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THE *IN VITRO* CULTIVATION OF CESTODES

T H E S I S

for the

Degree of Doctor of Philosophy

in the

University of Glasgow

by

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May, 1967

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Poor text in the original thesis.

CONTAINS PULLOUT

C O N T E N T S

Section III

Cultivation of Hymenolepis nana in vitro.

Section IV

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Declaration

The work on the effect of temperature on the growth and maturation of *Schistocephalus solidus* *in vitro* has been accepted for publication under joint authorship with Dr. C.A.Hopkins (Sinha and Hopkins,1967) but the majority of the experimental work was carried out by Sinha. The works on the *in vitro* cultivation of *Hymenolepis nana* and the *in vitro* excystation of the cysticercoids of this tapeworm were carried out by Sinha and are reported in this thesis.

SECTION I. HELMINTH CULTIVATION : GENERAL CONSIDERATION AND TECHNIQUES

A. Introduction

The fascination for culturing metazoan parasites *in vitro* has resulted in substantial progress towards the successful cultivation of many types in recent years. Workers in this field are fully aware that *in vitro* cultivation of parasites can lead to a better understanding of host-parasite relationships, host specificity, nature of parasitism and of the physiological and biochemical aspects of their life processes. These techniques can also be profitably applied to obtain valuable information on the metabolism, nutritional and physico-chemical requirements, antigens and secretions and excretions of parasites. Read (1950) has very clearly emphasised this idea in the following words, "It is imperative to develop techniques for cultivating intestinal helminths *in vitro* and to make further study of intestinal physiology. It seems apparent that the cultivation of parasitic helminths outside the host presents one of the most difficult and most challenging problems facing parasitologists today."

Weinstein (1966) has also outlined various uses of this tool for exploring many other aspects of parasite-biology.

Though it is well appreciated that the task of

culturing helminth parasites *in vitro* is not an easy one, there is little doubt that such organisms can be induced to survive, grow and reproduce *in vitro* with proper provision of nutrition and physico-chemical environments. However, up till now no helminth parasite has been cultured and grown indefinitely in a chemically defined medium, though recent literature contains a considerable number of descriptions of such attempts aimed at either mere "survival" of parasites or promoting growth, development and maturation *in vitro*.

There is no simple recipe known for growing helminth parasites in the laboratory axenically or otherwise. The basic problems confronting investigators in this field have been well outlined by many workers (Smyth, 1959, 1962a, 1966; Dougherty, 1959; Silverman, 1963, 1965). They may be briefly summarised here.

1. Difficulty in maintaining helminth parasites through their life cycle in the laboratory.
2. Complexity of the life cycle of many helminths, with larval stages in one or more intermediate hosts, each providing a distinct nutritional and physico-chemical environment.
3. Imperfect knowledge of the physico-chemical characteristics of biological habitats, *viz*. intestine,

blood stream, perivisceral cavity etc.

4. Incomplete understanding of histological, cytological and biochemical aspects of the life cycle of parasites.
5. Sterilization for subsequent cultivation.
6. Maintenance of aseptic conditions in culture.
7. Development of a suitable medium of known chemical composition.
8. Provision of a suitable matrix or physical support.
9. Problem of the removal of toxic metabolic wastes from the culture.
10. Establishment of criteria for the assessment of the response of parasites <u>in vitro</u>.

Out of these, among many other problems, sterilization of the organisms and maintenance of aseptic conditions in cultures are essential prerequisites to cultivation. Many parasitic helminths live in sterile habitats (such as body cavity, blood stream etc.) within their hosts; others, especially the intestinal ones, are surrounded by a bacterial and fungal flora, without the elimination of which, results would be difficult to interpret. Fortunately, the availability of a wide variety of antibiotics and various kinds of bacterial filters has greatly simplified the problem.

The factors determining the physico-chemical characteristics are temperature, osmotic pressure, pH, oxygen tension, gas phase, oxidation reduction potential, matrix or physical support, type of culture vessel and the medium. A knowledge of the conditions prevailing in vivo is quite helpful in bringing about similar conditions in cultures but it is not necessarily a guide to the in vitro requirements of the parasites. The importance of these factors can be well appreciated in the light of the findings of various investigators engaged in this field of study.

Temperature

Smyth (1946) demonstrated that raising the temperature of culture containing even a non-nutritive medium stimulated the differentiation of the progenetic plerocercoids of Schistocephalus to sexually mature adults.

Weinstein and Jones (1956) found that a rise in temperature to 37°C was essential for the early differentiation of the third stage larvae of Nippostrongylus brasiliensis in vitro.

Berntzen (1962) showed that a temperature of 39° ± 0.5°C must be maintained for the maximal growth of Hymenolepis nana. At 37° ± 0.5°C, growth was markedly reduced.

Osmotic pressure and pH.

Helminths show some tolerance to small variations in osmotic pressure and are remarkably indifferent to wide variations in pH. The maintenance of osmotic pressure is an energy process and considerable differences between the osmotic pressure of the medium and that of the parasite can result in the leakage of substances which may be essential for growth and development. The pH of a medium in which any organism is growing may be quite different from that of its habitat. This may be owing to the fact that some of the nutritive substances and growth promoting factors may be in a state readily utilisable by a growing organism at one pH but not at the other. A good example is Haemonchus contortus which develops in vitro at pH 6.4-6.5 but at pH 1.0-1.2 in vivo (quoted by Silverman, 1963).

Oxygen tension and gas phase

"The parasitic worms studied so far can all be classified as facultative aerobes, most of them showing a marked tolerance to lack of oxygen, but all being able to consume oxygen if available." (von Brand, 1966, p.336). The sensitivity of parasites to high oxygen tensions or their requirements of oxygen varies from

species to species, depending on the normal habitat of the parasite. Recent findings have indicated that CO_2 is an essential component of the gas phase in cultures.

Berntzen (1962) and Berntzen and Mueller (1964) have demonstrated the effect of oxygen tension and a suitable gas phase in their experiments on the cultivation of Hymenolepis nana and Spirometra mansonoides respectively. They found that a mixture of 5-10% CO_2 in nitrogen was the best gas phase and the presence of oxygen, even at a concentration of 5%, caused death of the worms.

Sommerville (1964, 1966) experimenting with Haemonchus contortus discovered that the third stage filariform larvae of this nematode formed an enlarged buccal capsule and moulted to 4th stage when exposed to high concentrations of CO_2.

On the other hand, Berntzen (1965) obtained most successful cultures of Trichinella spiralis with a gas phase of 85% N + 5% CO_2 + 10% O_2 and, recently, Smyth and Howkins (1966) have also shown that maturation of Echinococcus granulosus was most successful in a diphasic medium with a gas phase of 8% O_2 + 5% CO_2 in nitrogen.

Redox potential

Though this characteristic has been little investigated and its significance hardly understood, Berntzen and Mueller (1964) have shown that a negative oxidation-reduction potential of the value of -160mV to -190mV (direct reading on a Beckman Model G meter with a Beckman # 39186 platinum combination electrode) controlled by addition of appropriate amounts of reduced glutathione and cysteine hydrochloride, was essential for the growth and differentiation of Spirometra mansonoides plerocercoids to young adults. They also demonstrated that a redox value of less than -100mV or more than -250mV prevented either the differentiation of scolex or caused the death of the worms in culture. These negative values appear to be rather confusing. In fact, the values of -160mV and -190mV would be +62mV and +32mV respectively if the reference electrode used by these authors had a potential of +222 mV (see Hopkins, 1967).

Physical support

Helminths are equipped with various kinds of structures such as "teeth", hooks and suckers with which they attach themselves to the organ of the host in which they live. Provision of an organic support

in a culture may make the worms "feel" that they are in the right environment. Some workers have used such supports in their cultures. Berntzen (1962) lined culture tubes with filter paper to which, he claimed, Hymenolepis nana were found attached. Recently, Smyth (1967) has emphasised the importance of a nutritive solid substrate in his diphasic medium in which he has grown Echinococcus granulosus from the protoscolex stage to a 3-segment strobilate tapeworm.

Culture vessel

The selection and design of a culture vessel depends on the nature and size of the organisms to be cultured. Preferably it should be such as to permit an easy changing of the medium and rapid removal of metabolic wastes accumulating in the vicinity of the worms. Undoubtedly, roller tubes are the simplest type of culture vessel but in recent years Clegg (1961) and Berntzen (1962, 1965) have designed "continuous flow culture apparatus" permitting a controlled flow of medium over the worms, efficient equilibration of the medium with the gas phase, easy changing of the medium and observations of the worms.

Medium

Many well known media, for example 199, Eagle's or Waymouth's, have been used for tissue culture work but they have proved to be unsatisfactory and in some cases toxic for helminths, even if supplemented with tissue extracts or serum. It has always been the attempt of workers in this field to devise chemically defined media, to know about the nutritional requirements of worms and other aspects of their physiology, in strictly defined chemical terms, but success has been achieved by a few workers only with arthropods (House, Gordon, Sang etc., quoted by Dougherty, 1959). [With helminths, only oligidic (medium containing crude organic materials) or meridic (medium containing one ingredient of unknown structure) (Dougherty, 1959) have been successfully used so far.] Dougherty and his co-workers (1948 et seq.) have provided a considerable body of information in a series of papers on the cultivation of Caenorhabditis briggsae. Many helminths, e.g. plerocercoids of Schistocephalus and Ligula (Smyth, 1949, 1950, 1954) and the microphallid trematode Gynaecotyla adunca (Hunter and Chait, 1952) have a store of endogenous reserves which permit development to maturity in a non-nutritive medium if in a favourable physico-chemical environment, but for the majority, nutritive components of a medium are as

important as the provision of a suitable physico-chemical environment.

Apart from the problems encountered in the cultivation of helminths, cestodes present special difficulties because of being intestinal parasites as adults and therefore rich in microflora covering their bodies, and also because of having a large strobila and no alimentary canal. This means that they are difficult to sterilise if recovered from the alimentary canal of the host and also that the medium in which they are to be cultured must have components that can be readily assimilated without further breakdown which these animals are unable to perform (Smyth, 1962 a).

