Journal of the Oceanographical Society of Japan
Vol. 25, No. 1, pp. 36 to 47, February 1969

Deep-Sea Pressure Effects on Starch Hydrolysis by Marine Bacteria*

Claude E. ZoBELL** and Leslie L. HITTLE***

Abstract: Bacteria endowed with the ability to hydrolyze starch appear to be widely distributed in sea water, bottom deposits, and associated with marine animals. Several cultures recovered from depths exceeding 9,500 m were found to be able to grow and to hydrolyze starch at hydrostatic pressures around 1,000 atm, but the growth of most species from shallow depths was inhibited by pressures of 300 to 600 atm. Whole cultures grown at 1 atm, filtrates therefrom, and washed cells were shown to be able to hydrolyze starch at 1,000 atm. Starch hydrolysis by whole cultures in the logarithmic phase of growth was found to be an average of 85 % less rapid at 500 atm than at 1 atm and 82 % less rapid at 1,000 atm than at 1 atm, due largely to the inhibition of bacterial growth and enzyme formation. There was a slight tendency for deep-sea pressures to inactivate amylases at temperatures in the range of 4° to 35°C. Despite this tendency, the rate of starch hydrolysis by α-amylase was found to be accelerated by deep-sea pressures, particularly in the range of 25° to 60°C, presumably because increased pressure accelerates the absolute chemical reaction rate. The thermal inactivation of α-amylase was retarded by increased pressure.

1. Introduction

Starch occurs in significant quantities at certain times and places in the sea. Besides that which gets into the sea from terrigenous sources, starch is produced by certain marine algae. Most Green Algae, dinoflagellates, and cryptomonads produce true starches, *i.e.*, polysaccharides which are largely α-1, 4-D-glucopyranoses. Floridean starches, the principal photosynthetic storage products of Red Algae, are also D-glucopyranoses essentially identical with higher plant starches as indicated by their optical rotation, iodine reaction, and susceptibility to amylases (MEEUSE, 1962). Floridean starches consist almost exclusively of α-1, 4-glucosidic linkages. Myxophycean starches of Blue-green Algae are also polyglucans consisting predominantly of α-1, 4-linkages generally identical with the amylopectin fraction of higher plant starches (PERCIVAL and MCDOWELL, 1967). Higher plant starches like those of algae differ in structure from species to species, but all consist of various proportions of amylose and amylopectin, both of which are composed of D-glucopyranose units. In amylose the units are uniformly linked by 1→4 α-glucosidic bonds, whereas in amylopectin the majority of the units are linked with 1→4 α-glucosidic bonds but about 4% are linked with 1→6 α-glucosidic bonds at the branch points (WHISTLER and CORBETT, 1957). Both amylose and amylopectin combine with iodine to give a blue color. Amylases, also known as diastases, cleave amyloses and amylopectins into saccharides which do not give a blue color with iodine. β-amylases cleave starch with the rapid formation of maltose and glucose while α-amylases form mainly dextrins with some reducing sugars (MYRBÄCK and NEUMÜLLER, 1950).

Most of the starch in the sea appears to be produced by planktonic dinoflagellates and benthic Green Algae, with smaller quantities being contributed by Red Algae, Blue-green Algae, and Cryptophytes. BURSA (1968) reported finding starch grains in arctic waters off the coast of Canada, in the Baltic and Mediterranean Seas,

* Received Oct. 21, 1968
Contribution from the Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92037, U.S.A.
** Scripps Institution of Oceanography, University of California
*** Now at University of Oklahoma, Microbiology Department, Norman, Oklahoma 73069, U.S.A.

and in the North Atlantic and Indian Ocean. The highest concentration of starch reported by BURSA was 3,155 mg/m^3 during blooms of dinoflagellates. The maxima at most stations rarely reached 100 mg/m^3 and more often no starch was found.

Bacteria are believed to be largely responsible for the degradation of starch in sea water, bottom sediments, and in the digestive tracts of herbivorous metazoans. Representatives of various marine phyla ingest starch, but with few exceptions, notably lamellibranchs, it is not known whether the animals digest starch unaided by amylolytic microflora. Starch grains have been found in fecal pellets of certain metazoans which feed on dinoflagellates or Green Algae (BURSA, 1968). We have found the gut of feedy amphipods, copepods, euphausids, mussels, and barnacles to be particularly good sources of starch-hydrolyzing bacteria. Smaller numbers of amylolytic bacteria have been demonstrated in some of the most abyssal depths in the sea (ZOBELL and MORITA, 1959). Of the 60 new species of marine bacteria described by ZOBELL and UPHAM (1944), 22 were starch hydrolyzers. About half of the marine heterotrophs listed in Bergey's Manual (BREED *et al.*, 1957) are characterized as being diastatic or starch hydrolyzers, including *Pseudomonas ichthyodermis*, *Photobacterium harveyi*, *P. fischeri*, *Alginomonas alginovora*, *Mycoplana dimorpha*, *Vibrio marinopraesens*, *V. albensis*, *V. agarliquefaciens*, *V. beijerinckii*, *V. granii*, *Flavobacterium halohydrium*, *F. neptunium*, *Agarbacterium boreale*, *A. rhodomelae*, *A. mesentericum*, *A. reducans*, *A. polysiphoniae*, *A. ceramicola*, *A. delesseriae*, *Beneckea labra*, *B. ureasophora*, *B. hyperoptica*, *B. indolthetica*, *B. lipophaga*, *Brevibacterium sociovivum*, *B. immotum*, *B. marinopiscosum*, *Nocardia marina*, *N. atlantica*, and *Streptomyces marinus*.

Since the mere presence of amylolytic bacteria in the sea fails to tell whether they can hydrolyze starch *in situ*, the following studies were undertaken to determine the effects of hydrostatic pressure (a) on the growth of starch-hydrolyzing bacteria, (b) on their ability to produce amylolytic enzymes, (c) on the stability of such enzymes, and (d) on the rate of starch hydrolysis.

