**Alkali Resistance in a Strain of *Bacillus cereus*
Pathogenic for the Larch Sawfly
*Pristiphora erichsonii***

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SUMMARY: Upon repeated subculture in media of gradually increasing pH value a strain of *Bacillus cereus* developed the ability to grow under strongly alkaline conditions. Organisms with the highest alkali resistance could grow at pH 10·8, and stocks of intermediate resistance were also obtained. When growing on alkaline agar, resistant bacteria underwent marked morphological changes. In liquid media, the bacteria retained the ability to synthesize lecithinase. Resistance was not lost upon several transfers on a neutral medium.

Resistant bacteria when growing in alkaline media caused a rapid decrease in pH value. A similar pH decrease was caused by sensitive bacteria in media of the highest degree of alkalinity in which they could grow. The decline of pH value accompanied, but did not precede, growth; acid production by the resistant bacteria does not appear to be a primary mechanism of resistance.

Most bacterial species grow best in media of a relatively restricted pH range, near neutrality. *Topley and Wilson's Principles* (1955) stated that, with few exceptions, human pathogens grow in the range pH 5·0–8·0. To the extent that the pH range for growth of other bacterial species has been studied, there appear to be few major deviations from these figures. The most striking, on the acid side, is that of *Thiobacillus thiooxidans*, which can grow in '5–7 %' H₂SO₄ (Umbreit, 1951). On the alkaline side, *Streptococcus faecalis* was reported to grow up to pH 11, as well as in strongly acid media (Downie & Cruickshank, 1928). The effect of the pH value of the medium on the growth of *Bacillus cereus* Fr. & Fr. is one of the factors governing the pathogenicity of this organism for insects. In recent discussions of this subject (Heimpel, 1955*a*; Heimpel & Angus, 1958) it was pointed out that *B. cereus* ingested by mouth is a pathogen of larvae of the codling moth, *Carpocapsa pomonella* L. (Stephens, 1952), and of the larch sawfly, *Pristiphora erichsoni* Htg. (Heimpel, 1954), but not of the forest tent caterpillar, *Malacosoma disstria* Hbn. In the first two insects the midgut pH value is near neutrality and within the growth range of normal *B. cereus*. In the last organism, however, the midgut is at pH 9·5–10·5, a range outside that in which *B. cereus* usually grows.

The work described here arose from an attempt to increase the host range of micro-organisms which infect insects. For *Bacillus cereus* to cause disease in insect larvae the following sequence of events appears necessary: the bacteria or their spore must be swallowed; the spores, when present, must germinate; and the vegetative organisms must grow and damage the insect, possibly by lecithinase production (Heimpel, 1955*b*; Kushner & Heimpel, 1957). By whatever mechanism the bacteria kill the insect, in most cases it is likely that

they must first multiply inside it. Surveys of internal pH values of insects (Staudenmayer, 1940; Waterhouse, 1949) show that the larvae of several species of phytophagous lepidopterous insects have, as does the forest tent caterpillar, highly alkaline midgut contents. Alkaline midgut conditions have also been observed in the Trichoptera and in some species of Coleoptera. Although *B. cereus*, as well as many other bacteria, would not normally be expected to grow in these insects, it seemed possible that bacteria induced to grow under more alkaline conditions than usual might be able to grow in these insects and perhaps to kill them. As a first step in this work, we have produced an alkali-resistant strain of *B. cereus*, which is able to grow in media of higher pH value than can the parent strain.

METHODS

Organisms. *Bacillus cereus*, strain Mu-3055, a pathogen of the larch sawfly (Heimpel, 1955*b*), was used throughout this study.

Growth media. Buffered growth media were used in all experiments, the buffer concentrations chosen being the highest that would permit maximal bacterial growth. The following media were prepared:

(1) Nutrient broth + phosphate buffer. Phosphate buffer (0.1 M, pH 7.5) was prepared by dissolving 71.6 g. $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in water, adding 3.2 ml. conc. HCl, and bringing the volume to 2 l. with water. Dried nutrient broth (Difco) was dissolved in this to 8 g./l. The final pH value was 7.4.

