M.Sc. Parasitology

ABSTRACT

ORGANIC ACIDS IN *ASCARIS* HAEMOLYMPH: QUALITATIVE AND QUANTITATIVE CHANGES *IN VITRO*.

GILES LEIGH-BROWNE

When haemolymph is taken from *Ascaris lumbricoides* at the time the worms are collected from pigs, it contains acetic, propionic, 2-methylbutyric, *n*-valeric, 2-methylvaleric and succinic acid radicals: tiglic acid is absent.

After the worms have been kept for 3.5 hours in a saline medium, tiglate can be detected in the haemolymph, and after 7.5 hours, *n*-caproate and two other, unidentified compounds (6-C or greater) appear: meanwhile, the succinate concentration in the haemolymph decreases. Succinate added to the medium has no effect on haemolymph succinate concentrations, although the concentration of propionate and *n*-valerate may be lowered. Low oxygen tension decreases the concentration of *n*-valerate and it halves that of tiglate. It appears that oxygen is a factor in the appearance of tiglate when worms are kept *in vitro*.

Of interest is the discovery that succinate is present in the gut lumen of pigs (the host), rats and sheep.

SHORT TITLE:

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ORGANIC ACIDS IN *ASCARIS* HAEMOLYMPH: QUALITATIVE AND QUANTITATIVE CHANGES *IN VITRO*.

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

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PREFACE

The body cavity, or pseudocoelom, of nematodes contains a fluid which bathes all the internal organs. This fluid, the haemolymph (also, and perhaps more correctly, known as the perienteric or pseudocoelomic fluid), must obviously be of great physiological significance. The importance of the nematode haemolymph in functioning as a hydrostatic skeleton has been dealt with in several studies (e.g. Harpur, 1964; Harris and Crofton, 1957; Weinstein, 1960), and although the complex composition of this fluid has been studied (see Fairbairn, 1957, 1960) other functions of the haemolymph remain obscure.

As haemolymph is the only internal, extracellular fluid it must assume some, at least, of the functions of the blood, lymph and intercellular fluid of vertebrates. Thus, the haemolymph must be involved in the transport of solutes from one tissue to another, whether these be the products of digestion and absorption, mobilised storage products, metabolic wastes, or gases such as oxygen and carbon dioxide. One such group of solutes present in the haemolymph is the organic acids produced by the fermentative catabolism of glycogen in the muscle: these are equivalent to lactic acid in mammalian blood. Various types of blood analysis are often used to assess the state of health of an animal: in

the work reported here the fermentation acids of Ascaris haemolymph were investigated prior to, and at stages during in vitro maintenance.

That Ascaris under current in vitro conditions is an ailing organism is undoubtedly true, and death usually ensues within a few days, or at the most about a couple of weeks. However, studies on whole Ascaris or its tissues are usually made in the intervening period of in vitro existence and it is therefore important to know what changes are occurring in the animal during such studies.

Chapter I reviews the literature pertaining to the formation of the haemolymph organic acids and also deals with the problems of in vitro maintenance of Ascaris. Chapter II outlines the experimental procedure, Chapter III describes the experiments and Chapter IV discusses the significance of the experimental findings. A summary is given in Chapter V. Detailed statistical information is available in the appendix.

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A. CARBOHYDRATE METABOLISM OF PARASITIC HELMINTHS

1. Introduction

The object of this review is to present a resume of existing knowledge of the carbohydrate metabolism of parasites with emphasis on the intestinal helminths, particularly Ascaris lumbricoides. There is no suggestion that Ascaris is a stereotype through which the biology of other nematodes can be adequately understood, but, as an experimental animal, it has the advantages of size and availability which have made it particularly favourable in the eyes of physiologists and biochemists. It must, surely, be true that an approach to the less common or less easily obtained nematodes can better be made when there is in existence a reasonably complete picture of an organism such as Ascaris.

Because of the great variation in the physiological environments of parasitic worms, both between species and, within species, between the various stages of their life cycles, a study of their metabolism is of particular interest and, evidently, of particular complexity. It was largely through studies of Ascaris that it was first recognised that the metabolism of intestinal nematodes, and endoparasites in general, might be very different from that of the established

systems studied in vertebrates.

Recent reviews of the subject are comprehensive, covering parasites in general (von Brand, 1966, 1968), parasitic worms (Bueding, 1949b; Hobson, 1948), their carbohydrate metabolism in particular (von Brand, 1950, 1960; Bueding, 1962; Read, 1961), the physiology and biochemistry of nematodes (Fairbairn, 1960), and the biochemistry of Ascaris itself (Fairbairn, 1957). This list is only a fraction of the papers published recently on helminth metabolism: in 1952 von Brand quoted 122 relevant papers which had appeared between 1865 and 1951; eight years later he was able to add 115 more to the list. Bibliographies of the years 1951 to 1966, inclusive, have been prepared by Smith (1965, 1968).

In the following sections of this review the sequence of events leading to the production of the fermentation-acids is restated and, where possible, revised to bring the picture up to date. The involvement of the haemolymph is indicated wherever applicable.

2. Historical background

Many aspects of the metabolism of nematodes, as exemplified by Ascaris, remain a mystery. Although it is generally recognised that they differ markedly from vertebrates in respect of their metabolism, as recently as last year it was claimed (Smith, 1969) that some of the most

basic observations on Ascaris have been induced through an unnatural imposition of anaerobiasis on the organism in vitro.

A dilemma has existed for workers with Ascaris since the founder of parasite physiology, Ernst Weinland, produced his "classical series" (Weinland, 1901a, b, 1902a, b, 1903, 1904; Weinland and Ritter, 1902) and developed a picture of the overall metabolism of the worm. His conclusion was revolutionary: that Ascaris, a worm which may reach a length of 25 cm and a weight of 5 gm, was unable to utilise molecular oxygen and produced all its energy by fermentation alone, although he did concede that the eggs might use oxygen. The dilemma: whether Ascaris should be regarded as an aerobe or an anaerobe and which of these conditions should be provided for in vitro.

Although Weinland's was the first extensive study, an earlier worker, Bunge, had preceded him to the realisation that Ascaris had a fermentative type of metabolism, however, he came to this conclusion by a rather devious and roundabout route. Bunge believed that the oxidative processes in metabolism functioned primarily as a source of heat, and that fermentative, anaerobic processes were responsible for energy. His thesis followed that parasitic helminths of warm-blooded animals should have, at most, a minimal oxygen requirement as their heat would come from the host. To support his argument he was able to show experimentally that several ascarid

species could live under strict anaerobiasis, producing *in vitro* large amounts of carbon dioxide (but no hydrogen) and volatile acids to which he attributed the characteristic smell of *Ascaris* (Bunge, 1883, 1889).

Weinland, in the series already mentioned, made similar observations, correlating the rate of glycogen consumption with the production of carbon dioxide and volatile acids. His beliefs did not pass unchallenged, as will appear but he is on record as being the first to show that the metabolism of intestinal helminths is characterised by carbohydrate fermentation.

Knowledge of the significance of glycogen in animal tissues goes back to Claude Bernard who in 1850 discovered the polysaccharide in mammalian liver after realising that this organ served as a source of blood sugar. This study (Bernard, 1850) led to further work on the role of glycogen (Bernard, 1857) which he subsequently demonstrated in various invertebrates including tapeworms, ascarids and flukes (Bernard, 1859). Foster (1865), six years later, made the first quantitative glycogen analysis in an ascarid. Since Bernard's pioneering, a demonstration of the presence of glycogen in tissue has been accepted as indicative of an energy reservoir.

Weinland, as a result of his studies, naturally

supposed that Ascaris was an anaerobe and this view, along with his belief that the worm produced volatile fatty acids, was to give rise to controversy. This controversy was heralded by Slater in 1925 who attempted to show Weinland mistaken on both accounts. Slater believed that the volatile acids were produced by bacteria and that under anaerobic conditions the worm itself produced only lactic and, possibly, phosphoric acid. From his introductory remarks it is evident that Slater was biased, however he managed to isolate bacteria from Ascaris which did indeed produce volatile fatty acids, although he gave no indication that the amount of acid was equivalent to that obtained previously from Ascaris keeping medium. It was not until 1950, when axenised worms were shown to produce large amounts of volatile fatty acids, that a bacterial origin of these acids was finally dismissed (Epps et al., 1950).

Slater's second line of attack was to claim that the worms were aerobic, a conclusion based on the greater longevity of electrically stimulated worms in aerobic rather than anaerobic media. He pointed out that the diffusion of oxygen from capillaries in the intestinal wall might make the lumen rather more aerobic than was otherwise thought. Von Brand resolved this apparent dichotomy by suggesting that whether the lumen of the host's small intestine provided an aerobic

or anaerobic environment depended on the surface area/volume ratio of the worm concerned (von Brand, 1938). There was no absolute, it was a matter of degree: whereas a large nematode, such as Ascaris, might be predominantly anaerobic, a smaller worm, in the same environment, could be predominantly aerobic. Weight for this reasoning was furnished by Davey (1937, 1938) who found that small nematodes of the sheep intestine survived significantly longer in the present of oxygen.

The production by Ascaris of volatile fatty acids, and the worm's survival with little or no oxygen may be as envisaged by Weinland but his assertion that Ascaris cannot use oxygen has been disproved. Adam (1932) found that male Ascaris (i.e. without the complication of the egg's metabolism) consumed oxygen, and in 1935 Harnisch demonstrated oxygen consumption by anterior (without eggs) portions of female worms. Since that time, all parasitic worms studied have been found capable of oxygen uptake, though the interpretation of such an uptake, in terms of in vivo respiration, is often debatable.

3. Carbohydrate content of worms

Most endoparisites have a pronounced carbohydrate metabolism. This is reflected in their high level of stored

carbohydrate, their extensive use of endogenous carbohydrate and the rapidity with which they transport exogenous glucose into their tissue. For example: Hymenolepis diminuta may contain from 26 to 48% of its dry weight as stored glycogen (Fairbairn et al., 1961); under starvation conditions Fasciola hepatica uses up to 20% of its glycogen within 5 hrs. (Goil, 1961); glucose consumption by the filarial worm, Litomosoides carinii corresponds to from 50 to 80% of the worm's fresh body weight in 24 hrs. (Bueding, 1949a).

Von Brand has suggested (1950) that the amount of glycogen stored is associated with two features of the physiology of parasites. In parasites which do not have direct access to the host's carbohydrate reserve, or in which metabolism is predominantly anaerobic, large amounts of glycogen are stored. Examples are the large nematode parasites of the intestinal lumen, such as Ascaris lumbricoides, Parascaris equorum and Ascaridia galli. Conversely, tissue glycogen is usually low in parasites with direct access to the host's carbohydrate reserve, either because they suck blood and live in areas of high glycogen, or because they have a predominantly aerobic metabolism. Nematode examples of this type would be, Trichinella spiralis, Ancylostoma spp., Litomosoides carinii and Dirofilaria immitis.

The suggested correlation between anaerobiasis and carbohydrate content is thought to be due to the nature of

the intermediately oxidised carbon atoms .....

$$(H - \overset{|}{\underset{|}{C}} - OH)$$

.... which make carbohydrate a much better substrate for gaining energy anaerobically than either protein or fat, both of which contain less oxygen. Anaerobic fermentation is essentially an oxidation-reduction process involving electron transfer without the participation of molecular oxygen. Molecular groups which are neither completely oxidised nor completely reduced are ideally suited to anaerobic processes (Hellerman, 1947; Hungate, 1955).

There seems to be no doubt that carbohydrate is a useful reserve under oxygen-poor conditions. However, Read (1961) suggested that stored carbohydrate may be better correlated with growth and reproductive requirements. The cestodes Hymenolepis diminuta (Read, 1959), Oochoristica from rodents and Lacistorhynchus from dogfish (Read, 1957), the acanthocephalan Moniliformis dubius (Read and Rothman, 1958) and the nematode Ascaridia galli (Reid, 1945a, b) have all been shown to have a pronounced requirement of carbohydrate for growth and development.

It is perhaps fortuitous that those parasites in aerobic habitats in addition usually have access to the host's tissue carbohydrate; although these parasites may have a marked carbohydrate metabolism (e.g. Schistosoma, Bueding,

cited by von Brand, 1952) there may be no necessity to build up a reserve themselves. Parasites feeding on the gut contents, on the other hand, are probably obliged to store carbohydrate as the supply in this habitat is more sporadic. Such a habitat as the gut also happens to have lower oxygen tensions than the tissues of the host so that there is a possibility that the correlation of carbohydrate reserve with oxygen-poor surroundings is illusory, and this seems to be the distinction that Read's argument makes.

Perhaps the ease with which carbohydrate can be converted into energy, fat or protein makes it a useful short-term reserve material. But it should be remembered that carbohydrate is not as useful as fat on a weight/calorie basis and one might expect it to represent a reservoir for relatively short, "emergency" periods. It is interesting, in this respect, that free-living, embryonating Ascaris eggs employ a fat reserve (Fairbairn, 1955) which is derived, in part at least, from the adult's carbohydrate catabolism (Saz and Lescure, 1966). The developing eggs, being free of the host's intestine, have access to oxygen and are thus able to metabolise the fat produced as "waste" by the adult.

There seem, then, to be several factors determining the extent to which carbohydrate is stored in parasitic helminths. At this stage no simple explanation can be given

but both anaerobiasis and a distinct carbohydrate requirement appear to be implicated.

Of low molecular weight carbohydrates, trehalose appears to be most widespread. Fairbairn and Passey (1957) isolated this non-reducing dimer of glucose from unembryonated Ascaris eggs, reporting that this sugar comprised almost 8% of the total solids. In a subsequent survey of 71 species of invertebrate from various phyla, Fairbairn (1958) found this disaccharide to be widely distributed. It occurred in higher concentrations in the 8 species of nematode examined than in cestodes, trematodes and other invertebrates, with the possible exception of insects.

Free glucose has been identified as a constituent of the tissues of several species of nematodes, cestodes, trematodes and acanthocephalans (Fairbairn, 1958; Agosin, 1957).

In the haemolymph of Ascaris carbohydrates are abundant, consisting of glycogen (0.40%) and trehalose (0.77%). Glucose and other free carbohydrates occur only in traces (Fairbairn and Passey, 1957; Fairbairn, 1958).

4. End-products: fermentation acids

Biochemical variability characterises the parasitic helminths; as Bueding (1962) has pointed out, such diversity should receive as much attention as biochemical unity.

The fermentative use of carbohydrate leads uniformly in vertebrates to the production of lactic acid: in bacteria and yeasts, on the other hand, fermentation produces a great variety of compounds. Animal parasites appear to be intermediate between these two extremes.

As opposed to the aerobic respiration of carbohydrates, which results in complete catabolism to carbon dioxide and water, fermentative processes are incomplete. Many parasitic worms excrete incompletely oxidised derivatives of carbohydrate, although certain of them may oxidise a considerable proportion to carbon dioxide and water (Read, 1961).

Of the gaseous products, hydrogen, formed by many bacteria and some parasitic protozoa, is not known to be produced by helminths. Carbon dioxide, on the other hand, is an almost universal "end-product" of fermentation, and this is true of both protozoan and metazoan parasites, whether it is of an "inorganic" nature, liberated from bicarbonate, or of a truly respiratory nature, where the carbon atom is derived from an organic compound (von Brand, 1966).

The main organic end-products of carbohydrate metabolism in parasites are a variety of organic acids. Those excreted by some helminth parasites are shown in Table I. Von Brand (1966) points out that these probably demonstrate, at least in some cases, the "biochemical potential" of the organism concerned, rather than <u>in vivo</u> reality, and this

suggestion will be discussed later (Chapter IV) as it has bearing on the experimental findings of this study. As can be seen in Table I, lactic acid is the most commonly reported end-product. Some parasitic worms, like vertebrates, form lactic acid as the major end-product of fermentation: this is characteristic of several cestodes, schistosomes and filarial worms. Lactic acid is the major product of carbohydrate metabolism in the cestodes Hymenolepis diminuta, Oochoristica symmetrica and Echinococcus granulosus under both aerobic and anaerobic conditions (Agosin, 1957; Laurie, 1957; Read, 1956), although Fairbairn et al. (1961) found only small amounts of this acid in H. diminuta instead finding large amounts of succinic acid. Bueding (1950) found lactic acid to account for 81 to 91% of the glucose used by Schistosoma mansoni in the presence or absence of atmospheric oxygen, and lactate accounts for most of the carbon of the metabolised glucose in Dirofilaria uniformis (von Brand et al., 1963) and Litomosoides carinii (Bueding, 1949a).

