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PRELIMINARY OBSERVATIONS ON THE METABOLISM OF C^{14}-GLUCOSE BY INTACT LARVAE OF TRICHINELLA SPIRALIS UNDER NEAR ANEROBIC CONDITIONS

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PRELIMINARY OBSERVATIONS ON THE METABOLISM OF C^{14}-GLUCOSE BY INTACT LARVAE OF *TRICHINELLA SPIRALIS* UNDER NEAR ANAEROBIC CONDITIONS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PRELIMINARY OBSERVATIONS ON THE METABOLISM OF C^{14}-GLUCOSE BY INTACT LARVAE OF *TRICHINELLA SPIRALIS* UNDER NEAR ANAEROBIC CONDITIONS

CHAPTER I

INTRODUCTION, HISTORICAL REVIEW, AND STATEMENT OF THE PROBLEM

Introduction

Physiological studies of animal parasites have unquestionably developed considerably apart from invertebrate physiology in general. Basically, however, the intentions of such investigations are identical regardless of whether one is dealing with free-living terrestrial or aquatic animals or with the parasitic variety. One strives to determine by what mechanism such an organism survives in the habitat peculiar to it. Here one finds that the habitats of endoparasites diverge in many ways from those of their free-living relatives. Parasitic forms are exposed to an almost endless array of excretions and secretions of their hosts their sites may or may not be very poor in oxygen; and the pH may be uncommonly constant or markedly variable. To further tax the imagination of the investigator, one finds that parasites may live part of their life cycle in the "outside world" with fluctuating environmental conditions and the balance of their life cycle under the constant "host supervision" and controlled environmental provisions. Undoubtedly, these

conditions "leave their mark" on a parasitic animal, but the most amazing fact of all is not that parasites have adapted themselves to such given circumstances but that they have altered themselves so irreversibly as to have a "physiological need" for such an existence. Indeed, some forms cannot exist in the absence of what at first glance would seem to be almost insurmountable barriers. This is the realm of host-parasite relationship and is inseparable from parasite physiology.

In this investigation, the approach might appropriately be termed a biochemical one, but the intent is most certainly not to neglect the overall importance of the interplay of environment provided by each participant--the parasite and the host. Adequate consideration of these aspects dictates that one be continually mindful both of the life history of the form studied and of the environmental conditions supplied by the host animal.

Although the carbohydrate metabolism of parasites is one of the best known phases of parasite physiology, our knowledge is still far from complete, both as to the mechanism of carbohydrate utilization and the types of carbohydrate metabolism occurring in most groups. It is no coincidence that carbohydrate metabolism should currently be enjoying increased scrutiny in parasitic forms, since all forms living in anaerobic habitats (such as the intestine) utilize carbohydrate primarily because it is the best source of anaerobic energy. Anoxidative processes are primarily oxidation-reduction reactions and it has been pointed out that the intermediately oxidized carbon atoms of carbohydrate (H-C-OH) are ideally suited for such purposes. It is further noteworthy that most of the parasites studied to date do not completely oxidize sugar to carbon

dioxide and water. Although this is a necessary result of the lack of sufficient supply of oxygen when such forms are living in an area of reduced oxygen tension, the same observation is usually noted when such forms metabolize aerobically (Cheng, 1964). In other words, most endoparasites are characterized by the prevalence of anaerobic or aerobic fermentation. It is readily apparent that both anaerobic and aerobic fermentation is "wasteful" but this is not a disadvantage to parasitic forms since they occupy surroundings that offer them a surplus of readily available nutrients. Parasites may utilize the carbohydrate present in their environment for synthesis of cellular materials; for production of energy; or indirectly, for transformation into reserve energy stores. Glucose would seem to be most frequently encountered by intestinal parasites, and while its utilization has been shown for some endoparasites, Stannard, et al. (1938) were unable to demonstrate a glucose consumption by intact Trichinella spiralis larvae.

Ezymatic preparations from larval homogenates or acetone powders have revealed the presence of hexokinase, phosphohexose isomerase, aldolase, glyceral phosphate dehydrogenase, enolase and lactic dehydrogenase (Goldberg, 1957a, 1957b, 1958; Agosin and Aravena, 1959). This evidence is quite indicative of the presence of a classical glycolytic scheme. Goldberg (1958) postulates the occurrence of such a pathway similar to that found in mammalian muscle. His evidence is based on the occurrence of and the similar optima of the enzymes purified and characterized. These more elaborate studies are in good agreement with the earlier work of von Brand et al. (1951) which indicated that approximately the same amount of endogenous glycogen was consumed during aerobic metabolism as

in anaerobically metabolizing larvae. However, in the same report (von Brand et al. 1951), it was noted that whereas no endogenous lipids disappeared in anaerobiosis, the lipid content decreased about 21% per day under aerobic conditions. That the larvae have a functional hemoglobin, a complete cytochrome system, succinic dehydrogenase and most of (if not a complete) tricarboxylic cycle, is well documented (Agosin, 1956; Goldberg and Nolf, 1956; Goldberg, 1957b). The same workers reported the occurrence of these pigments in adult trichinae homogenates, but stated that the enzymes were apparently less active than in the larvae. These details, when coupled with the documentation that glycogen is apparently fermented to CO_2 and energy rich volatile fatty acids, illustrates an apparent idiosyncracy of these worms.

The reported presence of hexokinase and glucose-6-phosphate dehydrogenase enzymes (Agosin and Aravena, 1959) suggests that pathways for carbohydrate degradation other than the classical glycolytic scheme may be present in T. spiralis. No attempts to obtain further evidence that this may be true have been forthcoming.

Historical Review

Trichinella spiralis larvae were first described from the muscle of man in 1835 by Sir Richard Owens at a meeting of the Zoological Society in London. Credit for discovery of the worm, however, must be given to a student of Owens' named James Paget (Gould, 1945). The description of the worm from the flesh of hogs was published by Leidy (1846), but the great German scientists Leuckart (1860) and Virchow (1865) independently unraveled the main features of the complicated life history of Trichinella

spiralis. Numerous other investigators have contributed to our knowledge of the parasite. Notable in this group was Zenker (1860), who demonstrated that the worm could cause a fatal illness in man in the course of a few weeks. The presence of both intestinal and extraintestinal phases has made the study of this rather unique parasite a monumental task, and possibly for this reason, the species has continued to hold the fascination of investigators in ever-increasing numbers to the current time. Larsh (1962) expressed it in the following manner:

> "The infection produced by T. spiralis is very complex and, considered in the broad sense, is little understood even today. To advance our knowledge, more information is needed considering the physiology of both the parasite and the host. In recent years, biochemical studies of larvae under various conditions have been fruitful in providing information concerning chemical composition, certain metabolic pathways, excretory products, and so forth. However, the time has not arrived when such data can be used directly to explain the many complex interrelationships of the parasite and host...we may in time unravel the mysteries of their precise mechanism, especially if the chemical studies continue to provide basic data concerning the life needs and host-toxic metabolites of the parasite."

Thus, despite the tremendous efforts of scientists and the use of elaborate scientific equipment, many important aspects of the parasite and the disease it causes remain to be explained.

Life History of Trichinella spiralis

The following remarks concerning the life history of the trichina worm must be prefaced by the realization that, although the cycle discussed herein pertains primarily to experimental infections of laboratory mice and rats, Trichinella spiralis is perhaps the single species of parasitic nematodes least restricted in its range of hosts (Gould, 1945). It has been possible, on occasion, by experimentally altering

some of the host's environmental conditions, to infect an almost endless array of animals with this remarkably non-specific parasite. The principal factor in the successful infection of animals which are not found to be naturally infected seems to be body temperature. The worm develops most efficiently in hosts having a body temperature of 35 to 37°C. In general, homeothermic carnivores and omnivores are found to be naturally parasitized, at least on occasion, provided the host temperature range stated above is met. However, the principal natural hosts of Trichinella spiralis appear to be man, the pig and the rat (Gould, 1945).

Based upon the number of publications, the most popular hosts for experimental studies have been mice, rats, hamsters, rabbits, and guinea pigs, respectively (Larsh, 1962). Since mice have been used more commonly as experimental hosts, the pertinent details of the life history of the parasite in this animal are reviewed in order to better understand the overall host-parasite relationships and, hopefully, to gain some insight as to "important factors" provided by the host.

The life cycle is begun by ingestion of viable trichina larvae encysted in raw or undercooked meat, or more commonly in experimental infections, by the oral inoculation of larvae freed from infected muscle tissue by the action of artificial gastric juice. In either case, larvae freed in the stomach of the host pass into the small intestine where they anchor themselves to the intestinal mucosa. Gould (1945) suggests that it is possible that they obtain both nutriment (tissue juice) and oxygen for growth from the mucosa. The first parasitic phase is usually termed the pre-adult and it will undergo development to an adult in the 8th to 72nd postinfective hour according to various investigators (Gould, 1945;

Wu and Kingscote, 1957; Berntzen, 1965). The development of a pre-adult into a sexually mature adult worm in the small intestine has been interpreted to require from one (Hyman, 1951; Berntzen, 1965) to four molts (Weller, 1943; Gould, 1945; Kim, 1961, 1962), but it is generally agreed that the transformation is usually completed by the 72nd postinfective hour (Gould, 1945; Larsh, 1962; Berntzen, 1965). If the recent work of Berntzen (1965) is confirmed, the encysted trichinae (4th stage larva) require only a single molt to become mature intestinal worms and in a population of worms this molt occurs between the 8th to 72nd hour after ingestion of viable larvae. If what Berntzen reports is true, this would represent the fourth molt and would indicate that Trichinella has a typical nematode life cycle rather than an atypical cycle as suggested by the work of Weller (1943) and Kim (1961, 1962). Irrespective of the number of molts (interpreted or postulated), both the pre-adults and adults are deeply embedded in the intestinal mucosa and on occasion are reported to have penetrated into the crypts of Lieberkuhn (Larsh, 1962). The preferred site of the adult trichinae seems to be the posterior half of the small intestine (Roth, 1938; Larsh, 1962), which is a region abounding in both partially and totally digested nutriments (Read, 1950). Some investigators have reported that in young mice the anterior half of the small intestine is more heavily parasitized; but the same effect can be produced in older mice if any agent (such as morphine sulfate) is administered which drastically reduces the intestinal emptying time (Larsh and Hendricks, 1949). The effect of intestinal emptying time has likewise been noted with other intestinal parasites (Read, 1959).

After the adult stage of the worm is reached, copulation takes

place either in the lumen or within the mucosa of the intestine (Berntzen, 1965). This occurs approximately 48 hours after the ingestion of infected muscle, but only after the final molt of the participating worms has been accomplished (Gould, 1945; Berntzen, 1965). The adult male usually measures from 1.4 to 1.6 mm in length by 0.033 to 0.040 in diameter while the adult female is considerably larger, measuring from 2.2 to 3.6 mm in length and from 0.060 to 0.072 mm in diameter. After copulation, male worms are slowly lost from the intestine. The time required from fertilization of females until progeny are deposited is about 96 hours at minimum (Berntzen, 1965), and the total number of larvae produced per female varies markedly with the species of host. About 1000 to 1500 young trichinae are released by each female worm during her residence in the intestine of a guinea pig whereas in infected mice from 230 to 507 larvae are produced (Roth, 1939). The length of the female worm's residence in a particular host species varies from a usual maximum of 16 days in the mouse to 37 days in the guinea pig (Roth, 1938, 1939); thus, if reduced to a basis of larvae released per female worm per day, the rates are comparable and furthermore are in good general agreement with other earlier investigators who reported that after the onset of larviposition, approximately one larva was born from each female worm every half hour (Gould, 1945). The gravid female releases the second stage larvae directly in the mucosa (the first molt having occurred in utero) (Berntzen, 1965). Gould (1945), in citing the interpretations of other investigators, states that the discharge may be directly into the lymphatics or lacteals of the intestinal villi and most previous investigators accepted a hematogenous dissemination of the larvae to the

muscles. However, Berntzen (1965) refutes that any significant number of larvae are found either in blood or the lymphatic system. Many contemporary investigators have inadvertently strengthened Berntzen's argument (even before it was advanced) by the admission that it is usually extremely difficult to demonstrate larvae in the blood, even at expected peak intervals.

The route of juvenile migration from the intestine to the final location in striated muscle appears to occur primarily via abdominal and thoracic cavities and connective tissue. Examinations of fluid from the abdominal and thoracic cavities of host animals yield large numbers of second stage larvae (measuring about 100 microns in length by 5 μ in width) starting at the fifth postinfective day, and in experimental infections of rats direct microscopic examinations of fresh mesentery show larvae migrating through connective tissues (Berntzen, 1965). Microscopic blood examinations during the same period are usually fruitless, regardless of the host species involved (Gould, 1945; Kagan, 1960). As early as 1860, Zenker noted that the trichinae were especially numerous in the muscles at the sites of tendinous insertions. Examinations of rodent striated muscle reveal increasing numbers of second stage juveniles from about the 7th postinfective day onward until about the 17th day, after which the number increases slightly if at all (Phillipson and Kershaw, 1960, 1961; Berntzen, 1965). The size of newly deposited trichinae apparently remains unchanged during their transit from the intestine to the striated muscles (Gould, 1945; Berntzen, 1965). After arrival in and invasion of the muscles, the larvae grow at the rate of 0.02 mm per day (Roth, 1939) until a maximum size of about 1 mm in length and

35 microns in width is attained within the cyst. Berntzen (1965) observed the third and fourth molts during this period of rapid larval growth - the third molt occurred approximately four days after muscular invasion and the fourth molt occurred about the eighth day. The last ecdysis produces the fourth stage larvae, and these are infective to the next host. According to this scheme, the fourth molt occurs about the 16th postinfective day. This time correlates well with the occurrence of infective larvae in muscles of most experimental hosts.

