FERGUSON, Ann Elizabeth Hey, 1942-
CARBOHYDRATE METABOLISM IN
ACTINOMYCETES.

Rice University, Ph.D., 1969
Biochemistry

University Microfilms, Inc., Ann Arbor, Michigan
RICE UNIVERSITY

Carbohydrate Metabolism in Actinomycetes

by

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A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

Thesis Director's signature:

Houston, Texas

May, 1969
ACKNOWLEDGEMENTS

I wish to express my sincere thanks to the following people:
Dr. Alan D. Elbein for revealing to me the pleasure and challenge of research, and for help and encouragement above and beyond the call of duty.
Dr. R. V. Stevens for reading and commenting on this thesis.
My husband, Richard, who helped and encouraged me in ways too numerous to mention.
My parents, Mr. and Mrs. G. B. Hey, for showing me the value of education.
My parents-in-law, Mr. and Mrs. J. B. Ferguson, for boosting my morale at frequent intervals and for the use of their electric typewriter.
My fellow graduate students and coworkers in the Biology Department for making my stay at Rice such a pleasurable experience.
Mrs. A. D. Elbein for her interest and encouragement throughout the last four years.
Miss Roma Simons for typing this thesis.

I am grateful also to the National Institutes of Health for 18 months support from a Training Grant and 18 months support from a Predoctoral Fellowship.
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INTRODUCTION

Carbohydrate metabolism in the Actinomycetes is of interest, since a large number of these organisms produce antibiotics which contain unusual carbohydrate moieties. Furthermore, since carbohydrates form a readily usable source of energy for any organism, it is of interest to study the metabolism of these compounds, with regard to both the assimilation of external carbohydrate sources and the synthesis and utilization of carbohydrates within the organism.

Interest has been focused, for quite some time, upon the role of trehalose in microorganisms. It has generally been concluded that trehalose constitutes a reserve supply of carbohydrate, and therefore energy, in the organisms in which it occurs. Thus, it appears to play an important role in the germination process (Sussman and Lingappa; 1959; Ceccarini; 1967 and Roth and Sussman; 1968). The synthesis of trehalose-6-phosphate had been described in Streptomyces hygroscopicus (Elbein; 1967a). Furthermore trehalose had been isolated from various Streptomyces species (Elbein; 1967b). Under adverse conditions, S. hygroscopicus will sporulate. Germination will occur on reversal of these conditions. Thus an ideal system was present in which to investigate further the role of trehalose in microorganisms.

The utilization of exogenous carbohydrate sources other than glucose has been the subject of much recent investigation (Domack and Morecker; 1965 and Anderson and Allison; 1965). It was found that in the case of a strain of Mycobacterium smegmatis available in this laboratory, mannose was more readily assimilated than glucose. This presented a new system for the investigation of the utilization of mannose by microorganisms.
LITERATURE REVIEW

TREHALOSE, OCCURRENCE AND METABOLISM

A. α,α-TREHALOSE

The presence, synthesis and metabolism of α,α-trehalose has been reported in a great variety of organisms. For the sake of convenience, these will be considered in a number of groups.

1) Trehalose in Actinomycetes. Trehalose was first isolated from Mycobacterium phlei by Pangborn and Anderson (1933). Anderson and Newman (1933) showed that the neutral fat from human tubercle bacillus, which is soluble in cold acetone, is a complex of fatty acids and trehalose. Aebi, Asselineau and Lederer (1953) confirmed this discovery with the human "Brevannes" strain of M. tuberculosis. Noll et al. (1956) showed that the cord factor of M. tuberculosis is α,α-trehalose 6,6′ dimycolate. Cord factor was also isolated from Wax D of M. tuberculosis BCG by Nojima (1959). Azuma and Yamamura (1962) isolated toxic glycolipids from M. fortuitum and atypical mycobacteria strain P16. They identified a glycolipid from M. fortuitum as being identical to cord factor. In contrast, the fatty acid moieties of a trehalose diester isolated from P16 contained fatty acids with the probable molecular formula C₆₇H₁₃₄O₄. Azuma et al. (1962) showed that the fatty acids esterified with trehalose in M. smegmatis and M. phlei had the molecular formula C₆₇H₁₃₄O₄. Subsequently, Vilkas and Rojas (1964) showed that trehalose in M. fortuitum is esterified at the 6 and 6′ positions with palmitic acid. Narumi and Tsumita (1965) isolated trehalose 6,6′ diphosphate from a phosphorylated polysaccharide of M. tuberculosis. Later, they identified the main component of this polysaccharide as α,α-trehalose 6,6′ dimannosyl phosphate (Narumi and Tsumita; 1967). Trehalose phosphate synthesis from uridine diphosphate D-glucose and glucose-6-phosphate has been
described in *M. tuberculosis* (Loritzo and Goldman; 1964). Trehalose-6-phosphate can be synthesized from either uridine diphosphate D-glucose or guanosine diphosphate D-glucose in *M. smegmatis* (Liu, Patterson and Elbein; unpublished observations). Trehalase activity was detected in Mycobacteria by Bloch and Sullivan (1945) and by Hey and Elbein (1968). Winder and Brennan (1964) and Winder et al. (1967) showed that trehalase accumulates in Mycobacteria exposed to isoniazid.

Recently, the enzymatic synthesis of trehalose-6-phosphate from guanosine diphosphate D-glucose and glucose-6-phosphate was described in *Streptomyces hygroscopicus* (Elbein; 1967a) and the presence of trehalose has been detected in several species of *Streptomyces* (Elbein; 1967b).

2) **Trehalose in yeasts.** Trehalose has been isolated in large quantities from yeast. Tanret (1936) obtained 20g of α,α-trehalose from dried pressed yeast, and Myrback and Ortenblad (1936) found 10-15% from the same source. Trehalose phosphate was isolated from brewer's yeast by Robison and Morgan (1928). Bouthilet et al. (1949) found that several species of yeast could ferment trehalose. The synthesis of trehalose-6-phosphate from uridine diphosphate D-glucose and glucose-6-phosphate was first described in brewer's yeast by Cabib and Leloir (1958). Panek (1962) described the synthesis of trehalose-6-phosphate from uridine diphosphate D-glucose in baker's yeast. Frohwein and Leibowitz (1962) found two α-glucosidases in baker's yeast, one responsible for the hydrolysis of maltose and one responsible for the hydrolysis of trehalose. Panek and Souza (1964) purified a specific trehalase from baker's yeast. Avigad et al. (1965) isolated a specific trehalase from a hybrid yeast. Panek (1963) found that trehalose was utilized during the lag phase of growth of baker's yeast. Souza and Panek (1968) showed that in baker's yeast, trehalase activity remains in the soluble fraction of lysates prepared
from protoplasts, whereas trehalose remains associated with the particulate fraction. Alteration of the membrane permeability with toluene resulted in the hydrolysis of the trehalose by the trehalase. Kruger and Hess (1968) found two forms of trehalase in *Saccharomyces carlsbergensis*, one of which was cytoplasmic and one of which was bound to the mitochondrial membrane.

3) Trehalose in the slime molds. Clegg and Filosa (1961) found that trehalose made up 7% of the dry weight of the spores of *Dictyostelium mucoroides*. In a study of the carbohydrates of *D. discoideum*, Ceccarini and Filosa (1965) found that the only water soluble sugars present were glucose and trehalose. In the vegetative and migrating stages, trehalose was present in quantities less than 0.5%, but increased to 1.5% during the latter part of culmination. Trehalose comprised more than 5% of the dry weight of spores. Ceccarini (1966) purified a trehalase from the amebae of *D. discoideum* and later showed that trehalase activity increased rapidly during spore germination (Ceccarini; 1967). He further showed that there is a rapid disappearance of trehalose with a simultaneous increase in glucose. The glucose formed was then further metabolized. Roth and Sussman (1966) demonstrated the synthesis of trehalose-6-phosphate from uridine diphosphate D-glucose in *D. discoideum* and showed that the activity of trehalose phosphate synthetase was low in cells at an early stage of development. It started to accumulate at 5 hours, peaked at 16 hours and then began to disappear. The normal pattern in wild type was modified in various ways in mutants displaying morphogenetic aberrations (Roth and Sussman; 1968).

4) Trehalose in Neurospora. Lingappa and Sussman (1959) found that within minutes of exposure of ascospores of *Neurospora* to temperatures sufficient to break dormancy, there was a rapid decrease in the
endogenous carbohydrate, soluble in 80% ethanol. They later characterized this carbohydrate as trehalose (Sussman and Lingappa; 1959). It was later shown that trehalose makes up 15% of the dry weight of Neurospora ascospores (Sussman; 1961). Hill and Sussman (1963) crystallized a trehalase from the mycelia of Neurospora. Later they showed that trehalase activity is lowest in ascospores and highest in ungerminated conidia (Hill and Sussman; 1964). Yu et al. (1967) showed that the ascospores protected trehalase against heat inactivation and concluded that the spatial arrangement of the enzyme was probably responsible. Hanks (1967) showed that in Neurospora crassa Strain 69-1113A, trehalose began to accumulate in the vegetative mycelia on the second day of growth and reached a maximum on the third day. At this time conidiation occurred, followed by a rapid decrease in trehalose. Trehalase activity increased rapidly from the 4th to the 10th days with a concomitant cessation of growth and rapid production of aerial hyphae and conidia.

5) Trehalose in other fungi. Bourquelot (1889) isolated trehalose from a large number of fungi, including several species of mushrooms. He further identified the presence of a trehalase in Aspergillus niger (Bourquelot; 1893) and mushrooms (Bourquelot and Herrissey; 1904). Vining and Taber (1964) showed that two strains of Calviceps purpurea accumulated trehalose. Trehalose has been identified in an aqueous extract of Coriolus sanguinlus (Cambie and LeQueusne; 1966); in Schizophyllum commune (Williams and Niederpruem; 1968); and in the yeast-like fungus Pullularia pullulans (Merdinger and Kohn; 1967). Trehalose comprises 20% of the dry weight of the spores of Myrothecium verrucaria (Mandels et al.; 1965) and 4.3% of the weight of dry conidia of Penicillium chrysogenum (Ballio et al.; 1964). Trehalase activity has been detected in Schizophyllum commune (Williams and
Niederpruem; 1968) and in the spores of *Myrothecium verrucaria* (Handels et al.; 1965).

6) **Trehalose in insects.** Frerejacque (1941) first described the presence of a trehalase in the gut of adult insects and in larvae. Leibowitz (1944) found that the manna excreted by scale insects of the Suleimanya Desert contained 7% trehalose. Wyatt and Kalf (1956 and 1957) reported the presence of trehalose in several species of insects at various stages of development and crystallized it from the larval plasma of *Telea polyphemus*. Subsequently trehalose has been detected in the blood of the desert locust (*Schistocerca gregaria*) (Howden and Kilby; 1956); in the blood of the adult blowfly and honeybee (Evans and Dethier; 1957); in the intestine of the Tsetse fly (Geig et al.; 1959); in the prepupae of the sawfly *Trichocampus populi* (Asahina and Tanno; 1964); in the haemolymph and eggs of the differential grasshopper *Melanoplus differentialis* (Randall and Derr; 1965); and in the flight muscle of the housefly *Musca domestica* (Rockstein and Srivastava; 1967). A review of the carbohydrates in insect haemolymph has been made by Wyatt (1961).

