This dissertation has been
microfilmed exactly as received 69-10,604

MIGITA, Lloyd Kazuo, 1939-
THE PREPARATION OF A CELL FREE SYSTEM FROM BACILLUS SUBTILIS CAPABLE OF CARRYING OUT PROTEIN SYNTHESIS.

University of Hawaii, Ph.D., 1968
Chemistry, biological

University Microfilms, Inc., Ann Arbor, Michigan

THE PREPARATION OF A CELL FREE SYSTEM FROM BACILLUS SUBTILIS CAPABLE OF CARRYING OUT PROTEIN SYNTHESIS

A Dissertation Submitted to the Graduate Division of the

University of Hawaii in Partial Fulfillment

of the Requirement for the Degree of

Doctor of Philosophy

In Biochemistry

September 1968

by

Lloyd Kazuo Migita

Dissertation Committee:

Professor Kerry T. Yasunobu, Chairman
Professor Clair E. Folsome
Associate Professor Howard F. Mower
Associate Professor John B. Hall
Associate Professor Robert H. McKay

DEDICATION

To my family

and to my wife Jean

ACKNOWLEDGMENTS

To the National Defense Education Act

Fellowship for support, 1961 to 1964.

To Mrs. Tomi Haehnlen

for her competent assistance in drawing figures.

TABLE OF CONTENTS

Page

LIST OF TABLES ix

LIST OF FIGURES x

ABBREVIATIONS xii

ABSTRACT xiii

I. INTRODUCTION 1

A. α-Amylase 1

B. Protein Synthesis 3

C. α-Amylase Biosynthesis 6

II. MATERIALS AND METHODS 10

A. Materials 10

1. Materials obtained commercially 10

2. Materials obtained as gifts 11

B. Methods 12

1. Preparation of resins 12

a. DEAE-cellulose 12

b. Sephadex 12

c. Hydroxylapatite 13

2. Assay methods 13

a. Determination of α amylase activity 13

b. Determination of proteinase activity 15

c. Biuret method for proteins 15

d. Protein determination (α-amylase) 16

Page

3. Ultracentrifuge studies 16
a. Sedimentation velocity 16
b. Sucrose density gradient 17
4. Electrophoresis 17
a. Free-boundary electrophoresis 17
b. Gel electrophoresis 18
5. NH_2-terminal determination 18
6. Molecular weight determination 18
7. Bacterial cultures and harvesting 19
8. Lysozyme treatment of B. subtilis 21
9. Preparation and determination of radioactive samples 21
10. Incubation mixture 22
a. Protein synthesis 22
b. α-Amylase synthesis 22
c. Poly U directed phenylalanine incorporation 23
III. RESULTS 25
A. Purification and Homogeneity Studies 25
1. Purification of B. subtilis α-amylase 25
2. Purity studies 36
a. Ultracentrifugation 38
b. Electrophoresis 38
c. Gel electrophoresis 38
d. Amino terminal studies 43
e. Rechromatography 43

Page

3. Physical and chemical studies 43
a. Sedimentation velocity 43
b. The $E^{1\%}_{1cm}$ (280 mμ) of the B. subtilis α-amylase 54
c. Zinc analysis 54
d. Molecular weight determination 54
4. Discussion 58
B. Cell Free Protein Synthesis (B. subtilis) 60
1. B. subtilis culturing and fractionation 60
a. Growth of B. subtilis 60
b. Determination of optimum harvesting time 63
c. Fractionation of B. subtilis 63
2. Effect of subcellular fractions on incorporation of amino acids into proteins 72
a. Effect of 105,000 x g supernatant and pH 5.0 supernatant 72
b. Effect of ribosomes and pH 5.0 proteins 72
3. Effect of components of incubation mixture on protein synthesis 79
a. Magnesium ions 79
b. Ammonium ions 79
c. Sulfhydryl reagents 86
d. Amino acid mixture 86
e. C-14-labeled leucine 86
4. Other conditions for protein synthesis 93
a. Temperature 93

Page

b. pH 93

c. Incorporation time 93

d. Energy requirement 93

5. Inhibitors of protein synthesis 110

a. Enzymes and drugs 110

6. Study of _B._ _subtilis_ ribosomes 110

a. Stability of _B._ _subtilis_ ribosomes 110

b. Sucrose density studies 114

c. Electron microscope studies 114

C. α-Amylase Biosynthesis 114

1. The effect of ATP and ATP generating system on apparent synthesis of amylase 119

2. The effect of RNAse and puromycin on the production of amylase 122

3. Study of subcellular fractions 125

4. Effect of some cations and sucrose on the apparent synthesis of α-amylase 125

IV. DISCUSSION OF RESULTS 134

V. SUMMARY 142

VI. BIBLIOGRAPHY 144

LIST OF TABLES

Table		Page
I.	Amylase Purification Procedure	27
II.	Results of Amylase Purification	28
III.	Summary of Disc Polyacrylamide Gel Electrophoresis	38
IV.	Amino Terminal Amino Acid Analysis	46
V.	Summary of the Sedimentation Constants	51
VI.	Molecular Weight Determination by Sephadex Gel Filtration	55
VII.	Comparison of α-Amylase	59
VIII.	Summary of Energy Requirements for Cell Free Protein Synthesis in *B. subtilis*	101
IX.	Effect of Nucleotide Triphosphates on Cell Free Protein Synthesis	102
X.	Effect of Inhibitors of Protein Synthesis on the *B. subtilis* Cell Free System	111
XI.	Effect of Some Cations and Sucrose on Apparent Amylase Synthesis	133
XII.	Comparison of *B. subtilis* Cell Free Systems	136

LIST OF FIGURES

Figure		Page
1	*B. subtilis* α-amylase purification scheme	26
2	Chromatography on Duolite A-2 column	30
3	Chromatography on DEAE-cellulose column	32
4	Chromatography on hydroxylapatite	34
5	Photograph of crystalline *B. subtilis* α-amylase	37
6	Sedimentation patterns of α-amylase	39
7	Electrophoretic patterns of α-amylase	41
8	Chromatography on Sephadex G-75 column	44
9	Rechromatography on Sephadex G-75 column	47
10	Rechromatography on DEAE-cellulose column	49
11	Sedimentation constants of α-amylase as a function of concentration	52
12	Molecular weight determination of α-amylase by gel filtration	56
13	Submerged culture growth of *B. subtilis*	61
14	Effect of age of *B. subtilis* on subcellular protein synthesis	64
15	Effect of cell density of *B. subtilis* on subcellular protein synthesis	66
16	Lysozyme lysis of *B. subtilis*	69
17	Fractionation of *B. subtilis* components	71
18	Effect of 105,000 x g supernatant fraction on subcellular protein synthesis	73
19	Effect of the pH 5.0 supernatant fraction on subcellular protein synthesis	75

Figure		Page
20	Effect of 5.0 fraction on subcellular protein synthesis . . .	77
21	Effect of ribosomes on subcellular protein synthesis	80
22	Effect of magnesium ions on protein synthesis	82
23	Effect of ammonium ions on protein synthesis	84
24	Effect of sulfhydryl reagents on protein synthesis	87
25	Effect of amino acid mixture on protein synthesis	89
26	Effect of varying C-14-leucine on incorporation	91
27	Effect of temperature on protein synthesis	94
28	Effect of pH on protein synthesis	96
29	Effect of incubation time on protein synthesis	98
30	Effect of nucleotide triphosphates on protein synthesis . . .	103
31	Effect of GTP on protein synthesis	106
32	Effect of ammonium chloride washing of ribosomes and preincubation of ribosomes on GTP dependent protein synthesis	108
33	Effect of storing ribosomes at -20° on protein synthesis . .	112
34	Sucrose density centrifugation of _B. subtilis_ ribosomes . . .	115
35	Electron micrograph of _B. subtilis_ ribosomes	117
36	Energy requirement for apparent α- amylase synthesis	120
37	Effect of energy on protein synthesis and apparent α-amylase synthesis	123
38	Effect of RNAse and puromycin on apparent α-amylase synthesis	126
39	Effect of RNAse and puromycin on protein synthesis and apparent amylase synthesis	128
40	Effect of ribosomes and pH 5.0 fraction on the apparent α-amylase synthesis	130

ABBREVIATIONS

aa	Amino Acid
ATP	Adenosine Triphosphate
CPM	Counts Per Minute
CTP	Cytosine Triphosphate
DEAE-cellulose	Diethylaminoethyl Cellulose
DNA	Deoxyribonucleic Acid
DNAse	Deoxyribonuclease
DNP-	Dinitrophenyl-
FDNB	Fluorodinitrobenzene
GTP	Guanosine Triphosphate
M	Molar Concentration
mg	milligram
m-RNA	Messenger Ribonucleic Acid
mμ	millimicron
P.U.	Proteolytic Units
P.L.	Pacific Laboratories
RNA	Ribonucleic Acid
RNAse	Ribonuclease
s	sedimentation coefficient
$S_{20,w}$	Svedberg Units (10^{-13}), corrected to water and 20°
TCA	Trichloroacetic Acid
Tris	Trishydroxymethylaminoethyl methane
t-RNA	Transfer Ribonucleic Acid
ug	microgram
umole	micromoles
UTP	Uridine Triphosphate

ABSTRACT

α-Amylases have been obtained in highly purified crystalline forms from Bacillus subtilis (Yamamoto, Bull. Agr. Chem. Soc. Japan 19, 121, 1955; Fillig et al., Helv. Chim. Acta. 40, 529, 1957; Hagihara, Proc. Japan Acad. 27, 346, 1951).

A new purification method for obtaining crystalline α-amylase starting with a crude commercial enzyme preparation from the Pacific Laboratories strain B. subtilis has been developed. The purification method employs the use of chromatography on DEAE-cellulose, Duolite A-2, and hydroxylapatite. The crystalline enzyme is shown to be homogeneous by the criteria of ultracentrifugation, electrophoresis, amino terminal amino acid analysis, and rechromatography.

Unlike other B. subtilis α-amylase (Stein et al., J. Biol. Chem. 232, 867, 1958) which has been reported to occur as a dimer with molecular weight of 100,000 with a zinc ion holding the subunits together, Pacific Laboratories α- amylase yielded a molecular weight value of 48,700 by the Sephadex gel filtration method. Also, the amylase was shown to contain only trace amounts of zinc (0.11 moles zinc/50,000). Other studies on the chemical and physical properties are also reported.

A cell free extract capable of incorporating radioactive amino acids into hot trichloroacetic acid insoluble proteins has been prepared from the Pacific Laboratories strain B. subtilis. The endogenous activity of the subcellular system was dependent on the age of the B. subtilis culture. Thus, subcellular systems that are prepared from the exponentially growing bacteria yielded highly active systems.

The amino acid incorporation is dependent upon ATP and ATP generating system, magnesium ions, ammonium ions, an amino acids mixture, and a sulfhydryl reagent. Omission of GTP did not result in a decrease of incorporating activity as reported in other B. subtilis system (Taubman et al., 1964, In Antimicrobial Agents and Chemotherapy, p. 395).

The incorporation of amino acids is sensitive to ribonuclease and deoxyribonuclease which inhibited the system 92% and 20% respectively. The antibiotics puromycin (90% inhibition), chloramphenicol (31%), and mitomycin c (18% inhibition), also decreased the incorporation of amino acids.

The formation of α-amylase in the subcellular fractions of B. subtilis was demonstrated by following the increase of enzyme activity with time. Increase of α-amylase activity could be demonstrated in subcellular fractions isolated from both exponentially growing cells and stationary phase cells. This is in contrast to a previous report by Oishi et al. (Biochem. Biophys. Res. Comm. 8, 342, 1962) who reported increase of enzyme activity in subcellular fractions from stationary phase cells only.

The observed increase of α-amylase in the Pacific Laboratories B. subtilis system is shown to be partly due to an energy dependent system and partly to an energy independent process, presumably, the release of preformed enzyme. That part of the process is not a de novo synthesis of α-amylase is supported by the fact that addition of ribonuclease and puromycin increased the enzyme activity.

I. INTRODUCTION

A. α-Amylase

α-Amylase are enzymes catalyzing the hydrolysis of polysaccharides such as starch, glycogen, or their degradation products (Myrback and Neumuller, 1950). α-Amylase, which is classified as an endoamylase, acts randomly on the α-1-4 glucosidic linkages. However, the branching points of the polysaccharides (α-1-6 links) remain intact. The primary products of the enzymic cleavages are dextrins (oligosaccharides), maltose, glucose, isomaltose, and branched-chain low molecular weight products.

The term "alpha" was given to this endoamylase originally by Kuhn (1925), since enzymic hydrolysis of polysaccharides releases a reducing hemiacetal group which is in the α-optical configuration and mutarotates downward.

In 1811 Kirchhoff described a factor in wheat extracts which was responsible for the digestion of starch, thus making α-amylase one of the earliest known enzymes. It was then found to be present in saliva (Leuchs, 1831), in blood (Payen and Persoz, 1833) and in *Aspergillus oryzae* (Atkinson, 1881). Since these early discoveries, α-amylases have been obtained from many other sources until today they are known to exist throughout the animal and vegetable kingdom.

α-Amylases have been obtained in highly purified crystalline forms from human saliva (Meyer *et al.*, 1948), *Bacillus subtilis* (Yamamoto, 1955; Fellig *et al.*, 1957; Hagihara, 1954), *Pseudomonas saccharophilia* (Markowvitz *et al.*, 1956), hog pancreas (Meyer *et al.*, 1947), *A. oryzae* (Fischer and Montmollin, 1951), barley malt (Schwimmer and Balls, 1949),

A. candidus (Takaoka *et al.*, 1952), and rat pancreas (Heatley, 1958).

The α-amylases studied thus far constitute a rather homogeneous group of proteins whose molecular weights are approximately 50,000. They are all slightly acidic, water soluble proteins containing at least one gram atom of calcium ion, which is essential for their activity. The specific activities of the different α-amylases are of the same order of magnitude and they all seem to catalyze the same chemical reaction.

There are some distinct differences among the α-amylases. The amino acid composition of the α-amylases from different sources varies to a large extent. *B. subtilis* α-amylases differ from most other amylases so far investigated in that it contains no sulfhydryl groups or disulfide linkages (Junge *et al.*, 1959). Chloride ions or some other monovalent anions are needed for the activity of only the mammalian and possibly some bacterial α-amylases, whereas the other amylases show no such requirement. Also native *B. subtilis* α-amylase seems to occur as a dimer with molecular weight of 100,000 with zinc ion holding the subunits together, in contrast to other α-amylases which are obtained as monomers with molecular weights of 50,000 (Vallee *et al.*, 1959).

α-Amylases are stabilized to a great extent by calcium ions (Schwimmer and Balls, 1949). Calcium ions are routinely added during the purification of α-amylases (Hagihara, 1954) to stabilize the enzymes and also to promote its crystallization. Nothing definite is known about the groups involved in the binding of calcium or how this

metal protects the enzyme from chemical denaturation and proteolytic degradation. Likewise, nothing is known about the role of calcium ions, about the nature of the groups involved, or about the mechanism of action of this enzyme.

B. Protein Synthesis

Proteins are complex macromolecules, each with its own characteristic amino acid sequence, three dimensional structure, and its own specific function in cellular activities. How living cells synthesize the many different types of protein molecules has been one of the major problems in biochemistry during the last decade.

As many excellent review articles on protein biosynthesis have been published during recent years *e.g.* by Zamecnik (1962), Simpson (1962), Chantrenne (1961), Moldave (1965), Lengyel (1966), Singer and Leder (1966), Schweet and Heintz (1966) and in the Cold Spring Harbor Symposia of 1963, only the main principles will be outlined here. The evidence accumulated over the past few years supports the following hypothesis for protein synthesis. The base sequence of DNA contains the information for the amino acid sequence in a given protein. This message is transcribed into its complementary base sequence in the m-RNA. Thus, it is the m-RNA which transfers the genetic information from the DNA to the cytoplasm, where at the ultimate site of protein synthesis the base sequence is translated into a specific amino acid sequence of a protein molecule. The translation of the message is carried out by aminoacyltransfer-RNA's which bind to the m-RNA which is bound to a ribosome or polyribosome, at specific

positions determined by the nucleotide sequence of the template or messenger RNA. Nucleophilic attack by the α-NH_2 group of the incoming aminoacyl-t-RNA on the carboxyl carbon atom of peptidyl-t-RNA result in the formation of a new peptide bond. As this process is repeated, the polypeptide chains grow from the NH_2-terminal to COOH-terminal by the continued addition of amino acids. When the condensing site of a ribosome reaches the end of the m-RNA both ribosome and the finished polypeptide chain or protein falls off.

The elucidation of the mechanism of protein synthesis has come mainly from studies of subcellular systems. Using a subcellular system derived from rat liver, Zamecnik and Keller (1954) reported the incorporation of C-14-labeled amino acids into proteins. With such a system, the details of requirements for protein synthesis could be studied for the first time. Today, almost all studies on protein synthesis utilizes cell free systems.

Three different types of cell free protein synthesizing systems are commonly used.

1. The DNA-dependent systems, which are inhibited by DNAse and Actinomycin D. This system first synthesizes m-RNA which in turn directs the amino acid incorporation into proteins.
2. Systems utilizing endogenous m-RNA which is present as polysomal complex.

3. Systems in which m-RNA is added. These include systems in which homologous or heterologous messenger RNA, as well as synthetic m-RNA, is added to direct the incorporation of amino acids into proteins or polypeptides.

Cell free systems isolated from B. subtilis have not been well studied. An in vitro system which showed low activity of polynucleotide dependent amino acid incorporation into polypeptides was described by Nakai et al. (1965). These workers felt that hydrolytic nucleases and proteinases produced by this bacterium inhibited their protein synthesizing system. Recently, a more active subcellular fraction from B. subtilis strain M was described by Hirashima et al. (1967). These Japanese workers showed that the addition of diisopropylfluoro-phosphate, which is a known inhibitor of esterases, and polyamines, which are stabilizing reagents for ribosomes, to their incubation mixture, increased the activity of the system. Another cell free system from B. subtilis that has been described is the system of Taubman et al. (1963), who studied the effect of erythromycin on the in vitro system. Erythromycin was shown to partially inhibit the in vitro incorporation of amino acids into both trichloroacetic acid-insoluble material in this system.

The ultimate aim of all work on protein synthesis is to elucidate the mechanism of how a specific protein with biological activity is synthesized. The synthesis of specific proteins directed by endogenous m-RNA has been shown to occur in cell free systems derived from variety of sources, including α-galactosidase in E. coli

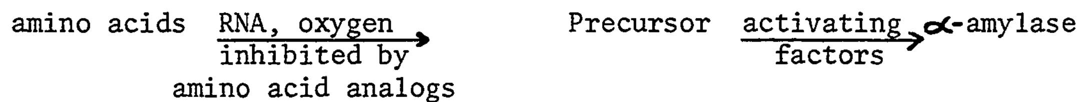
(Eisenstadt *et al.*, 1962; Bito, 1965; Nisman *et al.*, 1959), hemoglobin in rabbit reticulocytes (Knopf *et al.*, 1965; Kruh *et al.*, 1964), tryptophan synthetase in *Neurospora grassa* (Wrainwright, 1959) and *E. coli* (Murashige *et al.*, 1964) alkaline phosphatase in *B. subtilis* (Whiteley, 1963), α-amylase in *B. subtilis* (Oishi *et al.*, 1962) and α-amylase in pigeon pancreas (Redman *et al.*, 1962).

C. *α-Amylase Biosynthesis in Bacteria*

Fukumoto (1943, 1944) initiated the study of amylase biosynthesis in bacteria (*B. amyoliquefaciens*) 24 years ago. Amylase was shown to be produced at high concentrations after the stationary phase of cell growth was reached. Thus, Nomura *et al.* (1956) concluded that α-amylase formation occurs only in old cells in which normal cellular multiplication no longer occurs at the time just preceding autolysis of the cells. Optimum production of the enzyme by the bacteria required aerobic conditions, phosphate salts and specific carbon sources such as starch, lactose or galactose (Fukumoto *et al.*, 1957).

The first subcellular formation of α-amylase was described by Nomura *et al.* (1956, 1957). Using a lysozyme lysate, formation of amylase was shown to take place only if a boiled extract (shown to be related to RNA) of the bacteria is added. The amylase activity of the lysate was associated with a heavy fraction sedimenting in 15 minutes at 900xg. The lysate was still capable of oxidizing glucose or Krebs cycle intermediates (Tsuru *et al.*, 1958) and were also capable of incorporating glycine into proteins and nucleic acids.

Nomura *et al*. (1959) postulated the formation of a precursor in the synthesis of *B. subtilis* α-amylase from the following observation: (1) Incorporation of S-35-labeled methionine into amylase occurs only when intact cells are used. (2) p-Fluorophenylalanine, an inhibitor of amylase production in a washed whole cell suspension, does not inhibit the formation of α-amylase when a lysozyme lysate of *B. subtilis* is used. The following scheme has been proposed by Nomura:



Eisenstadt *et al*. (1959) and Schiff *et al*. (1959), however, could find no evidence for an amylase precursor during the induced biosynthesis of amylase by *P. saccharophil*.

The first report of α-amylase synthesis by a purified cell free system from *B. subtilis* was reported by Oishi *et al*. (1962). The Japanese workers reported a net increase in α-amylase activity when an ATP generating system, ribosomes, and a soluble fraction, prepared from cells in stationary phase was incubated for 40 minutes. In their system the boiled extract (RNA) of Nomura *et al*. (1956, 1957) was not needed. Amylase production, however, was reduced by the addition of RNAse or DNAse. Subcellular fractions prepared during the logarithmic phase of growth was found to be inactive in synthesizing α-amylase. It is evident from the above discussion that the biosynthesis of α-amylase in bacteria is poorly understood.

D. Statement of Problem

The purpose of the experiments to be described in this dissertation was to develop a cell free system from *B. subtilis* with which

both the incorporation of C-14 labeled amino acid into total protein and the synthesis of a specific enzyme could be studied.

α-Amylase was selected as the enzyme to be studied since a mutant strain of B. subtilis which produced high concentrations of α-amylase and neutral protease was available from Pacific Laboratories (Honolulu, Hawaii). The systematic study of the biosynthesis of α-amylase required that the physical and chemical properties of the enzyme isolated from the mutant bacteria be known. Determination of essential cofactors of metals as well as the amino acid composition would give some clue as to the requirements for the biosynthesis of the enzyme. In addition, the physico-chemical properties had to be determined in order to demonstrate that the enzyme synthesized by the in vitro system was identical to the enzyme produced by the intact bacteria.

Thus, work was started on the isolation of α-amylase from the Pacific Laboratories (P. L.) B. subtilis. Since large quantities of the enzyme were needed for characterization, crude commercial enzyme powder was obtained from Pacific Laboratories. At the outset, however, it became apparent that established procedures would not yield the crystalline material due to the presence of large amounts of colored impurities in the commercial product. Therefore, a modified procedure for obtaining the crystalline enzyme was worked out (see Section III A 1).

A cell free system was prepared from P. L. B. subtilis by modifying the procedure described by Bloemendal et al. (1964). Protein synthesis was measured by the ability of the in vitro system to

system to incorporate C-14 labeled as into a hot TCA insoluble fraction. When conditions had been worked out for obtaining an active *in vitro* system, studies designed to characterize the system and to determine optimum conditions for protein synthesis were carried out.

