spores at constant *p*H in neutral salt. Upper curve, 20 mg spores per 4 ml 0.025*M* $CaCl_2$ held at *p*H 7.9 for 16.8 hr at 18°C and for 5 additional hr at 50°C. Lower curve, 20.5 mg spores per 5 ml 0.02*M* $CaCl_2$ held at *p*H 5.7 for 17.4 hr at 18°C; for 2 hr longer at 25°C; then 4.0 hr at 50°C. The temperature changes required about 0.4 min per degree.

are correlated with the rates (Fig. 2) of the biologically measured heat adaptation under similar conditions of *p*H and temperature. Biological assays on portions removed from the titrations (Fig. 3) after the periods at 18°C and 50°C showed increases in heat resistance similar to those expected from the buffer incubation (Fig. 2).

The rates of cation uptake (Fig. 3) suggest at least two kinds of binding sites, different in accessibility to diffusion or in chemical nature. The initial rapid uptake results in a small increase of heat resistance compared to that resulting from the later slower stage. When calcium-loaded spores are subjected to a low *p*H for stripping, a large uptake of acid also occurs rapidly with a small effect on heat resistance, after which further stripping gives large reductions. In other words, a graph of heat resistance plotted against titrimetric uptake of base followed by acid would have the general appearance of a hysteresis loop over a loading and stripping cycle.

The usual large effect of cation valence on exchange affinity appears to operate in the exchange system of the spore. Incubation of stripped spores with sodium buffer at *p*H 5.7 gave only minor enhancement of heat resistance compared with that shown by calcium ion. The monovalent sodium ion apparently is unable to compete with protons for the exchange sites in this *p*H region. At higher *p*H on the other hand both metallic ions are effective in conferring heat resistance. Thus, in the commonly encountered *p*H region around 5 and 6, calcium ion becomes particularly important to heat sensitivity. This difference in affinity also appears in the ease of acid stripping of spores. Calcium-loaded *B. megaterium* spores require a lower *p*H to reduce heat resistance in a convenient time than do the untreated spores which have a high potassium load.

It would appear from the biological and chemical results that exponential mortality curves (straight lines on a graph similar to Fig. 1), rather than being the general case, might be expected to be possible only in situations where the heat adaptation reaction could not proceed at lethal temperatures at a rate appreciably relative to the killing rate. One such situation would consist in heating of spores at high lethal temperatures for a short time where the different temperature dependence of the two types of reactions

would mask the effects of the adaptation. Any arrangement of the chemical environment so that ion exchange between the spores and the external medium would be minimal during lethal heating should also reduce the curvature of the death-rate curves. We have obtained fairly straight log survivor curves at moderate lethal temperatures for water suspensions of stripped spores neutralized to various degrees with hydroxides. As mentioned previously, heating hydrogen-form (stripped) spores in calcium buffer at moderately lethal temperatures gives a survivor curve with upward curvature. When heat adapted (calcium loaded) spores freshly inserted into an acidic buffer are subjected to similar moderate lethal heat, the logarithmic survivor curve shows an accelerating death rate or downward curvature.

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5 August 1963

Vitamin D_3: Direct Action on the Small Intestine of the Rat

Abstract. *Vitamin D_3 placed directly into loops of rat duodenum in vitamin D deficient animals increases markedly the subsequent transport of calcium by slices of the duodenal loop in vitro. Under similar conditions the same dose of vitamin given intravenously or placed in a jejunal loop has little or no effect on the duodenal tissue. Thus the vitamin acts directly on the small intestine without prior activation in another organ.*

Studies with segments of small intestine in vitro demonstrate that vitamin D is required to maintain an active transport mechanism for calcium absorption in the mucosa (*1, 2*). Although this end result of the administration of vitamin D to intact animals is well-established, the biochemical mechanism and initial site of action of the sterol remain unknown. Addition of the vitamin to media containing intestinal segments in vitro has failed to affect the calcium transport detectably. Sallis and Holdsworth have suggested that the vitamin must be activated in the adrenal prior to its intestinal action (*3*). This hypothesis is untenable in the case of the rat, for adrenalectomized, vitamin D deficient rats respond normally to administration of the vitamin (*4*). More generally, the following experiments demonstrate that vitamin D_3 acts directly on the small intestine of the rat, and the vitamin need not be activated in another organ.