In the excreta of nematodes, lower fatty acids predominate. Lactic acid and other non-volatile acids are not excreted in large amounts by intact Ascaris (von Brand, 1934; Bueding and Yale, 1951; Toryu, 1936) and in muscle homogenates of this worm lactate is not produced in any quantity unless the system is fortified with pyruvate (Bueding and

TABLE I

ORGANIC METABOLITES EXCRETED BY PARASITIC HELMINTHS
(After von Brand, 1966)

	Lactic acid	Pyruvic acid	Formic acid	Acetic acid	Propionic acid	Butyric acid	C_5 acids	C_6 acids	Higher fatty acids	Succinic acid	Acetoin	Ethyl alcohol
Trematodes												
Fasciola hepatica	(X)			X	X				X			
Schistosoma mansoni	X											
Cestodes												
Echinococcus granulosus	X	X		X						X		X
Hymenolepis diminuta	X			X						X		
Moniezia expansa	X								X	X		
Taenia taeniaeformis	X	X		X						X		X
Nematodes												
Ascaris lumbricoides	X		(X)	X	X	X	X	X		X	X	
Heterakis gallinae	(X)	(X)		X	X	(X)				X		
Litomosoides carinii	X			X							X	
Parascaris equorum	X				X		X					
Trichinella spiralis larvae	(X)			X	X	X	X	X				
Trichuris vulpis	(X)		(X)	X	X	(X)	X	X	X			

X = compound excreted in significant amounts.
(X)= compound excreted in very small amounts only.
Source refs., von Brand, 1966, pp 116 & 117.

Yale, 1951; Rathbone and Rees, 1954). A similar situation exists in *Trichinella* larvae (Agosin and Aravena, 1959; von Brand *et al.*, 1952; Goldberg, 1958).

Of the nematodes studies, *Ascaris* produces perhaps the greatest variety of organic acids although whether the end-products of carbohydrate fermentation are the same under aerobic and anaerobic conditions is not always clear. Excreted acids have to be collected from keeping media and this, of course, entails maintaining the worm *in vitro* for periods of time, sometimes several days. Studies of this type, aimed at elucidating the physiology of the worm, of necessity oblige workers to assume knowledge of certain of its physiological requirements in order to establish *in vitro* conditions. The result seems often to have been a vicious circle.

Using bacteria-free worms, Epps *et al.* (1950), Bueding and Yale (1951) and Bueding (1953) separated and identified volatile fatty acids present in *Ascaris* keeping medium as acetic, propionic, butyric, 2-methylbutyric, *n*-valeric and tiglic (*cis*-2-methylcrotonic). A relatively large 6-carbon volatile acid reported by these authors was subsequently identified by Saz and Gerzon (1962) as 2-methyl-valeric, in contradiction with Ellison, Thomson and Strong (1960) who had identified it as *n*-caproic. Whitlock and Strong (1963) further substantiated the findings of Saz and

Gerzon concerning this acid.

Analysis of haemolymph by Bueding and Farrow (1956) showed succinic acid to be another important organic acid product of Ascaris, although of other dibasic or tricarboxylic acids, little oxaloacetic acid and no fumaric, malic, aconitic or citric acids could be isolated (Ueno et al.,1960).

Analysis of the volatile fatty acid content of haemolymph has shown results qualitatively similar to analyses of the keeping medium, with 2-methylbutyric and 2-methylvaleric as the major components. The first analyses of volatile fatty acids in Ascaris haemolymph, those of Moyle and Baldwin (1952), who used buffered silica columns for separation, identified acetic, propionic, n-butyric, 2-methylbutyric and an unidentified 6-carbon acid; and Puskarevs (1965), using paper-chromatography, obtained similar results with the additional finding of formic acid. By gas chromatography, Harpur (1969) separated and detected acetic, propionic, 2-methylbutyric, n-valeric, 2-methylvaleric, tiglic and succinic acids in Ascaris haemolymph.

Apart from the consistently reported acids, acetic, propionic, 2-methylbutyric, n-valeric, 2-methylvaleric and succinate, several others seem to have a more sporadic appearance in the literature and occur in low concentration or as traces. These acids include, formic, both iso- and n-butyric, tiglic, caproic and an "unknown" with a carbon-chain longer

than six. Some of the reports of these acids have already been mentioned. Others include Ellison et al.(1960), who found small amounts of isobutyric, n-butyric, tiglic, n-caproic and an unidentified 7 or 8-carbon branched-chain acid; and Greichus and Greichus (1966) who had almost identical results. Saz (1965) mentioned that, apart from 2-methlvaleric, traces of other 6-carbon acids appear occasionally, and Harpur (1969) reported occasional traces of isobutyric acid and two unidentified compounds appearing on chromatograms as unresolved peaks with retention times similar to n-butyric acid. Part of this thesis presents data suggesting that some of these acids are not normally formed by Ascaris but are symptoms of "in vitro-sickness".

5. Intermediary metabolism

Several methods can be employed in attempting a demonstration of functional metabolic pathways. One line of approach has been the isolation of the relevant enzymes; another one, the demonstration of intermediates; a third, whether intermediates can be utilised; and fourthly, by determining the fate of radio activity introduced as variously labelled substrates. However, data from studies of this type, whether positive or negative, must be interpreted with caution (see von Brand, 1966, p.134).

A case in point is the evidence for a functional Krebs tricarboxylic acid cycle in parasitic helminths. Apparently a functional cycle exists in Echinococcus granulosus scolices (Agosin and Repetto, 1963), Moniezia expansa (Davey and Bryant, 1969), Fasciola hepatica (Prichard and Schofield, 1968), Trichinella spiralis larvae (Goldberg, 1957) and in certain small intestinal nematodes (Massey and Rogers, 1950).

It has been generally considered unlikely that large intestinal nematodes, such as adult Ascaris lumbricoides, possess a complete Krebs cycle although the evidence for a partial cycle has been good (e.g. Rathbone, 1955; Seidman and Entner, 1961). These studies were conducted with particulate fractions obtained by differential centrifugation, an approach which was subsequently criticised by Ward and Schofield (1967) bearing out von Brand's cautionary words (see above). Ward and Schofield's (1967) study demonstrated all the Krebs cycle enzymes in Haemonchus contortus larvae, but of particular note was their finding that the activity of some of these enzymes, especially aconitate hydratase and the isocitrate dehydrogenases, was far higher in the supernatent than in the particulate fraction, where activity was sometimes very low. They point out that all the investigations which provided good evidence for the existence of all the

Krebs cycle enzymes had been carried out on whole homogenates. (Traditionally, of course, the Krebs cycle enzymes are all located in the mitochondria.)

The implication, then, is that a more careful look at the supernatant, as well as the sedimented portion of parasitic helminth homogenates, might well reveal all the enzymes of this cycle where previously only a partial cycle had been shown.

The exception, which may "prove the rule", is an analysis of an Ascaris particulate fraction by Oya et al. (1965) who demonstrated all the enzymes of the cycle but had considerable difficulty in showing the presence of aconitate hydratase.

The question of whether or not the Krebs cycle operates in vivo in Ascaris cannot, at this stage, be answered. The natural habitat of the worm, the small intestine, has a low oxygen tension and cytochrome oxidase is either absent in Ascaris (Bueding and Charms, 1952; Chance and Parsons, 1963; Katsume and Obo, 1962) or present only in low concentrations (Kikuchi and Ban, 1961; Kikuchi et al., 1959) so that the physiological role of the cycle, under these conditions, would probably be of minor importance unless there were some alternative terminal oxidation process.

For the operation of another metabolic system, the pentose phosphate pathway, there is some evidence in parasitic helminths. This pathway consists of a complex series of reactions involving the formation and degradation of phosphates of heptoses, hexoses, tetroses and trioses, a sugar lactone and gluconic acid. The end result is complete oxidation of glucose to carbon dioxide and water with the formation of 3 moles ATP per mole glucose utilized. In Ascaris some of the intermediates and enzymes have been demonstrated (De Ley and Vercruysse, 1955; Entner, 1957) so that a functional cycle seems to exist, but studies on the incorporation of variously labelled glucose into the end-product succinate indicate that this pathway is probably not of great significance in Ascaris (Saz and Vidrine, 1959).

There is, however, good evidence that Embden-Meyerhof glycolysis is an important pathway in parasitic helminths and a complete range of the enzymes of this system has been found in Ascaris muscle (Cavier and Savel, 1953; Rathbone and Rees, 1954; Saz and Hubbard, 1957).

Glycolysis, the lysis or breakdown of sugar, is an energy-producing sequence independent of oxygen and can consequently serve as a source of energy for anaerobic organisms. As realised in vertebrates, glycolysis leads from carbohydrate to pyruvate which, under oxygen deficiency, is reduced to

form lactate; the latter step reoxidising NADH produced earlier in the glycolytic sequence, thus recycling NAD. Many parasitic helminths, by contrast, produce only small or negligible amounts of lactate, instead characteristically forming succinate and volatile fatty acids. This feature and the modifications by parasitic worms of classical glycolysis, is elaborated in the remainder of this section.

A major modification is the incorporation or "fixation" of carbon dioxide. Carbon dioxide fixation is, of course, vital to the photosynthetic process in plants and it is known to occur in bacteria and other organisms including vertebrates (see review of the subject by Harland et al., 1965). Earlier work on this aspect in nematodes includes that of Rogers and Lazarus (1949b) showing a net disappearance of carbon dioxide from anaerobic bicarbonate medium after incubation of Nematodirus spp. and Ascaridia galli; and Glocklin and Fairbairn (1952) who noted a sparing action of carbon dioxide on endogenous carbohydrate utilisation by Heterakis gallinae. The uptake or fixation of carbon dioxide has since been shown to occur in a number of parasitic helminths (Fairbairn, 1954; Prescott and Campbell, 1965; Scheibel and Saz, 1966; Ward et al., 1968b) including Ascaris lumbricoides (Saz and Vidrine, 1959).

Paralleling parasitic worms, molluscs have also been found to fix carbon dioxide and produce succinate; a further

similarity is their ability to withstand anaerobiasis, in this case caused by periods of shell closure or aestivation. Working on three species of bivalve, Simpson and Awapara (1966) showed that carbon dioxide was incorporated into phosphoenolpyruvate (PEP), the immediate precursor of pyruvate in the glycolytic sequence. The relationship of this site of carbon dioxide fixation to glycolysis, and the ensuing pathways in molluscs and parasitic worms can be seen in Fig. 1.

Two years later, Ward et al. (1968a) suggested that in parasitic helminths also, PEP was probably the site of carbon dioxide fixation: previously it had been assumed that carbon dioxide was incorporated into pyruvate. The slightly later study of Bueding and Saz (1968) showed the former most likely to be the case in Ascaris where they found the activity of the enzyme converting PEP to pyruvate, pyruvate kinase, was low, whereas the activity of PEP-carboxykinase, which catalyses the incorporation of carbon dioxide into PEP (Fig.1) was relatively high.

In essence the primary steps of carbohydrate fermentation in Ascaris (and molluscs) have been outlined. To summarise: glycogen or glucose is dissimilated via the Embden-Meyerhof scheme of phosphorylating glycolysis as far as PEP, which instead of being reduced to pyruvate, is carboxylated to form a 4-carbon dicarboxylic acid, reduction of

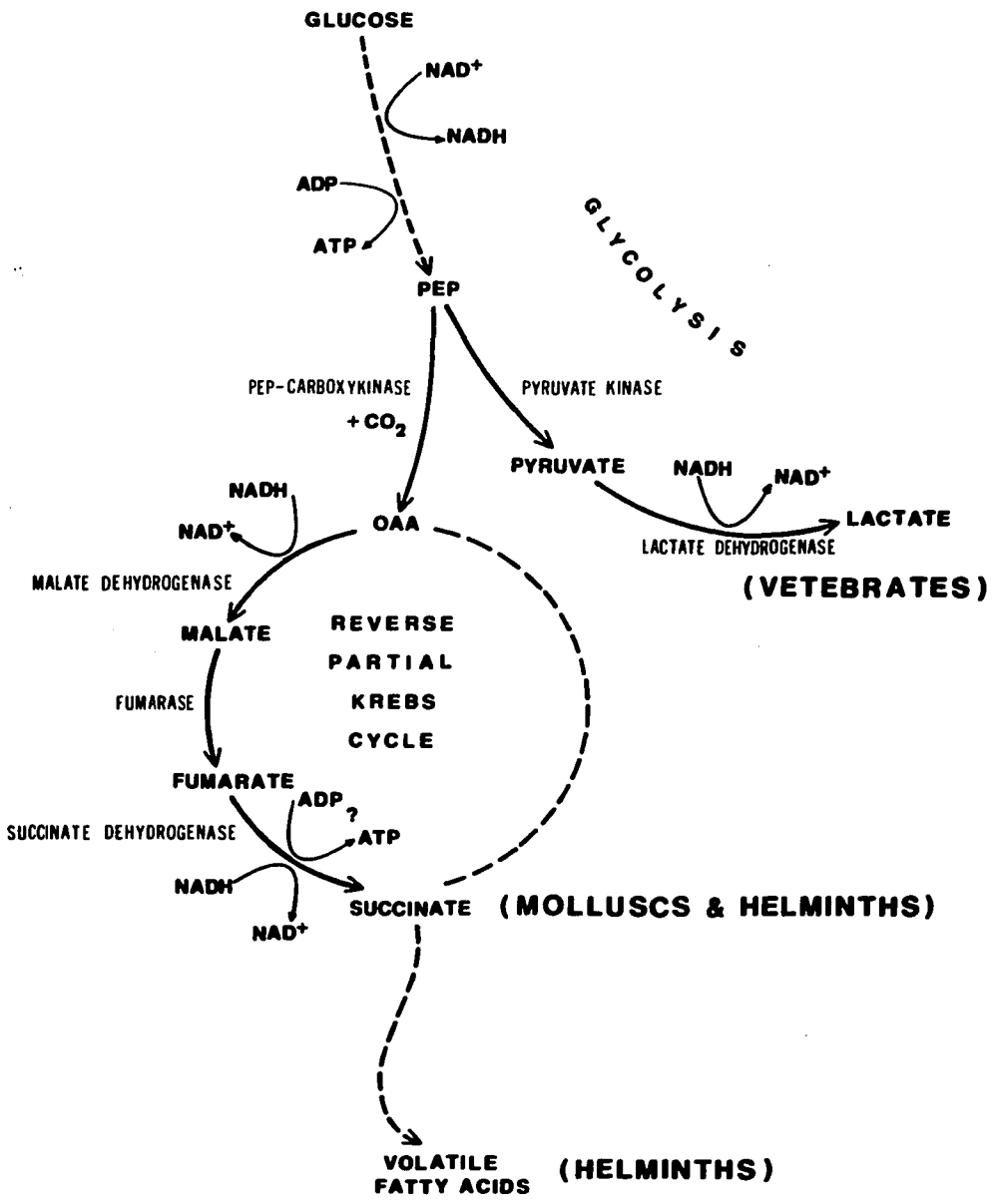


Fig. 1. Anaerobic pathways of carbohydrate catabolism in vertebrates, molluscs and parasitic helminths.

(Abbreviations: NAD, nicotinomide-adenine dinucleotide; NADH, reduced form of NAD; PEP, phosphoenolpyruvate; OAA, oxaloacetic acid.)

which produces succinate, one of the major fermentation products.

An interesting parallel has been revealed in a study by Opie and Newsholme (1967) of vertebrate red muscle and white muscle glycolytic enzymes. They found that, unlike red muscle, the white form has a high PEP-carboxykinase activity, and they suggest that this high activity is related to a greater dependence of white muscle on glycolysis, proposing that reduction of oxaloacetic acid (OAA, formed by the carboxylation of PEP) to malate could serve to reoxidise NADH formed during glycolysis.

In relation to the further reactions necessary to produce succinate, Saz and Hubbard (1957) have demonstrated the presence in Ascaris of malic dehydrogenase and fumarase; and Bueding et al. (1955) have partially purified a potent succinic dehydrogenase, so that all the necessary enzymes are accounted for. The terminal sequence is: PEP, OAA, malate, fumarate and succinate (Fig. 1).

The final step producing succinate is brought about by a mitochondrial system catalysing the transfer of electrons from NADH, via a flavoprotein, to fumarate, the latter being the terminal electron acceptor (Kmetec and Bueding, 1961). Evidence suggests that this reaction is also coupled with the anaerobic phosphorylation of ADP at the elecron transport

level (Chin and Bueding, 1954; Kmetec and Bueding, 1961; Seidman and Entner, 1961). Formation of succinate from fumarate is evidently of great importance in Ascaris, serving not only to reoxidise NADH but presumably also producing energy for muscular contraction.

The association of succinate production with muscular contraction is further evidenced by the marked inhibition of the formation of this dicarboxylic acid by Ascaris paralysed with the anthelminthic drug piperazine (Bueding et al., 1959). For similar reasons, succinate production may be important in other parasitic helminths.

Succinate is also the precursor of the volatile fatty acids in Ascaris and has been shown to give rise to propionate (Saz and Vidrine, 1959), 2-methylbutyrate (Saz and Weil,1960), and 2-methylvalerate (Saz and Weil, 1962).

A recent study by Saz and Lescure (1969) on enzyme distribution, and malate utilisation, by Ascaris mitochondria suggests that malate of cytoplasmic origin enters mitochondria where half of it is oxidised, via a malic enzyme, to pyruvate and acetate, while the remainder is reduced to succinate and propionate, with the accompanying generation of ATP. Saz and Lescure's proposed scheme is illustrated in Fig. 2. It should be noted in this scheme that NAD can be regenerated, independently, on either side of the mito-

chondrial membrane (no NADH is supposed to be able to traverse this membrane).