It became evident very early in the study that the cell free synthesis of α-amylase in this bacterium would be difficult. Oishi *et al*. (1963) reported that α-amylase was synthesized mainly during the late stationary phase of bacterial growth, however, preliminary experiments in our laboratory indicated that subcellular fractions from bacteria harvested during the stationary phase of growth showed very little protein synthesizing activity. On further studies, aa incorporating activity was found to be associated with subcellular fractions obtained from bacteria harvested during the logarithmic growth phase of the bacteria (Section III B 1a). Therefore, an attempt was made to resolve this contradiction. It was also our hope that the subcellular system isolated from P. L. *B. subtilis* would carry out the *de novo* synthesis of α-amylase.

II. MATERIALS AND METHODS

A. Materials

1. Materials and reagents obtained commercially

a. Aluminum Company of America, Pittsburgh, Pa.

Alumina A-305

b. Baker and Adamson, Morristown, N. J.

Calcium Acetate (H_2O), Reagent

Starch, Soluble, Powder ACS

c. Calbiochem, Los Angeles, Calif.

Adenosine 5'-triphosphate, disodium tetrahydrate

Creatine Phosphate, sodium salt

Deoxyribonucleic Acid (Pancreas)

Glutathione (reduced), cryst.

DL-Leucine-1-C-14

Lysozyme 3x cryst. (egg white)

DL-Phenylalanine-2-C-14

Ribonuclease (bovine pancreas)

DL-Valine-1-C-14

d. California Corp. for Biochemical Research, Los Angeles, Calif.

Spermidine Phosphate Trihydrate

e. Carl Schleicher and Schuell Co., New Hampshire

Diethylaminoethyl Cellulose

f. Difco Laboratories, Detroit, Michigan

Bacto Peptone

Bacto Yeast Extract

g. Fisher Scientific Co., Fair Lawn, N. J.

Cacodylic Acid

Sodium Deoxycholate (powder, purified)

h. Matheson Coleman and Bell, Cincinnati, Ohio

Orange G Indicator

i. Nutritional Biochemicals Corporation, Ohio

Casein (Hammersten Quality)

Mitomycin C

Puromycin

j. Pierce Chemical Co., Rockford, Ill.

2-Mercaptoethanol

k. Robinson Laboratory Inc., San Francisco, Calif.

Bentonite U.S.P.

l. Pharmacia Fine Chemicals, Inc., New York

Sephadex G-25, G-75, G-200

m. Sigma Chemical Co., Missouri

Creatine Phosphokinase (rabbit muscle)

Cytidine 5' triphosphate (CTP), Sodium salt

Guanosine 5' triphosphate (GTP), Type II

Polyuridylic Acid (5') Type I ammonium salt

Trishydroxymethylamino methane

Uridine 5' triphosphate (UTP), sodium salt, muscle

2. Materials obtained as gifts

B. subtilis Enzyme Powder (from Pacific Enzyme Laboratories, Honolulu).

Duolite A-2 anionic resin (from Pacific Enzyme Laboratories, Honolulu).

B. Methods

1. Preparation of resins

a. DEAE-cellulose

DEAE-cellulose, obtained as a dry powder was prepared for use as described by Peterson and Sober (1962). The dry material was allowed to sink into 1 N sodium hydroxide and the suspension was filtered on a coarse grade sintered-glass funnel and washed with additional 1 N sodium hydroxide until no more yellow color was removed. This was followed by the addition of sufficient HCl (1 N) to make a strongly acidic suspension which was filtered almost immediately and washed free of acid with deionized water. The filter cake was again suspended in 1 N NaOH, filtered and washed free of alkali with water before adjusting to the pH of the selected buffer.

b. Sephadex

All forms of Sephadex were treated as follows: The powder was suspended in 1.0% aqueous NaCl to disperse the aggregates, then allowed to stand until swelling was complete. The fines were removed by repeated decantations. The resin was then introduced to a column and washed continually until salt-free with deionized water or until the pH of the effluent was within 0.1 pH unit of the influent buffer.

c. Hydroxylapatite

Hydroxylapatite was prepared by the method of Levine (1962). Two liters of 0.5 M Na_2HPO_4 and 2 liters of 0.5 M $CaCl_2$ were run at speeds of 15 ml per minute each into a 5 liter vessel with mechanical stirring. The supernatant was sucked off and the precipitate washed four times with 3 liters of water. To the precipitate suspended in 3 liters of water were added 100 ml 40% (w/w) NaOH, and the contents were boiled for 1 hour with stirring. After 5 minutes settling the supernatant was sucked off, the precipitate stirred with 4 liters of water for 5 minutes and allowed to settle for 5 minutes. This was repeated three times. Four liters of 0.01 M phosphate buffer pH 6.8 were added, and the mixture just brought to the boil with stirring. After 5 minutes settling, the supernatant was sucked off and the operation repeated once with 0.01 M and twice with 0.0001 M phosphate buffer, each time with 15 minutes boiling. When stored in the 0.0001 M phosphate, it was stable for at least a year.

2. Assay Methods

a. Determination of α-amylase activity

Enzyme activity was determined by the method described by Stein and Fischer (1958), with slight modifications. Substrate: A 1% solution of soluble starch was made up in 0.05 M sodium acetate, pH 5.8.

Stopping Reagent: An alkaline solution of dinitrosalicylic acid was prepared as follows. Twenty g of 3,5-dinitrosalicylic were suspended in 400 ml water. Thirty-two g NaOH in 300 ml water were added dropwise with stirring and if necessary the mixture was gently heated on a water bath until a clear solution was obtained. Six hundred g of Rochelle salt (potassium sodium tartrate) and water was added to the mixture to a final volume of 2000 ml. The solution was stored at room temperature in the dark.

Assay: One ml starch was added to 1 ml enzyme solution which was diluted with 0.0005 M calcium acetate. After 3 minutes incubation at 25°, 2 ml of the stopping reagent was added. The tube was placed in a boiling water bath for 5 minutes, cooled to room temperature, and diluted with 20 ml water. The sample was read in a Beckman DU spectrophotometer at 540 mμ. The absorbance was corrected for absorbancy contributions of non-enzymatic nature by the use of an enzyme blank prepared by addition of stopping reagent before the addition of enzyme. The extinction value was converted to mg of maltose from a standard curve established with D (+) maltose hydrate. By definition, 1 unit of α-amylase released 1 mg maltose under the above conditions.

b. Casein digestion method for protease activity determination

The proteolytic activity of the *B. subtilis* proteases was determined by the method of Hagihara *et al.* (1958) using a 0.6% solution of Hammersten quality casein in suitable buffers. Most of the routine assays were made at 30° in 0.1 N NaCl pH 7.3. The reaction with casein was carried out in the following manner: After equilibrating the casein (5 ml) to bath temperature, 1 ml of the enzyme solution was added. Digestion was then allowed to proceed for ten minutes, after which the reaction was terminated by the addition of 5 ml of precipitating reagent (0.1 M trichloroacetic acid + 0.22 M NaAc + 0.33 M HAc). The mixture was allowed to stand for 30 minutes after which it was filtered by gravity. The absorbancy of the filtrate was then measured at 275 mμ in a Beckman DU spectrophotometer.

A unit of enzyme activity ($P.U.\ ^{casein}_{275\ m\mu}$) is defined as the enzyme activity which gives the extinction at 275 mu equivalent to 1 μg of tyrosine liberated in one minute at 30° (Hagihara *et al.*, 1958). The absorbance was corrected for absorbancy contributions of non-enzymatic nature by the use of an enzyme blank prepared by the addition of 5 ml of precipitating reagent to the casein before the addition of enzyme.

c. Biuret method for proteins

Proteins were measured by the method described by

Gornall *et al*. (1949) with the following modifications. Protein solution was made up to 1.5 ml with water. One and one-half ml of the biuret reagent was added and mixed. Incubation was carried out at 100° for 20 seconds, cooled to room temperature in an ice bath and read at 540 mμ in the Beckman spectrophotometer against a reagent blank of 1.5 ml H_2O plus 1.5 ml biuret reagent which had been similarly incubated. Bovine serum albumin was used as the standard protein for the standardization of the Biuret reagent.

d. Protein determination (α-amylase)

Protein concentrations for α-amylase solutions were determined spectrophotometrically with a Beckman DU spectrophotometer at 280 mμ using an $E_{1\ cm}^{1\%}$ (280 mμ) of 25.3 (Junge *et al*., 1959).

3. Ultracentrifuge studies

a. Sedimentation velocity measurements were made in a Spinco Model E ultracentrifuge equipped with a RTIC unit for temperature regulation within ± 0.1°C. The conventional 12-mm cell with a 4° centerpiece was used. The speed employed was 59,780 rpm. All runs were carried out at 22.5°. The sedimentation coefficient (s) was calculated according to the following equation:

$$s = dx/dt/xw^2$$

Where x is the distance from center of rotation to the peak, t is the time, and w^2 is the angular velocity.

The observed sedimentation velocity was reduced to standard conditions (water as solvent, 20°) according to Svedberg *et al*. (1940).

The sedimentation coefficients ($s_{20,w}$) were averaged and then plotted against initial concentration and the line extrapolated to infinite dilution by the method of linear least squares.

b. *Sucrose density gradient*

Sucrose density gradient sedimentation was carried out by the method of Britten and Roberts (1960), using 24 ml of 30-15% linear gradients centrifuging for 120 minutes at 25,000 rpm in the SW 25 rotor of a Spinco preparative ultracentrifuge. In some experiments 4.5 ml of 30-15% or 20-5% linear gradients were used at various speeds and times in the SW 39 rotor, depending on the experiment. Sucrose solutions were prepared in 0.1 M tris, pH 7.5, containing 0.14 M $MgAc_2$.

4. *Electrophoresis*

a. *Free-boundary electrophoresis*

Electrophoresis was conducted at 4° with a Perkin-Elmer electrophoresis apparatus, Model 38, equipped with a Schlieren optical system. A record of the electrophoretic behavior was obtained by use of a Polaroid Land-Back camera attached to the instrument.

The buffers were prepared by the method of Miller and Golder (1950).

b. Gel electrophoresis

Polyacrylamide gels (7.5%) were prepared in a Tris-glycine buffer system pH 8.3 according to Ornstein and Davis (1961) and run according to the procedure described by Ackrell *et al.* (1966). The maker dye was bromophenol blue. Electrophoresis of samples of proteins (about 100 to 300 μg) was carried out on gel columns (6 by 60 mm) at 2.5 ma per column at room temperature. When the marker dye had traversed approximately three-fourths of the gel column, the gel was removed from its glass tube and cut transversely at the position of the marker dye band. The ratio of the distance traveled by a protein band to that traveled by the marker dye was recorded as a R_f value.

5. NH_2-terminal determination

a. Dinitrofluorobenzene (FDNB) method

The method of Sanger as described by Fraenkel-Conrat (1958) was used for the determination of the NH_2-terminal amino acid residue. The ether phase was chromatographed in the upper phase of the solvent system, tertiary-amyl alcohol: 3% NH_3 in the first dimension and the 1.5 M phosphate buffer (pH 6.0) system in the second. No corrections were applied for manipulative loss or destruction during acid hydrolysis.

6. Molecular weight determination

a. Estimation of molecular weight by gel filtration

The molecular weight of α-amylase was estimated by a

modified gel filtration technique similar to that described by Andrews (1964, 1965) and Whitaker (1963). Sephadex G-100 was allowed to swell in 0.06 M phosphate buffer, pH 7.0 for five days. The gel was then packed in 2 x 50 cm column and equilibrated with the same buffer. Blue Dextran 2000, an artificial, colored dextran (M_W 2,000,000) which is completely excluded from all types of Sephadex was used to determine the void volume of the column. After calibrating the column with proteins of known molecular weights, the α-amylase was applied to the column.

The elution volume (V_e) of each substance was defined as the effluent volume corresponding to maximum concentration of the solute as determined by optical densities at 280 mμ. Fractions of approximately 1 ml were collected and the optical densities at 280 mμ were determined and plotted against the total effluent volume. The elution volume was estimated by extrapolation of both sides of the solute peak to an apex.

Calculation of the molecular weight was done by the method of Whitaker (1963).

7. Bacterial cultures and harvesting procedures

A stock culture of *B. subtilis* was obtained from Pacific Laboratories. The organism was first cultivated in 40 ml of medium containing 1% soluble starch, 0.5% Bactopeptone, 1%

yeast extract, 1.3% ammonium phosphate (dibasic), 0.2% sodium citrate, 0.1% NaCl, 0.05% magnesium sulphate, 0.05% potassium chloride, 0.005% calcium chloride, and 0.005% manganese sulphate, adjusted to pH 6.8. After 10 to 12 hours growth at 37°, the 40 ml were used to inoculate 6 liters of the same medium which was contained in a 25 liter bottle. The culture was grown at 28-30° with vigorous aeration and shaking. Growth was stopped by the addition of crushed ice to the culture at various times depending on the experiment. To obtain active amino acid incorporating activity, cells were harvested while in the early phase of logarithmic growth (7-8 hours with absorbance reading at 640 mμ about 0.9 to 1.4). Cells were usually harvested with a Sharples continuous flow centrifuge. The cells were washed once with deionized water at 4° and with standard buffer which consisted of 0.01 M Tris-HCl buffer, pH 7.5 containing 0.012 M magnesium acetate, 0.04 M ammonium chloride, and 0.004 M mercaptoethanol. Standard buffer is a modification of the buffer described by Taubman et al. (1964). The yield of cell paste harvested during the logarithmic phase ($O.D._{640\ mu}$ = 1.1) was approximately 8-12 grams wet weight.

Freshly harvested cells were used immediately in the preparation of subcellular fractions. All procedures were carried out at 4°C unless otherwise stated. Cells were ruptured in either of two ways.

In initial experiments, the cells were ground with alumina at 0° as previously described for the *B. subtilis* experiments (Taubman *et al*., 1963). Later, lysozyme treatment was more frequently used.

8. *Lysozyme treatment of B. subtilis*

To each gram of washed bacterial cells 2 ml standard buffer (II B 7), 3 mg lysozyme (Cal Biochem), 0.2 mg DNAse (Cal Biochem) and 4 mg bentonite (Robinson Laboratory, Inc.) were added. The mixture was then incubated for 45 to 60 minutes at 19°, until the optical density reading at 570 mμ reached a constant minimum level (see Results and Figure 16).

9. *Preparation and determination of radioactive samples*

After precipitating the radioactive samples with 1.2 ml 10% TCA containing carrier unlabeled amino acid (2 mg/ml) the samples were treated in the following way:

1) Incubated 15 minutes at 90°.
2) Washed samples three times with 5 ml of 5% TCA containing 1 mg/ml carrier amino acid.
3) Washed sample with 5 ml ethanol-ether (3:1 v/v).
4) Dried overnight in desiccator *in vacuo*.

All centrifugations during the washing procedure were done in the Servall centrifuge for 15 minutes at 12,500 x g.

The washed and dried radioactive samples were dissolved in 0.2 ml 98% formic acid. Samples were poured into nickel plated steel planchets, and dried under a heat lamp. Samples were counted with a Nuclear Chicago Model D 47 counter equipped

with an automatic sample changer. Efficiency of the gas flow counter is approximately 17%.

10. Incubation mixtures

a. Incubation mixture for incorporating amino acids into proteins

The reaction mixture was a modification of that described by Taubman *et al*. (1964). The subcellular system was composed of ribosomes and pH 5.0 fraction (about 2 to 3 mg protein) freshly isolated from *B. subtilis*. The reaction was carried out in a final volume of 1 ml containing the following in µmoles, unless otherwise stated. Tris pH 7.5, 100; ammonium chloride, 50; magnesium acetate, 14; mercaptoethanol, 4; ATP, 1; creatine phosphate, 5; creatine phosphokinase, 0.05 mg; GTP, 0.05; CTP, 0.05; UTP, 0.05; amino acid mixture (no leucine), 0.072 µmoles of each amino acid; DL-Leucine-1-C-14 ($\frac{12.1 \text{ mC}}{\text{mmole}}$), 1 µcurie or 82 mµmole.

The incubation was usually carried out at 37° for 15 minutes. The reaction was stopped by the addition of 1.2 ml 10% TCA containing 2 mg leucine per ml.

In some experiments, CTP and UTP were omitted from the reaction mixture.

b. Incubation mixture for α-amylase synthesis

Subcellular synthesis of α-amylase was carried out in a final volume of 2.0 ml containing 6 to 10 mg of

freshly isolated ribosomal fraction and the pH 5.0 fraction. Moreover the system contained the following in µmoles unless otherwise stated. Tris pH 7.5, 200; ammonium chloride, 100; magnesium acetate, 28; mercaptoethanol, 8; sucrose, 200; manganese sulphate, 10; calcium acetate, 10; ATP, 5.0; GTP, 0.44; creatine phosphate, 30; creatine phosphokinase, 0.15 mg.

The reaction was started by the addition of the ribosomal and pH 5.0 proteins at 37°. Aliquots were removed at various times and the activity of α-amylase determined by the procedure described in Section II B 2a under Methods.

c. Method for poly U directed phenylalanine incorporation

Various methods were used to follow the polyuridylic acid directed incorporation of labeled phenylalanine.

1. The mixture described in Section II B 10a was preincubated for 15 minutes. Unlabeled leucine was added in place of C-14-leucine. After adding polyuridylic acid (25.9 ug) and 1 µcurie C-14-phenylalanine, the mixture was incubated at 37° for 15 minutes. The reaction was stopped by the addition of TCA as previously described. In some experiments the mixture was dialyzed for 3 hours against standard buffer after the preincubation.
2. A 30,000 x g extract of *B. subtilis* was prepared. The 30,000 x g extract was incubated with the

added amino acid mixture, and energy source for 30 minutes at 37°. The mixture was then dialyzed against the standard buffer for 3 hours. Then the mixture described in Section II B 10a was prepared and incubated with the exception that the amino acid mixture and C 14-leucine was left out and in its place poly U and C 14-phenylalanine was used.

III. RESULTS

A. Purification and Homogeneity Studies

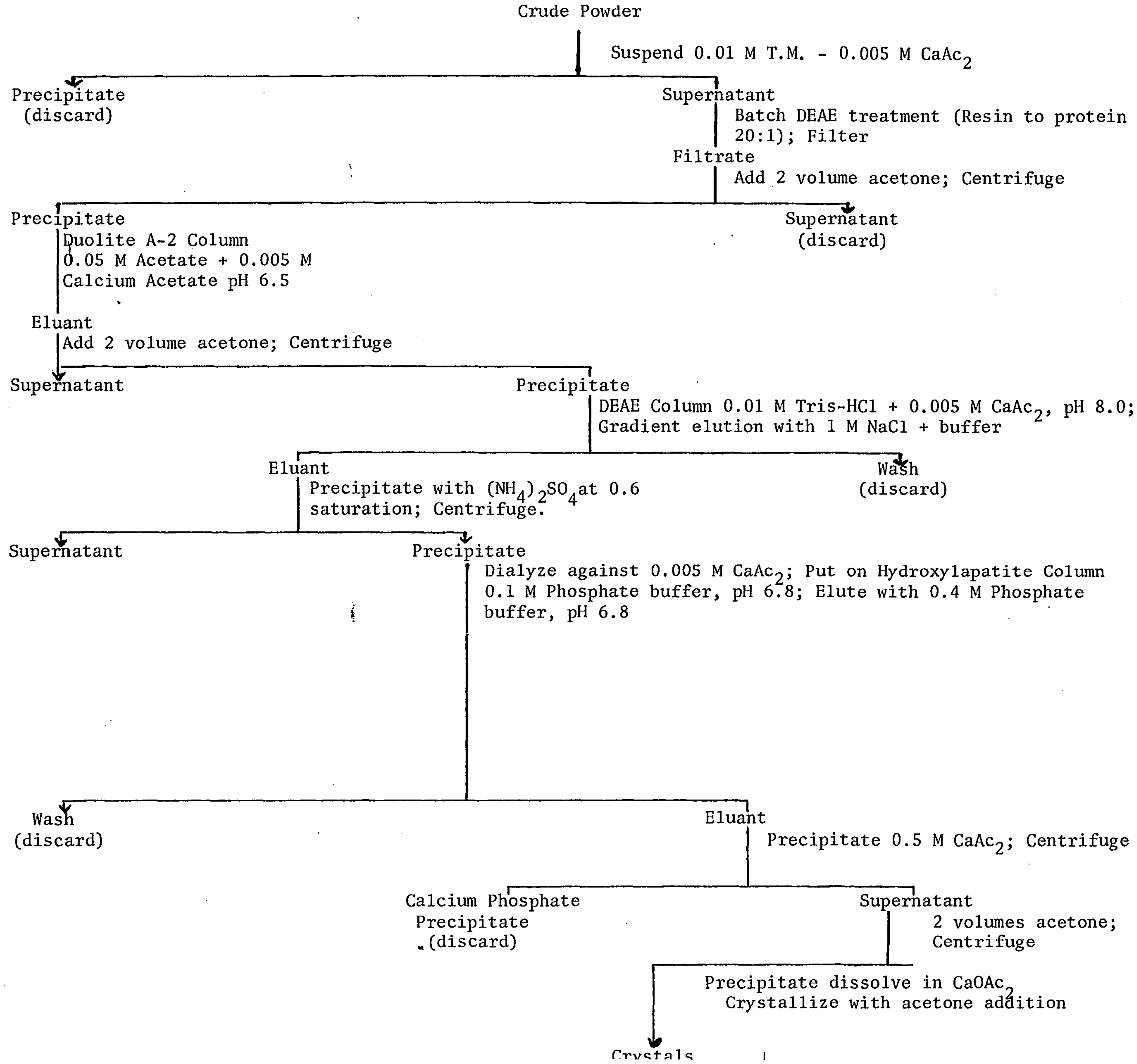
1. Purification

One of the major problems encountered during the purification of B. subtilis α-amylase was the presence of colored materials and pigments in the starting material. These colored contaminants in the commercial enzyme powder were introduced by the surface culture bacterial growth technique used and the enzyme extraction method employed. Preliminary investigation showed that the removal of these colored contaminants was necessary to obtain the crystalline enzyme. Thus, many of the steps employed in the purification procedure were directed toward the removal of these colored materials.

The flow sheet for the purification of B. subtilis α-amylase using a crude enzyme powder from Pacific Laboratories (Honolulu) is shown in Figure 1, while the detailed explanation of the procedure is given in Table I. Table II gives the results of one purification in which 50 grams of crude enzyme powder were used.

The batch-wise addition of DEAE cellulose to the black enzyme solution was an excellent method for the removal of much of the colored material. The DEAE cellulose absorbed much of the colored contaminant and extraneous protein (between 50-80%, depending on the experiment) while leaving most of the α-amylase in solution.

