A Study of the Nature of the Immediate Precursor of the Extracellular α -Amylase of *Bacillus amyloliquefaciens*

A REAPPRAISAL

By MARGARET A. GRANT* and G. COLEMAN†

Department of Biochemistry, John Curtin School of Medical Research,
Australian National University, Canberra, A.C.T., Australia

(Received 7 April 1972)

1. A defined medium was devised for use in washed-cell experiments with post-exponential-phase cultures of *Bacillus amyloliquefaciens*. The medium allowed α -amylase to be secreted, bacterial concentration to increase and L-[U- 14 C]valine to be incorporated into protein at a linear rate, which was the same as in a post-exponential-phase culture, for up to 6 h. 2. Determination of the specific radioactivity of L-[U- 14 C]valine in the medium, the intracellular amino acid pool, the cellular protein and the isolated α -amylase, after a 3 h incubation of washed cells in the defined medium, showed that at least 76% of the α -amylase secreted was synthesized *de novo*. 3. By isolating the α -amylase formed during a 6 h incubation in the presence of L-[U- 14 C]valine it was shown that the specific radioactivity of the N-terminal valine, within the limits of experimental error, was the same as that of the total valine residues from the complete α -amylase molecule. 4. A consideration of these results in relation to the whole literature on the subject strongly supports the idea that there is no reason to suppose that extracellular α -amylase is formed from a high-molecular-weight precursor in *B. amyloliquefaciens* and closely related organisms with identical characteristics of exoenzyme secretion.

The nature of the secretion of extracellular enzymes by *Bacillus* spp. has been the subject of a considerable amount of work (Lampen, 1965). In particular, the formation of α -amylase has been studied extensively in an attempt to determine the details of the mechanism of synthesis and its control and the mechanism of release of exoenzymes from bacteria (Kaiser, 1971).

The requirement for a source of energy and amino acids, inability to find enzyme activity within actively secreting cells and the effects of a wide variety of inhibitors of energy production and protein synthesis all support the idea that α -amylase is formed *de novo* immediately before its release (Fukumoto *et al.*, 1957; Coleman & Elliott, 1962; Welker & Campbell, 1963; Coleman & Elliott, 1965). Nomura *et al.* (1957) and Yoshida & Tobita (1960) reported the contrary view that α -amylase was formed from a high-molecular-weight precursor which was accumulated intracellularly before the phase of exoenzyme secretion. Lampen (1965) expressed the opinion that these reports of the existence of precursors must be viewed with some scepticism. However, it was considered important, particularly in relation to current studies on the regulation of extracellular enzyme formation, that further direct experimental verification in favour of one or the other of the two proposals should be obtained.

The work reported below consists of a study of the distribution of radioactivity between various cell fractions and in purified α -amylase labelled with

L-[U- 14 C]valine, during active secretion of the enzyme by *Bacillus amyloliquefaciens* maintained under controlled conditions in a defined environment. L-[U- 14 C]Valine was selected as the label, since valine occupies the N-terminal position in the extracellular α -amylase molecule (Yoshida & Tobita, 1960).

Materials and Methods

Chemicals

L-Amino acids were Mann Assayed grade (highest quality, chromatographically homogeneous) obtained from Mann Research Laboratories Inc., New York, N.Y., U.S.A.; maltose was Laboratory Reagent grade obtained from May and Baker Ltd., Dagenham, Essex, U.K.; casein hydrolysate and glycogen (from oysters) were both Laboratory Reagent grade products obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.; Bacto Difco Agar and Difco Complete Adjuvant were obtained from Difco Laboratories, Detroit, Mich., U.S.A.; DEAE-cellulose (DE 50) was a Whatman product (H. Reeve Angel and Co. Ltd., London, E.C.4, U.K.); L-[U- 14 C]valine (specific radioactivity 1.32 and 0.91 mCi/mg) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

* Present address: Department of Microbial Genetics, University of Sussex, Brighton BN1 9QG, U.K.

† Present address: Department of Biochemistry, University of Nottingham, Nottingham NG7 2RD, U.K.

All other chemicals were A.R. grade or the highest quality commercially available.