The scheme in Fig. 2 shows how acetate and propionate may be formed in Ascaris. From these volatile acid radicals the two branch-chained major end-products, 2-methylbutyrate and 2-methylvalerate, are formed. Using labelled compounds, Saz and Weil (1960, 1962) have shown that the former has its origin in the condensation of the carboxyl carbon of acetate and the carbon 2 of propionate (Fig. 3), while the latter is formed by a similar condensation of two propionate radicals. Tiglic acid, as can be seen in Fig. 3, is the immediate precursor of 2-methylbutyric acid. Although the precise mechanisms for the production of butyrate, n-valerate and caproate by Ascaris are not known they may be formed by condensations of various combinations of acetate and propionate, as they are in bacteria (Bueding, 1962).

Why are these volatile fatty acids produced at all? One possible function, suggested by Saz and Bueding (1966), is that further reductions of succinate, in a manner similar to this acids production, may be mediated by flavoprotein and may also reoxidise NADH. In female Ascaris, at least, the volatile fatty acids are not entirely wasted: although some acid is excreted, a small fraction is deposited in the neutral lipid fraction of the eggs, later to be metabolised or recon-

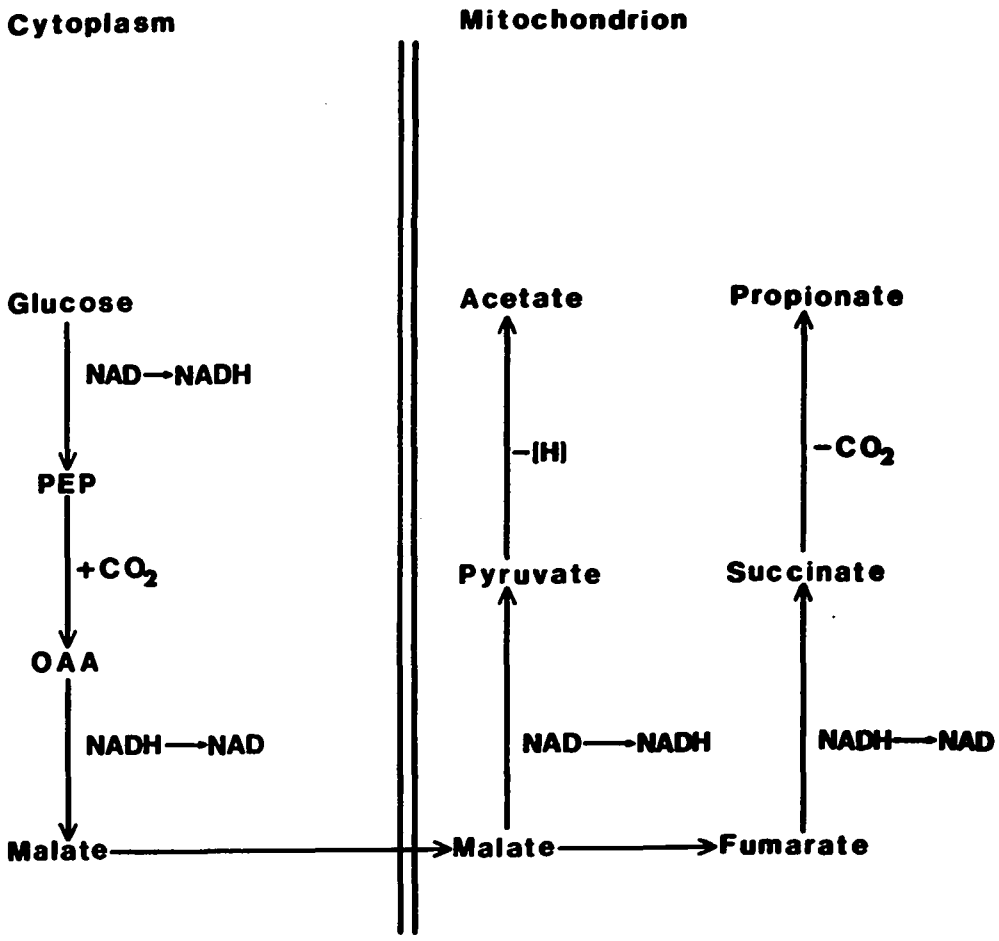


Fig. 2. Proposed pathway for the utilisation of carbohydrate in Ascaris muscle (after Saz and Lescure, 1969).

(Abbreviations: NAD, nicotinomide-adenine dinucleotide; NADH, reduced form of NAD; PEP, phosphoenolpyruvate; OAA, oxaloacetic acid.)

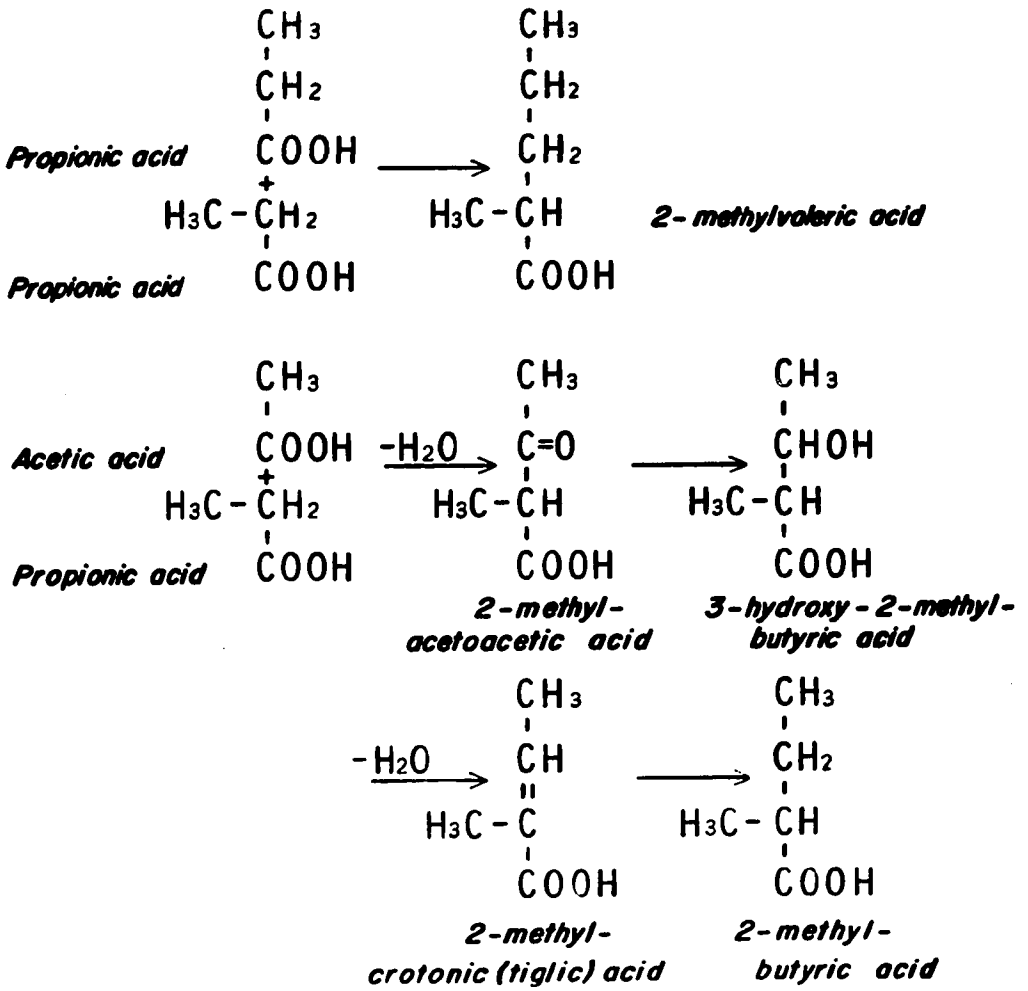


Fig. 3. Mechanism of formation of 2-methylvaleric acid (after Bueding, 1962) and 2-methylbutyric acid (after Saz and Weil, 1960).

verted to carbohydrate by the developing embryos (Saz and Lescure, 1966).

6. Nutrition and the feeding behaviour of Ascaris

"The nutritional relationship with a host is usually regarded as the hallmark of parasitism" (Read, 1968), and yet we know surprisingly little about this aspect. In nature, the feeding requirements of an animal are often more complex than the mere availability of adequately nutritious substances. House (1959) distinguished between nutritional requirements, which are the chemical factors making a diet adequate; chemical feeding requirements, again chemical factors but in this category important to the normal feeding behaviour; and physical feeding requirements such as dietary texture, position, light intensity and other physical factors that influence feeding behaviour. In Ascaris more is known about the first of these categories than the two influencing behaviour, and this lack of knowledge may be responsible for some of the difficulties of in vitro maintenance.

Apart from water, some ions and certain hydrophobic chemicals, it is accepted that entry of nutrients through the cuticle of Ascaris is negligible, the major path being through the intestine (Cavier and Savel, 1952; Mueller, 1929; Read, 1966; Rogers and Lazarus, 1949a; Zam et al., 1963). Since

foodstuff entering the intestine is partially predigested the function of the instestine is assumed to be primarily absorptive, although the following enzymes have been isolated in the gut: amylase, lipase, esterase, protease (Rogers,1940, 1941a, b), four peptidases (Carpenter, 1952), and alkaline and acid phosphatases (Rogers, 1947; Yamao, 1951).

Both sugars and amino acids have been shown to be absorbed by Ascaris. Cavier and Savel (1952) found that worms synthesised glycogen if glucose, fructose, sorbose, sucrose or maltose, but not mannose, galactose or lactose were added to a mineral medium. Similarly, Entner and Gonzales (1959) demonstrated the incorporation of C^{14}-glucose into glycogen by Ascaris in vitro. Cavier and Savel (1954) also showed that starving Ascaris could synthesise large amounts of protein if only four amino acids (glycine, alanine, glutamic acid and tyrosine) were added to the keeping medium, though Fairbairn (1957) commented that this finding warranted confirmation. Transport of nutrients by the gut itself has been demonstrated by Popkin (1967), who showed active transport of glucose from the mucosal to the serosal side; and Read (1966) who found that strips of Ascaris intestine actively accumulated certain amino acids, either by facilitated diffusion or by active transport.

Bearing in mind the above-mentioned categories of

nutritional factors (House, 1959), a study by Fernando and Wong (1964) of another nematode, the dog hookworm Ancylostoma caninum, is of interest. These authors demonstrated C^{14}-glucose incorporation into the glycogen of the worm incubated in Krebs-Ringer bicarbonate. However, when the worms were incubated in dog serum the incorporation of radioactivity into glycogen was enhanced 10 times, indicating that the serum factor is probably in the category of a "chemical feeding requirement". No such requirements have yet been shown in Ascaris, although they may well exist.

Transport of nutrients from the intestine to the muscle and reproductive organs must, in nematodes, be by way of the haemolymph. One would therefore expect this fluid to have some means of circulation, and yet there are no pumping organs in Ascaris so that the haemolymph would appear static unless the animal's movements as a whole are considered. With this angle of approach, Harpur (1964) proposed that a contraction in one portion of the worm accompanied by relaxation in another would cause volume changes tending to move haemolymph into the region of relaxation. Thus, to a certain extent, circulation within the body cavity may be affected.

Harpur (1969) has also pointed out that the intestine may play a role in movement of nutrients or metabolites from one part of the body of Ascaris to another: a substance

secreted into the gut lumen at the anterior end of the worm would move posteriorly and could then be reabsorbed into the haemolymph.

As mentioned earlier in this section, the behavioural aspect of nutrition in Ascaris is poorly understood. Ackert and Whitlock (1937), reviewing the feeding habits of vertebrate parasitic nematodes, concluded that Ascaris swims free in the host's intestinal lumen and feeds by ingesting the semi-liquid chyle. Although Ascaris is probably not "attached" in the same sense as hookworms and whipworms, it is hard to see how it can be regarded as "swimming free": its large size and undulating movements must keep it in constant contact with the intestinal wall. The radiographs in Makidono's (1956) paper illustrate this point. Additionally Ascaris seems to go through exploratory movements and has often been seen to jam its head into any small opening available (Akimoto, 1952; Slater, 1925; Yob, 1961). Finally, biliary ascariases are known to be fairly common: here worms have entered the bile duct and are obviously far from being free-swimming.

The above seems to indicate that although Ascaris may not be "attached", it could very easily bring parts of its body, especially the head, in contact with the intestinal epithelium.

Recently, Davey (1964) has shown that the buccal

cavity and intestine of Ascaris, taken directly from pigs, contains host epithelial cells at a concentration far greater than that of sloughed cells in the host's intestinal lumen. The mean density in the worms intestinal tract was 3.9 ± 1.2 S.D. $\times 10^6$ cells per cu.mm., while that in the host's intestinal tract was estimated to be 5.9 ± 2.8 S.D. $\times 10^4$ cells per cu.mm., a difference of two orders of magnitude.

There seem to be two possible explanations for this phenomenon: either the worm ingests intestinal contents and concentrates the epithelial cells by expressing the liquid portion, or the worm may browse on the intestinal epithelium, picking off the cells as they are being sloughed. The first explanation seems more likely, and in the light of what other evidence there is, more conservative, but the latter cannot be ignored as a possibility, although one might expect to see pathological evidence for this type of feeding.

B. ASCARIS IN VITRO: A SICK ANIMAL

1. Introduction

The setting up of an axenic culture is not an end in itself but a tool. Parasites, by the very nature of their intimate association with the host, are hard to study in their natural environment so that *in vitro* maintenance is often obligatory. This is particularly true for behavioural, physiological or biochemical studies and where screening of anti-parasitic drugs or the immunogenic properties of metabolic products of parasites are being investigated. Many such studies can only be conducted *in vitro* and, consequently, advances in these fields have run concurrently with improvement of culture methods.

Axenic culture of parasitic helminths involves problems not met with in free-living organisms (see Smyth, 1959). For example, the physiological properties of the host-environment are often incompletely known and may be hard to reproduce *in vitro*; the parasite's food may be imperfectly known, or when known, is of a complex nature (blood, bile, mucus, etc.) and is hard to reproduce artifically; the diffusion of waste material may require special provision *in vitro*.

The problem of *in vitro* maintenance of parasites is obviously very complex, essentially being an attempt to create,

artificially, the living environment provided *in vivo* by the host. Furthermore, difficulty is encountered in deciding which criteria to use in an assessment of the *in vitro* conditions. Criteria which may be used for this purpose in *Ascaris* include, the carbohydrate content of worms, their carbon dioxide production, and measurement of the haemolymph osmotic pressure. As will be shown in Chapters III, IV and V, the organic acid composition of the haemolymph may also be indicative of the *in vitro* conditions.

2. *In vitro* - health

Ascaris lumbricoides has not yet been successfully cultured outside its host. Yet many experimental studies have been conducted with *Ascaris in vitro*, some of these involving maintenance for several days. Death of the organism has frequently been the factor limiting the duration of such experiments but little consideration has been given to the state of health of worms before death. Subjective assessment has assumed the worms to be either alive, and presumably healthy, or dead. As Smith (1969) pointed out, "This is a situation quite beyond normal experience, unless there is a strange disease which can strike down its victims at various well separated intervals of time". However, there is now ample evidence to suggest, not surprisingly, that the health of the worm begins to decline as soon as it is removed from the host.

The importance of a knowledge of the health of an experimental animal is well understood by biologists: poor health may easily contribute to erroneous experimental findings. It has even been claimed (Smith, 1969) that healthy Ascaris does not produce fermentation acids at all, and that these only appear when the worm suffers oxygen deficiency in vitro. Although this is undoubtedly mistaken, it has to be admitted that studies in vitro, without any assessment of an organism's health, could result in gross errors of this nature.

3. Changes in vitro

Predictably, one of the major changes which takes place when Ascaris is kept in vitro is a depletion of the glycogen reserve. This is most rapid during the first 24 hrs. when it amounts to a loss of 23% of the initial glycogen content (a loss of more than 1% of the body weight) (von Brand, 1937) or according to Harpur (1963) 27% of the initial content after 18 hrs. Although glucose in the medium can be incorporated into the worm's glycogen (Cavier and Savel, 1952; Entner and Gonsalez, 1959) and has a sparing action on glycogen consumption (Harpur, 1963; Weinland and Ritter, 1902), there is no reversal of the tendency, and the sparing action does not come into effect until after the first 18 hrs. in vitro

(Harpur, 1963). This first 18 or so hrs. appears to be crucial, involving an as yet irreversible change in the glycogen content.

Also irreversible is the change in the osmolality of the haemolymph which occurs in Ascaris after only a few hours of in vitro existence. Hobson et al. (1952) found the osmolality of Ascaris haemolymph, a few hours after collection of the worms, to average 352 milliosmoles (mOsm). The osmolality of the pig's small intestinal contents they found to be 468 mOsm, so the worm was hypotonic to its surroundings in vivo. However, after about 2 days in vitro the osmotic gradient across the body wall of Ascaris had reversed, and it was found necessary to use a medium of lower osmolality (325 mOsm) than the pig's gut contents in order to keep the haemolymph osmolality at the original level.