Acetone fractionation of the proteins proved to be a valuable tool for the rapid concentration of the enzyme as well as for the removal of some colored contaminants. Acetone precipitation was


Crude Powder
Suspend 0.01 M T.M. - 0.005 M CaAc2
Precipitate (discard)
Supernatant
Batch DEAE treatment (Resin to protein 20:1); Filter
Filtrate
Add 2 volume acetone; Centrifuge
Precipitate
Supernatant (discard)
Duolite A-2 Column
0.05 M Acetate + 0.005 M Calcium Acetate pH 6.5
Eluant
Add 2 volume acetone; Centrifuge
Supernatant
Precipitate
DEAE Column 0.01 M Tris-HCl + 0.005 M CaAc2, pH 8.0;
Gradient elution with 1 M NaCl + buffer
Eluant
Wash (discard)
Precipitate with (NH4)2SO4 at 0.6 saturation; Centrifuge.
Supernatant
Precipitate
Dialyze against 0.005 M CaAc2; Put on Hydroxylapatite Column 0.1 M Phosphate buffer, pH 6.8; Elute with 0.4 M Phosphate buffer, pH 6.8
Wash (discard)
Eluant
Precipitate 0.5 M CaAc2; Centrifuge
Calcium Phosphate Precipitate (discard)
Supernatant
2 volumes acetone; Centrifuge
Precipitate dissolve in CaOAc2
Crystallize with acetone addition
Crystals

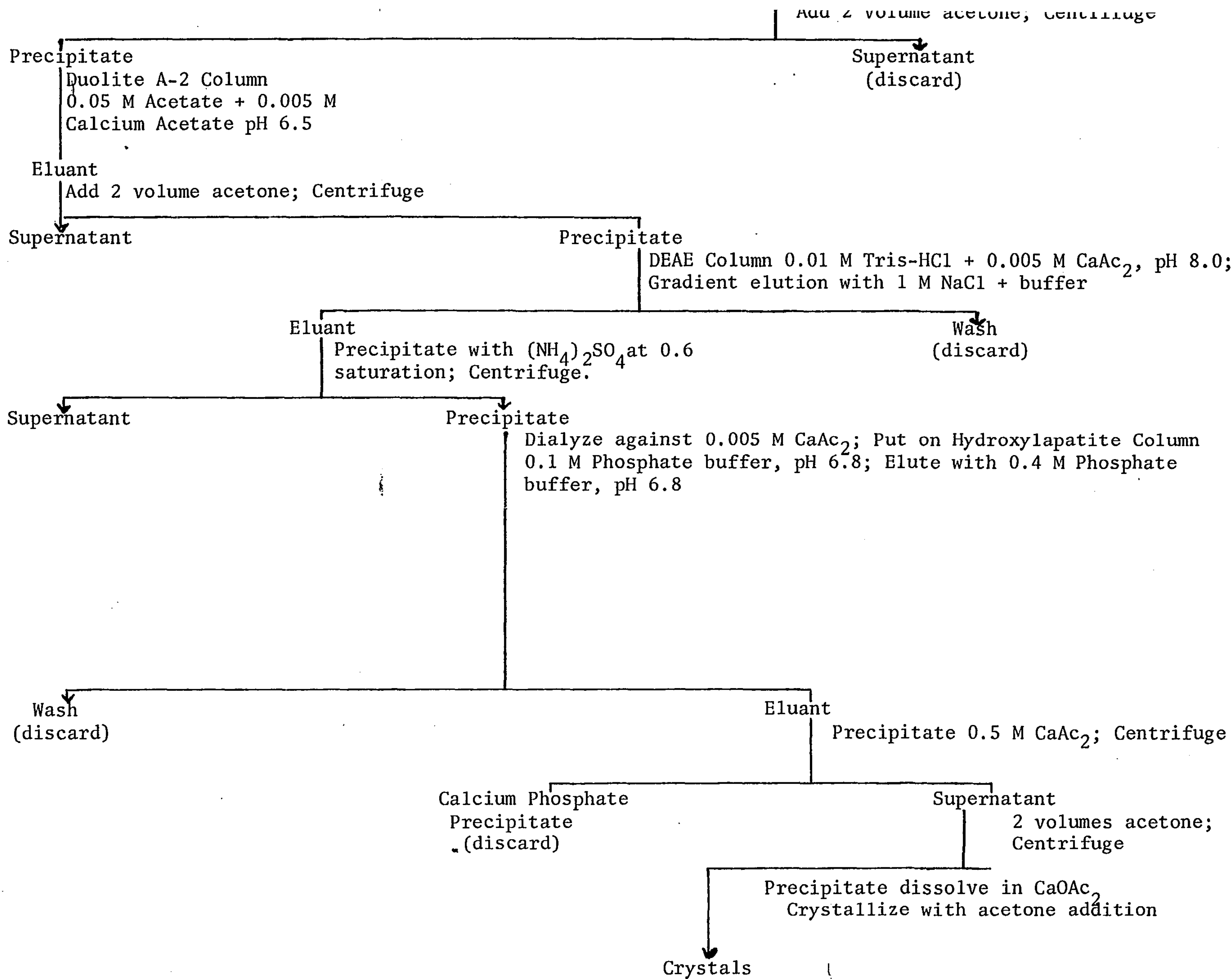


Figure I

Purification of B. subtilis of α-amylase

TABLE I

STEP NO.	DESCRIPTION
1	All steps were carried out at 4° unless otherwise stated. Crude powder suspended in 0.01 M Tris-maleate + 0.005 M calcium acetate, pH 6.8 (30 ml per gram of powder) for 1 hour. Centrifuge (16,000 x g) to remove insoluble material.
2	Add batchwise pH 6.8 equilibrated DEAE (with above buffer) at resin to protein ratio of 20:1. Filter by suction through sintered glass funnel after mixing suspension for 1 hour. Add more buffer (30 ml per gram of powder), stir 1/2 hour and filter.
3	Filtrate is made up to 66% with acetone (final v/v) by slow addition at 0°. Acetone precipitate is collected by centrifugation at 16,000 x g after standing overnight in the cold room.
4	Acetone was removed from the precipitate in an air stream at 4°. The precipitate was then dissolved in minimum amount of 0.05 M acetate + 0.005 M calcium acetate pH 6.5. Buffered protein was passed through a column of Duolite A-2 (3 x 50 cm) equilibrated with the above solution.
5	Partly decolorized eluant was precipitated with acetone 66% (final v/v) at 0°. After 4 hours, acetone precipitate is collected by centrifugation at 16,000 x g.
6	Acetone was removed in air stream at 4°. The precipitate was dissolved in minimum amount of 0.01 M Tris-HCl + 0.005 M calcium acetate, pH 8.0.
7	Buffered protein solution was applied on DEAE cellulose column (3 x 45 cm) equilibrated with the above buffer. After washing with 150 ml of buffer, gradient elution was started with 1000 ml buffer and 1000 ml 1 M NaCl + buffer. After discarding the wash, the following protein peak was precipitated by the addition of solid ammonium sulphate to 0.6 saturation (456 g/l). After standing overnight the precipitate was collected by centrifugation at 16,000 x g.
8	Precipitate was dialyzed against 0.005 M calcium acetate solution pH 7.0. Crystallization attempted by the dropwise addition of acetone. If the dialyzed protein solution is too dilute the protein is first concentrated by acetone precipitation as described above (Step 3). If there is too much colored material in the preparation crystallization is difficult so another purification step is added.
9	Colored protein solution was put on hydroxylapatite column (2x 30 cm) equilibrated with 0.1 M phosphate buffer pH 6.8. After passing the same buffer through the column, the first protein peak washed off, the enzyme was eluted off the column by the addition 0.4 M phosphate buffer. Calcium acetate 0.5 M was added to the eluted enzyme solution in a 1:1 (v/v) ratio. The pH was adjusted to 6.5 with 6 N NaOH and the solution was kept at -20° for half an hour. The calcium phosphate precipitate was removed by centrifugation. The precipitate was washed with 0.25 M calcium acetate. The wash and supernatant were precipitated with acetone, 66% (v/v), and crystallized as described in Step 8.

TABLE II

Amylase Purification

	Step	Volume ml	Activity units x 10^6	Protein (mg)	Specific activity ($\frac{units}{mg}$)	Recovery %
1	Crude Solution	1,450	17.4	46,990	474	100
2	DEAE cellulose treated enzyme	2,390	12.4	10,277	1,210	71
3	Acetone precipitate	225	12.8	10,200	1,244	74
4	Duolite A-2 column	500	10.4	7,060	1,473	60
5	Second Acetone precipitation	100	10.6	6,700	1,582	61
6	DEAE cellulose chromatography	300	5.4	2,880	1,840	34
7	Dialyzed $(NH_4)_2SO_4$	66	5.3	2,400	2,200	31
8	Hydroxylapatite Column	164	4.7	2,136	2,200	27
9	Supernatant $Ca_3(PO_4)_2$ precipitate	318	4.3	1,620	2,647	25
10	Crystalline Enzyme	40	2.1	750	2,800	12

carried out by the slow addition of cold acetone (cooled in freezer at -20°) so that the temperature did not rise above 0°. Acetone was usually removed from the precipitate by passing a gentle stream of air over it.

The protein solution was decolorized further by passing it through Duolite A-2, an anionic resin (Figure 2).

Some of the proteinase could be separated from the amylase by passing the protein solution through DEAE-cellulose column at pH 8.0. Figure 3 shows the elution pattern from the DEAE-cellulose column chromatography step. The first peak coming through with the wash showed low proteinase activity. The amylase activity was then eluted by gradient elution with increasing NaCl concentrations.

Addition of ammonium sulfate to 0.6 saturation removed most of the α-amylase activity while leaving most of the colored impurities in solution. After dialyzing the ammonium sulfate precipitate, crystalline enzyme could be obtained in some of the preparation by the dropwise addition of acetone. This, however, could be done only in preparations which were almost completely decolorized.

More often than not, another purification step had to be employed before crystalline enzyme could be obtained. Hydroxylapatite column chromatography was used for the last purification step. Figure 4 illustrates the chromatography of the enzyme on hydroxylapatite at pH 6.8. After removing the phosphate ions

Figure 2. Chromatography of crude enzyme on Duolite A-2 column. A Duolite A-2 column (3 x 50 cm) equilibrated with 0.05 M acetate buffer, pH 6.5 containing 0.005 M calcium acetate, was charged with 10 grams of protein containing 12.8×10^6 units. Elution was carried out with the equilibrating buffer mentioned above. The flow rate was 20 ml per hour and fractions of 10 ml were collected. 0-0-0 represents the absorbance at 280 mμ while x-x-x represents the α-amylase activity.

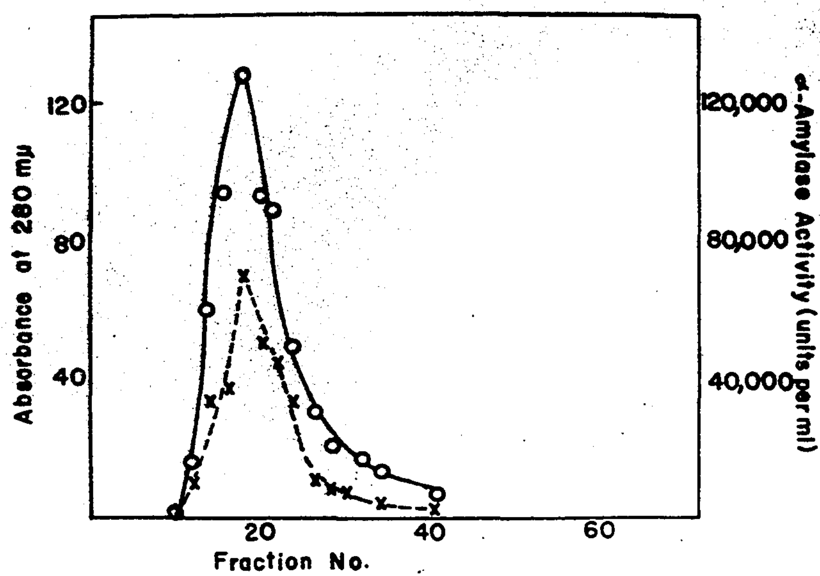


Figure 2

Figure 3. Chromatography of acetone precipitate on DEAE-cellulose column. A DEAE cellulose column (3 x 45 cm) was equilibrated with 0.01 M Tris-HCl buffer pH 8.0, containing 0.005 M calcium acetate. After charging the column with 6.7 grams of protein, the column was washed with 150 ml of the starting buffer. Gradient elution was then started at the point indicated on the figure with 1 liter of buffer and 1 liter of 1 M NaCl + buffer. The flow rate was 20 ml per hour and fractions of 5 ml were collected. 0-0-0 represents the absorbance at 280 mμ while x-x-x represents the α-amylase activity.

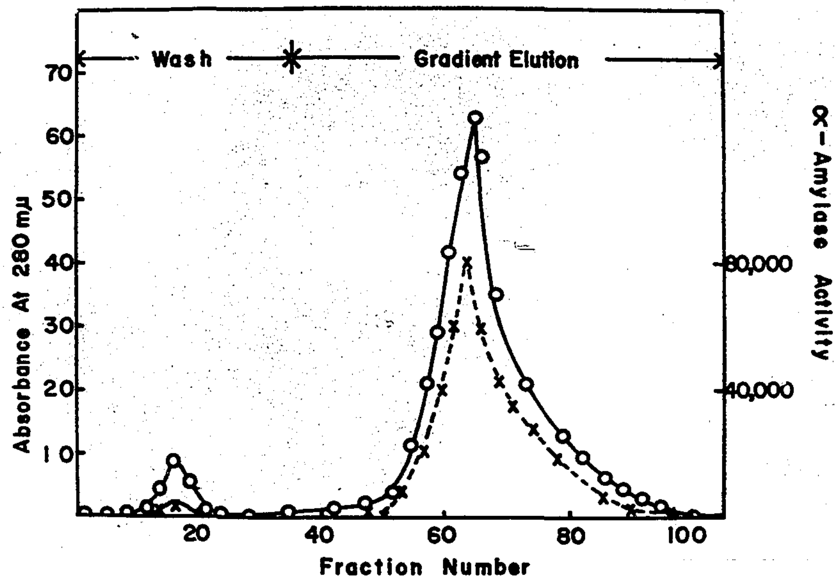


Figure 3

Figure 4. Chromatography of amylase on hydroxylapatite column. A hydroxylapatite column (2 x 30 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 6.8 was charged with 2.4 grams amylase containing 5.3×10^6 units. The flow rate was 4 ml per hour and fractions of 2 ml were collected. After washing the column with 120 ml of starting buffer, 0.4 M phosphate buffer, pH 6.8 was added. The 0.4 M eluant was collected and utilized in further studies. 0-0-0 and x-x-x represent absorbance at 280 mμ and amylase activity (units per ml), respectively.

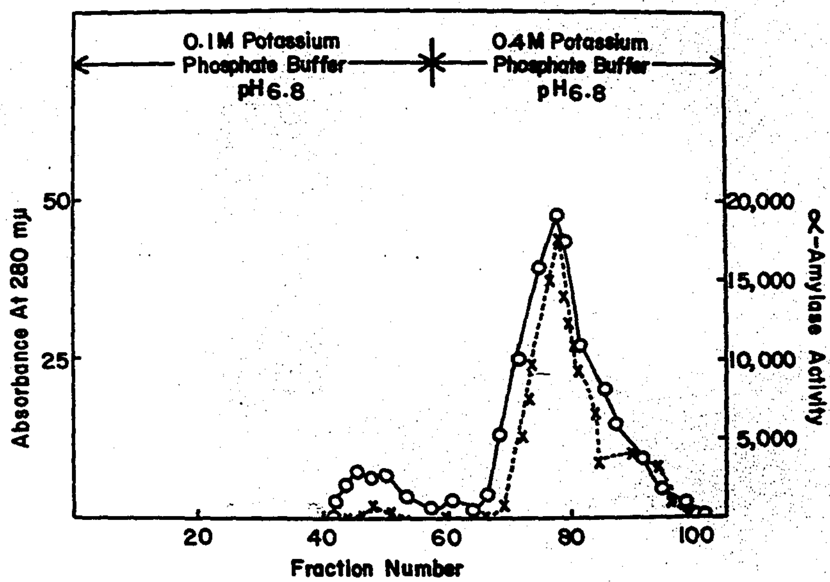


Figure 4

(phosphate buffer) from the eluant by precipitation with calcium ions, the enzyme could be readily crystallized.

The Pacific Laboratories *B. subtilis* α-amylase crystals were obtained as needles as shown in Figure 5. The crystalline enzyme represented approximately 10% of the starting enzyme activity.

The average time required for a large scale preparation (50 grams starting material) was approximately two weeks.

2. Purity studies

Although some degree of purity must be attained before an enzyme can be crystallized, crystallization of protein *per se* does not necessarily mean that it is uncontaminated with respect to other proteins. It has been demonstrated that some preparations of crystalline amylases, particularly those of hog pancreas and *B. subtilis* (Stein and Fischer, 1958), were still contaminated by traces of proteases even after repeated crystallization. Thus, independent physical and chemical methods must be utilized as criteria for indicating the homogeneity of a protein preparation. Li (1951) and Pirie (1940) have discussed the problems involved in the determination of purity of proteins.

In this present study the purity of Pacific *B. subtilis* amylase was determined by utilizing the criteria of ultracentrifugation, disc electrophoresis, free boundary electrophoresis, rechromatography on columns, and amino-terminal amino acid analysis.

Figure 5

B. subtilis α-amylase crystals.

a. Ultracentrifugation studies

Sedimentation patterns of α-amylase at pH 6.0 are shown in Figure 6. Examination of the sedimentation patterns show no gross heterogeniety during its sedimentation indicating that the once recrystallized α-amylase is relatively homogeneous with respect to size and shape.

b. Free boundary electrophoresis

Electrophoresis of once recrystallized α-amylase at pH 6.7 is shown in Figure 7. It is apparent from the migration patterns that the crystalline enzyme is monodispersed and homogeneous electrophoretically at this pH.

c. Gel electrophoresis

Disc electrophoresis using polyacrylamide gel is a very powerful tool in demonstrating the purity of proteins, as well as for separating multiple forms of the same enzyme. Under the conditions described in methods (Section II B 4b) at pH 8.3, α-amylase migrated as a sharp single band with an average R_f of 0.47 as shown in Table III.

TABLE III
Summary of Disc Polyacrylamide Gel Electrophoresis

Preparation no.	ug Enzyme[a*]	Distance to marker dye (cm)	Distance to amylase band (cm)	R_f
1	120	9.40	4.52	0.48
2	150	9.40	4.44	0.47
3	200	8.13	3.81	0.47

a* Protein concentration was determined by the absorbance at 280 mμ using a $E_{1}^{1\%}$cm of 25.3.

Figure 6. The sedimentation patterns of the B. subtilis α-amylase.

a. A 0.66% solution of 1 x recrystallized amylase (specific activity 2,400) was prepared in 0.1 M NaCl + 0.005 M calcium acetate pH 6.0 and was centrifuged at 59,780 rpm at 22.5°. The photographs were taken at 0.73, 8.73, 16.73, 24.73, and 40.73 minutes after reaching full speed. The bar angle in all photographs was 50°. In the series of photographs, the peak is sedimenting from right to left.

b. A 0.37% solution of amylase was prepared and centrifuged as described above. The photographs were taken at 1.32, 17.32, 33.32, 49.32, and 65.32 minutes after reaching full speed. The bar angle for photograph 1 was 45° and was 40° for the rest of the photographs.

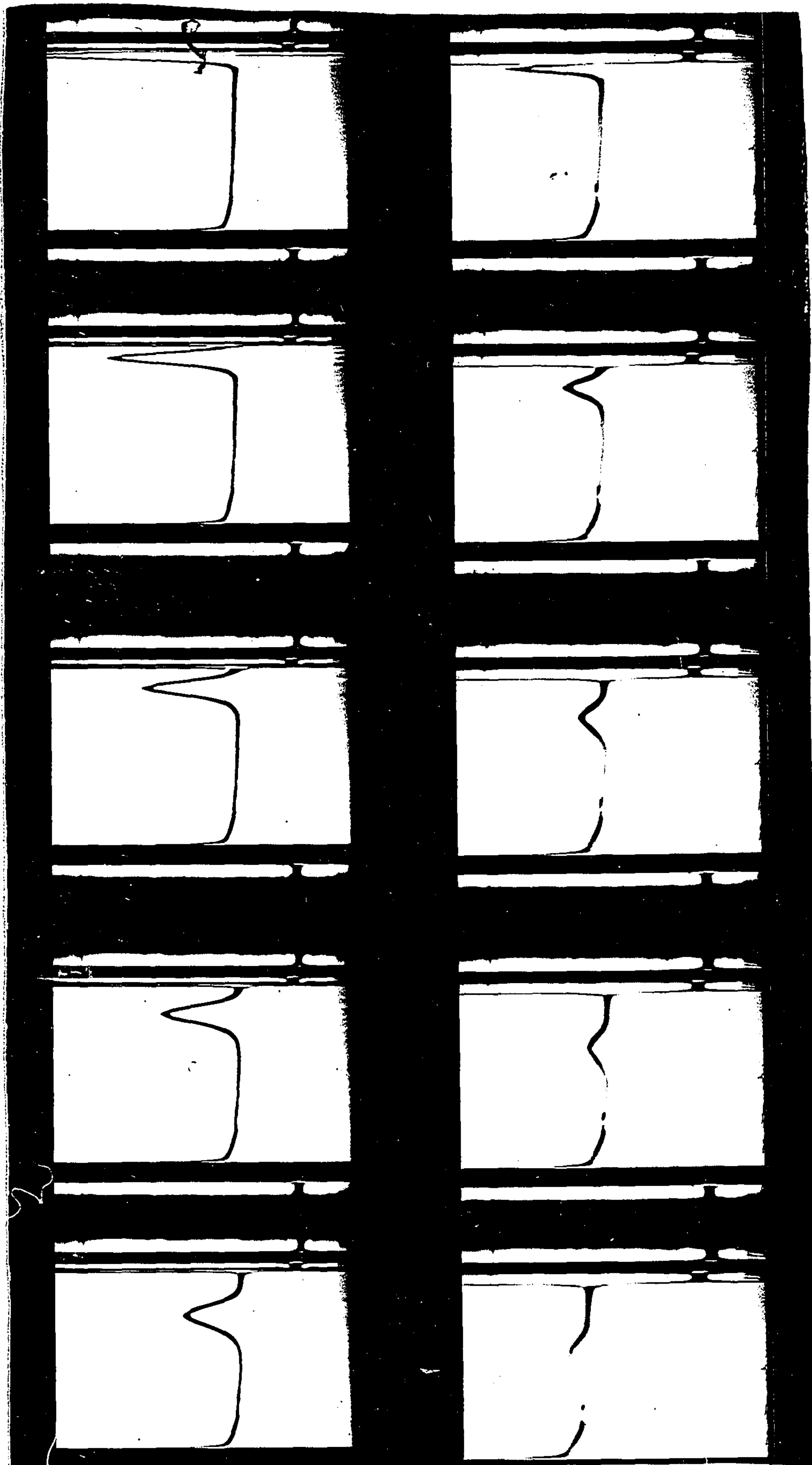


Figure 7. Electrophoretic patterns of Pacific Laboratories α-amylase. The 1x recrystallized α-amylase (specific activity 2500) was prepared in cacocylic buffer (0.1 ionic strength), pH 6.37 containing 0.005 M calcium acetate. Descending boundary at 0, 30, 60, and 90 minutes are shown below (a, b, c, and d, respectively).