Tapeworms have also evolved a number of physiological modifications as a result of obligate parasitism, viz:

1. dependence on the supply of carbohydrate by the host for growth and reproduction
2. ability to utilise only a few simple sugars
3. dependence on the chemical regulatory mechanisms of the host
4. specific response of egg, oncospheres and larvae to distinct physico-chemical factors during establishment within a host.

In spite of these difficulties, the structural simplicity of tapeworms renders them very suitable for investigation on parasite physiology. Studies in vivo with the use of radio-active isotopes and techniques of chemical analysis have yielded a considerable body of information but such results have always been open to criticism . Many attempts have been made to culture cestodes in vitro using everything from river water to complex media consisting of a multitude of ingredients of known and unknown chemical structure. The earlier workers reported mainly on the "survival" of cestodes in simple or complex media without applying any suitable criteria for the assessment of the normality, growth and differentiation of the worms in cultures and they are of little value today.

The literature on the in vitro cultivation of cestodes has been comprehensively reviewed by Smyth (1947 a) and supplementation of this with those of Baer (1952) and Wardle and McLeod (1952) completes the report on the work done before 1950. The period after this date has seen some real successes in the field of cestode cultivation and the reports have been adequately reviewed by Silverman (1965) and Weinstein (1966). A summary of these reports has been shown in Table 1.

TABLE 1. Cestodes cultivated *in vitro* with partial or complete success in recent years.

Cestode	Author	Initial stage	Result
Spirometra mansonoides	Mueller 1959	Procercoid	Growth from 0.1 to 10 mm in 28 days; plerocercoid infective to cat.
,,	Berntzen & Mueller 1964	,,	Young adult, 48-64 mm long with 249-301 proglottids.
Schistocephalus solidus	McCaig & Hopkins 1965	Plerocercoid	Definite growth (about 500% in small worms) as shown by increase in dry wt.
Taenia crassiceps	Robinson *et al.* 1963	Larvae	Budding, development of hooks, suckers, scolex; strobilation of mature cysticerci.
,,	Taylor 1963	,,	Growth up to 22-27 mm.
Hymenolepis diminuta	Schiller *et al.* 1959	scolex & a small part of neck	30-fold size increase with about 130 proglottids; no maturation
,,	Berntzen 1961	excysted cysticercoid	Worms with proglottids containing developing oncospheres.
,,	Schiller 1965	,,	Egg-bearing adults
Hymenolepis nana	Berntzen 1962	,,	Egg-bearing adults

TABLE 1 (contd.)

Cestode	Author	Initial stage	Results
Echinococcus multilocularis	Rausch & Jentoft 1957	Finely divided larval tissue	Numerous vesicles and scolices after 42 days; vesicles infective.
,,	Yamashita et al. 1962	Larval scolices	Hydatid cyst with germinal layer by 45th. day.
Echinococcus granulosus & E. multilocularis	Webster & Cameron 1963	Intact scolices, cysts,	segmented E. multilocularis
E. granulosus	Smyth 1962b	Protoscolices	Miniature hydatid cysts with laminated envelopes
,,	Smyth & Howkins 1966	28-38 days strobilated worm from dog	Maturation and egg production.
,,	Smyth 1967	Proto-scolices	Worms with one segment in 30 days; 3 segments in 60 days; production of sperms and infertile eggs if mixture 199 in the medium is is replaced by 'Parker 858'.

In the light of this information and the fact that an attempt, if successful, would be a rewarding one, it was decided to undertake the work on cultivation of cestodes *in vitro*. The work was started with the cultivation of the plerocercoids of *Schistocephalus solidus*, a pseudophyllidean, for the following reasons:

1. This cestode parasite has been under investigation for more than twenty years and sufficient information is available on its structure and life history (Hopkins and Smyth, 1951; Clarke, 1954; Hopkins and McCaig, 1963; McCaig and Hopkins, 1963), glycogen metabolism and utilisation (Hopkins, 1950, 1952) and cultivation *in vitro* (Smyth, 1946, 1950, 1952 and 1954; McCaig and Hopkins, 1965).
2. A medium (McCaig and Hopkins, 1965) in which plerocercoids of *S. solidus* would grow was known.
3. These plerocercoids, recovered sterile from the body cavity of sticklebacks, are quite suitable for a beginner to learn general laboratory techniques required in this field, simultaneously with obtaining data on the problem under investigation.

However, the seasonal availability of the plerocercoids in good numbers, and the difficulty in obtaining

plerocercoids of known age and weight without a method of maintaining the cestode through its life cycle in the laboratory made it difficult to continue with this tapeworm, although many interesting problems could have been investigated apart from attempting to develop a more suitable medium. It was also realised that work with a cyclophyllidean species would permit a comparative observation and extend the scope of the training. The dwarf tapeworm, Hymenolepis nana, was therefore selected and all attention centred around cultivation of this worm from artificially excysted cysticercoids to egg-bearing adults.

The results obtained from the in vitro cultivation of these two cestodes, Schistocephalus solidus and Hymenolepis nana are reported in this thesis which is divided into four sections. The first section is devoted to the information on the cultivation of helminths in general, routine procedures and laboratory techniques and preparation of medium ingredients. The second section deals with the growth and maturation of S. solidus in vitro. The third section is devoted to Hymenolepis nana, covering all experiments which led to the development of a successful medium promoting growth of excysted cysticercoids to egg-bearing adults.

In the fourth and last section are described the results of experiments on excystation of cysticercoids of H.nana in vitro. In each section, the experimental results have been discussed and summarised.

B. Cleaning and sterilization of glassware and other equipment

Cleaning

1. All glassware was placed in water immediately after use. The collected glassware was immersed in a warm solution of "Pyroneg" (Diversey Ltd., London) prepared by adding ½ oz. of the detergent to one gallon of hot tap water in the wash sink or a bowl, and allowed to soak for 1-2 hours. Flasks, bottles, and roller tubes were brushed to remove any adherent debris and rinsed several times to ensure complete removal of the detergent. Pipettes and continuous flow culture apparatus were rinsed for 1½-2 hours in a siphon type pipette-washer. All cleaned glassware was then dried in an oven at 100°C.

2. "Suba seal" turnover type closures, rubber liners from screw-capped bottles, and metal caps were cleaned in boiling tap water. "Esco" rubber bungs and silicone rubber tubing were cleaned for 30 min in boiling 1% $NaHCO_3$ solution, cooled, washed, placed for 2 hours in 1% HCl solution and then rinsed in 3-4 changes of tap water.

3. Gas filter tubes were cleaned by heating for 20 min at 90°C in a solution of 0.5% $NaClO_3$ and 0.5% $NaNO_3$ in concentrated H_2SO_4. The filters were left overnight in this solution and then washed thoroughly by

passing several litres of tap water.

Sterilization

1. Pipettes were plugged with non-absorbent cotton wool and their ends were wrapped in paper. They were then packed in glass cylinders the open ends of which were closed with non-absorbent cotton wool and covered with paper.
2. Crystallizing dishes and petri dishes were covered with their lids and wrapped in paper.
3. "Suba seal" turnover type closures and rubber bungs were packed in crystallizing dishes lined with filter paper.
4. The caps of screw-capped bottles were slackly screwed in place.
5. Continuous flow culture apparatus were assembled and their open ends were covered with paper. Each culture unit was then separately wrapped in paper.

All of these were sterilized by autoclaving at 15 p.s.i. for 20 minutes.

Syringes, needles, and dissecting instruments were sterilized in a boiling water sterilizer.

C. Preparation of medium ingredients

1. Balanced Salt solution (Hanks' formula, modified).

This was prepared in two parts using only 'ANALAR' grade (BDH) salts.

Hanks' I

NaCl	8.40g
KCl	0.40g
NaH_2PO_4	0.10g
$Na_2HPO_4.2H_2O$	0.10g
de-ionized water	900 ml
0.2% Phenol Red	10 ml

Hanks' II

$CaCl_2$ (dried)	0.14g
$MgCl_2.6H_2O$	0.10g
de-ionized water	100 ml

These two parts were dispensed separately in appropriate quantities into screw-top bottles and sterilized by autoclaving at a pressure of 15 pounds per square inch (p.s.i.) for 15 minutes. One part

of Hanks' solution II was mixed with nine parts of Hanks' solution I to obtain a desired quantity of normal Hanks' solution.

Hanks' original formula was modified as explained below:

$MgSO_4.7H_2O$ was omitted to prevent precipitation of calcium sulphate or Magnesium phosphate. To compensate osmotically the phosphates were increased from 0.06 g/l to 0.1 g/l. Glucose, 1.0 g/l in the original formula was omitted and 0.16 g/l NaCl added instead. Similarly, 0.35 g/l $NaHCO_3$ in the original formula was replaced by 0.24 g/l NaCl. NaH_2PO_4 was used in place of KH_2PO_4.

Phenol Red solution was prepared as recommended by Hanks (1955). 2.0g were dissolved in 100 ml of 0.05N-NaOH. The volume was made up to one litre by addition of de-ionized water and pH adjusted to 7.0 with 0.05N-NaOH. The solution was then stored at -15°C in 50 ml aliquots in screw-top bottles.

2. <u>Sodium bicarbonate solution</u> - was prepared as an isotonic 1.4% (w/v) solution in de-ionized water, dispensed in 5 ml and 10 ml aliquots into screw-top universal containers and sterilized by autoclaving at 15 p.s.i. for 15 minutes.

3. Glucose solution - this was prepared as an isotonic solution by dissolving 6.5g of 'ANALAR' D(+) Glucose (Dextrose) in 100 ml of de-ionized water, dispensed into screw-top universal containers in 5 ml and 10 ml quantities, and autoclaved at 15 p.s.i. for 15 minutes for sterilization.