Harpur and Popkin (1965) had similar findings but their initial value was taken from samples obtained in the abattoir as soon as worms were removed from the host's intestines. This figure, 323 mOsm, assumed to be close to the in vivo condition, was much lower than that of Hobson et al. (1952). However, after being in saline for only 3 hrs., the osmolality rose to 353 mOsm, agreeing with the earlier data. Harpur and Popkin's estimation of the osmolality of the duodenal contents of the pig was 320 mOsm, while that of the remainder of the

small intestine was found to be 380 mOsm. Again there had been an osmotic reversal: *in vivo* (i.e. immediately on collection) the worms were hypotonic to the environment, but in saline (320 mOsm, 39°C) they became hypertonic. The cause of this change is not known but worms do not seem to regain their original low level of osmolality; it is possible that traumatic shock may be a contributing factor.

Other fairly rapid changes occurring *in vitro* include Fisher's (1965) report that glucose transport across *Ascaris* intestine decreases after the worms have been *in vitro* for 4-6 hrs., and the observation (Harpur, 1962; Harpur and Waters, 1960) that carbon dioxide production decreases steadily.

C. CONCLUDING COMMENTS

Apart from providing background information, the purpose of this review has been to draw attention to the following major points of particular relevance to the remainder of this study:

(i) *Ascaris in vitro* is "abnormal".

(ii) The organic acid products of fermentation in *Ascaris* are variable *in vitro* and may not represent the "normal" condition.

(iii) There is a need to study the worm as soon as possible after collection to determine its "normal" condition.

(iv) There is obviously room for, and much need of, improvement of "culture" conditions.

II. MATERIALS AND METHODS

A. THE WORM

A major obstacle in the study of parasitic nematodes is their small size. Ascaris is a "giant", a rare phenomenon among nematode species. Adult, female Ascaris lumbricoides (var.suum), which was the species used throughout this study, is commonly 25 cm long and from 4-5 gm in weight: male worms, although still very large nematodes, are only about a third the size of females.

1. Collection and incubation

In the abattoir, worms were taken directly from the still warm, small intestines of pigs as soon as the viscera were available (i.e. within about half-an-hour of the host's death). Unless they were to be bled on the spot (see section 2), the worms were placed, for transportation (1 hr.), in insulated flasks containing either 3 or 1½ litres of 320 mOsm saline at a temperature of 37-39°C. During the transportation and subsequent incubation, at least 1 litre of saline was allowed for every 10 worms. On arrival at the laboratory, the worms were rinsed and incubated at 39°C in a similar, non-nutrient, saline medium. Antibiotics, except on one occasion, were not used in the medium as the experiments were of such short duration.

2. Haemolymph

It is important in taking haemolymph from Ascaris to avoid contamination with eggs or faeces. The more usual method is to cut through the body-wall close to the tail; consequently contaimination with faeces is likely. Harpur and Popkin (1965) took precautions to avoid contamination and their method was used for collection of haemolymph in this study. First, defaecation is encouraged by stroking the worm with a tissue. The worm is then bent double dorso-ventrally in the region of the genital girdle (where the intestine is narrowest) and an incision is made through a lateral canal. Usually, a loop of uterus erupts through the cut but remains intact, so that there is no contamination with eggs. The cut portion of the worm is placed in a funnel and the haemolymph drains into a test tube.

As the body-wall is cut through a lateral canal the vulva is avoided, however, any damage of the body-wall itself will result in contamination with that tissue's fluid and it is not known what effect release of the hydrostatic pressure has on the muscle bulbs within the body-cavity: there is a possibility that these may rupture into the haemolymph.

Haemolymph samples were either collected in the abattoir, as soon as worms were available, in which case they were chilled in ice for transportation, or else worms were

exsanguinated after periods of incubation in the laboratory. In either case the samples were kept frozen in the laboratory at -20°C until analysed.

The yield of haemolymph from a large female was usually 1-2 ml, which is ample for analysis by gas chromatography. Note was made of the variation in colour (presumably due to the haemoglobin content) of the haemolymph which ranged from pale straw to deep amber. Also noticeable was the turbidity of some haemolymph samples after freezing and thawing; others remained quite clear.

3. Muscle

Ascaris muscle was required for extraction of succinic dehydrogenase for the spectrophotometric estimation of succinic (see part D of this chapter). The method of obtaining muscle from Ascaris was basically that of Laser (1944). Worms were pinned through the head and an incision was made along a lateral canal (avoiding the vulva). The gut and reproductive organs were removed and the muscle layer was pared away from the cuticle with a blunt scalpel. The muscle so obtained was kept chilled in ice during collection and was then frozen at -20°C or used immediately for the extraction procedure.

B. VERTEBRATE GUT CONTENTS

In part of the experimental work succinate deter-

minations were made on the contents of the small intestine of various vertebrates. Preliminary studies were made on pigs, sheep and rats and, subsequently, rats were used in an experiment on this aspect.

In all cases the animals were mature, normally fed, adults and the samples were taken immediately after killing. The intestines were laid out, ligatured in sections, and then emptied, care being taken to avoid contamination with blood. The semi-liquid contents were mixed thoroughly, samples were collected (the entire contents in the case of rats), and these were filtered through glass-wool, centrifuged at low speed, chilled in ice and, as soon as possible, frozen at -20°C until analysed. Samples from 8 pigs, 4 sheep and 6 rats were obtained for the preliminary studies. The experiment employing rats is described, as an entity, in Chapter III, part D.

C. GAS-LIQUID CHROMATOGRAPHY

The procedure used has been described previously (Harpur, 1969) but it is felt that a detailed account is appropriate to the continuity of this presentation and some additional information is provided.

1. Chromatographic apparatus

The instrument used was an F and M model 700,

equipped with dual columns and hydrogen-flame-ionisation detectors, a Varian G-41 recorder (1 mv range, 10 inch chart-width), and an auxiliary oven-heater with a sensitive proportional controller. The column used throughout was made, in the laboratory, of coiled stainless steel tubing 6 m long with an i.d. of 2 mm and the packing material was 60-80 mesh firebrick (Chromosorb W) with a liquid phase of 20% neopentyl-glycol succinate (NPGS) and 2% phosphoric acid. The carrier gas was helium at a flow rate of 50 ml/min., with the addition of formic acid vapour during the separation of volatile fatty acids. This latter addition prevented "ghosts" appearing (Ackman and Burgher, 1963) in subsequent runs with volatile fatty acids, but it was unnecessary during ester separations. As standard procedure the oven temperature was kept at $140 \pm 5^{o}C$ with the detector block at $250^{o}C$ and the injection port at $148^{o}C$; however, for additional identification of certain of the volatile acids, their methyl esters were separated at the lower oven temperature of $75^{o}C$.

2. Calibration and detection

Before being used for analysis the column was conditioned for 48 hrs. by passing the gas phase through at the maximum temperature used. Mixtures of the following acids and esters were then injected into the column for calibration.

Volatile fatty acids: acetic, propionic, isobutyric, 2-methylbutyric, n-valeric, 2-methylvaleric and n-caproic. Dimethyl esters: malonic and succinic. These included all the organic acids expected in analyses of haemolymph plus internal standards (isobutyric for the volatile fatty acids and dimethyl malonate for the estimation of esters of non-volatiles). These internal standards produce peaks, on chromatograms, which do not coincide with the peaks formed by the other acids or esters.

Four mixtures, in methanolic solution, of both the acids and the esters were prepared, each containing a different concentration level of any one component. A fixed volume (2.2 microlitres) of each of the standard mixtures was injected into the column and the peak-heights were measured on the resulting chromatograms. Typical chromatograms of these known mixtures are shown in Fig. 8 B and Fig. 9 B. As shown in the examples in Fig. 4, the response (peak-heights) was linear: since there was no trend away from linearity, the small deviations noted are attributable to dilution and syringe errors.

More correctly, the concentration of a component is proportional to the area under the peak, but if operating conditions are fixed and small sample sizes are used, a measurement of the peak-height is sufficiently accurate. Peak-height was the measurement of response routinely taken for analyses reported in this study.

A quantitative assessment can easily be made by comparing a peak-height with graphs such as those in Fig. 4, provided the volume of the sample injected is the same as the volume of the standard (i.e. 2.2 microlitres). This is not practicable, however, and some other constant factor is required; hence the use of internal standards. Isobutyrate and malonate are therefore added, in known concentration, to the samples before chromatography. As the concentration of the internal standard present in the sample is known, a correction factor can easily be calculated to bring the peak-height of the internal standard in the chromatogram of the sample to the measurement obtained with exactly 2.2 microlitres of the same concentration of internal standard (i.e. if the peak-height of 2.2 microlitres of internal standard is 12 cm, and the peak-height of the same concentration of internal standard in a sample of unknown volume is 6 cm, then the correction factor = $\times \frac{12}{6} = \times 2$). This correction factor can then be applied to the peak-heights of all the other components. The result is a measure of the peak-heights which would have been obtained if exactly 2.2 microlitres of sample had been injected into the column. Since the standards were all of 2.2 microlitre volume, the concentration can be found by comparing the corrected peak-heights with graphs such as those shown in Fig. 4.

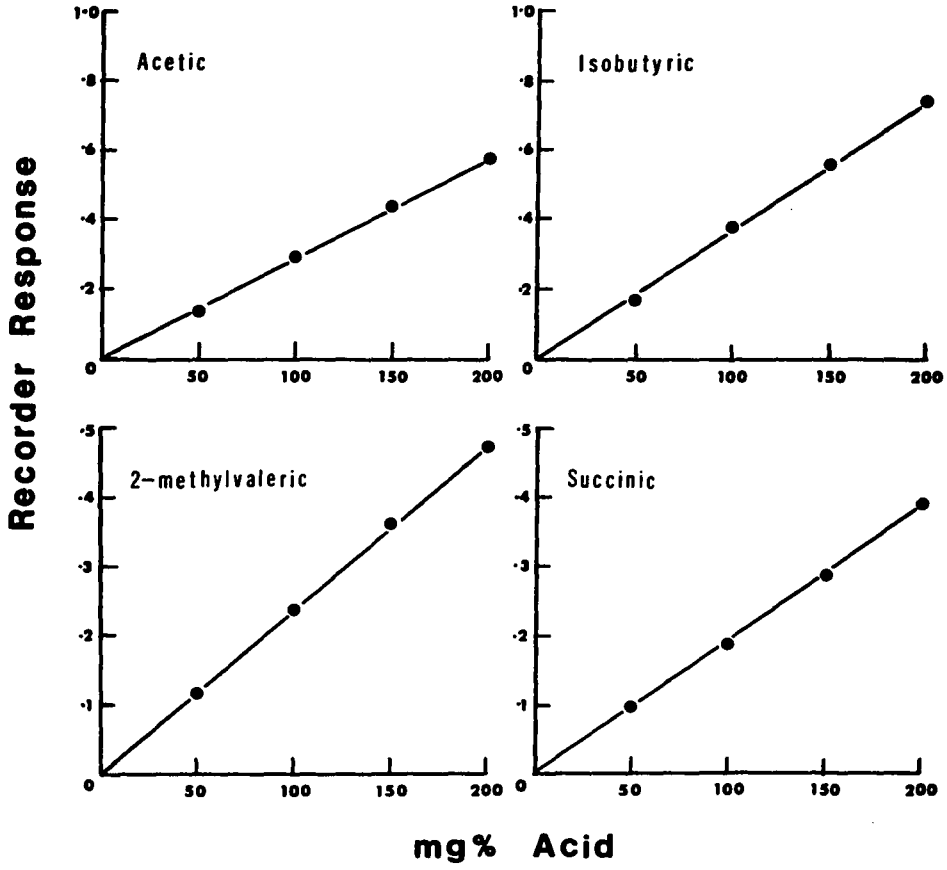


Fig. 4. Examples of linearity of recorder response to concentration of organic acids analysed by gas-liquid chromatography.

(N.B. For the non-volatile, succinic acid, the response shown is to the dimethyl ester; for the remainder, the response shown is to the acid.)

The incorporation of internal standards is particularly important when peak-height is the basis for quantitation as their use compensates for minor variations in operating parameters.

Qualitative estimation is made simply by comparing the retention times (times, after injection, of the elution of peaks) of the unknown peaks with those of known mixtures. It was found that the retention times became slightly shorter as the column aged but regular chromatography of the standard solutions served to correct any ambiguity in qualitative estimation and showed that although peak-heights increased slightly they remained proportionally the same, so that there was no interference with the quantitative estimation.

3. Sample preparation (see Fig. 5)

Risk of evaporation-loss was first minimised by the conversion of the acids to their sodium salts through the addition of 5 N sodium hydroxide (0.5 microlitres per 100 microlitres of sample). The samples were next deproteinised by filtering through cellulose membrane under centrifugal force (approximately 60 min. at 2500xg). A solution of the sodium salts of the internal standards, isobutyrate and malonate at concentrations of 114 and 385 mequiv per litre, respectively, was introduced at this stage (5 microlitres per 100 microlitres of original sample volume).

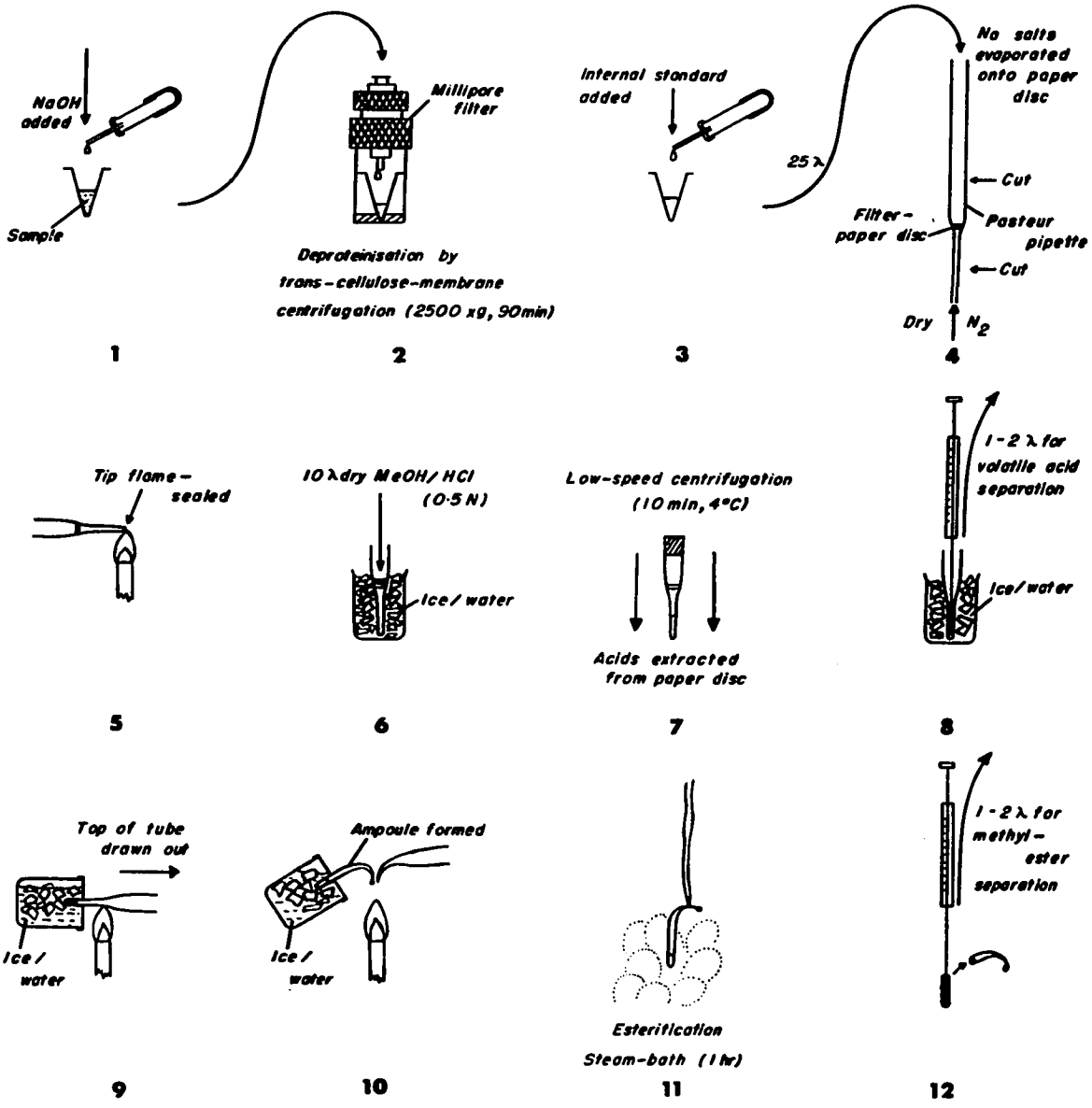


Fig. 5. Procedure for the preparation of samples for analysis of organic acids by gas-liquid chromatography (details in text).