Howden and Kilby (1956) demonstrated the presence of a trehalase in the blood and fat body of the locust. Soluble trehalases have also been isolated and purified from the larvae of the wax moth *Galleria mellonella* (Kalf and Rieder; 1958); from whole flies of *Phormia regina* (Friedman; 1960a); from the pupae of the silkworm *Bombyx mori* (Saito; 1960); from the differential grasshopper *Melanoplus differentialis* (Derr and Randall; 1966) and from the labial glands of *Formica polyctena* F (Paulsen; 1966). Membrane bound trehalases have been found in the thoracic muscle of the woodroach *Leucophaeae maderae* (Zebe and McShan; 1959) and in the flight muscle of *Hyalophora cecropia* (Gussin and Wyatt; 1965). A soluble trehalase was also isolated from
larval midgut; the pH optimum and $K_m$ of the membrane bound enzyme were considerably higher. Hansen (1966a) showed that in an extract of flight muscle from Phormia, 25% of the trehalase activity was soluble, while 75% was membrane bound. Zebe and McShan (1959) measured the trehalase activity of the mitochondria of the thoracic muscle of the woodroach and found that it was quite high. They concluded that the trehalase activity may be bound to the mitochondria. Hansen (1966b) demonstrated histochemically that a trehalase is indeed bound to the mitochondria of the flight muscle of Phormia regina.

Candy and Kilby (1961) described the synthesis of trehalose-6-phosphate from uridine diphosphate D-glucose and glucose-6-phosphate in the locust fat body. Murphy and Wyatt (1964 and 1965) found that trehalose inhibits the synthesis of trehalose phosphate in the silk moth fat body, thus diverting the uridine diphosphate D-glucose to glycogen synthesis. Therefore the levels of glycogen and trehalose are controlled by a feedback mechanism. In addition, Friedman (1968) found that trehalose increases the rate of hydrolysis of glucose-6-phosphate in various tissues of adult Phormia. Clegg and Evans (1961) found that the concentration of trehalose in the blood of Phormia regina determined the rate of energy expenditure during flight. Candy (1968) showed that both trehalose and glucose can be utilized by locust thoracic muscle. Since the presence of trehalose did not affect the rate of carbon dioxide production from glucose, but the presence of glucose inhibited the rate of carbon dioxide production from trehalose, he concluded that glucose was probably used during the first few minutes of flight. Friedman (1960b) purified a trehalose-6-phosphatase from Phormia regina Meig.

7) Trehalose in other organisms. Trehalose has been shown to be present in many other organisms, including lichens, higher plants,
oysters and shrimps and *Ascaris*. Thus, Lindberg (1955) isolated trehalose from the lichen *Dermatocarpon miniatum*. Trehalose has also been isolated from several species of *Selaginella* (Quillet and Soulet; 1964); from the fern *Ophioglossum* (Lohr; 1968) and from the cambial sap of *Fagus sylvatica* (Oesch and Meier; 1967). In a type of *Artemia salina* which undergoes dormancy as a blastula, there is 8 times as much trehalose after 10 hours of development as in a type that undergoes direct development. Trehalose comprises 15% of the dry weight of the dormant blastula (Clegg; 1965). Badman (1967) has also detected trehalose in the oyster *Crassostrea virginica*. Fairbairn and Passey (1957) isolated trehalose from the unembryonated eggs of *Ascaris lumbricoides* and detected the presence of trehalose in the perivitelline fluid of the embryonated infective egg. Trehalose was found in the muscle and integument and in the ovaries and uterus of females and in the testis and seminal vesicle of the male. Fukushima (1967) detected trehalase activity in the gut muscle and reproductive organs of *Ascaris lumbricoides*. Freezing doubled the trehalase activity of the gut, but had no effect on the muscle trehalase.

B. α,β-TREHALOSE

Until very recently, it was thought that α,α-trehalose was the only isomer of trehalose found in nature. Two other isomers of trehalose are possible, α,β-trehalose and β,β-trehalose. In 1966, Fischer and Kriegstain isolated a trisaccharide of glucose from *Streptococcus faecalis* grown on D-galactose. This trisaccharide was non-reducing and on partial acid hydrolysis yielded glucose, nigerose and α,β-trehalose, as identified by paper chromatography and high voltage electrophoresis. They identified the compound as 3-O-α-D-glucopyranosyl-α,β-trehalose. Further studies confirmed this structure (Kriegstein and Fischer; 1966b). Enzyme studies with D-glucose
oxidase showed that the trisaccharide was composed entirely of glucose. The alkali stability and absence of reduction with borohydride indicated that all the glucose molecules were joined by glycosidic linkages. Four moles of periodate were consumed per mole of trisaccharide and subsequent acid hydrolysis yielded intact glucose. The products of acid hydrolysis of the hendecacacetate were further identified and $\alpha,\beta$-trehalose was characterized by its physical data. (Fischer and Kriegstein; 1967).

C. TREHALASE ACTIVITY IN MAMMALIAN SYSTEMS

Trehalose has never been detected in mammalian systems. On the other hand, trehalase activity has been found in several mammalian tissues. Dahlqvist (1960) characterized a trehalase from hog intestine. He further found that there was no trehalase activity in the new born animal (Dahlqvist; 1961). Intestinal trehalase activity has also been detected in rats (Rubino et al; 1964; Reddy and Wostmann; 1966). Both groups of investigators found that there was no detectable trehalase in the new born animal, but that it appeared and increased during the third week of life. Trehalase activity has also been detected in the intestine of the goat and pig (Malhotra and Philip; 1964); the human and monkey (Swaminathan and Radhakrishnan; 1965) and in the cow (Siddons; 1968). Dahlqvist and Nordstrom (1966), using the microassay method of Messer and Dahlqvist (1966) showed that trehalase activity is concentrated in the tips of the villi.

Trehalase activity has been detected in the serum of several mammals, including man (Courtois and Demelier; 1966). Trehalase activity appears to increase with age (Courtois et al.; 1966). Trehalase activity has been detected in several organs of a number of mammals (Courtois and Demelier; 1966) and has been purified from the kidney of the pig (Courtois et al.; 1968). Sacktor (1968) has
described the presence of a trehalase in the renal cortical tubules of
ten mammals and in the small intestine of rabbits and rats. The
trehalase is membrane bound. Since the enzyme systems leading to the
formation of trehalose are also present, Sacktor speculates that
trehalose may have a role in the transport of hexoses across the
intestinal mucosa and in the reabsorption of glucose in the kidney.

METABOLISM OF MANNOSE

Since fructose-6-phosphate can be considered to be a key inter-
mediate in the metabolism of hexoses, it is worthwhile to review the
ways in which mannose can be converted to this compound. No attempt
at a complete review is made. Rather an attempt is made to discuss
the possible ways in which this transformation could occur and to
outline the experimental evidence, if any, for these pathways.

Mannose can be phosphorylated by a number of non-specific hexo-
kinasas. The phosphorylation of hexoses was first detected in yeast
and muscle by Meyerhof (1927). In 1943, Colowick and Kalckar showed
that the phosphorylation occurred by the donation of the terminal
phosphate of adenosine triphosphate to the hexose. Non-specific
hexokinases, which phosphorylate mannose, in addition to other hexoses,
including glucose and fructose, at the 6 position, have been isolated
by numerous investigators. Kunitz and McDonald (1946) crystallized
a hexokinase from bakers yeast and showed that glucose and fructose
were phosphorylated at about the same rate. Phosphorylation of mannose
to mannose-6-phosphate proceeded at about half the rate. Berger et al.
(1946) isolated a hexokinase from baker's yeast and showed that the
relative rates of phosphorylation of glucose, fructose and mannose
were 1 : 1.4: 0.3. Wiebelhaus and Lardy (1949) showed that brain
hexokinase could also phosphorylate glucose, mannose and fructose.
Slein et al. (1950) compared the hexokinases from yeast and brain.
They showed that the first product of fructose phosphorylation was fructose-6-phosphate. With both brain and yeast hexokinases, fructose phosphorylation was inhibited 100% by equimolar concentrations of mannose or glucose. There was also a mutual inhibition between mannose and glucose. It was concluded that all compounds acted as substrates and inhibitors at the same center. Saltman (1953) isolated both soluble and particulate hexokinases from wheat germ and detected hexokinase activity in several other higher plants. The insoluble hexokinase was capable of phosphorylating glucose, fructose, mannose and glucosamine. Gavard (1954) showed that hexokinase from Clostridium butyricum was capable of catalyzing the phosphorylation of glucose, fructose, mannose and galactose to their respective 6-phosphates in the ratio 100:32:75:32. Davidson (1960) isolated a hexokinase from Aspergillus parasiticus which phosphorylates a wide range of hexoses, including D-mannose. Moore and O'Kane (1963) found that a particulate hexokinase from Streptococcus faecalis was active towards glucose, fructose and mannose and to a lesser extent towards galactose, glucuronate and mannitol. Vinuela et al. (1963) found that both hexokinase and glucokinase from rat liver could catalyze the phosphorylation of mannose. A glucokinase from rabbit liver (Salas et al.; 1965) could also catalyze this reaction. An 870-fold purified glucokinase from rat liver was capable of catalyzing the phosphorylation of mannose and 2-deoxyglucose in addition to glucose (Parry and Walker; 1966).

In addition to these non-specific hexokinases which catalyze the phosphorylation of mannose, there are a number of hexokinases which are specific for mannose. Bueding and Mackinnon (1955) found that Schistosoma mansoni contains four distinct hexokinases, each one of which catalyzes the phosphorylation of glucose, fructose, mannose or glucosamine. Agosin and Aravena (1959) also found four hexokinases
in *Echinococcus granulosus* catalyzing specifically the phosphorylation of glucose, fructose, mannose and glucosamine. Moore and O'Kane (1963) found kinases in the cytoplasm of *Streptococcus faecalis*, which are specific for fructose, mannose and galactose. Sapico and Anderson (1967) purified to apparent homogeneity a hexokinase from *Leuconostoc mesenteroides*. D-mannose and D-fructose were phosphorylated at equal rates.

Mannose-6-phosphate can be isomerized to fructose-6-phosphate. The reaction is catalyzed by a specific isomerase, phosphomannose isomerase. The possibility of two phosphohexose isomerases, one catalyzing the interconversion of glucose-6-phosphate and fructose-6-phosphate and a second catalyzing the interconversion of mannose-6-phosphate and fructose-6-phosphate was first suggested by Gottschalk (1947). Slein (1950) purified a phosphomannose isomerase from rabbit muscle. Noltmann and Bruns (1958) purified phosphomannose isomerase 15-fold from brewer's yeast. The preparation was free from phosphoglucose isomerase activity and the product was shown to be fructose-6-phosphate. Recently, Gracy and Noltmann (1968a) have purified this enzyme to apparent homogeneity and characterized it as a zinc metalloenzyme (Gracy and Noltmann; 1968b).

Enzyme systems have also been described which catalyze the direct isomerization of mannose to fructose. Palleroni and Doudoroff (1956) were the first to describe this type of isomerization. A partially purified enzyme preparation from *Pseudomonas saccharophila* was found to catalyze the reversible isomerization of D-mannose and D-fructose. The enzyme was also active towards D-lyxose and D-rhamnose. The product of isomerization of D-lyxose was identified as D-xylulose. The product of isomerization of D-rhamnose was presumed to be D-rhamnulose. The enzyme preparation was inactive towards
mannose-6-phosphate. More recently, Anderson and Allison (1965) isolated a D-lyxose isomerase from Aerobacter aerogenes grown on D-lyxose. The enzyme preparation was active towards D-mannose, which was isomerized to D-fructose. However, the enzyme could not be induced by growing the cells on mannose.

