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The Biosynthesis of Trehalose
in
Moniliiformis dubius (Acanthocephala)

by
Robert O. McAlister

A THESIS SUBMITTED
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DOCTOR OF PHILOSOPHY

Thesis Director's Signature:

A large, stylized handwritten signature in black ink, written over a horizontal line. The signature is cursive and appears to be the name of the thesis director.

Houston, Texas

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ACKNOWLEDGEMENTS

On Wednesday, November 5, 1969, I was privileged to hear Professor Theodor von Brand present the Presidential Address at the annual meetings of the American Society of Parasitologists in Washington, D.C. I was indeed fortunate as a graduate student to have the opportunity to attend these meetings. In his address, "An Old-Timer's Look at Parasite Physiology," Dr. von Brand included one particularly relevant statement:

"...When I compare today's parasite biochemistry with what has been done in the old days, I find that a most striking development in depth has taken place. While then almost exclusively whole organisms were used to investigate one phase or the other of the overall metabolism, now studies on intermediate metabolism predominate. They involve such approaches as identification and characterization of enzymes, elucidation of catabolic and anabolic pathways, studies on electron transport mechanisms, or characterization of nucleic acids, to mention just some of the modern lines of biochemical studies done with protozoan and helminth parasites."

It has been my privilege as a graduate student at Rice to be associated with a department which has built a twenty-year tradition of research on the biochemistry and physiology of the host-parasite complex. Many of the "modern" approaches to the field which Dr. von Brand mentions had either direct or indirect origins in this department. The excitement of working in a young field among pioneer researchers has been for me, as a "green" graduate student, unforgettable.

The following study was conducted under the able tutelage of Professor Frank M. Fisher. As a teacher and as a friend, his guidance and enthusiasm were unparalleled. My experiments with him have taught me that no question, if properly inquired of nature, is too naive; if approached with a large measure of patience, no problem is too complex to fathom. It is for these lessons that I shall always be indebted to "The Boss".

Those persons who have donated to me their precious time and talent during my tenure at Rice are almost too numerous to mention; however, special thnaks are extended to the members of my guidance committee, Profs. J.W. Campbell, R.M. Eisenberg, and C.H. Ward. The hours spent in class and in discussions with Prof. C.P. Read were without question some of the most stimulating and informative of my entire experience. The constant support lent by my parents, Bill and Lucy McAlister, was indispensable for the completion of this study. The lessons which my friend Jane helped me to learn will not soon be forgotten; in the pursuit of a goal, the unselfish willingness of a friend to make sacrifices can often spell the difference between success and failure.

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INTRODUCTION

The disaccharide trehalose (1-(α -D-glucopyranosyl)-D-glucopyranoside) was first isolated and named by Berthelot in 1859. Although his source of the sugar was derived from the shed exoskeletons of scale insects, Wyatt (1967) points out that for the next one hundred years, it was generally considered that trehalose was a product of lower plants. In simultaneous research conducted in several laboratories (Wyatt and Kalf, 1956, 1957; Howden and Kilby, 1956; Evans and Dethier, 1957), trehalose was re-discovered as an insect sugar. It was found that the hemolymph of many insect groups contains high concentrations of this disaccharide, and indeed that it constitutes the major circulating carbohydrate in several of these groups (Wyatt, loc.cit.).

The biosynthesis of trehalose was first demonstrated in yeast (Cabib and Leloir, 1958). These workers showed that the pathway involved two enzymes:

(1) TREHALOSE - PHOSPHATE SYNTHETASE:

(2) TREHALOSE-6-PHOSPHATASE:

In this study, the enzyme system was subjected to partial purification and the product of reaction (1) was characterized as authentic trehalose-6-P. The purified enzyme preparation was also shown to contain a phosphatase specific for trehalose-6-phosphate.

¹The following abbreviations are used: ATP for adenosine-5'-triphosphate, UDPG for uridine diphosphoglucose, GDPG for guanosine diphosphoglucose, ADPG for adenosine diphosphoglucose, CDPG for cytidine diphosphoglucose, TDPG for thymidine diphosphoglucose, EDTA for ethylene-diaminetetraacetic acid, and Tris for tris(hydroxymethyl) aminomethane.

The biosynthesis of trehalose was subsequently shown to proceed by a similar pathway in insect fat body (Candy and Kilby, 1961). This same reaction was later found to occur in Mycobacterium tuberculosis (Goldman and Lornitzo, 1962), and Dictyostelium discoideum (Roth and Sussman, 1966). In 1967, Elbein (1967) demonstrated another enzymatic synthesis of trehalose-phosphate to be operative in Streptomyces hygroscopicus:

This reaction has since been shown to occur in a number of other species of Streptomyces (Elbein, 1968).

It was recently demonstrated (Liu et al., 1969) that several species of Mycobacterium have the capacity to synthesize the disaccharide phosphate using either UDPG or GDPG as a precursor. It was further shown that GDPG is the normal glucosyl donor for the synthesis of trehalose in Mycobacterium smegmatis, but in the presence of a polynucleotide fraction, the enzyme can be modified to utilize UDPG.

There seems to be general agreement (although exceptions to this do exist; Mandels et al.) that trehalose metabolism in biological systems is mediated by the hydrolytic enzyme, trehalase, which catalyzes the reaction:

Wyatt's review (loc.cit.) adequately covers the experimental evidence supporting this premise, and includes an hypothesis for explaining the

physiological role of trehalose in insects and the functional role of the hydrolytic enzyme. It should be added that there is at least one report implicating some other pathway for trehalose metabolism (Mandels, et al., 1965). Trehalase has been isolated from a variety of sources, and in several instances has been sufficiently purified and characterized to be used as a quantitative tool for assaying the quantities of trehalose present in tissue extracts (Fisher and McAlister, 1969).

The comprehensive nature of Wyatt's review (loc.cit.) would make redundant a more thorough discussion of the biological properties and occurrence of trehalose in this dissertation. It should be pointed out that among the insect, bacterial, and fungal groups, sufficient studies have been performed to leave little doubt as to the origin and functions of trehalose in these systems. The compound is exceptionally stable to hydrolysis by either mild acid or mild alkali, is highly soluble in aqueous systems, and is osmotically active. The simple conversion by hydrolysis into glucose makes for easy entry into the main pathways of carbohydrate metabolism, and, as Wyatt points out, can thus be regulated easily by control of the enzyme responsible for this one step. In short, this disaccharide is a circulating stored carbohydrate which is easily mobilized when the energy demands of the organism require it.

The organism selected for this study, Moniliiformis dubius, (Meyer, 1933) is an acanthocephalan parasite of the rat. The first comprehensive treatment of the phylum Acanthocephala appeared in 1933 in "Bronn's Klassen und Ordnungen des Tierreichs" (Meyer, 1932-33). In his review of the group, Nicholas (1967) points out that the phylum is a small one (600-650 species described), and, although entirely parasitic, has largely been neglected by many parasitologists, possibly due to its minor importance in human and veterinary medicine. Nicholas remarks, however, that in recent years more and more biologists have focused their studies on this group, largely because of an increased interest in a comparative approach to biological research (Nicholas, loc.cit.).

Hyman (1951) includes in her account of the Acanthocephala a good review of the early research done on this group, and points out the morphological and developmental differences between the different orders. It seems to be generally accepted that the phylum consists of three orders: the Archiacanthocephala, the Palaeacanthocephala, and the Eocanthocephala. Of these, the genus Moniliiformis falls under the Archiacanthocephala. Nicholas (loc. cit.) gives a good comprehensive review of the significant research done on the biology of the Acanthocephala.

The life history of the organism used in this study, Moniliiformis dubius, was first studied and described in detail by Moore (1946). Although Moore's description of the life history is adequate, the

following account will include the nomenclature used by Nicholas (loc.cit.) for the larval stages, which in this author's opinion is easier to follow and has been used by most authors in recent works on the group.

Life History of Moniliiformis dubius

The parasites are dioecious; following copulations, the fertilized oocytes are contained in amorphous structures termed ovarian balls, which float free in the body cavity of the female worm. As development ensues, the body cavity becomes filled with ovarian balls, developing embryos, and the fully infective acanthors. Upon completion of development, the acanthor is surrounded by several membranes, one or more of which become hard and impart to the acanthor considerable resistance to adverse environmental conditions (see Nicholas, loc.cit.) . At this point, the acanthor is released by the female worm and passes out of the rat in the feces. It undergoes no further development until it is ingested the invertebrate host, Periplaneta americana.

After ingestion by a cockroach, the acanthor hatches within a few hours. The outer shell of the egg is lost and the activated acanthors, presumably aided by the anterior hooks, frees itself from the inner egg membranes. The larva then actively begins a process of penetration of the midgut of the roach, aided by the rostellar hooks. This process is usually completed within forty-eight hours after ingestion. For the next 10-12 days, the larvae remain imbedded in the midgut wall, at the end of which time they appear as minute iridescent specks on the serosal wall (Moore, 1946). The acanthor then drops into the haemocoel of the roach and begins a period of growth and differentiation which eventually leads to a stage in which all the adult organs have appeared and the parasite, in the words of Nicholas (loc. cit.),

"....resembles a miniature version of the adult." This stage is referred to as the acanthella, and is present from five to six weeks after ingestion by the roach. The acanthella stage persists for 1-2 weeks, depending upon the environmental temperature, after which time the proboscis and neck region are inverted by muscular contraction, and come to completely fill the pseudocoel. In M.dubius, the larvae take on a semi-flattened, triangular appearance, and fluid accumulates between the parasite and the outer capsule, or cyst, which is a product of the roach's tissue response to the foreign material (Mercer and Nicholas, 1967). This dormant stage, referred to as the cystacanth, undergoes no further development, and persists in the haemocoel of the roach until the host dies or is eaten by a suitable vertebrate host.

Upon ingestion, the cyst wall of the cystacanth is partially digested in the stomach of the rat, (Graff and Kitzman, 1965). The cystacanth undergoes a process termed "activation", after passage into the small intestine, whereby the proboscis is everted, penetrating the surrounding membranes of the cyst wall. The activation process is an active one on the part of the parasite, but requires bile salts and is enhanced by the presence of CO₂ in vitro (Graff and Kitzman, loc. cit.). Following activation, the cystacanth, which I now propose to term a juvenile, attaches itself to the mucosa of the vertebrate small intestine, and begins to grow. The prepatent period, leading to the adult stage, ranges from five to six weeks (Burlingame and

Chandler, 1941).

Studies on the Acanthocephala have been, as was pointed out above, rather sparse. When one examines only those papers which deal directly with biochemical and physiological processes operative in this group, it becomes patently obvious that this group has been largely neglected. Such a situation presents an apparent dilemma to prospective investigators, because, while the area is "wide-open", there are few, if any, background data available as a foundation for a sophisticated study of some metabolic process in these worms. This fact, coupled with the relatively minor importance which the phylum contributes to human disease, probably accounts for the lack of interest in the group.

As a research animal, M.dubius presents many advantages. The worms grow to a size which is easily handled, and the life cycle requires a minimum of effort to maintain under controlled laboratory conditions. Both the invertebrate host, Periplaneta americana, and the final host, the albino rat, are easily procured and maintained. These two organisms, being so easy to rear in the laboratory, afford the experimenter the option of utilizing inbred strains for rearing the parasite, a factor which is highly desirable if reproducibility of data on growth and dietary requirements is to be maximized. The potential effects of strain differences arising from animals obtained from different populations can thus be minimized.

