5. There was a parallel increase in flavinadenine dinucleotide content and synthesizing activity for this compound with the progress of germination.

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The Effect of Alcohols on the Synthesis of Lipase, Lecithinase and other Enzymes by *Bacillus cereus*

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It was found previously that the presence of any one of several lower alcohols in the growth medium inhibited the synthesis of lecithinase by growing *Bacillus cereus* (Kushner, 1957a). A similar effect was observed for lecithinase production in *Clostridium perfringens* by Jayko & Lichstein (1958). In both these studies the alcohols, in the concentrations used, either did not inhibit bacterial growth or had a much greater inhibitory effect on lecithinase formation than upon growth. Thus they were clearly not acting as general toxic agents or as general inhibitors of enzyme synthesis.

Their differential effect suggested that alcohols might prove valuable in other investigations of enzyme formation, and their action has been studied further. The effect of alcohols on lecithinase synthesis was first demonstrated with standing cultures of *Bacillus cereus* (Kushner, 1957a), in which both growth and lecithinase synthesis were suboptimum. This work has now been repeated in vigorously growing aerated cultures. The extent of utilization by the growing bacteria of the added

alcohols has been determined. Finally, in order to assess the specificity of action of alcohols their effect on the formation of enzymes other than lecithinase in *Bacillus cereus* has been investigated. The enzymes studied include penicillinase, which, like lecithinase, is an extracellular enzyme, and the cell-bound enzymes phosphatase, catalase and lipase. The last-mentioned enzyme, whose synthesis was studied in some detail, was chosen because, like lecithinase, it also possesses a fatty substrate.

A preliminary account of these results has been given (Kushner, 1959).

MATERIALS AND METHODS

Bacteria. *Bacillus cereus* strain 569, which has been extensively used in experiments on penicillinase formation (Pollock, 1953), was used for most of this study. For certain experiments *B. cereus* strain Mu-3055, in which the inhibitory effect of alcohols on lecithinase synthesis was first demonstrated (Kushner, 1957a), and the penicillinase-constitutive mutant strain 569/H (Kogut, Pollock & Tridgell, 1956), were used.

Growth medium. S broth, made as described by Pollock & Perret (1953) except that the initial steaming before autoclaving was omitted, was used for all experiments with

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B. cereus strains 569 and 569/H. In most experiments 0.1 ml. of a spore suspension (containing 3×10^8 spores/ml.) was added to 10 ml. of S broth and incubated with shaking for 3–6 hr. The resulting suspension of bacteria was used as an inoculum, and amounts equivalent to 0.05 mg. of bacteria (about 3×10^7 viable cells) were added to 50 ml. of S broth in 500 ml. Erlenmeyer flasks (in some experiments proportionately smaller amounts of inoculum were added to 25 ml. or 10 ml. of broth in 250 ml. or 100 ml. flasks). For certain specified experiments the spore suspension (usually 0.05 ml./10 ml. of broth) was used as the sole inoculum. Alcohols had closely comparable effects on growth and on enzyme synthesis whether the inoculum consisted of spores or of vegetative cells. After inoculation, the flasks were incubated at 35° on a reciprocal shaker at 88 cyc./min. with a lateral throw of 3 in.

For the experiment with *B. cereus* strain Mu-3055 reported in Table 2, shaking (200 cyc./min.) and standing incubations were carried out as previously reported (Kushner, 1957a; Kushner & Lisson, 1959).

For lipase, phosphatase and catalase assays, the bacteria were harvested by centrifuging, washed once or twice with water and resuspended in a small volume of water, usually to a density of 10 mg. dry bacterial wt./ml. For penicillinase assays the whole bacterial culture was diluted tenfold with 0.83 mM-8-hydroxyquinoline (oxine) in 1% gelatin, at the desired time, to halt growth and enzyme synthesis. A portion (1 ml.) of this, or of a further dilution in oxine-gelatin if necessary, was used for assay.

Measurements of bacterial density were made turbidimetrically, with a Hilger Spekker absorptiometer, and are expressed as mg. dry wt. of bacteria/ml.

Enzyme assays. Lipase assays were carried out manometrically, essentially as described by Smith, Worrel & Swanson (1949). The main compartment of the Warburg vessel contained 2 ml. of 25 mM-NaHCO₃ and 0.5 ml. of bacterial suspension; the side arm contained 0.5 ml. of 0.2 M-tributyrin emulsified in 2% of gum acacia in 25 mM-NaHCO₃. Controls were run for each bacterial suspension with 25 mM-NaHCO₃ in the side arm to correct for endogenous output of CO₂, which was in some cases 20% of that observed in the presence of substrate. Addition of gum acacia alone did not raise the endogenous CO₂ output. For the gas phase CO₂ + N₂ (5:95) was used, and activity was measured at 38°. Measurements of CO₂ output were carried out for 30–60 min. Measurements on a suspension of bacteria grown in the absence of alcohols showed that in the range of activities studied lipase activity was proportional to bacterial concentration.