Presumably the isomerization of mannose to fructose is followed by the phosphorylation of fructose. The occurrence of non-specific hexokinases capable of catalyzing the phosphorylation of fructose has already been described. In addition, some examples of enzymes which specifically catalyze the conversion of fructose to fructose-6-phosphate have been mentioned. Cardini (1951) found that the ability of Escherichia coli hexokinase to catalyze the phosphorylation of glucose and fructose varied widely according to the method of preparation. Some preparations were incapable of phosphorylating fructose. He concluded therefore that there must be two enzyme systems present, one catalyzing the phosphorylation of glucose and one catalyzing the phosphorylation of fructose. Medina and Sols (1956) isolated a highly specific soluble fructokinase from peas.

Glucose can be phosphorylated to glucose-6-phosphate by the non-specific hexokinases described previously. There are also a number of specific glucokinases. Some of these have also been mentioned. Klein (1953) isolated a hexokinase from Pseudomonas putrefaciens and showed that it catalyzes only the phosphorylation of D-glucose and D-glucosamine. Phosphorylation of D-fructose proceeded at 0-10% of the rate and of mannose at 0-5% of the rate of phosphorylation of glucose. Highly stereospecific glucokinases have also been isolated from Brevibacterium fuscum (Saito; 1965); Aerobacter aerogenes (Kamel et al.; 1966); and Entameba histolytica (Reeves et al.; 1967).
Phosphoglucone isomerase catalyzes the interconversion of glucose-6-phosphate and fructose-6-phosphate. Lohmann (1933) was the first to demonstrate this interconversion in muscle extract. Tanko (1936) found that when fructose-6-phosphate was incubated with marrow pea flour, 62% was converted to an aldose ester. Somers and Cosby (1945) showed that this aldose ester was in fact glucose-6-phosphate.

Phosphoglucone isomerase has been isolated from a large number of sources. Only a few examples will be mentioned. Ramasarma and Giri (1956) isolated phosphoglucone isomerase from Phaseolus radiatus, and showed that at equilibrium, there was about 60% glucose-6-phosphate and 40% fructose-6-phosphate. Jagannathan and Singh (1953) detected the presence of phosphoglucone isomerase in Aspergillus niger and Singh (1959) purified the enzyme from this source. Noltmann and Bruns (1959) purified phosphoglucone isomerase from brewer's yeast. Later Nakagawa and Noltmann (1965) obtained this enzyme in crystalline form. They have since shown that yeast phosphoglucone isomerase consists of three isoenzymes (Nakagawa and Noltmann; 1967). Slein (1955) described a method for the isolation of phosphoglucone isomerase from rabbit muscle. Noltmann (1964) has recently obtained this enzyme in crystalline form. Phosphoglucone isomerase has also been purified to apparent homogeneity from lactating bovine mammary gland. (Balch et al.; 1960).

Glucose can therefore be phosphorylated to glucose-6-phosphate and glucose-6-phosphate isomerized to fructose-6-phosphate. Thus mannose and glucose can be converted to fructose-6-phosphate by exactly analogous reactions. Apparently it is also possible for glucose to be directly isomerized to fructose (Takasaki and Tanake; 1967).

Although there is a well documented alternate pathway for the conversion of glucose to fructose in many organisms, involving the
reduction of glucose to sorbitol and the reoxidation of sorbitol to
fructose, there is no evidence that mannose can be converted to
fructose by an analogous route.

The enzyme which catalyzes the reduction of glucose to sorbitol
was designated aldose reductase (Hers; 1960a). van Heynigan (1959)
found that dialyzed extracts of rat lens would reduce D-xylose,
D-galactose and D-glucose in the presence of reduced triphosphopyridine
nucleotide (TPNH). The corresponding polyols were oxidized in the
presence of triphosphopyridine nucleotide (TPNt). Hers (1960a) showed
that the seminal vesicles and placenta of sheep contain an aldose
reductase which catalyzes the reduction by TPNH of a large number of
aldoses and related compounds. Notably, glucose is reduced to
sorbitol and glucosone to fructose. Reduced diphosphopyridine nucleo-
tide (DPNH) could not replace TPNH. Kinoshita et al. (1963) isolated
an aldose reductase from the lens of calf, rabbit and rat. Hayman
and Kinoshita (1965) purified aldose reductase 1,000-fold from calf
lenses. The enzyme preparation required TPNH and was completely
inactive towards mannose. Pottinger (1967) has shown that glucose
can be reduced to sorbitol by extracts of lenses of a number of
different species. Chino (1960) found that silkworm eggs also contain
aldose reductase. Again TPNH and not DPNH was the active cofactor.

The oxidation of sorbitol to fructose has been demonstrated in
a large number of organisms. Blakely (1951) purified a sorbitol
dehydrogenase from rat liver and demonstrated that fructose was the
product by osazone formation. McCorkindale and Edson (1954) purified
L-iditol dehydrogenase from rat liver, and showed that diphospho-
pyridine nucleotide (DPN) is a cofactor. L-iditol dehydrogenase
was also crystallized from sheep liver (Smith; 1962). The enzyme
catalyzed the reversible oxidation of xylitol to D-xylulose,
ribitol to D-ribulose, sorbitol to fructose and L-iditol to sorbose.
Hers (1960b) found that sorbitol could be oxidized to fructose in the foetal liver of sheep and in the seminal vesicles. Williams-Ashman and Banks (1954) found that extracts of rat liver catalyzed the reduction of fructose by DPNH. The same preparations could also catalyze the reduction of DPN by sorbitol.

Enzymes catalyzing the reversible oxidation of sorbitol to fructose have been found in many microorganisms. Many microorganisms can apparently oxidize mannitol to fructose, in addition. In most cases, the enzymes are adaptive. Sebek and Randles (1952) found that Pseudomonas fluorescens could oxidize mannitol and sorbitol to fructose by different adaptive enzymes. Shaw (1956) found that extracts of an airborne bacterium, which he presumed to be a Pseudomonas, grown on sorbitol could oxidize sorbitol to fructose. There also appeared to be a labile mannitol dehydrogenase. Arcus and Edson (1956) isolated two polyol dehydrogenases from Acetobacter suboxydans. One of the enzymes was associated with the particles containing cytochrome. It was capable of converting both mannitol and sorbitol to fructose. A soluble DPN requiring enzyme could also catalyze the oxidation of both mannitol and sorbitol to fructose. This soluble enzyme was also detected in extracts of Candida utilis. Chakravorty et al. (1962) have purified 35-fold from extracts of Candida utilis, an enzyme which catalyzes the oxidation of sorbitol, mannitol and xylitol to their corresponding ketoheoses. Marcus and Marr (1961) purified two polyol dehydrogenases from Azotobacter agilis. The purified D-mannitol dehydrogenase catalyzed the oxidation of D-mannitol, D-rhamnitol and perseitol and the reduction of D-fructose and D-xylulose. Purified L-iditol dehydrogenase catalyzed the oxidation of sorbitol, iditol, xylitol and ribitol and reduced D-fructose, L-sorbose, D-xylulose and D-ribulose. Maurer and Batt (1962) found that
mannitol dehydrogenase and sorbitol dehydrogenase activity were induced in *Nocardia corallina* if the cells were grown on either mannitol or sorbitol. They found, however, that storage at 0°C for two months resulted in complete loss of enzymatic activity with sorbitol, but that with mannitol was only reduced. A specific mannitol dehydrogenase has been crystallized from *Lactobacillus brevis* (Martinez et al.; 1963). Horwitz and Kaplan (1964) isolated a specific sorbitol dehydrogenase from *Bacillus subtilis*.

Dehydrogenases which catalyze the oxidation of mannitol-1-phosphate and sorbitol-6-phosphate to fructose-6-phosphate have been detected in many organisms. For example, mannitol-1-phosphate dehydrogenase activity has been detected in *Eschericia coli* (Wolf and Kaplan; 1956a, b); *Bacillus subtilis* (Horowitz and Kaplan; 1964) and *Aerobacter aerogenes* (Liss et al.; 1962). Sorbitol-6-phosphate dehydrogenase activity has been detected in *Aerobacter aerogenes* (Liss et al.; 1962); in insect blood (Faulkner; 1956) and silkworm eggs (Chino; 1960). The reduction of glucose-6-phosphate to sorbitol-6-phosphate has been reported in insect blood (Faulkner; 1956) and silkworm eggs (Chino; 1960). The cofactor required is TPNH.

Presumably the polyols are phosphorylated before they are further metabolized. However, there is practically no mention in the literature of enzymes capable of phosphorylating these compounds. Moore and O'Kane (1963) mention that their particulate hexokinase from *Streptococcus faecalis* phosphorylated mannitol to a certain extent. Also adenosine triphosphate (ATP) was utilized by a cytoplasmic fraction in the presence of mannitol.

Thus, glucose can be metabolized in a number of different ways. It can be phosphorylated to glucose-6-phosphate, which can be further isomerized to fructose-6-phosphate. Glucose-6-phosphate can be reduced
to sorbitol-6-phosphate, which can be reoxidized to fructose-6-phosphate. Glucose can be isomerized to fructose, which can then be phosphorylated to fructose-6-phosphate. Glucose can also be reduced to sorbitol which can be phosphorylated to sorbitol-6-phosphate. The sorbitol-6-phosphate can be oxidized to fructose-6-phosphate.

The number of pathways for the utilization of mannose are more limited. Mannose can be phosphorylated to mannose-6-phosphate, which can be isomerized to fructose-6-phosphate. Mannose can also be isomerized directly to fructose, which is then phosphorylated. It has not been determined as yet, whether mannose can be converted enzymatically to mannitol. If this were possible, the mannitol formed could be oxidized to fructose, which could then be phosphorylated. Mannitol could also be phosphorylated to mannitol-1-phosphate and oxidized to fructose-6-phosphate. Recently, Kamel and Anderson (1966) have demonstrated a unique mechanism for the metabolism of mannose in Aerobacter aerogenes. Kamel and Anderson (1964) described the enzymatic phosphorylation of D-glucose with acetyl phosphate. They also found that Aerobacter aerogenes had a highly specific glucokinase which was inactive towards mannose (Kamel et al., 1966). However, the organism utilized mannose constitutively. There also appeared to be a 2-epimerization of mannose and glucose. What in fact was occurring was that glucose was being phosphorylated with either ATP or acetyl or carbamyl phosphate to form glucose-6-phosphate. The glucose-6-phosphate was then donating the phosphate group to mannose to form mannose-6-phosphate. The mannose-6-phosphate was then isomerized to fructose-6-phosphate. Fructose-6-phosphate could be reconverted to glucose-6-phosphate. Mannose could also be directly phosphorylated by acetyl or carbamyl phosphate.
MATERIAL AND METHODS

Materials. All chemicals were obtained from commercial sources unless otherwise indicated. A lyophilized culture of Leuconostoc mesenteroides NRRL B 1424, which produces a dextran from which kojibiose and isomaltose were isolated, was kindly supplied by Allene R. Jeanes of the U. S. Department of Agriculture. The culture was maintained as described by Jeanes (1965). Kojibiose and isomaltose were isolated by acetolysis (Torii et al.; 1963). Nigerose was obtained from the hydrolysis of nigeran (Barker et al.; 1957) and laminaribiose from the hydrolysis of laminarin (Peat et al.; 1958). Sophorose was a gift from H. G. Fletcher of the National Institutes of Health and trehalosamine was a gift from F. Arcamone, Laboratori Rierche, Farmitalia, Milano, Italy. Guanosine diphosphate D-glucose-\textsuperscript{14}C (GDPG-\textsuperscript{14}C) was prepared chemically by a modification (Elbein; 1966) of the method of Roseman et al. (1961). Guanosine diphosphate D-glucose (GDPG) was purchased from Calbiochem, Los Angeles, California. α,α-trehalose-6-phosphate-\textsuperscript{14}C was prepared enzymatically, by incubating GDPG-\textsuperscript{14}C and glucose-6-phosphate with purified trehalose phosphate synthetase (Guanosine diphosphate D-glucose : D-glucose-6-phosphate 1-glucosyl transferase) from from Streptomyces hygroscopicus (Elbein; 1968). The trehalose phosphate formed was purified by paper chromatography in ethyl acetate-acetic acid-water (3:3:1). Calcium phosphate gel was made by the method of Keilin and Hartree (1938) and hydroxyapatite by the method of Miyazawa and Thomas (1965). Amidex was obtained from Corn Products Refining Co., Argo, Illinois and N-Z Amine A (an enzymatic hydrolysate of casein) from Sheffield Farms Co., Inc., New York 19, N.Y. Curbay B G Liquid was obtained from United States Industrial Chemicals, Inc., Louisville, Kentucky and
Corn Steep Liquor from the A. E. Staley Co., Decatur, Illinois.