Although background information on the physiology of the

Acanthocephala is scanty, the author is fortunate that such is not the case with other intestinal helminths. Although members of different phyla, those cestodes and nematodes which parasitize vertebrates have been studied in considerable detail. As Read (1950) so eloquently points out, there are many unifying principles which a researcher may draw on when thinking of any parasite which has as its environment the vertebrate gut. Although one must hesitate to draw direct comparisons, the elegant studies of Read and co-workers (1963), on the rat tapeworm, Hymenolepis diminuta, as well as the abundant data compiled on the cosmopolitan intestinal roundworm, Ascaris suum (Fairbairn, 1957), certainly yield strong hints as to results which might be obtained in a physiological or biochemical study of another intestinal parasite. The host-parasite system affords the opportunity to draw such analogies based on similarities in environment which, in the opinion of this author, is almost unique among prospective areas of research in the biological sciences. The reasons for this seem to be two-fold:

(1) The environment of the parasite is under very delicate control mediated by the intricate homeostatic mechanisms of the host, especially in the mammals. This control is so concise that many examples abound of parasites having evolved to the point that the number of areas in which they show obligate dependence on host homeostatic mechanisms are so numerous, and often so subtle, as to preclude objective studies of the parasite's processes independent of the host-parasite system

(Read, 1970).

(2) Although members of phylogenetically distinct groups, the fact that two helminths spend the adult stage in the same environment, i.e., in the same host, would favor the evolution of parallel mechanisms for maintenance of the species and acquisition of necessary metabolites. The analogy becomes even stronger if one examines the fact that both Hymenolepis diminuta and M. dubius lack a mouth or alimentary tract, and therefore must depend on similar mechanisms for obtaining nutrients, i.e., by absorption through the external surface. The more steps in the life cycle which two parasitic organisms share in common, the more similar might be the processes which have evolved for coping with the environment and maintaining the species, if it is assumed that the organisms have undergone a somewhat parallel evolution after adoption of parasitism.

A review of the literature available on intestinal helminths in general would consume several volumes; such would be outside the province of this dissertation. However, an index of reference material will be included in the appendix, with references to sources of the large volume of information on nutrition, biochemistry, and physiology of parasites.

Concerning the Acanthocephala, Nicholas (loc.cit.) has adequately reviewed the literature available, limited as it is, on this subject. The author will only mention specific papers which are felt to hold special relevance to carbohydrate metabolism in M.dubius; other related topics are adequately discussed by Nicholas.

Metabolic Studies on Moniliformis dubius

The first detailed study on the carbohydrate metabolism of the worm was completed by Laurie (1957) as part of a comparative study which included the cestodes, Hymenolepis diminuta and Oochoristica symmetrica. Studying the capacity of the worms to ferment exogenously added substrates to acidic end-products, he concluded that M.dubius (males and females) could ferment glucose, galactose, mannose, fructose, and maltose, but had no measurable capacity to ferment cellobiose, lactose, melibiose, sucrose, trehalose or turanose. A broad spectrum of sugar alcohols, pentoses, and glucose analogs also were not metabolized. Laurie pointed out that these data are valid only for an atmosphere of nitrogen-CO₂ and did not rule out the possibility of the metabolism of one or more of the test substrates to non-acidic end-products. The fermentation of glucose and fructose in M.dubius was inhibited by p-chloromercurobenzoate, but was unaffected by iodoacetate (Laurie, 1957).

In a related paper (Laurie, 1959), Laurie studied the capacity of M.dubius to utilize exogenous carbohydrates under an aerobic atmosphere and attempted to identify the acidic end-products of metabolism. From these studies, he concluded that the major stored carbohydrate in M.dubius was glycogen. By this time, Fairbairn's survey paper (1958, see below) had appeared, and Laurie assayed the alkali-stable, ethanol-soluble fraction for trehalose. Glycogen and trehalose pool levels were established as 6.74% and 1.83%, respectively, of total worm dry weight. Only traces of free glucose were detected

in the soluble pool. Upon aerobic incubation, a large net synthesis of glycogen was detected, using exogenously added glucose, fructose, mannose, or maltose. No net trehalose synthesis was measured, and the author postulated a need for the use of isotopically labelled substrate in order to demonstrate synthesis of the disaccharide. The worm showed an appreciable aerobic respiration which was stimulated by mannose and maltose, but was unaffected by the addition of glucose. End-products of aerobic fermentation were acetic, formic, and lactic acids. Acetate and formate appeared to be the principal end-products, with lactate a minor component. Addition of maltose or fructose increased the quantity of lactate produced, at the expense of formate. Glucose and mannose were also shown to stimulate lactate production. In the discussion, the author speculates that trehalose, unlike glycogen, is neither synthesized nor degraded rapidly in M.dubius.

Read and Rothman (1958) studied the carbohydrate requirement of M.dubius, by measuring pool levels of carbohydrate reserves. These workers concluded that a drastic drop in worm polysaccharide occurs after starvation of the host. This trend could be reversed by allowing starved hosts to ingest starch. They further observed a diurnal fluctuation in polysaccharide content of worms from fed hosts, which was correlated with the feeding habits of the rat. The worms had an obligate requirement for dietary carbohydrate, and worms in starved hosts showed precipitous weight losses. Host animals maintained on a carbohydrate-free diet lost most of the female worms; males were still present, but ceased to grow.

In 1958, a paper appeared which created what was to become the single greatest impetus for the initiation of the research study described in this dissertation. Professor Donald Fairbairn at McGill University reported the occurrence of the non-reducing disaccharide, trehalose, to be present in 71 species of invertebrates representing the major phyla. The assays utilized left little doubt that the identity of the compound was trehalose. No obvious phylogenetic relationships appeared, based on the distribution of trehalose and glucose in the tissues, but nevertheless there were detectable and often large amounts of the disaccharide in almost every species examined. One of the species examined was M.dubius, which was found to contain trehalose in a concentration of 2.3% of total tissue solids. This figure was one of the highest measured in all the species surveyed, surpassed only by Ascaris hemolymph, which contained 4% trehalose. Fairbairn speculated that trehalose would eventually be accepted, like glucose, as a carbohydrate occurring widely in invertebrates. By this time, trehalose had been implicated as playing a fundamental role in the energy metabolism of several representatives from the Insecta (Wyatt, loc.cit.), and Fairbairn hinted that there may be reason to believe trehalose to be involved in a similar role in other species of invertebrates (Fairbairn, 1958).

In a related paper, Fairbairn and Passey (1957) examined the occurrence and distribution of trehalose and glycogen in the eggs of

Ascaris. The egg glycogen was almost entirely concentrated in the embryo proper, while a majority of the trehalose was confined to the perivitelline fluid. Trehalose was found to comprise 13.7% (w/v) of the fluid, and glycogen constituted 9% of the embryo. During embryonation, trehalose and glycogen (expressed as total alkali-stable carbohydrate) dropped to 56% of the original concentration during the first 10 days. This drop was followed by a resynthesis to the original level by the end of 25 days. The resynthesis was accounted for by the conversion of fat to carbohydrate, a phenomenon never before directly demonstrated in animal tissue. The authors offered no explanation for the resynthesis, but did point out that all of the resynthesized trehalose would be lost when hatching occurs in the host's intestine, as the perivitelline membranes rupture. It was postulated that trehalose may be concerned with the maintenance of a high osmotic pressure in the perivitelline fluid, and may be intimately involved in the hatching mechanism itself.

The final report on the role of trehalose in the biology of Ascaris appeared in 1965 (Feist, Read and Fisher, 1965). This study involved a survey of the relative capacity of various tissues of Ascaris to synthesize and hydrolyze trehalose. No synthesis of the disaccharide from glucose-C¹⁴ could be detected using whole adult male or female worms, anaerobically at pH 7.4. However, minced tissues incubated at pH 6.4 readily showed trehalose synthesis. The highest capacity for synthesis was found to reside in the male and

female reproductive tissues; muscle showed only a limited capacity for synthesis, and intestine and perienteric fluid proved negative. In all incubations which showed a capacity for synthesis, a large majority of the labelled disaccharide appeared in the incubation medium, rather than in ethanol extracts from the tissues. Efforts at demonstrating trehalose synthesis in a cell-free system fortified with Mg^{++} ions, ATP, and UDPG proved fruitless. These workers further attempted to detect the presence of the hydrolytic enzyme, trehalase. The data indicate that all such hydrolytic activity resided in the worm gut tissue. It was pointed out that Ascaris muscle, while containing relatively large amounts of the disaccharide, contained only a limited capacity for synthesis. The workers theorized that the sugar is synthesized elsewhere and is transported to the muscle.

In 1964, Fisher in this laboratory demonstrated the synthesis of trehalose in the acanthocephalans, M.dubius and Macracanthorhynchus hirudinaceus. Using body wall minces washed free of eggs and body fluid and homogenized, he showed a capacity for synthesis of trehalose from a radioactive glucose precursor. Addition of ATP and UDPG increased the synthetic capacity of the system. This effect, it was postulated, implies a mechanism of synthesis of the disaccharide similar to that seen in yeast (Cabib and Leloir, 1958) and insects (Candy and Kilby, 1961).

Wyatt (1967) has also reviewed the evidence that trehalose seems to be found in highest concentrations in the dormant stages of several

organisms. In Neurospora, trehalose is present in dormant ascospores in levels of up to 14% of the dry weight; after activation, the sugar is preferentially and rapidly used more than other carbohydrates and lipids. Similar phenomena have been observed in the spores of the cellular slime mould, Dictyostelium mucoroides, in the eggs of Ascaris, the cysts of the brine shrimp, Artemia salina, the statoblasts of the bryozoan, Plumatella, and the desiccation-resistant eggs of the mosquito, Aedes aegypti. The need for all of the above examples of dormant stages to withstand long periods of desiccation hints that trehalose, being osmotically active, may assist in imparting resistance to such desiccation in these forms.

Although the biosynthetic pathway leading to trehalose has been well established and, in some cases, well characterized in insects, bacteria and the lower plants, our knowledge of the pathway of trehalose biosynthesis and catabolism in other invertebrate groups is rudimentary. The present study was undertaken to contribute to our knowledge of the distribution and characteristics of the enzymes leading to the biosynthesis of trehalose in the acanthocephala. The organism chosen for study, Moniliiformis dubius, had originally been shown by Fairbairn (1958) to contain the disaccharide in appreciable amounts. Fisher's report (1964), while preliminary, did show that the biosynthesis could be demonstrated in a crude preparation of the worm. Since the author's interests lie primarily in the biochemistry of host-parasite systems, these reports formed the foundation for the present study.

MATERIALS AND METHODS

I. MAINTENANCE OF THE LIFE CYCLE OF M.dubius

Adult parasites raised in Sprague-Dawley albino rats (Holtzman) were kept on a diet of Purina Laboratory Chow (Ralston Purina Co.) and tap water ad libitum. Males weighing 80-90 grams at the time of infection were used exclusively. All rats were housed in air-conditioned animal quarters, at a temperature of 21-26°C. Infections were initiated by administering 25 cystacanths to each of 20 rats every two weeks. Infection was effected by a force-feeding method; counted cystacanths in water or KRT (Krebs-Ringer saline with 25 mM Tris (hydroxy-methyl) aminomethane-maleate, pH 7.4 ---- see appendix) were drawn into a pipette and carefully inserted past the mouth region into the esophagus. The cystacanths were then flushed from the pipette into the esophagus.

At approximately 60-day intervals, 10-20 rats carrying infections older than eight weeks were sacrificed for re-infection of the cockroach intermediate host. Rats were killed by a sharp blow on the head, and the worms were quickly removed to a beaker containing KRT at 37°C. After 2-3 rinses in fresh saline, the posterior end of each female worm was snipped off, and the entire contents of the body cavity was "milked" into a test tube. Each female worm yielded approximately 0.5-0.7 ml. of body fluid, containing fully infective acanthors, and immature eggs and ovarian balls. After sufficient material was collected (4-5 ml.), the eggs were washed 8-10 times in glass-distilled water to remove ovarian balls and cellular debris. The egg suspension

was then decanted onto a large piece of filter paper, which absorbed most of the water. There remained a white paste composed of numerous infective acanthors, as well as immature eggs at all stages. This paste was mixed 1 : 1 with crushed apple of a similar consistency, and placed in the cockroach colony.