For the subsequent esterification of samples an almost anhydrous, methanolic solution of the acids is required. A portion (20-25 microlitres) of the sample was evaporated onto a small disc of filter paper seated in the neck of a Pasteur pipette while a stream of dry nitrogen was played onto the lower surface of the paper. The rate of evaporation was increased by heating the Pasteur pipette above room temperature resting it against a heating-pad.

When evaporation was complete (about 1½ hr.) the Pasteur pipette was cut at points 2 cm above and below the paper disc and the lower end was flame-sealed. The resulting tube was seated in an ice-bath and 10 microlitres of dry methanol containing hydrogen chloride (0.5 N) was pipetted onto the filter paper. The acids were extracted from the paper by low-speed centrifugation in the cold, the paper was removed, and a 1-2 microlitre portion was injected into the column for the separation of the volatile fatty acids.

For esterification of the remaining solution, the top of the tube was flame-sealed, while the bottom of the tube was kept in an ice-bath, and the resulting ampoule was heated in a steam-bath for 1 hr., by which time esterification of the organic acids was complete. Again 1-2 microlitres were injected into the column, this time for the separation of the now-volatile succinic and malonic radicals. (At the normal

operating temperature of the oven (140°C) the esters of the volatile acids eluted close to the solvent peak, by which they were largely obscured).

D. ENZYMATIC DETERMINATION OF SUCCINATE (KMETEC, 1966)

Succinate may be oxidised to fumarate by the catalytic action of the enzyme succinic dehydrogenase. Ferricyanide can act as an electron acceptor for the above reaction and, when it does so, it is reduced to ferrocyanide:

$$Fe^{3+} + e \longrightarrow Fe^{2+}$$

This reaction is accompanied by a change in optical density which, when measured on a spectrophotometer, enables an assay to be made of the succinate present in the reaction mixture.

1. Extraction of succinic dehydrogenase (see Fig. 6.)

A prerequisite for the assay is the obtaining of the enzyme. This was done by following Kmetec's (1966) procedure which is summarised in Fig. 6. Either fresh or frozen *Ascaris* muscle was used as the enzyme source (*Ascaris* tissue was the obvious choice as the source of enzyme in this study and, according to Kmetec, yields a highly specific enzyme). Trial homogenisations were carried out using an all-glass Potter-and-Elvehjem homogeniser, and Virtis and Servall blenders. It was found that the highest level of enzyme activity was

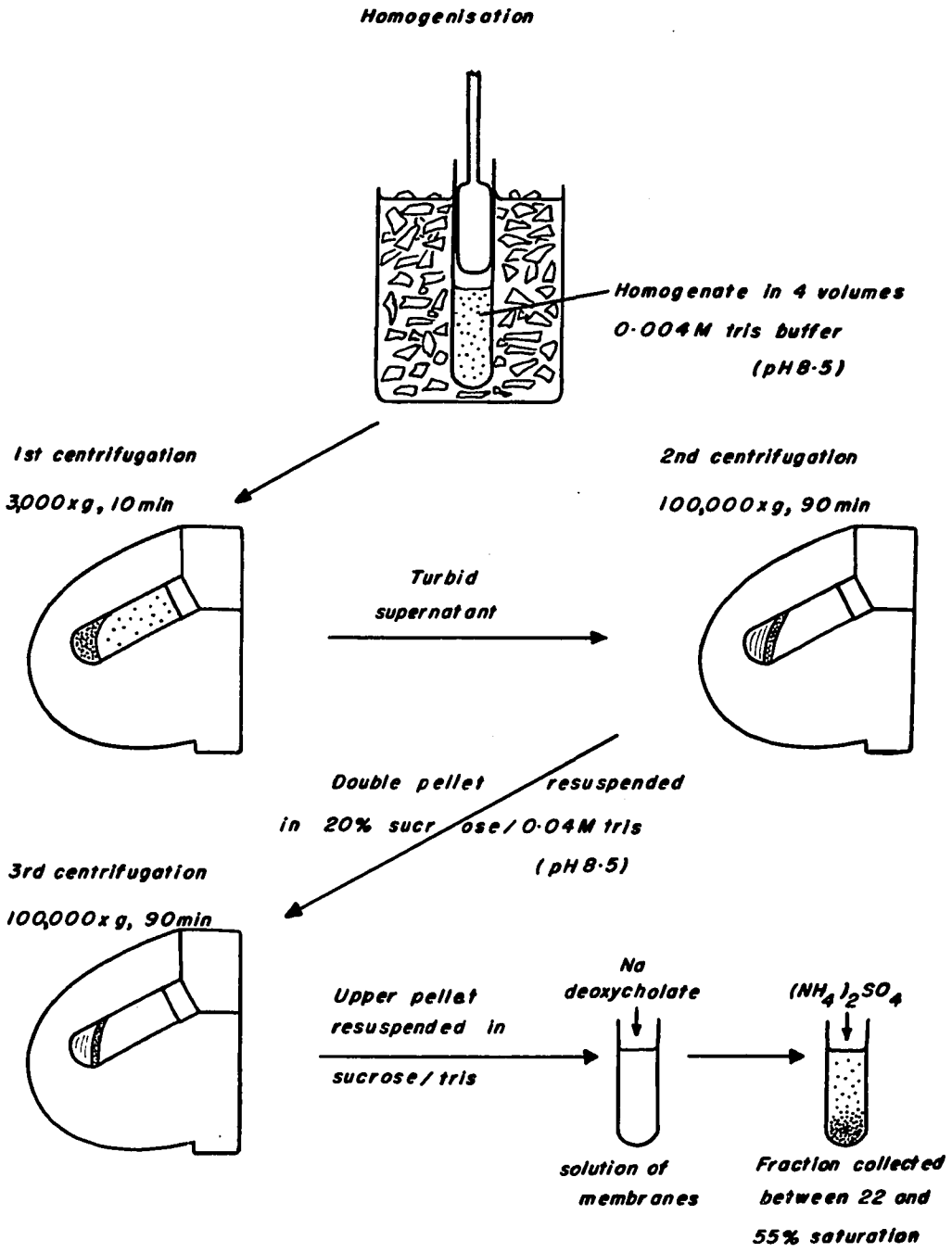


Fig. 6. Kmetec's (1966) procedure for the extraction of succinic dehydrogenase from Ascaris muscle (further detail in text).

obtained by mincing followed by homogenisation in the Potter-and-Elvehjem.

Homogenisation was followed by 3 centrifugations: the first removing course cellular material, such as nuclei and intact cells; the second and third (ultracentrifugations) separating two pellets, the upper a particulate layer, the lower of glycogen. The upper pellet was resuspended and the membranes were dissolved with sodium deoxycholate (2%). The ammonium sulphate fraction (collected between 22 and 55% saturation) was then dissolved in a solution of 20% sucrose in tris buffer (pH 8.5) to produce a "soluble enzyme preparation". Throughout the operation, temperatures were kept at 4°C.

2. Succinate assay

The drop in optical density, caused by the reduction of potassium ferricyanide, was measured at 410 nm on a Unicam spectrophotometer. Cuvettes with a 1 cm light path were used and the reaction mixture contained, in a final volume of 1.0 ml: 0.1 ml of 0.5% bovine serum albumin; 0.08 ml of 0.01 M potassium ferricyanide; 0.2 ml of 0.4 M tris buffer, pH 8.5; 0.2 ml of the soluble enzyme preparation; and 0.42 ml of the sample. Kmetec stipulates 0.1 M potassium ferricyanide but no optical density change could be measured at this concentration level and, consequently, 0.01 M was used instead. The reaction temperature was approximately 20°C (room temperature).

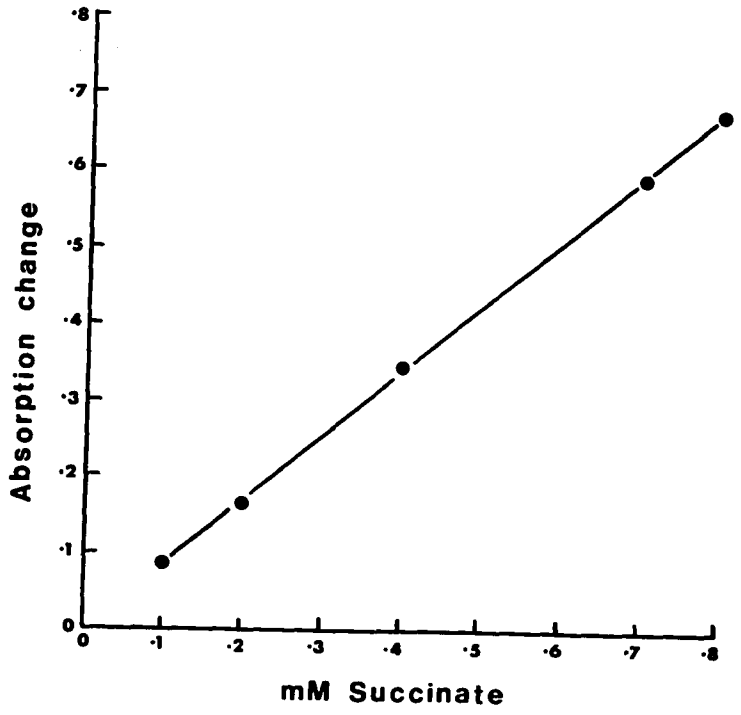


Fig. 7. Relation between change in absorbance, as measured at 410 nm, and concentration of succinate in the ferricyanide assay.

Samples were prepared by mixing with twice their volume of perchloric acid to precipitate proteins, the acid suspension being neutralised with 20% potassium hydroxide and cleared by centrifugation in the cold.

Assay of standards showed the optical density change to be linear in the succinate concentration range tested (0.1 - 0.8 mM) (Fig. 7) and samples were diluted, if necessary, to bring them within this range.

E. MEASUREMENT OF OSMOTIC CONCENTRATION

Osmolality is a convenient index of osmotic concentration; numerically it is equal to the molality of an "ideal" non-electrolyte with the same freezing point as the test solution and it is expressed in units of milliosmols per kilogram of water, abbreviated as mOsm.

In this study the osmolality of haemolymph was routinely determined before further analysis, 0.2 ml portions of the samples being used for this purpose. Freezing point depressions were measured using an Advanced Instrument osmometer (model 65 - 31) which reads directly in milliosmols per kilogram of water. The instrument was calibrated with standard solutions of sodium chloride (100 mOsm = 52.849 mmolal, 500 mOsm = 272.541 mmolal) as specified in the instruction manual.

F. STATISTICAL TREATMENT

Duncan's (1955) multiple range test and the analysis of variance were used to assess the significance of the results. The concentrations of organic acids were converted to logarithms for statistical treatment (to improve the homogeneity of variance and increase the rigour of the tests) but are presented in their original form in the main body of the text. Detailed tables are provided in the appendix.

III. EXPERIMENTAL RESULTS

The questions basic to the experimental work were:

(i) What changes occur in the production of haemolymph organic acids when Ascaris is taken from the host and incubated *in vitro*?

(ii) Can any changes that occur be correlated with the rapid rise in osmolality of the haemolymph of *Ascaris in vitro*?

(iii) Can any changes that occur be traced to a difference between *in vivo* and *in vitro* conditions and, if so, can they be corrected by manipulation of the keeping medium?

A. "NORMAL" AND *IN VITRO* HAEMOLYMPH ORGANIC ACIDS

1. Procedure

Following the methods laid out in the previous chapter, haemolymph samples were taken from 30 worms, 10 being exsanguinated at each of three times: immediately on collection (0.0 hrs. *in vitro* - these were to be considered "normal"); after 3.5 hrs. *in vitro*; and after 7.5 hrs. *in vitro*. (The latter times were chosen as being approximately 4 and 8 hrs, respectively, after the host's death.) The osmolality of these samples was measured (see part B of this chapter) and their organic acid content was then analysed by gas-liquid chromatography. It was found that sucrose interfered with

succinate estimation by gas chromatography, representative haemolymph samples were therefore also analysed for this acid by Kmetec's (1966) enzymatic method, but virtually no difference was found.

2. Results

A representative chromatogram of the volatile fatty acids of "normal" haemolymph is shown in Fig. 8A, together with that of a standard mixture for reference (Fig. 8B). Similarly, the separation of the dimethyl esters is shown in Fig. 9. Although expected, as it had been previously reported in haemolymph (Harpur, 1969), tiglic acid was present in none of these 10 analyses. However, acetic, propionic, 2-methylbutyric, n-valeric, 2-methylvaleric and succinic acid radicals were always present and, as in Harpur's (1969) analyses, a very small double peak (indicating only trace amounts) often occurred between the peaks of the isobutyric internal standard and 2-methylbutyric acid.

Fig. 10 shows a chromatogram of haemolymph volatile acids after worms had been *in vitro* for 3.5 hrs. Although otherwise qualitatively similar to the "normal" condition, these analyses showed tiglic acid present in all but one sample.

Finally, Fig. 11 shows the volatile fatty acids

present after 7.5 hrs. *in vitro*. Again tiglic acid was present, this time in all 10 samples, and in addition, 3 other peaks, with retention times longer than tiglic acid, put in an appearance. The first of these peaks, occurring after tiglic acid, coincided with the peak obtained for *n*-caproic acid in the chromatograms of known mixtures, but the remaining two peaks were not identified.

During separation of the methyl esters at the usual operating temperature of 140°C, the esters of the volatile acids elute close to the solvent peak by which they are largely obscured. To further define the unknown peaks, the methyl esters were therefore separated at the lower oven temperature of 75°C. The resulting chromatogram is shown in Fig. 12, again indicating that the first of the unknown peaks is probably *n*-caproic acid and also reinforcing the identification of tiglic acid, reported only once previously in *Ascaris* haemolymph (Harpur, 1969).

The quantitative data are summarised in Table II in which several other changes are evident. Most noticeable of these is a drop in concentration of succinic acid from an initial level of 13.7 mM to 5.4 mM after 7.5 hrs. *in vitro*. This overall drop is significant ($P < 0.01$) and the change between the 3 times, individually, is of probable significance ($P < 0.05$).

Other quantitative changes can be seen in Table II but these show no definite trends except in the case of the two branch-chained acids, 2-methylbutyric and 2-methylvaleric, which appear to be in negative correlation. However, the relation between these acids is probably more complex and is discussed in Chapter IV.

(On other occasions, haemolymph samples obtained in the abattoir, and after the two *in vitro* periods, were analysed by gas chromatography and in all cases confirmed the foregoing results.)

3. Summary

Haemolymph taken from *Ascaris* at the time the worm is collected from pigs contains: acetic, propionic, 2-methylbutyric, *n*-valeric, 2-methylvaleric and succinic acid radicals: tiglic acid is absent. However, after the worms have been kept for 3.5 hrs. in a saline medium, tiglate can be detected in the haemolymph, and after 7.5 hrs., *n*-caproate and two unidentified compounds (6-C or greater) appear. During this time the succinate concentration decreases to almost a third its original level.

TABLE II

ORGANIC ACID RADICALS IN ASCARIS LUMBRICOIDES HAEMOLYMPH

Each figure is the mean concentration (mM) of acid from ten worms

Time after death of host (hr.)	0.5	4.0	8.0	
(In vitro time (hr.)	(0.0)	(3.5)	(7.5)	P*
Acetic	13.6	17.0	8.4	0.05
Propionic	2.4	0.1	1.6	< 0.05
2-methylbutyric	28.2	32.0	36.5	0.05
n-valeric	8.8	8.9	3.9	< 0.05
2-methylvaleric	36.5	26.9	20.6	0.05
Tiglic	ND	3.0	1.8	0.05
n-caproic (?)	ND	ND	0.2	
Unidentified I	ND	ND	+	
Unidentified II	ND	ND	+	
Succinic	13.7	8.9	5.4	< 0.01
				< 0.05
Total	116.9	112.8	83.6	0.05

* At the given probability, a mean with an unbroken underline is not considered different from a mean similarly marked by the same line (cf. Duncan, 1955).