The pressure-depth gradient in the deep sea is approximately 0.106 atm/m, so at the greatest known depth (11,034 m, according to FISHER and HESS, 1963), pressures of nearly 1,200 atm occur. Laboratory experiments have demonstrated that many marine bacteria are killed in sea water by prolonged compression at 300 to 600 atm and that such pressures affect certain physiological reaction rates of nearly all species (JOHNSON *et al.*, 1954; HEDÉN, 1964; ZOBELL, 1964; MORITA, 1967).

Various units have been used by different authors to designate pressure: 1 atm = 1.0332 kg/cm^2 = 1.0133 bars = 1.0133×10^6 dynes/cm^2 = 14.696 psi or lb/in^2.

2. Methods

1) *Nutrient media and cultures*

Marine bacteria were grown in sea water starch broth consisting of 0.5 g soluble starch (LINTNER), 1.0 g Bacto-Casitone (Difco), 5.0 g yeast extract (Difco), 1.0 g KNO_3, 0.1 g $FePO_4.2H_2O$, 250 m*l* distilled water, and 750 m*l* aged sea water (ZOBELL, 1946). To this was added 1.5 % Bacto-Agar (Difco) when solid medium was required. Following autoclave sterilization for 15 min at 121°C, the reaction was pH 7.5 to 7.6.

Immediately prior to being used in the experiments, wild and stock cultures of bacteria were rejuvenated in sea water starch broth and streaked on nutrient agar plates to ensure their purity. The ability of various species to survive, reproduce, or to hydrolyze starch at different pressures was tested by inoculating starch broth with enough of a 48-hr old broth culture to give from 2 to 5×10^4 viable cells per m*l*. Such inocula did not cause any perceptible amount of starch hydrolysis in controls treated with 1 % toluol to inhibit growth. Colony counts on starch agar showed that, in the absence of toluol, most of the amylolytic species increased to 10^8 to 10^{10}/m*l* in starch broth at 25°C at 1 atm in from 2 to 10 days, during which time they degraded 0.5 mg/m*l* of starch to the achroic point (colorless with iodine reagent).

Bacterial biomass was determined with a Bausch & Lomb Spectronic 20 spectrophotometer at 420 mμ. The abundance of viable cells was determined by plating the cultures on nutrient sea water agar.

2) *Iodine test for starch*

The standard iodine reagent used to detect starch consisted of 0.3% iodine and 3% KI dissolved in distilled water. The 0.5 mg/m*l* starch broth gave a deep blue color when 1 m*l* was treated with 0.1 m*l* of the iodine reagent in a white porcelain spot plate. As the concentration of starch was decreased by dilution or by enzymatic degradation, about 5 μg/m*l* was the least amount that could be detected visually by the faintest blue color. When greater sensitivity to about 1 μg/m*l* was essential, 10 m*l* of iodine-treated starch solutions were examined either with the Bausch & Lomb Spectronic 20 or a Beckman DU spectrophotometer at 620 mμ. At this wavelength, light absorption due to the iodine reagent alone is negligible, although certain bacterial cell products cause some slight interference.

3) *High pressure apparatus and procedures*

Increased pressure was applied to cultures, cell suspensions, and enzyme solutions using the method described by ZoBELL and OPPENHEIMER (1950). Depending upon how many duplicates were required for incubation at various pressures and temperatures, usually 100 to 250 m*l* of each culture or mixture to be tested was dispensed in 3-m*l* culture tubes. The tubes were filled to capacity and sealed with Neoprene stoppers, which function as pistons when compressed with hydraulic fluid in the pressure cylinders. In families of five, held together with elastic bands, 20 such stoppered tubes were placed in each cylinder, the inside dimensions of which were about 3.5×28 cm (ZoBELL, 1959). When the experiment called for more than 3 m*l* of culture or enzyme mixtures, 10-m*l* piston-stoppered tubes were used, six per cylinder.

The pressure cylinders were filled with water treated with Lugol's solution to give 5 ppm free iodine. The iodine helps to maintain sterility and it also serves as an indicator of leakage in or out of the piston-stoppered tubes, something which rarely happens. Pressure was applied to the valved cylinders with an Enerpac model 11-430 hand pump (Enerpac Test Systems, Butler, Wisconsin), using a 1:1 mixture of glycerol and water as the hydraulic fluid. When compressed to the desired pressure, as indicated by the Bourdon gauge connected to the pump assembly, the needle valve was closed and the cylinder was placed in a constant temperature water bath.

Prior to being compressed, the pressure cylinders and all solutions to be placed therein were equilibrated at the temperature of incubation. Rapidly compressing gas-free solutions to deep-sea pressures under the described experimental conditions resulted in temperature increases ranging from 2° to 5°C, but when the pressure cylinders were submerged in a constant temperature water bath, thermistors inside the cylinder showed that the temperature returned to ambient within 15 to 20 min, with about 50% of the temperature equilibration occurring within 3 min (ZoBELL, 1959).

4) *Amylase activity and stability determinations*

Quantitative determinations of amylase stability and activity at various pressures were made using the method of SMITH and ROE (1949) modified as follows. Test tubes were constructed to hold 10 m*l* when tightly stoppered with air excluded. Immediately after putting the enzyme solutions in the tubes and stoppering them, they were placed in pressure cylinders, compressed to the desired pressure, and submerged in a water bath, operations which required about 2 min per cylinder. All such tests were made in triplicate.

Stock solutions of α-amylase (Calbiochem, bacterial, lot No. 503455) for testing the stability and activity of the enzyme at various pressures were prepared by dissolving 100 mg in 100 m*l* of pH 7.2 phosphate buffer. The buffer solution consisted of 7.62 g KH_2PO_4, 20.4 g Na_2HPO_4, and 1.57 g calcium acetate per liter of distilled water. Calcium ion has been shown to suppress the inactivation of α-amylase and to be essential for its maximum activity (MANNING and CAMPBELL, 1961; SUZUKI and KITAMURA, 1963; MEYAGAWA, 1965). The stock solution of α-amylase was diluted with the buffer to give the desired number of units per volume. As defined by SMITH and ROE (1949), a unit of α-amylase is the amount which will hydrolyze 10 mg of soluble starch in 30 min at 25°C to a stage where no color is given with iodine at 620 mμ. The amylolytic activity of crystalline amylase slowly decreased, even when stored near 0°C, so that from month to month progressively more was required to give a unit.