Methods

Growth of the organism. The aerobic spore-forming bacillus used in this work was previously described as an unclassified strain of *Bacillus subtilis*; however, it was more recently identified as *Bacillus amyloliquefaciens* strain T (Welker & Campbell, 1967). The conditions of growth of small batches of culture were as described by Coleman & Grant (1966). Thus 40 ml batches of medium contained in inverted T-tubes were inoculated from a suspension of spores in water by means of a platinum loop. The cultures were incubated at 30°C and aerated by rocking the vessels through an angle of 40° 40 times/min. When large amounts were required 500 ml batches of the same medium contained in 2-litre conical flasks were incubated at 30°C and 180 rev./min in a Gyrotory incubator-shaker (model G25; New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.).

Washed-cell incubations. Bacterial cells were harvested 30 h after inoculation of the culture medium, when exponential-phase growth had ceased, by centrifuging at 1800g for 45 s. The cells were washed in suspension medium, unless otherwise stated, and finally resuspended in suspension medium to the bacterial concentration of the 30 h culture. The suspension medium, unless otherwise indicated, was the same as the growth medium (Coleman & Grant, 1966) with the following changes in L-amino acid concentration: valine to 0.12 mg/ml, isoleucine to 0.2 mg/ml, methionine to 0.04 mg/ml and the remaining 16 amino acids (cysteine was omitted) to 0.005 mg/ml; in the text this is designated 'VIM+16AA' medium.

The entire resuspension procedure was done in 8 min or less at 30°C, at which temperature all solutions and apparatus had been equilibrated. The washed-cell suspensions (5 or 500 ml) were incubated with shaking at 30°C for the times indicated in the text, in either 100 ml conical flasks, in a water bath with a reciprocating platform, or in 2-litre conical flasks, in a Gyrotory incubator-shaker.

Bacterial concentration determinations. Bacterial concentrations were determined as described by Coleman & Elliott (1962).

α -Amylase assay. α -Amylase was determined by the procedure of Coleman & Elliott (1962).

Protein. This was assayed by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin (BDH Chemicals) as a standard.

Sedimentation-velocity studies. These were carried out on α -amylase preparations in a Beckman Spinco model E analytical ultracentrifuge with the schlieren optical system. The α -amylase preparations were made up to a concentration of 0.3–0.5% (w/v) protein

in 0.012 M-sodium veronal-veronal buffer, pH 7.5, containing 0.09 M-NaCl and 50 μ M-CaCl₂. A double-sector cell balanced against a counterpoise was used, the speed of rotation was 59740 rev./min and photographs were taken every 16 min after maximum speed was reached. During the runs the temperature, which was not controlled, was recorded with the R.T.I.C. unit and it was in the range 19.5–20°C.

Electrophoresis. Samples (20 μ g) of α -amylase were subjected to electrophoresis at 30 V/cm for 60 min on Sepharose III membranes in a Gelman rapid-electrophoresis chamber no. 51100 (Gelman Instrument Co., Ann Arbor, Mich., U.S.A.). Three different buffer systems were used: 0.12 M-sodium veronal-veronal, pH 8.6, 0.05 M-K₂HPO₄-KH₂PO₄, pH 6.5, and 0.05 M-sodium acetate-acetic acid, pH 4.5. The electrophoretograms were stained with Naphthalene Black 10 B by the method of Reisfeld *et al.* (1962), after which the background stain was minimized by washing in 3% (v/v) acetic acid.

Immunodiffusion. Two different antisera were prepared, one to a purified α -amylase preparation and the other to a crude-culture supernatant. In the first case a rabbit was injected with material eluted from a DEAE-cellulose column (see under 'Isolation of α -amylase' below); the inoculum consisted of enzyme protein at a concentration of 10 mg/ml in 0.85% (w/v) NaCl containing 5 mM-CaCl₂. The animal was injected intravenously with 0.5 ml of the preparation on the first day followed by 1 ml on alternate days for 5 days. On the 12th. and the 28th. day after the last injection 20 ml of blood was taken from the rabbit and the serum was separated. In the second case the rabbit was injected with a preparation of the crude supernatant fraction, containing α -amylase, from the post-exponential-phase culture, from which the purified preparation had been isolated. Thus, the supernatant fraction from a 39 h *B. amyloliquefaciens* culture was concentrated by freeze-drying until the α -amylase concentration was about 10 mg/ml; Difco Complete Adjuvant was then added to the resulting preparation to a final concentration of 50% (w/v). The animal was injected once with 1 ml of this inoculum and after 21 days 20 ml of blood was removed and the serum again separated.