(2) Nutrient broth + tris buffer. A 0.05 M solution of 2-amino-2-hydroxy-methylpropane-1:3 diol (tris: pH 7.5) was prepared by dissolving 12.1 g. tris in water, adding 6.25 ml. conc. HCl, and diluting to 2 l. Nutrient broth (dried, Difco) was made up in this, as for medium no. 1, the final pH value being 7.5.

(3) Casein hydrolysate + tris buffer. A 2% (w/v) solution of 'Enzymatic Casein Hydrolysate' (Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A.) was made in tris buffer solution as in medium no. 2. The final pH value was 7.5.

(4) Buffered nutrient agar. Nutrient agar (Difco) was dissolved in 0.1 M-phosphate buffer; or 1.5% (w/v) 'Bacto Agar' (Difco) was made up in nutrient broth + phosphate buffer.

For most of this work the liquid media were dispensed in 10 ml. lots in 1 in. $\times$ 8 in. culture tubes and autoclaved at 15 lb./sq.in. for 15 min. Immediately before use, the pH value of a tube of medium was brought to the desired level by adding N- or 10 N-NaOH, as given by previously determined titration curves. The buffered agar was sterilized in 5 or 10 ml. lots in screw-cap tubes. For use, the agar was re-melted by immersing the tubes in a hot water bath, and NaOH was added before making slopes or pouring plates. Addition of NaOH *after* sterilizing was necessary since autoclaving strongly alkaline media led to charring. It was necessary, furthermore, to adjust the pH value of each tube just before use, since these media do not maintain a high pH value indefinitely. The extent of the decrease in pH value observed when different samples of sterile alkaline media were shaken at 37° is shown in Table 1. Alkaline casein hydrolysate + tris buffer medium was the most stable

and alkaline nutrient broth + tris buffer medium the least so. Media of pH 8.0 or less did not change pH value on incubation in the absence of bacteria. The chemical changes which caused the pH to decrease were not investigated, but CO₂ absorption was probably involved.

Table 1. *Fall of pH in sterile alkaline media incubated at 37°*

Time (hr.)	pH								
	Nutrient broth + phosphate			Nutrient broth + tris				Casein hydrolysate + tris	
0	9.36	9.84	10.39	9.48	10.00	10.51	11.05	9.61	10.00 10.47
10	9.22	9.72	10.38	9.36	9.77	10.18	10.70	9.50	9.99 10.34
20	9.05	9.47	10.01	9.28	9.51	9.74	10.08	9.48	9.98 10.23
30	9.03	9.37	10.01	9.23	9.52	9.72	10.02	9.42	9.85 10.15

All media of initial pH 7.40 did not change pH on incubation.

In following bacterial growth the culture tubes were shaken on a rotary shaker in an incubator at 37°. The rate of shaking was 160 cycles/min. and each tube moved in a circle of diameter 2 in. Growth was measured directly in the culture tubes as turbidity, the changes in optical density at 650 m μ being followed by a Beckman Model DU spectrophotometer fitted with a test-tube adapter. In this system an optical density of 1.0 corresponded to 0.32 mg. dry wt. bacteria/ml. or to 8×10^7 viable bacteria /ml. as measured by plate counts. All figures given are corrected for the turbidity caused by the inoculum itself.

Measurements of pH value were made with a Beckman Model G pH meter and a glass electrode. Lecithinase activity was measured as previously described (Kushner, 1957).

RESULTS

Development of alkali resistance

In nutrient broth + phosphate buffer 9.5 was the highest pH value at which *Bacillus cereus* Mu-3055 grew, and there was an extended lag period before growth began (Fig. 2). The organism was induced to grow readily at pH values of 9.5 or higher by repeated subculture in media of increasing alkalinity, as follows: a 24 hr. culture of *B. cereus*, which had been maintained routinely on nutrient agar, was inoculated into a tube of nutrient broth + phosphate buffer, pH 7.4, and incubated with shaking until an optical density of about 1.0 was reached. One-half ml. of this culture was then placed in each of a series of tubes at graded pH values from 7.4 up. After shaking for 8–12 hr., the tube of highest pH value showing growth comparable to that found in the tube of pH 7.4 was selected for subculture in a series of tubes of the same and higher pH values. The process was continued, selecting each time bacteria from the tube of highest pH value showing vigorous growth. When necessary, serial subculture at the highest pH value possible was continued until the bacteria had acquired the ability to grow at a slightly higher pH value.