Penicillinase assays were carried out by the method of Henry & Housewright (1947) modified as previously described (Pollock, 1950), except that 20 000 units of penicillin were used as substrate.

Phosphatase activity was measured by following the hydrolysis of *p*-nitrophenyl phosphate under the following conditions: to 2 ml. of 0.01 M-phosphate buffer, pH 7.0, were added 0.2 ml. of a cell suspension and 1 ml. of *p*-nitrophenyl phosphate (1 mg./ml.). After incubation (usually for 30 min., but longer if the activity was low) at 38°, 1 ml. of M-K₂CO₃ was added, the bacteria were centrifuged down and the released *p*-nitrophenol was estimated spectrophotometrically at 420 mμ. Appropriate controls were carried out for colour in the substrate itself and for material absorbing at 420 mμ released from the bacteria.

Lecithinase assays were carried out as previously described (Kushner, 1957b). In this system, 1 unit of lecithinase activity is taken as equivalent to the liberation of 1 μmole of phosphorus from lecithin in 2 hr.

Catalase activity was measured spectrophotometrically by a method similar to that given by Chance & Maehly (1955). Silica cuvettes contained: water, 1.7 ml.; 0.05 M-H₂O₂, 0.3 ml.; 0.1 M-phosphate buffer, pH 7.0, 1 ml.; suspension of normal or toluene-treated bacteria (2 mg./ml.), 0.1 ml. After rapid mixing, the fall of *E* at 230 mμ was followed at room temperature (18–20°). The rate of decrease was found to be linear for 1–2 min., depending on activity. A cuvette containing water, buffer and bacteria but no H₂O₂ served as blank. Catalase activity is expressed as μmoles of H₂O₂ destroyed/mg. of bacteria/min.

A Unicam spectrophotometer model SP. 500 was employed for all spectrophotometric measurements.

Disruption of bacterial cells. Crushed-cell preparations for lipase assay were made with the Hughes press (Hughes, 1951), which was cooled to –35° before addition of the bacterial suspension. The crushed material was recovered quantitatively by washing the press with water and 50 mM-NaHCO₃, the final NaHCO₃ concentration being 25 mM. For determination of the amount of soluble-lipase activity, the crushed material was centrifuged for 15 min. at 14 750 g at 2°. A portion (1 ml.) of the supernatant was assayed manometrically, 1 ml. of 25 mM-NaHCO₃ and 0.5 ml. of water being added to the main vessel. The lipase activity of the total crushed material, before centrifuging, was also measured.

For phosphatase and catalase assays cells were disrupted by shaking the suspension in a stoppered tube with 0.1 vol. of toluene for 30 min. at 35°. In most cases, air was bubbled through the aqueous part of the suspension to remove excess of toluene before assay; it was found, however, that a small amount of remaining toluene did not affect the catalase assay.

Alcohol determination. All alcohols were measured by a variation of the method for ethanol described by Snell & Snell (1937). To a tube containing up to 0.15 m-mole of each alcohol in 1 ml. of water was added 5 ml. of 0.6% K₂Cr₂O₇ in conc. HNO₃-water (1:2, v/v). After heating for 2 hr. at 50° the tubes were cooled and *E* values at 630 mμ read against a blank prepared in the same way but containing no alcohol. Standard curves for each alcohol are given in Fig. 1. Alcohols could be measured directly in the

Fig. 1. Standard curves for alcohols. ●, Methanol; ■, ethanol; △, propan-1-ol; ○, butan-1-ol.

culture medium without prior distillation. Both S broth and Nutrient Broth (Difco) without added alcohols gave readings corresponding to a concentration of about 0.04M-methanol; these figures were reduced by 25–50% after bacterial growth. Recoveries of alcohols added to the growth media were quantitative. All alcohol analyses were corrected for substances present in the growth medium.

Chemical compounds. The methanol and ethanol used in most experiments had been dried and redistilled before use over sodium and dimethyl or diethyl phthalate respectively; commercial material gave the same results as the redistilled alcohols. Propan-1-ol, butan-1-ol and tributyrin were British Drug House Ltd. AnalaR grade, hydrogen peroxide was Hopkins and Williams Ltd. AnalaR grade. Gum acacia was supplied by Allen and Hanburys Ltd., London. *p*-Nitrophenyl phosphate was supplied by the Sigma Chemical Co., St Louis, Mo., U.S.A.

