SUMMARY

1. Autopsy samples of liver from three cases of cystinosis have been compared with normal liver with respect to the degradative metabolism of cystine and cysteine, with particular emphasis on the enzymic reduction of cystine.

2. Under the conditions of these experiments the reduction of cystine to cysteine by cystine reductase and by glutathione-cysteine transhydrogenase was normal in cystinotic liver. Also, no abnormality was found in the cystine-reductase activity of kidney.

3. Glutathione-reductase activity and reduced glutathione content of cystinotic liver were normal.

4. Cysteine, cystine and cysteinesulphinic acid were completely oxidized by liver of the cystinosis cases, and these findings were substantiated by the normal urinary excretion of inorganic sulphate by cystinosis patients.

5. The liberation of hydrogen sulphide from cysteine and cystine by cystinotic liver was normal, the reactivity of cystine depending on its prior reduction to cysteine.

6. No abnormality of cystinotic liver was observed for the transamination of cysteine, cysteic acid and cysteinesulphinic acid with α -oxo-glutarate.

7. Normal decarboxylation of cysteic acid and cysteinesulphinic acid was observed for cystinotic liver.

8. These findings are discussed with respect to the aetiology of cystinosis.

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Studies on α -Amylase Formation by *Bacillus subtilis*

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The synthesis of α -amylase (α -1,4-glucan 4-glucanhydrolase, EC 3.2.1.1) by cultures of *Bacillus subtilis* has been studied by several groups of

workers, who have defined the basic conditions required for the formation of high yields of the enzyme (Hagihara, 1951, 1954; Fukumoto, Yamamoto, Tsuru & Ichikawa, 1957*b*; Fukumoto, Yamamoto & Tsuru, 1957*a*). For α -amylase production a carbohydrate source is required and this

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may be starch or one of a variety of sugars, including maltose, lactose and galactose, but not glucose. Nitrogen can be supplied in the form of ammonium salts or as a complex organic source. High phosphate concentrations are required for maximal synthesis.

It was shown by Fukumoto *et al.* (1957*a*) that washed *B. subtilis* cells contain little α -amylase but that when suspensions of the organism are incubated aerobically in the presence of phosphate buffer, small amounts of the enzyme appear in the medium and this appearance is increased by the addition of carbohydrate. Nomura, Hosoda, Maruo & Akabori (1956*a*) also observed that washed cells produce α -amylase in the absence of carbon and nitrogen sources, and further that α -amylase formation under such conditions is inhibited by cyanide, chloramphenicol, streptomycin and toluene. These authors concluded that the appearance of the enzyme in the external medium during experiments with washed-cell suspensions was not due simply to liberation of preformed enzyme.

Several groups of workers have reported on different aspects of α -amylase synthesis in washed *B. subtilis* cell suspensions but the literature on this subject is very confused. This is partly because of the diversity of strains of the organism, growth media and experimental conditions employed, and partly because a considerable portion of the work was concerned with the effect on α -amylase synthesis of a factor, present in extracts of boiled *B. subtilis* cells, that stimulated the formation of the enzyme (Nomura, Hosoda, Yoshikawa & Nishimura, 1957; Nomura & Yoshikawa, 1959). The factor was believed to be related to nucleic acid but has been shown (Yoshikawa & Maruo, 1960) to be an amine, partially replaceable by compounds such as putrescine and cadaverine, which is associated with the nucleic acid fraction. Since the factor-stimulated formation of α -amylase was found in some respects to differ from the 'basic' enzyme production, it is not clear what relationship exists between the two.

For these reasons the characteristics of α -amylase formation in washed-cell suspensions have been reinvestigated and the results are reported in this paper. Some observations on α -amylase estimation and on a requirement for Fe^{3+} ions in its production by *B. subtilis* cultures are also described. The work forms part of an investigation into the synthesis of a specific protein with α -amylase formation used as a 'model' system.