Aqueous solutions of α-amylase were found to deteriorate more rapidly than the dry crystals. Consequently, fresh stock solutions had to be prepared and standardized for each series of experiments.

Unless otherwise stated, all tests for amylolytic activity were made and all cultures were incubated at atmospheric pressure and 25°C.

3. Starch hydrolysis by wild cultures

During a period of 3 years a total of 318 wild cultures, believed to represent more than a hundred different varieties of bacteria, were tested for their ability to hydrolyze starch. The wild cultures were picked from plates of nutrient sea water agar which had been inoculated with samples of raw sea water, bottom sediments, or the gut contents of various animals. When cultivated in sea water starch broth at 25°C, 124 of the wild cultures (39 %) were found to be starch hydrolyzers. About one-half of the amylolytic cultures, including 19 from the gut contents of invertebrates, were recovered from samples collected along the coast of southern California. The other half came from widely scattered locations, including 14 cultures collected during the Dodo Expedition to the Philippine and Marianas Trenches in 1964 and 24 collected during the Deepac X Expedition to the Japan Trench in 1966 (SEKI and ZOBELL, 1967). Several of the amylolytic cultures collected during these expeditions came from depths of 9,500 to 10,370 m. Another 24 amylolytic cultures were collected from depths of 5 to 4,200 m at stations along the 127° meridian about 600 miles west of Baja California. We are grateful to Dr. R. A. LEWIN for two cultures from Auke Bay, Alaska, and Point Noire, Congo.

Since all of the wild cultures were isolated from media initially incubated at 1 atm and were sub-cultured a few times at atmospheric pressure before being tested for amylolytic ability and pressure tolerance, it is not surprising that none of these isolates was able to grow at 1,000 atm. Only 11 of the 124 amylolytic cultures grew at 600 atm. Sixty-two grew at 400 atm, but not nearly as well as at 1 atm. All except six grew at 200 atm. Every one of the 124 amylolytic cultures was found to hydrolyze starch at the highest pressure at which it was able to reproduce. This was shown by the degradation of 0.5/m*l* of starch to the achroic point (iodine test) in nutrient broth in which the bacteria reproduced enough to produce perceptible turbidity.

Incidentally, when starch broth was inoculated with raw mud freshly collected from depths exceeding 9,000 m, bacterial growth and starch hydrolysis took place when certain samples were incubated at 1,000 atm. Two pure cultures designated *Bacillus G-14 and G-17*, which grew and degraded starch at 1,000 atm, were isolated from such deep-sea bottom-deposit enrichment cultures.

Out of 5,822 wild cultures of bacteria collected from various depths and latitudes in the Atlantic, Pacific, Indian, and Antarctic Ocean, 3,683 or 63.3 % were found by KRISS *et al.* (1967) to be amylolytic. The cultures were not tested for their ability to grow or to hydrolyze starch at increased pressures.

4. Growth of starch hydrolyzers at increased pressure

Nineteen species of marine bacteria, known to hydrolyze starch at 1 atm, were tested for their ability to grow in nutrient starch broth at deep-sea pressures. From an initial concentration of 2 to 5×10^4 viable bacteria per m*l* of inoculated broth, the bacteria reproduced to give viable populations of from 10^9 to 10^{10}/m*l* after 7 days' incubation at 25°C at 1 atm. The bacterial biomass, as indicated by turbidity, which developed at 1 atm was taken as a normal growth index of 100. The biomass which developed at increased pressures is expressed in Table 1 as a per cent of the normal growth index.

Nearly all of the species grew more rapidly and produced more total biomass at 1 atm than at increased pressures. There was substantial growth of all 19 species at pressures up to 300 atm, but four species failed to reproduce at 400 atm and eight failed to reproduce at 500 atm. Only two species, recently isolated from Marianas Trench mud samples, reproduced at 800 and 1,000 atm. Even these two barophilic species grew much better at 1 atm than at deep-sea pressures.

Twelve cultures were sterilized by 7 days'

Table 1. Relative amounts (%) of bacterial biomass produced in nutrient sea water broth during 7 days' incubation at different pressures, expressed on a basis of the biomass produced by each species at 1 atm being 100 %.

Species	Hydrostatic pressure in atm							
	100	200	300	400	500	600	800	1000
Bacillus abysseus	92	84	73	39	8	0	0	0
Bacillus G-14	100	100	97	89	85	74	61	42
Bacillus G-17	98	96	94	83	70	57	43	30
B. borborokoites	98	91	76	62	51	37	0	S
B. filicolonicus	89	79	60	42	11	S	S	S
B. imomarinus	63	48	16	0	0	0	0	S
B. submarinus	87	74	58	35	6	0	S	S
Flavobacterium neptunium	56	38	14	0	0	0	S	S
F. halohydrium	73	52	44	23	0	0	0	0
Pseudomonas pleomorpha	94	87	68	46	17	0	0	0
P. ichthyodermis	81	76	52	27	0	S	S	S
P. marinoglutinosus	86	80	63	38	12	0	0	0
P. marinopersica	79	53	28	6	0	0	0	0
P. membranula	68	42	20	0	S	S	S	S
P. perfectomarinus	95	92	83	67	59	31	0	S
Vibrio algosus	93	88	68	43	30	18	S	S
V. marinagilis	69	51	31	0	0	0	S	S
V. marinopraesens	96	90	74	56	14	0	0	S
V. ponticus	97	90	62	37	0	S	S	S

S=Sterilized, no viable bacteria

compression at 1,000 atm; one was sterilized in 7 days at only 500 atm.