The course of a typical procedure is shown in Fig. 1. It is seen that the bacteria were quickly induced to grow readily at pH 9.5, but the ability to

grow in media of higher pH developed much more slowly. Bacteria had to be serially subcultured several times at each pH increment before they showed any increased alkali resistance. Even with numerous subcultures the resistant *Bacillus cereus* could not be induced to grow in broth of pH value greater than 10.3.

Fig. 1. Development of alkali resistance in *Bacillus cereus*. The time during which subcultures at each pH were carried out is shown on the abscissa. The actual number of subcultures at each pH is shown in parentheses in the figure.

After the bacteria had begun to grow at the higher pH values, subcultures were continued at intermediate values from pH 9.5 up. This was essential, since it was frequently found that bacteria which at first grew well at the highest pH value tested died out after several subcultures at the same pH value. In these cases, it was necessary to continue the 'inducing' process with bacteria 'established' at a slightly lower pH value.

After bacteria had succeeded in growing at pH 9.5, 10.0 and 10.3, they were spread on buffered nutrient agar plates of these pH values and maintained by transfer every few days on alkaline plates or slopes. Normal bacteria were also maintained on buffered nutrient agar at pH 7.4 and were used as 'sensitive bacteria' in subsequent experiments. As was expected, normal bacteria, unable to grow in alkaline liquid media, were also unable to grow on alkaline agar plates. Resistant bacteria have been maintained in our laboratory for periods of up to one year.

Nutrient broth + phosphate buffer was used in all cases as a medium for inducing alkali resistance in the bacteria whose properties are described here. Preliminary experiments showed that with subculture in casein hydrolysate + tris buffer medium of gradually increasing pH values alkali resistance was acquired in approximately one-third the time, though the degree of resistance reached was no higher.

Colony formation and morphology of resistant organisms

On alkaline agar the resistant cultures grew more slowly than they (or sensitive organisms) grew on neutral agar. The colonies were smaller than those of normal *Bacillus cereus* Mu-3055 grown for the same length of time, but otherwise they appeared the same. The total quantity of bacteria growing when

resistant organisms were spread on an alkaline plate was relatively small. When resistant organisms were grown on nutrient agar at pH 7.4 there was no apparent difference between their growth and that of normal *B. cereus*.

Microscopically, resistant bacteria growing on alkaline agar exhibited marked morphological changes. The presence of the buffer itself caused no such change; sensitive bacteria grown on buffered nutrient agar at pH 7.4 appeared the same as those growing on unbuffered agar. The resistant bacteria growing at pH 9.5 had an appearance only slightly different from that of sensitive bacteria; the cells were a little thinner and some were longer than those of sensitive cultures. The decrease in cellular diameter was more marked in bacteria growing at pH 10.3 and the organisms differed considerably in length.

When resistant organisms from plates at pH 10.3 were subcultured at pH 7.4 the organisms appeared microscopically like normal *Bacillus cereus* and formed spores. As will be seen below, resistant organisms retained their alkali resistance when grown at pH 7.4. There is a possibility that resistance may be transmitted through the spores; this has not yet been investigated.

Growth of sensitive and resistant bacteria in alkaline liquid media

The effect of pH value on the growth of sensitive and alkali-resistant organisms in nutrient broth + phosphate buffer is shown in Fig. 2. Measurements of the maximal growth rates and final bacterial concentrations obtained in this and other similar experiments are given in Table 2. In this type of experiment, the inocula consisted of equal quantities of sensitive or resistant

Fig. 2. The effect of pH on growth of sensitive and resistant bacteria in liquid medium. Nutrient broth + phosphate buffer was used. The initial pH of each tube is shown in the figure. The resistant bacteria had been maintained on agar of pH 10.3. The inoculum used of sensitive or resistant bacteria was 0.36 mg.

bacteria from agar plates (18–24 hr. culture), washed twice with distilled water before being added to broth tubes. The actual weights of bacteria added in each experiment were as given; the volume of bacterial suspension used varied with the bacterial concentrations but was in no case greater than 0.10 ml.