Cockroaches were maintained in blackened aquaria of 25-gallon capacity, covered with stainless steel or plastic mesh. For infection purposes, a quantity (100-200) of cockroaches at all stages of development were removed to a 10-gallon blackened aquarium and held for one week without food or water. It was found that this period was ample time to ensure complete consumption of the egg-apple paste by the cockroaches. After infection, cockroaches were held for at least eight weeks before sacrifice for infection of the vertebrate host. All cockroaches were maintained on a diet of pulverized Purina Laboratory Chow, supplemented periodically with fresh apples. Tap water was administered ad libitum. Although good reproductive rates were attained in the laboratory, it was necessary to replenish the cockroach colony with individuals from the natural environment. These were obtained using traps consisting of a 1-gallon jar, the uppermost inner portion of which had been coated with a thin layer of petroleum jelly. Peanut butter or rotten bananas were found to be the baits of choice for use in the traps. The omnivorous nature and abundance of Periplaneta americana greatly facilitates trapping of specimens.

II. ANALYTICAL METHODS

A. Chemical estimation of carbohydrate

Assays to determine carbohydrate were in most cases preceded by alkaline hydrolysis of the tissue in question. Tissue was suspended in 30% (w/v) KOH and heated in a boiling water bath for 30 minutes. This process solubilizes all proteins and destroys all carbohydrates except polysaccharide and trehalose. Polysaccharide was then precipitated by the addition of 1.2 volumes of 95% ethanol containing 0.1% LiCl. Tubes were then heated to boiling and allowed to cool. The polysaccharide appears as a white flocculate, which is then removed by centrifugation. The pellet was washed three times with 70% ethanol and dissolved in an appropriate aliquot of distilled water for assay.

For estimation of carbohydrate, aliquots of the polysaccharide solution and the ethanol-soluble fraction were assayed using the phenol- H_2SO_4 method (Dubois et al., 1956) or, alternatively, by a modification (Fisher, personal communication) of the anthrone method (Dimler et al., 1951). Samples and appropriate standards were read using a Coleman Universal spectrophotometer

B. Protein determination

Protein was estimated using the Folin-Ciocalteu reagent (Lowry et al., 1951), using crystalline bovine serum albumin as a standard. Protein was precipitated from tissue and enzyme preparations using 5% trichloroacetic acid (TCA). Standards and unknowns were then dissolved

in 0.1 N KOH before treatment with the phenol reagent. Absorbance was read at 660 μ using a Coleman Universal spectrophotometer equipped with a red filter.

C. Chromatography

Descending chromatography was conducted using airtight cabinets equipped with glass solvent troughs and anti-siphon rods (Research Specialties Co.). Ascending chromatography necessitated the use of pyrex jars covered with airtight glass covers. The following chromatography solvents were employed, using Whatman #1 paper exclusively.

Solvent 1: N-butanol: ethanol: acetone: H₂O--50:40:30:20
(Gray and Fraenkel, 1954) (DESCENDING)

Solvent 2: Ethyl acetate: pyridine: methyl ethyl ketone: H₂O
80:36: 36: 30 (Friedman, unpublished) (ASCENDING)

Solvent 3: 1-propanol: ethyl acetate: H₂O--7:1: 2
(Baar, 1954) (ASCENDING)

Solvent 4: isopropanol: pyridine: H₂O: acetic acid
8:8:4:1 (Gordon and Nardi, 1954) (ASCENDING)

Solvent 5: ethanol: NH₄OH: H₂O -- 80: 5: 15
(Cheftel et al., 1953)

D. Paper electrophoresis

Paper electrophoresis was employed widely in this study; using either Whatman #4, #3, or #3MM paper, samples were subjected to 1500-2000 volts for $1\frac{1}{4}$ -2 hours using either 0.03 M sodium borate buffer pH 9.2, or 0.2 M ammonium formate buffer pH 3.6. The apparatus employed consisted of a Savant 3000 volt power supply and companion flat plate. The plate was cooled to a temperature of 5°C, using a refrigerated bath.

The non-reducing nature of trehalose prevents this compound from forming a charged complex with borate; thus, trehalose remains on the origin after electrophoresis in borate, while almost all other low-molecular weight carbohydrates form a charged borate complex and migrate in the electric field. This separation of carbohydrates was used repeatedly, as electrophoresis became one of the steps in the enzyme assay itself. Ammonium formate buffer was used to resolve glucose-6-P and glucose-1-P.

E. Chemical visualization of carbohydrates

Separated carbohydrates (standards and unknowns) were visualized on chromatograms and electrophorograms using the alkaline silver nitrate reagent of Trevelyan et al. (1950). Sugar phosphates were localized using the Bandurski and Axelrod (1951) modification of the Haynes-Isherwood molybdate reagent. Development of the latter system was effected with an ultraviolet lamp (Chromato-Vue, Ultraviolet products, San Gabriel, California).

F. Elution

For the elution of material from chromatograms and electrophorograms, a modification of the technique employed by Dimler et al. (1952) was employed. After elution, samples were transferred to 20 X 150 mm Kimax tubes and the volumes reduced to dryness with a Rotary-Evapo-Mix (Buchler Instruments).

G. Assay of radioactivity on chromatograms and electrophorograms

In preliminary studies of a qualitative nature, radioactivity was

expressed as a percentage of the total amount of label (CPM) applied. However, the quantitative characterization of the enzyme system necessitated expression of the amount of label present in a given compound as disintegrations per minute; this was necessary for ultimate conversion into standard units of $\mu\text{moles/mg protein/hr}$.

Radioactive areas on chromatograms and paper electrophorograms were localized using a Vanguard Autoscaner 880 gas-flow chromatogram scanner. Employment of this device was used in a qualitative fashion only; it enabled the author to quickly select those samples which contained sufficient radioactivity in appropriate compounds to warrant further study; this was especially convenient in preliminary experiments where only presence or absence of enzyme activity was sought.

Quantitative estimation of radioactivity was determined using a Packard 3000 series liquid scintillation spectrometer. When eluted from paper, samples were counted in 5 ml of toluene containing 4 g PPO plus 50 mg POPOP per liter ("Liquifluor"--New England Nuclear Corp.) and fortified with 20% BBS-3 solubilizer (Beckman Instruments). It was found that reproducible results could also be obtained by counting the labelled areas on the paper without prior elution, by simple immersion of 1 X 2.5 cm strips in scintillation fluid without solubilizer (Davidson, 1962). In this case, 10 ml of toluene "Liquifluor" were used per vial, and vials could be used repeatedly, since none of the radioactive compounds under study were soluble in toluene. Efficiency

was determined using the channels ratio method of quench correction (Herberg, 1958). In order to ascertain the amount of quenching on electrophorograms due to the presence of sodium borate, samples of known amounts of isotope were applied to 1 X 2.5 cm strips of appropriate paper which had been pre-treated with borate buffer. These strips, after drying, were immersed in vials and counted.

It was found that the efficiency was reduced by 15%; however, reproducible results could be obtained. This fact greatly facilitated the development of a quick assay system for enzyme activity (see Results).

H. Miscellaneous Techniques

Sonic disruption of acanthors was conducted using a Bronwill Biosonic II ultrasonic probe, using maximum deflection and 30-second bursts. Tissue pulverization was effected using a Bronwill MSK mill, employing glass beads of 0.05 mm diameter. Homogenization was conducted using a motor-driven Kontes all-glass homogenizer. Centrifugations were completed in either an International PR-1 refrigerated centrifuge, or a Sorvall RC-2B refrigerated centrifuge.

All enzyme assays, unless otherwise specified, were incubated for 1 hour at 37°C in a shaking water bath (Research Specialties Co.). Incubation mixtures, as well as zero time reactions, were stopped by immersion in a boiling water bath for 2 minutes. On occasion, samples were additionally treated with excess TCA to ensure enzyme denaturation.

Chemicals of reagent grade were obtained from a variety of commercial sources: Sigma, Pfanstiehl, Baker, Mallinkrodt, and Fisher. Radioactive

substrates were obtained from New England Nuclear Corporation.

I. Gel filtration

Gel filtration was conducted using a column filled with Sephadex G-25 (50-150 μ) equilibrated in 25 mM Tris-maleate buffer at pH 7.4. Two columns were employed; in earlier studies, a column containing a bed volume of 70 ml was used. For later studies, a larger column was utilized, which had a bed volume of 300 ml. Both columns were equipped with a gravity-feed head of one liter of buffer. Both columns were jacketed and cooled to 4°C using a recirculating refrigeration device. It was found that the Sephadex could be used repeatedly if, between runs, the column was unpacked and the gel washed exhaustively with distilled water. Gels were re-equilibrated with several changes of buffer prior to use.

EXPERIMENTAL PROCEDURE AND RESULTS

The first task in the elucidation of the biosynthetic pathway for trehalose was the selection of the appropriate parasite tissue for study. In view of the abundant evidence showing trehalose to be present in highest concentrations in dormant and resistant stages in other invertebrates (Wyatt, 1967), the decision was made to first study the ovarian-egg system of M.dubius, in an effort to begin work on a tissue which contains the sugar in appreciable amounts.

Female parasites at least 10 weeks old were used exclusively in this study. Rats were not starved prior to sacrifice; however, animals were always killed between 0900 and 1200 hours. Using a sharp blow on the head, rats were killed and the worms quickly removed into KRT at 37°C.

ENDOGENEOUS TREHALOSE LEVELS

In these experiments, worms were washed 2 times in KRT; the posterior end of each parasite was removed, and the entire contents of the pseudocoel squeezed into a centrifuge tube. When 4-5 ml of material (8-10 female worms) was obtained, the preparation was diluted to 20 ml with KRT. The diluted pseudocoel contents were decanted through a "Nitex" bolting cloth (Paul O. Abbe, Inc., Little Falls, New Jersey) with a pore size of 183 microns. The ovarian balls were retained, while acanthors of all stages of development passed through this cloth. The resultant acanthor suspension was then poured through a "Nitex" cloth with a pore size of 93 microns; fully mature acanthors possessing all outer membranes were retained by the filter, while immature eggs of

all stages passed through. In this fashion, three distinct fractions, i.e., ovarian balls, immature acanthors, and mature acanthors, were obtained. Each fraction was then suspended in 5-10 ml KRT. Millipore filters were pre-wetted with KRT and subjected to vacuum until a constant weight was attained (2 minutes). Aliquots of the three fractions were applied to a pre-weighed filter, and the tissue and filter subjected to a vacuum for 2 minutes. Weights of the tissue and filter were then recorded, and a reproducible technique for estimation of wet weights of the tissue in each fraction was thus obtained. The tissue was then dried in an oven at 100°C for 12 hours. After weighing, the tissue was washed into a test tube and subjected to alkaline hydrolysis. Following precipitation of the polysaccharide with ethanol, aliquots of the supernatant ethanol-soluble fraction were assayed for alkali-stable carbohydrate. The results of this study are compiled in Table I.

TABLE I
Endogenous Trehalose Levels

FRACTION	TREHALOSE <u>μmoles/g wet wt.</u>	TREHALOSE <u>% wet wt.</u>	TREHALOSE <u>% of tissue solids</u>
Body fluid	8.96	0.34	--
Ovarian balls	10.15	0.38	1.41
Immature acanthors	15.57	0.59	2.47
Mature acanthors	16.02	0.60	2.59

The small quantity of tissue represented by ovarian balls precluded the use of this tissue for a source of the enzymes involved in the biosynthetic pathway. Since mature and immature acanthors represent dormant stages in the life history of this worm, and these stages do contain trehalose, it was of interest to examine the trehalose synthesizing system in these tissues.