As with the wild cultures, the rate of starch hydrolysis by all stock cultures was found to be more or less directly proportional to the rate of growth. Since the initial inocula did not carry enough enzyme to catalyze any measurable starch hydrolysis, amylases must have been synthesized by all species at the highest pressure at which perceptible growth occurred.

5. Starch hydrolysis by washed cell suspensions

Washed cell suspensions were found to hydrolyze starch very slowly, probably because most diastases and amylases produced by bacteria are extracellular enzymes (MYRBÄCK and NEUMÜLLER, 1950; POLLOCK, 1962). Whereas whole cultures containing 1 to 5×10^9 amylolytic bacteria per m*l* degraded 0.5 mg/m*l* of starch in from 1 to 4 hr at 25°C, a like amount of starch was degraded by comparable populations of washed cells only after 16 to 72 hr (Table 2).

Amylolytic bacteria for these observations were grown in nutrient starch broth at 25°C for 48 hr, at which time the starch had been degraded to the achroic point and the cells had rendered the medium turbid. When treated with additional starch, aliquots of the whole cultures were found to degrade 0.5 mg/m*l* in less than 8 hr. Cells were harvested from the 48-hr old cultures in a refrigerated centrifuge at 12,000 rpm and washed three times with cold 75 % sea water (pH 7.6). The cells were resuspended in 75 % aged sea water to give populations of 1 to 5×10^9/m*l* and treated with starch to give 0.5 mg/m*l*. After different periods of incubation at 25°C, these mixtures were examined for the presence of

Table 2. Relative amounts of starch* remaining after different periods of time at 25°C in aged sea water that had been treated with 0.5 mg/m*l* starch and from 1 to 5×10^9 washed bacterial cells per m*l*.

Species	Pressure (atm)	Amount of starch after 8 hr	16 hr	32 hr	48 hr	72 hr
B. submarinus	1	++++	++++	+++	++	−
	1000	++++	++++	+++	++	−
F. neptunium	1	++++	++++	++	−	−
	1000	++++	++++	++	−	−
P. pleomorpha	1	++++	+++	−	−	−
	1000	++++	+++	+	−	−
P. perfectomarinus	1	+++	+	−	−	−
	1000	+++	+	−	−	−
V. ponticus	1	++++	++++	+++	+	−
	1000	++++	++++	+++	+	−

* ++++=0.25–0.5 mg/m*l*; +++=0.1–0.25 mg/m*l*; ++=0.025–0.1 mg/m*l*; +0.005–0.025 mg/m*l*; −=<0.005 mg/m*l*

starch, using the spot-plate method. The washed cells, preformed at 1 atm, were found to hydrolyze starch at 1,000 atm at about the same rate as at 1 atm (Table 2).

6. Amylolytic activity of bacterial filtrates

Filtrates of starch hydrolyzers were found to have greater amylolytic activity than the bacterial cells removed therefrom. The starch-hydrolyzing bacteria were grown in nutrient starch broth at 25°C for 48 hr, at which time the starch had been depleted and the cultures contained 1 to 5 $\times10^9$ cells/m*l*. The cultures were passed through HA Millipore filters (0.45 μ) to give cell-free filtrates. The filtrates were treated with 0.5 mg/m*l* of starch and then dispensed in 10-m*l* piston-stoppered tubes for incubation under different conditions. After different periods of time, samples were examined for the presence of starch with iodine reagent in spot plates.

Starch appeared to be degraded by the cell-free filtrates about as rapidly at 1,000 atm as at 1

Table 3. Relative amounts of starch remaining in cell-free, starch-enriched (0.5 mg/m*l*) filtrates after different periods of time at 25°C.

Species	Pressure (atm)	Amount of starch after 4 hr	8 hr	16 hr	24 hr	32 hr
B. submarinus	1	+++	++	+	−	−
	1000	+++	++	+	−	−
F. neptunium	1	++++	+++	++	+	−
	1000	++++	+++	++	+	−
P. pleomorpha	1	+++	++	−	−	−
	1000	+++	++	+	−	−
P. perfectomarinus	1	++++	+++	−	−	−
	1000	++++	+++	+	−	−
V. ponticus	1	++	+	−	−	−
	1000	++	+	−	−	−

atm (Table 3). Filtrates of most species depleted the starch in from 3 to 24 hr, whereas the cells harvested from identical preparations required 32 to 72 hr to degrade 0.5 mg/m*l* of starch (Table 2). Note that whereas washed cells of either *P. perfectomarinus* or *P. pleomorpha* degraded starch more rapidly than any other species tested, the filtrates from these two *Pseudomonas* species degraded starch more slowly than other species in the test series.

7. Starch hydrolysis by whole cultures

Starch added to dense populations of preformed cells was found to be degraded appreciably faster than by equivalent concentrations of either washed cells or the filtrates separated from whole cultures. Fig. 1 shows that whole cultures degraded from 0.12 to 0.38 mg/m*l* of starch in 4 hr, as compared with 8 to 48 hr required for filtrates or washed cells to degrade similar quantities of starch. Significantly, the data summarized in Fig. 1 show that whole cultures of all species tested degraded starch at pressures at which most of the species were unable to grow (see Table 1). In these experiments, 10 m*l* of 48-hr old whole cultures, containing from 1 to 5×10^9 cells/m*l*, were treated with starch (0.5 mg/m*l*) and the starch remaining after 4 hr was determined spectrophotometrically.