Resistant bacteria from alkaline agar plates were used in the first experiments. Once it was found, however, that resistant bacteria retained their resistance when grown on neutral medium (see below), neutral agar plates were used in certain specified experiments for preparing inocula of resistant organisms.

Differences in pH range for growth as between sensitive and resistant bacteria are immediately apparent (Fig. 2). At pH 7·4 the lag period for growth of both sensitive and resistant organisms was approximately the same and this was found in practically all experiments. The sensitive bacteria grew at pH 9·5 only after a lag period of 14 hr. and grew at no higher pH during a 25 hr. period. Sensitive bacteria did not grow at pH 10·0 in experiments lasting 30 hr. or more. The resistant bacteria, however, grew at pH 9·5 after a 4 hr. lag and grew up to pH 10·0. The resistant bacteria used in the experiment shown in Fig. 2 had originally grown at pH 10·3 in liquid media before subculture on alkaline agar. A small loss of resistance, therefore, occurred upon transfer to agar, or possibly upon washing the organisms.

Table 2. *Growth rates and final bacterial concentrations attained by sensitive and alkali-resistant bacteria in nutrient broth + phosphate buffer of different initial pH values*

Expt.	Bacteria	pH	Maximal growth rate (O.D./hr.)	Maximal bacterial concentration (O.D.)
1	Sensitive	7·4	0·253	—
		8·9	0·193	—
		9·5	0·140	—
	Resistant	7·4	0·183	—
		8·9	0·180	—
		9·5	0·150	—
		10·0	0·187	—
2	Sensitive	7·4	0·226	1·46
		9·3	0·210	1·24
	Resistant	7·4	0·266	1·41
		9·3	0·188	1·30
3	Resistant	7·4	0·234	1·42
		9·3	0·204	1·35
4	Sensitive	7·4	0·350	—
		9·5	0·253	—
	Resistant	7·4	0·306	—
		9·5	0·285	—

The maximal growth rate was taken as the rate of increase in optical density between O.D. 0·100–0·700.

Rates in Expt. 1 are from the experiment illustrated in Fig. 2. Figures for maximal bacterial concentration were obtained 15–20 hr. after growth began.

Weight of inocula: Expt. 1, 0·36 mg.; Expts. 2 and 3, 0·10 mg.; Expt. 4, 0·30 mg.

As indicated in Fig. 2, in media of high pH before the onset of growth there was a slight decrease in optical density. This suggests that under strongly alkaline conditions some lysis of bacteria occurred.

The measurements given in Table 2 show that, despite variations from one experiment to another, sensitive and resistant bacteria in general grew at

closely comparable rates at pH 7.4. Raising the pH tended to decrease the maximal growth rate to a varying degree; but even at the highest initial pH growth, if it occurred at all, proceeded relatively rapidly. The word 'initial' is stressed because, as will be seen, bacteria growing in the buffered alkaline media used here caused a rapid decrease in the pH value. An accurate estimate of the considerable effect of pH on growth rate can only be obtained if the initial pH is held constant by frequent additions of alkali.

Little difference was noted between the maximal concentrations reached by both sensitive and resistant bacteria (Table 2). In nutrient broth + phosphate buffer maximal growth was reached at an optical density of 1.3–1.4. In the other media used there was a slightly higher maximal optical density (1.5–1.6). Maximal growth rates were similar to those found in nutrient broth + phosphate buffer.

Table 3. *Effect of pH on growth of alkali-sensitive and resistant bacteria in different media*