RESULTS

Effect of alcohols on lecithinase synthesis in *Bacillus cereus* strain 569. In the earlier work on the effect of alcohols on lecithinase synthesis, *B. cereus* strain Mu-3055 was used, and growth was in standing tubes instead of shaken flasks (Kushner, 1957a). In shaken cultures, overnight growth was increased about tenfold and final lecithinase activity about threefold. The effects of alcohols on shaken cultures of strain 569 were closely comparable with those found in standing cultures of strain Mu-3055 (Kushner, 1957a) (Table 1). With shaken cultures of strain Mu-3055 similar results were also obtained.

Fig. 2 shows the relation between growth and lecithinase production in a shaken *B. cereus* culture, as well as the effect of ethanol on both processes. There was a lag in lecithinase production in relation to growth. Enzyme activity appeared only after the end of the logarithmic-growth phase, although at a time when the bacteria were still growing rapidly. A more detailed study of this relation, as well as of the relation between cell-bound and

extracellular lecithinase, is to be published separately. It is seen that in this experiment ethanol inhibited growth slightly and, as expected, lecithinase production completely.

Degree of alcohol destruction during bacterial growth. Experiments carried out with both *B. cereus* strain Mu-3055 and strain 569 showed that there was little loss of added alcohols during bacterial growth (Table 2). The loss that did occur was no greater in shaken than in standing cultures of strain Mu-3055, despite the greater growth in the former. In addition, the greater part of the alcohol loss in shaken cultures of strain 569 occurred also in media with no bacteria present, presumably through evaporation.

The bacteria, then, caused little destruction of alcohols during growth, and it seemed likely that alcohols acted as such rather than as their metabolic derivatives. If this were so, then in the relatively high concentrations used the alcohols might be expected to act on the cells as lipid solvents, and a few experiments were carried out to test this possibility. A suspension of *B. cereus* strain 569 (20 mg./ml.) incubated with shaking at 35° released 1 mg. of soluble material/ml. in 2 hr. This contained 45 µg. of phosphorus, all as inorganic phosphate. Ethanol (0.35M), propan-1-ol (0.13M) and butan-1-ol (0.05M) increased the phosphate (and total phosphorus) released by 10%; methanol (0.5M) had no effect. The alcohols caused no increase in the total soluble material released, nor in the material absorbing at 260 mµ. There was no ether-soluble material (measured by dry weight and by phosphorus analysis) in intact bacterial cells treated, or untreated, with alcohols. These experiments indicated that, in the concentrations

Table 1. *Effect of alcohols on lecithinase synthesis in Bacillus cereus strain 569*

S broth (50 ml.) in a 250 ml. flask, with additions as shown, was inoculated with 0.1 ml. of a spore suspension and incubated with shaking for 13 hr. Lecithinase activity was measured in 0.5 ml. of the culture supernatant. In this experiment, 0.1 unit of penicillin/ml. had been added to the culture medium. Similar results were obtained in the absence of penicillin.

Alcohol added and concn. (M)	Growth (mg./ml.)	Lecithinase activity (units/mg.)
Nil	2.12	4.5
Methanol (0.5)	1.85	2.3
Ethanol (0.35)	1.62	0.5
Propan-1-ol (0.13)	1.32	0.5
Butan-1-ol (0.054)	1.92	0.5

Fig. 2. Lecithinase production by growing *Bacillus cereus*; the effect of ethanol on growth and enzyme formation. Broth (50 ml.) was inoculated with 0.1 ml. of spore suspension and lecithinase activity of the whole culture determined at the times shown. Continuous lines represent bacterial growth; broken lines represent lecithinase activity. ○, Control; ●, 0.35M-ethanol.

used, alcohols had no measurable solvent action on intact bacterial cells.

Specificity of alcohol action on enzyme formation: lipase formation. Washed cells from overnight cultures of *B. cereus* strain 569 possessed a measurable lipase activity. No activity could be detected in the external medium, and the enzyme thus

appears to be wholly cell-bound. If alcohols were added to the growth medium the intracellular-lipase activity was markedly lowered, though not altogether abolished (Table 3). In the concentrations used, the alcohols had, in general, little effect on total growth in overnight experiments (Table 3). Shorter experiments (see Fig. 3) showed that

Table 2. *Destruction of alcohols by growing Bacillus cereus*

Strains Mu-3055 and 569 were grown for 14 and 17 hr. respectively. Final bacterial density (in absence of alcohol): strain Mu-3055, standing, 0.06 mg./ml., shaken, 0.6 mg./ml.; strain 569, 3.5 mg./ml. Effects of alcohols on growth of shaken cultures were as shown in later tables. Growth of standing cultures of Mu-3055 was not affected by alcohols. Mu-3055 cultures were shaken (200 cyc./min.) in 10 ml. of Nutrient Broth (Difco) in 1 in. x 8 in. tubes as described by Kushner & Lisson (1959). Cultures of 569 were shaken in 10 ml. of S broth as described in the Materials and Methods section.