Nematode Nutrition and Physiology

Although some recent general reviews are available (Bueding and Most, 1956; Bueding, 1960, 1962a, 1962b; Fairbairn, 1960; von Brand, 1960; Lee, 1965), most of the work on nematode physiology has been performed on *Ascaris*, usually from hogs, and an excellent review of these studies is available (Fairbairn, 1957). The reason for extensive study of this parasite is rather obvious; it is a large form and easily obtainable. Since there have been no metabolic studies on the adult *Trichinella spiralis* and there appear to be certain similarities between *Trichinella* and *Ascaris*, the information available primarily from metabolic studies of *Ascaris* will be utilized in attempting to explain certain physiological characteristics of the intestinal trichinae.

In the initial consideration of parasitic nematode nutrition, it should be pointed out that it is believed that nematodes are exceedingly peculiar in at least one respect since, in general, they undergo no somatic cell multiplication after hatching in spite of a very considerable increase in size (Hyman, 1951). The major growth requirements

therefore are for cytoplasmic rather than nuclear synthesis and the nucleic acids occur in rather small quantities relative to the total tissue weight (Smyth, 1962). Thus it is expected, and usually observed, that the most rapid growth phase does not occur until the parasite reaches the intestine with its abundant supply of food materials. However, the picture is not as clear as it might seem for with the adaptation to a parasitic existence, there is generally associated an increased reproductive capacity. This implies either a synthesizing capacity on the part of the parasite or a provision of precursors for nucleic acid synthesis by the environment. In the case of Trichinella, it is felt that the developed ovum in utero contains sufficient endogenous reserves to enable a fully formed active larva to develop without the absorption of further nutriment. However, any time a measurable increase in the overall size of the larva occurs, nutrients are required to supply energy and precursors for the synthesis of additional cytoplasmic material. Then, at the time of sexual maturation, additional demands are made either of the parasite or its environment. In the adult trichinae, this requirement may be met by: (1) the ready availability of all host dietary components; (2) de novo synthesis of genetically important compounds by the parasite; (3) ingestion and absorption of the required substances from intestinal cells; or (4) any combination of the above.

Digestion of foodstuffs by nematodes may be extracorporeal, intracorporeal, or a combination of the two, but it is always, so far as is known, extracellular (Rogers, 1962). Extracorporeal digestion is thought to occur in nematodes whose food must enter the body by way of a hollow stylet, the dimensions of which are such that even bacteria

would transverse it with difficulty. Such forms are usually phyto-parasitic, but demonstration of cytolysis of host tissue by zooparasites is certainly not uncommon. Intracorporeal digestion is thought to be more common in nematodes although in some cases some preliminary solubilization of food may occur externally (Hobson, 1948). Smyth (1962) regarded the general morphology of the pharynx found in intestinal trichinae to be "markedly adapted for sucking". In 1939, von Someren demonstrated that adult trichinae of both sexes possessed a functional buccal stylet which could be protruded and retracted, probably for lacerating host tissue and releasing tissue fluids for nutrition. The same investigator suggested that the stichosome (the cells imbedded in the esophagus) had a secretory rather than absorptive capacity, and also noted the presence of two unicellular digestive glands at the junction of the esophagus and the intestine. He believed the adult trichinae penetrated the mucosa with the stylet to release the cell contents and fed upon tissue fluids of the mucosa. The larvae also possess a stylet for boring and it is tempting to speculate that they may gain some nutriment by cytolytic action as they penetrate through connective tissue. Rogers (1941), in a study of Ascaris and Strongylus, and Carpenter (1952), in an examination of the digestive enzymes of Ascaris, were able to demonstrate the presence of amylase, lipase, protease and four different peptidases in the esophagus and to a lesser extent in the mid- and hind gut. Their findings add strength to the generalization that nematodes probably contain an array of digestive enzymes (Cheng, 1964). Some of these digestive enzymes undoubtedly are released to the exterior by the esophageal glands, but at least certain amounts would be added to the ingested

nutriment on its passage to the intestine where the foregut secretions augment the enzymatic array. In addition to cytolytic effects noted on cells, reports of circumoral precipitates about both larvae and adults by immune serum strongly suggests that some enzymes are released by these forms. From the above considerations it is evident that extracorporeal digestion is obligatory in some forms; in others it is useful but non-essential; and in other parasites it may play no part in the normal digestive processes, which are then entirely intracorporeal.

Absorption of digested foodstuffs by nematodes is even more poorly understood. It seems certain, however, that in adult nematodes absorption usually occurs by way of the intestine and not through the cuticle (Rogers and Lazarus, 1949). This interpretation becomes more hazardous in consideration of larval nutrition due to the less impervious nature of the newly formed cuticle (Bird and Rogers, 1956; Bird, 1958). The acid and alkaline phosphatases found in the intestine are thought to play an essential role in the absorption of simple sugars against a concentration gradient (Rogers, 1962). That these enzymes are obligatory in Trichinella larvae seems dubious as the tissues contain relatively small amounts of glucose (0.04% of total tissue solids) but contain instead considerable quantities of the dissacharide trehalose (1.76% of total tissue solids) (Fairbairn, 1958). Thus, the concentration gradient would favor glucose absorption and the enzymes would possibly be superfluous. However, no concrete evidence is available that either larval or adult trichinae utilize glucose. Stannard, McCoy, and Latchford (1938) reported glucose gave no stimulation of oxygen uptake in the aerobically metabolizing larvae.

Distribution of absorbed substances is mediated by the hemolymph which bathes all the tissues. This is also the transport system for the movement of solutes from one tissue to another as well as for removal of metabolic wastes. This fluid has a rather high osmotic potential but is always consistently lower than the osmotic potential of the host gut fluids (Hobson et al, 1952). The high osmotic pressure is due to a high ash content in which the cations of K, Na, Ca and Mg predominate. Cl is the principal anion but is present in insufficient quantity to balance the cations; the deficiency is made up by volatile and non-volatile organic acids (Bueding and Most, 1956) and bicarbonate (Fairbairn, 1957). Proteins, carbohydrates, and fats are all present. The proteins consist mainly of albumins and globulins which differ little in amino acid content from their host. Non-protein nitrogen consists chiefly of free amino acids, peptides, urea, and ammonia. Carbohydrates present consist chiefly of glycogen and trehalose with only traces of glucose and other free carbohydrates (Fairbairn, 1958). Fats are the least abundant and are represented mostly by phospholipids and triglycerides (Fairbairn, 1960). The preceding information is derived solely from the studies of Ascaris hemolymph as detailed analysis on Trichinella are not yet possible. It is likely that other parasitic nematodes may not vary greatly in the general composition of the perienteric fluid.

Hemoglobin is usually found in the hemolymph and this is sometimes cited as evidence of an aerobic metabolism (von Brand, 1952; Cheng, 1964). This is a misconception for hemocoel hemoglobin of Ascaris binds oxygen so tightly that it is almost impossible to dissociate it even by chemical means (von Brand, 1960) and so it is doubtful whether this

compound is of any value in oxygen transport. Regarding the influence of varying oxygen tensions on the rate of oxygen consumption, von Brand (1960) divides the invertebrates into two large groups: (1) those which can maintain an unaltered rate of oxygen consumption even at low tensions because of easy entry of oxygen into their bodies (i.e., high surface/volume ratio) and/or an efficient distribution mechanism (i.e., a functional pigments) and (2) those which have a respiration dependent upon oxygen tension. This concept makes meaningless the old debate regarding the aerobic or anaerobic nature of intestinal worms purely on the basis of habitat. Thus, it is theoretically possible to find two different species of worms lying side by side in the intestine with one leading a predominantly anaerobic existence while the other exhibits a primarily aerobic metabolism. The choice would depend on (1) an endowment of and (2) the functional quality of respiratory pigments. While Ascaris adults apparently would fall in category 2 mentioned above, Trichinella larvae contain functional respiratory pigments and a favorable surface/volume ratio which makes respiration independent of oxygen tensions as low as 6 mm Hg (Stannard et al, 1938). It is tempting to speculate that the same would be true for adults and pre-adults of this form in the intestine but the reports that marked changes in cytochrome content occur during the larval development of Ascaris does not permit this assumption (Costello and Oya, 1963). Fairbairn (1960) states that no obligate anaerobes have been found in the nematodes although some adult forms (especially Ascaris) appear to be microaerophilic. Although some eggs withstand lengthy periods of anoxia, there has been general agreement that the metabolism of nematode eggs is primarily, and even obligately,

aerobic (Fairbairn, 1960).

Oxygen at partial pressures up to at least 160 mm Hg is not toxic to most adult nematodes; in fact at these pressures it frequently aids their survival *in vitro* (Rogers, 1962). All nematodes can respire in oxygen, but the question is raised as to whether or not this respiration gives rise to energy which is useful in the economy of the parasites *in vivo*. Possibly, larvae of *T. spiralis* require the energy derived from oxidative metabolism for motility (von Brand, 1952) and perhaps it is used only for this function. There is some evidence that oxidative processes which use lipids as substrates are to some degree independent of the anaerobic processes and do not lead to the conservation of carbohydrate. It seems possible, therefore, that the oxidation of fats may be necessary for the migration of the larvae *in vivo*, but when the larvae lie quiescent in the muscular tissue of the host the anaerobic catabolism of carbohydrates probably provides sufficient energy for survival (Rogers, 1962). The importance of the same schemes to the intestinal trichinae is difficult to evaluate because of inadequate knowledge as to just how much mobility is necessary for survival. Fairbairn (1960) speculates that, in all nematodes, the amounts of energy required for motion and locomotion *in vivo* are small.

While this is not an attempt to negate the efforts of others, it has been pointed out (Fairbairn, 1960; von Brand, 1960; Rogers, 1962) that manometric determinations of oxygen uptake (Q_{O_2}) and respiratory quotients (RQ) of parasites *in vitro* may be quite misleading. For example, the ionic composition of the suspending medium may profoundly affect the respiration rate (Bueding, 1949) and removal of CO_2 may produce similar

alterations. To illustrate the latter statement, routine Warburg determinations on the oxygen uptake of Nippostrongylus muris indicated that the early, free-living larvae were unaffected by the presence of 5% CO_2 but this concentration of CO_2 doubled the oxygen uptake of the infective larvae (Schwabe, 1957). Measurements obtained in abnormal saline or buffer solutions may also be misleading due to a stimulation of activity (Rogers, 1962). In addition to measuring oxygen consumption, many workers have determined carbon dioxide production during respiration and from the results, have calculated the R.Q. As pointed out by Fairbairn (1960), carbon dioxide measurements are in themselves legitimate, but the interpretation of R.Q. values even in animals whose metabolism is comparatively well-known is hazardous because the interpretation presupposes that all CO_2 arises from the complete oxidation of the substrate and that no CO_2 fixation by the tissues has occurred. Thus such data should be examined with extreme caution and the assumptions involved should be recognized.

Biochemistry of Nematodes

Protein and Amino Acid Metabolism

The exact essential amino acids required by the parasitic nematodes are unknown due primarily to our inability to culture such forms until recently. It would appear that protein synthesis must be quite efficient, considering the increased reproductive capacities of adult females as well as the rapid growth phases following molting of larvae. There are indications that various transaminases are present in nematodes, thus allowing for efficient interconversions of amino acids (Rogers, 1962).

Reductive amination of pyruvate and α-ketoglutarate has been demonstrated in Ascaris tissues (Pollak and Fairbairn, 1955). The occurrence of both processes in nematodes would suggest that they are not totally dependent upon host dietary amino acids as was suggested as a possible explanation for strict host specificities of the cestodes (Read, 1965).

Haskins and Weinstein (1957, a,b,c) studied the end products of nitrogen metabolism in Trichinella spiralis larvae and reported that they excrete 2.8 mg. nitrogen per gram of wet body weight, of which 33% is ammonia, 20% is peptide nitrogen, 29% is amino acid nitrogen, and 7% is volatile amino nitrogen. Eleven amino acids were identified in the excreta and the majority of these are termed "essential" in mammalian metabolism. Most of the amines found are thought to represent decarboxylation products of corresponding amino acids and some of those identified are known to have profound pharmacological actions on tissues of higher animals (Fairbairn, 1960; Rogers, 1962). These nitrogenous substances are thought to be normally released although the in vitro amounts may be somewhat elevated (Fairbairn, 1960). The effects of these nitrogenous excrements on host physiology are rather obvious but just what they indicate in terms of parasite nutritional requirements remains to be evaluated.

Lipid Metabolism

Although lipases and esterases occur in the gut of certain intestinal nematodes, it is not known whether fatty acids can be absorbed through the intestinal wall (Cheng, 1964). Likewise, the synthesis of lipid in these forms has not been explored (Fairbairn, 1955, 1956).

Regarding lipid catabolism, there is some evidence that higher fatty acids give rise to trehalose and glycogen in embryonating Ascaris eggs (Passey and Fairbairn, 1957). Presumably this occurs by catabolism to a 3-carbon compound followed by condensation of the 3-carbon intermediates, possibly via glucose to glycogen. This conversion of fat to carbohydrate has not been conclusively demonstrated in animals but is known to occur in germinating seeds (Fairbairn, 1960).

During aerobic metabolism of larval trichinae, there is a significant decrease in tissue lipids of the parasite. Anaerobically, however, lipid appears to be metabolically inert (von Brand et al, 1951; von Brand, 1952). This decrease in lipid content during periods of aerobiosis appears to be associated with the energy requirements for motility. The assessment of this observation has been previously discussed.