Medium	Duration of expt. (hr.)	Bacteria in inoculum (mg.)	Initial pH*	$T_{0.4}$ (hr.)†		Final pH	
				Sensitive	Resistant	Sensitive	Resistant
Nutrient‡ broth + phosphate	25	0.86	7.4	4.0	3.5	7.5	7.5
			8.9	3.5	5.0	8.2	8.2
			9.5	16.0	7.2	8.3	8.3
			9.8	> 25	11.0	9.2	8.5
			10.0	> 25	24.8	9.4	8.7
			10.1	> 25	> 25	9.5	9.5
Nutrient broth + tris buffer	27	0.86	7.5	2.5	2.7	7.7	7.9
			9.2	3.2	4.0	8.6	8.7
			9.5	4.5	5.5	8.7	8.8
			9.8	9.2	6.2	8.7	8.9
			10.0 (9.5)	15.0	7.0	8.8	8.9
			10.2	22.5	10.5	9.0	9.0
			10.5 (9.6)	25.0	13.5	9.2	9.0
			10.9	> 27	19.0	9.7	8.9
Casein hydrolysate + tris buffer	25	0.28	11.2 (9.9)	> 27	> 27	9.8	9.8
			7.4	2.5	2.8	8.1	8.1
			8.9	3.2	3.4	8.7	8.6
			9.4	4.6	5.2	8.9	8.9
			9.8 (9.7)	25.0	16.0	9.7	9.2
			10.0 (9.9)	> 25	17.0	9.9	9.3
			10.3 (10.2)	> 25	> 25	10.2	10.1

Resistant bacteria as in Fig. 2; organisms growing in casein hydrolysate + tris buffer had been subcultured at pH 7.4 before use.

* Figures in parentheses show final pH after incubation in the absence of bacteria.

† Time at which cultures reach a (net) optical density of 0.4.

‡ The results given are those illustrated in Fig. 2.

In these experiments the most striking and consistent effects of the pH value of the medium were those produced on the lag period. Growth curves can conveniently be described in terms of this figure or, more accurately, in terms of the time necessary for the bacteria to reach a defined net optical density. Here, the value $T_{0.4}$, that is the time at which an optical density of 0.4 is reached, is used to describe bacterial growth. The $T_{0.4}$ values for the curves shown in Fig. 2 are given, with other data, in Table 3.

The pattern of growth of sensitive and resistant organisms in nutrient broth + tris buffer medium and in casein hydrolysate + tris buffer medium was similar to that found in nutrient broth + phosphate buffer (Table 3). Both sensitive and resistant organisms grew at a slightly higher initial pH range in nutrient broth + tris buffer medium than in the other two media. This was probably due to the more rapid spontaneous decrease of pH value found in the nutrient broth + tris buffer medium (Tables 1 and 3). In these experiments, sensitive bacteria were compared with bacteria maintained at pH 10.3. Resistant cultures maintained on buffered agar plates at pH values between 7.4 and 10.3 had, as was expected, intermediate resistance in liquid media. As the figures in Table 4 show, organisms from nutrient agars of progressively increasing pH values showed progressive shortening of the $T_{0.4}$ value in alkaline media. Within the weight range used in these experiments, altering the size of inoculum had little effect on the growth. It was found, in addition, that decrease of inoculum size from equivalent 0.30–0.02 mg. dry wt./tube increased the lag period and the $T_{0.4}$ value by about 25 % at all pH values tested; increase of inoculum size, up to equivalent 1 mg. dry wt./tube had relatively little effect.

Table 4. *Effect of pH on growth of bacteria of varying degrees of resistance in liquid medium*

pH of medium	$T_{0.4}$ (hr.) Bacteria from agar of pH		
	7.4	9.5	10.3
7.4	3.0	3.6	2.8
9.4	> 12	7.3	5.0
9.9	> 12	> 12	11.0

A 0.34 mg. inoculum of bacteria was used in all tubes. Growth medium was nutrient broth + phosphate buffer.

Acid production by sensitive and resistant bacteria

Both sensitive and resistant bacteria growing in media at pH 8.9 or higher caused the pH value of the medium to fall (Table 3). In nutrient broth + phosphate buffer (pH 7.4) no change in the pH value occurred with growth; in tris-buffered media (pH 7.4–7.5) growth caused a small increase in pH value. When the organisms did not grow, the pH value of alkaline media fell only to the same extent as in media to which no bacteria were added (Tables 1 and 3).

Acid production was in some cases quite vigorous; the decreases in pH value which took place indicate that the growing bacteria produced 0.1–0.3 m-equiv. acid (corresponding to 0.1–0.3 ml. N-acid). The actual acid(s) produced were not determined. Possibly, as with *Escherichia coli* (Gale, 1948), *Bacillus cereus* Mu-3055 growing in alkaline media lowered the pH by deaminating some of the amino acids present and forming carboxylic acids.