The membranes surrounding the acanthor were found to be highly resistant to efforts at mechanical disruption and solubilization. Suspensions of acanthors in buffer or KRT were subjected to homogenization in an all-glass motor-driven homogenizer, to sonic disruption with an ultrasonic probe, to grinding in sand with mortar and pestle and to mechanical breakage with a motor-driven ball mill. Using various combinations of the above techniques, the best results obtained were about 70% disruption of a suspension of immature acanthors; when mature acanthors were used, this figure dropped to a little more than 50%. The best method entailed 15 minutes of intermittent sonic disruption, followed by thirty minutes treatment with the ball mill. All treatments were performed at 1-5°C. The preparation was then subjected to low-speed centrifugation (1500 g, 15 minutes), and the aqueous supernatant fluid held for subsequent assays. All assays were conducted using U-C¹⁴ glucose (sp. act. 14.7 μ curies/ μ mole).

Various techniques were utilized in an effort to demonstrate trehalose synthesis in body fluid or aqueous suspensions of disrupted immature or mature acanthors. These included fortification of reaction mixtures with various nucleotide triphosphates, sugar nucleotides, divalent

ions, and various protein concentrations. A broad spectrum of temperature and pH were investigated, as were varying lengths of incubation time from one minute to three hours. Enzyme activity was present, as evidenced by the conversion of the radioglucose precursor into, depending on conditions used, hexose phosphate or acidic end-products. The nature and relative proportions of these end-products were not determined. All efforts to show a synthesis of trehalose under these conditions were negative. Efforts at dialysis of the preparations to remove low-molecular weight substances which could interfere with the synthetic pathway were carried out under a variety of conditions, using several different buffers as well as glass-distilled water. Subsequent assay of the dialyzed preparation fortified with varying concentrations of ATP, UDPG and other nucleotide triphosphates and sugar nucleotides, as well as several sources of Mg^{++} at several concentrations yielded no evidence for synthesis of trehalose.

Efforts were made to demonstrate the presence of the hydrolytic enzyme, trehalase, in order to explain the lack of measurable labelled disaccharide. Using an enzymatic assay (Fisher and McAlister, 1969) or radioactive trehalose prepared after the method of Murphy and Wyatt (1965), no trehalase activity could be detected in the above concentrations.

Aqueous preparations were collected as described above, using KR-phosphate, pH 7.4, phosphate buffer pH 7.4, or citrate-phosphate buffer pH 6.5. On assay, similar results were found as those recorded using KRT.

To preclude the possibility of the system being cold-labile, extracts and body fluid were prepared in KRT at room temperature. In this case, no detectable enzymic activity was present; all the label appeared on chromatograms and electrophorograms as glucose.

Another series of experiments was carried out using preparations obtained as those described above, using KRT containing 0.1 mM dithiothreitol (Cleland's reagent), which has been used with success in other systems to protect sulfhydryl groups during enzyme isolation. Although hexose phosphate was readily synthesized in these preparations, no disaccharide synthesis was demonstrable.

A similar series of experiments to those described was next performed on the low-speed pellet of the disrupted acanthors. Intact acanthors were removed with a "Nitex" filter after treatment with the ball mill. Centrifugation was then carried out (1500 g, 15 minutes) and the pellet was resuspended in Tris-maleate buffer, pH 7.4. Using a variety of cofactors, incubation times, and protein concentrations, no disaccharide synthesis was detected.

In those experiments where there was detectable enzymic activity as evidenced by formation of hexose phosphate, it was found that 3-18% of the added radioglucose appeared in the polysaccharide fraction upon assay. This fact introduced the possibility that in the crude system used, equilibria may be shifted towards polysaccharide synthesis. If such were the case, it seemed plausible that the enzymes responsible for polysaccharide synthesis might be competing for available substrate

and cofactors, although this system in this organism has not been examined in detail. A fresh fraction of each of the three systems (body fluid, immature acanthor disruptate, mature acanthor disruptate) was prepared as described above, using KRT. After the low-speed supernatant was collected from each fraction, it was subjected to centrifugation at 37,000 g for one hour. Assays were then performed on the supernatant and pellet. Using UDPG as the glucosyl donor, glycogen synthesis was readily demonstrable in the high-speed pellet; in the high-speed supernatant, however, polysaccharide synthesis was undetectable, as was trehalose formation. The supernatant did show enzymic activity, as a large percentage of the radioglucose was converted to hexose phosphate.

Since all previous experiments had been conducted using worms from infections which were greater than 15 weeks old, experiments were next conducted to measure trehalose levels in intact females of various ages. Worms were collected in KRT, blotted on damp filter paper, and the wet weights recorded. Each worm was then individually subjected to alkaline hydrolysis, and the polysaccharide fraction removed with ethanol. The supernatant fraction was then assayed for ethanol-soluble alkali-stable carbohydrate. The results are compiled in Table II.

TABLE II

TREHALOSE POOLS IN FEMALE M.dubius OF DIFFERING AGES

<u>INFECTION AGE</u> <u>(weeks)</u>	<u># WORMS</u>	<u>TREHALOSE</u> <u>μmoles/g wet wt. (avg.)</u>
6	10	31.49
8	8	29.29
12	5	29.87
14	11	28.77
16	9	20.00
18	5	19.03
20	5	22.11

The finding of such high concentrations of trehalose in parasites of all ages led to abandonment of the developmental stages as a source of the enzymes of the synthetic pathway. The data in Table II show no obvious trend concerning changes in the whole worm trehalose pool with age of the parasite. However, the level of trehalose in intact worms was found to average about 26 μmoles/g wet weight, which corresponds to about 0.9% wet weight. Assuming a wet weight/dry weight ratio of approximately four, this would correspond to a level of trehalose in the neighborhood of 3% of the tissue solids. It was thus assumed that the body wall musculature of female worms contains trehalose in concentrations comparable to those found in the body fluid and acanthors. Fisher's (1964) finding that trehalose biosynthesis is demonstrable in body wall preparations of M.dubius lent additional weight to the idea of

utilizing the body wall as a source of the enzymes under study.

BODY WALL EXPERIMENTS

Body wall musculature was prepared by collecting worms from unstarved hosts in KRT at 27°C. Pseudocoel contents were flushed from each worm, using a syringe filled (0.5 ml) with KRT. Carcasses were blotted on wet filter paper and transferred to a tared beaker for wet weight determination.

Qualitative experiments were performed by incubating the above intact tissue or homogenate of the body wall in buffer (Tris-maleate, pH 7.4) or KRT plus cofactors or substrates, depending on the experiment, for one hour at 37°C. Reactions were stopped by the addition of excess 95% ethanol and heating the ethanol mixture to boiling for 2 minutes. The protein-polysaccharide pellet was removed (1500 g, 15 minutes) and washed three times with 70% ethanol. Supernatants and washes were combined and evaporated to dryness. The residue was re-dissolved in 0.3 ml glass distilled water, and 0.05 ml subjected to paper chromatography (solvent 1) and paper electrophoresis (Whatman # 3mM paper, borate buffer). Radioactivity was localized on chromatograms and electrophorograms with the strip scanner. In addition, the strips were cut into 1 X 2.5 cm pieces and counted in scintillation vials. Radioactivity in the trehalose area was expressed as a percentage of the total label contained in each sample.

LIGATED CARCASS EXPERIMENT

Five worms from a 22-week old infection were collected and the pseudocoel contents removed as above. The ends of each worm were ligated with surgical thread, and the pseudocoel filled with 0.5 ml KRT containing μc (0.067 μmoles) U-C^{14} glucose. The filled "sacs" were then individually immersed in 2 ml KRT and incubated for one hour at 37°C . At the end of the incubation period, ligations were removed and the contents of the pseudocoel flushed into tubes containing an excess of 95% ethanol. The carcasses were placed in 70% ethanol and extracted by boiling for two minutes. The pseudocoel contents, after boiling, were allowed to cool and were centrifuged (1500 g , 15 minutes). The body wall extract was treated in a similar fashion. The supernatant and washes were pooled and evaporated to dryness. The dry residue was redissolved in water and subjected to borate electrophoresis and chromatography (solvent 1). Results are reported in Table III.

TABLE III
TREHALOSE SYNTHESIS IN LIGATED PARASITES

FRACTION	% RADIOACTIVITY		
	trehalose	glucose	glucose phosphate
Pseudocoel contents	62.1%	3.8%	31.1%
Body wall ethanol extract	69.0%	1.0%	24.2%
Medium	no detectable radioactivity		

In order to demonstrate that the product found in this experiment was, in fact, trehalose, portions of the suspected trehalose area were eluted from the chromatograms which had been developed in solvent 1. The eluate was evaporated to dryness, redissolved in a minimal quantity of water, and co-chromatographed with authentic trehalose in solvents 2-5. A portion of the eluate was subjected to alkaline hydrolysis, desalted and re-chromatographed. In all instances, the product co-chromatographed with trehalose. Further, 93% of the radioactivity was recovered in the trehalose area after treatment with strong alkali. These experiments provided strong evidence that the enzymes for trehalose synthesis are contained in the body wall. The low levels of glucose remaining in the pseudocoel and body wall after one hour indicate that the equilibria are shifted in the direction of hexose phosphate and trehalose formation. The recovery of virtually all of the radioactivity in low-molecular weight compounds was initially surprising; it was anticipated that a proportion of the substrate would be incorporated into polysaccharide. However, these worms were obtained from unstarved hosts. In this case, it would be expected that polysaccharide reserves would be reasonably high (Read and Rothman, 1958). Thus, polysaccharide synthesis would not be expected to occur at an appreciable rate. The finding that 25-30% of the radioglucose was present in glucose-6-phosphate implied that this compound may be a precursor of trehalose, as has been shown in other systems (Wyatt, loc.cit.; Elbein, 1968). That the compound containing the major portion of the radioactivity was in fact

trehalose was strongly supported by the co-chromatography studies. The stability of the compound in the presence of strong alkali lends additional support to this interpretation.

PROGRESSIVE DISRUPTION OF THE BODY WALL

Seven worms were collected, and carcasses prepared in the normal fashion. Worms were then progressively chopped up, so that each of the seven samples contained the mince of one worm. In the first sample, the flushed carcass was left intact. Sample 2 contained a carcass which had been chopped into ten pieces of approximately equal size. This gradation continued to sample 7, which represents a homogenate of the carcass. Incubations were conducted in the presence of 2 ml KRT containing 1 μ curie (0.067 μ moles) U-C¹⁴ glucose. Results are given in Table IV.

TABLE IV
TREHALOSE SYNTHESIS AFTER PROGRESSIVE DISRUPTION OF BODY WALL

<u>SAMPLE</u>	<u>% RADIOACTIVITY</u>		
	<u>glucose</u>	<u>glucose-6-P</u>	<u>trehalose</u>
1	<1	89.9	8.1
2	<1	79.8	18.7
3	<1	76.2	22.0
4	<1	75.2	23.2
5	<1	71.7	23.2
6	<1	66.7	30.6
7	<1	59.4	38.8

These findings indicate that as the body wall musculature is subjected to increasing disruption, increasing capacity for trehalose synthesis is obtained. This implies either the release of soluble substances necessary for enzymic activity (enzymes, substrates, or cofactors), or an increased accessibility to a membrane-bound component necessary for synthesis. The decreasing amount of label appearing in glucose-6-phosphate with increasing trehalose activity lends further support to the idea that glucose-6-phosphate is possibly a precursor for trehalose synthesis.

ISOLATION OF SOLUBLE ENZYMES

These experiments were designed to ascertain whether the trehalose-synthesizing system in the body wall was soluble or bound to membranes. An additional purpose was the establishment of conditions under which these preparations could be stored with no apparent loss in enzymic activity.

Crude homogenates of the body wall musculature of 3 parasites were prepared as described above (1:2 w/v KRT). The crude homogenate was then centrifuged at 37,000 g for one hour and the resultant amber super natant fluid was poured through a funnel containing glass wool, to remove the lipid layer. A total of 3.0 ml of cell-free high-speed supernatant fluid was thus obtained. One-half of the fraction was held in an ice-bath for assay; this was designated Fraction "A". The other 1.5 ml fraction was transferred to a 25ml Erlenmeyer flask and lyophilized.