<i>B. cereus</i> strain	Alcohol added and concn. (M)	Percentage remaining after growth	
		Standing culture	Shaken culture
Mu-3055	Methanol (0.5)	93	94
	Ethanol (0.35)	88	87
	Propan-1-ol (0.133)	83	77
	Butan-1-ol (0.054)	80	88
569		Shaken culture	Shaken medium without bacteria
	Methanol (0.8)	88	89
	Ethanol (0.2)	77	86
	Propan-1-ol (0.08)	62	80
	Butan-1-ol (0.08)	64	75

Table 3. *Growth of Bacillus cereus strain 569 in the presence of alcohols, and the lipase activity of intact cells*

Bacteria were grown in 50 ml. of S broth as described in the Materials and Methods section. Figures in parentheses represent the control lipase activity (in μ l. of CO_2 /mg. of bacteria/hr.) in each experiment.

Expt. no.	Alcohol added	Concn. (M)	Time of incubation (hr.)	Bacterial growth (mg./ml.)	Percentage of control lipase activity
1	Nil		16	4.24	100 (66.5)
	Methanol	0.4		3.34	75
		0.8		2.70	48
	Ethanol	0.1		4.40	33
		0.2		3.96	25
		0.4		3.60	21
2	Nil		17	3.50	100 (53.2)
	Methanol	0.8		2.32	64
		1.2		2.80	35
		1.6		3.10	30
	Ethanol	0.1		5.50	55
		0.4		5.10	82
3	Nil		17	4.44	100 (41.3)
	Ethanol	0.02		5.10	82
		0.04		5.50	52
		0.1		5.50	55
	Propan-1-ol	0.04		4.64	58
		0.08		3.88	46
4	Nil		16	4.64	100 (40.6)
	Propan-1-ol	0.08		3.88	46
		0.12		3.94	40
		0.02		4.44	88
	Butan-1-ol	0.04		4.64	86
		0.08		3.88	64

propan-1-ol increased the lag period and decreased the growth rate, and a similar effect was sometimes found with butan-1-ol (e.g. Table 5). Higher concentrations of propan-1-ol and butan-1-ol than those shown tended to inhibit growth completely.

Regardless of their effect on growth, the presence of each of these alcohols in the growth medium led to a lowered lipase activity/mg. of bacteria, in the washed cells. The most active alcohols in this respect (on a molar basis) were ethanol and propan-1-ol. The maximal effect of methanol was almost given by 1.2M concentration; ethanol had its maximal effect at 0.1–0.2M concentration. Propan-1-ol appeared to approach a similar maximal effect at 0.12M, the highest concentration it was possible to test. Because of its growth-inhibitory effects the maximal concentration for butan-1-ol could not be determined.

The lowered lipase activity of intact alcohol-grown cells did not by itself indicate a lowered lipase content. The cells might have been altered in such a way as to make the intracellular enzyme less accessible to its substrate. To assess this possibility, the lipase activities of cell-free extracts of normal and alcohol-grown cells were compared. Because of the relatively small effect of butan-1-ol, only methanol, ethanol and propan-1-ol were examined in these experiments. The pattern of lowered lipase activity found in intact alcohol-grown cells was also found in their extracts (Table 4).

After crushing the cells most or all of the lipase activity in the crushed material was solubilized (i.e. remained in the supernatant after centrifuging at 14 750 g), but recovery of lipase activity was not quantitative. Half or more of the lipase activity was lost on crushing. Attempts to avoid this loss by disrupting the cells with toluene or with ultrasonic vibration (25 kc., 250 w at the transducer) were unsuccessful. Both methods caused almost total inactivation of the enzyme. The loss of activity limits comparison between cell-free extracts of normal and alcohol-grown cells. From the figures

obtained, however, it seems very unlikely that the alcohols acted by masking the expression of lipase in the intact cell.

Effect of alcohols on the stability of formed lipase. Since washed bacterial cells were used in the above-described assays the question of inhibitory action of alcohols on lipase activity did not arise. The possibility remained, however, that the alcohols might have destroyed the lipase activity, once formed, or have acted on the cells to cause a release of lipase. These possibilities were tested in the following manner. To a culture of *B. cereus* grown overnight in the absence of alcohol, chloramphenicol (0.1 mg./ml.) was added to prevent further growth. The culture was then divided into 10 ml. lots and shaken in 100 ml. flasks, with and without the addition of alcohols. Lipase activity of these cultures was rather unstable. In the control flasks, a 30% loss of activity was found in 6 hr. and a 70% loss in 18 hr. The loss was no greater in the presence than in the absence of alcohols (in the highest concentrations shown in Table 3). Thus the alcohol effect on the final lipase activity of intact cells was not due to enzyme destruction or release. That alcohols did not cause the release of lipase from intact cells was also indicated by the fact that the cell-free medium from alcohol-grown and from normal cultures was devoid of lipase activity.