Carbohydrate Metabolism in Nematodes Other than Trichinella spiralis

Glycogen is the main type of reserve nutrient found in the bodies of parasitic nematodes and it is not known to differ from mammalian glycogen except for a slightly smaller molecular weight (von Brand, 1952). When parasitic nematodes are starved, glycogen is depleted rapidly and results in the production of metabolic end-products outlined in a succeeding paragraph. Nothing is known about the synthesis of glycogen in parasitic nematodes so it is assumed to follow pathways similar to those found in mammals (Rogers, 1962). The presence of monosaccharides, especially glucose, leads to a net synthesis of glycogen in Ascaris and

Entner and Gonzalez (1958) cited isotopic evidence to indicate that approximately 50% of the glucose utilized from the medium was channeled initially to glycogen synthesis.

The mechanism for synthesis of trehalose is uncertain although the relative abundance of this disaccharide is rather high in the parasitic nematodes studied to date. Trehalose cannot be directly converted by polymerization to glycogen. If it contributes to glycogen synthesis, an initial hydrolysis would be necessary for the rearrangement of the 1——1 to a 1——4 type of carbon linkage found in glycogen.

It has been suggested that nematodes depend upon carbohydrate metabolism as their source of energy primarily because proteins and lipids do not lend themselves to the internal oxido-reduction reactions characteristic of anaerobic processes. However, even in the more aerobically dwelling parasite nematodes, carbohydrate metabolism is the main method of producing energy. In such cases, carbohydrates are not oxidized completely to carbon dioxide and water which indicates that the process is of the fermentative type. Fermentation products typically include carbon dioxide, organic acids (Bueding and Yale, 1951; Bueding, 1953; Fairbairn, 1954; Fairbairn, 1960; Rogers, 1962), and various alcohols (Bueding, 1951; Fairbairn, 1960; Rogers, 1962). Carbon dioxide arises from pyruvate or other intermediates in the formation of acid and non-acid fermentation products (Fairbairn, 1960; Rogers, 1962). The organic acids typically range from C2 to C6 in length (both branched and non-branched) and, although they originate during anaerobic glycolysis, these products continue to be produced in approximately the same amounts during aerobic metabolism (Fairbairn, 1960; Rogers, 1962). Lactic acid

is a major excretion product only in a very few forms whereas fatty acid(s), especially valeric acid, is commonly produced as a result of nematode metabolism (von Brand, 1952; Rogers, 1962). Thus, it would appear that aerobic respiration is merely superimposed upon a constant basic fermentation. The identification of various alcohols during metabolism has not been extensively studied but acetylmethylcarbinol has been found as a normal excretory product in the nematodes, Ascaris and Litomosoides (Fairbairn, 1960; Rogers, 1962). It is suggested that release of such non-acid substances may occur fairly generally.

Aside from the carbohydrates primarily concerned in energy metabolism, others of a more or less complex nature are also present. These include the ribose and deoxyribose of nucleic acids as well as the amino sugars and uronic acids of glycoproteins and mucoproteins. A number of reports have appeared describing the histochemical distribution of nucleic acids and glycoproteins in nematodes (Fairbairn, 1960) but these will not be discussed at this point.

In concluding this brief survey of carbohydrate metabolism, it may be legitimate to ask why nematodes indulge in such metabolic acrobatics as the formation of energy-rich fatty acids, especially of 5 to 6 carbon length, when simple reduction of pyruvate to lactate suffices in most systems as the waste product. A doubtful possibility might be that energy is obtained in the conversion. More acceptable reasoning, however, might be based upon the chemical properties of the acids themselves. Giese (1962) points out that cell membranes are considerably more permeable to undissociated than dissociated forms. Since the dissociation constant of lactic acid is about ten-fold that of valeric

acid, this might be a plausible reason for the formation of fatty acids. Another closely related point is that the fatty acids, being only slightly dissociated, are excreted mainly in acid form whereas lactate excretion would possibly be a more severe drain on tissue cation reserves.

Carbohydrate Metabolism in *Trichinella spiralis*

Currently, there is general agreement that many of the earlier attempts to reveal metabolic traits in parasitic nematodes are open to some criticism because they were not conducted under bacteria-free conditions. More recent attempts to eliminate or control bacterial contamination in such metabolic studies have employed chemotherapeutic agents, either during preparatory or incubation phases. The effects of such agents on the metabolism of parasitic forms subjected to investigation remains largely unevaluated and thus has not completely solved the problem.

The respiratory metabolism of *T. spiralis* larvae has been previously studied by several investigators. Stannard *et al.* (1938) employed mainly manometric techniques in their experiments and pointed out the need for additional confirmation of their work with chemical studies. Such studies were pursued and elaborated by von Brand *et al.* (1951). The latter group conducted studies in the absence of drugs but used a 3 hour digestion method to free the larvae from muscle tissue. Isolation and characterization of various enzymes associated with carbohydrate metabolism have been performed by other workers. The results of these studies are summarized in Figures 1 and 2. The individual(s) isolating and characterizing the specific enzymes are indicated in each