The dry lyophilized powder was re-suspended in 0.6 ml glass-distilled water, and designated fraction "B". The total dissolved solids (protein, buffer, salts, etc.) had thus been subjected to a 2.5-fold concentration in fraction "B". The two fractions, for assay, were mixed in varying proportions and incubated in the presence of 1 μ curie (0.067 μ moles) U-C¹⁴ glucose per reaction mixture for one hour (total volume = 0.12 ml). Results are expressed in Table V.

TABLE V
TREHALOSE SYNTHESIS BY
FRESH AND LYOPHILIZED ENZYME PREPARATIONS

SAMPLE	ml Fraction "A"	ml Fraction "B"	% Radioactivity in trehalose
1	.10	---	8.1
2	.08	.02	9.4
3	.06	.04	9.6
4	.04	.06	10.7
5	.02	.08	10.9
6	---	.10	11.8
7	.10 _{t₀} (0 time control)	---	0.2
8	---	.10 _{t₀} (0 time control)	0.1

The finding that the enzymes for trehalose synthesis were in the supernatant portion of the body wall homogenate suggested the enzymes for this pathway were soluble and separable from the glycogen synthetase system in the pellet. The results in Table V also indicate the soluble

enzyme fraction could be removed from the particulate fraction with no apparent loss of trehalose synthetic capacity. Subsequent experiments showed that the soluble enzyme remained stable in the lyophilized state for at least 6 weeks when stored at -20°C . This finding greatly facilitated later studies; the enzyme could be prepared in quantity and stored.

Since all experiments using body wall preparations had been conducted using unfortified mixtures, an experiment was designed to ascertain the effect of exogenous ATP on the synthetic capacity of the preparation. The experimental procedure paralleled that described above; however, the total volume of each sample was 0.25 ml. Results are compiled below.

TABLE VI
EFFECT OF ATP ON ENZYMATIC SYNTHESIS OF TREHALOSE

SAMPLE	mM ATP	% RADIOACTIVITY IN TREHALOSE
1	---	8.4
2	1.0	31.5
3	2.0	39.5
4	3.0	34.1
5	4.0	38.4
6	5.0	44.9
7	10.0	41.2

The results in Table VI indicate ATP stimulates trehalose synthesis. A large part of the stimulation occurred at between zero and 1 mM, which suggests that endogenous levels of ATP in enzyme preparation are relatively low. Under the experimental conditions defined, no stable glucose carrier was included; the initial substrate concentration was therefore 0.27 mM. Assuming the ATP to be participating in the hexokinase reaction, deletion of ATP from reaction mixtures thus renders the phosphorylation of glucose the rate-limiting step in the pathway. At levels above 1 mM, this effect is negated, and some other step in the pathway becomes rate-limiting. Since this experiment included no fortification with exogenous sugar nucleotide, it was concluded that either a sugar nucleotide is not an obligate requirement for trehalose synthesis or that the crude high-speed supernatant contained sufficient quantities of necessary sugar nucleotide, as well as other cofactors, to allow the synthesis to proceed at the level observed. At this point, the enzymes had been localized in the body wall and purification had proceeded to a point where synthesis could be measured in a cell-free system. Stability was attained using lyophilization, but it was recognized that quantitative kinetic data were impossible using a preparation containing low-molecular weight substances which could activate, inhibit, or participate in the reaction pathway. Therefore, some method for the removal of these contaminants was called for. Several attempts were made to dialyze the preparation against water and various concentrations of KRT and Tris-maleate buffer. Under no conditions could synthesis be

demonstrated following dialysis, although addition of a broad spectrum of cofactors, divalent ions and substrates was tested.

GEL FILTRATION

As an alternative method to dialysis, the use of "Sephadex" G-25 was investigated. The gel was equilibrated initially with 25 mM KRT. Using a column with a bed volume of 70 ml, 1.0 ml of high-speed supernatant prepared as described above was introduced to the top of the column. Following passage of the void volume ($V_0 = 8.7$ ml), 5 ml of the protein fraction was obtained. This fraction was transferred to a 25 ml Erlenmeyer flask and lyophilized. The dry powder was re-suspended in 1.0 ml of glass distilled water, thus approximating the protein concentration of the original high-speed supernatant. In practice, about 70-75% of the protein was actually recovered. The lyophilization - re-suspension process led to a five-fold concentration of the KRT. Using 0.2 ml of the preparation per sample, incubations were conducted in the presence of various combinations of cofactors using 1 μ curie (0.067 μ mole) U-C¹⁴ glucose as the substrate. The results are given in Table VII.

TABLE VII
EFFECT OF SEPHADEX GEL FILTRATION ON
ENZYMATIC SYNTHESIS OF TREHALOSE

<u>CONTENTS-- cofactors</u>	<u>TREHALOSE (% RADIOACTIVITY)</u>
none (0 time control)	< 1
<u>5mM</u> ATP	< 1
<u>5mM</u> ATP, <u>5mM</u> MgSO ₄	< 1
<u>5mM</u> ATP, <u>2mM</u> UDPG	23.7
<u>5mM</u> ATP, <u>2mM</u> UDPG, <u>2mM</u> EDTA	34.1

The results indicated the enzyme preparation, following gel filtration, retained the capacity for trehalose synthesis from glucose, and that UDPG enhanced the synthetic rate since enzyme activity was significant only in those samples containing UDPG. The effect of Mg++ ions could not be determined, since the enzyme preparation itself contained 6 mM MgSO₄, which, in the reaction mixture, would persist in a concentration of 4 mM. The apparent additional activity obtained by the inclusion of EDTA suggested that other divalent ions (Ca++?) were present which, if unbound, exhibited a slight inhibitory effect. Although this study does not conclusively implicate UDPG as a glucosyl donor in the reaction pathway, activity was not demonstrable without it, which suggests at least an indirect role for this sugar nucleotide in the formation of trehalose. This experiment revealed the desirability of obtaining a protein preparation free of salts such as NaCl, KCl, etc., or at least one in which the salt concentration was considerable less

than 5X KRT (see appendix).

Subsequent studies completed with the protein preparation obtained above revealed the enzymes to be stable either in the frozen lyophilized state following Sephadex treatment, or re-suspended in water and frozen at -20°C .

Using this preparation, several attempts were made to establish a chemical assay for trehalose synthesis. The means for specific estimation of trehalose was available (Fisher and McAlister, 1969), but it was found that the amounts of trehalose synthesized by this system were too low to be detected using colorimetric techniques for estimation of carbohydrate.

Several attempts were made to utilize a Sephadex column containing gel equilibrated in buffer alone; an enzyme preparation from this column would contain only traces of salts. Enzyme preparations taken from columns of Sephadex G-25 equilibrated with 25 mM Tris-maleate pH 7.4 were employed in four experiments with incubation mixtures similar to those employed above. These experiments all showed no capacity of the system for trehalose synthesis. Efforts to explain this puzzling phenomenon led to a testing of a broad range of ATP concentrations in the reaction mixtures. The enzyme was removed from the column in 5.0 ml 25 mM Tris-maleate and lyophilized. This preparation was then re-suspended in 1.0ml H_2O , yielding a final buffer strength of 125 mM. In the presence of 30 mM MgSO_4 and 3.5 mM UDPG, ATP concentration was varied. Results are given in Table VIIT.

TABLE VIII
EFFECTS OF ATP CONCENTRATION ON TREHALOSE SYNTHESIS
FOLLOWING TREATMENT OF ENZYMES WITH SEPHADEX-KRT

<u>ATP CONCENTRATION (mM)</u>	<u>% RADIOACTIVITY AS TREHALOSE</u>
none	1.7
2	88.7
4	86.7
6	77.8
10	3.2
40	< 1
60	< 1
100	< 1

The fact that concentrations of ATP above 10 mM inhibited the system completely led to an explanation that removal of the salts present in 5X KRT rendered the enzymes susceptible to inhibition by ATP. The previous use of ATP concentrations between 5 and 10 mM for fortification of the preparation explained the lack of activity measurable in the earlier experiments done on the preparation taken from the column containing only Tris-maleate buffer. The previous assays had been fortified with 10 mM ATP, a concentration which proved in this experiment to be strongly inhibitory. An explanation for this apparent partial protection of the enzymic activity from ATP inhibition by one or more components of KRT (5X concentration) is not forthcoming.

Subsequent studies on this enzymic preparation revealed it to be

stable when frozen at -20°C in the lyophilized state or in 125 mM Tris-maleate. Following removal from the column, it was also found that the enzyme preparation could be dialyzed against water prior to lyophilization. Under these conditions the buffer concentration could be reduced to a negligible level, provided the preparation was dialyzed against 3 changes of 3 liters of distilled water. Following dialysis, the enzyme was stored in the lyophilized condition at -20°C until used for assay. It was therefore concluded that the preparative techniques had progressed to the point where quantitative data concerning the pathway could be obtained.

Before quantitative studies could be initiated, it was necessary to establish the fact that no competing side reactions involving the substrates under study were present in the protein preparation. An additional need arose to justify the use of glucose as the precursor for trehalose in several experiments. The cost differential between commercially available glucose and glucose-6-phosphate was such that the use of glucose as the precursor was highly desirable, if it could be shown that conversion to glucose-6-phosphate occurred when the system was fortified with ATP and Mg^{++} ions. Therefore, a preliminary time-course experiment was designed as follows: 0.4 ml of enzyme was incubated in the presence of 40 μmoles buffer, 2.5 μmoles ATP, and 1 μmole UDPG in a total volume of 0.5 ml. Aliquots of 0.05 ml were removed at various time intervals and the reaction stopped by the addition of excess 95% ethanol. After washing and evaporation, the dry residue was redissolved in 0.05 ml

washer and chromatographed in solvent 1. Radioactive areas on water chromatograms corresponding to glucose, glucose-6-phosphate, and trehalose were cut into 1 X 2.5 cm strips and counted. Results are expressed in Table IX.

TABLE IX
GLUCOSE-6-P AND TREHALOSE FORMATION WITH TIME

TIME (MINUTES)	% RADIOACTIVITY		
	GLUCOSE	GLUCOSE-6-PHOSPHATE	TREHALOSE
0	97.2	1.3	0
5	80.6	12.9	0.76
10	8.7	79.3	2.1
20	5.1	78.2	5.3
30	2.9	79.1	8.4
45	2.2	77.6	11.5
60	1.7	74.8	16.3

These results imply the hexokinase present in the preparation to be very active, i.e., the enzyme has a low apparent K_m and the formation of glucose-6-phosphate is strongly favored. The fact that 80% of the glucose was converted to glucose-6-phosphate during the first few minutes justified the occasional use of glucose and ATP as a substitute for glucose-6-phosphate, the normal substrate for trehalose phosphate synthetases in other systems (Wyatt, 1967). In this experiment, no other compounds were detectable as being radioactive. The probability of side

reactions occurring which would preclude kinetic studies by competition for available substrate had been minimized. Virtually all the radioactivity added could be accounted for in metabolites falling in the pathway described for trehalose synthesis in other systems (Wyatt, loc.cit.). The fact that glucose-6-phosphate formation occurred rapidly and attained apparent equilibrium within 10 minutes lends support to the idea that, in studies such as velocity vs. protein concentration which follow, glucose could be validly substituted for glucose-6-phosphate, the common immediate precursor for trehalose synthesis in other systems. Such a substitution would have no deleterious effects, provided incubations were carried out for one hour. Figure 1 represents a summary of the steps in enzyme preparation which were standardized for the quantitative studies which follow. All later preparations were treated with "Sephadex" in a column with a bed volume of 300 ml; 8-10 ml of high-speed supernatant could thus be treated at a time, and a large quantity of enzyme collected and stored in one preparation.