Possibility of selection of cells with low lipase-forming ability. It seemed possible that, instead of inhibiting lipase synthesis in a homogeneous bacterial population, alcohols had selectively inhibited the growth of cells with normal lipase-forming ability and permitted the growth of cells with lower ability to synthesize lipase. Such a selection might well have taken place without any measurable effect on total bacterial growth in long-term experiments. To test this alternative explanation, bacteria grown overnight in the presence of alcohols were then reinoculated into normal S broth. The amounts of alcohol carried over in the inoculum were too low to have any effect. After

Table 4. *Lipase activity in whole cells and cell-free extracts of Bacillus cereus strain 569 grown in the presence of alcohols*

Bacteria were grown for 13½ hr. (Expt. 1) or 12 hr. (Expt. 2) in 50 ml. of S broth; 60 mg. of bacteria were crushed; 10 mg. of bacteria, or the equivalent in crushed material or supernatant (obtained after centrifuging at 14 750 g for 15 min.), were assayed. Lipase activity: μ l. of CO₂/hr./mg. of original cells.

Expt. no.	Alcohol added and concn. (M)	Lipase activity		
		Intact cells	Crushed cells	Supernatant
1	Nil	32.6	14.3	11.1
	Methanol (0.8)	19.6	9.1	7.4
	Ethanol (0.2)	19.6	6.5	4.4
2	Nil	30.1	17.2	11.8
	Ethanol (0.2)	15.6	4.4	4.6
	Propan-1-ol (0.12)	13.0	4.4	4.6

growth for 16 hr., the cells had at least as much lipase activity as control cells cultured twice in the absence of alcohols. If one discounts the very unlikely possibility that a reverse selection of high producers of lipase occurred in the absence of alcohols these experiments indicate again that alcohols caused a true inhibition of lipase formation.

Time course of lipase production by growing bacteria and the effects of alcohols. Experiments in which the effects of alcohols on lipase synthesis were followed throughout the bacterial-growth cycle showed that lipase activity/mg. of bacteria varied greatly during growth (Fig. 3). It was lowest during the early stages of growth and the logarithmic phase and rose rapidly in the late stages of growth, continuing to increase when growth, as judged by turbidity measurements, had apparently ceased. In the earlier stages of growth the presence of alcohols did not affect the formation

of lipase activity; with longer experiments the higher control activity and the inhibitory effects of alcohols were seen. Thus the alcohols seemed to act preferentially on the mechanism responsible for the greatly increased rate of lipase formation in the late and stationary phases of bacterial growth. The fact that one stage of lipase synthesis was not affected by alcohols is consistent with the observation that alcohols did not completely inhibit lipase formation (Table 3).

In the experiment shown in Fig. 3 there was a marked decrease of *E* in the methanol-grown culture, and a slight decrease in the ethanol-grown culture after 12 hr. This may have been due in part to cell lysis, but was also partly caused by a clumping of the cells, which sometimes took place in the presence of alcohols.

Crushed-cell preparations of 6 hr. cultures had approximately half the lipase activity of the intact cells, the same proportion as found in older cultures (see Table 4). This indicates that the increased lipase activity found in the older cells was not due to increased accessibility of enzyme to substrate. In the control culture, addition of chloramphenicol (0.1 mg./ml. final concentration) after growth for 12 hr. completely prevented the subsequent increase in lipase activity in the stationary culture. Thus the increased lipase activity probably involved protein synthesis.

Effect of alcohols on penicillinase and phosphatase synthesis. The effect of alcohols on penicillinase synthesis was studied both in *B. cereus* strain 569, where its formation is inducible, and in the constitutive strain 569/H (Kogut *et al.* 1956). With the inducible strain, in short-term experiments (Table 5) the alcohols increased slightly the amounts of penicillinase activity obtained after induction. In the constitutive strain there was little or no effect of alcohols on penicillinase production during 2 hr. growth in their presence. The alcohols, in the con-

Fig. 3. Effect of alcohols on lipase formation in growing *Bacillus cereus* strain 569. S broth (50 ml.), with alcohols as shown, was inoculated with 0.25 ml. of a spore suspension (3×10^8 /ml.) and samples were taken at the times shown. ●, Control flask; ○, methanol (0.8M); △, ethanol (0.2M); ▲, propan-1-ol (0.08M). Continuous lines represent bacterial growth; broken lines represent lipase activity.