MATERIALS AND METHODS

α -Amylase. This was a crystalline preparation from *B. subtilis* supplied by Daiwa Kasei K. K., Osaka, Japan.

Starches. For growth media, soluble starch (A.R.;

British Drug Houses Ltd.) was treated according to Lintner (1886). For α -amylase assay, untreated soluble starch was used. Partially enzymically hydrolysed starch was prepared by allowing α -amylase to act on soluble starch until no starch-iodine colour remained. The solution was then boiled to destroy the enzyme.

Chloramphenicol. This was a product of Parke, Davis and Co. Ltd.

2,4-Dinitrophenol. Solutions of this compound (British Drug Houses Ltd.) were adjusted to pH 7.2 with KOH.

p-Fluoro-DL-phenylalanine and L-amino acids. These were products of the California Foundation for Biochemical Research (CfP grade).

DL-Phenylalanine. This was a product of British Drug Houses Ltd. Other reagents were of A.R. grade.

Phosphate buffers. These were mixtures of KH_2PO_4 and Na_2HPO_4 .

Buffered saline. This contained 0.15M-(NH_4) $_2\text{HPO}_4$, 0.02M-KCl, 2 mM-MgSO $_4$, 1 mM-CaCl $_2$ and 0.4 mM-ZnSO $_4$, adjusted to pH 7.2 with H_2PO_4 .

Medium A. This was essentially that of Nomura, Maruo & Akabori (1956*b*) and consisted of 0.15M-(NH_4) $_2\text{HPO}_4$, 0.02M-KCl, 2 mM-MgSO $_4$, 1 mM-CaCl $_2$, 0.4 mM-ZnSO $_4$, 0.04M-sodium citrate and 10% of soluble starch, pH 7.2. The medium as used contained sufficient Fe^{2+} ions to promote consistently high yields of α -amylase (see Results section). Later work with medium A prepared from new batches of reagents, however, resulted in negligible yields of α -amylase, although growth occurred. Addition of FeCl_3 (0.5 mM) restored enzyme production to a high value.

Medium B. This was the same as medium A, except that the citrate was replaced by 1.2% of Difco Bacto nutrient broth, and FeCl_3 (0.5 mM) was added.

Growth of the organism. The *B. subtilis* used was an unclassified strain kindly supplied by the Takamine Laboratories Inc., Clifton, N.J., U.S.A. Cultures were grown at 30° in inverted T tubes (van Heyningen & Gladstone, 1953), aerated by rocking the vessels through an angle of 40° 40 times/min. Cultures in medium A were grown in 40 ml. of medium in vessels of 180 ml. capacity (4 cm. $\times$ 19 cm.), and those in medium B were grown in 10 ml. of medium in vessels of 60 ml. capacity (2.5 cm. $\times$ 16 cm.). The organism was inoculated into medium B from nutrient-agar slopes and a 24 hr. culture was used in each experiment for further inoculations (0.4 ml. for medium A cultures and a platinum-loopful for those in medium B). Frequent serial sub-culturing was avoided since some indications of loss of α -amylase-producing ability after such treatment were noted; new cultures were prepared as needed from washed-spore suspensions stored in sterile water at 0°.

Washed-cell experiments. Cells from medium A were harvested after growth for 90 hr. and washed twice by suspension in buffered saline and finally suspended in the same medium. In all cases the α -amylase content of the growth medium was assayed; this was found to be 500–900 units of enzyme/ml. (see below for definition of enzyme unit).

Cells from medium B were harvested at 35 hr. and treated in the same way. These cells at the time of harvesting were well out of the phase of rapid growth, which ceased at 20 hr.; from 20 hr. until at least 37 hr. the cell dry weight of the cultures increased steadily at a rate of 3%/hr. (3.2 mg./ml. at 20 hr. to 5.0 mg./ml. at 37 hr.).