FIGURE 1

SUMMARY OF PROCEDURE FOR ENZYME PURIFICATION

Summary of Procedure for Enzyme Purification

QUANTITATIVE STUDIES ON THE TREHALOSE SYNTHETIC SYSTEM

1. The Standard Assay System

For these experiments, techniques were standardized as much as possible to ensure reproducibility. It was found that optimal activity occurred in the presence of 50-100 mM Tris-maleate buffer. Therefore, the lyophilized powder was suspended in 100mM buffer for these experiments. Total reaction volumes were standardized at 0.2 ml, of which 0.1 ml was composed of the protein solution. This led to an effective buffer concentration of 50 mM. A stock flask containing the lyophilized powder from 5ml post-dialysis enzyme was suspended in 1.0-1.6 ml buffer. This represented a final protein concentration of 4-6 mg/ml. Unless otherwise specified, samples were incubated for one hour at 37°C and heat-killed. After centrifugation, .05 ml of supernatant was subjected to borate electrophoresis (1500 volts, Whatman #4 paper) for 90 minutes. Following electrophoresis, areas around the origin were cut into 1 X 2.5 cm strips and counted in the liquid scintillation system. After corrections for efficiency and, in those cases where cold carrier was utilized, for specific activity, the data were converted to μ moles trehalose/mg protein/hour. As the supply of stock enzyme was depleted, more was prepared and stored in the fashion outlined in Fig. 1, in 25 ml Erlenmeyer flasks. Most assays consisted, with controls, of 10-16 incubation mixtures. If more than 16 samples were used, two flasks of stock enzymes were used.

After enzyme preparation was added to the reaction mixtures, a ✓ the

small aliquot (0.1 ml) of the enzyme preparation was treated with 5% TCA and frozen until the determination of protein.

The results described in the following sections represent average values of two determinations; each determination was run in duplicate.

2. Effect of Different Sugar Nucleotides on Trehalose Synthesis

These studies were designed to establish the substrate specificity of the step in the pathway requiring a sugar nucleotide as glucosyl donor. Reactions contained 0.57 mg protein, 0.4 μ curie (0.04 μ moles), 1-C¹⁴ glucose-6-P, 10 μ moles Tris-maleate, 5 μ moles MgSO₄, and 1 μ mole of the appropriate sugar nucleotide in a total volume of 0.2 ml. Results are expressed in Table X; net cpm after correction for background are included to add clarity.

TABLE X
EFFECTS OF DIFFERENT SUGAR NUCLEOTIDES

CONTENTS	CPM TREHALOSE	μ moles trehalose/mg/ mg protein/ hour
None	239	---
UDPG	7680	1.17×10^{-5}
CDPG	262	---
GDPG	270	---
TDPG	218	---
ADPG	304	---
None (0 time control)	230	---

These results indicate that the enzyme is specific for UDPG. The fact that heat-killed reactions and those allowed to incubate for the hour with no glucosyl donor showed no difference in the radioactivity incorporated into trehalose, suggests that the level of sugar nucleotides present in the enzyme preparation had been minimized by the gel filtration procedure. All other sugar nucleotides gave results identical to those of the samples containing no glucosyl donor. These results strongly indicate that the system has an obligate requirement for UDPG.

3. Substrate specificity

These studies were undertaken to establish the substrate specificity of the enzymes leading to trehalose synthesis. Reactions consisted of 0.62 mg protein, 10 μ moles Tris-maleate, 5 μ moles MgSO_4 , 1 μ mole UDPG, and 0.5 μ curie labelled precursor in a total volume of 0.2 ml. The spectrum of precursors used necessitated a departure from the standard assay system for those samples which contained labelled precursors which remained on the origin in borate eletrophoresis. Thus, resolution was effected using either descending chromatography (solvent 1) or paper electrophoresis in ammonium formate buffer. Results are given in Table XI.

TABLE XI
RESULTS, SUBSTRATE SPECIFICITY

Substrate	Sp. Act. (μ curie/ μ mole)	CPM Trehalose	μ mole trehalose/ mg prot/hour
* glycerol	9.0	229	---
mannose	33.0	194	---
* mannitol	10.0	296	---
galactose	1.6	237	---
fructose	2.1	532	---
ribose	2.3	312	---
arabinose	2.4	272	---
* glucosamine	6.8	199	---
* maltose	3.2	4195	0.64×10^{-5}
+ glucose-1-P	15.0	288	---
+ glucose-6-P	10.0	9774	1.49×10^{-5}
glucose-6-P (0 time control)		321	---

* Assayed using descending chromatography (solvent 1)
+ Assayed using ammonium formate electrophoresis

The samples in the above experiment which used C^{14} -maltose as precursor were eluted from chromatograms, reduced to dryness, and subjected to alkaline hydrolysis. Following this treatment, they were re-chromatographed in solvent 1 and counted. Following this treatment the radioactivity found in the trehalose--maltose area did not differ significantly from that of the control values.

These studies indicate that enzyme preparations from Moniliiformis dubius have the capacity for synthesis of trehalose from glucose-6-phosphate. The lack of synthetic activity when glucose-1-phosphate was used as a precursor implies that the enzyme is specific for the hexose-6-phosphate. Further, under conditions tested, phosphoglucomutase activity catalyzing the interconversions of glucose-1-phosphate and glucose-6-phosphate, was not detected. The apparent activity in the presence of maltose is highly suspect; trehalose and maltose are difficult to resolve using the available chromatographic and electrophoretic techniques. However, subsequent treatment of eluates with strong alkali revealed the labelled compound in these samples to be sensitive to KOH hydrolysis. This implies the compound originally termed trehalose in these samples was, in reality, maltose. The lack of label in trehalose after incubation with the spectrum of substrates tested strengthens the premise that the enzyme preparation was sufficiently pure to merit kinetic studies.

4. The Effect of Protein Concentration on Trehalose Synthesis

These studies were designed to evaluate the effects of protein concentration on reaction velocity. Reactions consisted of 10 μ moles Tris-maleate buffer pH 7.4, 3 μ moles MgSO_4 , 0.5 μ moles UDPG, 0.6 μ moles ATP, and 0.5 μ curie (0.034 μ moles) U-C^{14} glucose in a total volume of 0.2 ml. Results are expressed in Figure 2. Trehalose synthesis was found to be linear with respect to protein concentration when glucose was utilized as the labelled precursor.

5. The Effects of Magnesium Ions on Trehalose Synthesis

The effects of Mg^{++} were investigated using $MgSO_4$ and $MgCl_2$. The curve represents the average of four determinations, two using $MgSO_4$ and two using $MgCl_2$. Reactions contained 0.79 mg protein, 10 μ moles Tris-maleate, 0.5 μ moles UDPG, and 0.8 μ curie (0.08 μ moles) $1-C^{14}$ glucose-6-phosphate in a total volume of 0.2 ml. Figure 3 represents the results. The pathway has an apparent obligate requirement for divalent ions. Activation was found to increase with increasing Mg^{++} concentration; deletion of Mg^{++} resulted in no trehalose synthesis. The nature of the activation was not established. These results are similar to those reported by Murphy and Wyatt (1965) for silk moth fat body trehalose phosphate synthetase. These workers reported Mg^{++} to act by lowering the K_m for glucose-6-phosphate; however, activity was measurable in the absence of Mg^{++} in this system. Cabib and Leloir (1958) reported trehalose phosphate synthetase from yeast to be activated by concentrations of Mg^{++} up to 25 mM; at higher concentrations, Mg^{++} ions became inhibitory. These workers concluded the enzyme from yeast had no obligate requirement for magnesium.

6. The Effects of Divalent Ions on Trehalose Synthesis

The effects of two different concentrations of a number of divalent metals were determined in a system containing 0.52 mg protein, 10 μ moles Tris-maleate, 1 μ mole UDPG, 2 μ mole glucose-6-phosphate, and 0.4 μ curie (0.04 μ moles) $1-C^{14}$ glucose-6-P in a total volume of 0.2 ml. Results are shown in Table XII.

TABLE XII
THE EFFECT OF DIVALENT METALS ON TREHALOSE SYNTHESIS

CONTENTS	V (μ moles trehalose/mg protein/hour)
no divalent ions	49.23×10^{-5}
5 <u>mM</u> MgSO_4	121.69×10^{-5}
20 <u>mM</u> MgSO_4	253.89×10^{-5}
5 <u>mM</u> ZnSO_4	81.73×10^{-5}
20 <u>mM</u> ZnSO_4	118.80×10^{-5}
5 <u>mM</u> MnSO_4	44.6×10^{-5}
20 <u>mM</u> MnSO_4	98.9×10^{-5}
5 <u>mM</u> CaSO_4	0
20 <u>mM</u> CaSO_4	0
5 <u>mM</u> CuSO_4	0
20 <u>mM</u> CuSO_4	0
5 <u>mM</u> CdSO_4	0
20 <u>mM</u> CdSO_4	140.4×10^{-5}
5 <u>mM</u> FeSO_4	173.2×10^{-5}
20 <u>mM</u> FeSO_4	297.2×10^{-5}

Reaction mixtures fortified with ferrous ions supported trehalose synthesis at a rate similar to that found in those mixtures fortified with Mg^{++} ions. Substitution of Zn^{++} or Mn^{++} for Mg^{++} also supported trehalose formation, but at approximately 40-60% of the rate observed using Mg^{++} . Complete inhibition was observed in the presence of Ca^{++} or Cu^{++} ions; low levels of Cd^{++} inhibited synthesis, while higher concentrations somewhat stimulated activation. The finding that Ca^{++} exerts an inhibitory effect may explain the apparent increased capacity for synthesis observed in the earlier experiments done with Sephadex-KRT when EDTA was included in the incubations.

7. The Effect of Time on Trehalose Synthesis

Progress of trehalose formation with time was estimated using identical samples incubated for varying time intervals. Incubations contained 0.43 mg protein, 2 μ moles UDPG, 10 μ moles Tris-maleate, 5 μ moles $MgSO_4$, 0.4 μ curie (0.04 μ moles) $1-C^{14}$ glucose-6-P, and 4 μ moles stable glucose-6-P, in a total volume of 0.2ml. Results are plotted in Figure 4. It was found that trehalose formation increased with increasing incubation time; the curve was linear after the first forty-five minutes. The break in the curve corresponding to incubations of less than forty-five minutes is unexplainable at this time.

8. The Effect of pH on Trehalose Synthesis

The pH optimum of the system was determined using the following series of buffers: acetate (3.8 - 4.3), citrate (3.7 - 5.8), Tris-maleate (6.4 - 8.9), Tris (7.8 - 8.2), sodium glycinate (9.3 - 10.6), and barbital (8.8 - 9.4). Buffers containing phosphate were not used, because of the stimulatory effect of phosphate on the synthesis (see below). Reaction consisted of 0.65 mg protein, 0.4 μ curie (0.04 μ mole),

1-C¹⁴ glucose-6-P, 1 μ mole UDPG, 5 μ mole MgSO₄, and 10 μ moles of the appropriate buffer, in a total volume of 0.2 ml. Results are given in Figure 5. The pH optimum of the trehalose synthesizing system of M.dubius was found to be about pH 8.0. No inhibitory effects of the buffers were observed.

9. The Effect of Temperature on the Synthesis of Trehalose

Optimal temperature for trehalose synthesis was determined by assaying enzymic activity at nine different temperatures from 0° to 80°C. Reactions contained: 0.63 mg protein, 10 μ moles Tris-maleate, 5 μ moles MgSO₄, 0.4 μ curie (0.04 μ moles) 1-C¹⁴ glucose-6-P, and 1 μ mole UDPG in a total volume of 0.2ml. The linear portion of the Arrhenius plot was used for estimation of the energy of activation (Gibson, 1953). A sharp increase in the rate of trehalose synthesis was observed at 50°C (Figure 6). The energy of activation was calculated and found to be ca. 2606 Calories/mole. It should be pointed out that this figure is valid only for the enzyme catalyzing the rate-limiting step in the reaction sequence.