Table 5. *Effect of alcohols on penicillinase synthesis*

S broth (100 ml.) was inoculated with 0.3 ml. of spore suspension (3×10^8 /ml.) and shaken until a bacterial density of about 0.1 mg./ml. was reached (zero time); 10 ml. lots of the suspension were placed in 100 ml. flasks with 1% of gelatin (final concentration) and alcohols as indicated and shaken for 2 hr. Benzylpenicillin (10 units/ml.) was added to 569 cultures at zero time and at 1 hr. Pen-ase, penicillinase (expressed as units/mg. of bacteria).

Alcohol added and concn. (M)	<i>B. cereus</i> strain					
	569		569/H			
	Expt. 1		Expt. 2		Expt. 3	
	Growth (mg./ml.)	Pen-ase	Growth (mg./ml.)	Pen-ase	Growth (mg./ml.)	Pen-ase
Nil	0.66	330	0.75	2175	0.85	1972
Methanol (0.8)	0.52	360	0.62	2090	0.70	1982
Ethanol (0.2)	0.56	409	0.78	2155	0.78	1962
Propan-1-ol (0.04)	0.38	412	0.38	2975	0.50	2090
Butan-1-ol (0.08)	0.24	435	0.40	2190	0.54	1832

Table 6. *Effect of alcohols on phosphatase formation in growing Bacillus cereus strain 569*

For Expts. 1 and 3, 100 ml. of S broth was inoculated with 0.3 ml. of a spore suspension ($3 \times 10^8 \text{ ml.}$) and shaken for 4 hr., then divided into 10 ml. lots in 100 ml. flasks, and alcohols were added as shown. In Expts. 2 and 4 inoculations of spores (0.05 ml./10 ml. of broth) were made into control and alcohol-containing media. Phosphatase is expressed as $\mu\text{mole of } p\text{-nitrophenol/mg. of bacteria/hr.}$ Figures in parentheses show phosphatase activity of toluene-treated cells.

Expt. no.	Time of growth (hr.)	Alcohol added and concn. (M)									
		Nil		Methanol (0.8)		Ethanol (0.2)		Propanol (0.04)		Butanol (0.04)	
		Growth (mg./ml.)	Phosphatase	Growth (mg./ml.)	Phosphatase	Growth (mg./ml.)	Phosphatase	Growth (mg./ml.)	Phosphatase	Growth (mg./ml.)	Phosphatase
1	5	0.312	0.232	0.267	0.200	0.310	0.204	0.205	0.419	0.280	0.218
	6	0.820	0.107	0.660	0.139	0.840	0.100	0.074	0.343	0.720	0.120
2	6	1.28	0.116	0.660	0.193	1.04	0.117	0.840	0.233	0.780	0.149
	10	3.69	0.015	3.13	0.012	3.63	0.009	2.78	0.025	3.40	0.015
3	4	0.100	0.702 (0.711)	—	—	—	—	—	—	—	—
	6	0.720	0.238 (0.225)	0.620	0.209 (0.255)	0.740	0.183 (0.241)	0.110	0.440 (0.389)	0.700	0.177 (0.239)
4	16	2.5	0.021 (0.007)	—	—	—	—	—	—	—	—

Table 7. *Effect of alcohols on catalase formation in growing Bacillus cereus strain 569*

Conditions were as given in Table 6, Expts. 2 and 4. Catalase activity: $\mu\text{moles of } \text{H}_2\text{O}_2 \text{ destroyed/mg. of bacteria/min.}$ Figures in parentheses show catalase activity of toluene-treated cells.

Time of growth (hr.)	Alcohol added and concn. (M)									
	Nil		Methanol (0.8)		Ethanol (0.2)		Propanol (0.08)		Butanol (0.04)	
	Growth (mg./ml.)	Catalase	Growth (mg./ml.)	Catalase	Growth (mg./ml.)	Catalase	Growth (mg./ml.)	Catalase	Growth (mg./ml.)	Catalase
6	1.70	3.8 (3.1)	1.12	4.2 (2.8)	1.56	4.8 (3.3)	1.14	6.3 (6.6)	1.36	3.1 (3.1)
11	3.60	3.5 (3.1)	3.70	5.6 (6.1)	4.00	4.7 (4.8)	3.30	5.0 (4.2)	3.86	3.8 (4.8)
16	3.60	8.9 (21.9)	2.20	19.0 (26.8)	3.10	10.0 (23.0)	3.90	8.0 (29.0)	2.90	10.0 (24.5)

centrations used in these assays, had no effect on penicillinase activity.

Penicillinase production could not be measured accurately after overnight incubation. The amounts of penicillinase produced per mg. of bacteria were much lower than those shown in Table 5, and the results were too erratic to interpret. The loss of activity may have been due to penicillinase destruction by proteinase action (Kogut *et al.* 1956) but was not further investigated. It was concluded from these experiments that in short-term incubations alcohols had little or no effect on penicillinase synthesis.