4. Yeast extract solution - was prepared as a 5%(w/v) solution of the dry powder (from various sources) in de-ionized water, tubed in 5 ml and 10 ml aliquots, and autoclaved at 10 p.s.i. for 10 minutes. For many experiments, yeast extract was sterilized by filtration. This was done by passing the solution successively through a RA 1.2μ (with a microfibre glass prefilter), AA 0.8μ and an HA 0.45μ, and finally through a sterilized GS 0.22μ Millipore filter (Millipore (U.K.) Ltd., Wembley, England).

The solution sterilized by either method was stored at 4°C. Solutions more than three weeks old were not used.

5. Chick embryo extract (CEE_{50})

Chick embryo extract (CEE_{50}) was prepared using 11-day-old chick embryos. The method employed was similar to that described by Paul (1960). Twenty-four eggs were used at a time. All instruments and glass-

ware used were sterile and the processing of the eggs was done in a sterile cabinet.

1. Each egg was held with its blunt end up in a beaker of suitable size standing in the centre of a large perti dish.

2. A small amount of 95% alcohol was poured over the egg to wet the egg-shell, which was then flamed to hasten drying as well as to ensure sterilization.

3. The blunt end of the egg was carefully cut open with a pair of scissors and the membrane beneath the air space was removed using a pair of forceps.

4. The embryo was held by its neck with the help of a sterile steel hook, taken out of the egg and placed in a dish containing Hanks' saline.

5. The embryo was washed again in Hanks' contained in a second dish to free it from adhering blood and yolk, and transferred to the barrel of a 10 ml Luer syringe.

6. The tip of the syringe was inserted into a 250 ml centrifuge flask held in a clamp and the embryo was expressed by pressing the plunger. The process was repeated with each egg taking care that each instrument used was sterile.

7. An equal volume of Hanks' BSS was added to the pulp of the embryos in the centrifuge flask, stirred with a sterile glass rod and allowed to stand at room temperature for 30 minutes.

8. The mixture was then centrifuged for 20 minutes at nearly 2000 g at 10°C.

9. The supernatant (CEE_{50}) was dispensed in 10 ml aliquots into screw-top universal containers and stored at -15°C.

Before use, the frozen extract was thawed slowly in cold water and the precipitate formed was dispersed evenly. Extracts more than six weeks old were not used.

The methods of preparation of several other ingredients which were used for the preparation of media are described at appropriate places.

SECTION II. THE EFFECT OF TEMPERATURE ON THE GROWTH AND MATURATION OF *SCHISTOCEPHALUS SOLIDUS* IN VITRO

A. Introduction

The plerocercoids of some pseudophyllidean cestodes are especially suitable for cultivation as they can be collected aseptically from their fish intermediate hosts and cultured for long periods in simple media under controlled physico-chemical conditions. Those of Schistocephalus solidus Muller, 1776, commonly occurring in the perivisceral cavity of 3-spined sticklebacks (Gasterosteus aculeatus) are particularly suitable on account of being progenetic, possessing genitalia in a stage of development capable of maturing in the gut of the definitive host (a fish-eating bird) in about 36 hours, and also in vitro, even in non-nutrient media, within 48 hours at 40°C (Smyth, 1946). Secondly, they also represent the stage in the life-cycle of S. solidus most easily obtained in nature.

As a result of Smyth's work (summarised 1959), it became clear that it was the temperature and not the appropriate nutritional level that was required for the maturation of Schistocephalus. This was just a matter of tissue differentiation and the endogenous reserves of the plerocercoid were enough to provide energy for this purpose in a suitable physico-chemical environment. The nature of the mechanism which

switches development from the plerocercoid pathway to that of the adult is one of the most interesting problems in the life-cycle of the worm. Although it is a general problem in cestodes, it is particularly susceptible to investigation in Schistocephalus as the external signal which initiates the switch over is simply a rise in temperature.

It is well known that of all the environmental factors, temperature has diverse effects on living organisms, for it controls rate of chemical reactions, and determines growth, metabolism, survival, and distribution of animals on the earth. Because of inhabiting the perivisceral cavity of a fish, conforming passively to changes in temperature in the environment, the plerocercoids of S. solidus are subjected to a wide range of temperature variation in life, and, above all, they encounter a sudden rise in temperature of around 30°C on entry into the bird's intestine.

Smyth (1952) reported that below 35°C, plero-cercoids of Schistocephalus could not grow into normal sexually mature adults capable of producing eggs which would embryonate. At 33°C only highly abnormal eggs were produced by a few worms. This means that below

this temperature, the genetical coded system directing the production of sperms and ova is not operative. Hopkins and McCaig (1963) found that somatic growth of Schistocephalus ceased after entry into the intestine of a duck (i.e. after maturation), and that the worms lost weight in a duckling, about 10% in the first 48 hours and 20% in 96 hours. It is not known whether this cessation of growth is due to the rise in temperature causing suppression or denaturing of enzymes catalysing growth processes, or to a poor nutritional level in the host's intestine. These alternatives are not easily resolved with in vivo experiments.

In the absence of any published report on the effect of temperature on the growth of larval pseudophyllideans, it was decided to undertake an investigation on the effect of temperature on the growth and maturation of the plerocercoids of Schistocephalus solidus in vitro. The development of a medium (BSSG+HS+YE; McCaig and Hopkins, 1965) in which plerocercoids of Schistocephalus would grow, made it possible to investigate the effect of temperature more precisely as other conditions could be held constant.

In this investigation, answers to the following questions were sought:

1. What is the effect of low temperature on the rate of growth of the plerocercoids of Schistocephalus? This would give an idea of the growth rate of these worms in fish during the winter months, and would be especially useful to an ecologist interested in knowing about the duration of the life-cycle in nature, and also to an experimental parasitologist who would like to store the worms alive in the laboratory on account of their non-availability (due to difficulty of catching fish) during the colder months of the year and the difficulties involved in maintaining this cestode in the laboratory through all stages of the life-cycle.

2. At what temperature is the growth rate maximal? The adequacy of various types of media and several other variables of culture conditions can then be tested at that temperature.

3. What is the relationship between temperature and growth rate? Is the Q_{10} approximately constant over the whole temperature range at which growth occurs suggesting adaptation of the plerocercoid to specific temperature ranges?

4. What is the upper limit of temperature at which growth can occur?

5. Is the change from growth of larval tissues to maturation an "all or nothing" switch over at a specific temperature or is there a gradual decrease in growth rate above a certain temperature suggesting in the latter case that the enzyme system for larval growth gradually delcines in efficiency to be replaced by an anzyme system which canalises metabolites into gamete production?

6. Is maturation which occurs due to a rise in temperature to 35°-40°C an irreversible process, or will a drop in temperature cause suppression of gamete production and induce growth again? In other words, is the genetic code guiding larval growth destroyed once the step to adult maturation is taken?

7. Is the failure of the worms to grow and the loss of weight at temperatures at which maturation occurs, due to release of eggs or to some nutritional deficiency in the gut of the definitive host?

Cultivation of the plerocercoids in vitro seemed to be the ~~most ideal~~ best approach to find out the answers to these questions.

Finally, an attempt was also made to supplement the medium BSSG+HS+YE with chick embryo extract to see if that would support better growth for a longer period.

B. Material and Methods

Infected 3-spined sticklebacks (Gasterosteus aculeatus) carrying plerocercoids of Schistocephalus solidus were netted from ponds and reservoirs in and around Glasgow and maintained in the laboratory aquarium in running water in polythene tanks. On the day of setting up of cultures, the fish were killed by pithing, dipped twice in 90% ethanol, to which a few drops of I_2+KI solution was added, and allowed to dry. Using sterilized instruments, the plerocercoids were removed from the perivisceral cavity of the fish after making a ventral incision in the abdomen, and weighed immediately in closed sterile pouches made of aluminium foil, to determine the wet weight. The plerocercoids were then placed singly in 125 x 25 mm roller tubes each containing 5 ml of medium BSSG+HS+YE (McCaig and Hopkins, 1965; slightly modified formula) of the following composition:

(i) Hanks' balanced salt solution (BSS, modified formula see Section I).

(ii) Glucose (G) added as a 6.5% solution to give a final concentration of 0.65%.

(iii) Bacto Yeast extract (YE) added as a 5% solution (autoclaved) to give a final concentration of 0.5%.

(iv) Horse serum No. 2 (recalcified and heated at 56°C, as supplied by Burroughs Wellcome & Co. Ltd., London) to have a level of 20% in the final medium.

(v) 100 units of penicillin G (sodium salt) and 100µg of streptomycin sulphate per ml of medium (See Section I for the preparation and sterilization of medium constituents.).

Sufficient amount of medium for the whole experiment was prepared on the day cultures were to be set up. After adjusting its pH to 7.2-7.4 by addition of 1.4% $NaHCO_3$ solution, the medium was dispensed in suitable aliquots into screw-top bottles, stored at 4°C, and used for changing the medium throughout the period of the experiment.

The roller tubes containing medium were closed by sterile suba seals or E_{21} Esco rubber bungs [Esco (Rubber) Ltd., London] and were placed inside an incubator on a rotating roller drum (one revolution in 7 minutes) 2 hours before inoculation with the worms, to allow the medium to attain the desired experimental temperature. The experimental temperatures reported in this work were the actual medium temperatures, not the air temperature of the oven.

Each set of cultures was continued for 8 days, after which period the worms were removed from the culture tubes, blotted lightly on a filter paper and weighed to determine the fresh weight (F.W.). The worms were then dried at 100°C for 18-20 hours and weighed again to determine the dry weight (D.W.).

The medium was changed every 48 hours. This was done by withdrawing the medium from the culture tube with a sterile pipette and adding fresh medium (previously heated to the same temperature as that of the cultures under experiment) with another sterile pipette. The pH of the medium in the culture was maintained between 6.8-7.4 by addition of 1.4% $NaHCO_3$ or 0.2N-HCl whenever required (as indicated by Phenol Red indicator in the medium). Adjustment of pH seemed necessary especially in cultures at higher temperatures and containing large worms, in which cases the medium tended to become acid within a few hours. If pH readings were required, the medium from a culture tube was withdrawn with a sterile pipette, placed in a glass dish of 6-8 ml capacity, and pH measured within 2-3 minutes of the removal of the medium using a glass electrode and a Pye meter.