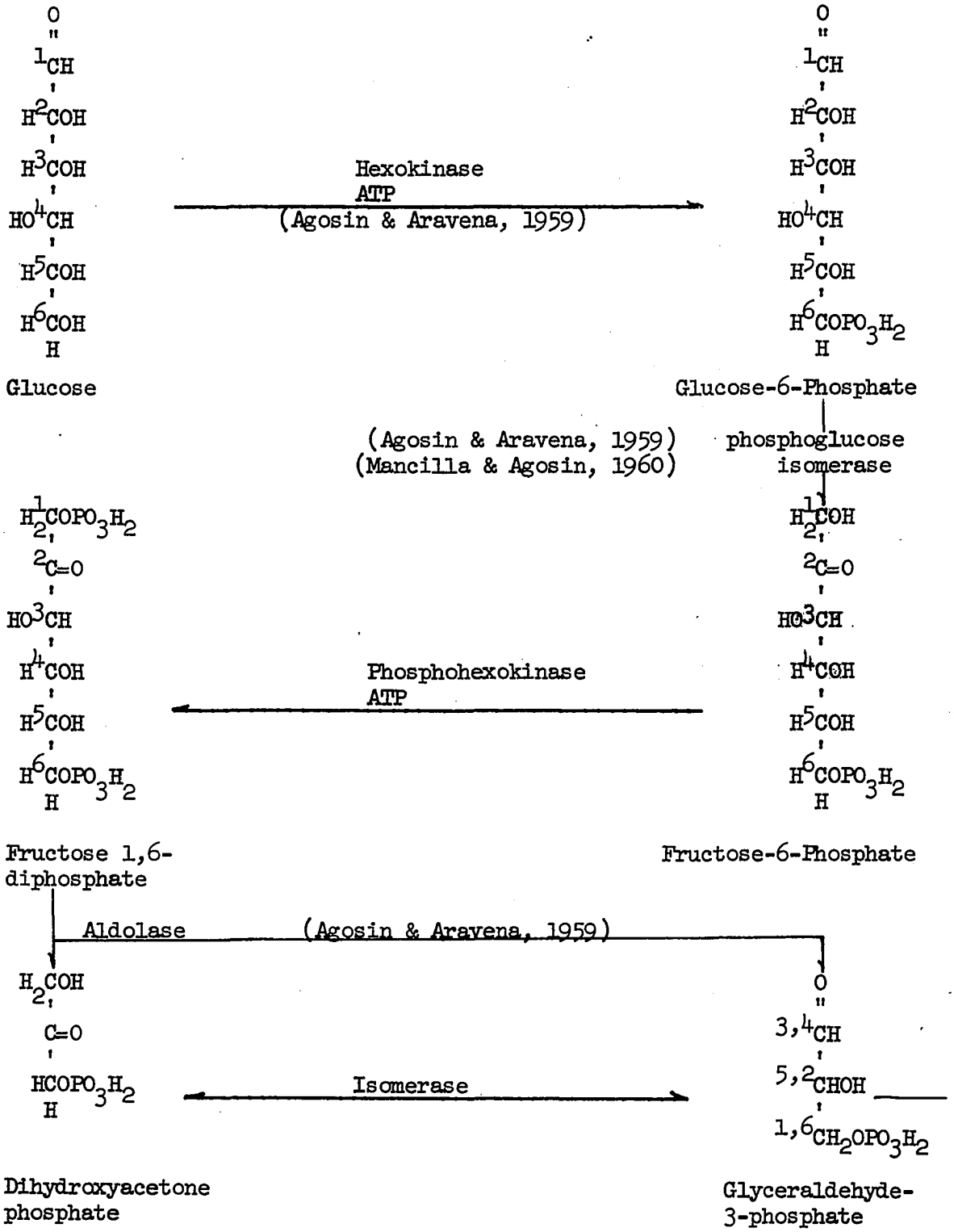


Figure 1.--Fates of C^{14} labels in anaerobic glycolysis

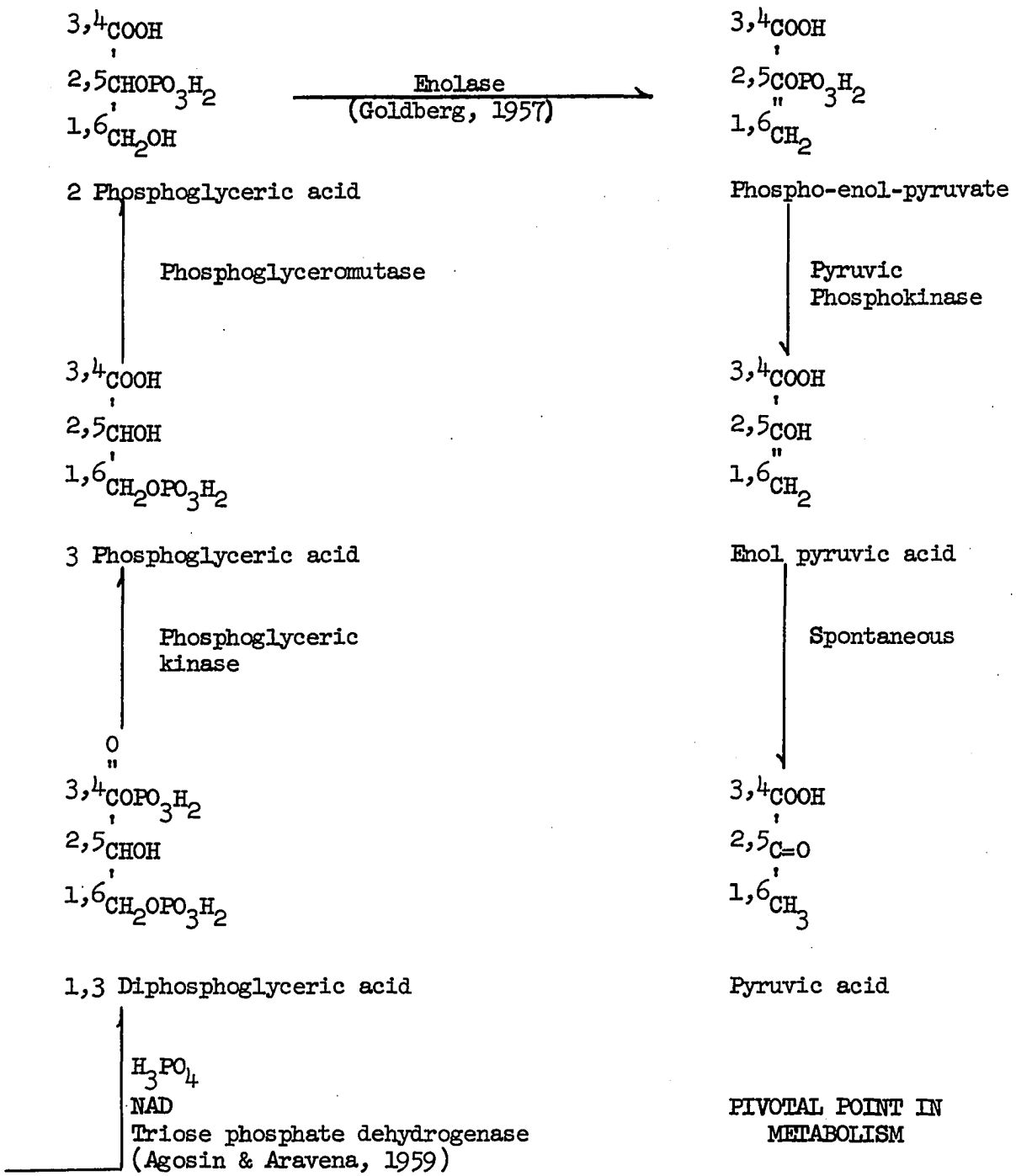


Figure 1.--Continued

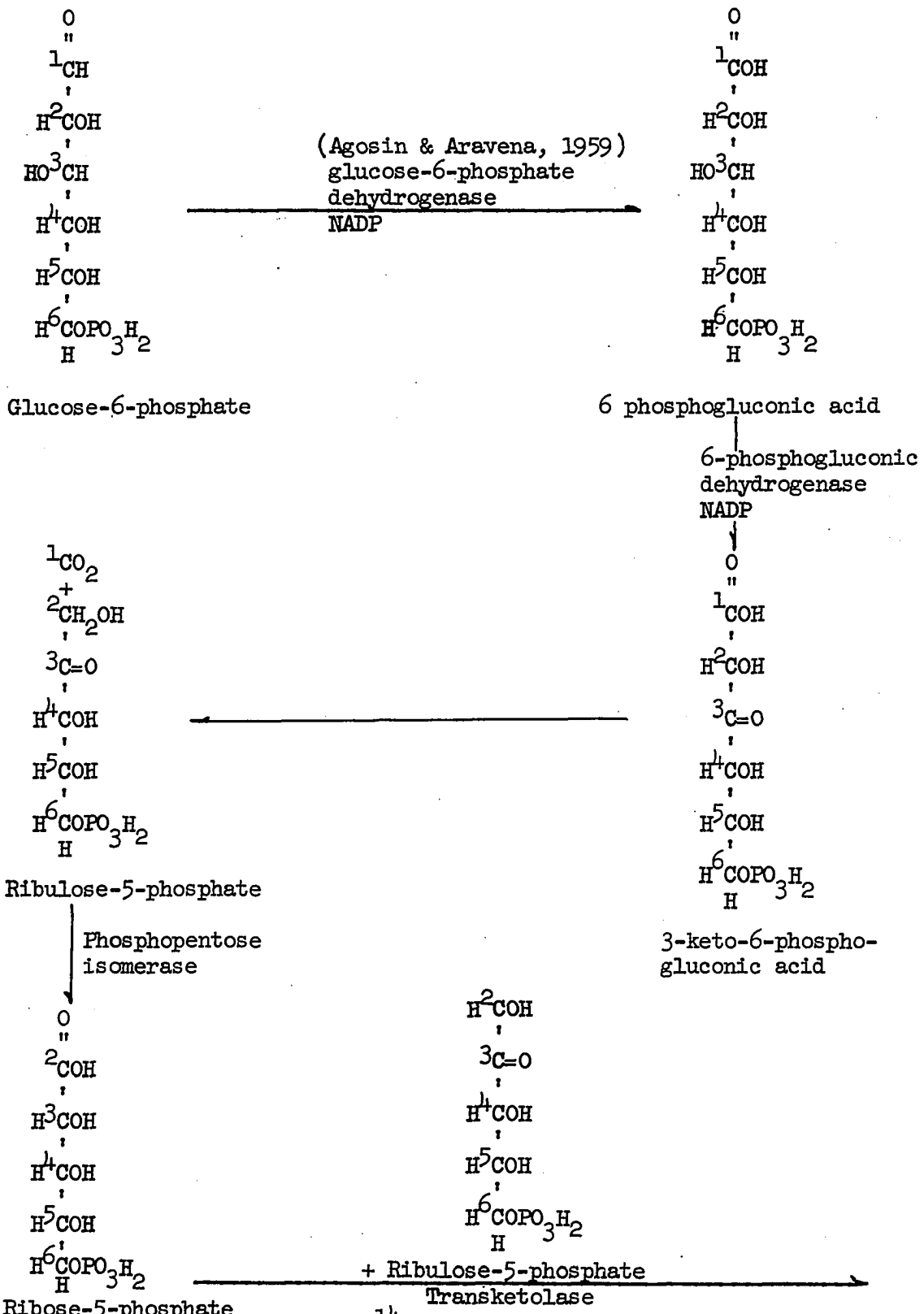


Figure 2.--Fate of C^{14} labels in hexose monophosphate shunt.

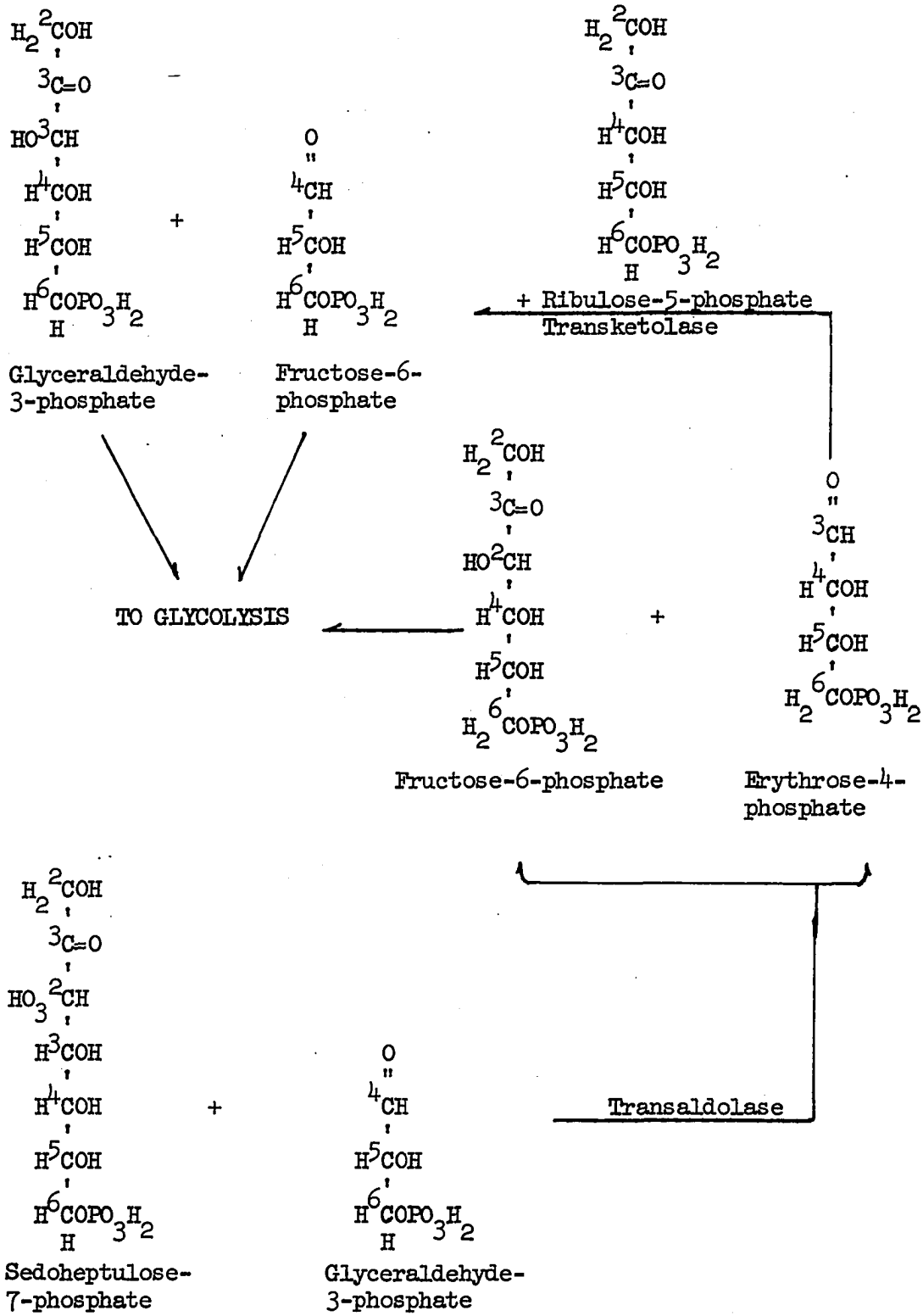


Figure 2.--Continued

figure. While these studies on worm homogenates do yield valuable information as to potential capabilities of the larvae, it is impossible to determine the value of the particular enzyme or enzymatic pathway to the intact worm in such analysis.

The hazards of carbon balances and respiratory quotients have been previously discussed. Since zooparasitic nematodes are not routinely cultured *in vitro* throughout their entire life cycle, many of the experimental procedures developed for the study of bacterial metabolism are not feasible. However, the use of radioisotopes to evaluate the preponderance of metabolic pathways should theoretically be applicable in parasite physiology studies. For example, Stoner and Hankes (1955, 1958) have employed C^{14}-amino acids to demonstrate the uptake of certain amino acids by parasites *in vivo* as well as the subsequent metabolism *in vitro*.

As yet, no attempts to study the carbohydrate metabolism of *T. spiralis* larvae with radioisotopes have been made. Consideration of the biochemical information regarding *T. spiralis* larvae suggests an ability to utilize exogenous glucose. This fact was contraindicated only by the report of Stannard *et al.* (1938). If an uptake of exogenous C^{14}-glucose did occur, it would then be possible to use variously labelled C^{14}-glucose to gain insight as to operative pathways found in the larvae. The rationale of the method is shown in Figures 1 and 2. It is apparent that if only anaerobic glycolysis occurs, the ratio of $C^{14}O_2$ released from glucose-1-C^{14} and glucose-6-C^{14} should be 1 since both labels would be found only in the methyl carbon of the glyceraldehyde-3-phosphate. On the other hand, if $C^{14}O_2$ is preferentially

released from glucose-1-C^{14}, when compared to glucose-6-C^{14}, this is presumptive evidence that the shunt mechanism outlined in Figure 2 is also operative. Such a situation would give a C1/C6 ratio greater than 1.

General Considerations

The importance of establishing a "total picture", in so far as possible, prior to initiating an experiment regarding parasite biochemistry is best described by Rogers (1962):

> "An example from outside parasitology may best explain what I mean here. The problem, let us say, is to discover the essential biochemical differences between muscle and liver. We might start, without any knowledge of the biology of these tissues, by studying their basic metabolism - aerobic and anaerobic respiration, the general processes of nitrogen catabolism and the mechanisms of synthesizing new tissues. The results would show that different parts of the basic ground plan of metabolism would have different emphasis in the different tissues as well as other minor variations. Even the synthetic mechanisms would probably be basically the same though the substances synthesized would differ. Gradually, however, information would accumulate which the inspired biochemist could interpret to show that the essential biochemical differences were those which concerned the contractile function of muscle and the storage function of the liver. Would this information have come more quickly if we had started with our biological knowledge about the functions of liver and muscle? I think so. This may seem a gross exaggeration of the situation of research on parasitism. But one must agree, for instance, that more information on the biology of infectiousness and the host as an environment for the parasite is needed as a basis for biochemical studies on parasitism."

Statement of the Problem

The present investigation was designed to obtain data to demonstrate or deny the ability of the intact organism to utilize exogenous glucose under anaerobiosis. Until this study no radioisotopic techniques

have been used to examine this facet of Trichinella spiralis larvae, although all investigators are aware of the merits of such a technique. For adequate evaluation, the initial research objectives were:

(1) to determine the necessary methods for the recovery of high numbers of viable muscle larvae in a short period of time following host death.

(2) to devise suitable techniques for the recovery of bacteria-free larvae without the use of antibiotics of chemotherapeutic agents.

(3) to establish reliable methods to quantitate larval populations.

(4) to select suitable radioisotopic methods for determination of biochemical pathways found in intact viable larvae.

Thus, after suitable preparatory techniques had been devised, labelled glucose-C^{14} would be used as a substrate in studies of larval carbohydrate metabolism. The analysis of the amount of labelling occurring in the $C^{14}O_2$ evolved from glucose-1-C^{14} as compared to glucose-6-C^{14} would give presumptive evidence as to the relative importance and pathway(s) of carbohydrate catabolism utilized by intact larvae. Further information about worm-bound C^{14}, in each instance, would add evidence to indicate the existent pathways.

It was further proposed to determine the amount of labelling which occurred in the various important biological compounds following nearly anaerobic incubation of the larvae in labelled glucose. The possible fates of such carbon atoms derived from glucose are outlined in Figure 3. From this figure it is seen that nucleic acid, glycogen and

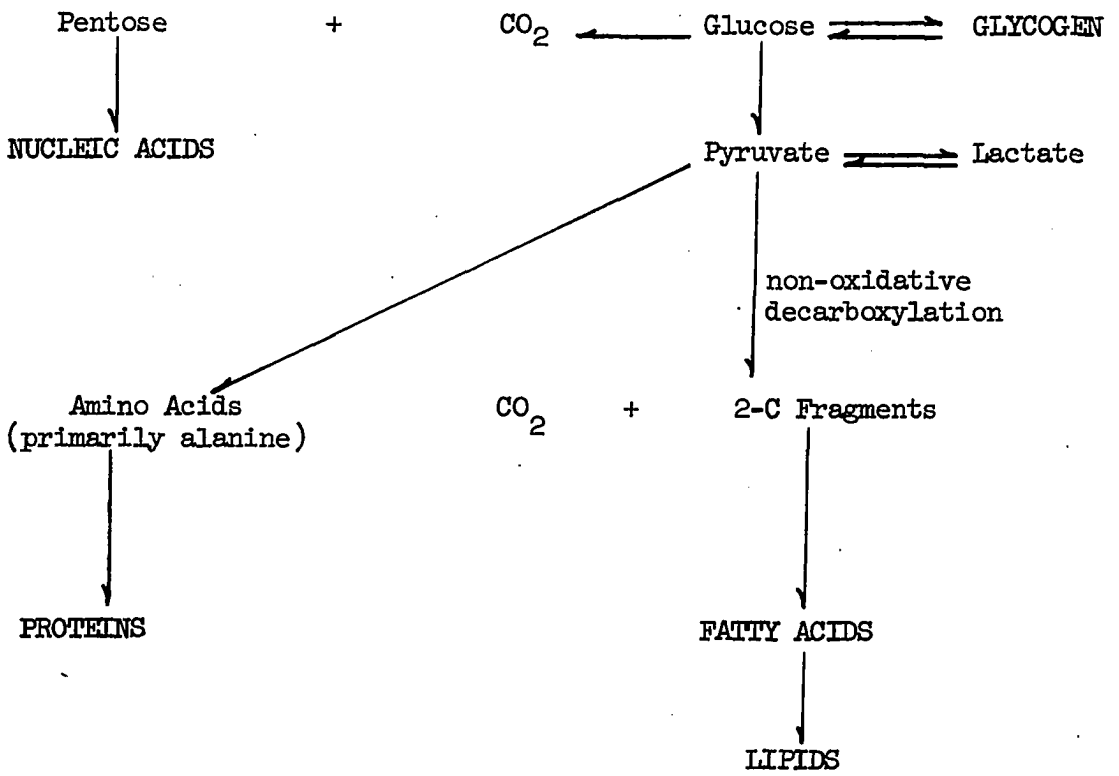


Figure 3.--Some possible fates of C^{14}-glucose

various carbohydrate fractions would probably be labelled if both the classical glycolytic pathway and pentose monophosphate pathway are present. Labelling of fatty acid and protein would not be expected to any major extent. Presumably, some fatty acid labelling could occur as a result of pyruvate degradation followed by conversion to acetyl Co A and its subsequent reactions. Protein could be labelled only if there were transamination of pyruvate to alanine. However, if anaerobic shunts (portions of the Kreb's cycle) were present to provide a suitable substrate for a given transaminase, other amino acids could also become labelled.