The time course of phosphatase synthesis and the effect of alcohols on this process are shown in Table 6. Phosphatase synthesis obviously took place very early in the growth cycle. The activity per mg. of cells decreased after the first measurement possible, and phosphatase synthesis, in terms of total activity, was ended 6 hr. after inoculation of the spores into the medium. Despite some variation between individual experiments, the alcohols, with the exception of propan-1-ol, had little effect on phosphatase synthesis; propan-1-ol appeared to stimulate this synthesis. Toluene treatment caused approximately the same small increase in the phosphatase activity of young control and alcohol-grown cells, whereas the very low activity of overnight cultures was further reduced by toluene treatment.

Effect of alcohols on catalase formation. The syntheses of lecithinase, penicillinase and phosphatase all take place relatively early in the bacterial-growth cycle. The effect of alcohols on the syntheses of these enzymes may not be directly comparable with their effect on lipase formation, a process that takes place in a later phase of growth. For this reason, catalase, an enzyme whose activity increases during growth in a manner similar to that of lipase, was of special interest in analysing the alcohol effect.

Table 7 shows the changes of catalase activity in intact and toluene-treated cells grown in the presence and in the absence of alcohols. As with lipase, catalase activity was low in the early stages of growth but increased rapidly between 11 and 16 hr., during the stationary growth phase; this late increase in catalase activity was completely prevented by 0.1 mg. of chloramphenicol/ml. Toluene treatment did not affect the activity of young cells, but increased by 100% or more the activity of 16 hr. cultures. Growth in the presence of alcohols had no, or a slightly stimulatory, effect on the catalase activity of toluene-treated cells, and, with the exception of methanol, little effect on the activity of intact cells. Intact cells grown in the presence of methanol for 16 hr. had a higher catalase activity than control cells,

but toluene had less stimulatory effect on their activity. Toluene-treated control and methanol-grown cells had, finally, approximately the same activity. This suggests that growth in the presence of methanol may have had the same effect as treatment with toluene.

DISCUSSION

Except for the experiments on lecithinase formation there appear to have been no systematic studies of alcohol action on the formation of specific enzymes, and few isolated observations. Baba, Hayashi & Oshima (1955) found that there was more formation of invertase in yeast when glucose was used as a carbon source than when ethanol was used. Satomura, Oi & Sawada (1958) observed recently that in the fungus *Sclerotinia libertiana* ethanol, together with a number of sugars, inhibited lipase formation. Ethanol enhanced mycelial growth and was thought to be utilized by the organism. If so, the action of ethanol on enzyme synthesis in both the above-mentioned organisms may have been fundamentally different from that found on lecithinase and lipase synthesis, since alcohols did not appear to be used as substrates for growth by the *B. cereus* strains studied.

To date, lecithinase formation in two bacterial species and lipase formation in one have been found to be inhibited by alcohols. The present survey has shown that penicillinase, phosphatase and catalase formations in *B. cereus* were not inhibited; nor, presumably, was the formation of many other enzymes since the bacteria grew in the presence of alcohols. One can obviously not draw a general rule of alcohol action from the few enzymes studied, but it is curious and may be significant that thus far the enzymes whose production has been found to be inhibited by alcohols are those concerned with the degradation of fatty substrates.

The fact that alcohols inhibit both lipase and lecithinase formation does not necessarily mean that the basis of action in each case is the same. One striking difference is that with lecithinase formation the inhibitory power of alcohols increases with increasing molecular weight (Kushner, 1957*a*), whereas with lipase formation butanol is less effective than propanol or even ethanol. A more important difference may lie in the fact that alcohols can inhibit lecithinase formation completely, whereas in the early stages of growth lipase formation is unaffected by alcohols.

The fact that most of the lipase activity appears late in the growth cycle and that alcohols appear to act specifically on this late formation makes comparison between lipase and other enzymes difficult.

During the period when alcohols inhibited lipase formation, lecithinase and phosphatase were no longer being formed and the formation of penicillinase could not easily be studied. It might be argued that the alcohol inhibition of lipase formation was in some way connected with this enzyme's increase late in the growth cycle, were it not that the formation of catalase, most of which also takes place late in the growth cycle, was not inhibited by alcohols.

It was, as stated, the apparent similarity between the substrates of lipase and lecithinase that led to the inclusion of lipase in this study. The substrate specificity of *B. cereus* lipase, however, has not yet been studied in any detail. Intact cells, which could hydrolyse tributyrin, were ten times as active against triacetin, both in solution (40mM) and as an emulsion (0.8M). They did not hydrolyse ethyl acetate (2 mM) or soluble ethyl butyrate (33 mM) but acted on an ethyl butyrate emulsion (0.45M). If the same enzyme were acting on all substrates then in some, though not all, of its properties *B. cereus* lipase resembles the pancreatic lipase described recently by Sarda & Desnuelle (1958), which does not hydrolyse water-soluble substrates but apparently requires an oil-water interface for its action.