The relation between acid production and the phase of bacterial growth is of importance in considering possible mechanisms of alkali resistance. This

relation was studied in the experiments illustrated in Fig. 3 in which the growth of resistant cultures and the pH changes were followed simultaneously. Before growth began the pH values of the medium declined slowly, the rate of decline being equal to the spontaneous rate already described (Table 1). After the onset of growth, the pH value fell much more rapidly and continued to fall while growth continued. When the pH of the medium was not permitted to fall freely but was held constant by periodic additions of NaOH solution, the resistant cultures still grew. The rate of growth was somewhat lower at pH 9.4, and considerably lower at pH 9.8, than in media in which the pH value was permitted to fall. Significant growth did take place when the pH was held at 9.8.

Figs. 3 *a, b*. Growth of resistant bacteria in alkaline media of varying and constant pH. Solid lines represent bacterial growth, and broken lines represent the pH of the medium. Curves *A* show bacterial growth that took place when the pH fell freely, and curves *B* the growth that took place when the pH was held at the initial value by periodic additions of NaOH (indicated by arrows). *Bacillus cereus* resistant to growth on agar of pH 10.3 was subcultured at pH 7.4 before use; 1.2 mg. inocula were placed in 200 ml. lots of nutrient broth + phosphate buffer in 500 ml. Erlenmeyer flasks, incubated with shaking, and samples taken at the times indicated for pH and optical density readings. (*a*) Initial pH of 9.4. (*b*) Initial pH of 9.8.

In an experiment similar to that shown in Fig. 3 *a* an inoculum of 4.2 mg. resistant organisms was used. The rate of pH change relative to growth was the same. The organisms used in the experiment shown in Fig. 3 had been subcultured once at pH 7.4. Resistant bacteria taken directly from agar of pH 10.3 caused the same rate of fall of pH when grown on liquid medium of pH 9.5.

The relation between growth phase and acid production by sensitive organisms multiplying in nutrient broth + phosphate buffer at pH 9.4 was similar to that found in the resistant cultures. The pH value remained almost constant during the prolonged lag phase and fell rapidly when growth began (Fig. 4). Comparison of Fig. 3 *a* with Fig. 4 shows that the rate of pH decline caused by growing sensitive organisms was similar to that caused by growing resistant organisms. Between pH 9.0 and 8.5, when the bacteria were growing at the maximum rate, the respective rates of pH decline were identical.

Stability of alkali resistance

That alkali resistance was not lost when resistant organisms were subcultured on agar of pH 7.4 was indicated by the fact that such organisms grew when replated on alkaline agar. A more detailed experiment on this point is shown in Table 5. Resistant cultures of the organism were serially subcultured 6 times at pH 7.4, and the effect of pH on the growth of these subcultures was tested. There was no loss of alkali resistance during growth at pH 7.4. Indeed, the $T_{0.4}$ values at pH 9.5 and especially at pH 10.0 for three of the four

Fig. 4. Changes in pH during growth of sensitive bacteria in alkaline medium. Conditions and legend as in Fig. 3a except that a 4.4 mg. inoculum of sensitive organisms was used and the pH was not held constant.

Table 5. *Retention of alkali resistance during subculture on agar of pH 7.4*

pH	Sensitive bacteria	$T_{0.4}$ (hr.)				
		No. of subcultures				
		0	1	2	5	6
		Resistant bacteria subcultured on agar of pH 7.4				
7.4	4.0	4.4	3.2	3.2	3.9	2.0
9.5	16.0	7.2	6.2	4.8	7.2	4.3
10.0	> 25	21.5	12.0	11.2	19.0	13.0

A 0.28 mg. inoculum of bacteria was used in each experiment. Resistant *B. cereus* had been maintained previously on agar of pH 10.3, and was serially subcultured on buffered agar of pH 7.4 at 24 hr. intervals. The 5th subculture was stored overnight at 5° before testing. Growth medium for testing pH sensitivity was nutrient broth + phosphate buffer.

subcultures tested were lower than those of the parent culture. It may be significant that the one member of the series shown in Table 5 virtually indistinguishable from the parent culture had been held overnight in the cold before testing, but these findings have not been further investigated.