By using a Beckman DU spectrophotometer, which provided for an accuracy of $\pm2\,\mu$g/m*l*, it was demonstrated that starch was degraded by 48-hr old whole cultures an average of 85% less

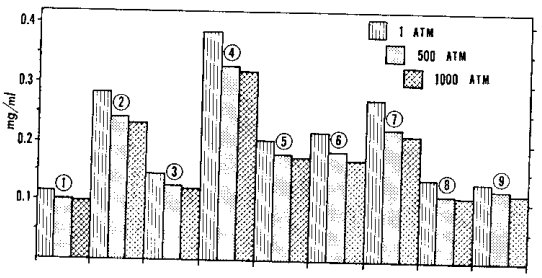

Fig. 1. Quantities of starch hydrolyzed in 4 hr at 25°C by 48-hr old whole cultures: 1) *Bacillus imomarinus*, 2) *B. submarinus*, 3) *B. filicolonicus*, 4) *Vibrio ponticus*, 5) *V. marinopraesens*, 6) *Pseudomonas perfectomarinus*, 7) *P. pleomorpha*, 8) *Flavobacterium neptunium*, and 9) *Bacillus G-14*.

rapidly at 500 atm than at 1 atm and 82% less rapidly at 1,000 atm than at 1 atm (Fig. 1). The reduced rate of starch degradation at high pressures was found to be attributable largely to the inhibition of growth and amylase formation at high pressure rather than to the direct retardation of amylase activity by deep-sea pressure. This was demonstrated (a) by determining the bacterial population of similar whole cultures after 4 hr under comparable conditions, (b) by determining the starch content of similar whole cultures treated with 1% toluol to inhibit bacterial growth, and (c) by investigating the activity and stability of pure bacterial α-amylase in buffered solutions.

Being in the logarithmic phase of growth, it is not surprising that the 48-hr old whole cultures were found to grow and to produce amylase during the additional 4-hr incubation at 1 atm. Some, but not all of the species reproduced enough at 500 atm to account in part for the more rapid rate of starch hydrolysis at 500 atm than at 1,000 atm (Fig. 1). Only one species, *Bacillus G-14*, grew at 1,000 atm and it grew very little.

When 48-hr old cultures were treated with 1% toluol and then enriched with starch (0.5 mg/m*l*) in 10-m*l* tubes, about as much starch was degraded in 4 hr at 1 atm as at 1,000 atm. Indeed, a few toluol-treated cultures appeared to degrade starch somewhat more rapidly at 1,000 atm than at 1 atm. Since the differences in most cases were little more than the differences between duplicates, this was believed to be intrinsic experimental error until later experiments demonstrated that the amylolytic activity rate of pure α-amylase was increased slightly by deep-sea pressures.

8. Amylases produced by marine bacteria

Whereas the principal product of starch hydrolysis by α-amylase is dextrin plus a little maltose and sometimes glucose, the action of β-amylase is mainly saccharifying, converting up to 60% of the starch hydrolyzed into maltose and often appreciable glucose (FUKUMOTO, 1943; MYBRÄCK and NEUMÜLLER, 1950). Many bacteria produce both types of amylases. The relatively small quantities of reducing sugars

produced by the starch hydrolyzers listed in Table 1 indicates that these marine bacteria produce mainly α-amylase. No difference could be detected in the proportion of reducing sugars and dextrins produced by the bacteria at different pressures.

9. Activity of α-amylase at increased pressure

Although the foregoing experiments clearly demonstrate that deep-sea pressures retard or inhibit bacterial growth and the concurrent production of amylolytic enzymes, it was indeterminate the extent to which the rate of starch degradation by whole cultures, filtrates therefrom, or washed cells at deep-sea pressures was attributable to the effects of pressure on (a) the stability of amylases, (b) the absolute chemical reaction rate, or (c) other conditions. Therefore, in an effort to eliminate as many complicating factors as possible and to minimize the time during which enzyme inactivation could occur, experiments were conducted with highly purified α-amylase in concentrations which degraded appreciable quantities of starch in 15 min. It was impractical to employ shorter incubation periods, because around 2 min was required to mix and to compress the reactants to 1,000 atm and another 2 min to decompress and to stop the reaction.

With all solutions, reagents, and receptacles in readiness, including temperature equilibrations, 1.0 m*l* of α-amylase solution equivalent to 20 units was mixed with 9.0 m*l* of phosphate buffer containing 60 mg of starch in 10-m*l* piston-stoppered tubes. After 15 min incubation at various temperatures and pressures, enzyme action was stopped by pouring the contents of each tube into a 50-m*l* beaker which contained 2 m*l* of 1 N HCl. Starch was determined by the iodine method as described by SMITH and ROE (1949), using a Beckman DU spectrophotometer at a wave-length of 620 mμ. Under these conditions, 2 units of α-amylase per m*l* hydrolyzed from 1.02 mg/m*l* (at 4°C) to 5.88 mg/m*l* (at 55°C) of starch. At all temperatures, starch was hydrolyzed somewhat more rapidly at deep-sea pressures than at 1 atm as shown by Fig. 2.

The results obtained at the lower temperatures (4° to 15°C) are believed to be more meaningful

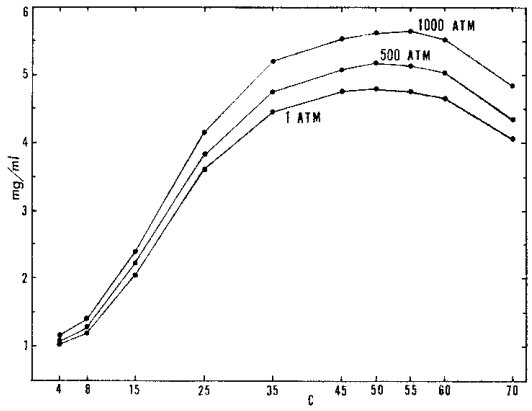

Fig. 2. Quantities of starch hydrolyzed in 15 min at various temperatures and pressures by 2 units/m*l* of α-amylase in buffer solution.

than the results obtained at the higher temperatures. In the first place, the lower temperatures are more representative of conditions in marine environments with which this study is primarily concerned. In the second place, amylases are more stable at the lower temperatures and consequently the results are believed to be more indicative of the effects of deep-sea pressure on the absolute chemical reaction rate. The deceleration in the rates of starch hydrolysis with increasing temperature observed above 25°C is attributed to the higher temperatures (GUZHOVA and LOGINOVA, 1966; KUO and HARTMAN, 1967), although α-amylases of certain bacteria are stable for short periods of time at temperatures as high as 90° (MANNING and CAMPBELL, 1961).