(ND = not detectable; + = compound detected)

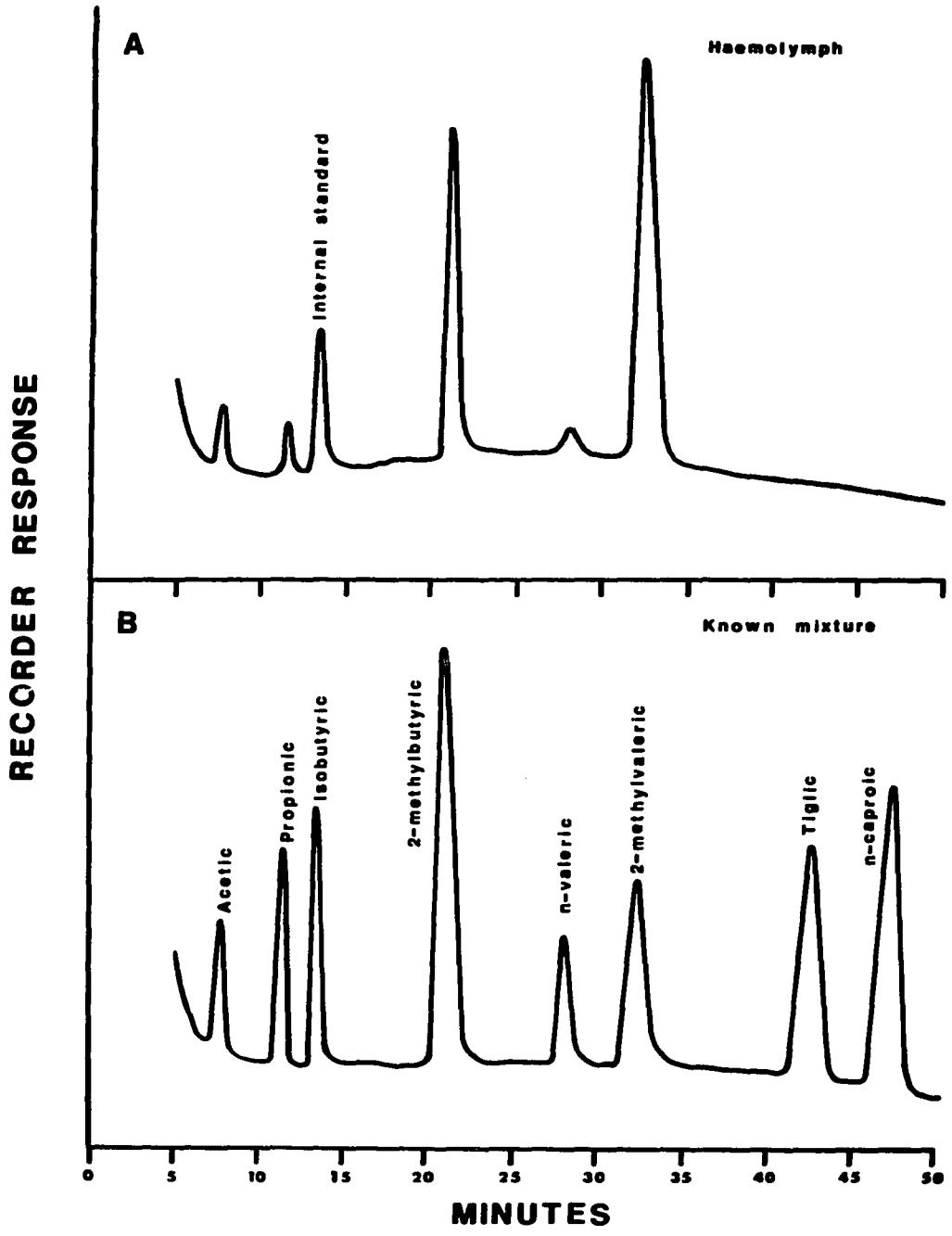


Fig. 8. Chromatograms of volatile fatty acids. A. Separation of acids in "normal" haemolymph of Ascaris. B. Separation of known mixture. Oven temp. 140°C. (Details of column conditions in Chapter II part C section 1.)

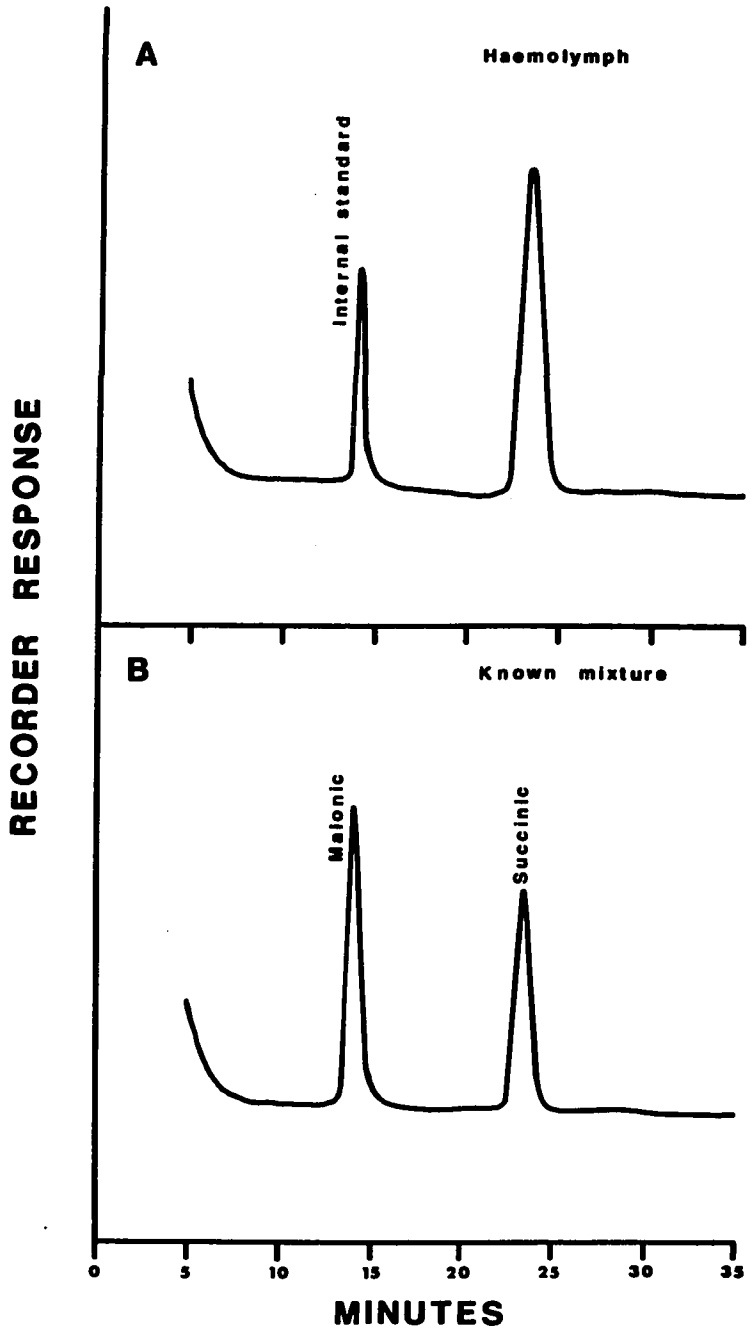


Fig. 9. Chromatograms of dimethyl esters.
A. Separation of the internal standard (malonic ester) and succinic ester of "normal" haemolymph of Ascaris. B. Separation of a known mixture. Oven temp. 140°C. (Details of column conditions in Chapter II part C section 1.)

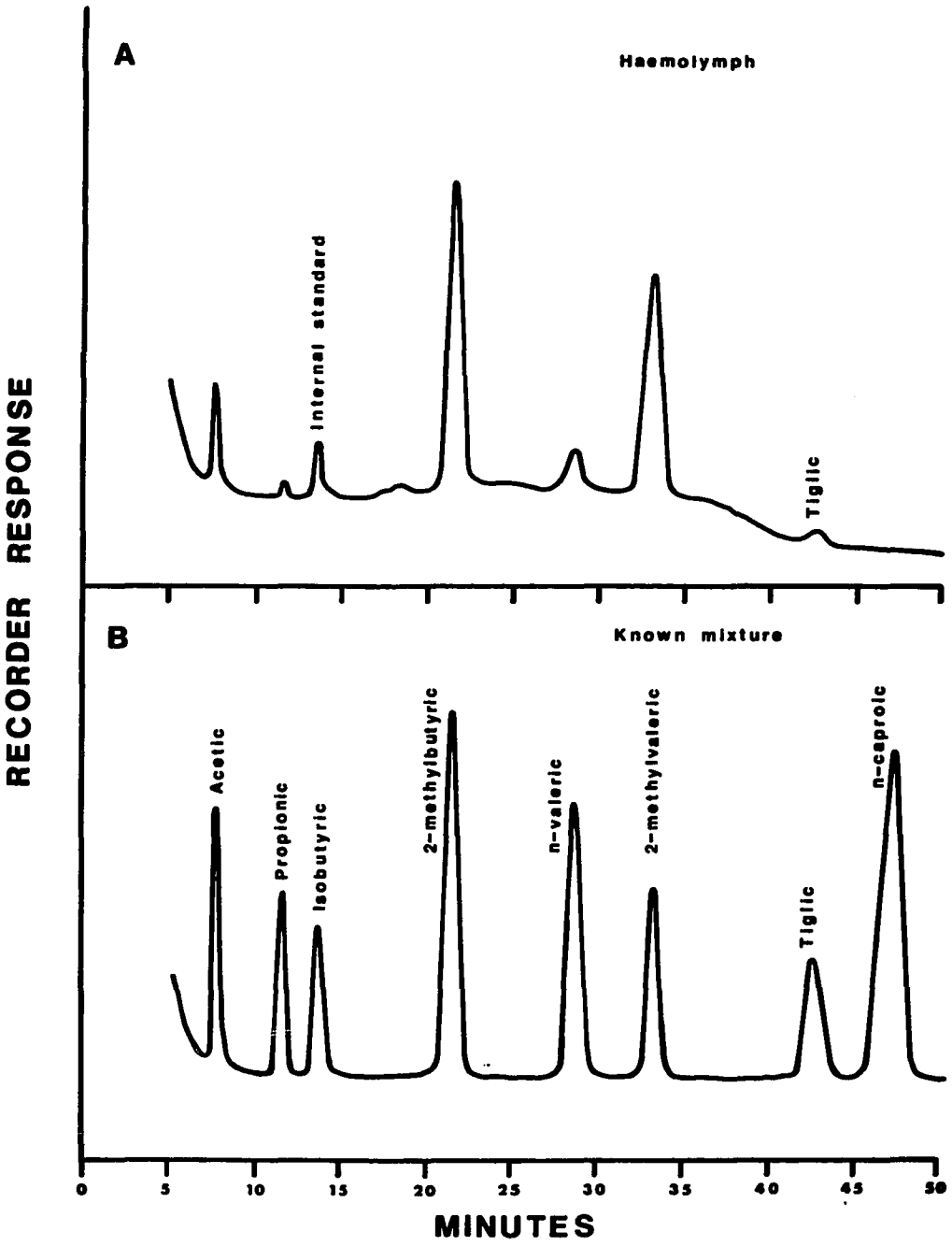


Fig. 10. Chromatograms of volatile fatty acids. A. Separation of acids from haemolymph of Ascaris after 3.5 hrs in vitro. B. Separation of known mixture. Oven temp. 140°C. (Details of column conditions in Chapter II part C section 1.)

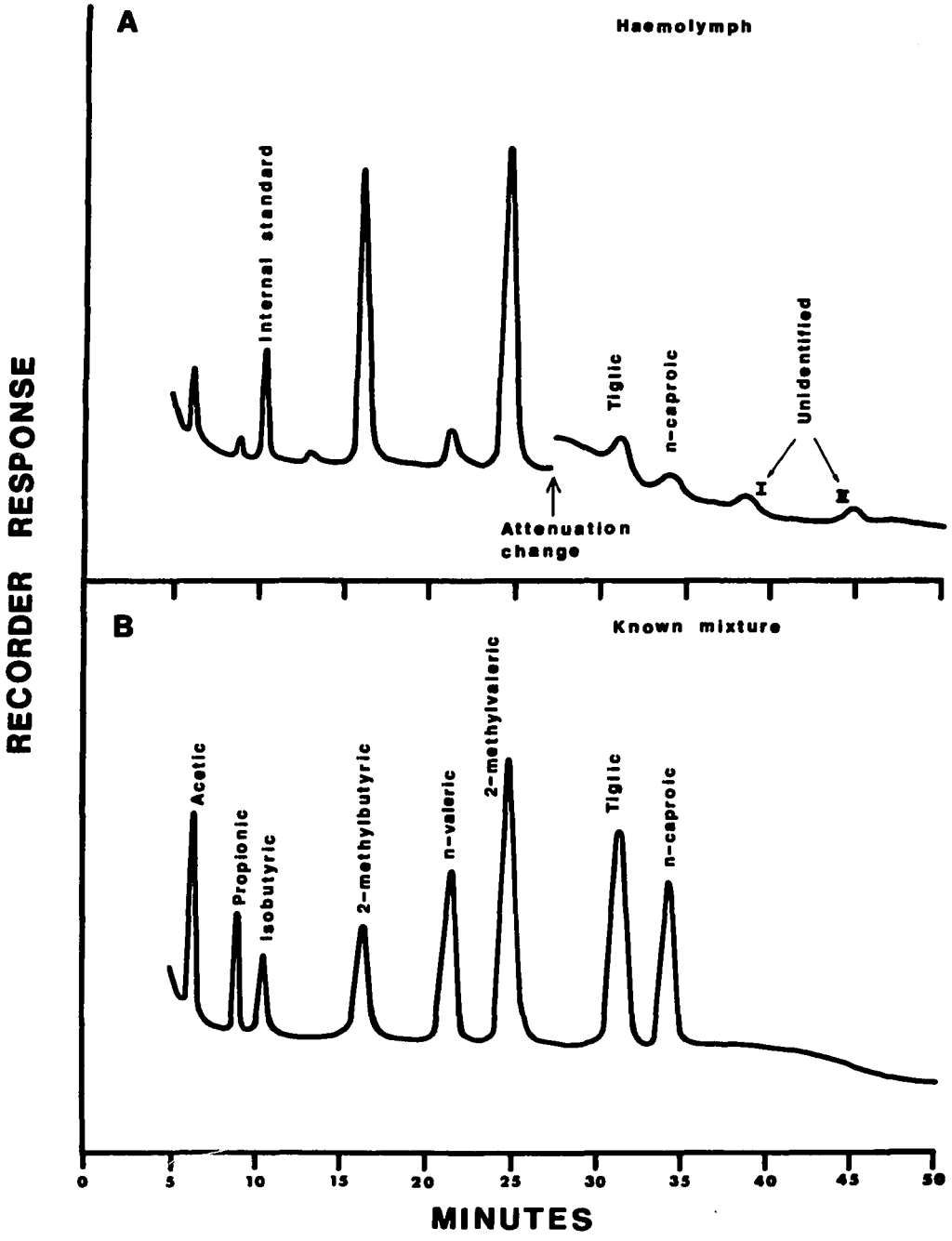


Fig. 11. Chromatograms of volatile fatty acids. A. Separation of acids from haemolymph of Ascaris after 7.5 hrs in vitro. B. Separation of known mixture. Oven temp. 140°C. (Details of column conditions in Chapter II part C section 1.)

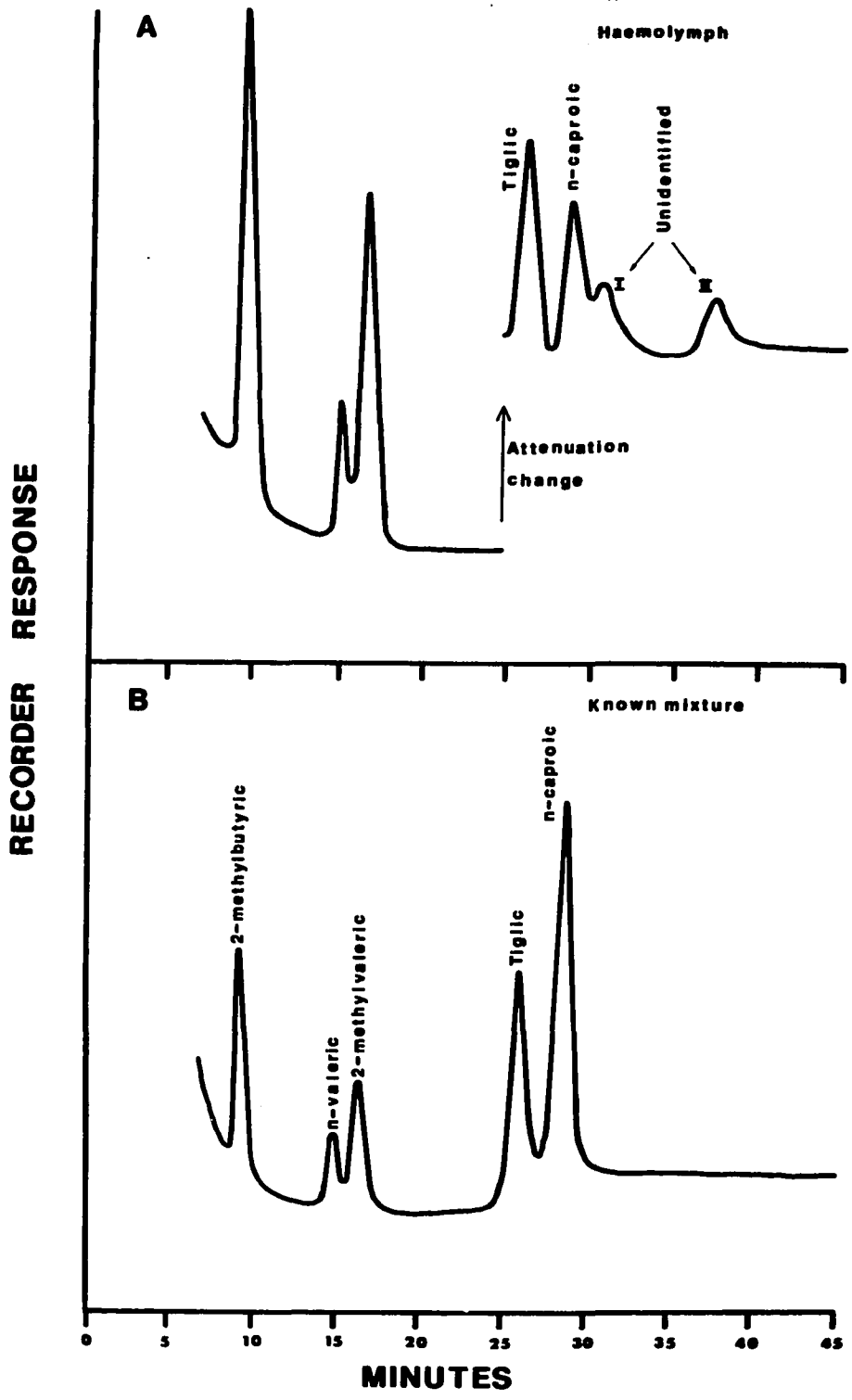


Fig. 12. Chromatograms of methyl esters of volatile fatty acids (oven temp. 75°C). A. Separation of esters of haemolymph of Ascaris after 7.5 hrs in vitro. B. Separation of known mixture. (Details of column conditions in Chapter II part C section 1.)