Two possible mechanisms of alcohol action have been considered. By the first, both lecithinase and lipase synthesis would depend on the presence in the cell of an internal inducer, either phospholipid or triglyceride in nature. Although the concentrations of alcohols used were not high enough to extract lipids from the bacterial cell, it might be possible that the presence of alcohols prevented fatty inducers from making effective contact with the appropriate enzyme-forming systems.

As an alternative, the necessity for an enzyme acting on a fatty substrate to overcome a phase barrier may provide a clue to the mechanism of alcohol action. Much attention has been given recently to the special conditions necessary for enzyme action on fatty substrates. In addition to pancreatic lipase one can cite the phospholipase B of *Penicillium notatum*, which requires the presence of certain lipids in order to attack lecithin (Bangham & Dawson, 1959; Dawson & Bangham, 1959), the role of the lipids being to introduce negative groups essential for enzyme action on the surface of the lecithin particle. Phospholipases of different types and from various sources have been found to be stimulated by the presence of ether and other organic solvents (Hanahan, 1952; Kates, 1957; Hanahan & Vercamer, 1954; Kushner, 1957b), these being necessary for contact between enzyme and substrate.

By the second hypothesis, alcohols would not necessarily inhibit the total synthesis of an enzyme

protein. Instead, it may be that the active enzyme contains as an essential part a group, possibly a lipid group, enabling it to come into effective contact with its phospholipid or triglyceride substrate, and that during growth in the presence of relatively high concentrations of alcohols attachment of this group to the inactive protein does not take place.

SUMMARY

1. The inhibitory effects of lower alcohols on lecithinase synthesis, first observed in standing cultures of *Bacillus cereus* strain Mu-3055, have now been found in shaken, vigorously growing cultures of this organism and of *B. cereus* strain 569.

2. During growth in the presence of alcohols both *B. cereus* strain 569 and *B. cereus* strain Mu-3055 caused little alcohol destruction. Alcohols, in the concentrations used in these experiments, had little or no detectable chemical action on intact bacterial cells.

3. Intact cells and cell-free extracts of *B. cereus* strain 569 grown in the presence of each of four lower alcohols had less lipase activity than cells or extracts of cells grown in the absence of alcohols. Ethanol and propan-1-ol were more active in this respect than methanol or butan-1-ol. The alcohols had no effect on the stability of lipase, once formed. They did not selectively promote the growth of cell lines with a lowered lipase activity. Their inhibitory effect appeared to be on the formation of the active enzyme.

4. The lipase content of *B. cereus* strain 569 varied greatly during bacterial growth. It was lowest in the logarithmic phase of growth and increased after the end of this phase; the greatest increase in lipase activity/mg. of bacteria took place during the stationary growth phase. Alcohols did not inhibit the early formation of lipase activity, but appeared to act specifically on that which took place in the late growth phases.

5. Penicillinase formation, both induced (in *B. cereus* strain 569) and constitutive (in *B. cereus* strain 569/H), was not inhibited by alcohols during and immediately after the logarithmic phase of growth.

6. Phosphatase formation took place early in the bacterial-growth cycle. With the exception of propan-1-ol, which had a stimulatory action, the alcohols were without effect on phosphatase formation.

7. The catalase activity of *B. cereus* strain 569, which increased late in growth in a manner similar to lipase, was not inhibited by alcohols.

8. So far, alcohols have been found to inhibit the formation of only those enzymes concerned with the degradation of fatty substrates. Possible mechanisms of alcohol action are discussed.

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The Biological Activity of Pure Peptides obtained by Enzymic Hydrolysis of Insulin

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Although much research has been done on the biological activity of material obtained by the action of enzymes on insulin, none of these investigations has been carried out with highly purified peptides of known structure. The determination of the structure of insulin by Sanger and his colleagues (Ryle, Sanger, Smith & Kitai, 1955) has made possible the assignment of known structures to the products of the proteolysis of insulin under con-

trolled conditions. The present investigation was undertaken to assess the biological activity, by more than one criterion, of purified peptides obtained from insulin by the action of carboxypeptidase and trypsin.

Treatment with carboxypeptidase liberates alanine from the C-terminal end of the phenylalanyl chain (Fig. 1). The residual molecule (DA insulin) was reported by Lens (1949) to have only slight hypoglycaemic activity in rabbits, whereas Harris & Li (1952) found it to be fully active by the mouse-convulsion assay. The present results confirm those of Harris & Li.

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Fig. 1. Amino acid sequence in ox insulin. ↑, Site of action of trypsin; ↗, site of action of carboxypeptidase.