10. Stability of α-amylase at deep-sea pressures

Data summarized in Fig. 3 show that α-amylase dissolved in phosphate-calcium acetate pH 7.2 buffer solution slowly lost its activity at 4°C. Inactivation took place progressively more rapidly at higher temperatures, but α-amylase is surprisingly stable at 55°C. The enzyme lost its activity more rapidly when compressed to 1,000 atm at 4°C than at 1 atm. Increased pressure also promoted the inactivation of α-amylase at 25°C, but not nearly to the same extent as at 4°C. At 55°C the effect of pressure is reversed, the rate of heat inactivation of the enzyme being

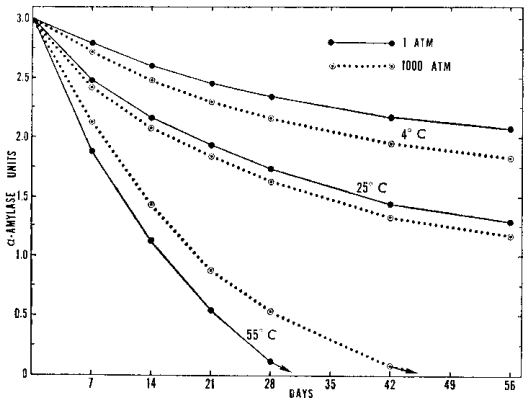

Fig. 3. Units of α-amylase after different periods of time in a buffer solution at different temperatures and pressures.

retarded appreciably at 1,000 atm. Similarly, SUZUKI and KITAMURA (1963) found that moderate pressures of 1,000 atm or less retard the heat inactivation of α-amylase.

The data depicted in Fig. 3 were obtained by holding aliquots of a buffer solution of α-amylase prepared to contain 3 units per m*l*. This stock solution was dispensed in several dozen small piston-stoppered tubes. Controls were examined at once for amylase activity and the others were held at different temperatures and pressures for from 7 to 56 days. Amylase activity was determined by mixing 1 m*l* of the enzyme solution with a solution containing 30 mg of starch and then determining the amount of starch remaining after 30 min at 25°C by the method of SMITH and ROE (1949). The complete degradation of 30 mg of starch indicated the presence of 3 units of active amylase and proportionately fewer units when smaller quantities of starch were degraded. The data recorded in Fig. 3 are averages of triplicate determinations, most of which agreed within ± 0.1 unit.

MEYAGAWA (1965) observed that in the absence of calcium ions, α-amylase was slowly inactivated by a pressure of 500 kg/cm^2, but in the presence of 0.01 M Ca^{++} α-amylase was not appreciably inactivated until a pressure of 3,000 kg/cm^2 was reached.

11. Bacterial hydrolysis of natural dinoflagellate starch

Although standard soluble starch was used in all of the experiments reported in this paper, a few significant observations were made on natural starch produced by marine dinoflagellates. Dr. O. HOLM-HANSEN of the Scripps Institution of Oceanography provided us with several grams of freshly grown *Cachonina niei* (LOEBLICH, 1968), the dry weight of which was found to contain about 27% starch. The natural raw starch was readily attacked by several different amylolytic bacteria at 1 atm and also at 400 atm, but not nearly as rapidly as soluble starch.

12. Summary and discussion

Deep-sea pressures affect starch hydrolysis chiefly by the suppression of bacterial growth. The vast majority of the amylolytic species as well as other kinds of bacteria found in the sea are unable to grow when compressed to 600 atm. The growth of most species is retarded by pressures exceeding 100 atm. Various conditions, including temperature, chemical composition of the medium, duration of compression, and the nature of the species, which influence the response of bacteria to increased pressure have been reviewed by ZOBELL and JOHNSON (1949), OPPENHEIMER and ZOBELL (1952), JOHNSON (1957), HEDÉN (1964), ZOBELL (1964), MORITA (1967), and YAYANOS (1967).

As compared with the pronounced effects of increased pressure on bacterial growth and the concurrent production of amylases, the influence of deep-sea pressure on the activity of preformed (at ambient pressure) bacterial amylases is relatively small. However, increased pressure does influence starch hydrolysis by affecting (a) the stability or inactivation of amylases, (b) the absolute chemical reaction rate, and (c) a variety of pertinent physicochemical conditions such as the ionization of weak electrolytes, pH, viscosity, etc. (GONIKBERG, 1963; HAMMAN, 1964).

BASSET and MACHEBOEUF (1932) and MACHEBOEUF *et al.* (1933) are often misquoted as having demonstrated that various amylases are stable at pressures of 10,000 to 15,000 atm. This generalization is based on results obtained

by an exposure for only 45 min, during which time the activity of amylases was reduced about one-third at 9,000 atm and the amylases were completely inactivated at 15,000. Pancreatic amylase was found to be partly inactivated in 30 min at 15,500 atm. Moderate pressure below 1,000 atm was found by SUZUKI and KITAMURA (1963) and MEYAGAWA (1965) to affect the inactivation of bacterial amylases, the pressure required for complete inactivation being largely a function of temperature, pH, calcium ion concentration, and duration of exposure.

Several examples of the protective effect of moderate pressure against the thermal destruction of enzymes and other biological materials have been reported by JOHNSON and EYRING (1948) and JOHNSON (1957). Moderate pressure up to 680 atm was found by FOSTER *et al.* (1949) to retard the thermal destruction of certain bacteriophages. JOHNSON and ZOBELL (1949) observed that whereas buffered salt solutions containing from 10^4 to 10^5 *Bacillus subtilis* endospores per m*l* were disinfected in 60 min at 93.6°C at 1 atm, from 10^2 to 10^3 endospores per m*l* remained viable at this temperature for 90 min when compressed to 680 atm. Such a protective effect of pressure is believed to account for the increasingly greater stability of α-amylase at temperatures in the range of 25° to 70°C (Figs. 2 and 3).