CHAPTER II

EXPERIMENTAL METHODS

Parasite Preparatory Techniques

The techniques utilized in cleaning and preparing the infected mice before the digestion procedure described later, were not evaluated. Since few bacteria survive passage through gastric juice, the outlined procedure may be of little importance or perhaps not even necessary in preventing such contaminations. However, these techniques are relatively rapid, easily performed, and were therefore incorporated into the experimental procedure without further evaluation.

The method outlined for the recovery of larvae from the infected animals described below was found to be quite satisfactory in most aspects. The use of blenderized tissue instead of larger fragments seemed to be indicated by the work of Kozar and Kozar, 1963. Obviously, the larger the fragments, the longer the period required for complete digestion of the tissue. Kozar and Kozar found a definite loss of larval infectivity to be associated with the lengthy digestions perscribed by other investigators and therefore, in this study, it was decided rather arbitrarily to minimize the digestion period. Unfortunately, blenderizing does increase the number of damaged larvae and thereby reduce the total larval yield per animal. To remove these inactive larvae, the added step of requiring the larvae to crawl through the four layers of bandage gauze

into a Petri dish lid was included. This did increase the time required for preparations but had the added benefit that only active larvae, virtually free of debris, were available in subsequent manipulations.

The subsequent passage of larvae through a relatively large volume of sterile saline successfully removed most, if not all, contaminating bacteria. While the final result in this case cannot be taken as evidence of any single procedure, the final preparations revealed only 9 contaminated tubes out of a total of 72 prepared in this manner. It is possible that the 9 tubes were contaminated in the later manipulations as surface growth, most likely as an aerobic contaminant, was evidenced in the thioglycollate medium.

Recovery of Viable Larvae

Trichinella spiralis larvae used in all experiments were obtained from female mice infected with 200 larvae approximately 3 to 4 months prior to necropsy. The strain of T. spiralis used in this study has been maintained in rats and mice in this laboratory for several years. The host animals were initially from the Jax strain and have been inbred in our animal house for many years. The female mice were infected at one month of age.

Three to four months post-infection, mice were sacrificed and the larvae freed from host tissue using artificial gastric juice. The procedure was as follows: the mouse was killed by a single blow to the head, the feet were amputated just above the ankle, the tail was clipped off at the base and a small cut was made through the hide just behind the ears. It was then possible to skin the mouse by pulling the pelt

posteriorly while holding on to the head with the other hand. The head was then removed and the animal eviscerated. Care was taken not to spill any of the gut contents on the carcass. All excess adipose tissue was then removed, the carcass was washed in running tap water and dipped in 70% ethanol. The carcass was then placed in a Waring blender with about 200 ml. of artificial gastric juice (7 ml. HCl and 10 grams powdered pepsin per liter of distilled water), and blenderized for 30-45 seconds. The resulting puree was poured into a 4000 ml. beaker, the blender container was washed several times with artificial gastric juice and this wash material was added to the digestion beaker until the approximate concentration in the beaker was one liter of gastric juice to ten grams of blenderized tissue. Digestion of the carcass material was hastened by placing the beaker on a magnetic-stir heating plate at 37°C with constant stirring for forty-five minutes, at which time digestion was usually complete. The freed larvae were recovered by pouring the contents of the beaker thru two layers of gauze into a large funnel. After about twenty to thirty minutes, most of the larvae were concentrated in the bottom fifty milliliters of the fluid in the funnel. This approximate volume of fluid was withdrawn into a 500 ml. beaker, 400 ml. of Hank's basal salt solution was added, and four layers of bandage gauze were secured over the top of the beaker with a rubber band. A Petri dish cover was placed over the beaker and the entire apparatus quickly inverted. About 10 ml. of Hank's solution were then added to the Petri dish. Actively moving larvae were found in large numbers in the Petri dish about 15 minutes later and these were easily removed with an oral pipette while observing the manipulation under a dissecting microscope.

Larvae obtained in the manner described above were then pipetted into a vertical series of two separatory funnels which previously had been autoclaved and filled with sterile Hank's salt solution (Figure 4). The volume of this apparatus was about 2500 ml. By gravity, the washed larvae became concentrated in the 500 ml. Erlenmeyer flask and, as determined by inoculation of a bacteriological medium, were bacterially sterile. All glassware and/or materials used after this point were sterilized by autoclaving or filtration through a Seitz filter.

By increasing the total amount of artificial gastric juice as well as amount of carcass material in each digestion beaker, and by using more sedimentation funnels, it was possible to recover large numbers of actively moving larvae. The entire procedure, from sacrifice of the host until "clean" larvae were available for incubation in experimental studies, generally required about four hours.

Estimation of Larval Quantity

In order to estimate the number of larvae present, the 500 ml. Erlenmeyer flask containing the washed larvae and a magnetic stirring bar were removed from the washing appartus (Figure 4). About 200 ml. of the salt solution was carefully but quickly decanted and the flask stoppered with a cotton plug. The flask was then placed on a magnetic stirring-heating plate at 37°C. Using a pro-pipette and a sterile 1 ml. pipette, samples were removed and placed in Syracuse watch glasses. The number of larvae in each container was counted under a dissecting microscope and the concentration of larvae per ml. of salt solution was adjusted either by adding or removing some of the suspending fluid.

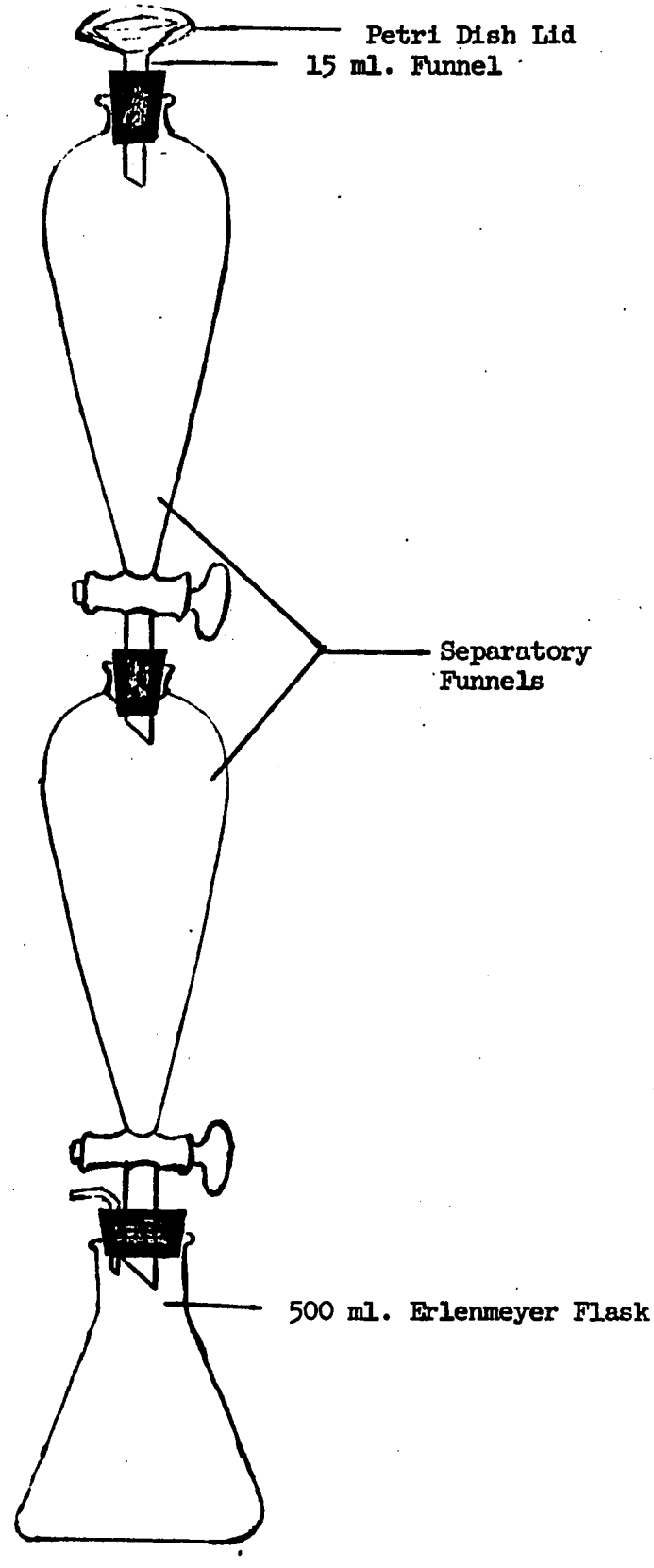


Figure 4.--Larval washing apparatus

Preparation of Radioactive Substrates

Fifty μC amounts of each glucose-U-C^{14} (CFAX 217 lot 2), glucose-1-C^{14} (CFAX 204 lot 1), glucose-2-C^{14} (CFAX 154 lot 27), and glucose-6-C^{14} (CFAX 205 Lot 1) were purchased from Nuclear-Chicago Corporation. Carrier glucose (D-glucose, Curtin Company) of 1.0 M concentration was prepared by weighing 90 grams of the sugar into a 500 ml. volumetric flask. The flask was brought to total volume and thoroughly mixed. A 25.0 ml. sample of the carrier glucose was then pipetted to each of four 50 ml. volumetric flasks. The radioactive isotope sample was opened and rinsed with distilled water into the 50 ml. flask. After six such rinsings, the flask was brought to volume with distilled water and labelled to indicate the respective position of the C^{14} label. These radioactive substrates were utilized in all the incubations performed in this study and had approximately 1 μC/ml. total activity.

Metabolism of Variously Labelled Glucose-C^{14} Substrates

Washed larvae were incubated anaerobically in physiological saline for varying time intervals (3, 6, and 18 hours) in a medium containing either glucose-U-C^{14}, glucose-1-C^{14}, glucose-2-C^{14} or glucose-6-C^{14}. The expired CO_2 was trapped in 5.0 ml. of 20% NaOH to determine the relative amount of $C^{14}O_2$ respired from the variously labelled substrates. Duplicate samples were incubated concurrently for each time interval with the specifically labelled substrate. Two loopfuls of the incubation medium were inoculated into glycollate medium at the close of the incubation period to determine if bacterial contamination had occurred. If there was contamination, the sample was discarded. If no bacterial colonies were evident after 48 hours, the sample was considered to be

free of bacterial contamination.

The incubations were performed in a specially designed apparatus (Figure 5) which could be autoclaved prior to use and then placed in a water bath maintained at 37°C during the experiment. The procedure in this study was as follows: 10 ml. of the washed larval suspension (recovered and assayed as previously stated) were pipetted into each of the 15 ml. conical base centrifuge tubes, allowed to settle, and 6 ml. of the supernate removed with a sterile pipette. One ml. of 0.5 M specifically labelled glucose (containing 1 μC/ml.) was then added to each tube, bubbling of N_2 gas into each container was begun (the flow to each container could be regulated by an individual three-way valve), and the incubation apparatus was placed in the water bath of a Warburg apparatus. Nitrogen gas was continuously bubbled through the incubation medium until the expiration of the pre-set time interval. At this time, duplicate incubation tubes were removed, the CO_2-trapping solutions were tightly stoppered and stored. The larvae were centrifuged from the incubation medium, washed three times with 1.5 ml. of sterile saline, suspended in 0.5 ml. of Hank's salt solution, frozen in an alcohol-dry ice bath and stored in the deep-freeze. The wash water was added to the incubation medium, quick frozen and stored in the deep-freeze.

C^{14} Counting Procedures

The trapped CO_2 was precipitated with an excess of saturated $BaCl_2$ solution and the precipitated $BaCO_3$ washed four times with CO_2-free distilled water. Re-suspension of the precipitated material after each centrifugation was facilitated by the use of a Lab-Line Super Mixer.