Initial experiments compared the effects of vitamin D_3 given intravenously with equal doses placed in loops of duodenum tied *in situ*. The duodenum is the segment of maximal active transport of calcium in the rat, and the oxygen-dependent accumulation of Ca^{45} (or Ca^{47}) by full-thickness slices of duodenum in vitro provides a sensitive method for the quantitative measurement of the action of the vitamin (*2*). When given to deficient rats, large doses of vitamin D act rapidly and small doses act slowly (*2*). Intermediate doses in the range of 100 to 400 I.U. of vitamin D_3 per rat yield suboptimal but detectable effects in 5 hours. In this period of time less than 5 percent of an intravenous dose of tritium-labeled vitamin D_3 accumulates in the duodenum, whereas over 80 percent of the same dose placed in a duodenal loop is absorbed into the mucosa (*5*). Under these conditions, therefore, vitamin D_3 placed in the loop would be much more effective than an intravenous dose if the action were direct and less effective if the sterol required prior activation in another organ.

The results of four experiments demonstrate that the action is direct (Table 1). Weanling male rats were maintained on the USP rachitogenic diet No. 2 (*6*) for 4 to 5 weeks in cages shielded from light. In each experiment three groups of eight depleted rats were anesthetized with ether. In one group (controls) a duodenal loop was tied in each rat (*7*) and the loop was filled with 0.25 ml of 0.85 percent NaCl and 0.01*M* sodium taurocholate (*8*); the common bile duct was ligated. The second group was treated similarly but given 100 to 400 international units (I.U.) of vitamin D_3 intravenously in 0.25 ml of the saline-taurocholate vehicle. The solutions were clear. The third group was treated similarly but the dose of vitamin D_3 was included in the solution used to fill each duodenal loop. Five hours after the operation the animals were sacrificed, and slices were prepared from the duodenal loops and tested for calcium accumulation in vitro (*2*). At 100 and 200 I.U., a distinct increase in calcium transport over the control values was observed only in loops exposed directly to the vitamin, with no increase after intravenous administration. At 400 I.U., some increase followed the intravenous dose, but a greater effect was observed in the loops exposed directly (Table 1).

Table 1. Effect of vitamin D_3 given intravenously or placed directly in a loop of duodenum on the oxygen-dependent accumulation of calcium by slices of duodenum in vitro.

Dose of D_3 (I.U./rat)	Oxygen-dependent accumulation of calcium (mμmole/g of slices per hour)		
	No D_3	Intravenous D_3	Loop D_3
100	63	55	102
200	76	78	177
200	86	62	114
400	108	148	175

A second type of experiment confirmed the foregoing results. Inasmuch as vitamin D is absorbed from the intestine into the chylomicron fraction of lymph (*5*), it was desirable to compare the effects on calcium transport in the duodenum when vitamin D_3 was placed either in a duodenal or a mid-jejunal loop of intestine. Absorption of vitamin D_3 from mid-jejunal loops is more rapid than from duodenal loops (*5*). Three groups, each containing eight vitamin-D deficient rats, were tested in each of two experiments. A duodenal and a mid-jejunal loop were tied in each rat

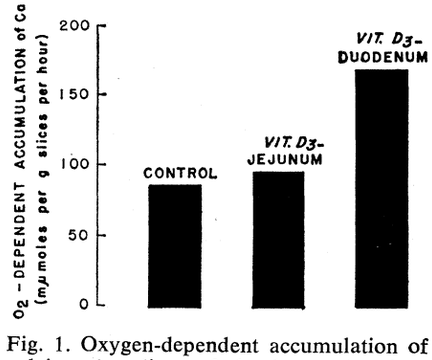

Fig. 1. Oxygen-dependent accumulation of calcium by slices of duodenum in vitro. The slices were prepared from duodenal loops tied 5 hours before the assay. When the loops were tied, the control rats received no vitamin D_3, the "vitamin D_3–jejunum" group was given 200 I.U. per rat in a jejunal loop, and the "vitamin D_3–duodenum" group was given 200 I.U. directly in each duodenal loop.