Owing to the presence of starch in the experiments on the rate of its hydrolysis at different pressures (Fig. 2), the rate of amylase inactivation was probably more rapid than the rates of inactivation indicated by data depicted in Fig. 3. For this reason and also because the percentage concentration of starch was decreasing considerably as it was being hydrolyzed, increased pressure probably accelerated the absolute chemical reaction rate (STEARN and EYRING, 1941) considerably more than indicated by data depicted in Fig. 2. This presumption is based (a) on the relatively large volume of activation, $\Delta V^{\ddagger}$, for the hydrolysis of starch, and (b) on the fact that pressure effects on biological reaction rates depend critically upon whether the substrate concentration is high or low (LAIDLER, 1951). According to LAIDLER (1955), the $\Delta V^{\ddagger}$ values for the amylase hydrolysis of starch range from -22 to -27 cm^3/mole. Ordinarily, negative $\Delta V^{\ddagger}$ values indicate an acceleration of the reaction by increased pressure (WEALE, 1967). Theoretically, under steady-state conditions a $\Delta V^{\ddagger}$ value of 1 cm^3/mole may give a change of rate constant of as much as 4 % for each kilobar (WHALLEY, 1964). But rate changes of this magnitude are rarely, if ever, realized in biological systems owing to the effects of extraneous chemicals and other conditions which influence biochemical reactions. As pointed out by BENSON and BERSON (1962), the value of $\Delta V^{\ddagger}$ is a composite quantity, being made up of effects of volume due to short-range changes in packing and geometry of molecules and effects due to changes in long-range forces between solute and solvent.

Acknowledgments

This investigation was supported by research grant GB-4240 from the U.S.A. National Science Foundation. The authors also appreciate the contributions of Dr. Humitake SEKI and Jean S. ZOBELL. Dr. N. TAGA, Ocean Research Institute of the University of Tokyo, and Dr. A. KAWAI, Kyoto University, helped in obtaining cultures from the Japanese Trench during Deepac X Expedition.

References

BASSET, J. and M. A. MACHEBOEUF (1932): Études sur les effets biologiques des ultra-pressions: Résistance des bactéries, des diastases et des toxins aux pressions très élevées. Compt. Rend. Acad. Sci., Paris, **195**, 1431–1433.

BENSON, S. W. and J. A. BERSON (1962): The effect of pressure on the rate and equilibrium constants of chemical reactions. The calculation of the activation volumes by application of the Tait equation. J. Amer. Chem. Soc., **84**, 152–158.

BREED, R. S., E. G. D. MURRAY, and N. R. SMITH (1957): Bergey's Manual of Determinative Bacteriology. Williams & Wilkins Co., Baltimore, Maryland, 7th Ed., 1094 pp.

BURSA, A. S. (1968): Starch in the oceans. J. Fish. Res. Bd. Canada, **25**, 1269–1284.

FISHER, R. L. and H. H. HESS (1963): Trenches. *In* M. N. HILL (ed.), The Sea. Interscience Publishers, New York, **3**, 411–436.

FOSTER, R. A. C., F. H. JOHNSON, and V. MILLER

(1949): The influence of hydrostatic pressure and urethane on the thermal inactivation of bacteriophage. J. Gen. Phys., **33**, 1–16.

FUKUMOTO, J. (1943): Studies on bacterial amylase. V. Optimum pH and temperature and heat resistance. J. Agr. Chem. Soc. Japan, **19**: 833–861.

GONIKBERG, M. G. (1963): Chemical equilibria and reaction rates at high pressure. Israel Program for Scientific Translations, Jerusalem, 212 pp.

GUZHOVA, E. P. and L. I. LOGINOVA (1966): Thermophilic bacteria forming active amylase. Microbiology, **35**, 361–373.

HAMANN, S. D. (1964): High pressure chemistry. Ann. Rev. Phys. Chem., **15**, 349–370.

HEDÉN, C. G. (1964): Effects of hydrostatic pressure on microbial systems. Bacteriol. Rev., **28**, 14–29.

JOHNSON, F. H. (1957): The action of pressure and temperature. *In* R. E. O. WILLIAMS and C. C. SPICER (ed.), Microbial Ecology, Cambridge Univ. Press, 132–167.

JOHNSON, F. H. and H. EYRING (1948): The fundamental action of pressure, temperature, and drugs on enzymes, as revealed by bacterial luminescence. Ann. New York Acad. Sci., **49**, 376–396.

JOHNSON, F. H., H. EYRING, and M. J. POLISSAR (1954): The Kinetic Basis of Molecular Biology. John Wiley & Sons, Inc., New York, 874 pp.

JOHNSON, F. H. and C. E. ZOBELL (1949): The retardation of thermal disinfection of *Bacillus subtilis* spores by hydrostatic pressure. J. Bacteriol., **57**, 353–358.

KRISS, A. E., I. E. MISHUSTINA, N. MITSKEVICH, and E. V. ZEMTSOVA (1967): Microbial Population of Ocean and Seas. (Translated from Russian by K. Syers and edited by G. E. Fogg), St. Martin's Press, New York, 287 pp.

KUO, M. J. and P. A. HARTMAN (1967): Purification and partial characterization of *Thermoactinomyces vulgaris* amylase. Can. J. Microbiol., **13**: 1157–1163.

LAIDLER, K. J. (1951): The influence of pressure on the rates of biological reactions. Arch. Biochem., **30**, 226–236.

LAIDLER, K. J. (1955): Some kinetics and mechanistic aspects of hydrolytic enzyme action. Faraday Soc. Discussions, **20**, 83–96.

LOEBLICH, A. (1968): A new marine dinoflagellate genus, *Cachonina*, in axenic culture from the Salton Sea, California. Proc. Biol. Soc. Wash., **81**, 91–96.

MACHEBOEUF, M., J. BASSET, and G. LEVY (1933): Influence des pressions très élevées sur les diastases. Ann. Physiol., **9**, 713–722.