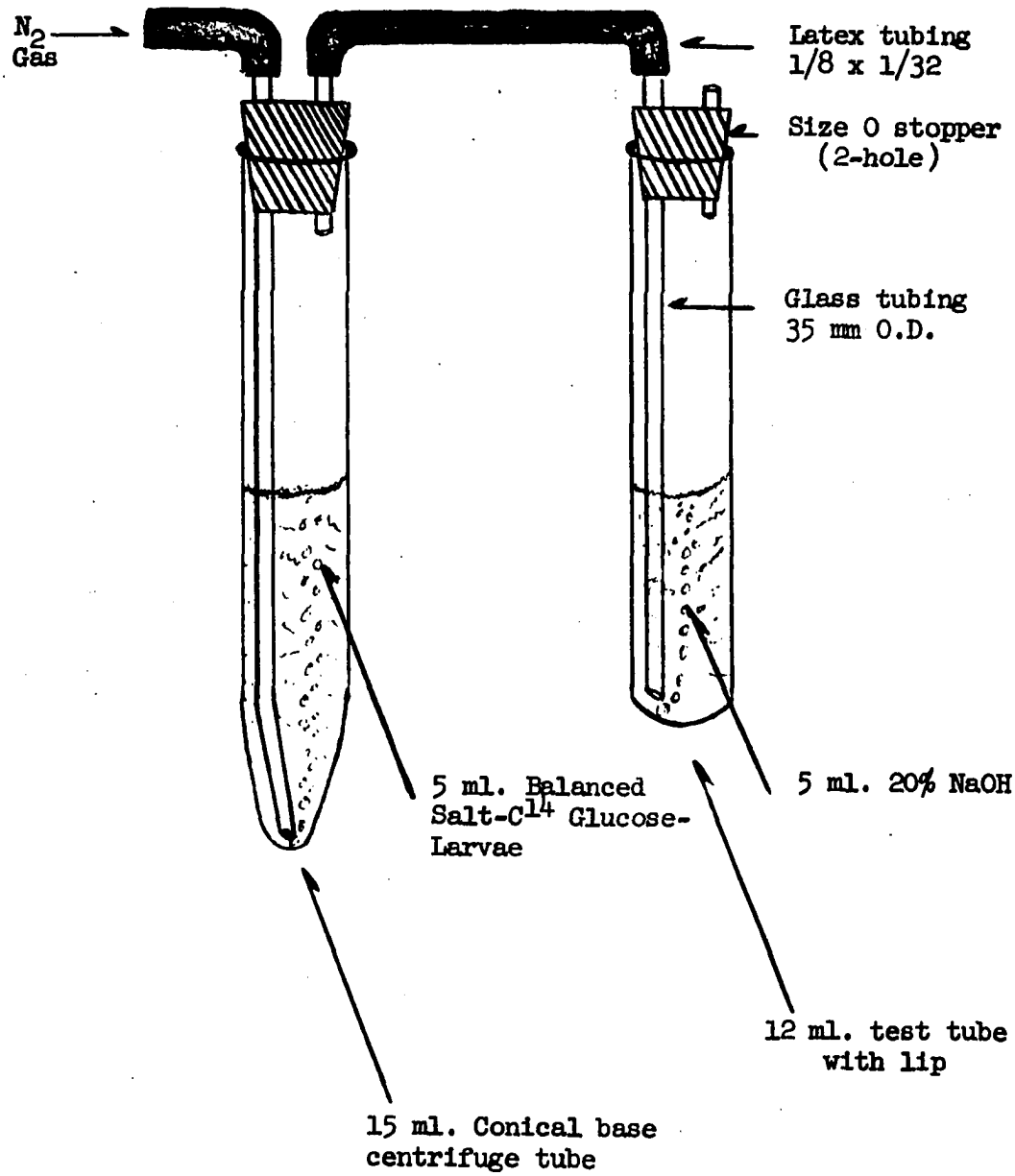


Figure 5.--Individual incubation tube with CO_2 trap

After the final wash, the precipitate was suspended in absolute ethanol and 1.0 ml. of the samples pipetted to previously weighed, concentric stainless steel planchets (Atomic Accessories, Inc.). The sample on the planchet was evaporated to dryness and weighed. The radioactivity of the expired CO_2 was determined in a Nuclear-Chicago continuous gas flow counting system. Corrections were made for self-absorption of the sample as well as for the background activity and the specific activity per 100 mg of $BaCO_3$ was calculated.

The larvae were removed from the deep-freeze and washed into standard scintillation vials (Nuclear-Chicago Corporation) of known weight. After addition of the larval material to the vials, they were placed uncapped in a large dessicator attached to a vacuum pump and dried for 48 hours. Vials were again weighed and 1.0 ml. of hydroxide of Hyamine (10X) (Packard Instrument Co., Inc.) added to each vial. The larvae were then digested by incubation in the capped vials for five hours at 45-50°C. Fifteen ml. of standard scintillation solution (4 grams 2,5 diphenyloxazole and 50 ml. 1,4-bis-2 [5-phenyloxazolyl] - benzene, Nuclear-Chicago, Inc. per 1000 ml. of spectro-quality toluene, Matheson Coleman and Bell) were added. To effect solution of the Hyamine-worm complex in the toluene, 1.0 ml. of absolute ethanol was placed in each vial before the addition of scintillation solution. The mixture was then counted in a Nuclear-Chicago room temperature liquid scintillation system (Model 723). Correction for quenching was calculated by the channels-ratio method (Nuclear-Chicago Tech. Bull. No. 13). If colored solutions resulted, 0.1 ml. of C^{14}-benzoic acid in toluene (activity 22,581 dpm/ml.) was added and the correction obtained by plotting a

separate color-quench curve. After correction of the count for quenching and background activity, the total activity per 10 mg of larvae was recorded.

Fate of Glucose in Intact Larvae

Determination of the fate of exogenous glucose metabolized by the intact larvae was attempted in the following manner: A 200 ml. suspension of washed larvae (containing an average of 790 larvae per ml.) in Hank's basal salt solution was pipetted into a sterile 250 ml. flask. The larvae were allowed to settle for 10 minutes, and 155 ml. of supernate was removed with a pipette. The supernatant fluid was examined to verify that no larvae were removed from the suspension. Oil pumped nitrogen gas was then bubbled through the larval suspension for 5 minutes, and then 5 ml. of 0.5 M glucose-U-C^{14} (Nuclear-Chicago CFAX 206 lot 1) containing 4.25 μC/ml. was added to the incubation flask. Nitrogen was bubbled continuously through the mixture during incubation at 37° for 24 hours with continuous shaking. The expelled gaseous mixture was bubbled through two carbon dioxide traps, each consisting of a fritted glass filter in 75 ml. of 10% NaOH solution. The NaOH used for the trapping solution was initially prepared as a 50% solution, centrifuged at a high speed to sediment any bicarbonate present, and diluted to 10% with CO_2-free water. At expiration of the pre-determined incubation phase, the trapped-CO_2 was stored in tightly stoppered flasks and worms were centrifuged from the suspending supernate. Prior to centrifugation of the incubation mixture, two loops of the mixture were inoculated into tubes of thioglycollate medium and incubated for 48 hours at 37°C to

check for bacterial contamination. The worms were then washed four times with physiological saline. Both worms and supernatation, in separate containers, were quick-frozen in an ethanol-dry ice bath and stored in the deep-freeze.

After determining that no bacterial contamination had occurred, the CO_2-trapping solution was treated as previously described. In the radioactive determinations, samples of known activity were included so that corrections could be made for self-absorption of the sample, background activity and machine efficiency. The determined counts per minute in the expired CO_2 could then be adjusted to absolute counts. This data could then be compared directly with that obtained with the liquid scintillation system used in the balance of the radioactive determinations.

The worms were suspended in 0.05 M phosphate buffer (pH 7.2) and homogenized in a Virtis '45' microhomogenizer. The homogenate was centrifuged at 300X g in an International Model V centrifuge to remove large fragments. The supernatant fluid was treated with three volumes of cold 10% trichloroacetic acid (TCA). The precipitate that resulted contained protein, lipid, nucleic acid, and some glycogen. The supernatant fluid contained glycogen and "acid soluble" substances. Both the supernatant fluid and the precipitate were fractionated further as shown in Figure 6. Precipitate II was washed a total of five times with cold 10% TCA (3X) and 5% TCA (2X) to remove the glycogen. The combined washes were pooled to form supernatation II, from which glycogen was precipitated by the addition of two volumes of 95% ethanol in an ice bath. Lipid materials were then removed from precipitate II by first

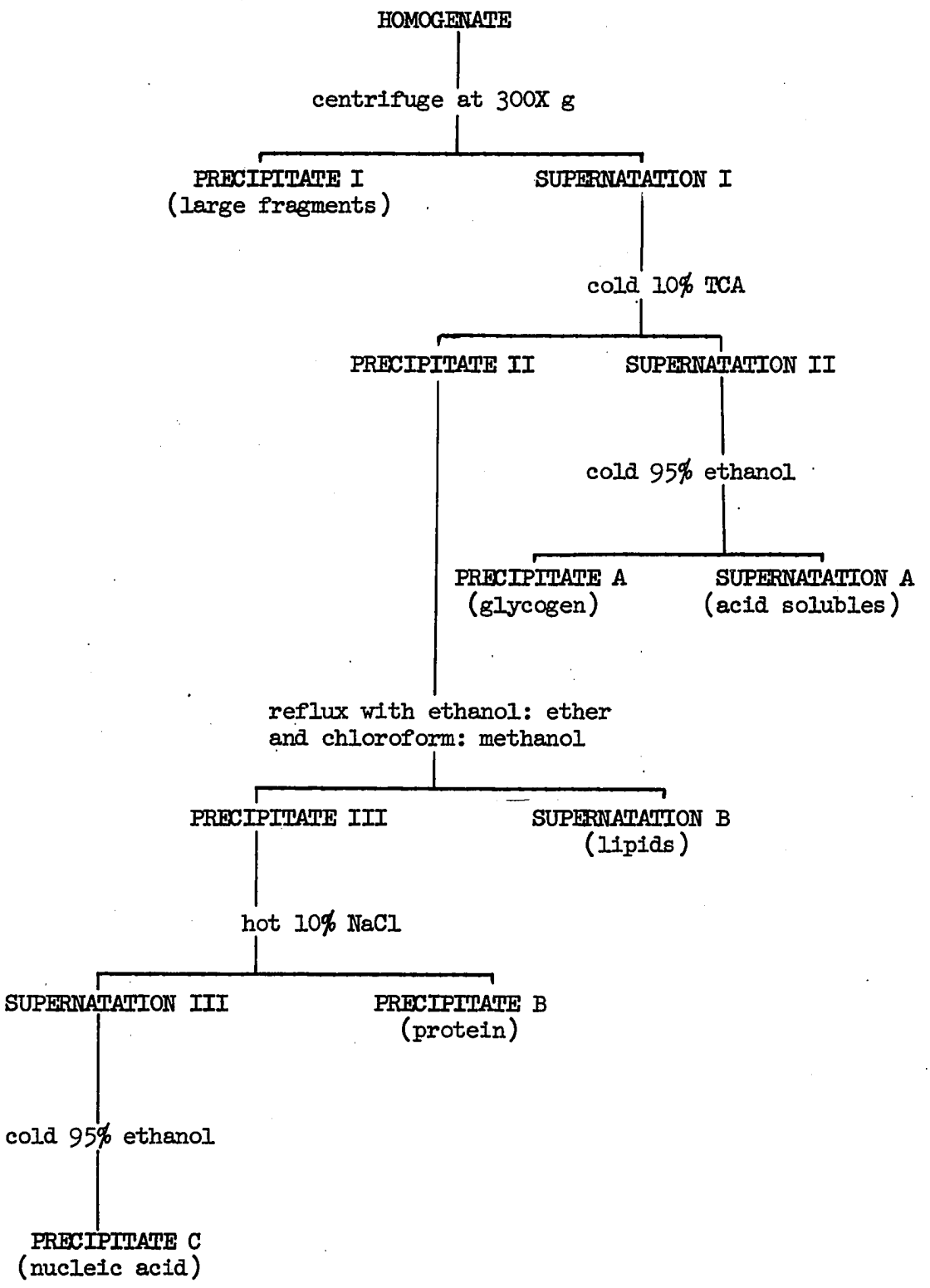


Figure 6.--Procedure flow sheet for larval fractionation

refluxing with 50 ml. of ethanol: ether (3:1) for two hours and then with 50 ml. of chloroform:methanol (1:1) for one hour. The supernatant fluids were combined and concentrated. Thus supernatation B represented the extractable lipid fraction of the homogenate. The remaining precipitate (precipitate III) was next extracted with hot 10% NaCl, two extractions each of one hour duration were performed in a boiling water bath. The remaining precipitate (precipitate III) was taken to represent protein while the nucleic acid in supernatation III was precipitated with two volumes of cold 95% ethanol, washed and redissolved.

Aliquots of precipitates I, A, B and C as well as supernatations A and B were placed in standard scintillation vials. Fifteen mls. of standard scintillation solution were added to each vial and the samples were then counted in a Nuclear-Chicago Corporation 723 room temperature liquid scintillation system.

CHAPTER III

RESULTS

Estimating Parasite Populations

The data presented in Table 1 indicate the number of larvae per 1 ml. of Hank's basal salt solution obtained by the larval estimation method set forth in Chapter II. Twenty samples of 1 ml. volume were removed from each of three flasks (A, B, and C) and placed in Syracuse watch glasses. Each flask contained a different number of larvae. The samples in the watch glasses were placed in the refrigerator overnight to immobilize the larvae and thereby expedite counting. The larvae were counted under a B & L Zoomoscope at maximum magnification.

As this procedure appeared to be reasonably reproducible, it was selected as the method of choice for the subsequent manipulations requiring standard numbers of larvae for comparisons. In order to adjust the total number of larvae per tube prior to incubation, a single 1.0 ml. sample was withdrawn and counted. Then, during the dispensing procedures after the initial adjustment, a total of twenty 1.0 ml. samples were withdrawn at random. These samples were refrigerated overnight and counted the succeeding day in order to obtain the average number of larvae in each incubation tube.

TABLE 1

ESTIMATION OF LARVAL POPULATIONS

Sample No.	Flask A	Flask B	Flask C
1.	309	496	607
2.	291	453	586
3.	273	450	633
4.	263	519	576
5.	282	504	592
6.	301	449	597
7.	255	502	575
8.	289	489	613
9.	282	474	550
10.	276	493	626
11.	301	467	605
12.	316	500	579
13.	281	487	574
14.	254	469	612
15.	286	503	690
16.	292	478	606
17.	267	467	616
18.	308	495	639
19.	287	497	574
20.	311	483	622
Total/20 ml.	5724	9675	12072
Average Number/ml.	286.2	483.75	603.6

Effects of the Experimental Environment on Larval Weights

An experiment was conducted to ascertain the effect of the nitrogen atmosphere on the weight of the larvae when incubated in 0.1 M glucose and Hank's basal salt solution (5 ml. total volume). Accordingly, duplicate samples from the same larval batch were placed in the incubation tubes for 3, 6, 12, 24 and 48 hour periods. At the expiration of the predetermined interval, the duplicate samples were removed from the bubbler and washed three times with 2.0 ml. of Hank's salt solution. The excess saline was removed from the sample with a pipette after the final wash. The samples were quick frozen in an alcohol-dry ice bath and then washed into weighing vials with distilled water. The vials containing samples were dried *in vacuo* and then weighed on a Mettler Model HV-6 balance to determine the dry weight of the sample. The results of this study are shown in Table 2 and suggest that a slight but measurable decrease in the weight of the larvae occurs during the 48 hour incubation period. It should be pointed out that not all of the salt solution can be removed by pipetting after centrifugation so the dissolved salts in this solution do contribute to the determined dry weights. The average number of larvae per sample was estimated to be 4838.