Ouchterlony plates were prepared with 0.75% Bacto Difco Agar containing 0.15 M-Na₂N₃, in which a central well was cut surrounded by three others. A purified α -amylase preparation, containing approx. 5 mg of protein/ml, was placed in the centre well. The outer wells contained the two antisera and serum from an untreated rabbit as a control. The reaction was allowed to proceed at 37°C for 5 days.

Extraction of the free amino acids from the bacterial cells. The method used was similar to that of Hancock (1958). Thus, the bacterial cells were washed in 0.1 M-KCl, after which they were suspended in aq. 25% (v/v) ethanol to a concentration of 1.5 mg dry wt./ml. The

suspension was kept at 5°C overnight, after which it was centrifuged at 2500g for 10min at the same temperature. The pellet was stored for subsequent protein extraction. The supernatant fraction was freeze-dried, and then the residue was resuspended in 5ml of 0.75M-HClO₄. The extract obtained was neutralized with KOH and, after removal of any precipitate which might have been formed, applied to a Dowex 50 (H⁺ form) column (10cm×2cm). After washing with water the column was eluted with 2.5M-NH₃ and the eluate, containing the free amino acids, was dried down by rotary film evaporation.

Extraction of the intracellular bacterial proteins. The cell residue from the extraction of the free amino acids (see above) was suspended in 50ml of 5% (w/v) trichloroacetic acid, the suspension was centrifuged for 3min at 2500g and the supernatant fraction was discarded. The pellet was resuspended in 50ml of 70% (v/v) ethanol and maintained at 55°C for 30min; the preparation was again centrifuged and the residue suspended in 50ml of ether–70% (v/v) ethanol (1:1, v/v) for 30min at 50°C. The insoluble material was reisolated and maintained at 95°C for 30min in 50ml of 5% (w/v) trichloroacetic acid. The preparation was again centrifuged and the pellet extracted with 50ml of acid ethanol (400ml of ethanol, 100ml of water and 5ml of acetic acid) and finally with 50ml of ether. The extracted pellet was then dissolved in formic acid, dialysed exhaustively against water and freeze-dried.

Extraction of amino acids from the 'initial' medium. A 15ml sample of the 'initial' medium was adsorbed on a Dowex 50 (H⁺ form) column (10cm×2cm). After the column had been washed exhaustively with water amino acids were eluted with 2.5M-NH₃ and isolated from the eluate by rotary film evaporation.

Extraction of amino acids from the 'final' medium. A 15ml sample of the 'final' medium was mixed with 15ml of 1.5M-HClO₄, cooled to 0°C, and centrifuged. The supernatant fraction was then neutralized with KOH, the preparation was again cooled and the KClO₄ filtered off. The subsequent treatment of the filtrate was as described for 'initial' medium above.

Isolation of α -amylase. The method is based on that described by Loyter & Schramm (1962), in which the enzyme was specifically precipitated while complexed with glycogen. All operations were done at between 0°C and 3°C unless otherwise stated.

Bacterial cells were removed from their amylase-containing medium by centrifuging at 5000g for 2min. The resulting preparation was concentrated fourfold by ultrafiltration (Everall & Wright, 1958) and centrifuged at 10000g for 10min. The clarified supernatant fraction was then dialysed against 0.02M-potassium phosphate buffer, pH6.9, containing 6.6mM-NaCl and 2.5mM-CaCl₂. For convenience, the dialysed preparation was processed as described below in batches not exceeding 280ml in volume.