All cultures were run with air as gas phase as

gassing of the medium with nitrogen or a mixture of 95% nitrogen and 5% CO_2 in some early experiments had failed to produce any significant difference in results. Aseptic precautions were taken at all stages. Medium transfers, dispensing of ingredients and setting up of cultures were done in a Hanovia cabinet fitted with an ultraviolet lamp. All cultures contaminated with bacteria or fungi were discarded. Contamination in cultures was detected in the following ways:

(a) appearance of cloudiness in the medium

(b) significant changes in pH

(c) examination of a small sample of medium under microscope (oil immersion)

The initial dry weight (D.W.) of the plerocercoids was calculated from the fresh weight (F.W.) using the data plotted by McCaig and Hopkins (1965, Text-Fig. 2). Studies on the effect of temperature on the growth of the plerocercoids were made in culture at 4°, 7°, 12°, 17°, 23°, 27°, 30°, 33°, 35° and 40°C.

C. Results

The specific growth rates of the plerocercoids of Schistocephalus at various temperatures ranging from 4° to 40°C after 8 days of cultivation in BSSG+HS+YE have been expressed as percentage changes in dry weight. Individual figures showing, for each plerocercoid cultured, the change in fresh and dry weights and the dry wt./fresh wt. ratio expressed as percentages are shown in Tables I-XIII of the Appendix.

(1) Growth at low temperatures (4°-27°C)

The specific growth rates of worms of different weights, expressed as percentage increases in dry weight after 8 days in culture at 4°, 7°, 12°, 17°, 23° and 27°C have been shown in Figs. 1-5. Regression lines were calculated for the points on each individual plot to demonstrate the degree of fit and facilitate the comparison of growth rates at different temperatures. They have been shown together in Fig. 6. At temperatures above 7°C, the results failed to show a good fit to a single regression line and therefore, the points were divided into two groups. The two regression lines have been shown as broken lines near the point at which they meet to indicate the doubt as to whether there is a sharp or gradual decrease in that region.

Table 2.

Q_{10} of growth rate of a 10 mg D.W. plerocercoid of Schistocephalus.

$$\log M_2 = \log M_1 + \frac{T_2-T_1}{10} \cdot \log Q_{10}$$

where M_2 and M_1 are the specific growth rates at two temperatures T_2 and T_1 respectively.

T_1(°C)	T_2(°C)	T_2-T_1	M_2(%)	M_1(%)	Q_{10}
4	7	3	15	12	2.0
7	12	5	29	15	3.7
12	17	5	60	29	4.3
17	23	6	115	60	2.9
10	20	10	84	20	4.1
20	30	10	70	84	0.8

mean Q_{10} between 7° and 23° = 3.6

Figures 1 - 6

The effect of temperature in the range 4^{o} - 27^{o}C, on the growth rate of Schistocephalus plerocercoids of different sizes. Each point is the mean rate of one worm during 8 days in culture; regression lines were calculated.

Fig.1 - Growth rate at 4^{o}C
Fig.2 - ,, ,, ,, 7^{o}C
Fig.3 - ,, ,, ,, 12^{o}C
Fig.4 - ,, ,, ,, 17^{o}C
Fig.5 - ,, ,, ,, $23/27^{o}$C
Fig.6 - Composite diagram of data in Figures 1-5 to illustrate the effect of temperature on the growth rate of Schistocephalus.

Figure 1.

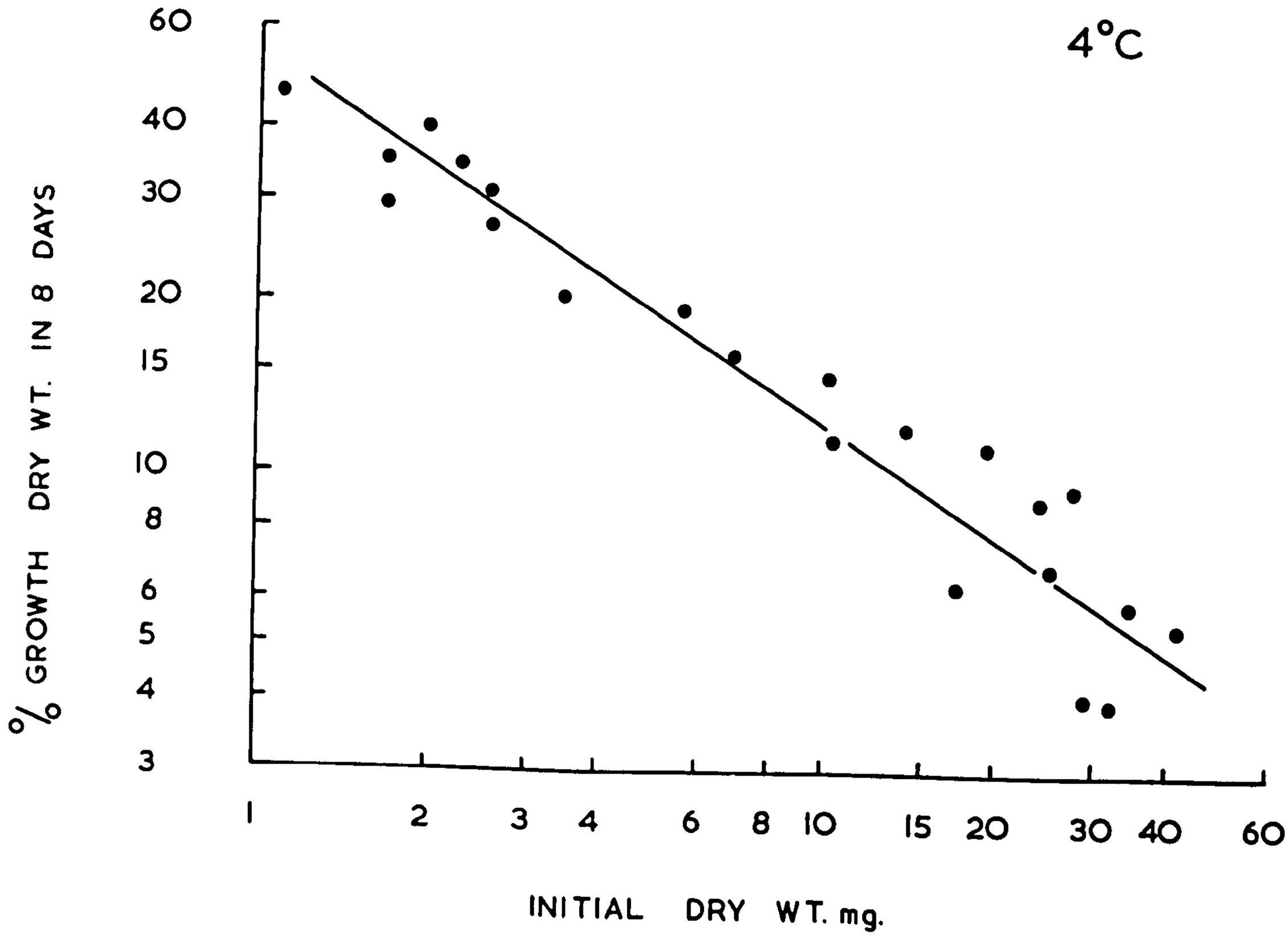

4°C
% GROWTH DRY WT. IN 8 DAYS
60
40
30
20
15
10
8
6
5
4
3
1
2
3
4
6
8
10
15
20
30
40
60
INITIAL DRY WT. mg.