Specific Activity of $C^{14}O_2$ Released During Incubation in Variously Labelled Glucose Substrates

Duplicate samples of larvae were prepared and incubated in glucose-U-C^{14}, glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14}. The total volume of the incubation mixture was 5 ml. Glucose concentration in

TABLE 2

DRY WEIGHT OF *TRICHINELLA* LARVAE AFTER INCUBATION IN 0.1 M GLUCOSE-HANK'S SALT SOLUTION FOR PERIODS UP TO 48 HOURS

Length of Incubation	Sample Number	Net Weight (mg.)	Average Dry Weight (mg.)*
3 hours	1	5.0	5.25
	2	5.5	
6 hours	1	5.0	5.1
	2	5.2	
12 hours	1	5.0	5.1
	2	5.2	
24 hours	1	4.8	5.0
	2	5.2	
48 hours	1	4.5	4.65
	2	4.8	

*Estimated 4800 larvae.

all flasks was 0.1 M. The saline solution employed was Hank's basal salt solution. The incubation mixture was prepared by adding 1.0 ml. of labelled glucose to 4.0 ml. of salt solution containing the freed larvae. Prior to the addition of substrate, the larval suspension was exposed to bubbling N_2 gas for 5 minutes. Glucose was then added and bubbling of N_2 gas continued for 3, 6, and 18 hours. The CO_2 was trapped in individual tubes containing 5 ml. of 20% NaOH. The NaOH trapping solution was initially prepared in a 50% concentration and centrifuged to remove any dissolved bicarbonate. The solution was then diluted to 20% concentration with CO_2-free distilled water. The average number of larvae per tube was estimated to be 2862.

At the close of each respective incubation period, the CO_2-trapping tube was tightly stoppered and stored in the refrigerator. Two loops of the supernate were withdrawn and inoculated on thioglycollate medium. All cultures were still negative after 48 hours.

The trapped CO_2 was then precipitated with a standard solution of $BaCl_2$, washed five times with CO_2-free water and suspended in absolute ethanol. Aliquots were subsequently plated on pre-weighed planchets and evaporated to dryness. The samples were again weighed and placed in a Nuclear-Chicago open-window continuous gas-flow counter. The determined counts were corrected for self absorption and background activity. Tables 3, 4, and 5 present the results of these studies, and Figure 7 shows these results plotted as curves. No attempt was made to correct the $BaCO_3$ activity to indicate the absolute activity. However, the counting device employed has a usual efficiency of about 30%. The counts as presented are comparable because of the standard conditions

TABLE 3

SPECIFIC ACTIVITY OF EXPIRED CO_2 AFTER THREE HOURS INCUBATION IN 0.1 M GLUCOSE- HANK'S BASAL SALT SOLUTION

Incubation Time	Substrate	Cpm/100 mg. $BaCO_3$	Average
3 hours	G-U-C^{14}	143	148
	G-U-C^{14}	153	
	G-1-C^{14}	142	146
	G-1-C^{14}	150	
	G-2-C^{14}	65	63.5
	G-2-C^{14}	62	
	G-6-C^{14}	65	77
	G-6-C^{14}	87	

TABLE 4

SPECIFIC ACTIVITY OF EXPIRED CO_2 AFTER SIX HOURS INCUBATION IN 0.1 M GLUCOSE-HANK'S BASAL SALT SOLUTION

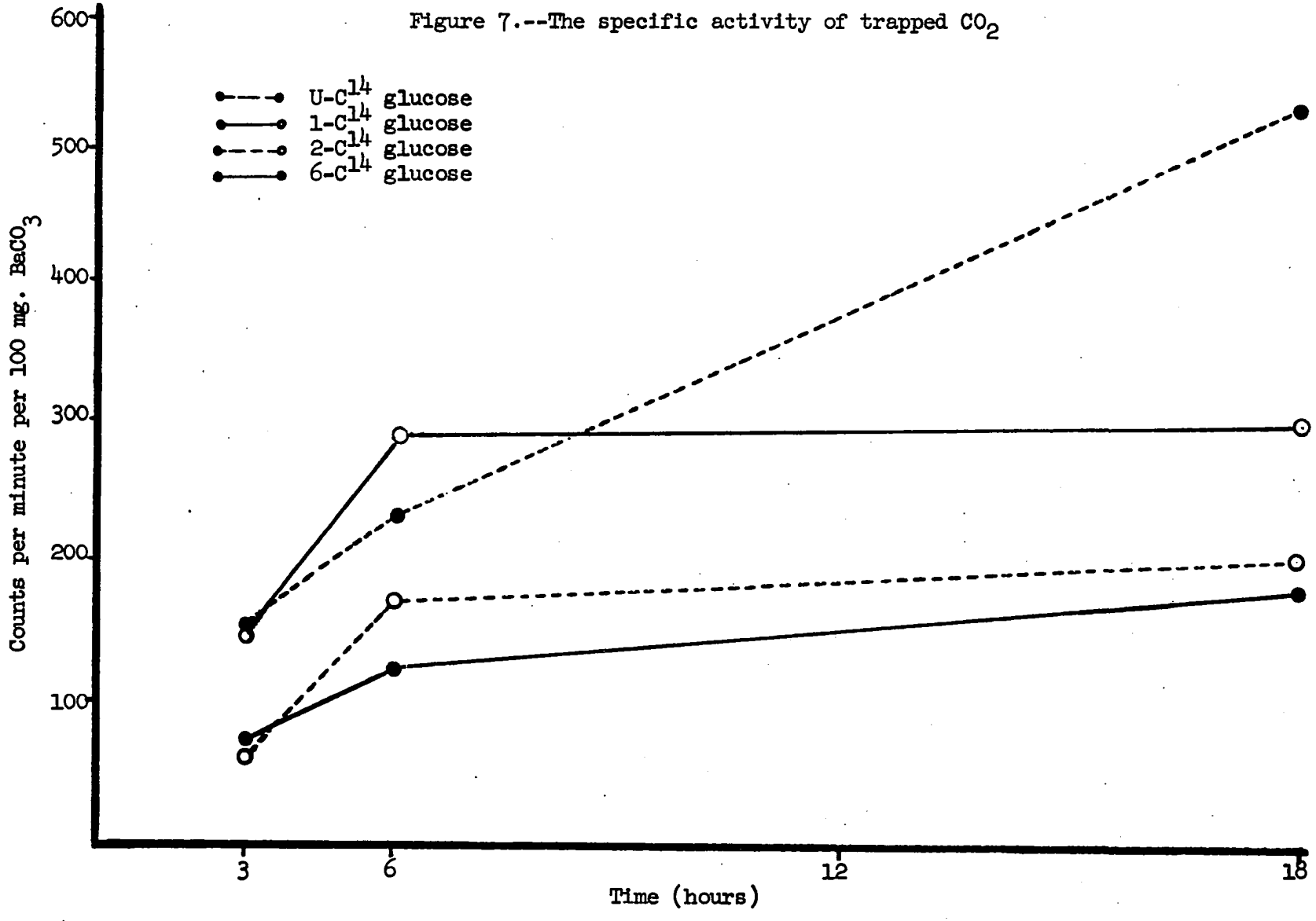
Incubation Time	Substrate	Cpm/100 mg. $BaCO_3$	Average
6 hours	G-U-C^{14}	226	236
	G-U-C^{14}	245	
	G-1-C^{14}	279	289
	G-1-C^{14}	298	
	G-2-C^{14}	157	163
	G-2-C^{14}	170	
	G-6-C^{14}	125	130
	G-6-C^{14}	136	

TABLE 5

SPECIFIC ACTIVITY OF EXPIRED CO_2 AFTER EIGHTEEN HOURS INCUBATION IN 0.1 M GLUCOSE-HANK'S BASAL SALT SOLUTION

Incubation Time	Substrate	Cpm/100 mg. $BaCO_3$	Average
18 hours	G-U-C^{14}	485	535
	G-U-C^{14}	571	
	G-1-C^{14}	286	296
	G-1-C^{14}	305	
	G-2-C^{14}	195	197
	G-2-C^{14}	200	
	G-6-C^{14}	185	177
	G-6-C^{14}	170	

Figure 7.--The specific activity of trapped CO_2



utilized in the incubation procedure. Further comment regarding the different specific activities of the trapped CO_2 will be deferred to the chapter entitled DISCUSSION.

C^{14}-Bound in Larval Tissue

Larvae were incubated in the presence 0.1 M glucose which was labelled either uniformly or in the 1, 2 and 6 positions. The incubations were conducted under commercially available nitrogen gas which was continuously bubbled through the liquid phase. After exposure to the substrate for either 3, 6 or 18 hours, duplicate tubes were removed, and two loopfuls of supernate inoculated into thioglycollate broth. The larvae were then centrifuged from the supernatation and washed five times with 1.5 ml. of saline solution. Excess saline was removed with a pipette. The larvae were suspended in 0.5 ml. of Hank's salt solution, quick frozen in an alcohol-dry ice bath, and stored in the deep-freeze.

After determining that bacterial contamination had not occurred, the larvae were thawed and transferred into previously weighed standard scintillation vials. The vials were then placed in a dessicator, a vacuum pump attached and vial contents dried in vacuo for 48 hours. The scintillation vials were again weighed on a Mettler HV-5 balance and 1.0 ml. of Hyamine Hydroxide (10X) added to each container. The capped containers were next placed in an oven heated to 50-55^{o}C for five hours to digest the larvae. One ml. of absolute ethanol was added to each vial to effect the solution of the Hyamine-worm complex. Fifteen ml. of standard scintillation fluid was added to each vial and the samples were counted in the Nuclear-Chicago Model 723 room temperature liquid

scintillation system. The corrected total counts per 10 mg of vial contents are listed in Tables 6, 7 and 8.

In order to correct for the quenching effect of the Hyamine and ethanol in the vials, a standard quench curve was established by the channels-ratio method and all counts were corrected to indicate total disintegrations occurring in the dissolved materials. The background correction was that obtained from a blank containing appropriate amounts of Hyamine, ethanol and scintillation solution. On occasion, some colored solutions were observed, in which case a separate quench curve was calculated by the addition of C^{14}-benzoic acid of known radioactivity to these samples. Such samples were again counted in the scintillator in order to obtain the corrected total activity of the container.

It was apparent that the larval bound C^{14} activity varied with the position of the label in the glucose substrate as seen in Figure 8. Plausible explanations of this data will be covered in DISCUSSION.

Larval Fractionation

In order to further pinpoint the reasons for the observed differences in labelling patterns, attempts have been made to fractionate the larvae into protein, lipid, glycogen, nucleic acid and acid soluble fractions according to the scheme shown in Figure 6 (Page 43). Due to the very small quantity of material presented by several thousand larvae as well as to their low specific activity, no success was realized in these attempts. The last attempt to fractionate the larvae involved the use of a Virtis '45' microhomogenizer which appears suitable for fractionation of the larvae. However, higher total numbers of larvae

TABLE 6

C^{14} BOUND IN TRICHINELLA LARVAE AFTER THREE HOURS INCUBATION IN 0.1 M GLUCOSE-HANK'S BASAL SALT SOLUTION

Incubation Time	Substrate	Cpm/10 mg. Worm	Average cpm/10 mg.
3 hours	G-U-C^{14}	224	244
	G-U-C^{14}	264	
	G-1-C^{14}	93	103
	G-1-C^{14}	113	
	G-2-C^{14}	184	190
	G-2-C^{14}	196	
	G-6-C^{14}	85	120
	G-6-C^{14}	160	