Ethanol at –80°C was added to the dialysed supernatant fraction at –4°C to a final concentration of 40% (v/v). The preparation was stirred throughout the addition of the ethanol and for a further 30min afterwards. It was then centrifuged at 20000g for 20min and the clear supernatant fraction removed. Then 0.2M-potassium phosphate buffer, pH8.0, was added to the clear supernatant fraction to a final concentration of 0.01M, followed by glycogen reagent, an aqueous 1.8% (w/v) solution prepared by the method of Loyter & Schramm (1962), such that there was 1mg of glycogen per 1000 enzyme units. The enzyme unit is defined by Coleman & Elliott (1962). Ethanol (–80°C) was added immediately to a final concentration of 40% (v/v); the preparation was stirred constantly during the addition of reagents and for 5min afterwards. The precipitate of enzyme–glycogen complex was isolated by centrifuging at 4000g for 5min and washed twice by resuspension in 40% (v/v) ethanol containing 0.01M-potassium phosphate buffer, pH8.0; then 1ml of buffered ethanol/mg of glycogen was added for the first wash and 0.5ml/mg for the second. Then 0.02M-potassium phosphate, pH6.9, containing 7mM-NaCl and 3mM-CaCl₂ was added to the washed precipitate to give a protein concentration of 0.3% (w/v). The mixture was then shaken gently at 25°C for 60min to allow the α -amylase to digest the glycogen. After this incubation the pH of the preparation was adjusted to 8.5 with 1M-NH₃, after which it was centrifuged at 6000g for 5min at room temperature. The supernatant fraction was removed and 1M-acetic acid added to lower the pH to 7.0; it was then made 10 μ M with respect to ZnSO₄ and dialysed against 0.02M-tris–HCl buffer, pH8.0, containing 10 μ M-ZnSO₄. After this treatment it was applied to a column (46cm×2.8cm) of DEAE-cellulose equilibrated with 0.02M-tris–HCl buffer, pH8.0, containing 5mM-CaCl₂. The column was washed with 150ml of the equilibration buffer and then eluted with a linear salt-gradient provided by 800ml of equilibration buffer and 800ml of 1.6M-NaCl in the same buffer.

Only one peak emerged from the column and the specific activity of α -amylase was constant throughout this peak. All the fractions containing enzyme activity were combined and dialysed against water before being concentrated by freeze-drying until the protein concentration was about 4% (w/v). The final specific activity of α -amylase isolated in this manner was 6900 units/mg of protein.

Determination of L-[U-¹⁴C]valine incorporation into bacterial protein. This was done as described by Coleman & Elliott (1965).

Determination of the specific radioactivity of L-[U-¹⁴C]valine in various fractions of a washed-cell suspension. All the fractions were hydrolysed by heating in 6M-HCl at 110°C for 22h in sealed tubes as described by Crestfield *et al.* (1963). The amount of

L-valine in each hydrolysate together with the radioactivity in the L-valine was determined by the method of Spackman *et al.* (1958) by using a Beckman 120B amino acid analyser with the 2ml flow cell of a Nuclear-Chicago liquid-scintillation spectrometer (model 701B) inserted between the column and the reaction coil. The specific radioactivity was calculated as counts/ μ mol of L-valine.

Determination of the specific radioactivity of the N-terminal L-valine of purified α -amylase. A sample of α -amylase (25mg) was dissolved in 1ml of 0.1M-N-allylpiperidine (prepared by the method of Men-shutkin, 1899) in pyridine and the pH was adjusted to 9.0 with 2M-acetic acid. Phenylisothiocyanate (50 μ l) was then added and the 3-phenyl-2-thiohydantoin derivative of the N-terminal valine (Val > PhNCS) was isolated as described by Niall & Edman (1962). This compound was hydrolysed by the method of Edman (1950) and the specific radioactivity of the L-valine was determined as described above.

Results

Characteristics of α -amylase formation by washed cell suspensions in the presence of different L-amino acid supplements

It was considered important that throughout this investigation the possibility of introducing unknown components into the system should be eliminated by growing and resuspending the bacterial cells in defined media.

B. amyloliquefaciens was grown as described by Coleman & Grant (1966) to the stage at which secretion of extracellular enzyme was occurring at the maximum rate. The bacterial cells were then harvested by centrifuging, washed and resuspended to the original density in a medium which differed from the growth medium by the substitution of different L-amino acids at different concentrations. The progress of α -amylase secretion in the presence of these L-amino acid supplements shows that in the absence of added amino acids a low, though progressively increasing, rate of α -amylase secretion was achieved during a 3h incubation period (Fig. 1). A low concentration of a mixture of 16 amino acids caused a doubling of the initial rate of enzyme formation, and a mixture of valine, isoleucine and methionine alone produced a further increase in rate during the latter part of the incubation. The combined effect of valine, isoleucine and methionine plus low concentrations of all the other amino acids involved in protein synthesis with the exception of cysteine ('VIM+16AA' medium) was to produce a high and linear rate of secretion throughout the incubation.