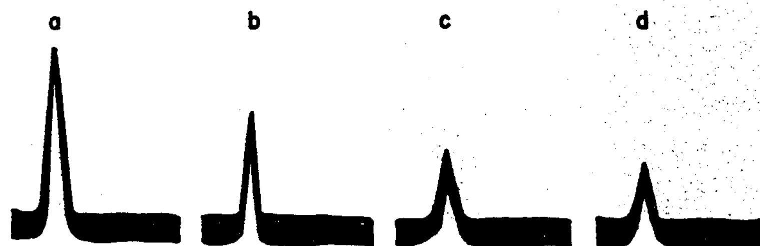


Figure 7

d. Amino terminal studies

Initial qualitative amino terminal analysis on the one time recrystallized α-amylase showed valine as the major amino terminal amino acid. However, the enzyme preparation was contaminated with traces of serine, threonine, aspartic and glutamic acids. These minor components could be partially removed by passing the enzyme through Sephadex G-75 (Figure 8) and almost completely eliminated by first precipitating the enzyme with 5% TCA, followed by two 5% TCA and four acetone washes of the precipitate. This suggests that the minor components represent low molecular weight peptides or amino acids which are adsorbed to the crystalline enzyme. Table IV shows the results of the amino terminal analysis of different preparations of TCA washed α-amylase.

e. Rechromatography of α-amylase

The rechromatography of α-amylase on Sephadex G-75 and DEAE cellulose is shown in Figures 9 and 10, respectively. Its rechromatography as a single symmetrical peak and a high recovery of the activity are indicative of the homogeneity of the enzyme.

3. Physical and chemical properties of α-amylase

a. Sedimentation velocity studies

Table V summarizes the values obtained for the sedimentation coefficient of the *B. subtilis* α-amylase in 0.1 N NaCl + 0.005 M $CaAc_2$, pH 6.0 at 22.5°C. Figure 11 illustrates these

Figure 8. Chromatography of one time recrystallized amylase on Sephadex G-75 column. Sephadex G-75 column (2 x 35 cm) equilibrated with 0.005 M calcium acetate was charged with 8 mg of the protein. The flow rate was 2 ml per hour and fractions of 2 ml were collected. Fraction 7 to 13 were pooled and used for further studies. 0-0-0 and x-x-x represent absorbance at 280 mμ and amylase activity per ml respectively.

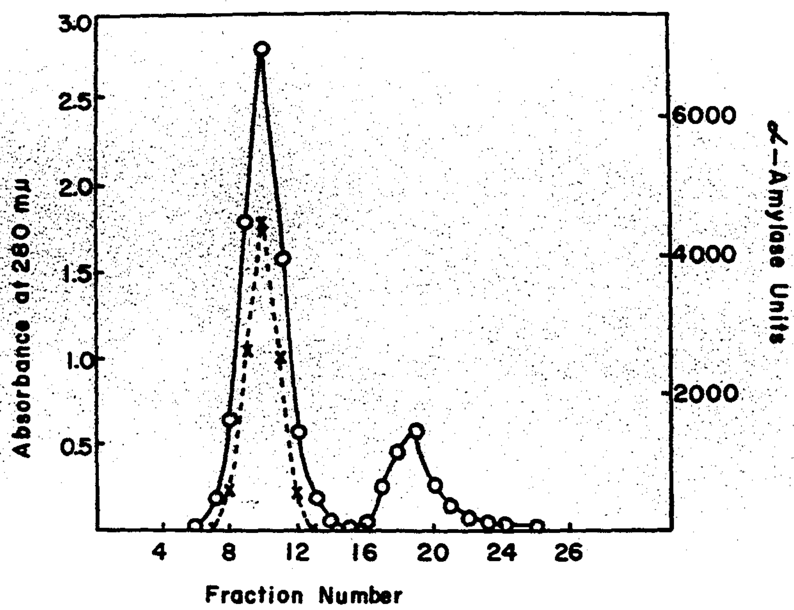


Figure 8

TABLE IV

Amino Terminal Analysis of α-Amylase

DNP prep. no.	Chroma- tography method used	umoles enzyme	Specific activity ($\frac{units}{mg}$)	Condition of hydrolysis	DNP AA	Yield* %
1	paper	0.23	2400	6 N HCl 18 hours	valine	22
2	paper	0.204	2500	6 N HCl 15 hours	valine	44
3	thin layer	0.01	2200	6 N HCl 34 hours	valine	--
4	thin layer	0.008	2600	6 N HCl 20 hours	valine	--

* Uncorrected for destruction or handling losses and the molecular weight of the enzyme was assumed to be 49,000.

Figure 9. Rechromatography of one time recrystallized amylase on Sephadex G-75 column. Sephadex G-75 column (2 x 37 cm) equilibrated with 0.005 M calcium acetate was charged with 7 mg of protein. The flow rate was 2 ml per hour and fractions of 2 ml were collected. 0-0-0 and x-x-x represent absorbance at 280 mμ and amylase activity per ml respectively.

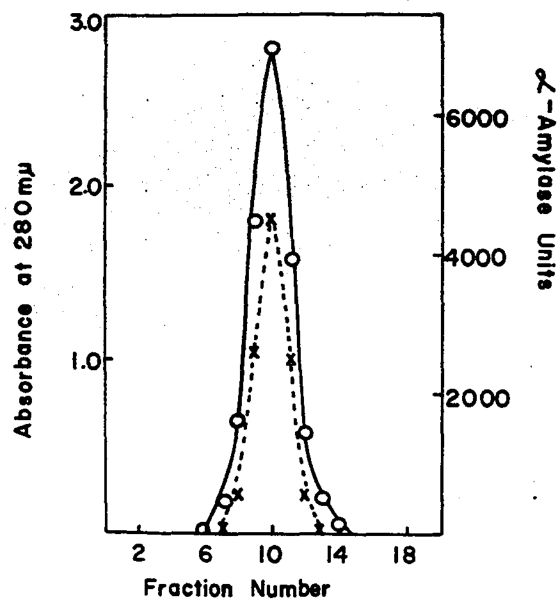


Figure 9

Figure 10. Rechromatography of 1x recrystallized α-amylase on a DEAE-cellulose column. A DEAE-cellulose column (2 x 35 cm) was equilibrated with 0.01 M Tris-HCl buffer pH 8.0, containing 0.005 M calcium acetate. After charging the column with 20 mg of protein, the column was washed with 80 ml of the starting buffer. Gradient elution was than started at the point indicated on the figure with 500 ml of 0.5 M NaCl + buffer and 500 ml of buffer in the mixing chamber. The flow rate was 8 ml per hour and fractions of 4 ml were collected. 0-0-0 represents the absorbance at 280 mμ while x-x-x represents the α-amylase activity.

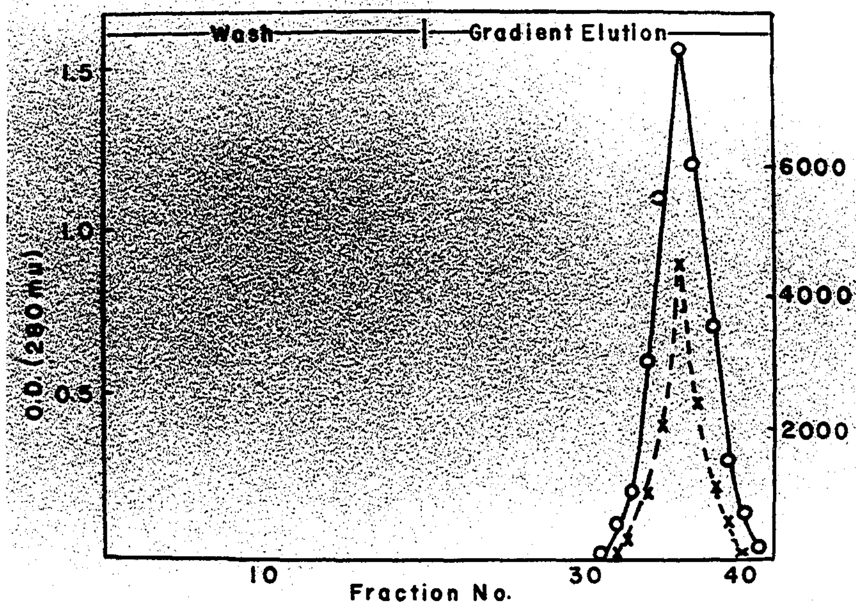


Figure 10

TABLE V

Summary of the Sedimentation Coefficients

No.	Temp. °C	pH	Solvent	Protein conc. (%)	$s_{obs.} \times 10^{-13}$
1	22.5	6.0	0.1 N NaCl 0.005 $CaAc_2$	0.88	5.45
2	22.5	6.0	"	0.66	5.19
3	"	"	"	0.495	5.02
4	"	"	"	0.414	5.15
5	"	"	"	0.37	5.13

Figure 11. The sedimentation coefficients of the Pacific Laboratories α-amylase as a function of protein concentration. The α-amylase was studied in 0.1 M NaCl + 0.005 M calcium acetate pH 6.0 at 22.5°. The line was obtained by the linear least squares method and was extrapolated to zero protein concentration.

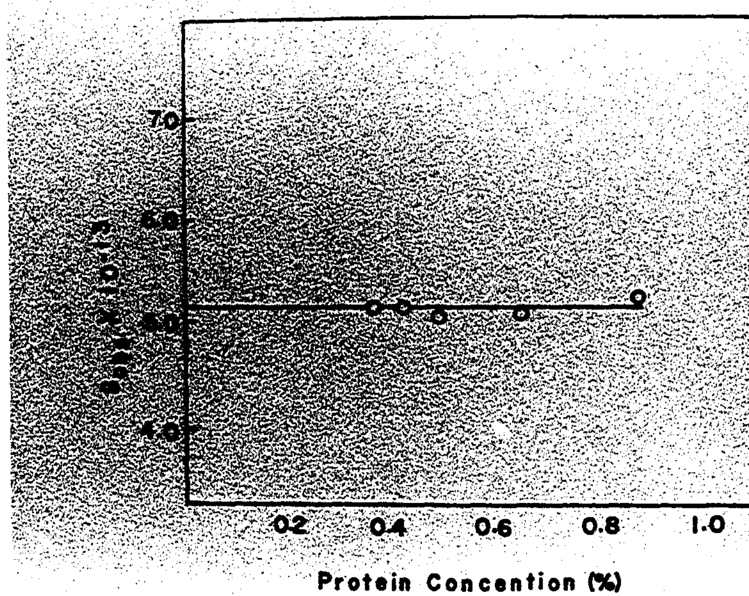


Figure 11

values as a function of protein concentration. The value of the sedimentation coefficient, corrected to 20°C in water, is 4.75 ± .08.

Under the conditions used and the range of protein concentration studies, the values of sedimentation coefficients did not exhibit concentration dependence.

b. The $E^{1\%}_{1cm}$ (280 mμ) of the *B. subtilis* α-amylase

The $E^{1\%}_{1cm}$ (280 mμ) was determined by relating the absorbancy at 280 mμ to a known weight of protein. The value obtained was 22.5 ± 0.2.

c. Zinc analysis of α-amylase

B. subtilis α-amylase has been reported to occur as a dimer with a molecular weight of 100,000 with a zinc ion holding the subunits together (Stein *et al*., 1958). Zinc analysis by the atomic adsorption method of Oppenheimer *et al*. (1967), on Pacific Laboratories α-amylase showed a zinc content of 0.11 moles/mole of enzyme.

d. Molecular weight determination by gel filtration

The molecular weight of Pacific Laboratories α-amylase was estimated by the gel filtration technique using a Sephadex G-100 column. Table VI lists the molecular weights and elution volumes of the standard proteins and the test protein, α-amylase. A void volume of 78.5 ml was determined by the elution of Blue Dextran 2,000.

Figure 12 illustrates the relationship of the log of molecular weight to the ratio of elution volume of the proteins

TABLE VI

Relationship between Molecular Weights and Elution Volume in Gel Filtration on Sephadex G-100

Protein	Molecular weight	Log mol. weight	Elution vol. (ml)	V_e/V_o
Blue dextran	2,000,000	---	78.5	---
BSA*	70,000	4.8451	98.0	1.25
Ovalbumin	40,000	4.6021	111.0	1.42
Cyt. C*	12,400	4.0934	147.6	1.88
α-Amylase	48,700	4.6870	106.0	1.35

*BSA. Bovine Serum Albumin.

Cyt. C Beef Heart Cytochrome C.

Figure 12. Determination of the molecular weight of α-amylase by gel filtration. Relationship between elution volume, V_e/V_o, and log of molecular weight of standard proteins. One and one-half to two mg of each protein was applied to the Sephadex G-100 column (1-1/2 x 110 cm) which was equilibrated with 0.05 M potassium phosphate buffer, pH 7.4 at 4.5°. Elution was carried out with the above buffer at a flow rate of 40 ml/hour.

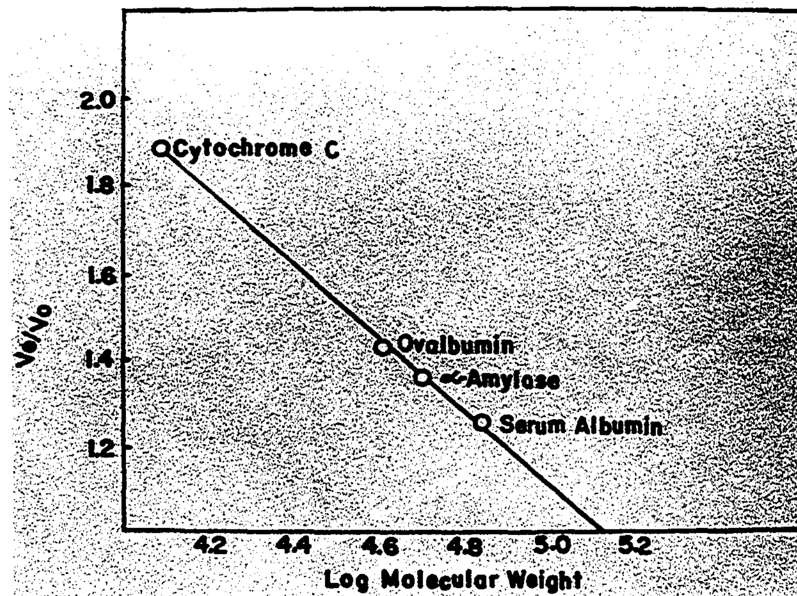


Figure 12

to void volume (V_e/V_o). The ratio V_eV_o for α-amylase was 1.35 as shown in Table VI. This value was found to correspond to a log molecular weight of approximately 4.687 on the slope of the line in Figure 12. Therefore, the molecular weight of amylase was estimated to be 48,700, suggesting mainly the presence of monomers.

4. Discussion

A comparison of the Pacific Laboratories *B. subtilis* amylase with other α-amylases seems warranted since it would show the differences and similarities between these amylases.

Table VII compares some physical and chemical properties of Pacific Laboratories α-amylase with an amylase from *Aspergillus oryzae* (Taka-amylase) and an α-amylase from another strain of *B. subtilis* (Takamine Laboratories). All three α-amylases are similar in their crystalline forms (needles), molecular weights (of the monomer), and their relatively high extinction coefficient at 280 mμ, which is due to their high tyrosine and tryptophan content (Junge *et al.*, 1959).

The *B. subtilis* α-amylases differ from Taka-amylase and most other α-amylases investigated so far in that they contain no sulfhydryl groups. Also the amino terminal residue in Pacific Laboratories *B. subtilis* α-amylase (valine) differs from that of the Taka-amylase (alanine). Valine has also been found in the amino terminal position of other *B. subtilis* α-amylases such as that of the leucine requiring mutant of *B. subtilis* described by Yoshida *et al.* (1960) and the *B. amyloliquefaciens* α-amylase.

TABLE VII

Comparison of α-amylase of *B. subtilis*

	Pacific Laboratories *B. subtilis* amylase	Takamine Laboratories *B. subtilis* amylase	*A. oryzae* Taka-amylase
Crystalline form	needle	needle	needles
Amino terminal	valine	---	alanine
Carboxyl terminal	---	---	serine, alanine glycine
Extinction at 280 mμ	22.5	22.3	19.7
Molecular weight			
a) monomer	48,700	48,900	51,000
b) dimer	none	96,900	none
$s_{20,w}$			
a) monomer	4.75	4.56	4.67
b) dimer	none	6.47	none
Zinc $\frac{(\text{gm atom})}{(50,000)}$	0.11	0.5	0.10
Calcium $\frac{(\text{gm atom})}{(50,000)}$	---	3	2-3

One difference between the two *B. subtilis* amylases compared in Table VII is the quaternary structure. Stein *et al.* (1960) have reported that the Takamine α-amylase, under normal conditions exists in the form of a dimer with one atom of zinc per molecule of dimer. Zinc analysis on the Pacific α-amylase gave only 0.11 gm atom of zinc per mole of enzyme, which is similar to the amount of zinc that was detected in the Taka-amylase. Also, the sedimentation coefficient ($s_{20,w}$) and molecular weight by Sephadex chromatography of the Pacific α-amylase correspond to the monomeric species.

The α-amylases from different strains of *B. subtilis* seem to differ from one another in their ability to dimerize in the presence of zinc. Thus, Pacific Laboratories amylase seems to occur as a monomer in its native state. Another *B. subtilis* strain K, has been reported to produce a monomeric amylase with a $S_{20,w}$ of 4.25 (Yoshida *et al.*, 1959). On the other hand, Biolase, a German strain (Stein *et al.*, 1960) and the Takamine *B. subtilis* produce amylases which bind zinc firmly, to form a dimer species.

B. Cell Free Protein Synthesis (*B. subtilis*)

1. *B. subtilis* culture and fractionation of subcellular components

a. Growth of *B. subtilis*

The growth of the Pacific Laboratories *B. subtilis* under our routine conditions is shown in Figure 13. Under our conditions of vigorous aeration and shaking (Section II B 9)

Figure 13. Submerged culture growth of *B. subtilis*. Six liters of a 1% starch medium contained in a 25 liter bottle were inoculated with 40 ml of a 12 hour old culture of *B. subtilis*. The culture was grown at 28° to 30° with vigorous aeration and shaking. At the times indicated, 10 ml aliquots were withdrawn. The cell density (absorbance at 640 mμ), amylase activity, and proteinase activity were determined as described in Methods. 0-0-0 represents the corrected absorbance at 640 mμ, △-△-△ represents the proteinase activity per ml and x-x-x represents the amylase activity per ml.

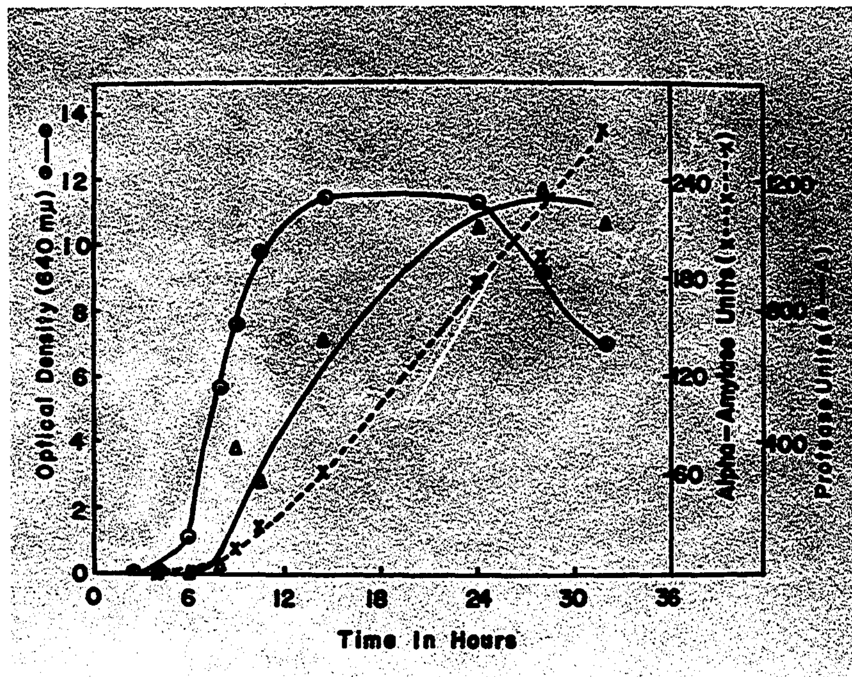


Figure 13

the logarithmic phase of growth was reached 6 hours after the initial inoculation, and the stationary phase in 13 hours. As reported for other strains of *B. subtilis* (Fukumoto *et al.*, 1957), the α-amylase production reached its maximum production during the end of the stationary growth phase. The protease activity also reached its maximum production during the end of the stationary growth phase of the cells.

b. *Study of optimum harvesting time*

Studies were carried out to determine the optimum harvesting time for preparing cell free extracts from *B. subtilis* which were active in incorporating labeled amino acids into TCA insoluble proteins. Figure 14 illustrates the results obtained from such a study. The results show that the cells harvested during the early to the middle of the logarithmic phase were most active in protein synthesis. This period is 6 to 7 hours after the initiation of bacterial growth.

In the course of our studies, some of the *B. subtilis* cultures harvested between 6 to 7 hours were inactive or had very low protein synthesizing activity. Careful reexamination of our results indicated that protein synthesis by *B. subtilis* was closely related to the cell density (absorbance at 640 mμ) of the bacteria. Figure 15 demonstrates the correlation of cell density (O.D. at 640 mμ) to protein synthesizing activity.

c. *Fractionation of B. subtilis components*

The use of sand and glass as abrasives for rupturing *B. subtilis* cells was not very successful for obtaining

Figure 14. Effect of age of B. subtilis on incorporation of C-14-leucine. At the times indicated in the figure the B. subtilis cultures were harvested and subcellular fractions were prepared. The cell free systems obtained were then measured for its ability to incorporate C-14-leucine into the TCA insoluble fraction. The protein synthesizing activities of the cell free systems expressed as CPM/mg are plotted with respect to the harvest times of the bacteria. Each (O) represents one experiment. The various growth phases of B. subtilis are indicated at the top of the figure. The different phases correspond to the growth curve obtained from Figure 14.

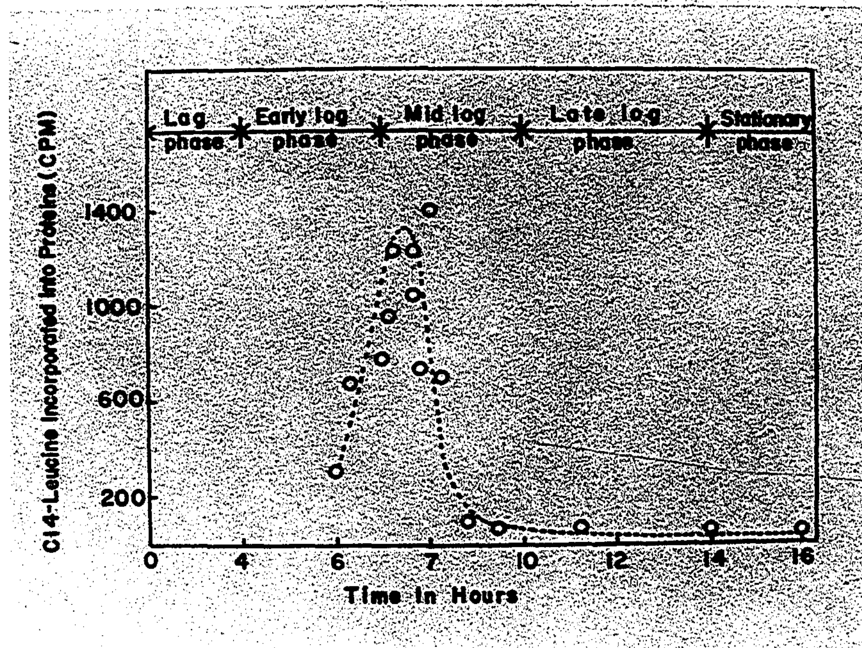


Figure 14

Figure 15. Cell density vs. incorporation of C-14-leucine. *B. subtilis* cultures were harvested at various times and subcellular fractions were prepared as described in Figure 18. The cell free systems obtained were then measured for their ability to incorporate C-14-leucine into the TCA insoluble fractions. The absorbance at 640 mμ of the bacterial cells from which the different subcellular fractions were obtained are plotted with respect to their incorporating activities. Absorbance greater than 2 was calculated from the measured values obtained from diluted samples.