In all cases incubation mixtures contained approx. 5 mg. (dry wt.) of cells/ml. Actual dry weights were determined on samples of the cell suspension used, and results are expressed throughout in terms of a cell density of 5 mg. (dry wt.)/ml. of medium.

Cell densities. These were measured by dry-weight determination; the cells were centrifuged down and washed twice in water. They were then suspended in acetone, transferred to a weighed beaker and dried at 110° to constant weight.

Disruption of bacterial cells. Cell suspensions were treated in the apparatus described by Nossal (1953). Quantitative disruption of the cells, as judged by microscopic examination, was achieved in 1 min.

Estimation of α -amylase. Incubated cell suspensions, or bacterial cultures, were centrifuged at 5000g for 5 min. and the supernatant fractions assayed for enzyme content. The method of assay was essentially the Hokin (1951) modification of the method of Smith & Roe (1949), except that the reaction mixtures contained mM-CaCl₂. Smith & Roe (1949) defined their unit of α -amylase in terms of weight of starch hydrolysed to the point at which it no longer gave a colour reaction with iodine. These authors observed variation in apparent activities from one starch substrate to another. We found that the latter troublesome feature was due to variations in the value of the initial iodine colour of the starches and that it was avoided by expressing a unit of activity in terms of a decrease in extinction of the starch-iodine colour at 620 m μ . Accordingly we define the unit of activity as that amount of enzyme which, under the standard test conditions, gives $\Delta E_{620\text{m}\mu}$ 0.0284. The unit so defined is quantitatively the same as the Smith & Roe unit, determined on an arbitrarily chosen batch of starch. Tests showed that activities were proportional to α -amylase content up to amounts of 1 μ g. of crystalline enzyme. The method gives satisfactory measurements of α -amylase in the presence of bacterial culture media (Table 1).

The crystalline bacterial α -amylase had an activity of 38800 units/mg. of protein nitrogen; this figure is of an order similar to that of crystalline α -amylase measured in the units of the Hagihara (1954) assay method and used by Nomura and co-workers, e.g. 37000 for *B. subtilis* H enzyme (Nomura, Maruo & Akabori, 1956c) and 17300 for *B. subtilis* N enzyme. The latter figure was calculated from the value of Nomura & Wada (1958), a nitrogen content of 16.2% being assumed for the protein (Akabori, Okada, Fujiwara & Sugae, 1956).

RESULTS

Formation of α -amylase in growing cultures of Bacillus subtilis

In the absence of organic nitrogen (medium A) growth of the organism was slow and α -amylase did not start to accumulate in the medium until after incubation for 60 hr., which corresponded to the beginning of the phase of most rapid cell growth. The cells grew at a constant rate from 60 hr. to the end of the experimental period, at 120 hr., at which stage a cell density of 7.1 mg. dry wt./ml. was attained. During this same period the α -amylase content of the medium increased from 0 to 1200 units/ml. After incubation for 80 hr. the enzyme was produced at a constant rate on a parallel with cell growth for at least 20 hr. This parallelism suggested that from 80 to 100 hr. cells were producing enzyme at a constant rate. Cells used for washed-cell suspensions were therefore harvested at 90 hr., when the rate of α -amylase production was estimated to be approx. 33 units/5 mg. dry wt. of cells/hr.

Growth in medium B, containing nutrient broth, was much faster, the rapid-growth phase being over by 20 hr. The formation of extracellular enzyme lagged behind cell growth, reaching a maximum after the end of the rapid-growth phase, as had been observed by Fukumoto *et al.* (1957b) with a similar growth medium; the α -amylase activity at 35 hr. was usually of the order of 1000 units/ml.