Analytical methods. Glucose was determined either with the reducing sugar method of Nelson (1944) or with glucose oxidase (Huggett and Nixon; 1957). Hexose was determined by the anthrone method (Loewus; 1952). Fructose was measured by the method of Roe (1934); other ketoses by the cysteine-carbazole reaction (Dische and Borenfreund; 1951). Protein was determined by the method of Sutherland et al. (1949).

Descending paper chromatography was performed with either Whatman no. 1 or no. 3MM filter paper. Chromatographic solvents were solvent I, propan-1-ol-ethyl acetate-water (7:1:2); solvent II, butanone-acetic acid-water (8:1:1); solvent III, butan-1-ol-pyridine-water (6:4:3); solvent IV, phenol-water (4:1 v/v); solvent V, ethyl acetate-acetic acid-water (3:3:1). Sugars were detected on paper chromatograms using the alkaline silver nitrate reagent (Trevelyan et al.; 1950) or using the acid aniline phthalate spray reagent (Partridge; 1949). Ketoses were detected on paper chromatograms with the orcinol-trichloracetic acid spray (Bevenue and Williams; 1951). Radioactivity was determined quantitatively in a Packard liquid scintillation spectrometer. Nucleotides were detected on paper by their U.V. absorption.

Culture of organisms. Cultures of *Streptomyces hygroscopicus* were maintained on slants of the following composition (Elbein, 1960)

<table>
<thead>
<tr>
<th>Compound</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble starch</td>
<td>0.50</td>
</tr>
<tr>
<td>Dextrose</td>
<td>0.50</td>
</tr>
<tr>
<td>Tryptone</td>
<td>0.50</td>
</tr>
<tr>
<td>Betaine</td>
<td>0.05</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Curbay B G liquid 0.10
K$_2$HPO$_4$ 0.01
MgSO$_4$ 7H$_2$O 0.01
Mineral mixture A 0.10
Agar 2.00

The pH was adjusted to 7.0 - 7.2.

Mineral mixture A had the following composition:

<table>
<thead>
<tr>
<th>Compound</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO$_4$·7H$_2$O</td>
<td>0.20</td>
</tr>
<tr>
<td>ZnSO$_4$·7H$_2$O</td>
<td>0.10</td>
</tr>
<tr>
<td>CuSO$_4$·5H$_2$O</td>
<td>0.05</td>
</tr>
<tr>
<td>MnCl$_2$·4H$_2$O</td>
<td>0.05</td>
</tr>
<tr>
<td>CoCl$_2$·6H$_2$O</td>
<td>0.01</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>4.00</td>
</tr>
</tbody>
</table>

Growth of organisms. Five hundred ml flasks containing 100 ml of vegetative medium were inoculated from the slants. The vegetative medium had the following composition:

<table>
<thead>
<tr>
<th>Compound</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>1.5</td>
</tr>
<tr>
<td>Soy bean meal</td>
<td>1.5</td>
</tr>
<tr>
<td>Corn steep liquid</td>
<td>0.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5</td>
</tr>
<tr>
<td>CaCO$_3$</td>
<td>0.2</td>
</tr>
</tbody>
</table>

After 2 days of growth at room temperature on a New Brunswick rotary shaker, 10 ml of the culture were transferred to a two liter flask containing 1 liter of fermentation medium. The fermentation medium had the following composition:

<table>
<thead>
<tr>
<th>Compound</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>8.0</td>
</tr>
</tbody>
</table>
(NH₄)₂SO₄  0.1
NH₄NO₃  0.5
MgCl₂·6H₂O  0.5
K₂HPO₄  0.3
CaCO₃  0.1
Mineral Mixture C  1.0

Mineral mixture C had the following composition:

<table>
<thead>
<tr>
<th>Compound</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.28</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.27</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.0125</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>0.1</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.01</td>
</tr>
</tbody>
</table>

The cells were allowed to grow for 8 days at room temperature on a New Brunswick rotary shaker and then either harvested or transferred to solid medium for sporulation.

The cells were harvested by filtration on a Buchner funnel and washed several times with ice-cold distilled water; the cell paste was stored at -10°C until used.

For sporulation, 5 ml of a cell suspension were transferred aseptically to 100 X 15 mm petri dishes containing approximately 20 ml of the following medium (Hickey and Tresner; 1952):

<table>
<thead>
<tr>
<th>Compound</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amidex</td>
<td>1.0</td>
</tr>
<tr>
<td>Z - Z Amine A</td>
<td>0.2</td>
</tr>
<tr>
<td>Beef extract</td>
<td>0.1</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.1</td>
</tr>
<tr>
<td>Co⁺⁺</td>
<td>0.002</td>
</tr>
<tr>
<td>Agar</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Incubation at 37°C for 7 days gave good yields of spores.
Cultures of *Mycobacterium smegmatis* were maintained on Tryptic Soy Agar (2% agar added to Tryptic Soy Broth). One hundred and twenty five ml flasks containing 50 ml of Tryptic Soy Broth were inoculated from the slants. After 48 hours of growth at 37°C, 5 ml of this culture were used to inoculate 2 liter flasks, containing 1 liter of the following medium, a modification of the medium of Heath and Ghalambor (1962):

<table>
<thead>
<tr>
<th>Compound</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl</td>
<td>0.5</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.0</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.3</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.02</td>
</tr>
<tr>
<td>CaCl₂·6H₂O</td>
<td>0.002</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.0001</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.25</td>
</tr>
</tbody>
</table>

The mannose was autoclaved separately as a 50% solution.

The cells were grown at 37°C for 72 hours on a New Brunswick rotary shaker. The cells were harvested by centrifugation at 15,000g for 20 min, washed twice with 0.15 M KCl and stored at -10°C until used.
TREHALOSE METABOLISM IN ACTINOMYCETES

High voltage paper electrophoresis of incubation mixtures of sonic extracts of *Streptomyces hygroscopicus* with GDPG-\(^{14}\)C and glucose-6-phosphate revealed the presence of a negatively charged radioactive compound with a lower mobility than GDPG. Radioactive neutral compounds were also present. The charged compound was identified as trehalose-6-phosphate (Elbein; 1967a). The neutral compounds were eluted and subjected to paper chromatography. Two compounds were detected and identified as trehalose and glucose. Therefore, the following scheme for the metabolism of trehalose in *S. hygroscopicus* was postulated:

1) GDPG + glucose-6-phosphate \(\rightarrow\) trehalose-6-phosphate
2) trehalose-6-phosphate \(\rightarrow\) trehalose + P\(_i\)
3) trehalose \(\rightarrow\) 2 glucose

Further evidence for the presence of an enzyme system catalyzing reaction 3) above was obtained by incubating trehalose with sonic extracts of *S. hygroscopicus*. Twenty g of a cell paste of *S. hygroscopicus* were suspended in 100 ml of 0.01 M potassium phosphate buffer, pH 7.0 containing 0.005 M \(\beta\)-mercaptoethanol. The cell suspension was sonicated using a Bronwill Biosonik ultrasonic disruptor for a total time of 15 min, each 3 min period of sonication being followed by a 3 min period of cooling. The mixture was centrifuged at 30,000g for 10 min. The supernatant liquid was used in the following experiment.

Incubation mixtures were set up containing the following components in a final volume of 0.1 ml: sodium cacodylate buffer, pH 6.5, 20 \(\mu\)moles; trehalose, 30 \(\mu\)moles and supernatant liquid in various amounts. Controls contained the same components, except that the supernatant liquid had previously been heated for 10 min in a boiling water bath. The mixtures were incubated for varying amounts
of time at 37°C. The reaction was stopped by heating the tubes in a boiling water bath for 10 min. Glucose was determined by the reducing sugar method or by glucose oxidase. Considerably more glucose was present in those mixtures which contained the supernatant liquid which had not previously been heat treated. The amounts of glucose present increased with the concentration of the supernatant liquid and the time of incubation, thus indicating that the hydrolysis of trehalose to glucose was enzyme catalyzed. In other words, this experiment indicated the presence of a trehalase (α,α'-glucoside 1-glucohydrolase) in Streptomyces hygroscopicus. This reaction is shown in Figure 1.

At this stage, it was decided to purify the enzyme and to determine its specificity.

**Partial Purification of Trehalase**

**Assay of enzyme.** For routine assay, incubation mixtures contained the following components in a final volume of 0.1 ml: sodium cacodylate buffer, pH 6.5, 20 μmoles; trehalose, 3.0 μmoles; and an appropriate amount of enzyme. The mixtures were incubated at 37°C for 15 min. The reaction was stopped by heating the tube for 10 min in a boiling water bath. The release of glucose was measured either by a Nelson method or by glucose oxidase. Both methods gave comparable results.

One unit of enzymatic activity is defined as that amount of enzyme which catalyzes the hydrolysis of 1 μmole of trehalose in 1 min at 37°C.

**Preparation of enzymatic extract.** It was found, during preliminary experiments, that a more efficient extraction of trehalase could be accomplished using lysozyme (Walker and Hnilica; 1964). A 60g amount of cell paste was suspended in 180 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 0.5 mg of ethylenediaminetetraacetic acid and 1 mg of egg white lysozyme per ml. After incubation at room temperature for 1 hour, the suspension was centrifuged at
Figure 1. Hydrolysis of trehalose to glucose
$\alpha, \alpha$ Trehalose $\rightarrow$ 2 Glucose
30,000g for 10 min. The supernatant liquid contained the enzymatic activity. All the following steps were conducted at 0°C.

Manganese precipitation and first ammonium sulfate fractionation. To 180 ml of crude extract, 7.2 ml of 1 M MnCl₂ were added slowly with stirring. The mixture was allowed to stand for 5 min and then was centrifuged. The precipitate was discarded and 31.5g of solid ammonium sulfate was added to the supernatant liquid (30% saturation). After centrifugation, an additional 38.7g of solid ammonium sulfate was added to the supernatant liquid (60% saturation). The precipitate was isolated by centrifugation, dissolved in 30 ml of distilled water and dialyzed overnight against distilled water.

Treatment with calcium phosphate gel. To 33 ml of the ammonium sulfate fraction, 49.5 ml of calcium phosphate gel (15 mg/ml) were added. The mixture was allowed to stand for 15 min and then was centrifuged. The supernatant liquid was discarded and the gel resuspended in 50 ml of 0.04 M potassium phosphate buffer, pH 6.5. The gel suspension was stirred mechanically for 30 min and then was centrifuged. The enzymatic activity was released from the gel and was recovered in the supernatant liquid.