Figure 2

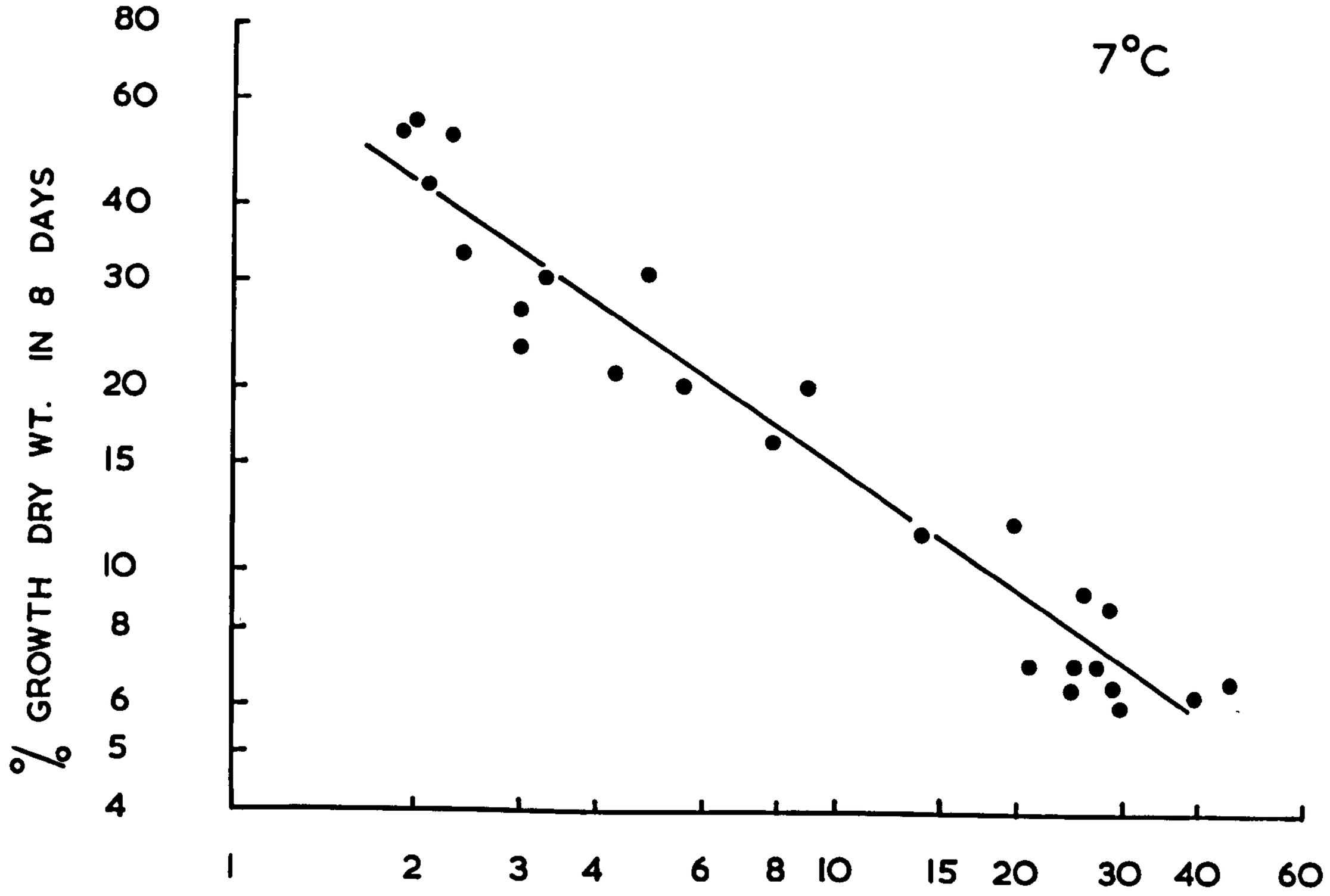

7°C
% GROWTH DRY WT. IN 8 DAYS
80
60
40
30
20
15
10
8
6
5
4
1
2
3
4
6
8
10
15
20
30
40
60
INITIAL DRY WT. mg.

Figure 3

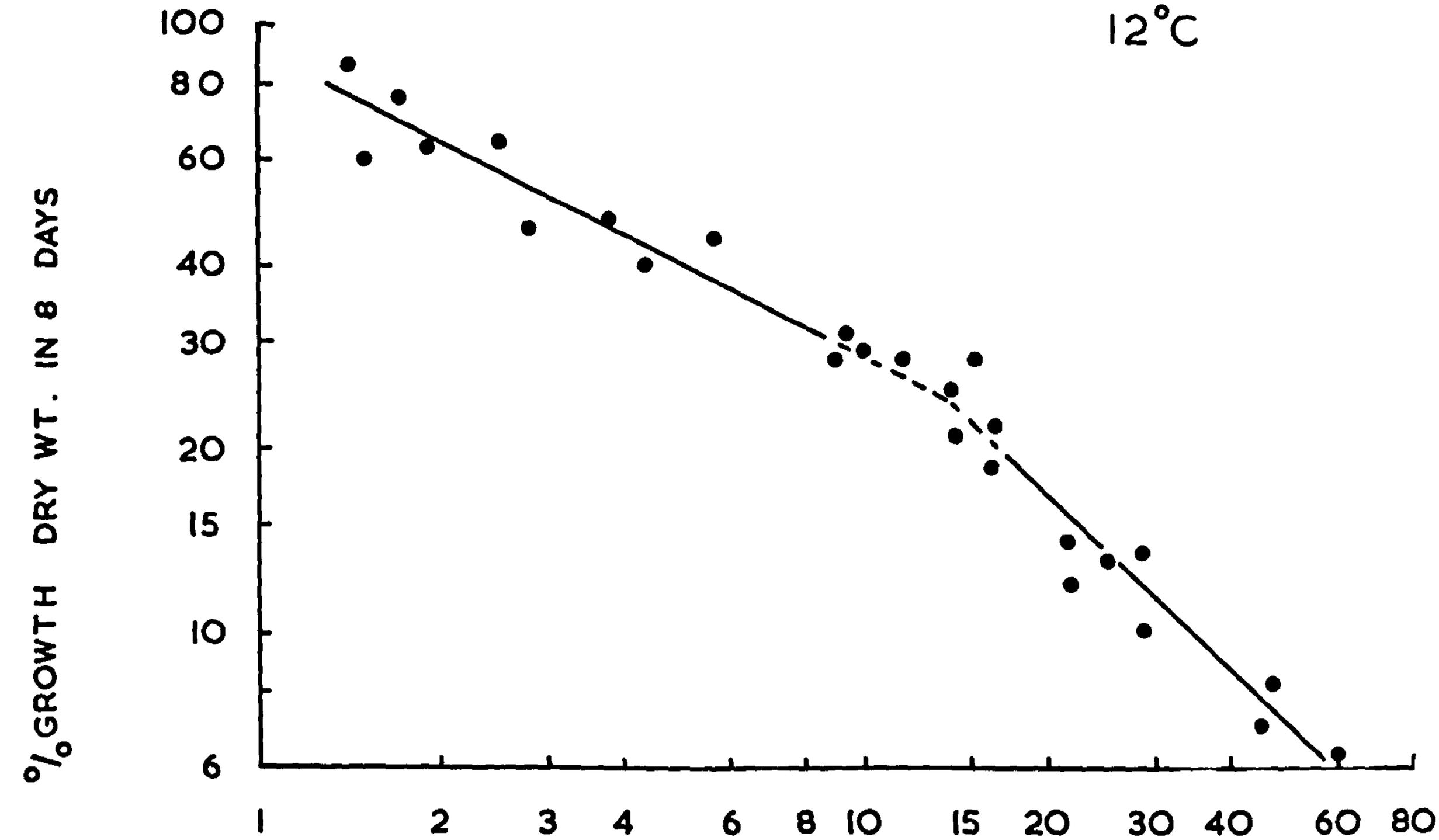

12°C
100
80
60
40
30
20
15
10
6
% GROWTH DRY WT. IN 8 DAYS
1
2
3
4
6
8
10
15
20
30
40
60
80
INITIAL DRY WT. mg.

Figure 4

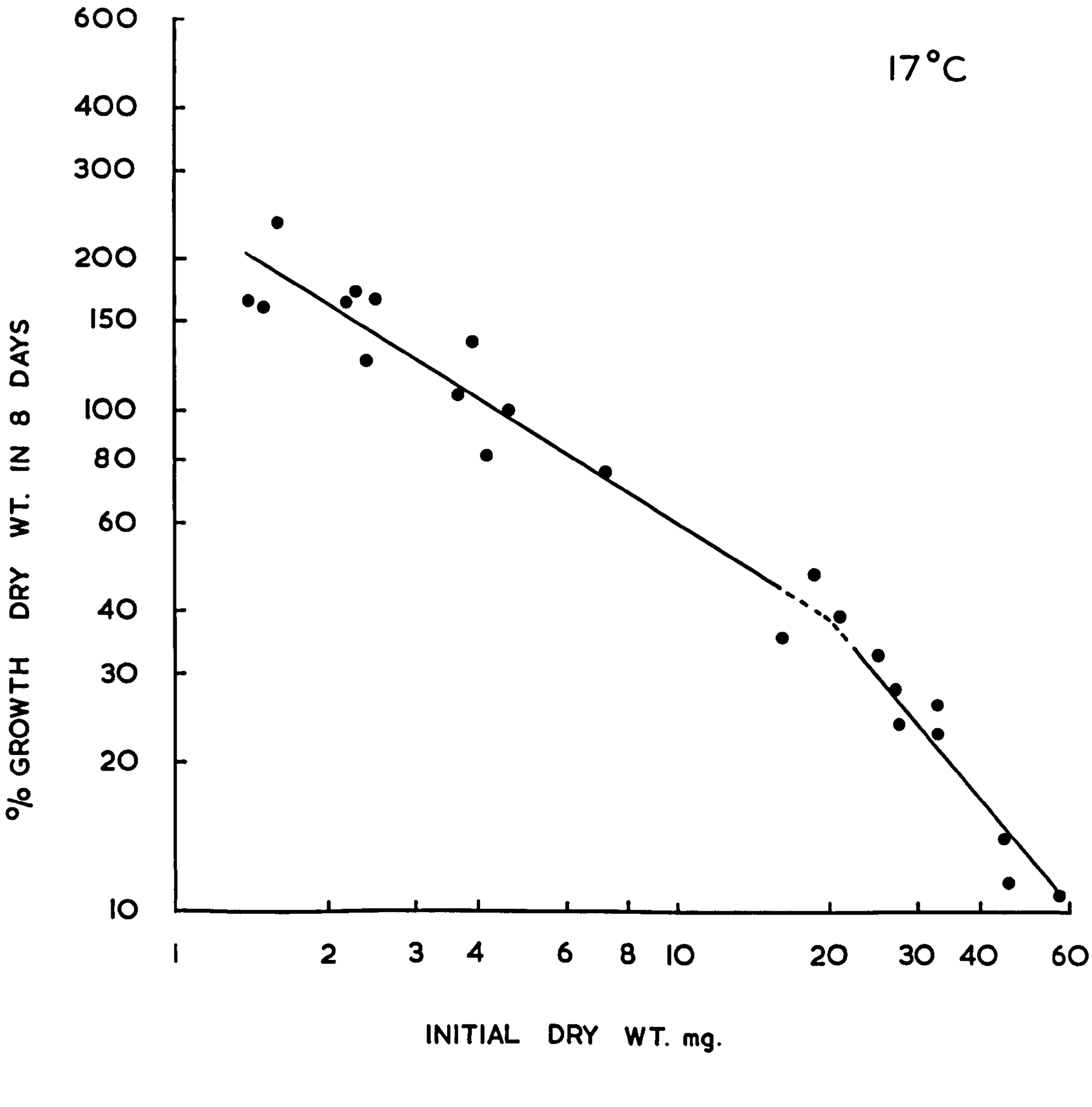

17°C
600
400
300
200
150
100
80
60
40
30
20
10
% GROWTH
INITIAL DRY WT. IN 8 DAYS
1
2
3
4
6
8
10
20
30
40
60
INITIAL DRY WT. mg.