Effect of ferric ions on α -amylase formation in growing cultures of Bacillus subtilis

Formation of α -amylase both in medium A and in medium B requires the presence of Fe³⁺ ions, negligible amounts of the enzyme being produced in their absence. Fig. 1 shows the relationship between cell growth, enzyme formation and concentration of these metal ions. In the absence of added Fe³⁺ ions growth occurred but gave rise to bulky masses of cells (the packed-cell volume was

Table 1. *Estimation of α -amylase added to the supernatant fraction of a culture of Bacillus subtilis*

Samples of a solution of crystalline α -amylase (approx. 1.5 μ g./ml.) and of the supernatant from a 12 hr. culture of *B. subtilis* (low in α -amylase) grown in medium B were mixed and the enzyme activity was determined. Separate determinations were made on samples of the solution of crystalline enzyme.

Composition of mixture		Total $\Delta E_{620\text{m}\mu}$ for mixture	Increase in $\Delta E_{620\text{m}\mu}$ due to added enzyme	$\Delta E_{620\text{m}\mu}$ of crystalline enzyme determined separately	Recovery of added enzyme (%)
Supernatant fraction (ml.)	Crystalline enzyme soln. (ml.)				
0.4	0	0.034	—	—	—
0.4	0.1	0.055	0.021	0.022	95.5
0.4	0.2	0.078	0.044	0.043	102.4
0.4	0.3	0.101	0.067	0.064	104.8
0.4	0.4	0.122	0.088	0.087	101.3

Fig. 1. Effect of concentration of Fe^{3+} ion on cell growth (●) and extracellular α -amylase formation (○) by cultures of *B. subtilis*. Cultures were grown in medium B containing Fe^{3+} ions (as FeCl_3). Cell densities and extracellular α -amylase were determined after 24 hr.

greater than with normal growth but the dry weight smaller). When such cells, harvested from medium A after growth for 90 hr., were tested in experiments in washed suspensions for α -amylase production, they were found to be almost completely inactive.

Formation of α -amylase in washed-cell suspensions

Cells grown in medium A were used in all but the last experiment, for which the cells were harvested from medium B.

Time-course of α -amylase formation. Production of extracellular α -amylase occurred at a low and fairly constant rate when washed cells, suspended in phosphate buffer, were shaken in the absence of carbon and nitrogen sources. This formation of enzyme in the absence of a carbon source is probably related to the observation by Clifton (1957) that washed cells of *B. subtilis* have large reserves of oxidizable substrate. The presence of maltose and ammonium salts had little effect during the first hour of incubation, but thereafter the rate of enzyme formation increased until it reached a constant high value (Fig. 2). The reason for the increase in the rate of enzyme appearance is not known since cell-density determinations at different time intervals showed that growth did not occur during the experimental period. This lack of growth was observed consistently and is presumably related to the slow rate of growth encountered with this organism in media containing no organic nitrogen.

The rate of α -amylase production in the experiment shown in Fig. 2 was, at its maximum, 25 units/5 mg. dry wt. of cells/hr. at 30° . This agrees reasonably well with the rate in growing 90 hr. cultures of approx. 33 units/5 mg. dry wt. of cells/hr.

Fig. 2. Extracellular α -amylase formation by washed-cell suspensions of *B. subtilis*. Cells were harvested after growth for 90 hr. in medium A and washed as described in the Materials and Methods section. Incubations were carried out at 30° , with shaking, in air in flasks containing 1% of maltose in buffered saline and 5 mg. (dry wt.) of cells/ml. At appropriate times the suspensions were centrifuged and α -amylase activity in the supernatants was determined. All values are corrected for a zero-time blank of 10.0 units of α -amylase/ml. ○, With 1% of maltose and NH_4^+ ions; ●, without maltose and NH_4^+ ions (ammonium phosphate was replaced by KH_2PO_4 - Na_2HPO_4 , pH 7.2).

Table 2. Distribution of α -amylase between extracellular and intracellular phases of *Bacillus subtilis* washed-cell suspensions

Washed cells (50 mg. dry wt.), harvested from 90 hr. cultures in medium A, were suspended in buffered saline containing 1% (w/v) of maltose, the total volume being 10 ml., and were incubated for 4 hr., with shaking, in air at 30° . After centrifuging, the supernatants were used for determining extracellular enzyme; cell pellets were twice washed and disrupted in a solution containing 0.5% NaCl-0.5% KCl and, after centrifuging at 10 000g for 10 min., the supernatants were used for intracellular-enzyme assays.