Second ammonium sulfate fractionation. To 50 ml of the supernatant liquid from the above treatment, 12.1g of solid ammonium sulfate (40% saturation) were added. The precipitate was removed by centrifugation and discarded. An additional 5.1g of solid ammonium sulfate (55% saturation) was added to the supernatant liquid. The precipitate was isolated by centrifugation, dissolved in 10 ml of distilled water and dialyzed overnight against distilled water.

Hydroxyapatite chromatography. A 10 ml amount of the second ammonium sulfate fraction was applied to a column (1.5 X 6cm) of hydroxyapatite, which had previously been equilibrated with 0.001 M
potassium phosphate buffer, pH 6.5. The column was washed with 50 ml of 0.001 M potassium phosphate buffer, pH 6.5. The protein was eluted stepwise with 50 ml portions of 0.01 M, 0.02 M and 0.03 M potassium phosphate buffer, pH 6.5. Enzymatic activity was eluted at 0.03 M.

With this procedure, the enzyme was purified approximately 80-fold with 10% recovery (Table I). This enzyme fraction was used in all the following experiments. The enzyme was found to be stable to freezing at all stages of purification. The purified enzyme showed no loss in activity after storage at -10°C for 4 weeks and could be frozen and thawed several times with no significant loss in activity.
<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PURIFICATION OF TREHALASE</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>TOTAL UNITS</th>
<th>SPECIFIC ACTIVITY (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRUDE EXTRACT</td>
<td>35.0</td>
<td>0.022</td>
</tr>
<tr>
<td>MANGANOUS CHLORIDE + AMMONIUM SULFATE</td>
<td>30.0</td>
<td>0.072</td>
</tr>
<tr>
<td>CALCIUM PHOSPHATE GEL</td>
<td>28.3</td>
<td>0.148</td>
</tr>
<tr>
<td>AMMONIUM SULFATE</td>
<td>15.8</td>
<td>0.218</td>
</tr>
<tr>
<td>HYDROXYAPATITE</td>
<td>3.3</td>
<td>1.667</td>
</tr>
</tbody>
</table>
RESULTS

Effect of time, enzyme concentration and substrate concentration on the rate of hydrolysis of trehalose. The rate of hydrolysis of trehalose was linear with respect to time and enzyme concentration (Figure 2). The $K_m$ was estimated to be $1.8 \times 10^{-2}$ (Figure 3).

Effect of pH. Figure 4 illustrates the effect of pH on the rate of hydrolysis of trehalose. The optimal pH in sodium cacodylate buffer was found to be approximately 6.5. Potassium phosphate buffer at the concentration used in the standard assay procedure (0.2 M) caused approximately 20% inhibition of enzymatic activity. Tris buffer at the same concentration completely inhibited enzymatic activity.

Stoichiometry of the reaction. Incubation of trehalose with the purified enzyme resulted in the formation of 2 moles of glucose for each mole of trehalose consumed (Figure 5).

Characterization of the product. As would be expected, the product of the reaction was easily identifiable as glucose. Approximately 12 $\mu$moles of the product were isolated, by paper chromatography in solvent I, from a large incubation mixture. On development of the chromatogram with alkaline silver nitrate, only two spots were observed, corresponding to glucose and trehalose. The area corresponding to glucose was cut out and eluted. On further chromatography of this area in two further solvents, it was again found to have the same mobility as authentic D-glucose (Table I). In addition, samples tested for reducing sugar, total hexose and activity with D-glucose oxidase gave a ratio of hexose to reducing sugar to glucose oxidase positive substance of 1.02 : 1 : 0.93.

Substrate specificity. The purified enzyme was found to be completely specific for trehalose (Table III). None of the other
Figure 2. Effect of time and enzyme concentration on the rate of hydrolysis of trehalose. Incubation mixtures contained the following components in a final volume of 1.0 ml: sodium cacodylate buffer, pH 6.5, 200 μmoles; trehalose, 30 μmoles; and trehalase, 0.0134 units (o--o), 0.0268 units (x--x) and 0.0536 units (●--●). At the times indicated, 0.1 ml samples were withdrawn and analyzed for reducing sugar formation.
Figure 3. Effect of substrate concentration on trehalase activity. Incubation mixtures contained the following components in a final volume of 0.2 ml: sodium cacodylate buffer, pH 6.5, 20 μmoles; trehalase, 0.0067 units; and trehalose in varying amounts. The mixtures were incubated at 37°C for 15 min and glucose formation was measured by the reducing sugar method.
Figure 4. Effect of pH on trehalase activity. Incubation mixtures contained the following components in a final volume of 0.1 ml: Trehalose, 3 μmoles; trehalase 0.0267 units; and 20 μmoles of the appropriate buffer. Acetate buffer was used from pH 4.5 to pH 6.0 (o--o) and sodium cacodylate buffer from pH 6.0 to pH 7.5 (x--x). The mixtures were incubated at 37°C for 15 min and glucose formation was measured by the reducing sugar method.
Figure 5. Stoichiometry of the reaction. Incubation mixtures contained the following components in a final volume of 1.0 ml: sodium cacodylate buffer, pH 6.5, 200 μmoles; trehalose, 30 μmoles; and 0.117 units of trehalase. At the times indicated, 5 μliter samples were withdrawn and assayed for glucose by the reducing sugar method (●--●). A further 5 μliter sample was removed at each time and assayed for trehalose by the anthrone method, after destruction of glucose by heating in 0.25 N sodium hydroxide at 100°C for 15 minutes (○--○).
MICROMOLES UNHYDROLYZED Trehalose

0.15

0.10

0.05

TIME (HOURS)

MICROMOLES GLUCOSE FORMED

0.15

0.10

0.05

0.079 MICROMOLES Trehalose HYDROLYZED

0.158 MICROMOLES GLUCOSE FORMED
### TABLE II

**PAPER CHROMATOGRAPHIC IDENTIFICATION OF THE PRODUCT FORMED FROM Trehalose**

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>( \cdot_{R_{glucose}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>GLUCOSE</td>
<td>1.00</td>
</tr>
<tr>
<td>GALACTOSE</td>
<td>0.92</td>
</tr>
<tr>
<td>MANNOSE</td>
<td>1.40</td>
</tr>
<tr>
<td>UNKNOWN</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* Mobility relative to glucose*
### TABLE III

**SUBSTRATE SPECIFICITY OF TREATALASE**

<table>
<thead>
<tr>
<th>DISACCHARIDE</th>
<th>LINKAGE</th>
<th>UMoles SUBSTRATE HYDROLYZED PER HR.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trehalose</td>
<td>α 1 → 1</td>
<td>0.36</td>
</tr>
<tr>
<td>Kojibiose</td>
<td>α 1 → 2</td>
<td>0.00</td>
</tr>
<tr>
<td>Sophorose</td>
<td>β 1 → 2</td>
<td>0.00</td>
</tr>
<tr>
<td>Nigerose</td>
<td>α 1 → 3</td>
<td>0.00</td>
</tr>
<tr>
<td>Laminaribiose</td>
<td>β 1 → 3</td>
<td>0.00</td>
</tr>
<tr>
<td>Maltose</td>
<td>α 1 → 4</td>
<td>0.02</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>β 1 → 4</td>
<td>0.00</td>
</tr>
<tr>
<td>Isomaltose</td>
<td>α 1 → 6</td>
<td>0.00</td>
</tr>
<tr>
<td>Gentiobiose</td>
<td>β 1 → 6</td>
<td>0.00</td>
</tr>
<tr>
<td>Trehalosamine</td>
<td>α 1 → 1</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Incubation mixtures contained the following components in a final volume of 0.1 ml: sodium cacodylate buffer, pH 6.5, 20 μmoles; trehalase, 0.0667 units and 3 μmoles of disaccharide. Mixtures were incubated for 15 min and glucose formation was measured by glucose oxidase.
naturally occurring glucose disaccharides exhibited any significant activity. No α- or β-glucosidase activity was detectable when the enzyme was tested with the α- or β-p-nitrophenyl glucosides. The enzyme did however exhibit a slight activity towards trehalosamine.

**Detection of trehalase activity in other Actinomycetes.** Table IV shows the specific activities of trehalase from various species of Streptomyces and also in two other Actinomycetes. It can be seen that, to a greater or lesser extent, the enzyme is present in all of the species so far examined. It should be noted, however, that although the relative specific activities differed as much as 100-fold, all of the values were significant.

**Trehalose metabolism in germinating spores.** Spores of *Streptomyces hygroscopicus* were prepared as previously described. Nutrient Broth was chosen as a suitable substrate for germination, since it contains no carbohydrates. It was not possible to produce germination in either water or mineral salts. Spores were harvested from the plates by suspending them in 5 ml of sterile Nutrient Broth. Any spores remaining on the plate were recovered by washing the plate with a further 5 ml of Nutrient Broth. Approximately 300 mg of spores were added to each of 7 two-liter flasks containing 1 liter of Nutrient Broth. At three hourly intervals, 250 ml aliquots were withdrawn from each of two flasks at random. Germination and growth were followed visually, by observing small quantities of the cells in the microscope. The first signs of germination of some of the spores was observed at 9 hours. Figure 6 shows the extent of germination at various intervals after transfer to Nutrient Broth. The remainder of the cells were harvested by centrifugation and stored as a frozen paste until used. The mixtures of spores and mycelia at 0, 6, 12, 18, 24 and 30 hours after transfer to Nutrient Broth were suspended in
<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>SPECIFIC ACTIVITY (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STREPTOMYCES HYGROSCOPICUS</td>
<td>1.55 \times 10^{-2}</td>
</tr>
<tr>
<td>S. ANTIBIOTICUS</td>
<td>0.07 \times 10^{-2}</td>
</tr>
<tr>
<td>S. AUREOFACIENS</td>
<td>0.10 \times 10^{-2}</td>
</tr>
<tr>
<td>S. GRISEUS</td>
<td>0.18 \times 10^{-2}</td>
</tr>
<tr>
<td>S. LAVENDULAE</td>
<td>0.12 \times 10^{-2}</td>
</tr>
<tr>
<td>S. RIMOSUS</td>
<td>2.25 \times 10^{-2}</td>
</tr>
<tr>
<td>S. VENEZUELAE</td>
<td>0.05 \times 10^{-2}</td>
</tr>
<tr>
<td>S. VIRGINIAE</td>
<td>0.10 \times 10^{-2}</td>
</tr>
<tr>
<td>MICROMONOSPORA CHALCÆ</td>
<td>0.02 \times 10^{-2}</td>
</tr>
<tr>
<td>MYCOBACTERIUM SMEGMATIS</td>
<td>0.03 \times 10^{-2}</td>
</tr>
</tbody>
</table>

Incubation mixtures contained the following components in a final volume of 0.2 ml: sodium cacodylate buffer, pH 6.5, 40 \( \mu \)moles; trehalose, 6 \( \mu \)moles; an appropriate amount of the dialyzed sonic extract of the organism indicated. Mixtures were incubated for 15 min, and the glucose formed was measured by the Nelson method for reducing sugar.
Figure 6. Germination of *Streptomyces hygroscopicus* spores
volumes equal to 10 times their weight of Tris-HCl buffer, pH 7.0, and were disrupted by sonication.