A more detailed examination of the effect of suspending post-exponential-phase cells in 'VIM+16AA' medium showed that a low but linear increase in cell density occurred over a period of up to 6h,

accompanied by a high linear rate of α -amylase secretion (Fig. 2), both processes occurring at the rates observed on the further incubation of the post-exponential-phase culture from which the washed cells had been isolated. Thus resuspending the bacterial cells in 'VIM+16AA' medium represents neither a 'shift-up' nor a 'shift-down' transition but a smooth, lag-free change to a defined environment resulting in no apparent disturbance of the biosynthetic processes of the bacteria. The further observation that L-[U- 14 C]valine was incorporated at a linear rate into the bacterial protein (Fig. 2) supports this conclusion and is further consistent with the presence of a small intracellular pool of valine with which the labelled material equilibrated instantaneously.

These characteristics seem to make the conditions defined ideal for the study of the distribution of L-[U- 14 C]valine in cellular proteins and α -amylase during the phase of active secretion of the enzyme by *B. amyloliquefaciens*.

Fig. 1. Progress of α -amylase formation by washed cells in the presence of various L-amino acid supplements

Incubations were carried out on 5-ml batches of cell suspension as described in the Materials and Methods section. The L-amino acids were present as indicated at the following concentrations: valine, 0.12mg/ml; isoleucine, 0.02mg/ml; methionine, 0.04mg/ml; 16 amino acids, each at 0.005mg/ml. Δ , No additions; o, valine, isoleucine and methionine; $\square$, 16 amino acids; $\bullet$, valine, isoleucine, methionine and 16 amino acids.

Incorporation of L-[U- 14 C]valine into various fractions of a washed-cell suspension

Post-exponential-phase cells were washed and re-suspended in 'VIM+16AA' medium containing L-[U- 14 C]valine. Samples of the various valine-containing components of the cell suspension were taken for analysis before and after a 3 h incubation period. The experiment was done twice, on separate occasions, and the results are shown in Table 1. Comparison of the specific radioactivities of L-[U- 14 C]valine in the various fractions expressed relative to the mean of that of the 'initial' and 'final' media and to the 'initial' medium alone shows that there is close agreement between the results of both experiments. The specific radioactivity of the α -amylase valine was lower than that of the free valine in the medium but it was much higher than that of the valine in the intracellular fractions. Since the rate of protein turnover and the behaviour of the amino acid pool within the cells, over the 3 h incubation period, is not known, the exact amount of α -amylase synthesized *de novo* in this washed cell suspension cannot be calculated. However, the minimum amount of enzyme synthesized from free amino acids during this period can be calculated, since the maximum specific radioactivity which valine in α -amylase could possibly have reached, even if all had been produced during the incubation, was that of the valine in the 'initial' medium. Hence the minimum fraction of the enzyme synthesized *de novo* would correspond to the

Fig. 2. Progress of L-[U- 14 C]valine incorporation into protein (○), increase in cell density (□) and α -amylase formation (Δ) by washed-cell suspensions

The cells were taken from 30h cultures grown, as described in the Materials and Methods section, under the large-scale incubation conditions in a Gyrotory incubator-shaker. Cell suspensions were prepared in 'VIM+16AA' medium containing 0.033 μ Ci of L-[U- 14 C]valine/ml as described in the Materials and Methods section except that L-valine was omitted from the washing medium. Incubations were done under the large-scale conditions and samples were taken for assay at various times over a 6h period.

Table 1. Specific radioactivity of L-[U- 14 C]valine contained in various fractions of a washed-cell suspension

Incubations were done for 3 h on a 3×500 ml scale; L-[U- 14 C]valine (specific radioactivity 0.91 and 1.32 mCi/mg) was added in Expts. 1 and 2 at zero time, in addition to unlabelled L-valine (0.12 mg/ml), to final concentrations of 0.033 and 0.048 μ Ci/ml, respectively. Experimental details were as described in the Materials and Methods section, except that L-valine was omitted from the medium used for washing the cells. The specific radioactivities for the α -amylase fraction were corrected for 'zero-time' contamination corresponding to 2.2 and 3.5% respectively of final concentrations of α -amylase reached. The purified α -amylase preparations had specific enzymic activities of 6960 and 6900 units/mg of protein respectively. Valine in the amino acid pool, intracellular protein and α -amylase was extracted after 3 h of incubation.