TABLE 7

C^{14} BOUND IN TRICHINELLA LARVAE AFTER SIX HOURS INCUBATION IN 0.1 M GLUCOSE-HANK'S BASAL SALT SOLUTION

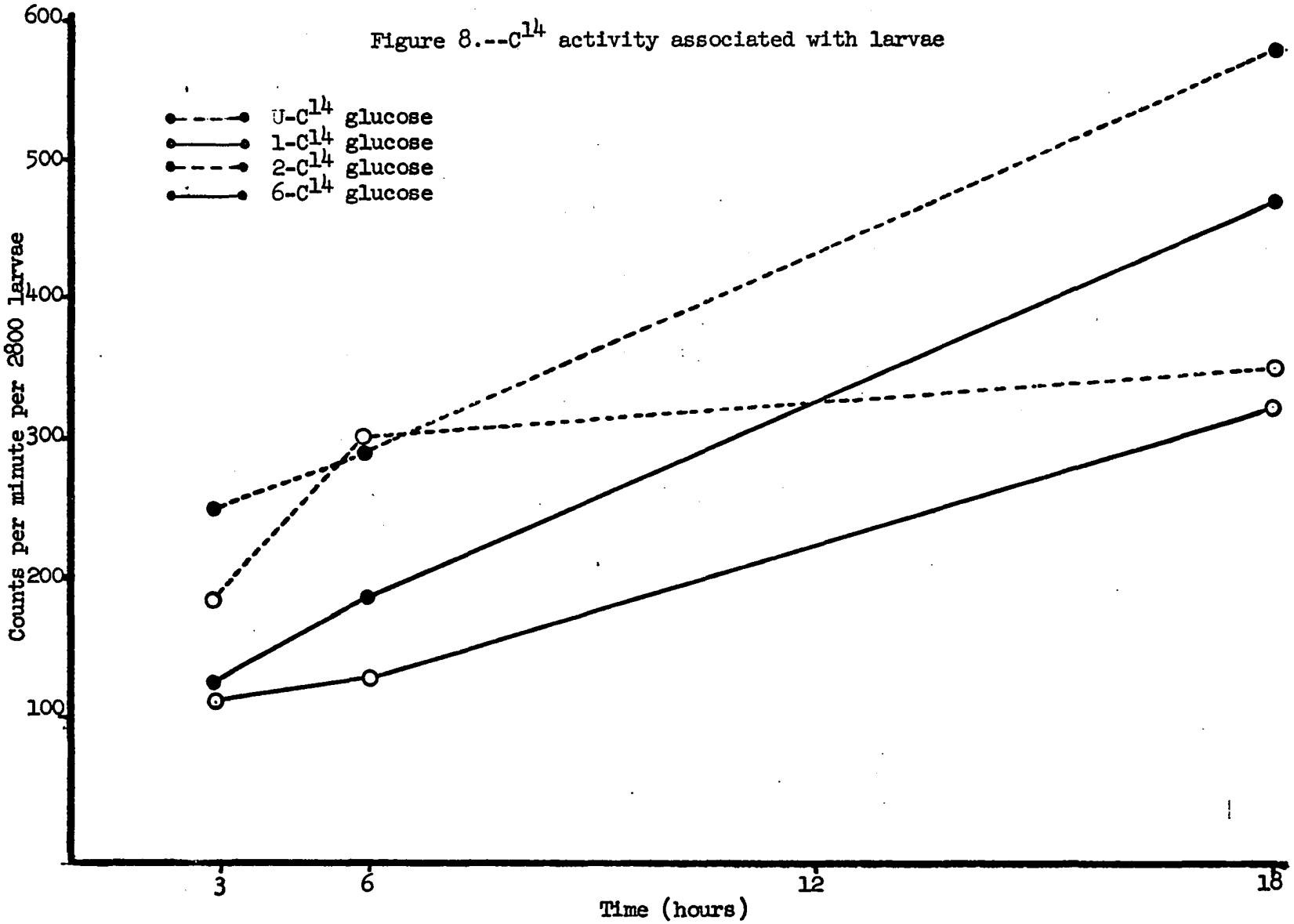
Incubation Time	Substrate	Cpm/10 mg. Worm	Average cpm/10 mg.
6 hours	G-U-C^{14}	275	292
	G-U-C^{14}	308	
	G-1-C^{14}	114	133
	G-1-C^{14}	151	
	G-2-C^{14}	250	300
	G-2-C^{14}	350	
	G-6-C^{14}	278	291
	G-6-C^{14}	304	

TABLE 8

C^{14} BOUND IN TRICHINELLA LARVAE AFTER EIGHTEEN HOURS INCUBATION IN 0.1 M GLUCOSE-HANK'S BASAL SALT SOLUTION

Incubation Time	Substrate	Cpm/10 mg. Worm	Average cpm/10 mg.
18 hours	G-U-C^{14}	616	579
	G-U-C^{14}	541	
	G-1-C^{14}	300	329
	G-1-C^{14}	357	
	G-2-C^{14}	360	354
	G-2-C^{14}	348	
	G-6-C^{14}	467	473
	G-6-C^{14}	485	

Figure 8.---C^{14} activity associated with larvae



and higher specific activity substrates will be required before radioactivity determinations are sufficiently above background levels.

CHAPTER IV

DISCUSSION

This study indicated that glucose was not actively transported into the cells of intact larvae. As determined by the count rate of both $C^{14}O_2$ and C^{14} associated with the larvae, less than 1% of the exogenous glucose was utilized. This resulted in low specific activity of the fractions counted. However, some glucose catabolism was evident from the activity of the trapped CO_2. As the larval tissues contain only minor amounts of glucose, the entry of glucose into the cells of the larvae was probably in response to a concentration gradient. However, once this substrate entered the cells, it was then metabolized via the functional pathway(s). The differences in specific activities of the $C^{14}O_2$ and bound-C^{14}, after incubation in variously labelled C^{14} glucose, were therefore evidence of the pathways of carbohydrate metabolism present in the intact larvae.

Theoretically, if a glucose molecule is absorbed and catabolized directly, and the data plotted on a curve, the specific activity of $C^{14}O_2$ should remain parallel to the abscissa after reaching maximal enzyme velocities. A graphic analysis of the $C^{14}O_2$ trapped during incubation of larvae in C^{14} glucose substrates was presented in Figure 7. Noting initially the plot obtained with U-C^{14} glucose, it is of interest to point out that one would not expect the observed increase in specific

activity of the expired CO_2 (with time) under near anaerobic conditions. This was interpreted to indicate the probable incorporation of the labelled compound into glycogen as well as the direct catabolism of glucose. In such a situation, it is obvious that glycogen would become more highly labelled with the isotope as the time intervals are lengthened. If the glycogen is also being mobilized to supply energy, and certainly a decrease in glycogen content of T. spiralis larvae under anaerobiosis seems well documented (von Brand, et al., 1951), then the increase in specific activity of expired $C^{14}O_2$ would be in order. A similar occurrence was reported for Ascaris (Entner and Gonzalez, 1958) where it appeared that the exogenous glucose, upon entry, was channeled approximately 50% to glycogen and 50% to catabolic processes. In the catabolism of U-C^{14} glucose, the major portion of the $C^{14}O_2$ evolved apparently originated from carbon positions three and four, especially after six hours incubation. As the radioactivity per specific carbon atom in this uniformly labelled substrate is only one-sixth that of the other isotopic substrates, it was apparent that the glycolytic pathway was the primary scheme for degradation of glucose.

Another interesting observation was that the C1/C6 ratio for 3 and 6 hour periods is approximately 2. This strongly suggests that the shunt mechanism is operative during this interval, at least to the point of pentose formation. That the balance of this enzymatic pathway is present seems to be indicated by the release of $C^{14}O_2$ from glucose-2-C^{14} which, although lower in the resulting specific activity, closely parallels the curve obtained with glucose-1-C^{14} as substrate. After 6 hours, it appears that the specific activity of $C^{14}O_2$ from both 1-C^{14} and 2-C^{14}

glucose has stabilized. This suggests that either: (1) an enzyme inhibition has occurred or (2) near anaerobic conditions have been established. It is essential to recognize that the shunt mechanism as outlined is primarily aerobic, but as long as re-oxidation of reduced nicotinamide-adenine dinucleotide phosphate (NADP) can be accomplished without linkage to molecular oxygen, the pathway would remain functional. It is possible that the generated NADP-H_2 is then coupled to the reduction of other organic compounds, most likely resulting in synthesis of fatty acid (such as n-valeric acid). Other problems do exist, such as the difficulty of completely removing oxygen from the larvae as well as their unusually high affinity for oxygen, even at very low O_2 tensions. Thus, it may be that only after about 6 hours had near anaerobic conditions been established. The very small percentages of oxygen usually found in commercially available nitrogen could also suffice to partically repay the oxygen debt. It is also possible that a "physiological shift" has occurred within the larvae. Pentose is required for the synthesis of nucleic acids. As the larvae under observation are only about 10 hours old, this is possibly a time when nucleic acid synthesis may be very high in order to prepare for rapid protein synthesis and growth. The presence of such a shunt may be significant in order to insure that these biologically important materials are available when needed. This may also help to explain why the larvae can infect such a wide range of hosts.

The graphic analysis of total amounts of C^{14}-bound in the worms was shown in Figure 8. Here, it is again noted that the increase in the worm-associated-C^{14} from incubation in glucose-U-C^{14} is linear with time.

This adds emphasis to the observation regarding incorporation into a metabolic pool, probably glycogen, which would serve to increase the specific activity of $C^{14}O_2$ expired as the time of incubation was increased. Rather interestingly, glucose-1-C^{14} incorporation is the lowest as would be expected from $C^{14}O_2$ data. However, the graph does begin to show an increase in total activity that appears to possibly parallel the plot for glucose-6-C^{14} after the sixth hour. Rather notably, incorporation of glucose-2-C^{14} rises remarkably until the sixth hour at which time it appears to plateau. This is somewhat puzzling. From the parallel lines observed in U-C^{14} and 6-C^{14}, it would appear that mainly anaerobic glycolysis is occurring and the resulting trioses are now in equilibrium. Probably the 2-C^{14} bound material is pentose and, if the balance of larval development is inhibited at this stage (such as by inadequate conditions for promoting incorporation of pentose into nucleic acid), then another metabolic pool would exist. Assuming the balance of the pentose shunt to be present, this pentose pool could be slowly metabolized and released as CO_2 because these steps are not dependent upon O_2 presence.

In the overall pursuit of this study, every attempt was made to perform experiments as closely as possible to physiological conditions. Since this parasite was not successfully cultivated in vitro until recently and there was no indication of growth here in incubated larvae, it must be conceded that these conditions were hardly physiological. That much is true, but the use of balanced salt solutions and relatively low concentrations of glucose does represent more nearly a physiological situation than high glucose concentrations and Ringer's solution. Giese

(1960) points out the importance of the various ions to the integrity of the cell membrane and solute absorption. Thus, although more difficult to prepare and sterilize, Hank's basal salt solution was chosen as the fluid phase. Also, the digestion procedures employed are possibly not too unlike the condition which occurs in animals acquiring an infection in nature.

The period of near anaerobiosis employed may be considered to be non-physiological, as it is now generally accepted that location of the adults in the gut mucosa presents an environment with only reduced oxygen tension. It appears likely that the presence of a very active hemoglobin transport system as well as the very high surface to volume ratio in the trichinae allows them to conduct an essentially aerobic respiration. This complicated the study, for it was originally planned to incubate the larvae for varying time intervals under strict anaerobic conditions in order to effectively block the aerobic pathways. However, the commercial nitrogen gas used during the incubation phase does contain a limited amount (probably 0.2%) of oxygen. This fact, coupled with the information that Trichinella larvae have a respiration rate independent of oxygen tensions down to 6 mm Hg (McCoy et al, 1938), indicates that some aerobic pathways were probably at least partially operative under the experimental conditions. Inadvertently, this may have produced more nearly the physiological conditions encountered by the larvae in natural infections. Another point of consideration is that the larvae do accrue an oxygen debt and as a result would show a burst of metabolic activity during the washing procedures after the close of the incubation phase. This could alter the amount of C^{14} bound

in the larvae but would be absent in the $C^{14}O_2$ data. It is felt that this effect is minimal due to the excellent correlation between bound C^{14} and expired CO_2 with respect to the position of the label in the substrate.

The linear time increase in the specific activity content of both CO_2 and bound C^{14} might be due to a concentration effect because of the evaporation of water during the incubation period. However, the decrease in the incubation volume over the 18 hour period studied is slight--only about 4%. Future studies to evaluate this effect should be made more precise by the inclusion in each flask of a labelled non-metabolite, preferably a tritiated compound. It would then be possible, by observing an increase or decrease in specific activity per unit volume, to correct for the effects of concentration. With a C^{14}-utilizable substrate and an H^3-nonmetabolite, the single counting of a given sample could yield information both as to disappearance of labelled substrate and changes in total volume (by the increase in specific activity/ml. of nonmetabolites). Even if labelled by-products were released, one could obtain data as to just how much labelling is due to products by the selection of a specific assay method for the labelled substrate.

The fractionation of larvae into crude extracts of protein, lipid, glycogen, nucleic acid and acid solubles is an absolute requirement to demonstrate unequivocally the postulated pathways. Future studies must be made with high specific activity glucose substrates as the relative amount of C^{14} associated with the larvae is very low. Also very large quantities of the larvae will be required in order to make

available sufficient material in each fraction to assay and count as was initially attempted. However, the rapid developments in the field of liquid scintillation counting may greatly reduce this problem due to the high counting efficiency and the potential of recovering the sample after the radioactive determination for quantitative assay.

CHAPTER V

SUMMARY AND CONCLUSIONS

A method was devised to permit the recovery of large numbers of actively moving Trichinella spiralis larvae in a short period of time following the sacrifice of the host animal. This method did not involve the use of drugs or chemotherapeutic agents during the washing or subsequent incubation phases. Microscopically, the larvae were free of foreign material and were shown to be bacterially sterile when inoculated into thioglycollate medium. Such larvae, when transferred into Hank's basal salt solution with 0.1 M C^{14}-glucose, were shown to produce $C^{14}O_2$. The specific activity of the $C^{14}O_2$ was observed to increase with time, suggesting possibly that some glucose is channelled to the synthesis of glycogen. The increase in the specific activity of the $C^{14}O_2$ was further shown to be linear with time from 3 hours to 18 hours. Studies on the amount of C^{14}-associated with larvae also showed a linear increase with time.

Metabolism of glucose-1-C^{14} and glucose-6-C^{14} as substrates demonstrated a preferential release of $C^{14}O_2$ from glucose-1-C^{14}. This was interpreted as evidence for a functional pentose phosphate pathway in intact larvae. The amount of C^{14}-associated with the larvae was found to be inversely related to the specific activity of the $C^{14}O_2$ when glucose-1-C^{14} and glucose-6-C^{14} were used as substrates.

On the basis of the counts obtained, the larvae utilized approximately 1% of the glucose in the incubation mixture during an 18 hour period. Thus, using a modern metabolic tool, this study demonstrated that exogenous glucose was not an important substrate for Trichinella spiralis under the experimental conditions. Future studies using a higher specific activity substrate will be required to unequivocally demonstrate the postulated sequences. Additional efforts to fractionate the larvae into crude components of glycogen, protein, nucleic acid and lipid will also require high specific activity C^{14}-glucose. Therefore, this study constitutes only a survey of the general characteristics of carbohydrate catabolism by intact Trichinella larvae as shown by isotopic techniques. Details of the precise mechanisms involved await further investigations.

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