To determine trehalose phosphate synthetase activity, incubation mixtures contained the following components in a final volume of 0.02 ml: Tris-HCl buffer, pH 7.5, 10 μmoles; Mg²⁺, 0.75 μmoles; glucose-6-phosphate, 1 μmole; GDPG⁻¹⁴C 0.75 μmoles (7,200 cpm) and 100 μliters of extract. The mixtures were incubated for 30 min at 37°C and the reaction stopped by heating the tube in a boiling water bath for 10 min. To each tube was added 0.1 ml of a solution (10 mg/ml) of intestinal alkaline phosphatase (Worthington Biochemical Corp.) in 0.02 M Tris-HCl buffer, pH 8.0. The tubes were incubated at 37°C for 1 hour. One ml of water was added to each tube and salts were removed by treatment with mixed-bed ion exchange resin. The supernatant liquid was chromatographed in solvent III and the trehalose areas were cut out and counted in a liquid scintillation counter.

For the determination of the activity of a phosphatase capable of catalyzing the hydrolysis of trehalose phosphate incubation mixtures contained the following components in a final volume of 0.2 ml: Tris-HCl buffer, pH 8.0, 20 μmoles; trehalose-6-phosphate⁻¹⁴C, 0.02 μmoles (2,200 cpm); and 100 μliters of extract. The mixtures were incubated at 37°C for 30 min and the reaction stopped by heating the tubes in a boiling water bath for 10 min. One ml of water was added to each tube and the mixtures applied to columns (0.5 X 6cm) of Dowex-1-Cl⁻. Each column was eluted with 10 ml of water. The effluents were evaporated to dryness, redissolved in 0.2 ml of water, applied to pieces of Whatman #3MM filter paper, 1 cm square, and counted in a liquid scintillation counter.

For estimation of trehalase activity, small aliquots of extract were dialyzed overnight against distilled water. This removes the
Tris, which would otherwise interfere with the determination of trehalase activity. Incubation mixtures contained the following components in a final volume of 0.2 ml: sodium cacodylate buffer, pH 6.5, 40 μmoles; trehalose, 6 μmoles; and 100 μliters of extract. The mixtures were incubated for 30 min and the reaction stopped by heating the tubes in a boiling water bath. Glucose was determined by the reducing sugar method.

Figure 7 shows how the levels of these enzymes change during the germination process. It can be seen that trehalose phosphate synthetase activity is constant for about 18 hours and rises to double its initial value at 30 hours. Phosphatase activity is greatest in the spores, but decreases rapidly between 12 and 30 hours to undetectable levels. The specificity of this phosphatase has not been determined. Trehalase activity is low in the spores, and increases rapidly between 12 and 30 hours to approximately 20 times its original value.

Mixtures of spores and mycelia at 3, 9, 18, 21 and 27 hours were suspended in a volume of 70% ethanol equal to 10 times their weight, heated almost to boiling and cooled in the refrigerator. The debris was removed by centrifugation. The ethanol extracts were evaporated to dryness in a rotary evaporator and redissolved in water. The mixtures were applied to columns (0.5 X 6cm) of Dowex-1 Cl⁻ and eluted with 10 ml of water, followed by 10 ml of 1 N HCl. The water and acid eluants were collected separately, evaporated to dryness and redissolved in 1 ml of water. The acid eluant was assayed for trehalose phosphate. Aliquots were taken, heated at 100°C for 13 min with 0.25 N HCl, then 0.3 N NaOH to remove any interfering compounds, and assayed for trehalose by the anthrone method. The water eluants were chromatographed in solvent III, on Whatman #3MM
Figure 7. Levels of trehalose phosphate synthetase (1) phosphatase (2) and trehalase (3) activities in germinating *Streptomyces hygroscopicus* spores.
1. Synthetase

2. Phosphatase

3. Trehalase

(time (hours) of germination)

(moles/mg/min)

Specific activity
filter paper. The areas corresponding to trehalose and glucose were cut out and eluted. Glucose was measured using glucose oxidase. Trehalose was assayed using the specific trehalase, prepared as previously described. The glucose formed in this reaction was assayed by glucose oxidase. Figure 8 shows the levels of trehalose, trehalose phosphate and glucose in germinating spores. It can be seen that the level of trehalose is greatest initially, and drops rapidly between 9 and 27 hours. Very small amounts of trehalose phosphate and glucose were present. A slight increase in the amounts of these compounds were observed at 27 hours.

The levels of trehalose and trehalose phosphate were also determined in the spores. 10g of spores were treated in the manner described above, except that adjustments were made to compensate for the increase in weight. The acid fraction was assayed for trehalose phosphate as described above. The water fraction was assayed for trehalose in the same manner. The concentrations of trehalose phosphate and trehalose per g wet weight of spores were 0.68 μmoles and 6.5 μmoles respectively.
Figure 8. Levels of trehalose (●—●), trehalose phosphate (○—○) and glucose (x—x) in germinating *Streptomyces hygroscopicus* spores.
MANNOSE METABOLISM IN MYCOBACTERIUM SMEGMATIS

Effect of substrate on growth of Mycobacterium smegmatis. One hundred and twenty five ml flasks, containing 50 ml of a 1% solution of sugar or sugar alcohol in mineral salts medium were inoculated with 0.2 ml of a 24 hr culture of Mycobacterium smegmatis, grown on Tryptic Soy Broth. At the times indicated in Figure 9, 1 ml aliquots were withdrawn, diluted to 3 ml with distilled water, and the optical density at 620 μ was determined, using a Zeiss spectrophotometer. It can be seen that growth on fructose, mannose, sorbitol and mannitol were essentially the same, whereas the lag period was considerably longer when the substrate was glucose.

It was suspected from these results that there was an enzyme system present in the cells grown on mannose, which allowed the conversion of mannose (or a mannose phosphate) to fructose (or a fructose phosphate). To test this hypothesis, sonic extracts of M. smegmatis, grown on mannose-mineral salts medium were incubated with mannose, and fructose formation was determined by the Roe method.

A 5g amount of a cell paste of M. smegmatis, grown on mannose-mineral salts medium was suspended in 25 ml of 0.005 M Tris-maleate buffer, pH 7.5 and sonically disrupted, using a Bronwill Biosonik ultrasonic disruptor, for a total time of 10 min, each period of 2 min sonication being followed by an equal period of cooling. The suspension was centrifuged at 30,000g for 10 min and the supernatant liquid was used in the following experiment.

Incubation mixtures were set up, which contained the following components in a final volume of 0.2 ml: Tris-maleate buffer, pH 7.5 5 μmoles; mannose 6 μmoles; and varying amounts of sonic extract prepared as described above. Controls contained the same components
Figure 9. Effect of substrate on growth of *Mycobacterium smegmatis*. 
except that the supernatant liquid had previously been heated for 10 min in a boiling water bath. The mixtures were incubated at 37°C for varying amounts of time. The reaction was stopped by heating the tubes in a boiling water bath for 5 min. Four ml of water were added, and 1 ml taken for the determination of fructose by the Roe method. It was found that the presence of fructose could be detected in all cases, except where heat treated supernatant liquid was used. Increased amounts of fructose were detected when either the amount of supernatant liquid or the time of incubation was increased. This indicated that extracts of M. smegmatis were indeed capable of converting mannose to fructose. In other words a mannose isomerase (D-mannose ketol-isomerase) was present, since an apparent direct isomerization of mannose to fructose was involved. The reaction is shown in Figure 10. One unit of enzymatic activity was defined as that amount of enzyme which will convert 1 μmole of mannose to fructose in 1 min at 37°C.

In a similar experiment, where glucose was substituted for mannose in the incubation mixture, no fructose formation was detected.

Effect of growth substrate on the ability of sonic extracts of M. smegmatis to convert mannose to fructose. Table V summarizes the results obtained when extracts of M. smegmatis, grown on various substrates were tested for their ability to convert mannose to fructose. There were very low levels of activity in all the extracts except those grown on mannose and fructose. There was an extremely high rate of conversion of mannose to fructose in the cells grown on mannose and a considerable rate of conversion of mannose to fructose in extracts of cells grown on fructose.

At this point, it was of interest to purify the mannose isomerase and to determine its properties.
Figure 10. Isomerization of mannose and fructose.
D (+) MANNOSE  D (-) FRUCTOSE
TABLE V

MANNOSE ISOMERASE ACTIVITY IN SONIC EXTRACTS OF
MYCOBACTERIUM SMEGMATICI GROWN ON VARIOUS SUBSTRATES

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>SPECIFIC ACTIVITY (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NUTRIENT BROTH</td>
<td>0.3 x 10^{-2}</td>
</tr>
<tr>
<td>GLUCOSE</td>
<td>0.2 x 10^{-2}</td>
</tr>
<tr>
<td>MANNOSE</td>
<td>3.3 x 10^{-2}</td>
</tr>
<tr>
<td>FRUCTOSE</td>
<td>1.1 x 10^{-2}</td>
</tr>
<tr>
<td>SORBITOL</td>
<td>0.2 x 10^{-2}</td>
</tr>
<tr>
<td>MANNITOL</td>
<td>0.0 x 10^{-2}</td>
</tr>
</tbody>
</table>

Sonic extracts were made as described in the text. Incubation mixtures contained the following components in a final volume of 0.2 ml: Tris-maleate buffer, pH 7.5, 5 μmoles; mannose, 6 μmoles; and 40 μl liters of sonic extracts of M. smegmatis grown on the substrates indicated above. Assay of enzymatic activity was performed as described in the text.
Partial Purification of Mannose Isomerase

Assay of Enzyme. For routine assay, incubation mixtures contained the following components in a final volume of 0.2 ml: Tris-maleate buffer, pH 7.5, 5 μmoles; mannose, 6 μmoles; and an appropriate amount of enzyme. The mixtures were incubated at 37°C for 30 min. The reaction was stopped by heating the reaction mixtures for 5 min in a boiling water bath. Four ml of water were added and 1 ml was taken for the assay of fructose by the Roe method.

Preparation of enzymatic extract. A 20g amount of a cell paste of M. smegmatis, grown on mannose-mineral salts medium was suspended in 100 ml of 0.005 M Tris-maleate buffer, pH 7.5, and was sonicated for a total time of 15 min, each 3 min period of sonication being followed by a 3 min period of cooling. The suspension was centrifuged at 30,000g for 10 min to remove cellular debris. As described above, the supernatant liquid contained the enzymatic activity. All the following steps were performed at 0°C.

Streptomycin sulfate fractionation. To 100 ml of the extract were added, slowly with stirring, 10 ml of an aqueous solution of streptomycin sulfate (100mg/ml). The mixture was allowed to stand for 10 min and the precipitate was removed by centrifugation.

pH and heat treatment. Twenty five ml aliquots of the supernatant liquid from the above step were placed in each of four 125 ml Erlenmeyer flasks. To each flask were added 1.7 ml of 1 M sodium acetate buffer, pH 5.0. Each flask was then gently shaken in a 55°C water bath for 90 sec and cooled rapidly in ice. The precipitate from this step was removed by centrifugation.

Treatment with calcium phosphate gel. To 100 ml of the supernatant liquid remaining from the above step were added slowly with stirring, 100 ml of calcium phosphate gel (15mg/ml). The mixture was
allowed to stand for 10 min and then centrifuged for 10 min at 30,000g. The enzymatic activity remained in the supernatant liquid.

Ammonium sulfate fractionation. To 185 ml of this supernatant liquid were added, slowly with stirring, 57.9g of solid ammonium sulfate (50% saturation). The precipitate was removed by centrifugation and to the supernatant liquid were added a further 28.8g of solid ammonium sulfate (70% saturation). The precipitate was isolated by centrifugation, dissolved in 12 ml of 0.01 M Tris-maleate buffer, pH 7.5, and dialyzed overnight against two liters of the same buffer.