Fraction assayed	Total α -amylase activity/ 50 mg. dry wt. of cells	
	0 min.	240 min.
Extracellular	99	616
Intracellular	61	48
Extra- + intra-cellular (cells disrupted in medium)	157	609

Distribution of α -amylase between extracellular and intracellular phases. The evidence given before suggests that α -amylase was truly extracellular and had not accumulated within the cells to be finally liberated by cell lysis. However, no quantitative report on the distribution of enzyme between the intracellular and extracellular phases has been published. The amounts of intra- and extra-cellular α -amylase in washed-cell suspensions that were actively producing the enzyme were therefore examined (Table 2). Over an experimental period

of 4 hr. the cell-bound α -amylase remained almost constant at a low value (approx. 8% of the final total), in fact, decreasing slightly whilst a large increase in the extracellular enzyme occurred. This gives strong support to the concept that α -amylase is 'secreted' by the cell as it is formed, a conclusion which is also in keeping with the work described below on the properties of the α -amylase-forming system of washed cells.

It was further observed that the extracellular activity of catalase, a truly intracellular enzyme, determined at 60 min. intervals during the experiment, did not amount to more than 2% of the total intracellular amount of this enzyme. Although the possibility that catalase destruction occurred in this system was not completely excluded this result again makes it unlikely that any large-scale lysis of the cells accompanied by the release of intracellular constituents took place.

Requirements for α -amylase formation in washed-cell suspensions. The addition of a carbohydrate source to washed-cell suspensions more than doubles the formation of α -amylase over a period of 4 hr. (Table 3). Maltose, glucose and hydrolysed starch were all effective; sodium citrate alone was ineffective and was slightly inhibitory in the presence of sugars.

Fukumoto *et al.* (1957*a*) found that lactose and galactose were effective in stimulating α -amylase formation in the absence of a nitrogen source, but that glucose (0.4%) was not. However, if glucose was added repeatedly in low concentration, it was as effective as lactose. Further studies by these workers showed that, in the presence of peptone, 0.5% of glucose promoted almost maximal enzyme formation.

Addition of NH_4^+ ions to washed-cell suspensions

Table 3. *Effect of different carbon sources on extracellular α -amylase formation by washed-cell suspensions of Bacillus subtilis*

Incubation mixtures consisted of buffered saline containing washed *B. subtilis* cells (5 mg. dry wt./ml.), harvested from 90 hr. cultures in medium A, with carbohydrate (1%) and sodium citrate (0.04M) as indicated. Flasks were shaken aerobically at 30° for 4 hr. The cells were removed by centrifuging and α -amylase activity in the supernatants was determined. All values are corrected for a zero-time blank of 9.0 α -amylase units/ml.

Carbon source	α -Amylase activity (units/ml.)
Glucose	49.8
Maltose	59.0
Hydrolysed starch	48.2
Glucose + sodium citrate	40.4
Maltose + sodium citrate	48.8
Hydrolysed starch + citrate	46.6
Sodium citrate	22.4
None	20.2

in the presence of maltose stimulated α -amylase formation to the extent of 27%, and no other nitrogen source tested was as effective. Supplementation of the ammonia with single amino acids resulted in partial inhibition of α -amylase production whereas mixtures of amino acids or casein hydrolysate showed a more marked inhibitory effect, as might be expected from the additive effect of their concentrations (Table 4).