MANNING, G. B. and L. L. CAMPBELL (1961): Thermostable α-amylase of *Bacillus stearothermophilus*. I. Crystallization and some general properties. J. Biol. Chem., **236**, 2952–2957.

MEEUSE, B. J. D. (1962): Storage products. *In* R. A. LEWIN (ed.), Physiology and Biochemistry of Algae. Academic Press, Inc., New York, 289–313.

MEYAGAWA, K. (1965): Reactivation of pressure-inactivated α-amylase of *Bacillus subtilis* under moderate pressure. Arch. Biochem. Biophys., **110**, 432–437.

MORITA, R. Y. (1967): Effects of hydrostatic pressure on marine microorganisms. Oceanogr. Mar. Biol. Ann. Rev., **5**, 187–203.

MYRBÄCK, K. and G. NEUMÜLLER (1950): Amylases and the hydrolysis of starch and glycogen. *In* J. B. SUMNER and K. MYRBÄCK (ed.), The Enzymes. Academic Press, Inc., New York, **1**, 653–724.

OPPENHEIMER, C. H. and C. E. ZOBELL (1952): The growth and viability of sixty-three species of marine bacteria as influenced by hydrostatic pressure. J. Mar. Res., **11**, 10–18.

PERCIVAL, E. and R. H. MCDOWELL (1967): Chemistry and Enzymology of Marine Algal Polysaccharides. Academic Press, Inc., New York, 219 pp.

POLLOCK, M. R. (1962): Exoenzymes. *In* I. C. GUNSALUS and R. Y. STANIER (ed.), The Bacteria. Academic Press, Inc., New York, **4**, 121–178.

SEKI, H. and C. E. ZOBELL (1967): Microbial assimilation of carbon dioxide in the Japan Trench. J. Oceanogr. Soc. Japan, **23**, 182–188.

SMITH, B. W. and J. H. ROE (1949): A photometric method for the determination of α-amylase in blood and urine, with use of the starch-iodine color. J. Biol. Chem., **179**, 53–59.

STEARN, A. E. and H. EYRING (1941): Pressure and rate processes. Chem. Rev., **29**, 509–523.

SUZUKI, K. and K. KITAMURA (1963): Inactivation of enzyme under high pressure. Studies on the kinetics of inactivation of α-amylase of *Bacillus subtilis* under high pressure. J. Biochem. (Tokyo), **54**, 214–219.

WEALE, K. E. (1967): Chemical Reactions at High Pressure. E. & F. N. Spon, Ltd., London, 349 pp.

WHALLEY, E. (1964): Use of volumes of activation for determining reaction mechanisms. *In* Advances in Physical Organic Chemistry. Academic Press, Inc., London, **2**, 93–162.

WHISTLER, R. L. and W. M. CORBETT (1957):

Polysaccharides. *In* W. PIGMAN (ed.), The Carbohydrates. Academic Press, Inc., New York, 641–708.

YAYANOS, A. A. (1967): A study of the effects of hydrostatic pressure on macromolecular synthesis and thiamineless death in *Escherichia coli* and the compression of some solutions of biological molecules. Dissertation, Pennsylvania State Univ., 101 pp.

ZoBELL, C. E. (1946): Marine Microbiology. Chronica Botanica Co., Waltham, Mass., 240 pp.

ZoBELL, C. E. (1959): Thermal changes accompanying the compression of aqueous solutions to deep-sea conditions. Limnol. Oceanogr., **4**, 463–471.

ZoBELL, C. E. (1464): Hydrostatic pressure as a factor affecting the activities of marine microbes. *In* Y. MIYAKE and T. KOYAMA (ed.), Recent Researches in the Fields of Hydrosphere, Atmosphere and Nuclear Geochemistry, Maruzen Co., Ltd., Tokyo, Japan, 83–116.

ZoBELL, C. E. and F. H. JOHNSON (1949): The influence of hydrostatic pressure on the growth and viability of terrestrial and marine bacteria. J. Bacteriol., **57**, 179–189.

ZoBELL, C. E. and R. Y. MORITA (1959): Deep-sea bacteria. Galathea Report, Copenhagen, **1**, 139–154.

ZoBELL, C. E. and C. H. OPPENHEIMER (1950): Some effects of hydrostatic pressure on the multiplication and morphology of marine bacteria. J. Bacteriol., **60**, 771–781.

ZoBELL, C. E. and H. C. UPHAM (1944): A list of marine bacteria including descriptions of sixty new species. Bull. Scripps Inst. Oceanogr., **5**, 239–292.

海洋細菌の澱粉加水分解作用に対する深海水圧の影響

Claude E. ZoBELL and Leslie L. HITTLE

要旨 澱粉加水分解能をもった細菌は，海水や海底堆積物中に，または海産動物と共に広く分布しているように思われる．9,500 m にもおよぶ深さから分離されたいくつかの菌株は，1,000 気圧前後の水圧下で増殖し，しかも澱粉を加水分解しうることが判明したが，浅所からの大半の分離種の増殖は，300～600気圧の圧力で阻害された．1 気圧で増殖させられた細菌培養液やその培養液から菌体を除いた上澄液，および洗条菌体は，1,000 気圧のもとで澱粉を加水分解しうることが示された．菌の対数増殖期における全培養液による澱粉加水分解作用力は，1 気圧の作用力に対し，500 気圧では平均 85% に，また 1,000 気圧では82%に低下することが判明した．この原因は，大部分細菌の増殖と酵素生成に対する阻害に起因している．4°～35°C の温度範囲では，深海水圧がアミラーゼを不活性化する傾向が多少あった．この傾向があるにもかかわらず，α-アミラーゼによる澱粉加水分解速度は，とくに25°～60°C 範囲で深海水圧によって促進されたが，この理由は多分高圧が絶体化学反応速度を促進するためであろう．圧力上昇に伴って，α-アミラーゼの熱不活性化は遅延させられた．