DEAE-cellulose chromatography. DEAE-cellulose was purified by consecutive treatments with 1 N NaOH and 1 N HCl. It was washed with distilled water until neutral and then suspended in 1 M KCl until used. Before use, the DEAE-cellulose was washed with 0.01 M Tris-maleate buffer, pH 7.5, until the effluent was free of chloride ion.

The ammonium sulfate fraction was applied to a column (2 X 24cm) of DEAE-cellulose. The column was washed with 250 ml of 0.01 M Tris-maleate buffer, pH 7.5, and then eluted with 250 ml of 0.1 M KCl in 0.01 M Tris-maleate buffer, pH 7.5, and 250 ml of 0.2 M KCl in the same buffer. 5 ml fractions of the 0.2 M KCl eluant were collected and monitored for protein and enzymatic activity. A typical elution profile is given in Figure 11. The fractions containing the enzymatic activity were pooled and concentrated to about 10 ml using the Amicon Diaflow apparatus, with a UM-1 filter (molecular weight cut off of 10,000). Any residual KCl was removed by overnight dialysis against 0.01 M Tris-maleate buffer, pH 7.5.

Using this procedure, the enzyme was purified approximately 60-fold, with 16% recovery (Table VI). This enzyme fraction was
Figure 11. Chromatography of mannose isomerase on DEAE-cellulose.
5 ml fractions of the 0.2 M eluant were collected and monitored for protein by determination of optical density at 280 m\(\mu\) (o--o) and for mannose isomerase activity as described in the text (●--●).
<table>
<thead>
<tr>
<th>FRACTION</th>
<th>TOTAL UNITS</th>
<th>SPECIFIC ACTIVITY (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRUDE EXTRACT</td>
<td>30.60</td>
<td>0.039</td>
</tr>
<tr>
<td>STREPTOMYCIN SULFATE, pH and HEAT</td>
<td>28.30</td>
<td>0.044</td>
</tr>
<tr>
<td>CALCIUM PHOSPHATE GEL</td>
<td>25.00</td>
<td>0.064</td>
</tr>
<tr>
<td>AMMONIUM SULFATE</td>
<td>10.35</td>
<td>0.396</td>
</tr>
<tr>
<td>DEAE-CELLULOSE</td>
<td>4.86</td>
<td>2.250</td>
</tr>
</tbody>
</table>
used in all the following experiments, unless otherwise indicated.

The enzyme was found to be stable to freezing at each stage of purification and could also be stored overnight in ice, without any detectable loss in activity. The purified enzyme could be stored at -10°C for at least two weeks and could be frozen and thawed several times without loss of activity.
RESULTS

Effect of time, protein concentration and substrate concentration on the isomerization of mannose and fructose. The rate of isomerization of mannose to fructose was linear with time and enzyme concentration, provided a large excess of mannose was present (Figure 12). However it could be demonstrated that eventually an equilibrium between mannose and fructose was established. At equilibrium the ratio of fructose to mannose was approximately 65:35. (Figure 13). The \( K_m \) for the conversion of mannose to fructose was estimated to be approximately \( 7 \times 10^{-3} \, M \) (Figure 14). It was not attempted to determine the \( K_m \) for the conversion of fructose to mannose, since small decreases in fructose in the presence of a large amount of fructose could not easily be detected.

Effect of pH. Figure 15 illustrates the effect of pH on the rate of fructose formation. The optimal pH in Tris-maleate buffer was found to be approximately 7.5.

Substrate specificity. The purified enzyme was found to be active towards D-mannose and D-lyxose. No activity could be detected with D-glucose, D-galactose or D-arabinose, showing that the enzyme must be specific for the D-mannose configuration. No activity could be detected towards D-mannitol, showing that the mannose was not reduced to mannitol and reoxidized to fructose (Table VII).

Characterization of products. The formation of a ketose when mannose was incubated with the purified enzyme was indicated by the positive reaction with cysteine-carbazole. Further, the positive reaction with the Roe method indicated fructose. When fructose was incubated with purified enzyme, a decrease in the amount of this compound could be detected using the Roe method. Paper chromatography
Figure 12. Effect of time and enzyme concentration on the rate of isomerization of mannose to fructose. Incubation mixtures contained the following components in a final volume of 1.0 ml: Tris-maleate buffer, pH 7.5, 25 μmoles; mannose, 30 μmoles; and 0.044 (o--o); 0.088 (x--x) or 0.176 (⁻⁻⁻) units of enzyme. At the times indicated, 0.1 ml aliquots were withdrawn and analyzed for fructose formation by the Roe method.
Figure 13. Kinetics of approach to equilibrium of mannose-fructose isomerization. Incubation mixtures contained the following components in a final volume of 1.5 ml: Tris-maleate buffer, pH 7.5, 25 µmoles; mannose (●--●) or fructose (○--○), 10 µmoles and 0.22 units of enzyme. At the times indicated, 0.1 ml aliquots were withdrawn and assayed for fructose by the Roe method.
Figure 14. Effect of mannose concentration on the rate of isomerization of mannose to fructose. Incubation mixtures contained the following components in a final volume of 0.2 ml: Tris-maleate buffer, pH 7.5, 5 μmoles; mannose isomerase, 0.044 units and mannose in varying amounts. Mixtures were incubated for 10 min at 37°C and assayed for fructose formation by the Roe method.
Figure 15. Effect of pH on mannose isomerase activity. Incubation mixtures contained the following components in a final volume of 0.2 ml: mannose, 6 μmoles; mannose isomerase, 0.04 units and 5 μmoles of Tris-maleate buffer at the pH values indicated. Mixtures were incubated for 30 min and fructose formation determined by the Roe method.
TABLE VII

SUBSTRATE SPECIFICITY OF MANNOSE ISOMERASE

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>µMOLES KETOSE FORMED</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-MANNOSE</td>
<td>0.124</td>
</tr>
<tr>
<td>D-GLUCOSE</td>
<td>0.000</td>
</tr>
<tr>
<td>D-GALACTOSE</td>
<td>0.002</td>
</tr>
<tr>
<td>D-LYXOSE</td>
<td>0.520</td>
</tr>
<tr>
<td>D-ARABINOSE</td>
<td>0.005</td>
</tr>
<tr>
<td>D-SORBITOL</td>
<td>0.001</td>
</tr>
<tr>
<td>D-MANNITOL</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Incubation mixtures contained the following components in a final volume of 0.1 ml: Tris-maleate buffer, pH 7.5, 2.5 µmoles; 0.004 units of enzyme; and 3 µmoles of the substrate indicated. Mixtures were incubated for 30 min and the amount of ketose formed was measured by the cystein-carbazole reaction. Fructose was used as standard.
in solvent IV of aliquots of the reaction mixtures, when either D-mannose or D-fructose were used as substrates revealed the presence of two and only two compounds in each reaction mixture. The mobilities relative to glucose of these two compounds were identical with those of authentic mannose and fructose (Table VIII). Further the compounds corresponding to fructose could be shown to be ketoses using the orcinol spray.

Use of purified mannose isomerase as an analytical tool. The only compound that the purified mannose isomerase will convert to fructose is mannose. Thus, it was thought possible that the use of this enzyme coupled with the Roe method for fructose determination would provide a rather elegant tool for the rapid quantitative determination of the amounts of mannose present in a mixture of sugars. A tool such as this would prove invaluable for the analysis of heteropolysaccharides and other carbohydrate-containing compounds. It would be especially useful if small quantities of mannose could be detected in the presence of large quantities of glucose. The only method currently available for the determination of mannose in the presence of glucose is the determination of total hexose by the anthrone method, and the determination of glucose by glucose oxidase. The difference between the two values will give the amount of mannose. The errors inherent in this method are obvious, if the amount of glucose greatly exceeds the amount of mannose. Further, glucose and mannose are difficult to separate, except as their borate complexes. In this case, the borate must be removed, before colorimetric reactions can be performed.

Use of mannose isomerase as an analytical tool would have further advantages. The enzyme is easy to prepare, requiring no equipment not usually available in most biochemical laboratories. It is stable
TABLE VIII  
PAPER CHROMATOGRAPHIC IDENTIFICATION OF THE PRODUCTS  
FORMED BY INCUBATION OF MANNOSE AND FRUCTOSE WITH  
MANNOSE ISOMERASE  

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>$R_{glucose}$ in SOLVENT IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUCOSE</td>
<td>1.00</td>
</tr>
<tr>
<td>MANNOSE</td>
<td>1.23</td>
</tr>
<tr>
<td>FRUCTOSE</td>
<td>1.45</td>
</tr>
<tr>
<td>UNKNOWNS 1*</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>1.45</td>
</tr>
<tr>
<td>UNKNOWNS 2†</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>1.47</td>
</tr>
</tbody>
</table>

* From incubation of mannose with purified mannose isomerase.  
† From incubation of fructose with purified mannose isomerase.
to freezing for extended periods of time, and could therefore be prepared and stored until required. Further, very small amounts of mannose could be detected. The interconversion of mannose and fructose is an equilibrium reaction. At equilibrium, approximately 65% of the initial mannose has been converted to fructose. Therefore, it should be theoretically possible to detect amounts of mannose equal to approximately 1.5 X the smallest amount of fructose that can be accurately determined by the Roe method. As this amount is approximately 0.02 μmoles, it should be possible to detect amounts of mannose as low as 0.03 μmoles. In practice, however, it would be more convenient to compare the amount of fructose formed when the sample of unknown composition was incubated with the enzyme, with the amount formed when mannose solutions of known concentrations were incubated with the enzyme. Thus an increase in enzyme concentration would lead to the reduction of the incubation time.

In view of these interesting possibilities, it was decided to determine if the presence of other naturally occurring common sugars would affect the assay system. Table IX shows the effect of D-glucose, D-galactose, N-acetyl-D-glucosamine, D-glucuronic acid and D-arabinose when present at either the same or at 10 times the concentration of mannose. It can be seen that only glucuronic acid and high concentrations of arabinose significantly changed the amount of fructose that was formed, as determined by the Roe method. It should be noted, however, that high concentrations of some of the above compounds interfered to a greater or lesser extent with the colorimetric reaction. This difficulty was easily overcome by running controls, in which the mannose isomerase had been omitted.

Thus, it would appear that purified mannose isomerase can indeed be applied as an analytical tool to measure the amount of mannose present in a mixture of sugars, unless fructose, glucuronic acid or
**TABLE IX**

**EFFECT OF VARIOUS SUGARS ON MANNOSE ISOMERASE ACTIVITY**

<table>
<thead>
<tr>
<th>ADDITION</th>
<th>AMOUNT (µmoles)</th>
<th>µMOLES FRUCTOSE FORMED</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>0.5</td>
<td>0.028</td>
</tr>
<tr>
<td>GLUCOSE</td>
<td>0.5</td>
<td>0.034</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.025</td>
</tr>
<tr>
<td>GALACTOSE</td>
<td>0.5</td>
<td>0.034</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.028</td>
</tr>
<tr>
<td>N-ACETYL GLUCOSAMINE</td>
<td>0.5</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.034</td>
</tr>
<tr>
<td>GLUCURONIC ACID</td>
<td>0.5</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.009</td>
</tr>
<tr>
<td>ARABINOSE</td>
<td>0.5</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Incubation mixtures contained the following components in a final volume of 0.1 ml: Tris-maleate buffer, pH 7.5, 2.5 µmoles; mannose isomerase, 0.002 units and amounts of the sugars indicated above. The mixtures were incubated for 15 min at 37°C, and fructose formation measured by the Roe method. Controls contained all of the above components, except for mannose isomerase.
high concentrations of arabinose, are present.