10. The Effect of Glucose Concentration on Trehalose Synthesis

Velocity of trehalose formation with respect to glucose concentration was measured using a system containing 0.12 mg protein, 10 μ moles Tris-maleate, 3 μ moles MgSO₄, 0.8 μ moles ATP and varying concentrations of chemical glucose, containing 0.5 μ curie (0.034 μ moles) U-C¹⁴ glucose in a total volume of 0.2ml. Results are given in the lower section of Figure 7. The upper curve represents a Lineweaver-Burk plot of the

same data. The lower curve represents a shape identical to that expected for a system exhibiting saturation kinetics; from the double-reciprocal plot, a K_m of 6.8 mM and a V_{max} of 4.95×10^{-3} μ moles trehalose/mg protein/hour were calculated. It must be added that the reactions were fortified with 4mM ATP in order to insure phosphorylation. The addition of ATP, while obligatory for phosphorylation and therefore for ultimate trehalose synthesis, sheds some doubt as to whether the values for the apparent K_m and maximum velocity may be compared with the stoichiometry measurements done with respect to UDPG or glucose-6-phosphate, which contained no ATP. This will be discussed in more detail below.

11. The Effect of UDPG Concentration on Trehalose Synthesis

Velocity of trehalose formation in the presence of varying concentrations of glucosyl donor was determined using samples containing 4 μ moles glucose-6-P, 0.4 μ curie (0.04 μ mole) 1-C¹⁴ glucose-6-P in a final volume of 0.2 ml. Results are given in the lower half of Figure 8. The upper curve represents the double-reciprocal plot. Calculations taken from the Lineweaver-Burk plot yielded an apparent K_m of 6.7 mM, and a maximum velocity of ca. 1.1×10^{-3} μ moles trehalose/mg protein/hour.

12. The Effect of Glucose-6-Phosphate on Trehalose Synthesis

Changes in velocity with increasing glucose-6-phosphate were monitored in an assay system containing 0.48 mg protein, 10 μ moles Tris-maleate, 5 μ moles $MgSO_4$, 2 μ moles UDPG, 0.4 μ curie (0.04 μ mole) 1-C¹⁴ glucose-6-P and varying amounts of stable glucose-6-P in a total

volume of 0.2 ml. A curve (Figure 9) exhibiting saturation kinetics was obtained. The upper curve represents the double-reciprocal plot, from which were obtained values of 13.5 mM for the apparent K_m and 1.25×10^{-3} μ moles trehalose/mg protein/hour maximum velocity.

When comparing the kinetic properties of the enzyme with respect to the two primary substrates, glucose-6-phosphate and UDPG, it should be pointed out that the observed maximum velocities were estimated to be essentially the same, while the apparent K_m with respect to glucose-6-phosphate was observed to be approximately twice that measured for UDPG. There is a temptation to infer that the affinity of the enzyme is approximately twice as strong for UDPG as for the hexose phosphate. However, such a conclusion is invalid due to the fact that the constant level of co-substrate was maintained at 20.2 mM glucose-6-phosphate in the case of UDPG, whereas the UDPG concentration was maintained at only one-half this level for the reciprocal study where glucose-6-phosphate was varied. Thus, direct comparisons between the values obtained are not possible, although the values themselves are valid under the defined experimental conditions.

13. The Effects of ATP Concentration on Trehalose Synthesis

The effects of ATP on the purified preparation were determined using varying concentrations of ATP in a system containing 0.67 mg protein, 10 μ moles Tris-maleate, 5 μ moles $MgSO_4$, 0.5 μ moles UDPG, 4 μ moles stable glucose-6-phosphate, and 0.8 μ curie (0.08 μ moles) $1-C^{14}$ glucose-6-phosphate in a total volume of 0.2 ml.

In the presence of 20.4 mM glucose-6-phosphate and 2 mM UDPG, it was found that increasing concentrations of ATP showed an initial stimulatory effect of trehalose synthesis (Figure 10). However, at concentrations above 1 mM ATP increasingly inhibited the system; at 20 mM ATP, this inhibition approached 100%. The initial stimulatory effect may furnish an explanation for the rather high maximum velocity observed in the stoichiometry experiments conducted using glucose as the disaccharide precursor.

14. The Effects of Inorganic Phosphate Concentration on the Synthesis of Trehalose

It was found during preliminary experiments designed to determine the pH optimum of the system, that buffers containing inorganic phosphate exhibited a stimulatory effect on reaction velocity. In order to establish this as being true stimulation rather than inhibition by Tris-maleate, an experiment was designed as follows: a stock flask of enzyme was prepared in 100 mM phosphate buffer. Samples were prepared containing concentrations of Tris-maleate varying from zero to 100 mM, under otherwise standard assay conditions, in the presence of 1 μ mole UDPG and 0.4 μ curie (0.04 μ moles) 1-C^{14} glucose-6-P. Reaction velocities were not found to vary significantly; velocity was independent of Tris-maleate concentration in the range tested. Then, the reverse experiment was prepared, by suspending a stock flask of enzyme in 100 mM Tris-maleate, and preparing samples containing phosphate buffer in concentrations of from zero to 100 mM (Figure 11). Each sample contained

0.59 mg protein, 1 μ mole UDPG, and 0.4 μ curie (0.04 μ moles) 1-C¹⁴ glucose-6-phosphate in a total volume of 0.2 ml. From the results of the ATP and inorganic phosphate experiments (Figs. 10 and 11), it could be postulated that the inorganic phosphate released during trehalose-phosphate conversion to trehalose by the phosphatase reaction could be exerting a stimulatory effect which could account for the high observed maximum velocity. Such an effect could occur in the experiments reported in Figure 10 if ATP was undergoing hydrolysis, either enzymatically or spontaneously. Thus, at low ATP concentrations, a rather large proportion of the nucleotide triphosphate may be spontaneously hydrolyzed to ADP and inorganic phosphate. Assuming this hydrolysis is spontaneous, this effect would only be detectable at very low ATP concentrations. At higher concentrations, the proportion of ATP to ADP-inorganic phosphate would be much larger, and the small amount of phosphate stimulation would be masked by the increasingly inhibitory effect of higher concentrations of added ATP.

This notion would be supported by the finding that phosphate buffer (Figure 11) does indeed exert a stimulatory effect on trehalose formation. Although the nature of the inhibition was not determined, stimulation approached 6-fold when the phosphate concentration reached 100 mM. This finding precluded the use of buffers containing inorganic phosphate for this study. That the effect was really stimulatory is substantiated by the results of the reciprocal experiment, where the enzyme was suspended in 100 mM phosphate buffer and incubated in

increasing amounts of Tris-maleate. The finding that velocity was independent of Tris-maleate concentration confirmed that phosphate is stimulatory, in contrast to the alternate hypothesis that Tris-maleate has an inhibitory effect on trehalose synthesis which is overcome by phosphate. The fact that inorganic phosphate has a stimulatory effect on measurable trehalose synthesis greatly complicates efforts at reaching conclusions about the kinetic behavior of the system, since there is good reason to believe that inorganic phosphate is released when trehalose phosphate is hydrolyzed to trehalose. The formation of trehalose phosphate as the first product in the biosynthetic pathway has been shown to occur in all other systems studied where the workers used means to detect it (Wyatt, 1967). Although the product was never isolated or detected in this study, it is likely that the M.dubius system, as those reported elsewhere, actually consists of two enzymes, trehalose phosphate synthetase, and trehalose phosphate phosphatase (trehalose-6-phosphatase). Friedman (1960) studied the properties of trehalose-6-phosphatase obtained and purified from Phormia regina. He found the enzyme to have an obligate requirement for divalent ions. The enzyme had a strong affinity for the substrate, and Friedman concluded that any trehalose-6-phosphate which reaches the blood of the insect would be immediately converted to trehalose. Murphy and Wyatt (1965) found the trehalose synthetic system present in silk moths to contain trehalose-6-phosphatase (although they never described the assay) which they termed "highly active". All analysis of the

kinetic data described in the paper of Murphy and Wyatt are based on the assumption that the initial product is trehalose-phosphate; this is immediately hydrolyzed to the free sugar.

Efforts at detecting synthesis of trehalose phosphate in the present study were unsuccessful. The unavailability of trehalose-6-phosphate from commercial sources prevented the comparison of its mobility with that of trehalose and glucose-6-phosphate in the chromatographic and electrophoretic methods employed in this study. The possibility that the disaccharide phosphate was present in these areas described as being trehalose or glucose-6-phosphate cannot therefore be entirely ruled out. The precedent set by Murphy and Wyatt (loc.cit.) of treating trehalose formation as a reflection of the properties of trehalose phosphate synthetase, the rate-limiting step, is therefore followed in this study. It should be added, however, that in view of Friedman's (1960) finding that Phormia trehalose-6-phosphatase has a divalent ion requirement, the finding in the present study of a similar requirement may, in actuality, relate to the disaccharide phosphate phosphatase rather than the synthetase. If this is the case, then the mobility of trehalose phosphate must be identical to that of glucose-6-phosphate in solvent 1 and in paper electrophoresis using sodium borate buffer.

15. The Effects of Trehalose Concentration on Trehalose Synthesis

The effects of varying concentrations of the end-product, trehalose, on reaction velocity were determined in a system containing .51 mg protein,

10 μ moles Tris-maleate, 5 μ moles MgSO_4 , 2 μ moles UDPG, 4 μ moles glucose-6-P, and 0.4 μ curies (0.04 μ moles) 1-C^{14} glucose-6-P in a total volume of 0.2 ml. Results are demonstrated in Figure 12.

The finding that trehalose exhibits end-product inhibition (Figure 12) of its own synthesis in M.dubius body wall has several implications. Under the experimental conditions, trehalose exerted an inhibitory effect which approached 50% at concentrations above 30 mM. A similar effect was observed by Murphy and Wyatt (1965) in the silk moth preparation. These workers postulated that accumulation of trehalose in the organism would inhibit further disaccharide synthesis; UDPG would therefore accumulate and permit increased synthesis of glycogen. The rise in circulating carbohydrate would thus promote increased synthesis of energy reserves in the form of polysaccharide. These mechanisms, it is suggested, would promote the rapid production of both trehalose and glycogen when blood glucose levels rise, followed by readjustment to initial blood trehalose levels.

The above-mentioned scheme, although hypothetical, could be operative in a modified form in the acanthocephalan, M.dubius. Although the function of trehalose in these worms is unclear, the level appears to be high and rather stable regardless of infection age (Table III). Maintenance of the trehalose pool could thus be controlled by the observed end-product inhibition demonstrated by the enzymes localized in the body wall. A survey of the changes in worm trehalose pools after starvation of the host would contribute much to our understanding of the function and significance of the observed end-product exhibition.

DISCUSSION

The concentration of trehalose estimated in the various developmental stages of M.dubius (Table I) compares favorably with those values reported by Fairbairn (1958) for whole worm trehalose. The acanthor stages seem to contain the largest amounts of disaccharide; this observation may be related to Fairbairn and Passey's (1957) findings that in Ascaris eggs, trehalose is present in high concentrations and may play an osmotic role in the hatching process.