Fraction	Specific radioactivity of L-[U- 14 C]valine (counts/ μ mol)		Relative specific radioactivity of L-[U- 14 C]valine			
			(% of mean value for 'initial' and 'final' media)		(% of value for 'initial' medium)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
'Initial' medium	59298	75575	—	—	—	—
'Final' medium	49198	52016	—	—	—	—
Amino acid pool	27953	27201	52	43	47	36
Intracellular protein	14731	17827	27	28	25	24
α -Amylase	39429	50285	73	79	66	67

Table 2. *Specific radioactivities of L-[U-¹⁴C]valine in the N-terminal position and in the whole of the purified α -amylase molecule*

An incubation was done for 6 h on a 17 $\times$ 500 ml scale. L-[U-¹⁴C]Valine (specific radioactivity 0.91 mCi/mg) was added at zero time to give a concentration of 0.033 μ Ci/ml in the suspending medium. After 6 h of incubation the bacterial cells were removed by centrifuging and the resulting supernatant fraction was divided into two equal parts (a and b); α -amylase was isolated from each part separately as described in the Materials and Methods section. The two α -amylase preparations (a) and (b) have specific enzymic activities of 6800 and 6681 units/mg of protein respectively.

Source of L-[U- ¹⁴ C]valine	Specific radioactivity of L-[U- ¹⁴ C]valine (counts/ μ mol)	
	Preparation (a)	Preparation (b)
Total	39 675	38 907
N-terminal	39 740	37 521

ratio of the specific radioactivity of the valine in the α -amylase over the specific radioactivity of that in the 'initial' medium. The mean value of this ratio was 66.5%. However, if, for example, one took the specific radioactivity of the external valine to be the average of that in the 'initial' and 'final' media, then the mean value for minimum percentage synthesized *de novo* was 76%. Since the valine in the amino acid pool had a specific radioactivity which was only 47.5% (i.e. the mean of 52 and 43%) of the mean specific radioactivity of the valine in the external medium it is clear that all the α -amylase could have been synthesized *de novo* during the post-exponential phase of the growth cycle.

Specific radioactivity of N-terminal L-[U-¹⁴C]valine compared with that of the total L-[U-¹⁴C]valine in α -amylase synthesized in washed-cell suspension

The results in Table 1 do not support the idea of the presence of an α -amylase precursor as proposed by Yoshida & Tobita (1960). Further, on comparing the specific radioactivity of N-terminal L-[U-¹⁴C]valine with that of the total L-[U-¹⁴C]valine in the α -amylase molecule there was no significant difference (Table 2), confirming that any α -amylase formed after the addition of labelled valine was formed completely *de novo*. In this case there can be no α -amylase formation from high-molecular-weight precursor during the period of the experiment nor, indeed, is there any evidence of the existence of such material, contrary to the re-

sults of Yoshida & Tobita (1960). In view of the importance of this experiment the α -amylase preparations analysed were subjected to rigorous tests for homogeneity. Thus application of the following tests, (a) sedimentation-velocity studies, (b) immunodiffusion studies with two different antisera, (c) electrophoresis at three different pH values and (d) co-chromatography of the phenylthiohydantoin derivative of the single N-terminal amino acid with Val>PhNCS, revealed only a single component in each case.

Discussion

The evidence leading to the suggestion that α -amylase is not formed *de novo* during the phase of active secretion of the enzyme by bacterial cells but from a high-molecular-weight precursor comes from two sources.