**Possible Induction of enzymatic activity.** Attempts were made to try to determine whether mannose isomerase activity was constitutive or inducible. Four liters of cells were grown on Tryptic Soy Broth. Growth was followed by optical density at 620 mμ. At the times indicated in figure 16, one ml aliquots were taken, diluted to three ml and the optical density at 620 mμ was determined. When growth was in the logarithmic phase, the cells were harvested in sterile centrifuge bottles and resuspended in four liters of mannose-mineral salts medium. At the times indicated, 500 ml aliquots were taken, the optical density at 620 mμ determined as described above, and the cells were harvested by centrifugation. The cells were washed twice with ice-cold 0.15 M KCl and stored as a cell paste at -10°C until used. Extracts were made and enzymatic activity determined as described previously. It can be seen that there was an immediate increase in the activity of the mannose isomerase on transfer to mannose. However, since there was a low level of activity in cells grown on Nutrient Broth (see Table V) and in cells immediately after transfer, there appears to be only an increase in activity when the cells are grown on mannose. It would appear, therefore, that mannose isomerase activity is constitutive in this strain of *M. smegmatis* and is only fully active in the presence of mannose. To further clarify this situation, it should be determined whether or not there is an increase in mannose isomerase activity when cultures of *M. smegmatis* are transferred to mannose-mineral salts medium, containing puromycin, an inhibitor of protein synthesis. Increase in activity would indicate that mannose isomerase activity is constitutive. On the other hand, no increase in activity would indicate de novo synthesis of mannose isomerase.
Figure 16. Effect of transferring *Mycobacterium smegmatis* grown on Nutrient Broth to mannose-mineral salts medium. Solid lines indicate growth on Nutrient Broth (○—○) and mannose-mineral salts (●—●). The broken line represents mannose isomerase activity (× — ×).
DISCUSSION

The trehalase isolated from *Streptomyces hygroscopicus* bears marked similarities to that isolated from the hybrid yeast by Avigad *et al.* (1965). They reported a $K_m$ of $1.02 \times 10^{-2}$ M and a pH optimum of 6.9. The trehalase isolated from *Streptomyces hygroscopicus* has a $K_m$ of $1.8 \times 10^{-2}$ M and a pH optimum of 6.5. The $K_m$ values reported for intestinal trehalases are of the order of $10^{-3}$ M and those for insects are generally of the order of $10^{-4}$ M. The pH optima for trehalases isolated from many organisms range from 4.5 for *Schizophyllum commune* (Williams and Niederpruem; 1968) to the value of 6.9 reported by Avigad *et al.*

The absence of phosphate in the incubation medium precludes the possibility of a phosphorolytic cleavage of trehalose to glucose and glucose-1-phosphate.

The slight activity of the purified trehalase towards trehalosamine is of interest. Preliminary studies have shown that trehalosamine can also act as an inhibitor of trehalase activity, when trehalose is used as substrate. Presumably, the inhibition is competitive. In this regard, it is of interest to note that trehalosamine exerts an antibacterial effect towards mycobacteria (Arcamone and Bizzioli; 1957). Trehalase activity has also been demonstrated in *Mycobacterium smegmatis*. Trehalosamine has also been found to inhibit the germination of spores of *Dictyostelium discoideum*. Trehalase activity increases rapidly in the germinating spores of this organism (Ceccarini; 1967).

The discovery that trehalose is present in high concentrations in spores of *Streptomyces hygroscopicus* but decreases rapidly on germination, indicates that it is possibly a storage compound, which serves as a rapidly available source of energy. The concomitant
Increase in trehalase activity shows that this enzyme is responsible for its breakdown. It would be expected that the increase in trehalase activity and corresponding decrease in trehalose would result in the formation of glucose. However, no detectable glucose was observed until about 27 hours. Almost certainly, any glucose that is formed is rapidly metabolized.

It is of interest to note that there is a small quantity of trehalose-6-phosphate present in the spores, but that it is almost immediately broken down. At the same time, there is possibly a slight increase in trehalose. The level of phosphatase activity is highest in spores, but decreases rapidly on germination.

It would therefore be of interest to attempt to determine the levels of trehalose and trehalose phosphate and phosphatase activity as sporulation commences and in spores of different ages. Possibly any trehalose phosphate formed during the vegetative phase of growth is hydrolyzed in the spores, thus providing a more immediately usable energy source.

The trehalose phosphate synthetase activity starts to increase at about 24 hours. It is possible that this activity will continue to increase to a maximum, thus increasing the supplies of trehalose phosphate and trehalose.

The finding that trehalose and trehalase activity decreases and increases, respectively, in germinating spores, is in good agreement with work done by other groups on Neurospora and Dictyostelium discoideum.

Lingappa and Sussman (1959) and Sussman and Lingappa (1959) showed that the endogenous trehalose of dormant ascospores of Neurospora tetrasperma decreased within a few minutes after exposure of the spores to temperatures sufficient to break dormancy and was
almost completely exhausted by the time the germ tube was protruded. Hill and Sussman (1964) showed that trehalase activity was lowest in ascospores. Trehalose also appears to play a role in the formation of conidia of Neurospora crassa. Hanks (1967), showed that trehalose is accumulated in the vegetative mycelia of Neurospora crassa Strain 69-1113A. It decreased rapidly with conidiation. If the production of conidia was delayed, trehalose continued to accumulate until spores were formed. Total trehalase activity was 6-10 times as great in heavily conidiating strains as in strains which do not produce conidia, strains which produce conidia slowly or in strains in which conidiation is suppressed. Trehalose thus would again appear to act as an energy source.

Ceccarini and Filosa (1965) showed that trehalose is present in high concentrations in the spores of Dictyostelium discoideum. Ceccarini (1967) further showed that on germination of these spores, trehalase activity increased rapidly. The trehalose is hydrolyzed rapidly, and the glucose formed is further metabolized.

Roth and Sussman (1966 and 1968) demonstrated that trehalose phosphate synthetase activity was low in the cells of Dictyostelium discoideum at an early stage of development. It started to accumulate at 5 hours, peaked at 16 hours and then began to disappear. Mutants blocked at different stages of development accumulated trehalose phosphate synthetase to a lesser extent or not at all.

An investigation of the levels of trehalose phosphate and phos- phatase activity has never been reported previously.

The enzyme catalyzed isomerization of mannose and fructose in Mycobacterium smegmatis is of interest, since only a few instances of the direct isomerization of these compounds have been reported. Palleroni and Doudoroff (1956) isolated a D-mannose isomerase from
Pseudomonas saccharophila grown on fructose. Anderson and Allison (1965) isolated a D-lyxose isomerase from Aerobacter aerogenes grown on D-lyxose, which was active towards mannose. However, the enzyme was not induced by growing the organisms on mannose.

The utilization of unusual compounds by microorganisms has posed some interesting problems. Usually sugars have to be phosphorylated in order to undergo further metabolism. Many workers have found that kinases active towards the substrate on which the organism is growing are absent. Several pathways for the metabolism of these compounds have been found.

For example, Hiatt and Horecker (1956) found that a strain of Alcaligenes faecalis which would grow on erythrose could form erythrose-4-phosphate. Domagk and Horecker (1965) showed that there was no kinase present in these organisms which would catalyze the phosphorylation of erythrose. However, a fructokinase and transketolase were present. Thus erythrose could be metabolized as follows:

\[
\text{fructose} + \text{ATP} \rightarrow \text{fructose-6-phosphate}
\]

\[
\text{fructose-6-phosphate} + \text{erythrose} \rightarrow \text{erythrose-4-phosphate} + \text{fructose}
\]

Kamel and Anderson (1966) showed that although mannose could be utilized constitutively by a strain of Aerobacter aerogenes, no kinase capable of catalyzing the phosphorylation of mannose was present. However, mannose and glucose could be phosphorylated with either acetyl or carbamyl phosphate. In addition, glucose could be phosphorylated with a specific glucokinase. They found that the phosphate group from glucose-6-phosphate could be donated to mannose to give mannose-6-phosphate. A phosphomannose isomerase present catalyzed the isomerization of mannose-6-phosphate to fructose-6-phosphate. The presence of phosphoglucone isomerase was also detected. Thus the
scheme proposed for the utilization of mannose was as shown in Figure 17.

The most likely pathway for the utilization of mannose in _M. smegmatis_ is:

\[ \text{D-mannose} \rightarrow \text{D-fructose} \]

\[ \text{D-fructose} + \text{ATP} \rightarrow \text{D-fructose-6-phosphate} + \text{ADP} \]

In order to show this conclusively, it should be determined if a kinase capable of catalyzing the phosphorylation of fructose is present.
Figure 17. The utilization of mannose by *Aerobacter aerogenes*.

(Kamel and Anderson; 1966).
D-MANNOSE

ACETYL or CARBAMYLM PHOSPHATE

D-MANNOSE-6-PHOSPHATE

D-GLUCOSE-6-PHOSPHATE

D-GLUCOSE

ATP

ACETYL or CARBAMYLM PHOSPHATE

D-FRUCTOSE-6-PHOSPHATE

EMBDEN-MEYERHOFF PATHWAY
SUMMARY

1. The metabolism, by Actinomycetes, of an endogenous carbohydrate source, α,α'-trehalose, and an exogenous carbohydrate source, D-mannose, has been examined.

2. The enzyme, α,α'-glucoside 1-glucohydrolase, which catalyzes the hydrolysis of trehalose, was isolated from Streptomyces hygroscopicus and was purified approximately 80-fold. The enzyme was completely specific for trehalose. None of the other naturally occurring glucose disaccharides exhibited any significant activity. The pH optimum for enzymatic activity was found to be 6.5 and the $K_m$ estimated to be $1.8 \times 10^{-2}$ M. The product of the reaction was identified as glucose by chemical, chromatographic and enzymatic methods. The presence of this enzyme was demonstrated in several species of Streptomyces and related organisms.

3. The level of this enzyme and two other enzymes involved in the metabolism of trehalose, trehalose phosphate synthetase and a phosphatase were examined in germinating spores of S. hygroscopicus. Trehalose phosphate synthetase activity remained low and constant during the initial stages of germination, but began to increase by 24 hours after induction of germination. Phosphatase activity was greatest in ungerminated spores, and decreased rapidly to undetectable amounts as germination commenced. Trehalase activity was lowest in spores and increased rapidly to 20 times the original value with the onset of germination. A small amount of trehalose phosphate was detectable in spores, but this disappeared almost immediately after induction of germination. At the same time, a small increase in endogenous trehalose was observed. There was a rapid decrease in trehalose concomitant with increase in trehalase activity.
4. An enzyme, D-mannose ketol isomerase, catalyzing the isomerization of D-mannose and D-fructose was isolated from *Mycobacterium smegmatis*, grown on mannose as the sole carbon source, and purified approximately 60-fold. The enzyme was shown to catalyze the conversion of D-mannose and D-lyxose to ketoses. The product of the reaction with D-mannose was identified as D-fructose by chemical and chromatographic methods. The reaction was shown to be reversible, the equilibrium ratio of fructose to mannose being 65 : 35. The pH optimum for the reaction was found to be 7.5 and the *K_m* estimated to be $7 \times 10^{-3} \text{ M}$. Mannose isomerase activity was found to be greatest in cells grown on mannose; cells grown on fructose exhibited approximately 30% as much activity. Very low levels of activity were detected in cells grown on other substrates. There was an immediate increase in activity on transfer of cells from nutrient broth to a mannose-mineral salts medium.

5. A method for the use of the purified mannose isomerase for the determination of mannose in a mixture of sugars is presented.
BIBLIOGRAPHY