Figure 5

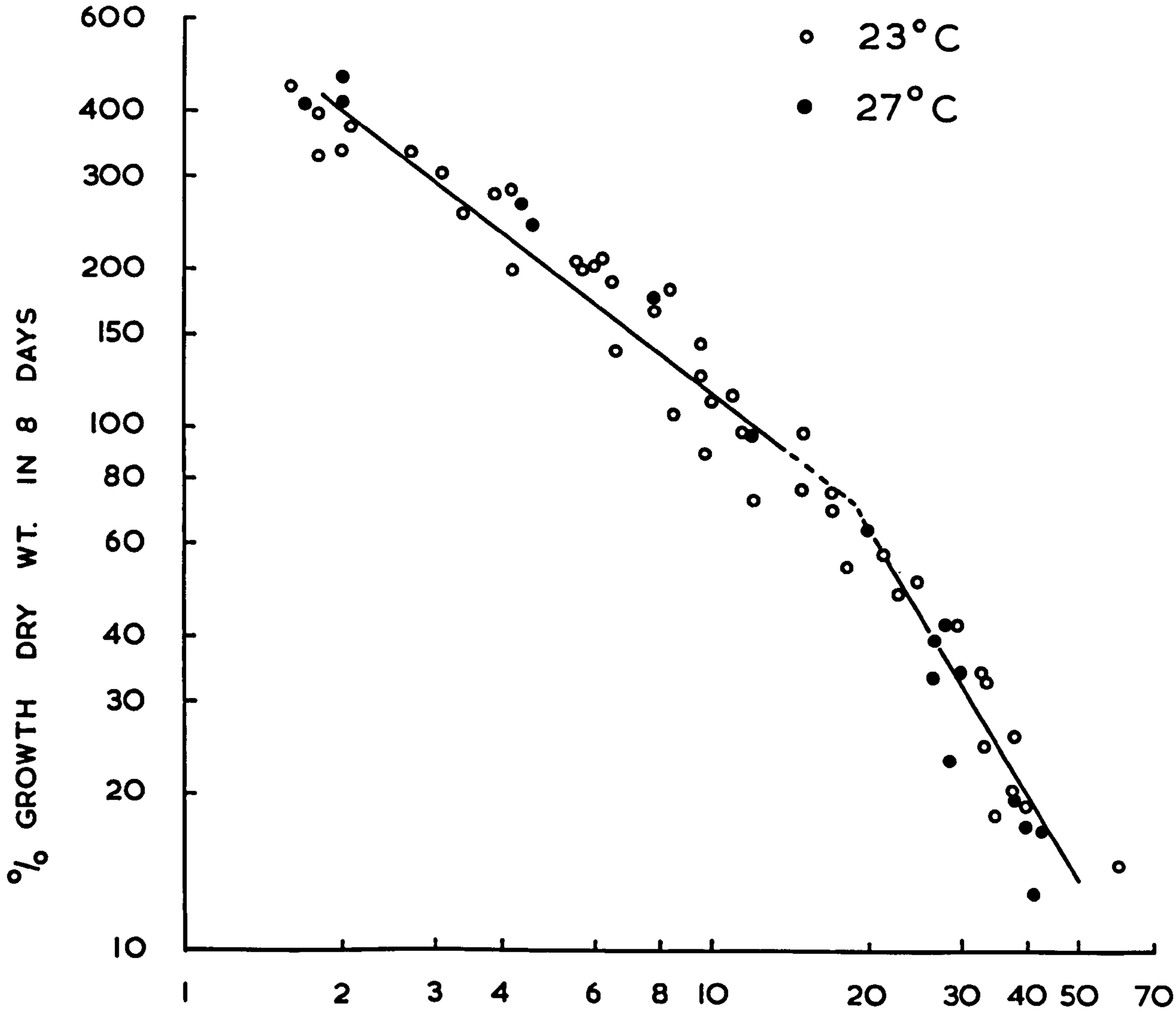

600
400
300
200
150
100
80
60
40
30
20
10
% GROWTH DRY WT. IN 8 DAYS
1
2
3
4
6
8
10
20
30
40
50
70
INITIAL DRY WT. mg.
23°C
27°C

Figure 6.

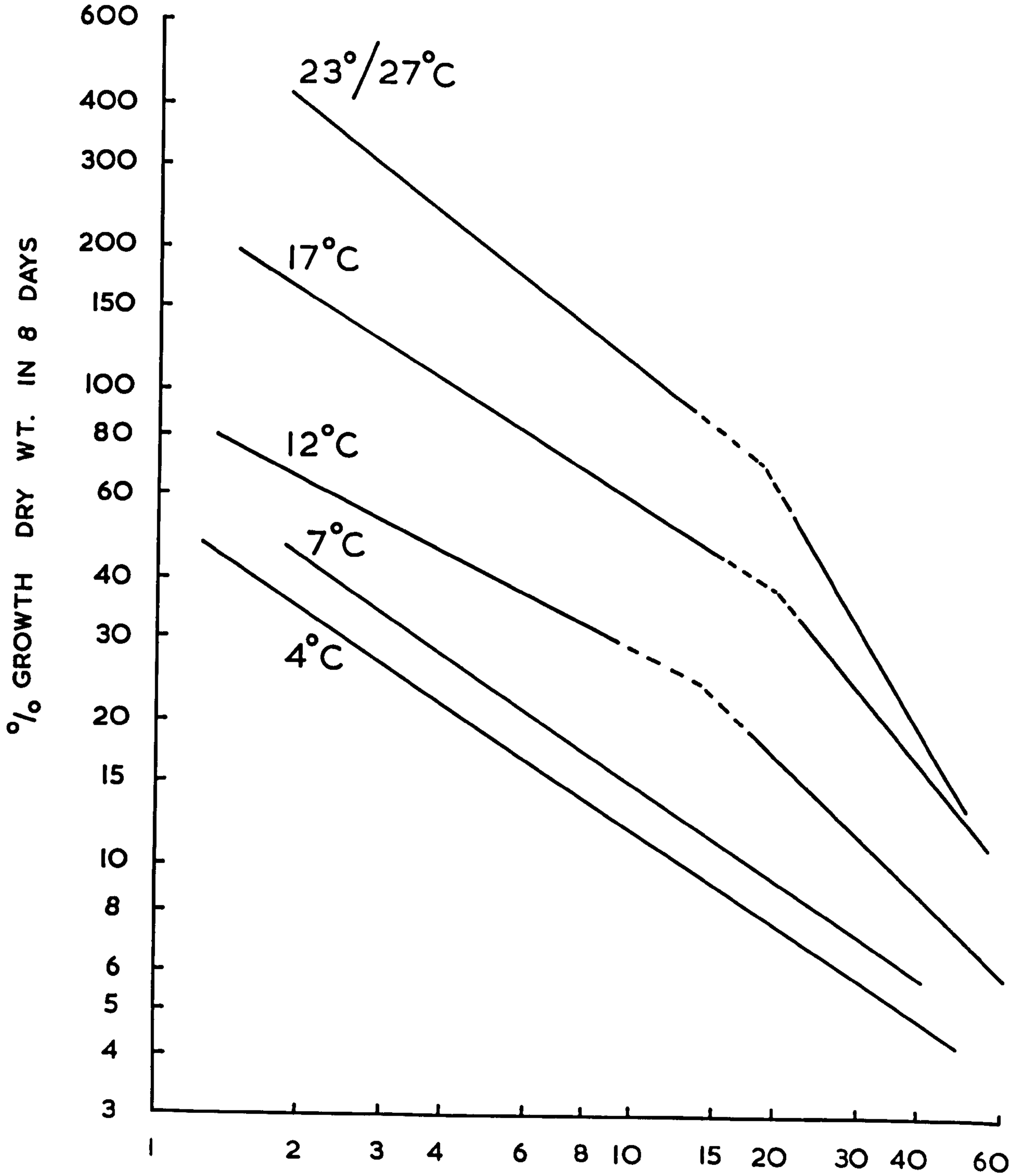

600
400
300
200
150
100
80
60
40
30
20
15
10
8
6
5
4
3
% GROWTH DRY WT. IN 8 DAYS
23°/27°C
17°C
12°C
7°C
4°C
1
2
3
4
6
8
10
15
20
30
40
60
INITIAL DRY WT. mg.

Figure 7

Growth rate of Schistocephalus plerocercoids at 23°/27°C during 8 days in culture : a plot of specific growth rate against the mean dry weight of the plerocercoid. Regression lines were calculated.

Figure 8

A log. velocity of growth against temperature plot to show the effect of increased temperature on the rate of growth of a 10 mg dry weight plerocercoid of Schistocephalus.

Figure 7.