Nomura *et al.* (1956*a*) reported that addition of tryptic hydrolysate of casein, plus glucose, had complex effects in that it did not affect the 'basal' α -amylase formation but markedly inhibited that formation of the enzyme that was dependent on the addition of boiled-cell factor referred to earlier. In subsequent work (Nomura *et al.* 1957), however, it was shown that the addition of casein acid hydrolysate, fortified with tryptophan, cysteine and methionine, inhibited α -amylase formation in the presence of glucose as carbohydrate source although these two nitrogen sources are said to be equivalent. Fukumoto *et al.* (1957*a*) found α -amylase synthesis to be stimulated fourfold by casein hydrolysate as the sole nitrogen source and to a lesser extent by single amino acids. These experiments were, however, of 15 hr. duration at a temperature of 37°, with 0.75M-phosphate buffer present to suppress growth, so that the conditions are not directly comparable with those reported in the present work.

Effects of anaerobiosis and inhibitors on α -amylase formation. Formation of α -amylase by washed-cell suspensions of *B. subtilis* was almost completely suppressed by incubation under nitrogen instead of air (Table 5), indicating that respiratory energy was probably necessary for enzyme production (this probability was confirmed by the inhibitory effects of 2,4-dinitrophenol). The lack of any inhibition after the addition of fluoride is possibly explained by the presence of a direct oxidative pathway for carbohydrate metabolism that has been reported to occur in *B. subtilis* cells grown in a synthetic medium (Gary, Klausmeier & Bard, 1954). Chloramphenicol and *p*-fluoro-DL-phenylalanine inhibited α -amylase formation, although the effect of the latter was not very marked at the concentration used. Experiments to demonstrate reversal of fluorophenylalanine inhibition by phenylalanine are complicated by inhibitory effects of the amino acids themselves. Nevertheless the results (Table 5) demonstrate competition between the analogue and phenylalanine reasonably clearly. In separate experiments, it was established that none of the inhibitors used above had any effect on the estimation of α -amylase. A check was also made that cells did not liberate an α -amylase inhibitor during incubation in the presence of dinitrophenol.

Nomura *et al.* (1956*a*, 1957) have also observed the inhibition by chloramphenicol. The previously reported effects of dinitrophenol on α -amylase formation by *B. subtilis* are conflicting. Thus Nomura & Hosoda (1958) reported that α -amylase production by stationary-phase cultures grown in the presence of polyethylene glycol were affected little by 0.05–0.15 mM-dinitrophenol, although

incorporation of labelled inorganic phosphate into nucleic acid was reduced by 35 % by this inhibitor at a concentration of 0.05 mM. However, Fukumoto & Yamamoto (1957), studying the effects of metabolic inhibitors, found that 0.2 mM-dinitrophenol inhibited enzyme production without affecting respiration and they concluded that oxidative phosphorylation is necessary for

Table 4. *Effect of nitrogenous supplements on extracellular α -amylase formation by washed-cell suspensions of Bacillus subtilis*

Experiments were carried out as described in Table 3. Incubation mixtures consisted of buffered saline containing 1 % of maltose, washed *B. subtilis* cells (5 mg. dry wt./ml.) grown in medium A for 90 hr. and the additions shown. Values are corrected for zero-time blanks of 15.1 and 10.8 units of α -amylase in Expts. A and B respectively. The concentration of each amino acid in the experiments was 5 mM.

Expt. A	Nitrogen source	α -Amylase activity	
		(units/ml.)	(%)
	None*	46.1	79
	NH ₄ ⁺ ions	58.5	[100]
	NH ₄ ⁺ ions + L-isoleucine	54.8	94
	NH ₄ ⁺ ions + L-phenylalanine	52.9	90
	NH ₄ ⁺ ions + L-proline	51.4	88
	NH ₄ ⁺ ions + L-tryptophan	50.9	87
	NH ₄ ⁺ ions + L-methionine	48.6	83
	NH ₄ ⁺ ions + L-threonine	47.0	80
	NH ₄ ⁺ ions + L-tyrosine	44.4	76
	NH ₄ ⁺ ions + L-aspartic acid	41.3	71
	NH ₄ ⁺ ions + a mixture of above amino acids	28.5	49
Expt. B			
	NH ₄ ⁺ ions	61.3	[100]
	NH ₄ ⁺ ions + 0.5 % of casein hydrolysate + L-tryptophan	37.4	61
	NH ₄ ⁺ ions + 0.1 % of yeast extract	55.8	91

* Ammonium phosphate in saline was replaced by KH₂PO₄–Na₂HPO₄.