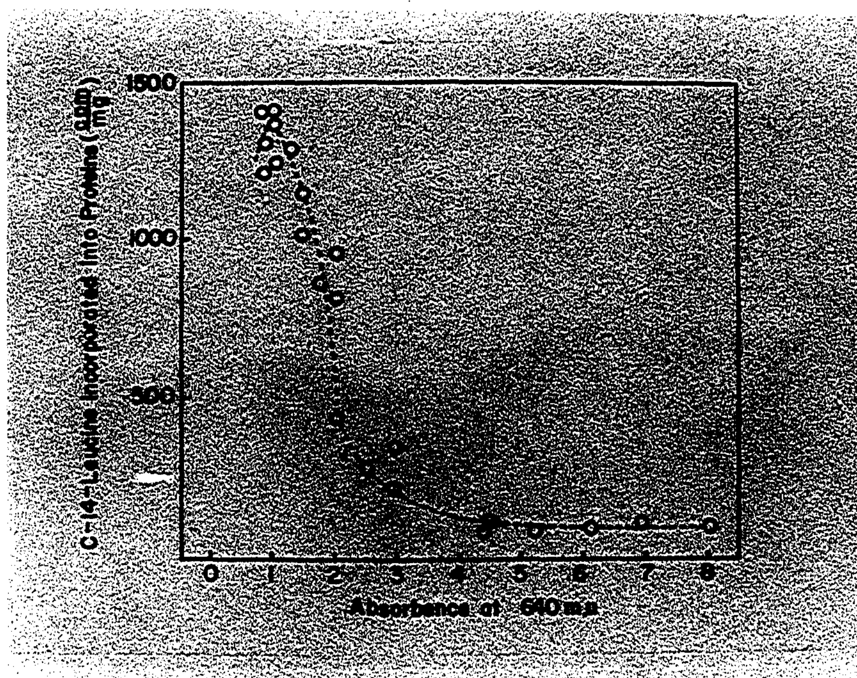


Figure 15

subcellular fractions active in protein synthesis. Some success was obtained when alumina (Alcoa) was used as the abrasive agent. Although alumina grinding by the method described by Taubman *et al*. (1963) gave active subcellular fractions the yield of ribosomal and pH 5.0 fraction proteins was relatively low. A possible explanation for the low yields by this method might be due to the adsorption of proteins and ribosomes to alumina.

The use of lysozyme (Section II B 10) for rupturing the cells was found to be an excellent method. Under the conditions worked out in our laboratory, active subcellular fractions could be obtained readily in good yield. The lysis was carried out at 19°C and the progress of the reaction was followed in a Beckman DU spectrophotometer. The reaction was stopped when the absorbance at 570 mμ reached a minimum level. Figure 16 illustrates a typical lysozyme lysis experiment.

Figure 17 outlines the scheme used for obtaining subcellular fractions from *B. subtilis*. Treatment of the cell debris (residue 1, Figure 17) with sodium deoxycholate greatly increased the yield of ribosomes. This was probably due to the release of polyribosomes from the cell membranes as reported by Schlessinger (1963) in his work on *B. megaterium*.

Clear ribosome pellets could be obtained by sedimenting the ribosomes through a layer of 1 M sucrose (Supernatant 3, Figure 17). On the other hand, ribosomes not sedimented

Figure 16. Lysozyme lysis of *B. subtilis*. The reaction mixture consisted of 2 ml of standard buffer pH 7.5, 3 mg lysozyme, 0.2 mg DNAse and 4 mg bentonite for each gram (wet weight) of bacterial cells. The mixture was then incubated with constant stirring at 19°. One-tenth ml aliquots were removed every 15 minutes, diluted to 3 ml with water, and the absorbance at 570 mμ determined in a Beckman DU spectrophotometer. The reaction was considered complete when the absorbance reached a constant minimum reading.

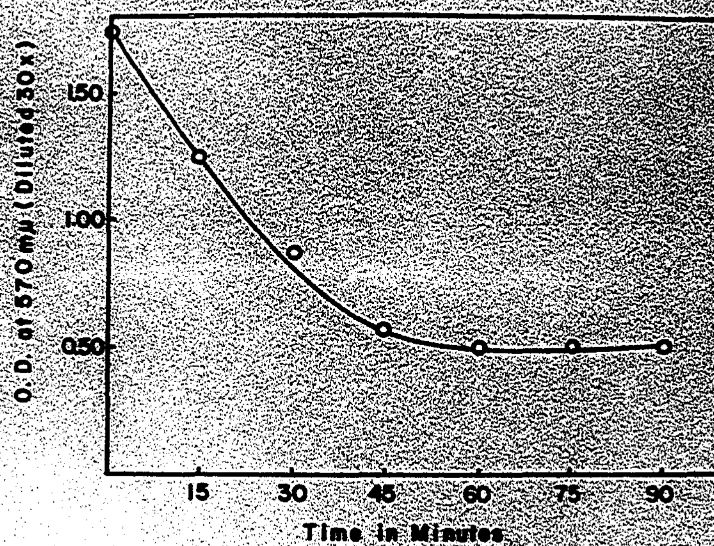


Figure 16

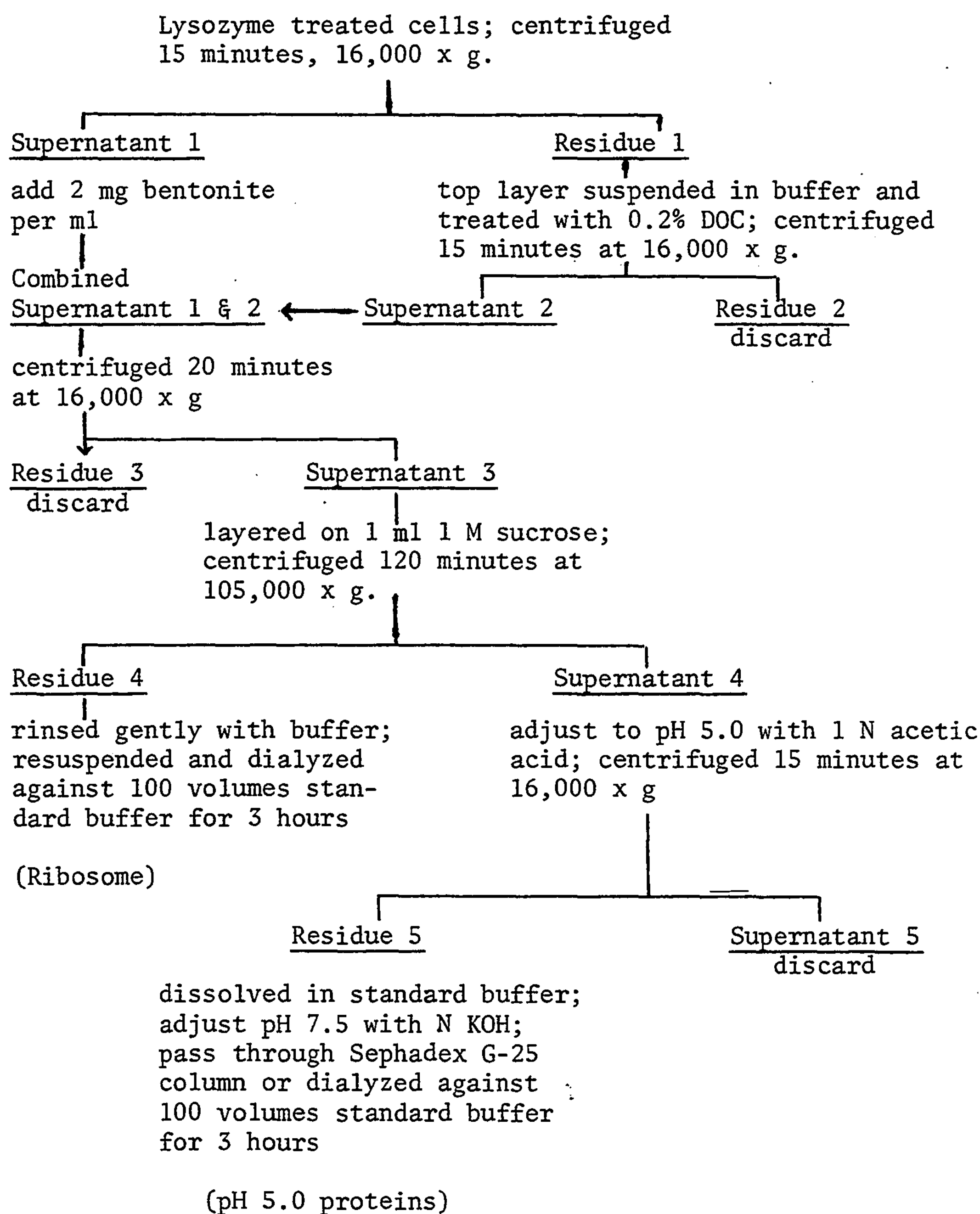


Figure 17

Fractionation of Subcellular Components

through sucrose were cloudy and amber in color.

Starting with the freshly harvested cells, active subcellular fractions could be obtained in 7 to 10 hours.

2. Effect of *B. subtilis* subcellular fractions on protein synthesis

a. Effect of 105,000 x g supernatant on protein synthesis

Figure 18 illustrates the effect of increasing amounts of the unfractionated 105,000 x g supernatant on labeled amino acid incorporation into hot TCA insoluble proteins. The results indicate that the 105,000 x g supernatant fraction contains an inhibitor of protein synthesis, since high concentrations of the supernatant inhibit the incorporation of the C-14-labeled amino acid into the protein fraction. This inhibitor can be removed from the active supernatant fraction by the precipitation at pH 5.0. After centrifugation, the inhibitor remains in the pH 5.0 supernatant (Supernatant 5, Figure 17). This is illustrated in Figure 19, where increasing concentrations of the pH 5.0 supernatant decreases the incorporation of the labeled amino acid into TCA insoluble fraction. The nature of the inhibitor(s) is not known.

b. Effect of pH 5.0 and ribosomal proteins

The effect of pH 5.0 proteins on incorporation is shown in Figure 20. By comparing the results in Figures 18 and 19 with 20, it is obvious that the inhibitor(s) has been removed by the pH 5.0 precipitation step.

Figure 18. Effect of the concentration of 105,000 x g supernatant fraction on the incorporation of C-14-leucine with a constant ribosomal protein concentration. Subcellular fractions were prepared from (O.D.$_{640\ m\mu}$ = 1.5) *B. subtilis* cells as described in Figure 17. The incubation mixtures were prepared as described in Section II B 10a. The ribosomal proteins were kept constant at 0.5 mg in each tube while the concentrations of the 105,000 x g supernatant proteins were varied as indicated. The incorporation of C-14-leucine is plotted with respect to increase of the I05,000 x g supernatant.

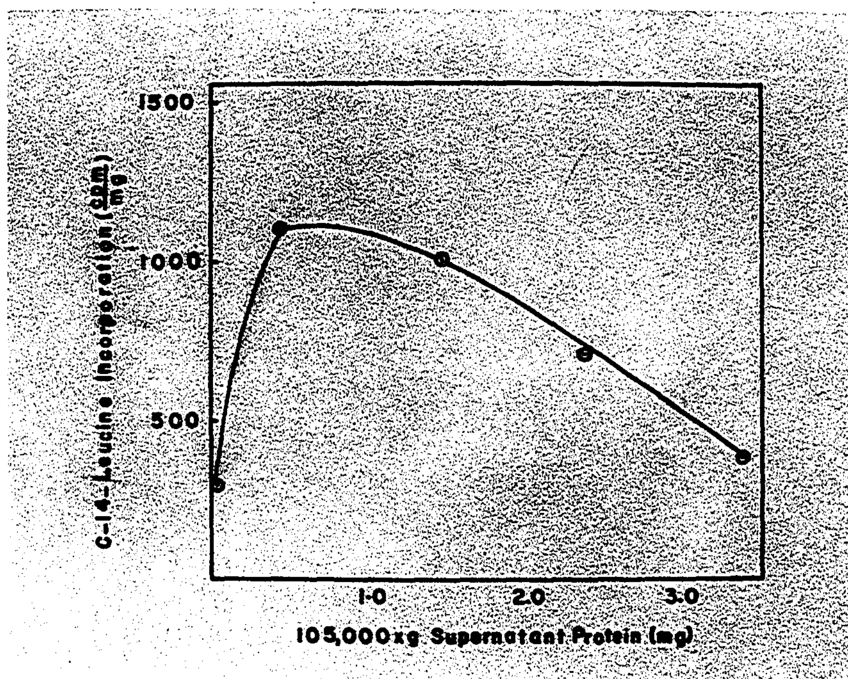


Figure 18

Figure 19. Effect of the concentration of pH 5.0 supernatant fraction on C-14-leucine incorporation with a constant ribosomal protein concentration. Subcellular fractions were prepared from ($O.D._{640\ m\mu} = 1.5$) *B. subtilis* cells as described in Section II B 10a. The ribosomal proteins were kept constant at 0.5 mg in each reaction tube while the pH 5.0 supernatant protein concentrations were varied as indicated. The incorporation of C-14-leucine is plotted with respect to increasing amounts of pH 5.0 supernatant.

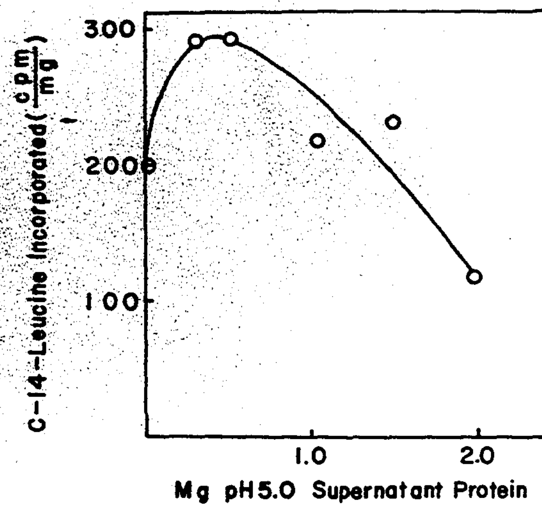


Figure 19

Figure 20. Effect of the concentration of pH 5.0 proteins on C-14-leucine incorporation with a constant ribosomal protein concentration. Subcellular fractions were prepared from (O.D.$_{640\ m\mu}$ = 1.5) *B. subtilis* cells as described in Figure 18. The incubation mixtures were prepared as described in Section II B 10a. The ribosomal proteins were kept constant at 0.5 mg in each reaction tube while the pH 5.0 protein concentrations were varied. The incorporation of C-14-leucine is plotted against increasing amounts of pH 5.0 fraction.

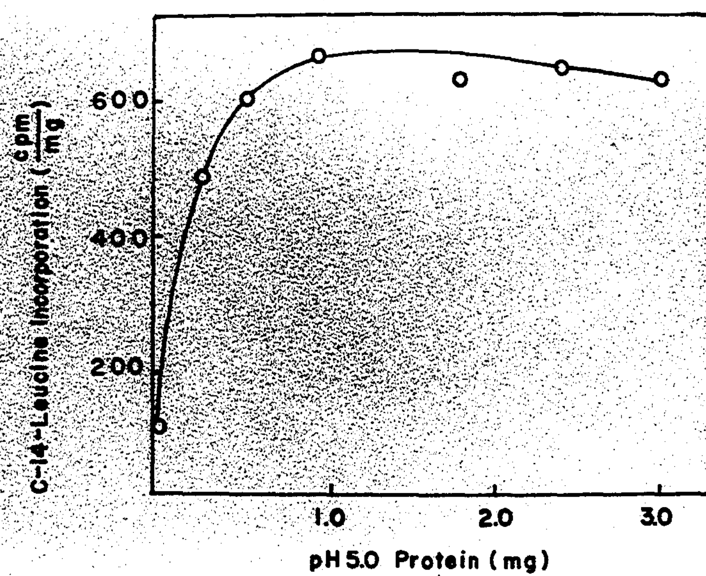


Figure 20

Figure 20 also shows that pH 5.0 fraction is required for incorporation of the labeled amino acid into proteins.

The effect of increasing the ribosome concentration on incorporation is shown in Figure 21. Maximum incorporation requires a certain minimum amount of ribosomes. The optimum ratio of ribosomes to pH 5.0 proteins is approximately 1:2 on a weight basis. Addition of more ribosomes after reaching this saturation point did not increase the incorporation of labeled amino acids into the protein fractions.

3. Effect of various components of the incubation.

a. Effect of magnesium ions

Magnesium ions are known to stabilize the structure of the ribosomes as well as the polyribosomes (Watson, 1965). The effect of varying the concentration of magnesium ions is shown in Figure 22. Maximum incorporation is obtained when 10 to 20 umoles of magnesium ions is used in 1 ml of the incubation mixture.

b. Effect of ammonium ions

Monovalent cations have been shown to be required in protein synthesis. The presence of K^+ or NH_4^+ ions is required for the attachment of amino acyl-t-RNA to the amino acyl-t-RNA binding site (Spyrides, 1964).

The effect of ammonium ions has been studied in our *B. subtilis* cell free system. Maximum incorporation of the labeled amino acid was obtained when 50 µmoles of the ions were added to the incubation system (Figure 23).

Figure 21. Effect of the concentration of ribosomes on C-14-leucine incorporation with a constant pH 5.0 protein concentration. Subcellular fractions were prepared as described in Section II B 10a. The pH 5.0 proteins were kept constant at 2.2 mg in each reaction tube while the ribosome concentrations were varied. The incorporation of C-14-leucine into TCA insoluble fraction is plotted against increasing amounts of ribosomal fraction.

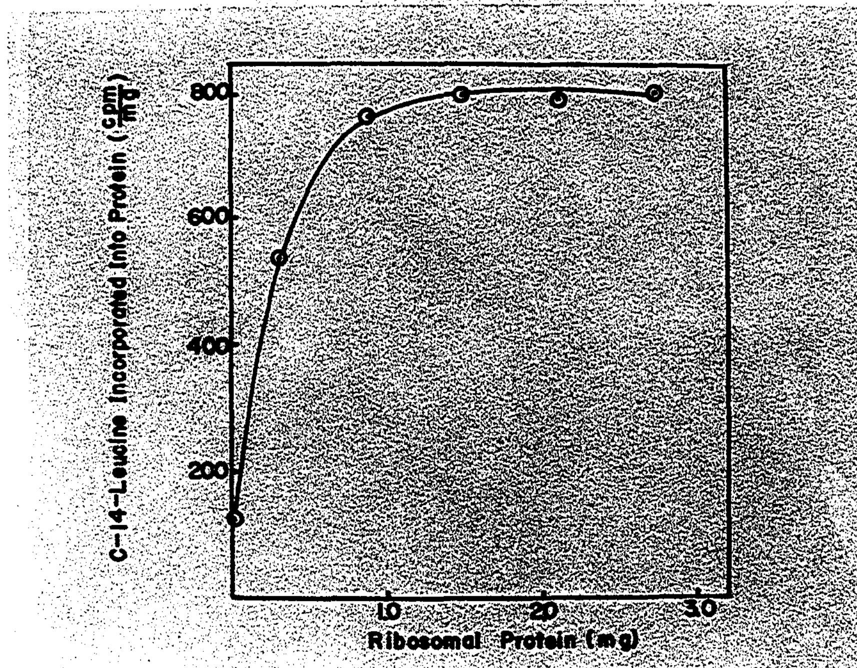


Figure 21

Figure 22. Effect of magnesium ions on protein synthesis. Subcellular fractions were prepared from ($O.D._{640\ m\mu} = 1.0$) *B. subtilis* cells as described in Figure 17. The incubation mixtures were prepared as described in Section II B 10a. One mg of ribosomes and 1.9 mg pH 5.0 fraction were used in each reaction tube. The incorporation of C-14-leucine is plotted against the magnesium acetate concentration.

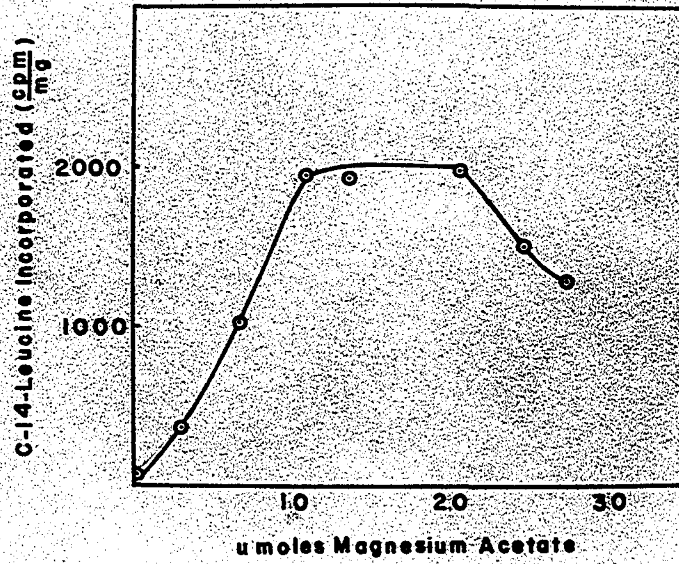


Figure 22

Figure 23. Effect of ammonium ions on protein synthesis. Subcellular fractions were prepared from (O.D.$_{640\ m\mu}$ = 1.3) B. subtilis cells as described in Figure 17. The incubation mixtures were prepared as described in Section II B 10a, except that the ammonium chloride concentrations were varied in each reaction tube. The incubation was carried out for 15 minutes at 37°. The incorporation of labeled leucine into hot TCA insoluble protein is plotted against the concentration of ammonium ions.

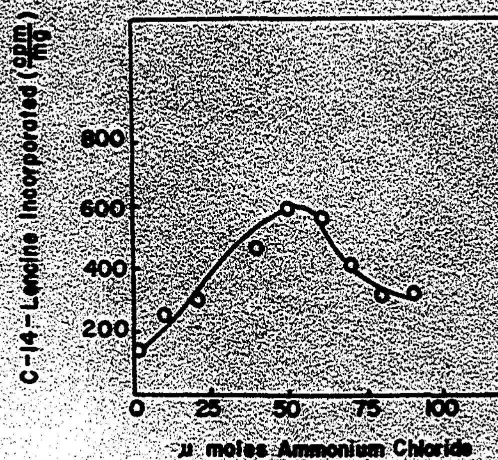


Figure 23

c. Effect of sulfhydryl reagents

During the synthesis of peptide bonds GTP Transferases I and II, and sulfhydryl compounds are required. It has been shown by Sutter *et al*. (1965) that transferase II is activated by sulfhydryl compounds.