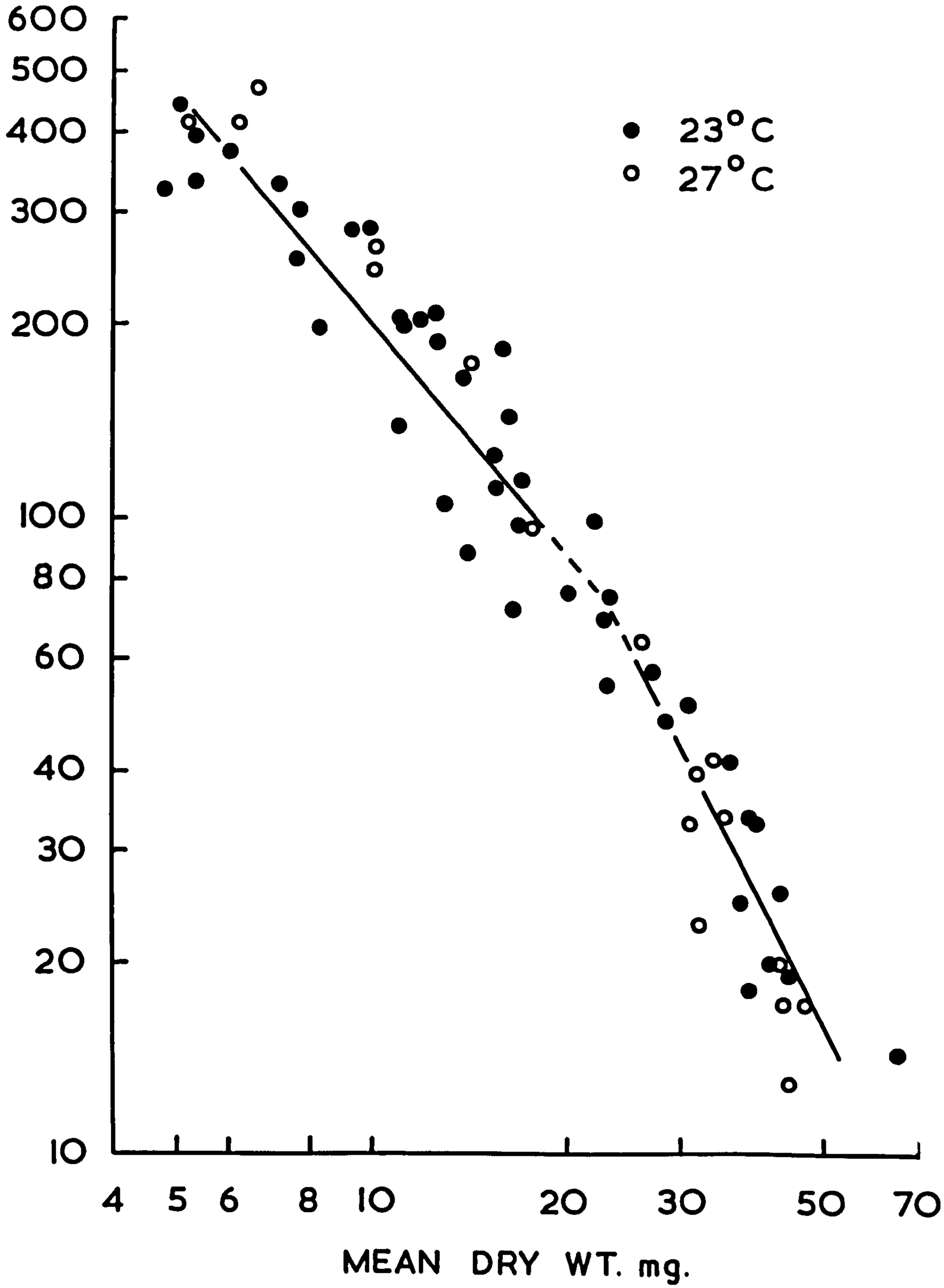
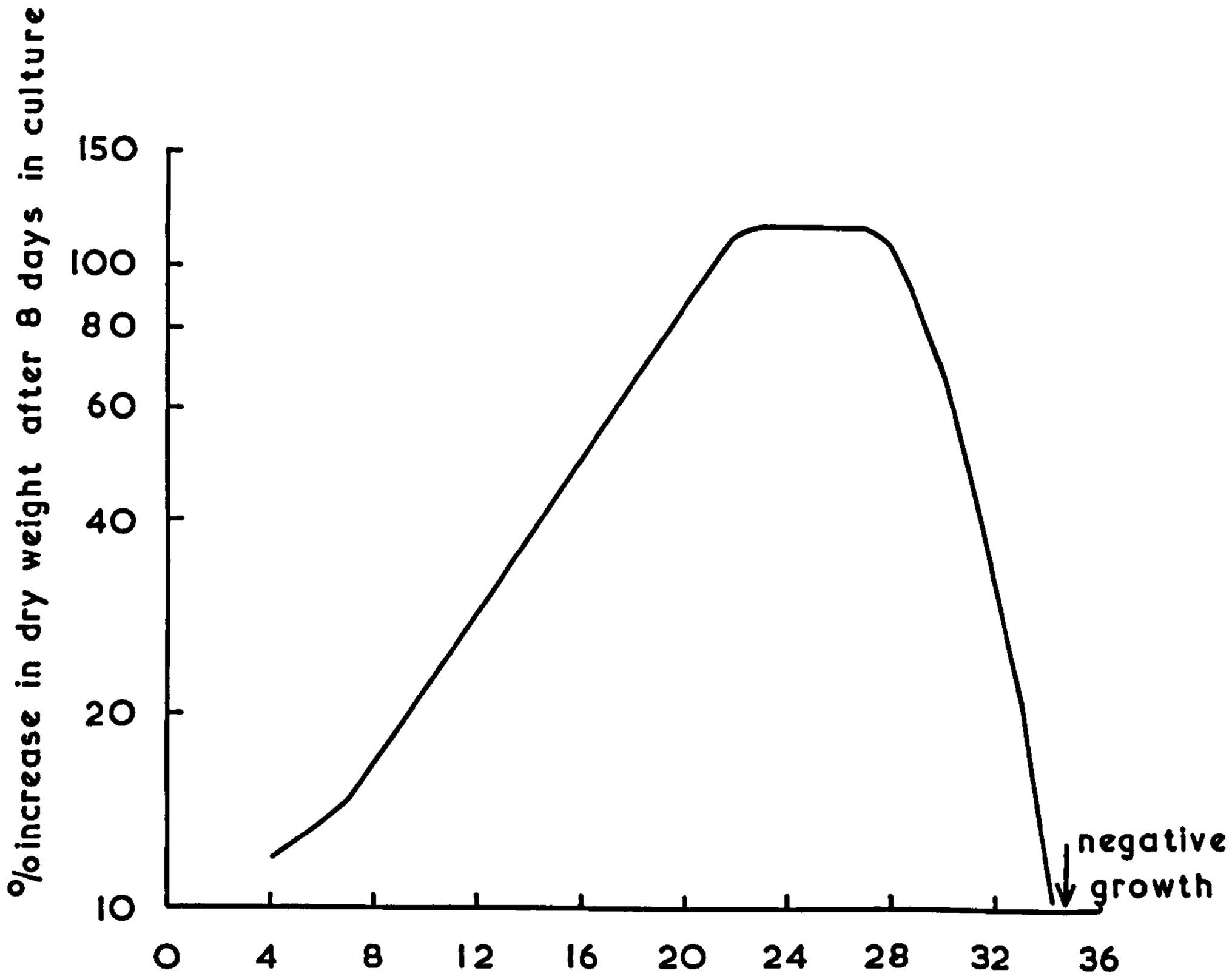

600
500
400
300
200
100
80
60
40
30
20
10
% GROWTH DRY WT. IN 8 DAYS
4
5
6
8
10
20
30
50
70
MEAN DRY WT. mg.
23°C
27°C

Figure 8.

A velocity of growth against temperature plot to show the effect of increased temperature on the growth rate of Schistocephalus



A single regression line fitted moderately well the points on 4° and 7° plots, but it is difficult to say whether this indicates a definite difference from the growth response at higher temperatures or whether this is due to the probable errors in accurate measurements of the very low rates of growth found in larger worms at these temperatures.

As previously observed by McCaig and Hopkins (1965), the specific growth rate of the plerocercoids was found to decrease with increase in size.

The calculated Q_{10} values of growth rate between various temperature ranges are shown in Table 2.

(2) Growth at high temperatures (27°-40°C)

The effect of high temperaturs in the range 27° to 40°C is shown in Fig. 9. At temperatures above 27°C, the rate of growth of Schistocephalus decreased; the higher the temperature the lower the specific growth rate. At 33°C worms over 15 mg D.W., at 35°C above 6 mg, and at 40°C worms above 3.5 mg D.W. were found to have lost weight after 8 days in culture. In order to show this negative percentage change or loss in weight, the abscissa of the plot has had to be changed from log. to linear scale to reach zero. The argument put forth by

Medawar (1945) that "what results from biological growth is itself typically capable of growth" justifies plotting of growth rate on a logarithmic scale, but this does not hold good for negative growth. Therefore, a linear scale has been used for showing loss in weight. This change in scale would obviously create a difference in the shape of the line as it passes to a different scale, and hence it should be ignored. The solid and broken lines shown in Fig. 9 have been fitted by eye. The line for 23/27° has been taken from Fig. 5.

At 33°C and over, the plerocercoids matured and produced eggs in culture, the response of the worms being dependent on their size (Hopkins and Sinha, 1965; Sinha and Hopkins, 1967). Worms 40-60 mg F.W. (10.7-18 mg D.W.) commenced maturation at once. The time of the appearance of eggs depended on the culture temperature. The relationship between the size of plerocercoids and egg production at various temperatures during 8 days of culture is shown in Table 3. After maturation no growth occurred although the worms were cultured in the same medium which supported growth at low temperatures. After 8 days in culture, worms about 60 mg F.W. (18 mg D.W.) showed a loss in weight of 15% at 33°C, 25% at 35°C, and 55% at 40°C.

Table 3.