Lecithinase production by resistant bacteria

Because of the possibility that the lecithinase which is produced by normal *Bacillus cereus* plays a part in its pathogenicity to insects (Heimpel, 1955*b*; Kushner & Heimpel, 1957), the ability of resistant *B. cereus* to produce this enzyme was measured. The resistant organisms when grown in unbuffered 2 % (w/v) casein hydrolysate produced amounts of lecithinase comparable to those produced by a normal culture of sensitive *B. cereus*.

DISCUSSION

In considering alkali resistance it is natural to refer to the work already carried out on the nature and development of drug resistance in bacteria (reviewed, for example, by Bryson & Szybalski, 1955; Davis, 1957; Schnitzer & Grunberg, 1957). However, two differences must be noted between drug resistance and the resistance to alkaline pH values described here. First, with alkali resistance one is not dealing with a chemical compound foreign to the cell but simply with an excess concentration of hydroxyl ion. Secondly, a drug may affect or be thought to affect only one enzyme, but pH changes might affect the action of many enzymes.

Since such a general inhibitor as [OH] is involved we may, at the start, rule out two theoretical mechanisms of resistance. Neither the formation of some metabolite in sufficient concentrations to overcome a specific metabolic block produced by a high pH value, nor the production of alternative metabolic pathways to circumvent such a block appears to be likely. The role that acid production by the growing bacteria plays in overcoming the toxic effects of high pH values deserves special consideration, and it seems important to distinguish between what may be termed primary and secondary mechanisms of resistance. If acid production were completely responsible for resistance, then we would expect the resistant inoculum to lower the pH value of the medium before growth began. This, however, did not occur. Except for a slight fall, found even in the absence of bacteria, the decrease of pH value accompanied, but did not precede, growth. When the pH value was maintained by periodic additions of alkali, growth still took place, though at a lower rate than in media of decreasing pH value. It should be noted that sensitive bacteria may grow in media of pH 9.4–9.5, though after a much longer lag period than resistant bacteria, and that during growth their rate of acid production is much the same as that of the growing resistant bacteria. Resistant bacteria, then, are distinguished from sensitive bacteria not by their ability to produce acid during growth in alkaline media but by their greater ability to begin to grow in these media. Acid production by the growing bacteria may be regarded as a secondary mechanism of resistance, which increases the rate

of growth once this has begun. It is this acid production that appears responsible for the fact that growth curves in media of initially different pH values differ mainly only in the length of the lag period.

Different possible primary mechanisms of resistance, which might enable resistant organisms to begin growing in highly alkaline media, have been considered. One possibility is that the resistant organisms have a limited permeability to hydroxyl ion or have an active mechanism that regulates the passage of hydroxyl ion into the cell. If either of these mechanisms were in operation, then a marked difference between intra- and extracellular pH might occur. In this case, individual enzymes in intact resistant cells might be less affected by external alkaline conditions than the same enzymes in intact sensitive cells. This possibility should be open to direct investigation.

Another hypothetical possibility is that of a shift in the pH-activity curves of several enzymes in the resistant cell. Kocholaty & Weil (1938) and Kocholaty & Hoogerheide (1938) noted a shift toward the acid side of extracellular proteinase produced by *Clostridium histolyticum* and of several dehydrogenases in *C. sporogenes* as these organisms were grown in more acid media. A similar shift in pH-activity curves towards the alkaline side is conceivable in alkali-resistant *Bacillus cereus*.

The detailed genetic aspects of alkali resistance have not yet been studied. Resistance appears to be a heritable property, transmitted from subculture to subculture, and persisting in neutral media. Its slow and gradual development suggests that resistance to the highest alkalinities does not arise through selection of a few resistant organisms originally present in the sensitive population, nor through a single-step mutation. A series of mutations, each leading to slightly increased resistance, might be involved. As Bryson & Szybalski (1955) pointed out: 'In a strict sense, proof that clones of drug-resistant cells depend in origin upon mutation can be supported only by genetic tests, for example, study of bacterial recombination, transduction, transformation, or analysis of the segregation of nuclear hereditary determinants.' This statement must apply as well to any genetic interpretation of our results.

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