B. HAEMOLYMPH OSMOLALITY CHANGES *IN VITRO*: THE EFFECT OF BICARBONATE

As previously mentioned, the osmolality of haemolymph samples was routinely measured before gas chromatography. One of the objects in studying the haemolymph organic acids was to see whether any change *in vitro* could be correlated with the rise in osmolality: none could. However, although the mean osmolality of haemolymph taken directly from *Ascaris* in the abattoir, 324 mOsm, was in close agreement with Harpur and Popkin's earlier (1965) figure of 323 mOsm, that from *Ascaris* after 3.5 hrs. *in vitro*, 360 mOsm, was much higher than Harpur and Popkin's measurement under similar conditions (335 mOsm).

The keeping medium used by Harpur and Popkin differed in that it contained potassium chloride, sodium bicarbonate and kanamycin. It was proposed to include sodium bicarbonate in the medium to see whether it would depress the haemolymph osmolality of *Ascaris* *in vitro*.

1. Procedure

Two batches of 10 worms were collected and maintained by the usual method except that the keeping medium for one batch contained 10 mM sodium bicarbonate, the sodium chloride content being adjusted to give the usual 325 mOsm strength.

After 3.5 hrs. *in vitro* the worms were exsanguinated and the osmolalities were recorded.

2. Results (see Table III)

The mean osmolality of the haemolymph from worms kept in the bicarbonate saline was 343 mOsm, still not as low as the figure given by Harpur and Popkin but significantly ($P = < 0.01$) lower than the mean osmolality, 355 mOsm, of haemolymph from worms kept in plain saline medium. The bicarbonate (or carbon dioxide) level in the medium would appear, then, to be one factor modifying the haemolymph osmolality *in vitro*.

TABLE III

THE EFFECT OF BICARBONATE ON THE OSMOLALITY OF THE HAEMOLYMPH OF *IN VITRO* *ASCARIS*

Treatment	Osmolality (mOsm) S.E. = ± 2.25 (10)
Bicarbonate in medium	343
No bicarbonate in medium	355

C. SUCCINATE IN THE HOST'S SMALL INTESTINAL CONTENTS

The rapid drop in concentration of haemolymph succinate already noted, coupled with the observation (Harpur,1969) that bile-stained Ascaris faeces (by implication, of host origin) may contain much higher concentrations of succinate than unstained faeces from worms in saline medium, led to the notion that some of the succinate might have an exogenous origin. Accordingly the small intestinal contents of various vertebrates (pigs, sheep and rats) were analysed for succinate. The methods of collection and analysis have already been described in Chapter II.

The results were positive. Table IV shows succinate concentrations as analysed by gas chromatography. As this was the first known report of succinate in the vertebrate small intestine, confirmatory analyses were made by the enzymatic method (Kmetec, 1966) and these analyses are summarised in Table V. Enzymatic assay gave consistently higher concentrations of succinate than gas chromatography but the difference was found to be linear.

TABLE IV

SUCCINATE CONCENTRATIONS (mM) IN THE SMALL INTESTINAL CONTENTS OF VARIOUS VERTEBRATES AS MEASURED BY GAS CHROMATOGRAPHY

	Animal No.	1	2	3	4	5	6	7	8
	Portion of small intestine								
Pigs	Ant. 1/3	-	-	-	-	11.1	4.4	1.4	1.4
	Mid 1/3	9.3	7.4	4.8	1.0	5.0	2.7	+	2.7
	Post. 1/3	-	-	-	-	0.4	0.5	+	0.5
Rats	Entire length	3.5	3.9	3.5	3.6	2.6	7.4	-	-
Sheep	Mid 1/3	0.5	+	+	+	-	-	-	-

(+ = trace only; - = no analysis made

TABLE V

SUCCINATE CONCENTRATIONS (mM) IN THE SMALL INTESTINAL CONTENTS OF VARIOUS VERTEBRATES AS MEASURED BY KMETEC'S (1966) ENZYMATIC METHOD

		Animal No.	1	2	3	4	5	6
	Portion of small intestine							
Pigs	Ant. 1/3		-	-	-	-	11.8	5.3
	Mid 1/3		9.8	-	5.4	1.2	5.8	3.6
	Post. 1/3		-	-	-	-	0.5	0.8
Rats	Entire length		4.3	4.7	-	-	-	-
Sheep	Mid 1/3		-	0.3	0.4	-	-	-

(- = no analysis made.)

D. WHAT IS THE ORIGIN OF SUCCINATE IN THE VERTEBRATE SMALL INTESTINE?

The origin of succinate in the contents of the small intestine of adult vertebrates, reported above, is unknown, and, indeed, succinate has not been previously reported in this locality. One possible source is the food; another is leakage or secretion from the intestinal lining. An experiment was designed to investigate the former possibility.

1. Procedure

Sixteen (2 replicates of 8), 150-200 gm male rats were used in the experiment, comparisons being made between starved animals, animals on a succinate-free diet, and animals given sodium succinate in their drinking water. In order to widen the scope of this investigation the experiment was expanded to determine whether prevention of coprophagia could modify the succinate content of the gut: thus, half the animals were fitted with anti-coprophagia tail cups of the type designed by Barnes *et al.* (1957), including their modification (Barnes *et al.*, 1963).

A succinate-free diet, suitable for feeding to rats, was made up of the following:

Ingredient	%
Dried egg white	9.75
Purified soybean protein	9.75
Starch/sucrose (1:1)	72.30
Soybean oil	2.00
Vitamin diet fortification mixture *	2.20
Mineral salt mixture RH *	4.00
Total	100.00

*Nutritional Biochemicals Corporation.

The above components were mixed together with water, pelleted, and dried. Sodium succinate, when present in the drinking water, was at a concentration of 30 mM. All the rats were starved for 48 hrs. and then put on the treatment schedule for 72 hrs., at the end of which they were killed and their small intestinal contents were collected, filtered through glass wool, and frozen until analyzed. Succinate concentrations were determined by the enzymatic method (Kmetec, 1966).

2. Results

Table VI summarises the major results. Although succinate concentrations in the 16 samples of gut contents ranged from 0.10 mM to 4.13 mM, statistical analysis showed no differences between treatments. These data bring out two points of major importance (Table VI):

1. Succinate was always present in the gut contents whether or not it was included in the drinking water.

2. There was no significant difference in the concentration of succinate whether the rats were starved, or whether they were fed a succinate-free diet.

The experiment indicates that the succinate in the gut lumen is of internal origin. It is interesting, in this respect, that starved rats were found to drink only a fifth the water drunk by fed rats (approximately 19 ml and 96 ml per rat, respectively), so that although the experiment could have been designed to eliminate this event, it can be concluded that wide variation in the amount of succinate-containing water consumed had no effect on the succinate concentration in the gut.

TABLE VI

EFFECT OF EXOGENOUS SUCCINATE SUPPLY AND STARVATION ON THE CONCENTRATION OF SUCCINATE IN THE SMALL INTESTINE OF RATS

Concentration (mM). S.E. = ± 0.53 (4)

		Succinate-free diet	
		Starved	Fed
Succinate in drinking water	0.0 mM	1.69	2.16
	30.0 mM	1.32	1.55

(Eight rats were permitted coprophagia and eight rats were fitted with anti-coprophagia tail cups. There was no difference between these treatments.)

E. EFFECT OF ANAEROBIASIS AND SUCCINATE *IN VITRO*

This experiment was conducted to see whether the major changes, *in vitro*, of the organic acids of *Ascaris* haemolymph could be corrected. Is the appearance of tiglate related to aerobic conditions and can succinate in the medium (as it is present in the host's gut contents) augment the haemolymph concentration of this acid?

1. Procedure

Upon collection from the host, 5 large, female worms were placed in each of 4 insulated flasks containing 1 litre of saline (325 mOsm) with kanamycin sulphate (5 mg %). Two of the flasks contained succinate (10 mM, 1/3 acid, 2/3 sodium salt) and, prior to collection of the worms, one of these, and one of those containing simple saline, were flushed with nitrogen. The temperature of all the flasks was adjusted to 39°C by adding warm saline of appropriate composition. The gassing and temperature-adjusting procedures were repeated following collection of the worms and upon arrival at the laboratory. The worms were exsanguinated 4 hrs. after the estimated time of the host's death (3.5 hrs. *in vitro*).

2. Results

Three acids, propionic, *n*-valeric, and tiglic showed

concentration changes in relation to the treatments (Table VII). Succinate, although apparently increasing slightly in concentration when this acid was also present in the medium, showed no significant change. The presence of succinate in the medium, however, tended to depress the concentration of all three acids shown (Table VII) as did nitrogen, although the effects were not additive.

TABLE VII

EFFECTS OF AIR AND SODIUM SUCCINATE ON CONCENTRATION (mM) OF ORGANIC ACID RADICALS IN HAEMOLYMPH

Each figure is the mean value from five worms

Factors		A	Air		Nitrogen		P†
		B	Succinate		Succinate		
Acid radicals			0	10 mM	0	10mM	
Propionic	AB*		4.27	2.22	3.75	2.94	<0.05
n-valeric	AB*		3.16	2.45	2.43	2.65	<0.05
Tiglic	A* AB*		1.31	0.81	0.50	0.53	<0.05

* Probability <0.05 for the factor and/or interaction shown.

† Probability for difference between means. A mean with an unbroken underline is not considered different from a mean similarly marked by the same line. (cf. Duncan, 1955).

IV DISCUSSION

"It is well known that nematodes are usually very resistant to unfavourable environmental conditions, and it is possible to keep some parasitic species alive and active in the laboratory for periods ranging from a maximum of about 1 month in the case of intestinal parasites of warm-blooded animals to several months in the case of some blood and tissue forms. This characteristic hardiness has made possible many valuable observations, but the comparatively short life of the worms under experimental conditions and the changes which occur in their behaviour, metabolic and chemical composition are reminders that we can hardly regard most of the specimens which have been investigated experimentally as "normal and healthy". Results obtained from them should therefore be interpreted with caution." (Hobson, 1948). The relevance of these remarks, as they apply to a study of Ascaris, will become apparent in this discussion.

Before continuing to the main body of the discussion, two possible sources of change in the haemolymph organic acids of in vitro Ascaris should be mentioned. Although at first sight, these may appear to be of importance, they are considered unlikely to have been influential in the experiments repeated in this presentation. The first of these possibilities

is that the changes in organic acids are due to imbalance of the excretory process rather than metabolic malfunction. The final excretion products show some selection (von Brand, 1966) and the excretory mechanism may be in the pattern of ultra-filtration followed by selective reabsorption (Harpur, 1969; Weinstein, 1960) so that any interference with this process could alter the composition of the haemolymph causing an uncontrolled build-up or depletion of organic acids. In connection with this possibility, the hydrostatic pressure of Ascaris haemolymph (which may well influence excretory function) is thought to be lower than usual at the time of collection of worms and to rise during subsequent in vitro maintenance (Harpur, 1964). However, as there was no significant change in the total concentration of organic acids in the haemolymph (Table II) and as the experiments were of relatively short duration it is not thought likely that any change in haemolymph organic acids was affected by excretory malfunction.

Similarly, objection might be made on the ground that the worms were starved during the in vitro periods. But this is not strictly true as Ascaris draws very readily on its glycogen reserve: Greichus and Greichus (1966), in a 7-day starvation experiment, did not expect to find that the haemolymph sugar concentration would be lowered until the worm's glycogen was completely depleted. Glycogen consumption by day

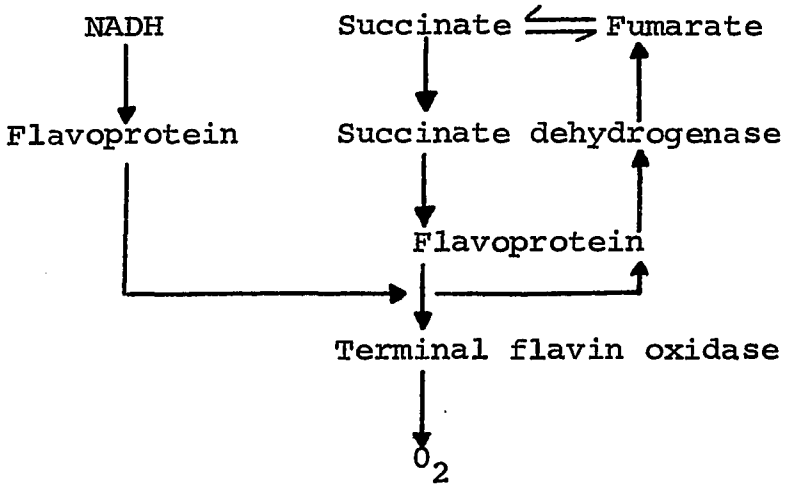
7 amounted to 71.3% of the original reservoir, so that even after several days starvation the reserve is not depleted. Starvation for a period as short as 8 hrs. should not, then, be an important factor influencing the fermentation products.

A. HAEMOLYMPH CONCENTRATION OF SUCCINATE

There are several possible reasons why the succinate concentration in Ascaris haemolymph drops in vitro. It is possible that in the laboratory the worms become less active, succinate produced endogenously during muscular contraction might then decrease. This effect has been observed in Ascaris paralysed by the anthelminthic drug piperazine (Bueding et al., 1959). However, fluoroscopic examination of human infections suggests that in their natural habitat, only about 8% of the worms are progressing (either orally or anally) and that the majority are more or less stationary (Makidono, 1956).

The second, and perhaps most likely explanation might be that oxygen interfered with the reduction of fumarate, the immediate precursor of succinate in Ascaris. Cestodes are known to excrete less succinate in aerobic media (Agosin,1957; von Brand and Bowman, 1961) and succinate can stimulate the oxygen uptake of Ascaris muscle (Rathbone, 1955). Furthermore, with the electron transport system proposed by Kmetec and Bueding (1961) and Bueding (1962) for the succinate dehy-

drogenase system of Ascaris, electrons would be directed to oxygen, rather than fumarate, under aerobiasis:



(NADH = reduced nicotinomide-adenine dinucleotide).

In Ascaris muscle, electrons from NADH may be directed either to oxygen or, in the absence of that gas, to fumarate. Despite the foregoing, low oxygen tension was found to have no effect in maintaining the level of succinate, so that aerobiasis is not (as yet) implicated.

A third possibility is that, in vivo, the worm has access to an exogenous source of succinate. This notion was originally suggested by the observation (Harpur, 1969) that bile-stained faeces (by implication, of host origin) of Ascaris may contain much higher concentrations of succinate than unstained faeces from worms in saline medium. The discovery of succinate in the host's small intestinal contents, reporte

earlier in this presentation, adds weight to this idea, but sodium succinate in the keeping medium did not significantly influence the haemolymph content.

Although the haemolymph succinate concentration was not modified by succinate in the keeping medium, the concentration of three other haemolymph organic acids was depressed (Table VII), producing an effect similar, though not additive, to low oxygen tension. Succinate presumably exercised this effect by behaving as a proton-donor, though whether this was mediated by the worm itself or by bacteria (despite the presence of kanamycin) cannot be told from the experiment.

The failure of succinate to be absorbed by worms *in vitro* could be due to a lack of some additional factor required for absorption in general by the intestine. For instance, the cestode *Schistocephalus solidus* will only absorb nutrients *in vitro* when glucose is added above a certain threshold (Hopkins, 1952). Also, studies on trypanosomids by von Brand and Agosin (1955) and Zeledon (1960) have shown that the Krebs cycle acids are not readily absorbed from neutral or alkaline surroundings and the requirement of the dog hookworm for host serum has already been mentioned (Chapter I, Part A, Section 6).