The failure to demonstrate the biosynthesis of trehalose in body fluid or acanthors merits discussion. The fact that trehalose is present in these tissues in high concentrations is consistent with other reports (Wyatt, 1967) concerning dormant and resistant stages of invertebrates. However, presence or absence of a given compound in a tissue does not necessarily indicate that synthesis occurred in that particular tissue. The body fluid presents an effective means for the transport of water-soluble metabolites between various tissues and organs of the worm. The fact that the acanthor stage, when fully mature, is prepared to undergo long periods of inactivity might shed doubt on the concept that the enzymes themselves are located in these tissues. The fact that Fairbairn (loc.cit.) and Laurie (1959) found trehalose to be present in high concentrations in whole worms indicates that the compound is not limited to the developmental stages. Thus, even if the enzymes are present inside the acanthor membranes, these would probably only function in the synthesis of the disaccharide for the needs of the acanthor, since the membranes appear to be relatively impermeable. There should be some other site of the necessary enzymic

complement. Of course, the presence of the enzymes in body fluid or acanthors cannot be entirely eliminated; under the conditions used in this investigation, synthesis could not be demonstrated in these tissues. The possibility of enzyme repression or inhibition does exist. The finding that crude homogenates of acanthors incorporated radioglucose into polysaccharide suggests the presence of glycogen synthetase. The enzyme seemed to be associated with the particulate fraction of the acanthor homogenate; it was removed by centrifugation at 37,000 g. The repeated demonstration of this enzyme, as well as the capacity for hexose phosphorylation, shows that disruption techniques were not producing a generalized denaturation of proteins. It can be argued that the mature acanthors would not be expected to possess significant enzyme activity, since the tissue represents a dormant stage. Indeed, many enzymes would probably be difficult if not impossible to detect, unless the acanthors had been artificially induced to undergo activation and hatching.

The results of this study indicate that trehalose synthesis in Moniliiformis dubius body wall occurs in a fashion similar to that originally reported to occur in yeast (Cabib and Leloir, 1958), and later shown to be operative in insects (Candy and Kilby, 1961). UDPG operates as the glucosyl donor, and glucose-6-phosphate is required as co-substrate. Trehalose-6-phosphate is presumably formed as the first product; however, this compound was not identified in the current study.

Although the function of trehalose in the biology of M.dubius was not investigated, some remarks can be made concerning possible functions which would merit further study. . . The high concentrations of this disaccharide present in the acanthor may imply that the sugar is playing an osmotic role similar to that proposed to be operative in the eggs of Ascaris (Fairbairn and Passey, 1957). The localization of the enzymes responsible for trehalose synthesis in the body wall musculature of adult female worms may bear some special functional significance. Moniliiformis dubius shares in common with many intestinal helminths the entire lack of a digestive tract. All essential nutrients must therefore pass through the surface of the worm, either by diffusion or by some energy-requiring mechanism. Indeed, the surface of the worm seems to be highly specialized to facilitate acquisition of substances from the surrounding medium (Byram, 1969). That glucose appears to be the primary carbohydrate precursor for reserves and energy requirements in most helminths appears to be a well-established fact (Read, 1966). The mechanism for uptake of glucose seems to be obscure, although good evidence exists that this phenomenon occurs at a high rate and requires metabolic energy in some species (Phifer, 1960). Our knowledge of the possible mechanisms involved in the uptake of carbohydrate against a concentration gradient has been adequately reviewed (Read, loc. cit.). There remains little doubt that active absorption and accumulation of glucose is a real and widespread phenomenon.

The use of the biosynthetic pathway leading to trehalose as a possible means of obtaining essential carbohydrate from the host by M.dubius is a tempting hypothesis. The enzyme is localized near the environment, in the body wall; conversion of glucose into trehalose at the cell surface would thus promote diffusion of glucose into the worm, since internal glucose would be removed from exerting an effect on diffusion rate. Thus, glucose could continue to diffuse into the worm as long as trehalose synthesis was occurring. The disaccharide hydrolyzed to glucose prior to metabolism in insects; a demonstration of this process would greatly support such an hypothesis. Trehalose is freely water-soluble, is osmotically active, and, in the presence of trehalase, easily convertible into a metabolite which is readily available for entry into either anabolic pathways leading to storage as glycogen or catabolic routes for the ultimate generation of ATP. The end-product inhibition observed in this study would feasibly act as a control mechanism mediating glucose accumulation. When trehalose levels rise, its synthesis from incoming glucose would be reduced, and glucose would begin to accumulate inside the worm, slowing diffusion and thus uptake. The added observation that ATP at high concentrations also inhibits the system strengthens this idea; when adequate ATP was available, trehalose synthesis and thus concomitant glucose uptake would be inhibited. Such a scheme would be energy dependent, since ATP is required for phosphorylation of glucose prior to synthesis of trehalose. Such a system would show saturation kinetics

which would be a function of the rate-limiting step in the reaction sequence, presumably trehalose phosphate synthetase. It must be added that this hypothesis is not an original one. Fairbairn proposed that just such a mechanism may be operative in nematodes. This premise was based on evidence that Ascaris rapidly converts glucose into trehalose (Fairbairn, 1960).

The first major point which needs clarification in attempting to resolve this question is the metabolic fate of trehalose in those helminths where its synthesis has been demonstrated. The only report in the literature concerning the existence, properties, or distribution of trehalase in helminths was that of Feist, et al. (1965). These workers found gut tissue to be the only site of trehalase activity in Ascaris. Activity was present in both fresh and frozen homogenates of this tissue, but the hydrolase was not demonstrated in male and female reproductive tissues or in muscle preparations.

The importance of the demonstration of this enzyme, or some other mechanism for trehalose conversion into anabolic or catabolic intermediates, cannot be overemphasized. Without some explanation for the metabolic fate of the disaccharide, proposals concerning function which indirectly or directly link trehalose synthesis with energy assimilation are of questionable value.

Figure 2
THE EFFECT OF PROTEIN CONCENTRATION
ON
TREHALOSE SYNTHESIS

Each reaction contained:

10.0 μ moles Tris-maleate buffer pH 7.4

3.0 μ moles MgSO_4

0.6 μ moles ATP

0.5 μ moles UDPG

0.5 μ curie (0.034 μ moles) U-C¹⁴ glucose

Total Volume: 0.2 ml

Incubations run at 37°C for one hour.

Figure 3
THE EFFECTS OF MAGNESIUM IONS
ON
TREHALOSE SYNTHESIS

Each reaction contained:

0.79 mg protein

10.0 μ moles Tris-maleate buffer pH 7.4

0.5 μ moles UDPG

0.8 μ curie (0.08 μ moles) 1-C¹⁴ glucose-6-P

Total volume: 0.2 mL

Incubations were run at 37°C for one hour.

FIGURE 4

THE EFFECT OF INCUBATION TIME ON
TREHALOSE FORMATION

Each reaction contained:

0.43 mg protein

10.0 μ moles Tris-maleate buffer pH 7.4

5.0 μ moles MgSO_4

0.4 μ curie (0.04 μ moles) 1-C^{14} glucose-6-P

4.0 μ moles glucose-6-P

Total volume: 0.2 ml

Incubations were run at 37°C for one hour.

FIGURE 5

THE EFFECT OF pH ON TREHALOSE SYNTHESIS

Each reaction contained:

0.65 mg protein

10.0 μ moles Tris-maleate buffer, pH 7.4

5.0 μ moles MgSO_4

1.0 μ moles UDPG

0.4 μ curie (0.04 μ moles) 1-C¹⁴ glucose-6-P

Total volume: 0.2 ml

Incubations were run at 37°C for one hour.

FIGURE 6
THE EFFECT OF TEMPERATURE ON TREHALOSE SYNTHESIS
AND
ARRHENIUS PLOT

Each reaction contained:

0.63 mg protein
10.0 μ moles Tris-maleate buffer pH 7.4
5.0 μ moles MgSO_4
1.0 μ mole UDPG
0.4 μ curie (0.04 μ moles) 1-C^{14} glucose-6-P
1.0 μ mole glucose-6-P

Total volume: 0.2 ml.

Incubations were run for one hour.

FIGURE 7

THE EFFECT OF GLUCOSE CONCENTRATION ON TREHALOSE SYNTHESIS
AND
LINEWEAVER-BURK PLOT

Each reaction contained:

0.12 mg protein

10.0 μ moles Tris-maleate buffer pH 7.4

3.0 μ moles MgSO_4

2.0 μ moles UDPG

0.5 μ curie (0.034 μ moles) U- C^{14} glucose

Total volume: 0.2 ml

Incubations were run at 37°C for one hour.

FIGURE 8

THE EFFECT OF UDPG CONCENTRATION ON TREHALOSE SYNTHESIS
and
LINEWEAVER-BURK PLOT

Each reaction contained:

0.73 mg protein

10.0 μ moles Tris-maleate buffer pH 7.4

5.0 μ moles MgSO_4

0.4 μ curie (0.04 μ moles) 1-C^{14} glucose-6-P

4.0 μ moles glucose-6-P

Total volume: 0.2 ml

Incubations were run at 37°C for one hour.

FIGURE 9

THE EFFECT OF GLUCOSE-6-PHOSPHATE CONCENTRATION ON TREHALOSE
SYNTHESIS
AND
LINEWEAVER-BURK PLOT

Each reaction contained:

- 0. 48 mg protein
- 10. 0 μ moles Tris-maleate buffer pH 7.4
- 5.0 μ moles MgSO_4
- 2.0 μ moles UDPG
- 0.4 μ curie (0.04 μ moles) 1-C^{14} glucose-6-P

Total volume: 0.2 ml

Incubations were run at 37°C for one hour.

FIGURE 10

THE EFFECT OF ATP CONCENTRATION ON TREHALOSE SYNTHESIS

Each reaction contained:

- 0.67 mg protein
- 10.0 μ moles Tris-maleate buffer pH 7.4
- 5.0 μ moles MgSO_4
- 0.5 μ mole UDPG
- 0.8 μ curie (0.08 μ moles) 1-C^{14} glucose-6-P
- 4.0 μ moles glucose-6-P

Total volume: 0.2 ml

Incubations were run at 37°C for one hour.

FIGURE 11

THE EFFECT OF INORGANIC PHOSPHATE CONCENTRATION
ON
TREHALOSE SYNTHESIS

Each reaction contained:

0.59 mg protein

10.0 μ moles Tris-maleate buffer pH 7.4

5.0 μ moles MgSO_4

1.0 μ mole UDPG

0.4 μ curie (0.04 μ moles) 1-C¹⁴glucose-6-P

2.0 μ moles glucose-6-P

Total volume: 0.2 ml

Incubations were run at 37°C for one hour.

FIGURE 12

THE EFFECT OF TREHALOSE CONCENTRATION ON TREHALOSE SYNTHESIS

Each reaction contained:

0.51 mg protein

10.0 μ moles Tris-maleate buffer pH 7.4

5.0 μ moles MgSO_4

2.0 μ moles UDPG

0.4 μ curies (0.04 μ moles) 1-C¹⁴ glucose-6-P

4.0 μ moles glucose-6-P

Total volume: 0.2 ml

Incubations were run at 37°C for one hour.

APPENDIX

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APPENDIX (cont.)

K.R.T.

(1) Krebs-Ringer (10X) saline:

NaCl	70.2 gm
KCl	3.59 gm
CaCl ₂ ·2H ₂ O	3.79 gm
MgSO ₄ ·7H ₂ O	3.19 gm

Dissolve NaCl, KCl and MgSO₄ in about 800 ml H₂O and adjust volume to 1000 ml.

(2) Tris (hydroxymethyl aminomethane buffer) 1.0 M

(a) 1.0 M Tris-maleic acid solution

121.1 gm Tris

116.0 gm maleic acid

Dissolve in H₂O and adjust volume to 1000 ml. Filter through coarse filter paper.

(b) .25 M Tris-maleate buffer pH 7.4 (10X)

Combine 500 ml of (a) and 800 ml H₂O; adjust pH to 7.5* with NaOH (slightly over 500 ml of 1N NaOH). Adjust volume to 2000 ml with H₂O.

* pH drops about 0.1 unit with dilution
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APPENDIX (cont.)

The final resultant concentrations of KRT are as follows:

NaCl 120mM

KCl 4.8 mM

CaCl₂ 2.6 mM

MgSO₄ 1.2 mM

Tris-maleate pH 7.4 25.0 mM

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