The effect of sulfhydryl compounds mercaptoethanol and glutathione on the *B. subtilis* cell free system is shown in Figure 24. Addition of 2 µmoles of mercaptoethanol per ml of the reaction mixture increased the incorporation of labeled amino acid by 16%, while the addition of 3.2 µmoles of glutathione stimulated the incorporation by approximately 27%.

d. Effect of amino acid mixture on the incorporation of C-14-leucine into TCA insoluble proteins

The effect of varying the concentration of the amino acid mixture on incorporation of C-14-leucine is shown in Figure 25. The results show an increase in incorporation of labeled leucine into proteins on the addition of up to 100 mµmoles of each of the amino acids. Further addition of the amino acid mixture resulted in a decrease of incorporation. Seventy-two mumoles of each amino acid were added in our routine incubation mixture.

e. The effect of varying the amount of C-14-leucine

The effect of increasing the amount of C-14-leucine in the presence of 72 mµmoles of each of the other amino acids is shown in Figure 26. Under the routing conditions employed, the incubation system is saturated with the labeled amino acid

Figure 24. Effect of sulfhydryl compounds on protein synthesis. Subcellular fractions were prepared from *B. subtilis* cells as previously described (Figure 17). The incubation mixtures were prepared as in Section II B 10a except that mercaptoethanol was left out. The effect of adding increasing concentrations of glutathione and mercaptoethanol on the incorporation of C-14-labeled leucine is represented by x-x-x and 0-0-0 respectively.

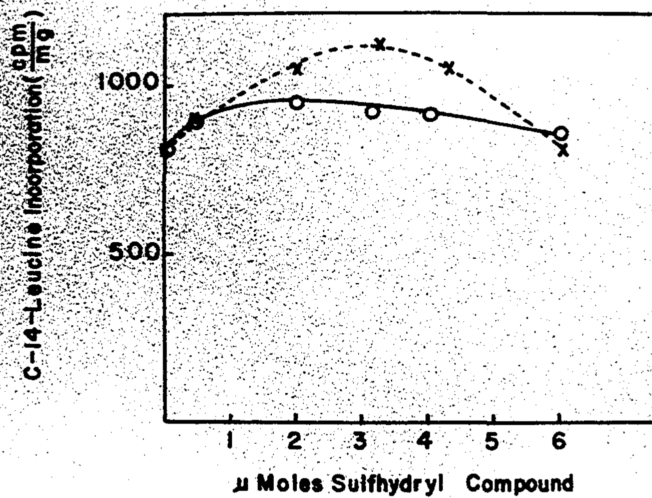


Figure 24

Figure 25. Effect of amino acid mixture on incorporation. Subcellular fractions were prepared from *B. subtilis* as described in Figure 17, and the incubation mixtures prepared as in Section II B 10a except for the amino acid mixture concentration which was varied as shown. Seventy-two mμmoles of each of the 20 common amino acids except leucine and asparagine were included in the amino acid mixture. The extent of incorporation of C 14-labeled leucine with increasing concentrations of the amino acid mixture is illustrated.

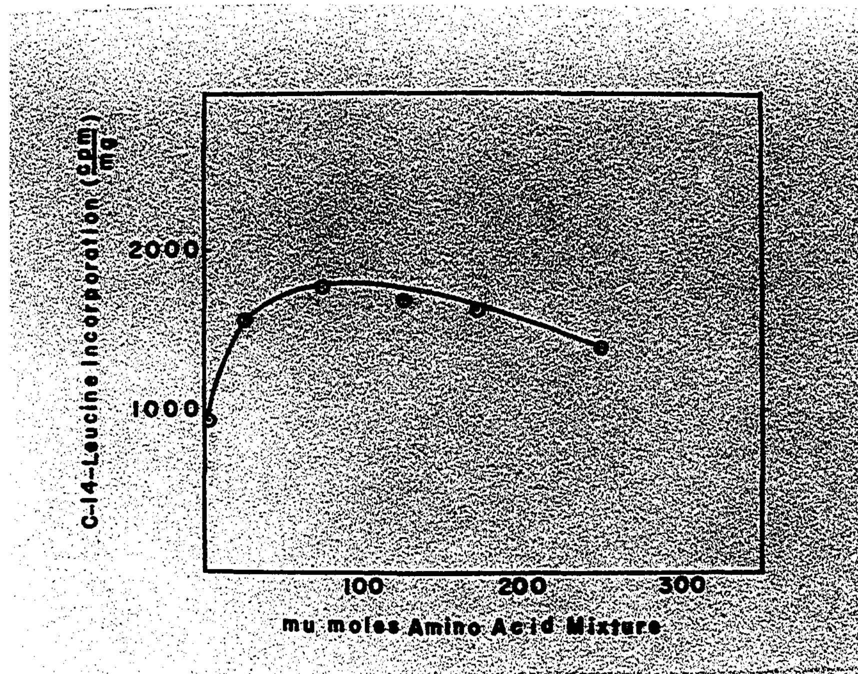


Figure 25

Figure 26. The effect of varying C-14-leucine concentrations on incorporation. Subcellular fractions were prepared from _B. subtilis_ cells as described in Figure 17, and the incubation mixtures prepared as in Section II B 10a except that the amount of C-14-leucine was varied in each reaction tube. The incorporation of the labeled leucine into hot TCA insoluble proteins is plotted versus increasing concentrations of the C-14-leucine added to the incubation mixture.

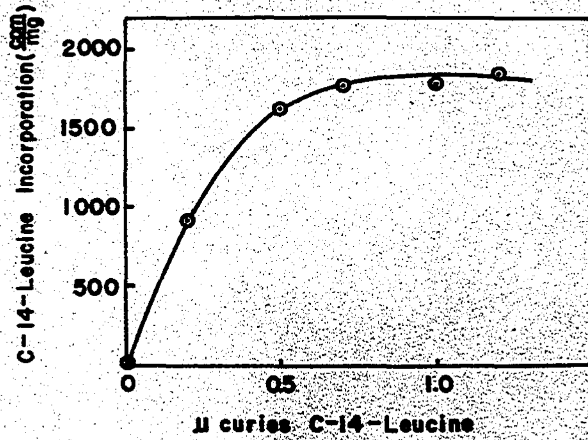


Figure 26

when 0.75 μcuries (62 mμmoles) is added per reaction tube. In our routine assay, 1 μcurie (82 mμmoles) of the labeled amino acid is added.

4. <u>Other conditions for protein synthesis</u>

a. <u>Incubation temperature</u>

The effect of varying the incubation temperature on the incorporation of labeled amino acid into proteins is shown in Figure 27. The results show that the incorporating activity varies drastically with the incubating temperature, with the optimum temperature being about 37°.

b. <u>The effect of pH on protein synthesis</u>

The effect of pH on the incorporation of C-14-leucine into proteins is illustrated in Figure 28. The optimum pH for incorporation lies between 7.2 and 7.7. The incorporating activity slowly decreases when the pH is lowered below pH 7.2 or raised above pH 7.7. Most of the experiments described in this section on protein synthesis were carried out at pH 7.5.

c. <u>The effect of incorporation time on protein synthesis</u>

The effect of incubation time on protein synthesis is shown in Figure 29. The <u>B. subtilis</u> subcellular system incorporates amino acids rapidly into proteins during the first 15 to 20 minutes, after which the rate of incorporation of the labeled amino acid gradually slows down.

d. <u>Energy requirement</u>

The effect of nucleotide triphosphates and the ATP

Figure 27. Effect of temperature on incorporation. Subcellular fractions were prepared from *B. subtilis* as described in Figure 17, and the incubation mixtures prepared as in Section II B 10a. The reaction mixtures, minus the subcellular protein fractions, were preincubated at the different experimental temperatures for 10 minutes. The reactions were then started by the addition of ribosomes and pH 5.0 proteins and carried out for 15 minutes. The incorporation of C 14-labeled leucine into hot TCA insoluble proteins is plotted against the different temperatures at which the incubations were carried out.

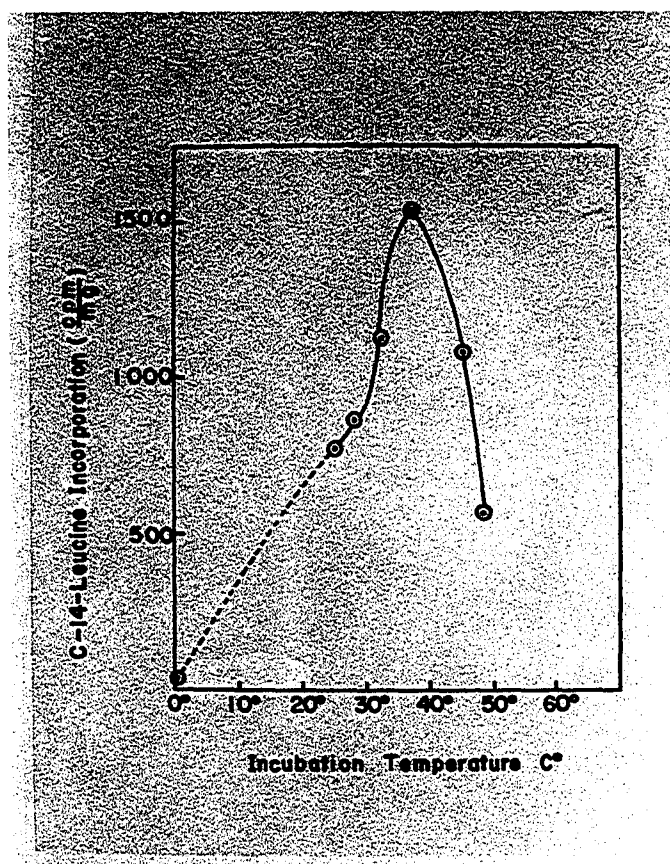


Figure 27

Figure 28. Effect of pH on incorporation. Subcellular fractions were prepared from *B. subtilis* as described in Figure 17, and the incubation mixtures prepared as in Section II B 10a, except that the pH of the reaction mixtures were varied. The incorporation of C-14-leucine was then measured at the different pH's and plotted as shown.

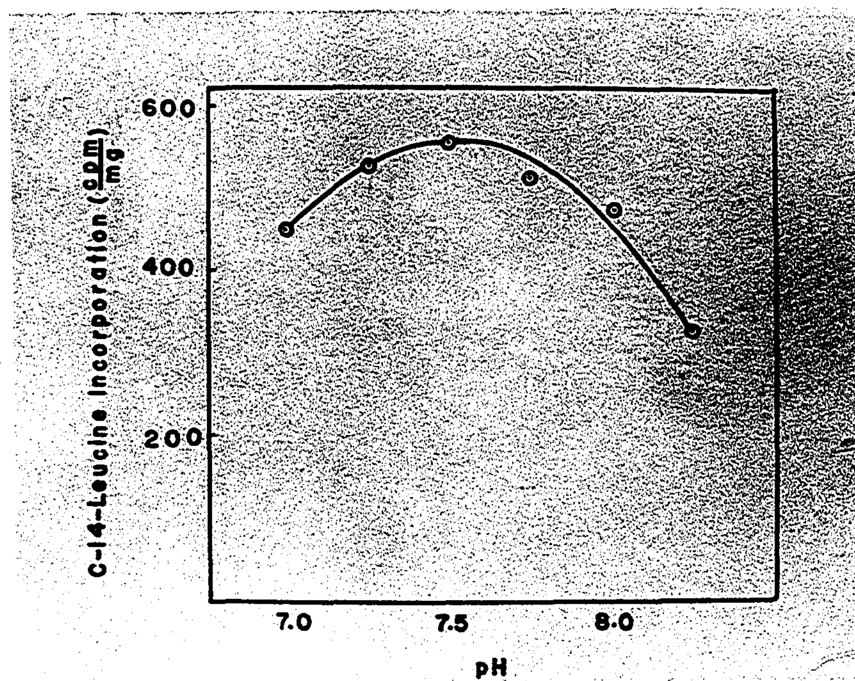


Figure 28

Figure 29. The effect of incubation time on incorporation. Subcellular fractions were prepared from *B. subtilis* as described in Figure 17, and the incubation mixtures prepared as in Section II B 10a. The reactions were started by the addition of ribosomes and pH 5.0 proteins and the incubation carried at 37° for various lengths of time as indicated in the figure. The incorporation of the labeled leucine is plotted versus the incubation time of the reaction.

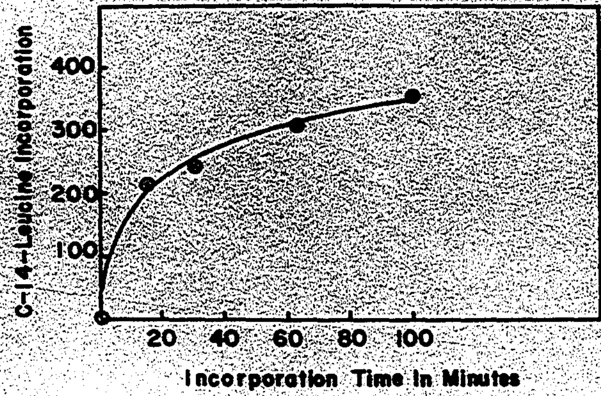


Figure 29

generating system (creatine phosphate and creatine phosphokinase) is summarized in Table VIII. The results show that the ATP generating system is needed for maximum incorporation. In the absence of the ATP generating system, the nucleotide triphosphates acting alone or in various combinations could not support maximum incorporation under the conditions employed.

An odd observation was made when some of the nucleotide triphosphates were omitted from the complete system. Assuming that the complete system (CP, CK, ATP, GTP, UTP and CTP) gave 100% incorporation, the incorporating system in which CTP and UTP have been deleted incorporated 63%. However, the system in which CTP, UTP, and GTP have been deleted gave a 115% incorporation. This unusual observation was investigated further. Table IX summarizes the results of 5 experiments that were carried out to check this point. In all cases higher incorporations were observed (107% to 130%) when the three nucleotide triphosphates were deleted from the incubation mixture, and lower incorporation (52% to 79%) when only the two pyrimidine nucleotide triphosphates were omitted. Figure 30 graphically illustrates the results obtained when the ATP generating system and nucleotide triphosphate concentrations were increased. Once more the results show that the energy mixture in which three nucleotide triphosphates (UTP, CTP, and GTP) were deleted incorporated labeled leucine just as well or better than the complete

TABLE VIII

Summary of Energy Requirement

Condition	CPM/mg	% Incorporation
Complete system: CP, CK ATP, GTP, UTP, CTP	1832	100
CP, CK, ATP, GTP	1149	63
CP, CK, ATP	2111	115
UTP, CTP, GTP	42	2
UTP, CTP	90	5
ATP, GTP	260	14
ATP	214	12
GTP	44	2
No Energy	40	2

Subcellular fractions were prepared from *B. subtilis* as described in Figure 17. The incubation mixtures were prepared as in Section II B 10a, except for the energy sources which were varied in different ways. In the above experiments the different nucleotide triphosphates and components of the energy generating system were present in the following concentrations expressed in umoles per ml unless otherwise stated. CP, 5; CK, 0.05 mg; ATP, 1.0; GTP, 0.05; CTP, 0.05; UTP, 0.05.

TABLE IX

Experiment	Percent Incorporation		
	Complete system: CP, CK, ATP, GTP, UTP, CTP	CP, CK, ATP	CP, CK, ATP, GTP
1	100	115	63
2	100	107	79
3	100	130	52
4	100	113	70
5	100	115	75

The effect of nucleotide triphosphate on incorporation. Subcellular fractions were prepared from (O.D.$_{640\ m}$ = 0.9 to 1.2) B. subtilis cells as described in Figure 17. Incubation conditions were as described in Methods (Section II B 10a). The components of the energy system when added were present in the following concentrations in moles unless otherwise stated in a final volume of one ml. CP, 5; CK, 0.05 mg; ATP, 1; GTP, 0.05; CTP, 0.05; and UTP, 0.05.

Figure 30. The effect of ATP generating system and nucleotide triphosphates on incorporation of C-14-leucine. Subcellular fractions were prepared from *B. subtilis* as described in Figure 17, and the incubation mixtures prepared as in Section II B 10a, except that the energy sources were varied in different ways. Stock solutions of the different energy sources were prepared as follows. Solution 1 contained the following in umoles per ml unless otherwise stated. CP, 50; CK, 0.5 mg; ATP, 10; GTP, 0.5; CTP, 0.5; and UTP, 0.5. Solution 2 contained CP, 50; CK, 0.5 mg; ATP, 0.5; and GTP, 0.5. Solution 3 contained CP, 50; CK, 0.5 mg; and ATP, 0.5. Reaction mixtures were made with varying amounts of one of the three energy solutions described above. The incorporation of labeled leucine was measured against increasing amounts of the energy solution added. △-△-△ represents incorporation due to increasing Solution 1 (complete system). x-x-x represents the incorporation due to increasing amounts of Solution 2 (no UTP and CTP) and 0-0-0 represents the incorporation due to increasing Solution 3 (no GTP, UTP, CTP).

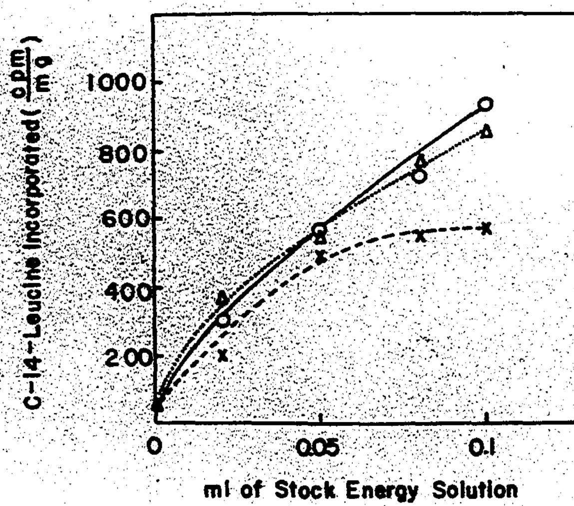


Figure 30

system, whereas the deletion of two nucleotide triphosphates (UTP and CTP) resulted in lower incorporation. Thus far, no satisfactory explanation for these results is known.

The role of GTP in peptide bond formation has been well documented. It has been shown that one GTP molecule is utilized per peptide linkage formed (Nishizuka and Lipmann, 1966).

The subcellular fractions as isolated from the Pacific Laboratories *B. subtilis* according to our procedure, showed very little stimulation in protein synthesis upon the addition of GTP. In many different experiments that were carried out, no increase of activity could be noted upon the addition of GTP (Figure 31). In a few preparations, however, slight stimulation was observed by low concentrations of GTP (i.e. below 0.05 umoles) but this stimulation was not observed if the GTP was increased further (Figure 31 b). Dependence on the addition of GTP for incorporation could be demonstrated if ribosomes were washed with 0.5 M ammonium chloride (Figure 32 a) or if the endogenous GTP was destroyed by preincubation, followed by a second incubation after the addition of a synthetic messenger and labeled amino acid (Figure 32 b). These results suggest that endogenous GTP is still available in subcellular fractions isolated from *B. subtilis*, and that the GTP is probably tightly bound to the enzyme since dialysis or Sephadex G-25 column treatment during the isolation did not seem to remove the GTP.

Figure 31 A and B. The effect of GTP on B. subtilis cell free protein synthesis. Subcellular fractions were prepared from B. subtilis as described in Figure 17, and the incubation mixtures prepared as in Section II B 10a, except that the GTP concentrations were varied in the experiment. The two types of typical results are shown in the figure, where the incorporation of labeled leucine is plotted against increasing concentrations of GTP.

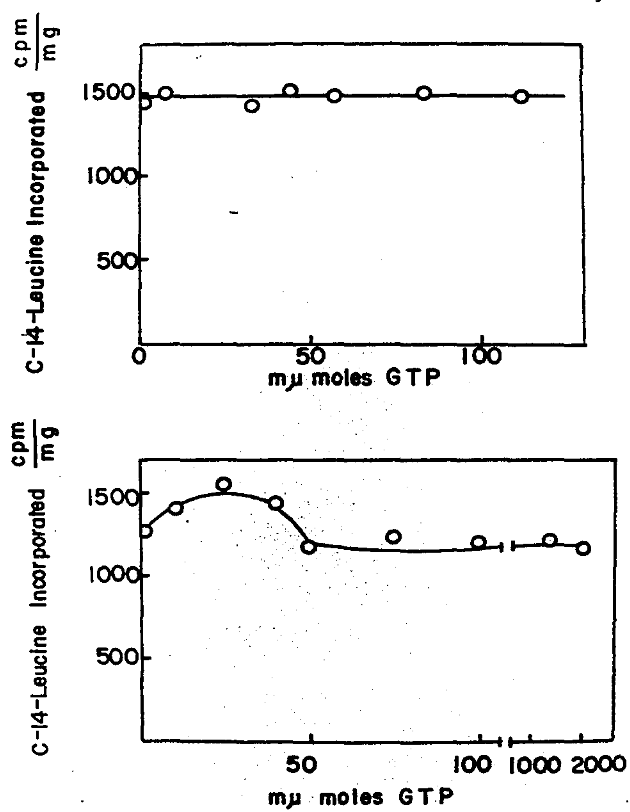


Figure 31

Figure 32 A. Effect of GTP on protein synthesis using ammonium chloride washed ribosomes. Subcellular fractions were prepared as described in Figure 17, except that the ribosomes were washed once with 0.5 M ammonium chloride and the ribosomes resedimented at 105,000 x g for 1 hour. The incubation mixtures were prepared as in Section II B 10a, using ammonium chloride washed ribosomes. The incorporation of labeled leucine is plotted against increasing GTP concentration.

Figure 32 B. The effect of GTP on polyphenylalanine synthesis using polyuridylic acid as messenger. A crude subcellular fraction was prepared from _B. subtilis_ by modifying the procedure described in Figure 17. A 30,000 x g crude extract was prepared with no further fractionation into ribosomes and pH 5.0 proteins. The 30,000 x g extract was then preincubated as described in Section II B 10c, except that unlabeled leucine was used and GTP left out. The preincubated 30,000 x g extract was dialyzed against standard buffer for 3 hours. The reaction mixture for the incorporation studies consisted of the following in µmoles per ml unless otherwise stated. Tris, pH 7.5, 100; ammonium chloride, 50; magnesium acetate, 14; mercaptoethanol, 4; ATP, 1; creatine phosphate, 5; creatine phosphokinase, 0.05 mg; polyuridylic acid, 26 µg; C-14-phenylalanine, 1 ucurie; and varying amounts of GTP as shown in the figure. The incorporation of the labeled amino acid was plotted versus the concentration of GTP.