Table 5. *Effect of anaerobiosis and inhibitors on α -amylase formation by washed-cell suspensions of Bacillus subtilis*

Experiments were carried out as described in Table 3. Incubation mixtures consisted of buffered saline containing 1 % of maltose and washed *B. subtilis* cells (5 mg. dry wt./ml.), harvested from 90 hr. cultures in medium A, and with the additions shown. Values are corrected for a zero-time blank of 5.4 units of α -amylase/ml. (Expt. A) and 4.8 units/ml. (Expt. B).

Expt. A	Experimental conditions	α -Amylase activity	Inhibition
		(units/ml.)	(%)
	No additions	64.0	—
	Incubated anaerobically	4.6	93
	With NaF (20 mM)	69.0	0
	With 2,4-dinitrophenol (10 mM)	14.2	78
	With 2,4-dinitrophenol (0.1 mM)	39.1	39
	With chloramphenicol (100 μ g./ml.)	5.9	91
	With chloramphenicol (10 μ g./ml.)	8.7	86
	With chloramphenicol (1 μ g./ml.)	28.0	56
Expt. B			
	No additions	40.8	—
	With <i>p</i> -fluoro-DL-phenylalanine (mM)	38.0	7
	With <i>p</i> -fluoro-DL-phenylalanine (10 mM)	22.9	44
	With <i>p</i> -fluoro-DL-phenylalanine (10 mM) + DL-phenylalanine (10 mM)	28.8	30
	With DL-phenylalanine (10 mM)	35.5	13

Table 6. *Characteristics of α -amylase formation by washed cells of Bacillus subtilis grown in medium B*

Experiments were carried out as described in Table 3. Incubation mixtures consisted of buffered saline containing 1% of maltose, washed cells of *B. subtilis* (5 mg. dry wt./ml.), harvested at 35 hr. from medium B, and with the additions shown. Values are corrected for zero-time blanks as follows: Expt. A, 5.7 units/ml.; Expt. B, 4.2 units/ml.; Expt. C, 5.3 units/ml.

Experimental conditions	α -Amylase activity (units/ml.)	Inhibition (%)
Expt. A		
No additions	39.4	—
Incubated under nitrogen	1.0	98
With L-amino acid mixture*	34.9	11
Expt. B		
No additions	68.3	—
With chloramphenicol (2 μ g./ml.)	33.8	51
With chloramphenicol (20 μ g./ml.)	0.5	99
Expt. C		
No additions	81.1	—
With 2,4-dinitrophenol (0.05 mM)	68.9	15
With 2,4-dinitrophenol (0.1 mM)	56.1	31
With 2,4-dinitrophenol (0.2 mM)	35.3	57
With 2,4-dinitrophenol (0.5 mM)	12.3	85
With 2,4-dinitrophenol (mM)	2.4	97
With <i>p</i> -fluoro-DL-phenylalanine (10 mM)	43.1	47
With DL-phenylalanine (10 mM)	83.0	0
With L-phenylalanine (5 mM)	69.2	15
With <i>p</i> -fluoro-DL-phenylalanine (10 mM) + L-phenylalanine (5 mM)	67.9	16

* Mixture shown in Table 4.

α -amylase formation. Nomura *et al.* (1957) also reported that α -amylase formation was unaffected by *p*-fluorophenylalanine, the action of this analogue being studied in the presence of 0.1 mM-dinitrophenol.