B. THE APPEARANCE OF TIGLATE

One of the most remarkable differences between pre-

viously recorded data (Harpur, 1969) and the analyses of fresh or "normal" haemolymph of Ascaris reported in this study is the absence of certain acids. It can be seen in Table II that the analysis after 7.5 hrs. in vitro includes nearly all the acids that have been reported for Ascaris. By contrast, the analysis of fresh material gives a much shorter list. Chief among the absentee acids is tiglic: this acid (limit of detection in the method used: approximately 0.05 mM) can easily be detected and measured after incubating the worms for 3.5 hrs. Tiglic acid, as mentioned in Chapter I, has been reported as an end-product of carbohydrate fermentation in Ascaris (Bueding, 1953) and has been reported in haemolymph analyses (Harpur, 1969).

It has been proposed that tiglic acid in Ascaris is a precursor of 2-methylbutyric acid (Saz and Weil, 1960) and its appearance at detectable levels in vitro may be due to reaction of oxygen with a hydrogen donor. In accord with this is the recent suggestion by Ward and Fairbairn (1970) that the reduction of tiglate to 2-methylbutyrate is catalysed by a flavorprotein and may be susceptible to the presence of oxygen. The repression of tiglate by nitrogen reported in this study (Table VII) indicates that exposure to oxygen is indeed involved in the appearance of tiglic acid de novo when Ascaris is kept in vitro.

C. CHANGES IN THE BRANCH-CHAINED ACIDS

Another change in the haemolymph organic acids is worth noting. The data in Table II suggest a negative correlation between the 2-methylbutyric and 2-methylvaleric acid radicals, the former progressively increasing in concentration while the latter is decreasing. The relation between these two acid radicals in individual worms is shown in Fig. 13 where it can be seen that the correlation is, in fact, positive for each sampling time. The apparently negative correlation, with time, is expressed by the change in position, relative to the axes, of the regression lines. Interpretation of these regression lines is difficult but they do, perhaps, clarify one point. The analyses in the literature show 6-carbon and 5-carbon acids to be the major metabolic end-products of Ascaris but sometimes 5-carbon acids (Greichus and Greichus, 1966; Harpur and Waters, 1960; Moyle and Baldwin, 1952; Puskarevs, 1965) and at other times 6-carbon acids (Ellison et al., 1960; Harpur, 1969) have been found to have quantitative dominance. The evidence here suggests that they are produced in equal amounts but the final balance is altered by an as yet unknown factor or factors (i.e. the slope indicates a 1:1 ratio but the shift of the intercept away from the origin superimposes a constant difference giving one or other of the acids dominance). For line C two plots were

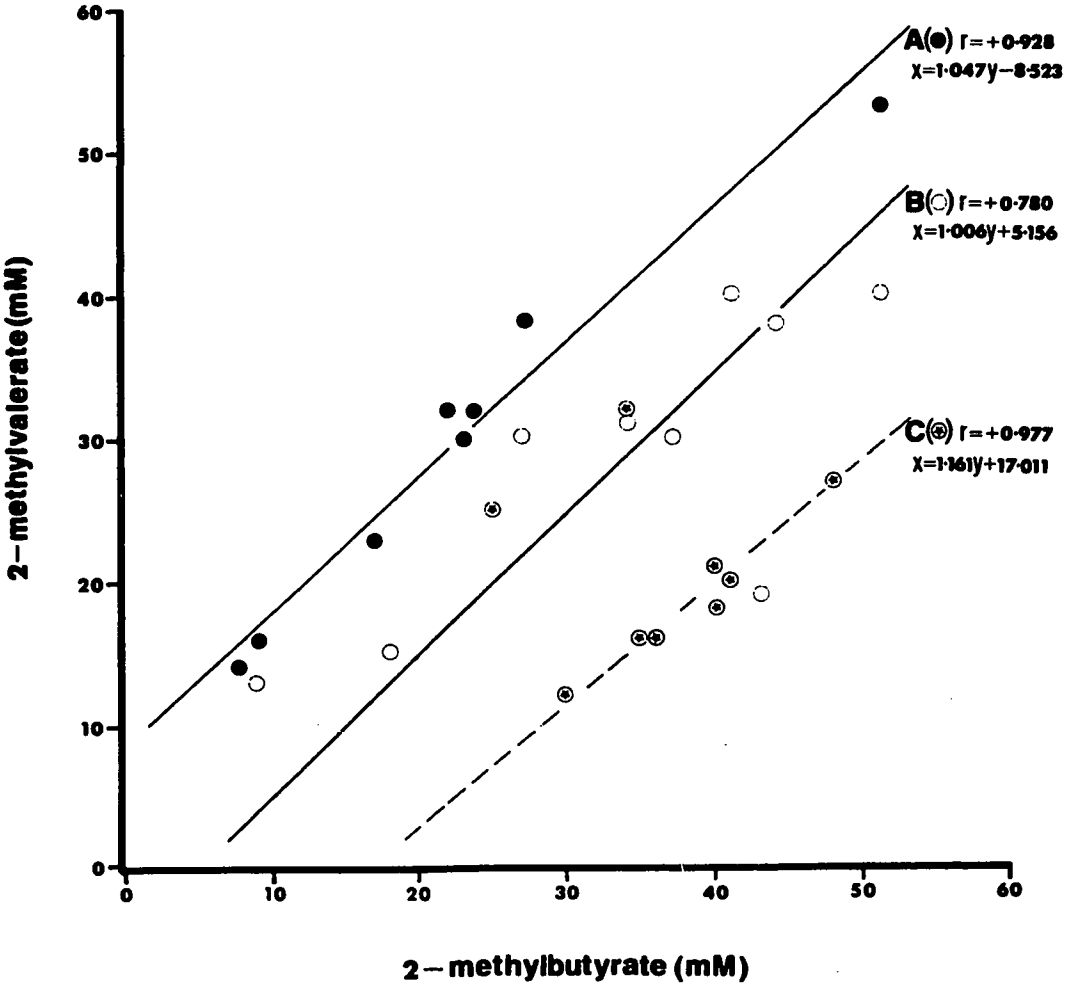


Fig. 13. Relation between the haemolymph concentration (mM) of 2-methylbutyrate and 2-methylvalerate. A: worms 0.5 hr after host-death. B: worms 4.0 hr after host-death. C: worms 8.0 hr after host-death. (The correlation coefficient and regression for C is tentative because the two plots belonging to C but located between lines A and B were omitted from the calculation.)

arbitrarily omitted from the calculation as the remainder give a slope with an angle similar to A and B. These two plots may represent worms which were not as "unhealthy" as the other "C" worms: similarly, one of the plots for B, which was included in B's calculation but makes little difference to its slope, appears close to the slope of C and may represent a worm "unhealthier" than the remainder of the "B" worms.

D. SUCCINATE AS A NUTRIENT

It is appropriate, here, to summarise the evidence for the use, by Ascaris, of an exogenous succinate supply. It has been suggested (Harpur, 1969) that the intestinal contents of Ascaris may contain much more succinate in vivo than in vitro. Subsequently, as reported in this study, considerable concentrations of succinate were found in the contents of the small intestine of pigs and lower concentrations were found in rats and sheep (Tables IV and V). There is, then, a source of succinate available to worms in their natural habitat. Finally, the haemolymph succinate concentration was found to drop rapidly during incubation of worms in saline medium (Table II).

The evidence therefore suggests that Ascaris has a requirement for exogenous succinate, but although sodium

succinate in the keeping medium may tend to maintain the haemolymph concentration of succinate (Appendix Table VIIA) this effect did not achieve statistical significance.

Possible reasons for the failure of worms to absorb succinate from the keeping medium have been mentioned earlier in this discussion (part A) and will not be repeated here, but some mention should be made of the possible use to Ascaris of a succinate supply augmented in the above manner. Several possibilities come to mind: the further reduction of succinate in Ascaris may be accompanied by reoxidation of NADH (Saz and Bueding, 1966) and the resulting fatty acids may be deposited in the eggs as a part of the food supply for the embryos (Saz and Lescure, 1966); additional supply of succinate could boost these processes. Further, it should be remembered that the Krebs cycle intermediates, of which succinate is one, may serve as precursors for other compounds, such as the amino acids.

E. SUCCINATE IN THE HOST'S INTESTINAL LUMEN

What, then, is the origin of the lumenal succinate? Perhaps the most likely source is in the desquamated epithelium.

An obvious solution would be to isolate intestinal epithelial cells and assay for succinate. This was tried on

one occasion using rats and following the method of Perris (1966). The result was negative: however, the yield of cells was inadequate and any succinate present in the cells may have diffused into the medium during the incubation procedure.

It has been shown that there is a rapid loss and replacement of the intestinal epithelium in mammals (see review by Leblond and Walker, 1956; Hooper, 1956), the cells being shed into the gut lumen. It is also known that the intestinal epithelial cells are very active and, among other enzymes, contain large amounts of succinic dehydrogenase (Padykula, et al., 1961). Once free in the lumen, the cells may lose some of their contents to the surroundings. The succinate concentrations in Tables IV and V appear to show a gradation along the pig's small intestine, the concentrations decreasing distally, so that,whatever its origin, succinate may be reabsorbed by the gut wall. Coincidentally, Ascaris has been found to consume large quantities of host epithelial cells, as explained earlier (Chapter I, part A, section 6) and, apart from providing a rich source of nutrients in general (Davey, 1964), these cells may be the origin of the high concentrations of succinate sometimes found in the intestine of Ascaris (Harpur, 1969)

As far as is known (an extensive search of the literature was made), succinate has not previously been reported

present in the small intestinal contents of vertebrates, although its occurrence there is not necessarily surprising. Experimental results shown in this presentation (Chapter III, part D) suggest that this concentration is independent of a dietary supply of succinate, indeed, it appears to be independent of food altogether.

V SUMMARY

A perspective of this presentation may be gained from the following:

1. The fermentation products of Ascaris, reported in the literature, constitute a variety of organic acids (Chapter I, part A, section 4) but it has been suggested (von Brand, 1966) that some of these may not normally be produced in vivo: such results should thus be interpreted with caution.

2. It was therefore decided, as part of this study, to analyse the organic acid content of the haemolymph of Ascaris obtained immediately on collection of the worms from the host: this would then establish, as closely as possible, the "normal" condition.

3. Gas chromatography showed the following organic acid radicals to be present in this (worms 0.0 hrs. in vitro) haemolymph: acetate, propionate, 2-methylbutyrate, n-valerate, 2-methylvalerate, and succinate (Fig. 8 and 9).

4. Similar analyses, after a 3.5 hrs. period of incubation of worms in a saline medium, detected an additional acid radical, tiglate (Fig. 10), which has been previously

(Bueding, 1953) shown to be excreted by Ascaris in vitro, and has been detected in haemolymph (Harpur, 1969). This result suggested that tiglate is not produced in vivo but is formed under the "unnatural" conditions of in vitro incubation.

5. After worms had been kept in saline medium for 7.5 hrs., three more peaks, in addition to that for tiglate, were obtained on chromatograms (Fig. 11). The first of these, eluting soon after tiglate, is tentatively identified as n-caproate. In this connection, Saz (1965) has mentioned that other C_6 acids, in addition to 2-methylvalerate, appear occasionally during in vitro maintenance of Ascaris. The other two peaks were not identified but, in all probability, represent organic acid radicals with a carbon chain length of 6 or greater.

6. Further definition of these compounds was obtained by gas chromatography of their methyl esters, again identifying the first to be eluted as n-caproate (Fig. 12).

7. Quantitative changes (Table II) which occur in the haemolymph organic acids of "normal" and in vitro Ascaris include a drop in the concentration of succinate and changes in the branch-chained acids (see Chapter IV, part C and Fig. 13).

8. No correlation was found between the organic acid content and the rise in osmolality of Ascaris haemolymph in vitro but bicarbonate in the medium may be a factor in supressing this rise (Table III).

9. Evidence suggested that Ascaris might make use of an exogenous supply of succinate (Chapter IV, part D). Considerable quantities of succinate were subsequently found in the small intestinal contents of pigs, rats and sheep (Tables IV and V) and, in rats, this was found to be independent of a dietary source of sodium succinate (Table VI). The source of this succinate is discussed in Chapter IV (part E).

10. Finally, incubation of worms under various medium conditions showed that oxygen may be a factor in the appearance of tiglate in the haemolymph of Ascaris in vitro (Table VII).

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APPENDIX

TABLE IIA.

EFFECT OF *IN VITRO* INCUBATION ON ORGANIC ACID RADICALS IN *ASCARIS LUMBRICOIDES* HAEMOLYMPH

1. Acetic

In vitro time (Hr.)	0.0	3.5	7.5
Mean log. concentration (mM) & No. of samples.	1.139(8)	1.205(8)	0.988(9)

Source	d.f.	variance estimate	F
Treatments	2	0.112	-
Error	23	0.452	
Total	25		

2. Propionic

In vitro time (Hr.)	0.0	3.5	7.5
Mean log. concentration (mM) & No. of samples	2.977(9)	0.688(9)	1.814(9)

Source	d.f.	variance estimate	F
Treatments	2	55.796	15.211**
Error	24	3.668	
Total	26		

** P = <0.01

3. 2-methylbutyric

In vitro time (hr.)	0.0	3.5	7.5
Mean log. concentration (mM) & No. of samples	1.349(9)	1.484(9)	1.556(9)

Source	d.f.	variance estimate	F
Treatments	2	0.096	1.740
Error	24	0.054	
Total	26		

4. n-Valeric

In vitro time (hr.)	0.0	3.5	7.5
Mean log. concentration (mM) & No. of samples	0.324(9)	0.957(9)	0.580(9)

Source	d.f.	variance estimate	F
Treatments	2	0.324	5.890*
Error	24	0.055	
Total	26		

*P = <0.05

5. 2-methylvaleric

In vitro time (hr.)	0.0	3.5	7.5
Mean log. concentration (mM) & No. of samples.	1.494(8)	1.421(9)	1.295(9)

Source	d.f.	variance estimate	F
Treatments	2	0.092	2.421
Error	24	0.038	
Total	26		

6. Tiglic

In vitro time (hr.)	0.0	3.5	7.5
Mean log. concentration (mM) & No. of samples.	0.000(9)	1.349(9)	1.095(8)

Source	d.f.	variance estimates	F
Treatments	1	0.255	1.328
Error	15	0.0192	
Total	16		

7. Succinate

In vitro time (hr.)	0.0	3.5	7.5
Mean log. concentration (mM) & No. of samples	1.084(8)	0.898(8)	0.707(9)

Source	d.f.	variance estimate	F
Treatments	2	0.163	6.037*
Error	22	0.027	
Total	24		

* P = <0.05

8. Total acids

In vitro time (hr.)	0.0	3.5	7.5
Mean log. concentration (mM) & No. of samples	1.956(9)	2.017(9)	1.896(9)

Source	d.f.	variance estimate	F
Treatments	2	0.033	-
Error	24	0.027	
Total	26		

TABLE IIIA

THE EFFECT OF BICARBONATE ON THE OSMOLALITY OF *ASCARIS* HAEMOLYMPH *IN VITRO*: STATISTICAL ANALYSIS

Source	d.f.	variance estimate	F
Between samples	1	720.0	14.20**
Within samples	18	50.6	
Total	19		

** P = <0.01

TABLE VIA

EFFECT OF EXOGENOUS SUCCINATE SUPPLY AND STARVATION ON THE CONCENTRATION OF SUCCINATE IN THE SMALL INTESTINE OF RATS

Source	d.f.	variance estimate	F
Replicates	1	2.747	2.412
Treatments	7	1.416	1.243
Error	7	1.139	
Total	15		

TABLE VIIA

EFFECT OF ANAEROBIASIS (A) AND SODIUM SUCCINATE (B) ON THE CONCENTRATION (mM) OF ORGANIC ACID RADICALS IN *ASCARIS* HAEMOLYMPH

1. Acetate

Source	d.f.	variance estimate	F
A	1	0.001	-
B	1	0.017	2.428
AB	1	0.027	3.857
Error	16	0.007	
Total	19		

2. Propionate

Source	d.f.	variance estimate	F
A	1	0.174	19.333***
B	1	0.004	-
AB	1	0.221	24.555***
Error	16	0.009	
Total	19		

***P = <0.001

3. 2-methylbutyrate

Source	d.f.	variance estimate	F
A	1	0.001	-
B	1	0.000	-
AB	1	0.020	2.857
Error	16	0.007	
Total	19		

4. n-valerate

Source	d.f.	variance estimate	F
A	1	0.014	1.750
B	1	0.013	1.625
AB	1	0.038	4.750*
Error	16	0.008	
Total	19		

* P = <0.05

5. 2-methylvalerate

Source	d.f.	variance estimate	F
A	1	0.001	-
B	1	0.006	-
AB	1	0.007	-
Error	16	0.008	-
Total	19		

6. Tiglic

Source	d.f.	variance estimate	F
A	1	0.041	-
B	1	0.260	5.200*
AB	1	0.354	7.080*
Error	16	0.050	
Total	19		

* P = <0.05

7. Succinate

Source	d.f.	variance estimate	F
A	1	0.012	-
B	1	0.006	-
AB	1	0.031	1.722
Error	16	0.018	