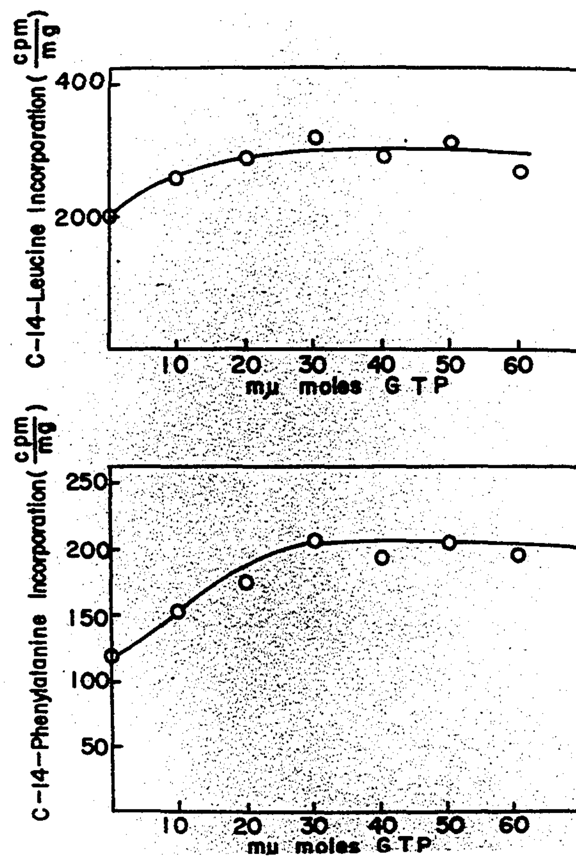


Figure 32

5. Inhibitors of protein synthesis

a. Effect of drugs and enzymes

The effect of various drugs and enzymes on protein synthesis by *B. subtilis* subcellular fractions were studied. Table X summarizes the results of such a study. Puromycin and RNAse which are known inhibitors of protein synthesis lowered the amino acid incorporation by the *B. subtilis* system approximately 90% whereas chloramphenicol lowered the incorporation by approximately 31%.

DNAse and Mitomycin c which interrupt the functioning of DNA, inhibited the system up to 20% depending on the individual preparation. This indicates that the subcellular fractions isolated from *B. subtilis* still contained endogenous DNA which was responsible for some of the incorporating activity, although DNAse was added to destroy the DNA during the preparation of the subcellular components.

6. Study of *B. subtilis* ribosomes

a. Effect of storage of ribosomes

The effect of storing the *B. subtilis* ribosomes in 0.25 M sucrose and in 15% (v/v) dimethyl sulfoxide at -20°C, on their ability to incorporate C-14-leucine into TCA precipitable material was studied (Figure 33). After 25 days the ribosomes stored in both the dimethyl sulfoxide and sucrose solution retained approximately 72% of their original activity.

TABLE X

Effect of Inhibitors of Protein Synthesis on the

B. subtilis Cell-free System

Condition	micrograms Inhibitor per ml	cpm/mg	% Inhibition
Complete	--	1,070	--
0 Time	--	58	--
RNAse	50	84	92
Puromycin	50	103	90
Chloramphenicol	50	741	31
Mitomycin C	30	874	18
DNAse	30	851	20

Subcellular fractions were prepared from *B. subtilis* as described in Figure 17, and the incubation mixtures prepared as in Section II B 10a, except that various drugs and enzymes were added to the incubation mixtures at zero time at concentrations described in the table above. The incorporation of C-14-leucine is reported as the counts per minute per mg of TCA insoluble protein, and the effectiveness of the inhibitor reported as % inhibition.

Figure 33. The effect of storage of ribosomes at -20° on protein synthesizing ability. The subcellular fractions were prepared as described in Figure 17. The ribosomes were then treated and stored in the following manner. Freshly prepared ribosomes were suspended in dimethyl sulfoxide (15% by volume) as described by Downey _et al_. (1956) or in 0.25 M sucrose. The ribosomes were frozen rapidly in an ethanol-CO_2 bath and stored at -20° for various lengths of time. Before use, the ribosomes were thawed and washed with standard buffer. Incubations were carried out as described in Section II B 10a. The activity of the ribosomes as measured by the incorporations of labeled leucine is plotted against the age of the ribosomes. 0-0-0 and x-x-x represent the protein synthesizing activity of the ribosomes stored in dimethyl sulfoxide and 0.25 M sucrose respectively.

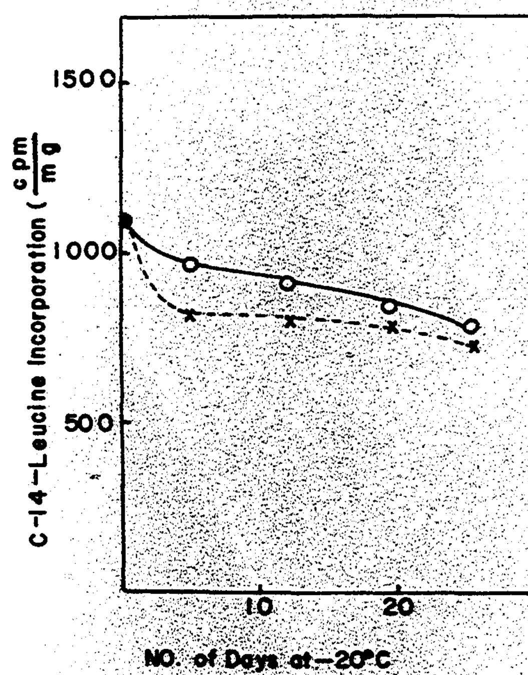


Figure 33

b. Sucrose density studies

The solid line in Figure 35 illustrates the distribution of ribosomes on sucrose gradient density centrifugation. The sedimentation pattern shows that polyribosomes exist. After treatment of the ribosomes with RNAse, there is a breakdown of the polyribosomes to smaller components (dotted line in Figure 34).

c. Electron microscope studies

The existence of polyribosomes in subcellular fractions from *B. subtilis* is further demonstrated in the electron micrograph shown in Figure 35. This photograph shows that the ribosomes isolated from *B. subtilis* extracts for routine assays in protein synthesis exist as large polysome clusters.

C. α-Amylase Biosynthesis

The demonstration of a specific enzyme synthesis proved to be a very difficult and complicated task, as will be seen in the results presented below. Oishi *et al.* (1962) reported that α-amylase biosynthesis could be demonstrated in subcellular fractions from *B. subtilis* cells harvested during the stationary phase of growth, but not in fractions isolated from exponentially growing cells. Contrary to their findings, our results indicate that the "apparent" synthesis of α-amylase can be demonstrated in subcellular fractions isolated from exponentially growing cells as well as in stationary phase cells.

Figure 34. Sucrose density gradient sedimentation was done by the method of Britten and Roberts (1960) using 4.5 ml 20-5% linear gradients and centrifuging for 100 minutes at 28,000 rpm in the SW 39 rotor of a Spinco preparative ultracentrifuge. Sucrose was prepared in 0.1 M Tris, pH 7.5, containing 0.14 M magnesium acetate. Ribosomes were prepared as described in Figure 17, and 2 mg samples of ribosomes were subjected to the centrifugation. For RNAse treatment experiments, RNAse (100 μg) was added to the ribosomes at 4° for 10 minutes prior to centrifugation. After centrifugation, 5 drop fractions were collected, diluted with 3 ml of H_2O, and the O.D. at 260 mμ determined. The top of the gradients is indicated by the arrow. The dashed line represents ribonuclease treated samples and the solid line the untreated sample.

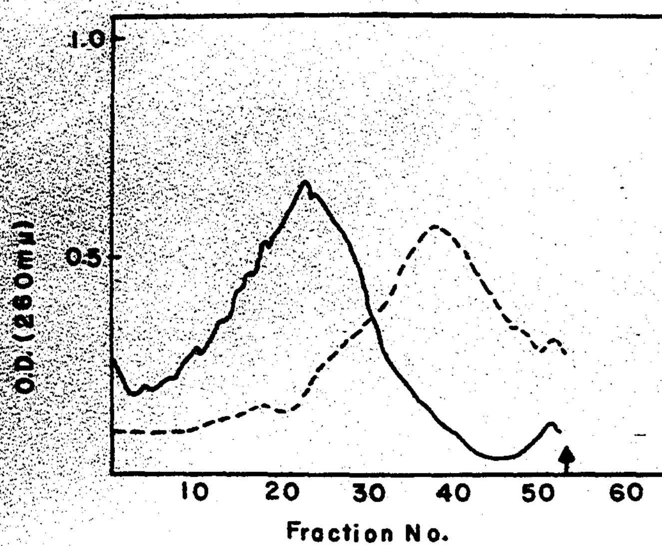


Figure 34

Figure 35. Electron microscope studies of *B. subtilis* ribosomes. *B. subtilis* ribosomes were prepared as described in Figure 17. The ribosomes were then fixed with 2% phosphotungstic acid and examined under the electron microscope at 150,000 times magnification. The electron microscopy was carried out by Robert W. Rongey of the Department of Microbiology, University of Hawaii.

B. subtilis ribosomes

In the experiments to be described, the biosynthesis of α-amylase was determined by following the increase of enzyme activity when subcellular fractions from *B. subtilis* were incubated under various conditions.

1. *The effect of ATP and ATP generating system on apparent synthesis of amylase*

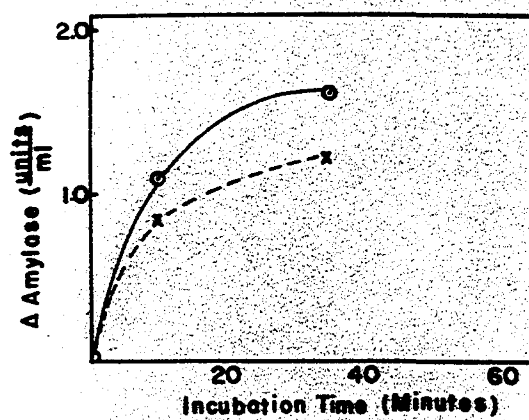
As ATP and GTP are known to be necessary for the *de novo* protein synthesis, the increase in α-amylase activity was determined in the presence and absence of added ATP generating system (i.e. ATP, GTP, creatine phosphokinase and creatine phosphate).

Even in the absence of the ATP generating system, incubation of ribosomes and pH 5.0 protein fractions from both exponentially growing cells as well as stationary phase cells showed increase of α-amylase activity with increasing time of incubation (Figure 36 A and B).

With the addition of ATP and the ATP generating system an increase of α-amylase activity over that of the energy depleted system could be demonstrated in about 80% of the experiments. The amount of ATP-induced stimulation of α-amylase activity varied in different experiments. In about 20% of the experiments no ATP stimulation of amylase activity was observed. It should also be pointed out that in a few experiments complete dependence on the addition of ATP and ATP generating system for the apparent enzyme synthesis was observed.

Figure 36 A. Effect of ATP and ATP generating system on apparent synthesis of α-amylase in subcellular fractions obtained from logarithmically growing cells. Subcellular fractions were prepared as described in Figure 17 from exponentially growing cells. Incubation mixtures were prepared for α-amylase synthesis as described in Section II B 10b. The incubation was carried out at 37° in the presence and absence of the energy system. At the times indicated, 0.5 ml aliquots of the reaction mixture were withdrawn and assayed for α-amylase activity at 25° as described in Section II B 2a. The increase of α-amylase units/ml of the incubation mixture is plotted against the incubation time with x-x-x representing the increase of α-amylase in the absence of the energy generating system and 0-0-0 representing the increase of enzyme in the presence of the energy generating system.

B. Effect of the energy generating system on apparent synthesis of α-amylase in subcellular systems isolated from _B. subtilis_ cells harvested during the stationary growth phase. Experiments similar to those described above were carried out on the stationary phase cells.



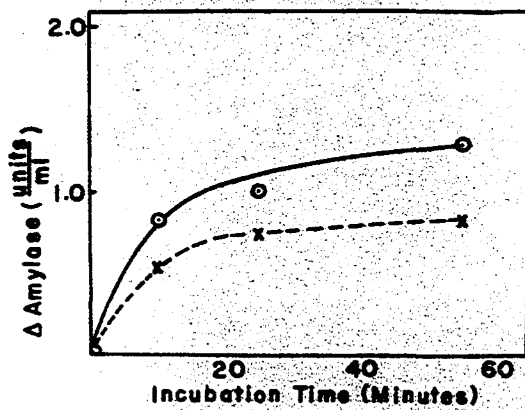


Figure 36

Figure 37 demonstrates the effect of the energy system on the apparent amylase biosynthesis and general protein synthesis as measured by the incorporation of labeled amino acids into hot TCA insoluble precipitate. As shown in Figure 37 A, omission of ATP and ATP generating system from the cell free system isolated from logarithmic growing cells prevents the incorporation of C-14 amino acids into proteins almost completely. On the other hand, significant increase of amylase activity could be seen. Addition of energy increased the activity of α-amylase but not as dramatically as it did the amino acid incorporating activity of the system.

The results of an analogous experiment, utilizing the subcellular fractions isolated during the stationary phase of bacterial growth are illustrated in Figure 37 B. As in the logarithmic phase cells, α-amylase activity increased with the addition of the energy system. However, differences in the amino acid incorporating activity were found. Even with the added energy, no significant increase of incorporating activity resulted. This is in agreement with the results in Figure 14, that subcellular fractions isolated from stationary phase cells were relatively inactive in amino acid incorporating activity compared to exponentially growing cells.

2. The effect of RNAse and puromycin on the production of α-amylase

The effect of inhibitors of protein synthesis on the α-amylase producing system was studied. Surprisingly, addition of RNAse or

Figure 37 A. Comparison of the effect of the energy generating system on α-amylase formation and the amino acid incorporating activity of *B. subtilis* subcellular fractions isolated from exponentially growing cells. Incubation mixtures for α-amylase formation and amino acid incorporation into proteins were prepared as described in Sections II B 10b and II B 10a, respectively. Assays for enzyme formation and amino acid incorporation into proteins were carried out as previously described. The increase of amylase units per ml in the presence and absence of the energy generating system is shown on the left side of the graph. The right side of the figure illustrates the effect of the energy generating system on the incorporation of C-14-leucine into proteins.

B. The effect of the energy generating system on amylase formation and amino acid incorporating activity of subcellular fractions isolated from *B. subtilis* cells in the stationary growth phase. Experiments identical to those described above were carried out on the stationary phase cells.

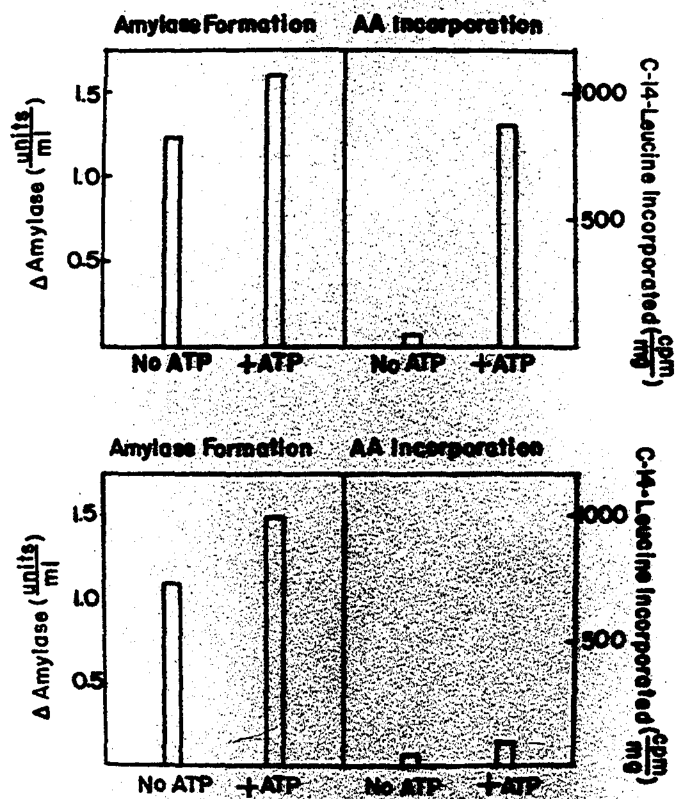


Figure 37

puromycin increased the activity of α-amylase in the cell free system (Figure 38). Figure 39 compares the effect of RNAse and puromycin on α-amylase synthesis and general protein synthesis. Addition of RNAse and puromycin to the subcellular fractions from logarithmically growing cells almost completely inhibits general protein synthesis while the apparent α-amylase synthesis is stimulated. RNAse also stimulates the α-amylase activity in the stationary phase subcellular system, but the effect on general protein synthesis is negligible due to the low incorporating activity of this system.

The above results seem to indicate that the increase of amylase activity does not represent a *de novo* synthesis of the enzyme.

3. *The study of subcellular fractions*

When pH 5.0 or ribosome fractions are incubated separately in the presence of ATP and ATP generating system, some increase of amylase activity was observed. However, maximum production of amylase required the presence of both fractions as illustrated in Figure 40. Similar observations were found for the subcellular fractions isolated from both logarithmic and stationary phase *B. subtilis* cells.

4. *Effect of some cations and sucrose on the apparent synthesis of α-amylase*

Calcium, manganese, and sucrose have been reported to stimulate the production of α-amylase (Oishi *et al.*, 1962). The effect of

Figure 38. The effect of inhibitors of protein synthesis on the formation of α-amylase. Subcellular fractions were prepared as described in Figure 17, and the incubation mixtures were prepared as in Section II B 10b. RNAse or puromycin when added to the incubation mixtures were present at 0 time at a concentration of 50 μg/ml. At the times indicated 0.5 ml aliquots were withdrawn and assayed for α-amylase activity as described in Section II B 2a. The increase of α-amylase units in the absence and presence of the inhibitors was plotted against the incubation time. The effect of puromycin and RNAse is represented by △ - △ and x - x, respectively, whereas 0 - 0 represents the enzyme formation in the absence of any inhibitors. The effect of the inhibitors on the enzyme formation in subcellular fractions from logarithmically growing cells and stationary phase cells are illustrated in the top and bottom portion of the figure respectively.

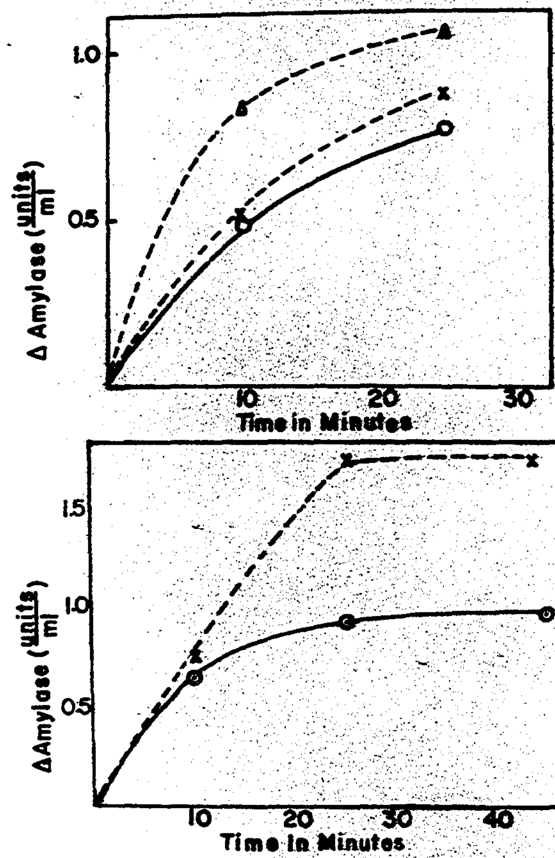


Figure 38

Figure 39. Effect of inhibitors of protein synthesis on amylase formation and incorporation of amino acids into proteins. Incubation mixtures for amylase formation and for amino acid incorporation were prepared as described in Sections II B 10b and II B 10a respectively. RNAse or puromycin when added to the incubation mixtures were present at 0 time at a concentration of 50 µg/ml. The figures on the left side of the graph illustrate the formation of amylase in units/ml, in the presence and absence of the inhibitors, in subcellular fractions isolated from the logarithmically growing cells (top) and stationary phase cells (bottom). The right side of the figure similarly illustrates the effect of these inhibitors on the incorporation of C-14-leucine into proteins (cpm/mg).

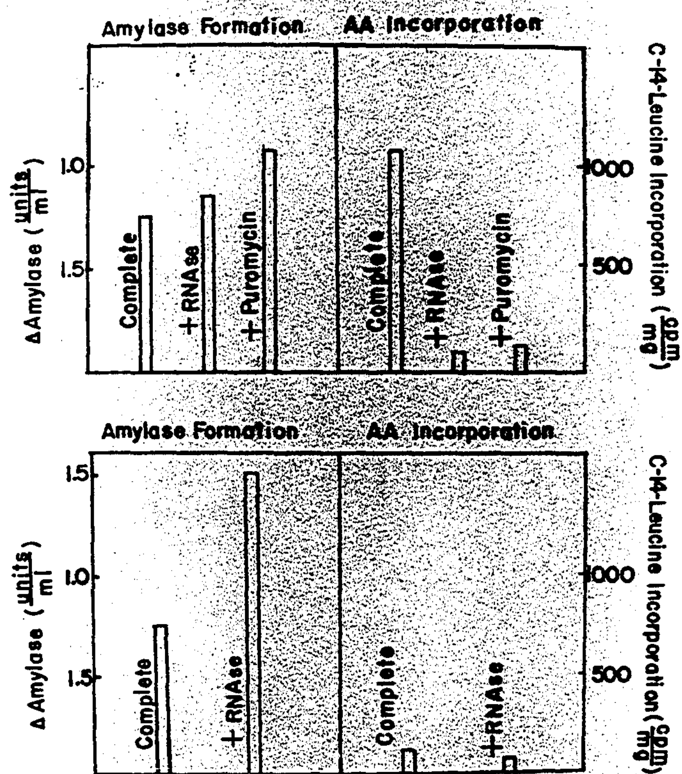


Figure 39

Figure 40. Effect of subcellular fractions on the formation of α-amylase. Subcellular fractions from _B. subtilis_ harvested from both logarithmically growing cells and stationary phase cells were prepared as described in Figure 17. Incubation mixtures were prepared as in Section II B 10b except that the ribosomes or pH 5.0 proteins were omitted from the incubation mixture. The effect of omitting ribosomes or pH 5.0 proteins on the increase of amylase activity (units/ml) with incubation time is shown. The increase of amylase in the complete system is represented by 0-0, the ribosome omitted system by x-x-x and the pH 5.0 omitted system by △-△ in experiments with both logarithmic cells (top) and stationary cells (bottom).

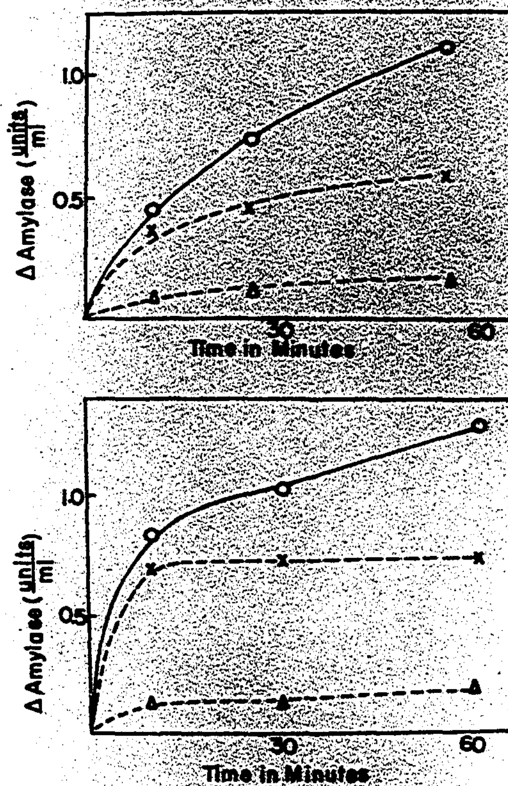


Figure 40

these compounds on the apparent synthesis of amylase in the cell free system is shown in Table XII. The omission of calcium ions, which are known to be required for α-amylase activity and stability (Schwimmer and Balls, 1949), from the incubation mixture decreased the production of the enzyme to between 50 to 80% of the complete system. Similarly, the omission of sucrose or manganese ions from the incubation mixture decreased the production of α-amylase to between 40 to 58% for the sucrose deleted system and 70 to 85% for the manganese deleted system.