Characteristics of α -amylase formation by cells grown in medium B. Some of our results were, as indicated, at variance with the findings of other workers studying α -amylase production in washed-cell suspensions. However, Nomura and his co-workers have been principally concerned with *B. subtilis* cells grown in the presence of organic nitrogen and harvested at the end of the phase of rapid growth. Consequently the different effects obtained may have been related to the different histories of the bacterial cells used. Accordingly the properties of the α -amylase-forming system of washed cells harvested from medium B at 35 hr., at which time the cells were past the phase of rapid growth, were examined (Table 6).

In general, the characteristics were the same as those already observed with cells grown in medium A. Thus formation of the enzyme was completely dependent on the presence of oxygen and was completely inhibited by chloramphenicol at a concentration of 20 μ g./ml. Dinitrophenol also inhibited and the effects of *p*-fluorophenylalanine on stationary-phase cells were essentially the same as those previously observed. Amino acids again inhibited, though not as much as with cells from

the synthetic medium. DL-Phenylalanine (10 mM) inhibited from 0 to 9% in different experiments and the L-isomer alone was a more effective inhibitor than the racemate (Table 6). The experiments shown in Table 6 provide evidence of a reversal of fluorophenylalanine inhibition by the natural analogue.

DISCUSSION

The question arises whether α -amylase formation in these experiments represents new synthesis of protein or is due to the conversion of a pre-existing protein-like precursor to the active enzyme, as has been suggested by Nomura *et al.* (1957) and supported by the work of Yoshida & Tobita (1960) and Yoshida, Tobita & Koyama (1960). It is not yet possible to answer this question with certainty but our work clearly shows that formation of the enzyme is dependent on a supply of energy. The results obtained with *p*-fluorophenylalanine suggest (but do not prove) that amino acids are involved in the process of α -amylase formation, both in the cells harvested from the synthetic medium and in the cells in the phase after that of rapid growth from the nutrient-broth medium. The sensitivity of the system to chloramphenicol is in accord with these observations, since, at the concentrations used, this compound is believed to be a relatively specific inhibitor of synthesis of bacterial protein. However, Straub,

Ullmann & Venetianer (1960) have reported that α -amylase formation in pancreas preparations, by a process postulated to involve conversion of an enzyme precursor, is also sensitive to chloramphenicol.

The above findings are not in accord with those of Nomura & Hosoda (1958) and Nomura *et al.* (1957), whose results implied that α -amylase formation by stationary-phase cells of *B. subtilis* does not require oxidative phosphorylation. The reasons for these discrepancies are not evident, though it is perhaps noteworthy that the α -amylase formation measured in many of the experiments reported by this group of workers was considerably lower (approx. 90% lower in some cases; cf. Nomura, Hosoda & Nishimura, 1958, and Fig. 2, above) than that obtained in the present work. The systems should not therefore necessarily be regarded as entirely comparable. The different findings may also be due to the fact that Nomura and his co-workers tested the various inhibitors at lower concentrations than have been found necessary here for obtaining marked effects on the system.

SUMMARY

1. α -Amylase formation has been studied in growing cultures of *Bacillus subtilis* and a requirement for Fe^{3+} ions demonstrated.

2. The distribution of α -amylase between the extra- and intra-cellular phases in washed-cell suspensions that were actively producing the enzyme has been examined. The cell-bound α -amylase remained constant at a low value whereas the extracellular enzyme rapidly increased in amount. The failure of the cells to accumulate α -amylase supports the concept that this enzyme is 'secreted' as a truly extracellular product.

3. Formation of the enzyme by washed-cell suspensions of *B. subtilis* is stimulated by a carbohydrate source and to a much lesser extent by NH_4^+ ions. Amino acids are inhibitory.

4. Formation of α -amylase by washed-cell suspensions is strongly inhibited by anaerobiosis, 2,4-dinitrophenol, chloramphenicol and, to a small extent, by *p*-fluorophenylalanine.

5. The relationship of these results to the mechanism of α -amylase formation and to the findings of previous workers is discussed.

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