First, Nomura *et al.* (1956, 1957) observed that α -amylase formation by washed cell suspensions of *Bacillus subtilis* was not inhibited by ethionine at concentrations that produced a 40–60% decrease in the incorporation of [³⁵S]methionine into cellular protein. Further, labelling was found in only 24% of the methionine residues of α -amylase, formed in the presence of ethionine and [³⁵S]methionine. It was concluded that in washed-cell suspensions α -amylase is produced from a complex precursor by a process that does not involve free amino acids. This conclusion was rendered unconvincing when Yoshida (1958) found that a methionine-requiring mutant of *B. subtilis* was capable of efficiently using ethionine, in place of methionine, for α -amylase synthesis without affecting the properties of the enzyme so formed.

More formidable evidence for the existence of an α -amylase precursor was published by Yoshida & Tobita (1960). These authors studied the formation of α -amylase by a leucine-requiring mutant of *B. subtilis* grown in surface culture. They observed that if, when growth ceased after 50 h, at which point the amount of α -amylase in the medium was 25% of the amount ultimately reached, [¹⁴C]leucine was added to the culture and incubation continued for a further 24 h then the specific radioactivity of the leucine in the α -amylase was only 13% of that of the free leucine in the medium and only 33% of that of the leucine residues in the general cellular protein. Yoshida & Tobita (1960) concluded that the enzyme must have been formed from a complex precursor which had been synthesized during the growth phase and stored within the cells before being converted into the active enzyme. However, their report contains ambiguities arising from the growth of the organism in surface culture which render the apparently well-founded conclusion less certain.

The first concerns whether or not the α -amylase produced by the cells diffuses rapidly and completely

out of the intact bacterial pellicle into the medium. If enzyme were retained within its structure a falsely low value for the 'zero time' enzyme activity would be obtained. However, at the time of harvesting when, presumably, the pellicle was broken up, any pre-formed 'pellicle-bound' enzyme could be extracted and equilibrated with the α -amylase finally isolated.

A second point of doubt is concerned with the rate at which the added [14 C]leucine would equilibrate with the metabolic pool of amino acids within the cells. In the work of Yoshida & Tobita (1960) the organism was, as mentioned above, grown in surface culture (in 1-litre batches of medium 1 cm deep) and the labelled amino acid was, at best, introduced beneath the pellicle and in some way dispersed evenly throughout the liquid. Although equilibration of an amino acid with cells grown in submerged culture would probably be rapid, as was illustrated in the present study, it is not obvious how quickly the [14 C]leucine would equilibrate throughout the cells of the pellicle. The situation is made even more complex, for example, by the further consideration that the uppermost cells might produce α -amylase more rapidly, owing to better aeration etc. The fact that the general cellular protein was labelled more than the α -amylase might seem to invalidate this criticism, but α -amylase synthesis occurred predominantly during the first 12 h period after addition of label, whereas 'turnover' of protein would have proceeded during the entire 24 h incubation. Any delay in the equilibration of [14 C]leucine with the cells would accordingly have a more pronounced effect on the labelling of α -amylase than on that of the cellular protein. It would seem essential that if they were to be substantiated, the experiments of Yoshida & Tobita (1960) should be carried out by using submerged cultures of the organism to eliminate these difficulties. The present study represents such an investigation and the results clearly differ from those of Yoshida & Tobita (1960).

The minimum percentage of *B. amyloliquefaciens* α -amylase synthesized *de novo*, calculated from the specific radioactivities of valine in the enzyme and external medium of a washed-cell suspension incubated in the presence of L-[U- 14 C]valine, was markedly higher than that reported by Yoshida & Tobita (1960). As the present work was done with a shaken, washed-cell suspension it was possible to estimate accurately the amount of α -amylase present in the culture at the time of the addition of the labelled amino acid, to ensure rapid distribution of the [14 C]-valine throughout the culture and constant, uniform aeration of all the cells present.

It was shown that an absolute minimum of 66.5% of the enzyme was synthesized from free amino acids in the post-exponential phase of growth. This value was less than the actual amount of enzyme that was synthesized *de novo* because it was based on the assumption