TABLE XI

Effect of Cations and Sucrose on the

Apparent Amylase Synthesis

Condition	Amylase production in percent*
Complete system[a]	100
minus Calcium ions	50 to 80
minus Manganese ions	70 to 85
minus Sucrose	40 to 58
minus all of above	20 to 40

*These results were obtained in experiments utilizing subcellular fractions from both logarithmic and stationary phase cells.

[a] Incubation mixtures were prepared as described in Section II B 10b. The cations and sucrose were present in the incubation mixture in the following concentrations expressed in umoles/ml. Manganese, 5; Calcium, 5; and Sucrose, 100 umoles.

IV. DISCUSSION OF RESULTS

A. Characterization of Amino Acid Incorporating System

Most of the details of cell free protein synthesis in bacterial cells have come from studies with the *E. coli* system. It was found that the general requirements of Pacific Laboratories *B. subtilis* cell free systems are similar to those previously described for *E. coli* (Matthaei and Nirenberg, 1961; Lamborg and Zamecnek, 1960). Thus, maximum amino acid incorporation is dependent upon ATP, an ATP generating system, magnesium ions, ammonium ions, a sulfhydryl reagent, ribosomes and the pH 5.0 fraction prepared from 105,000 x g supernatant. The incorporation of radioactive amino acids is sensitive to nuclease activity, with pancreatic ribonuclease and deoxyribonuclease inhibiting the system 92% and 20% respectively. The antibiotics puromycin (90% inhibition), chloramphenicol (31% inhibition), and mitomycin C (18% inhibition) also inhibited the system.

Recently, subcellular systems for *B. subtilis* have been characterized. Cell free amino acid incorporating systems from *B. subtilis* strain 168 (Taubman *et al*., 1964) and from Marburg the strain (Hirashima *et al*., 1967) have been prepared and described. A comparison of our cell free system with other *B. subtilis* systems seems warranted in order to point out the similarities and differences between various *B. subtilis* strains and for the purpose of obtaining a better understanding of the complex mechanism of protein synthesis. At the outset it should be pointed out that a direct comparison between the

different *B. subtilis* systems will be difficult at times since the methodology used by the different groups varied. For example, the subcellular system isolated from the Marburg strain of *B. subtilis* (Hirashima *et al.*, 1967) was used to study polyuridylic acid directed incorporation of C 14 phenylalanine into TCA insoluble polyphenylalanine peptides. On the other hand, the subcellular systems isolated from strain 168 (Taubman *et al.*, 1964) and our Pacific Laboratories strain *B. subtilis* were used to study the endogenous messenger RNA directed incorporation of radioactive amino acids into TCA insoluble proteins.

A summary of some of the properties of the cell free systems prepared from various strains of *B. subtilis* is shown in Table XIII. It was found that the general requirements for all three systems were similar, except for differences in DNAse sensitivity and nucleotide triphosphate requirements of the strain 168 and Pacific Laboratories *B. subtilis* subcellular fractions. Whereas a cell free system from Taubman's strain 168 was very sensitive to DNAse (83% inhibition), our system was inhibited only 20%. This difference in DNAse sensitivity can be attributed to the different methods used in isolating the subcellular fractions. In our isolation of subcellular fractions, DNAse was used to remove the endogenous DNA (Section II B 10), whereas the nuclease was not used in the preparation of Taubman's system. Thus, our subcellular system would be expected to contain less DNA than Taubman's preparation, making our system less sensitive to DNAse inhibition.

TABLE XII

Properties of *B. subtilis* Cell-free System

Conditions	Percent incorporation		
	Marburg[a]	*B. subtilis* 168[b]	Pacific Laboratories[b]
Complete	100	100	100
-ATP generating system	2	10	2
-GTP, UTP, CTP	32	70	115 (59)*
-UTP, -CTP	-	-	63
-Ribosomes	2	-	16
-pH 5.0 or supernatant	-	-	11
-Magnesium	-	4	7
-Ammonium	-	-	30
-Sulfhydryl compounds	-	-	70-80
-Amino acid mixture	-	-	50
+RNAse	-	1	8
+DNAse	-	17	80
+Puromycin	4	3	10
+Chloramphenicol	40	41	69
+Mitomycin C	-	-	82

[a] Amino acid incorporation by the Marburg strain (Hirashima *et al.*, 1967) was directed by synthetic messenger polyuridylic acid.

[b] Amino acid incorporation by Strain 168 (Taubman *et al.*, 1964) and that from Pacific Laboratories was dependent on endogenous messenger RNA.

\- Indicates that the data are not available.

*Value in parenthesis is for result obtained in polyuridylic acid directed phenylalanine incorporation.

Unlike Taubman's system, where incorporation of radioactive amino acid was reduced about 32% in the absence of three nucleotide triphosphates (GTP, UTP and CTP), our system was stimulated 15%. On the other hand, omitting two nucleotide triphosphates (UTP and CTP) from our system reduced the incorporation by 37%. This unusual behavior by the system has been pointed out previously in Section III B 4b. Until further investigations are made, no definite explanation for this unusual observation can be given.

The subcellular system isolated by our procedure did not require the addition of GTP for maximum amino acid incorporating activity when endogenous messenger RNA directed protein synthesis was measured (Figure 32). However, GTP dependence could be demonstrated if the isolated ribosomes were washed with 0.5 M ammonium chloride (Figure 33a) or if endogenous GTP and messenger RNA were first destroyed by preincubation, followed by the incubation with synthetic messenger RNA (polyuridylic acid) and labeled phenylalanine (Figure 33b). In this manner, a 41% reduction of phenylalanine incorporation could be demonstrated in the absence of GTP, as compared to 68% reduction for the system isolated by Hirashima (Table VIII).

In the preparation of active subcellular fractions from the bacteria the harvesting time was found to be important (Figure 15 and 16). Our studies indicate that cells harvested during the exponential growth phase were active for protein synthesis studies, while the older cells obtained during the stationary phase were relatively inactive. These results are in agreement with those observed by

Hirashima et al. (1967). The depression of protein synthesis in the stationary phase cells was probably due to the abundant production of ribonuclease (Nishimura and Nomura, 1959) and proteinase (Fukumoto et al., 1957) during the later stages of B. subtilis growth.

Ribosomes isolated from B. subtilis have been shown to be unstable compared to those isolated from E. coli (Takeda and Lipmann, 1966). Our studies have indicated that the ribosomes can be obtained as polysomes as seen by ribosome distribution on sucrose density gradients, and by electron microscopy (Figures 35 and 36, respectively). The ribosomes quickly lose their activity on standing at 4°C but could be stored for about one month at -20°C in 0.25 M sucrose or in 15% (v/v) dimethyl sulfoxide with only 25% loss of activity (Figure 34).

B. α-Amylase Discussion

There have been a number of reports that cell free preparations from microorganisms are capable of enzyme synthesis. In all of the work that has been reported for bacterial systems, the assays for specific enzyme synthesis have been done by following the increase of enzyme activity. Thus, increase of activity or galactosidase in E. coli (Eisenstadt et al., 1962) and Neurospora crassa (Wrainwright, 1959) has been reported. In the B. subtilis cell free systems, an increase of α-amylase (Oishi et al., 1962) and alkaline phosphatase (Whitely et al., 1963) has been reported.

To demonstrate a truly de novo synthesis of a specific enzyme, incorporation of radioactive aa into a specific protein which has

biological activity should be carried out. The radioactive amino acid should be randomly distributed through the protein molecule. The use of radioactive amino acids allows us to discriminate between newly synthesized polypeptides and pre-existing proteins in the subcellular system.

In our study of the cell free biosynthesis of α-amylase in extracts of B. subtilis, increase of the enzyme activity with time could be demonstrated. However, at least part of this increase of activity did not represent a de novo synthesis of amylase for the following reasons (Section III c):

1. Increase of the enzyme activity could be demonstrated in the absence of added energy.
2. Although inhibiting the incorporation of amino acids into proteins, RNAse did not inhibit the increase of enzyme production; in fact, the addition of the nuclease stimulated the production of amylase in most of the experiments (Figure 40).
3. Puromycin behaved like RNAse in stimulating the production of amylase, although it effectively blocked the incorporation of amino acids into TCA insoluble proteins (Figure 40).
4. The incorporation of radioactive amino acid into amylase could not be demonstrated.

Thus, this increase of activity which is dependent on both ribosomes and pH 5.0 fractions for maximum activity (Figure 41) is partly due to processes other than the de novo synthesis of amylase.

The fact that ribosomes are involved in this process implies that this apparently energy-independent increase of amylase activity is due to a release or conformational changes of the preformed enzyme. Similar arguments hold for the increase of enzyme activity when the system is treated with puromycin (which leads to a premature release of unfinished peptide chains from the ribosomes) and RNAse. Some of these aborted peptides may be not yet completed amylase molecules which are still able to show enzyme activity. It should be pointed out that many other enzyme molecules show enzyme activity when the entire molecule has not yet been synthesized or released from the polysome (Zipser, 1963; Kihara *et al.*, 1961; Cowie *et al.*, 1961; Duerksen and O'Connor, 1963).

From the experiment shown in Figure 37 it can be concluded that part of the increase of amylase activity on incubation of the subcellular system is due to an energy dependent process. However, that this energy dependent increase is due to the synthesis of new peptide bonds in unfinished amylase could not be conclusively demonstrated in the experiments that were carried out.

The only other report of α-amylase synthesis in a purified cell free extract of *B. subtilis* came from workers in Japan (Oishi *et al.*, 1962). They demonstrated that the increase of amylase activity in their system required an energy source, ribosomes, and 105,000 x g supernatant fraction. Their system was inhibited by RNAse, DNAse and chloramphenicol. They found, however, that α-amylase production could be demonstrated only in subcellular fractions that were prepared from stationary phase cells. Subcellular fractions prepared

from exponentially growing cells were inactive in producing α-amylase.

It is well documented that the maximum production of α-amylase by whole cells occurs near the end of the stationary phase growth (Fukumoto *et al.*, 1957; Coleman and Elliot, 1962; and in Figure 14 of this report). Nomura, Maruo, and Akabori (1956) reported experiments which showed that no amylase production was observed during the logarithmic growth phase of the cells. They concluded that α-amylase formation occurs only in old cells in which normal cellular multiplication no longer occurs. Thus, this report would support in part the experimental results of Oishi, who demonstrated an increase in subcellular amylase activity only in old cells of *B. subtilis*.

Contrary to their findings, we have detected amylase from the beginning of the exponential phase to the end of the stationary growth phase of the bacteria (Figure 14). This observation is in agreement with the recent report of Coleman and Grant (1966). Also as reported above (Figure 37 a) an increase of amylase activity could be observed in our subcellular system isolated from logarithmically growing cells as well as from stationary phase cells.

V. SUMMARY

The primary intent of this investigation was to prepare a cell free system from the Pacific Laboratories *B. subtilis* which would carry out the incorporation of amino acids into hot TCA insoluble fractions and to attempt the demonstration of a *de novo* synthesis of a specific enzyme, α-amylase. The systematic study of the biosynthesis of amylase required that the physical and chemical properties of the enzyme isolated from the mutant bacteria be known. Thus, the isolation and characterization of the α-amylase of the P. L. *B. subtilis* was carried out.

A new purification method for obtaining the crystalline α-amylase starting with a crude commercial enzyme preparation from the Pacific Laboratories strain *B. subtilis* was developed. The purification method employed the use of chromatography on DEAE-cellulose, Duolite A-2, and hydroxylapatite. The crystalline enzyme was shown to be homogeneous by the criteria of ultracentrifugation, electrophoresis, amino terminal amino acid analysis, and rechromatography.

The *B. subtilis* α-amylase had a molecular weight of 48,700 as determined by the Sephadex gel filtration method, a $s_{20,w}$ of 4.75 and had valine as its amino terminal amino acid residue.

A cell free extract capable of incorporating radioactive amino acids into hot trichloroacetic acid insoluble proteins was prepared from the Pacific Laboratories strain *B. subtilis*. The activity of the subcellular system was dependent on the age of the *B. subtilis*

culture from which the subcellular fraction was prepared. The exponentially growing cells yielded subcellular fractions which were highly active as compared to the subcellular fractions from the older cells. The amino acid incorporation was dependent upon ATP and ATP generating system, magnesium ions, ammonium ions, an amino acids mixture, and a sulfhydryl reagent for maximum activity. The optimum pH for the incorporation was found to be 7.5, and the optimum temperature was 37°. Omission of GTP did not result in a decrease of amino acid incorporating activity. GTP dependence could be demonstrated if the ribosomes were first treated with ammonium chloride or if the ribosomes were first preincubated to destroy the endogenous GTP.

The existence of *B. subtilis* polysomes was demonstrated by sucrose density gradient centrifugation and by the electron microscopy studies.

The incorporation of labeled amino acid into hot TCA insoluble fraction was inhibited 90% by both puromycin and RNAse. Mitomycin c and DNAse also inhibited the incorporation by 20%.

The increase of α-amylase activity was observed in the *B. subtilis* subcellular system. This increase of enzyme activity occurred in subcellular systems isolated from both exponentially growing cells as well as from stationary phase cells. The increase in enzyme activity in the subcellular system, however, was not a *de novo* synthesis of the enzyme since puromycin and RNAse (which are potent inhibitors of protein synthesis) stimulated the increase of the activity of the enzyme. Thus, it was concluded that this observed increase of enzyme activity represented mainly a release of pre-formed enzyme.

VI. BIBLIOGRAPHY

Ackrell, B. A. C., Asato, R. N., and Mower, H. F. (1966). J. Bacteriol. 92, 828.

Andrews, P. (1964). Biochem. J. 91, 222.

Andrews, P. (1965). Biochem. J. 96, 595.

Atkinson, R. W. (1881). Mem. Tokyo Imp. Univ., Sci. Dept. 6, 1.

Bito, Y. (1965). Exptl. Cell Res. 37, 338.

Britten, R. J., and Roberts, R. B. (1960). Science 131, 32.

Coleman, G., and Elliott, W. H. (1962). Biochem. J. 83, 256.

Cowie, D. B., Spiegelman, S., Roberts, R. B., Duerksen, J. D. (1961). Proc. Natl. Acad. Sci. U. S. 47, 114.

Downey, K. M., So, A. G., and Davie, E. W. (1965). Biochem. 4, 1702.

Duerksen, J. D., O'Connor, M. L. (1963). Biochem. Biophys. Res. Comm. 10, 34.

Eisenstadt, J. M., Kameyama, T., and Novelli, G. D. (1962). Proc. Natl. Acad. Sci. U. S. 48, 652.

Eisenstadt, J. M., and Klein, H. P. (1959). J. Bacteriol. 77, 661.

Fan, D., Higa, A., and Levinthal, C. (1964). J. Mol. Biol. 8, 210.

Fellig, J., Stein, E. A., and Fischer, E. H. (1957). Helv. Chim. Acta 40, 529.

Fischer, E. H., and de Montmollin, R. (1951). Nature 168, 606.

Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1958). In Methods of Biochemical Analysis (D. Glick, ed.), Vol. II, p. 359. Academic Press, New York.

Fukumoto, J. (1943). J. Agr. Chem. Soc. Japan 19, 487, 634, 689, 789, 853.

Fukumoto, J. (1944). J. Agr. Chem. Soc. Japan 20, 231, 121, 309.

Fukumoto, J., Yamamoto, T., and Tsuru, D. (1957). Symposia on Enz. Chem. (Japan), 12, 259.

Goodwin, T. W., and Morton, R. A. (1946). Biochem. J. 40, 628.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949). J. Biol. Chem. 177, 751.

Hagihara, B. (1951). Proc. Japan Acad. 27, 346.

Hagihara, B., Matsubara, H., Nakai, M., and Okunuki, K. (1958). J. Biochem. (Japan). 45, 185.

Heatley, N. G. (1958). Nature 181, 1069.

Hirashima, A., Asano, K. and Tsugita, A. (1967). Biochim. et Biophys. Acta 134, 165.

Junge, J. M., Stein, E. A., Neurath, H., and Fischer, E. A. (1959). J. Biol. Chem. 234, 556.

Kihara, H. K., Hu, A. S. L., Halverson, H. O. (1961). Proc. Natl. Acad. Sci. U. S. 47, 489.

Knopf, P. M. Dintzis, H. M. (1965). Biochem. 7, 1327.

Kruh, J., Schapira, G., Lareau, J. and Dreyfus, J. C. (1964). Biochim. et Biophys. Acta 87, 669.

Kuhn, R. (1925). Ann. Chem. 443, 1.

Lamborg, M. R., and Zamecnek, R. C. (1960). Biochim. Biophys. Acta 42, 206.

Lengyel, P. (1966). J. General Physiology 49, 305.

Leuchs, E. F. (1931). Poggendorff's Ann. Phys. U. Chem. 22, 623.

Levin, O. (1962). In Methods in Enzymology (S. P. Colowick and N. O. Kaplan, eds.), Vol. V, p. 27. Academic Press, New York and London.

Li, C. H. (1951). In Amino Acids and Proteins (D. M. Greenberg, ed.), p. 487. Charles Thomas, Springfield, Ill.

Markovitz, A., Klein, H. P., and Fischer, E. H. (1956). Biochim. Biophys. Acta 19, 267.

Marushige, K., Yura, T., and Imai, M. (1964). Biochim. et Biophys. Acta 87, 90.

Matthaei, J. H., and Nirenberg, M. W. (1961). Proc. Natl. Acad, Sci. U. S. 47, 1580.

Meyer, K. H., Fischer, E. H., and Bernfeld, P. (1947). Helv. Chim. Acta 30, 64.

Meyer, K. H., Fischer, E. H., Staub, A., and Bernfeld, P. (1948). Helv. Chim. Acta 31, 2158.

Miller, G. L., and Golder, R. H. (1950). Arch. Biochem. 29, 420.

Moldave, K. (1965). In Annual Rev. Biochem., Vol. 34, p. 419. Annual Reviews, Inc., Palo Alto, Calif.

Myrback, K., and Neumuller, G. (1950). In The Enzymes (1st ed.), Vol. I, p. 653.

Nakai, M., Minami, Z., Yamazaki, T., and Tsugita, A. (1965). J. Biochem. (Tokyo), 57, 96.

Nishizuka, Y., and Lipmann, F. (1966). Proc. Natl. Acad. Sci., U. S. 55, 212.

Nisman, B., Fukuhara, H. (1959). Compt. Rend. 249, 2240.

Nomura, M., Hosoda, J., Yoshikawa, H., and Nishimura, S. (1957). In Proc. International Symp. on Enz. Chem. Tokyo-Kyoto, I. U. B. Sym. Series. Vol. 2, p. 359.

Nomura, M., Maruo, B., and Akabori, S. (1956). J. Biochem. (Tokyo) 43, 143.

Nomura, M., and Yoshikawa, H. (1959). Biochim. et Biophys. Acta 31, 125.

Oishi, M., Takahashi, H., and Maruo, B. (1962). Biochem. Biophys. Res. Comm. 8, 342.

Oppenheimer, H. L., Green, R. W., and McKay, R. H. (1967). Arch. Biochem. and Biophys. 119, 552.

Ornstein, L., and Davis, B. J. (1961). Disc Electrophoresis. Canalco Industrial Corp., Bethesda, Md.

Payen, A. M., and Persoz, J. (1933). Ann. Chim. et Phys. 53, 73.

Peterson, E. A. and Sober, H. A. (1962). In Methods of Enzymology (S. P. Colowick and N. O. Kaplan, eds.), Vol. V, p. 3. Academic Press, New York and London.

Pirie, N. W. (1940). Biol. Revs. 15, 377.

Redman, C. M., Siekevitz, P., and Palade, G. E. (1966). J. Biol. Chem. 241, 1150.

Schiff, J. A., Eisenstadt, J. M., and Klein, H. P. (1959). J. Bacteriol. 78, 124.

Schlessinger, D. (1963). J. Mol. Biol. 7, 569.

Schweet, R., and Heintz, M. (1966). In Annual Reviews of Biochemistry. Vol. 35, p. 723. Annual Reviews, Inc., Palo Alto, Calif.

Schwimmer, S., and Balls, A. K. (1949). J. Biol. Chem. 179, 1063.

Simpson, M. V. (1962). In Annual Reviews of Biochemistry. Vol. 31, p. 333. Annual Reviews, Inc., Palo Alto, Calif.

Singer, M. F., and Leder, P. (1966). In Annual Reviews of Biochemistry. Vol. 35, p. 195. Annual Reviews, Inc., Palo Alto, Calif.

Spies, J. G., and Chambers, K. (1948). Anal. Chem. 20, 30.

Spyrides, G. J. (1964). Proc. Natl. Acad. Sci., U. S. 51, 1220.

Stein, E. A., Fischer, E. H. (1958). J. Biol. Chem. 232, 867.

Sutter, R. P., Gasior, E., and Moldave, K. (1965). Biochim. et Biophys. Acta 108, 719.

Svedberg, T. and Pederson, K. O. (1940). The Ultracentrifuge. Oxford University Press, London, England.

Takaoka, K., Fuwa, H., and Nikuni, Z. (1952). Mem. Inst. Sci. Ind. Research, Osaka, Univ. 10, 199.

Taubman, S. B., So, A. G., Young, F. E., Davie, E. W., and Corcoran, J. W. (1964). In Antimicrobial Agents and Chemotherapy. (J. C. Sylvester, ed.), p. 395. Ann Arbor, Michigan.

Tsuru, D., Yamamoto, T., and Fukumoto, J. (1958). Bull. Agri. Chem. Soc. (Japan), 22, 168.

Vallee, B. L., Stein, E. A., Sumerell, W. N., Fischer, E. H. (1959). J. Biol. Chem. 234, 2901.

Watson, I. D. (1965). In Molecular Biology of the Gene. W. A. Benjamin, Inc. New York, Amsterdam.

Whitaker, J. R. (1963). Anal. Chem. 12, 1950.

Whiteley, H. R., and Oishi, M. (1963). Biochem. Biophys. Res. Comm. 13, 6.

Wrainwright, S. D. (1959). Can. J. Biochem. 37, 1417.

Yamamoto, T. (1955). Bull. Agr. Chem. Soc. (Japan) 19, 121.

Yoshida, A., and Tobita, T. (1960). Biochim. et Biophys. Acta 37, 513.

Yoshida, A., and Yamazaki, M. (1959). Biochim. et Biophys. Acta 34, 158.

Zamecnik, P. C. (1962). Biochem. J. 85, 257.

Zamecnik, P. C., and Keller, E. B. (1954). J. Biol. Chem. 209, 337.

Zipser, D. (1963). J. Mol. Biol. 7, 739.