that the valine in the external medium was incorporated into α -amylase without dilution with unlabelled, intracellular valine. This latter would comprise the free unlabelled valine within the cells before the addition of the label and that arising from protein 'turnover' during incubation of the washed-cell suspension. It has been shown that the freely extractable amino acids of *Candida utilis* (Cowie & McClure, 1959), *Escherichia coli* (Kempner & Cowie, 1960) and *Neurospora crassa* (Zalokar, 1961) exist in two different pools. The composition of one of these pools reflects the relative amino acid concentrations of the extracellular medium and is variable in size. The second pool, which is more constant in size, has an amino acid composition which approximates to that of protein. However, it is uncertain to what extent the composition of each of these pools is influenced by the external medium and by protein 'turnover' and to what degree each contributes its amino acids to protein synthesis. It is evident that *B. amyloliquefaciens* must also have these two pools of intracellular amino acids; hence, even if the initial amount of valine present within the cells and the rate of protein 'turnover' were known it would not be possible to estimate more accurately the amount of the enzyme that was synthesized *de novo*. To this extent the results shown in Table 1 are inconclusive. Nevertheless, because the specific radioactivity of the α -amylase valine was 76% of the mean specific radioactivity of the valine in the external medium (almost three times greater than that in the cellular protein and much greater than that of the total extractable, free, intracellular valine), the results leave little doubt that the concept of α -amylase synthesis as put forward by Yoshida & Tobita (1960) must be open to question.

This conclusion is reinforced by the fact that the specific radioactivity of the N-terminal valine of the isolated enzyme, synthesized by washed-cell suspensions of post-exponential-phase cells in the presence of [14 C]valine, was the same as that of the valine of the whole protein, which shows clearly that in these cells the enzyme is synthesized *de novo*, after the addition of the labelled amino acid, and during the phase of active secretion.

This work was done during the tenure by M. A. G. of an Australian National University Research Scholarship. The authors are indebted to Professor W. H. Elliott for his constant advice and encouragement, to Professor L. W. Nichol for ultracentrifugal analyses and to Dr. Per Edman for the hospitality of his laboratory and supervision of the N-terminal amino acid analyses.

References

- Coleman, G. & Elliott, W. H. (1962) *Biochem. J.* **83**, 256
- Coleman, G. & Elliott, W. H. (1965) *Biochem. J.* **95**, 699
- Coleman, G. & Grant, M. A. (1966) *Nature (London)* **211**, 306

- Cowie, D. B. & McClure, F. T. (1959) *Biochim. Biophys. Acta* **31**, 236
- Crestfield, A. M., Moore, S. & Stein, W. H. (1963) *J. Biol. Chem.* **238**, 622
- Edman, P. (1950) *Acta Chem. Scand.* **4**, 283
- Everall, P. H. & Wright, G. H. (1958) *J. Med. Lab. Technol.* **15**, 209
- Fukumoto, J., Yamamoto, T. & Tsuru, D. (1957) *Proc. Int. Symp. Enzyme Chem.* 366
- Hancock, R. (1958) *Biochim. Biophys. Acta* **28**, 402
- Kaiser, P. (1971) *Bull. Inst. Pasteur Paris* **69**, 153
- Kempner, E. S. & Cowie, D. B. (1960) *Biochim. Biophys. Acta* **42**, 401
- Lampen, J. (1965) *Symp. Soc. Gen. Microbiol.* **15**, 115
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265
- Loyter, A. & Schramm, M. (1962) *Biochim. Biophys. Acta* **65**, 200
- Menshutkin, N. (1899) *Zh. Russ. Fiz.-khim. Obshchest.* **31**, 43
- Niall, H. & Edman, P. (1962) *J. Gen. Physiol.* **45**, 185
- Nomura, M., Hosoda, J., Maruo, B. & Akabori, S. (1956) *J. Biochem. (Tokyo)* **43**, 841
- Nomura, M., Hosoda, J., Yoshikawa, H. & Nishimura, S. (1957) *Proc. Int. Symp. Enzyme Chem.* 359
- Reisfeld, R. A., Lewis, U. J. & Williams, D. E. (1962) *Nature (London)* **195**, 281
- Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30**, 1190
- Welker, N. E. & Campbell, L. L. (1963) *J. Bacteriol.* **86**, 1202
- Welker, N. E. & Campbell, L. L. (1967) *J. Bacteriol.* **94**, 1131
- Yoshida, A. (1958) *Biochim. Biophys. Acta* **29**, 213
- Yoshida, A. & Tobita, T. (1960) *Biochim. Biophys. Acta* **37**, 513
- Zalokar, M. (1961) *Biochim. Biophys. Acta* **46**, 423