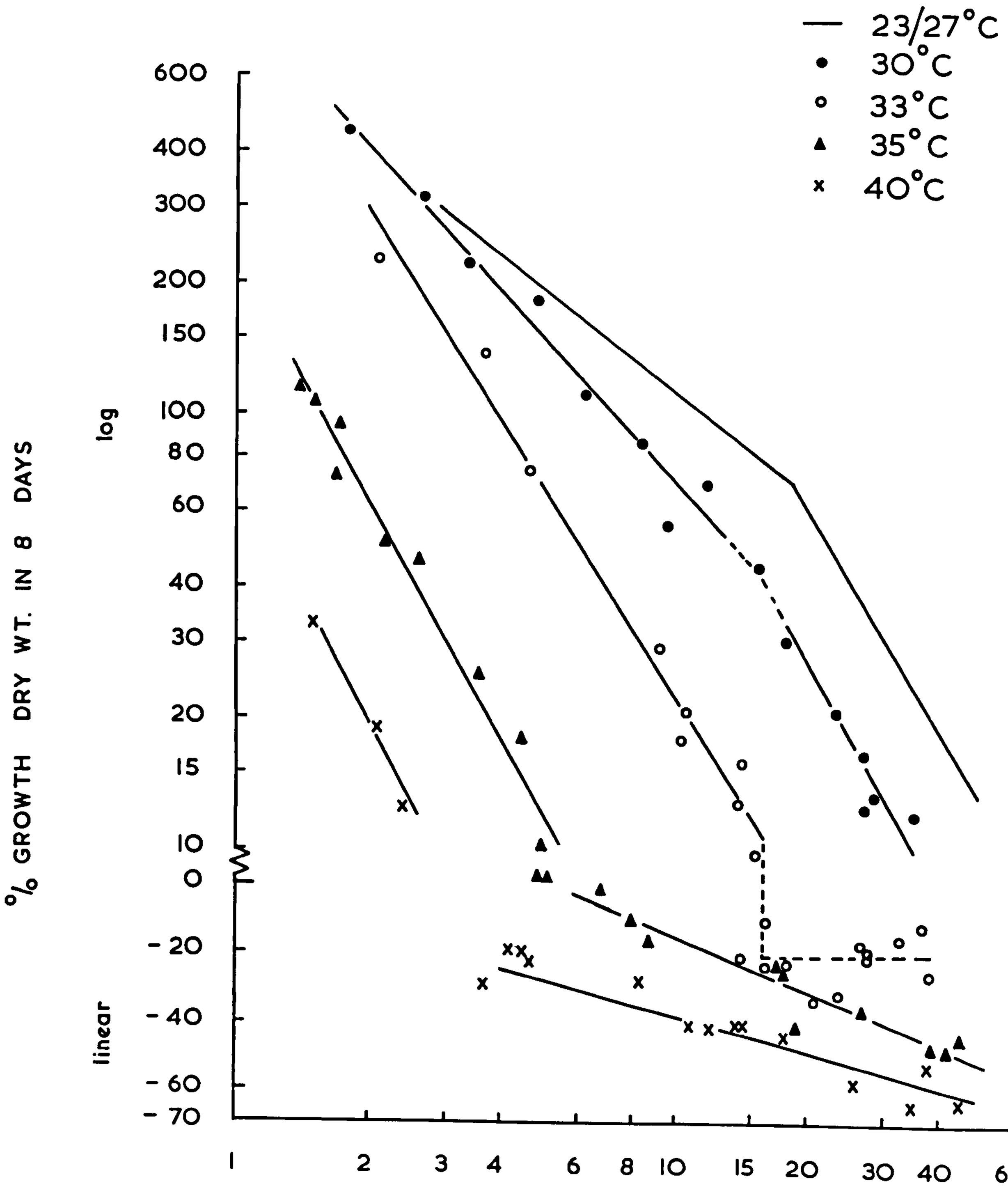
The relationship between size of the plerocercoids and commencement of egg production at various temperatures during 8 days in culture.

Wt. of plerocercoid(mg)			
F.W.	D.W.	Temperature	Eggs first in medium
<40	<10.7	33°	None
>40	>10.7	33°	72 to 96 hours
<22	< 5.0	35°	None
22-33	5.0-8.0	35°	Egg production rare
>33	> 8.2	35°	48 to 72 hours
<18	< 3.7	40°	None
18-33	3.7-8.2	40°	Some produced eggs
>33	> 8.2	40°	42 to 56 hours

Figure 9

The effect of high temperature (30°-40°C) on the growth rate of Schistocephalus. Regression lines were fitted by eye. Note change in scale of ordinate (see text).

Figure 9.


— 23/27°C
• 30°C
° 33°C
▲ 35°C
× 40°C
% GROWTH DRY WT. IN 8 DAYS
log
linear
600
400
300
200
150
100
80
60
40
30
20
15
10
0
- 20
- 40
- 60
- 70
1
2
3
4
6
8
10
15
20
30
40
60
INITIAL DRY WT. mg.

(3) Growth at 23°C after incubation at 35°C

To test whether maturation was an irreversible process or whether a drop in temperature after maturation would suppress sperm and egg production and induce growth again, 36 plerocercoids were weighed and culture singly in 3 groups.

Group I - 12 worms incubated at 35°C for 8 days.

Group II - 11 worms at 23°C for 8 days.

Group III - 13 worms at 35°C for the first four days and then at 23°C for the next four days.

All worms were weighed initially and on days 4 and 8. After recording the F.W. on day 8, the worms were dried and reweighed to determine the D.W. and also to see if they had a water content normal for cultured worms (McCaig and Hopkins, 1965; Text-Fig. 5). One plerocercoid of group II was rejected as abnormal.

The results of this experiment are shown in Fig.10 (details in Table XI, see Appendix).

The worms in Group II grew at the expected rate at 23°C. Their growth during 5th to 8th day was 10-20% less

Figure 10

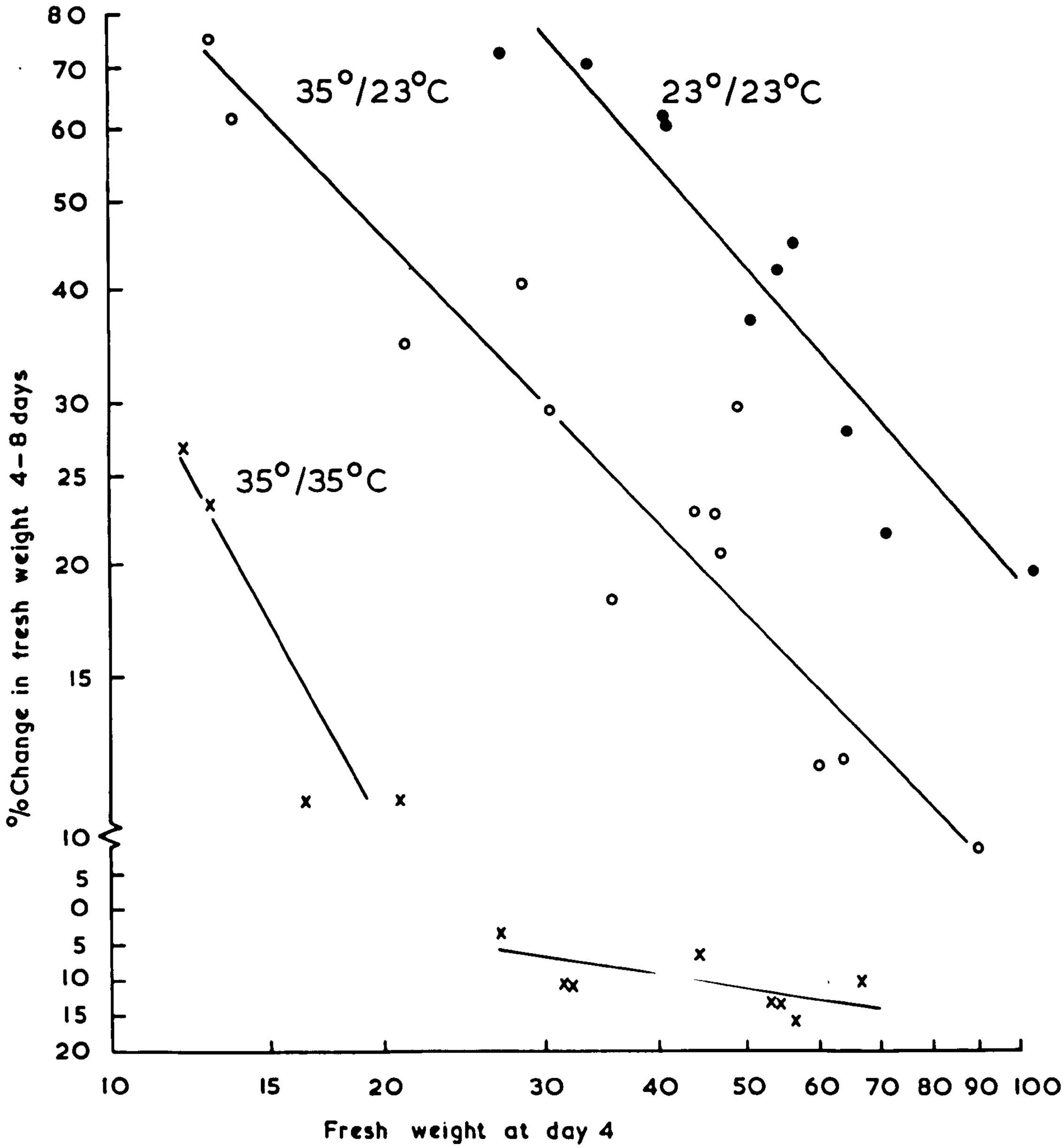
Growth of Schistocephalus plerocercoids at 23°C and 35°C during the 5th - 8th day in culture after different treatments during day 1 - 4.

Closed circles - Growth of worms at 23°C after previous culture at 23°C.

Open circles - Growth at 23°C after previous culture at 35°C.

Crosses - Growth at 35°C after previous culture at 35°C.

Figure 10.


35°/23°C
23°/23°C
35°/35°C
%Change in fresh weight 4–8 days
80
70
60
50
40
30
25
20
15
10
5
0
5
10
15
20
10
15
20
30
40
50
60
70
80
90
100
Fresh weight at day 4

than that of worms of similar weights during the first four days in culture. At 35°C(Group I) response of the worms was normal. Worms over 23 mg F.W. (approx. 5 mg D.W.) showed loss in weight, both during the initial and the second half of the 8-day culture period. Worms under 23 mg F.W., which do not mature at this temperature, showed small gains in weight.

Worms of Group III, held at 35°C during the first four days all lost weight except those under 20 mg F.W. (approx. 4.3 mg D.W.) which showed small gains. However, all gained weight when incubated at 23°C during 5th to the 8th day period (Fig.10). The growth rate of such worms was poor, being slightly less than half of the expected rate for worms in culture from day 4-8 at 23°C.

(4) Supplementation of medium BSSG+HS+YE with chick embryo extract.

McCaig and Hopkins (1965) observed that the growth rate of Schistocephalus plerocercoids during a second culture period of 8 days (between day 8 and 16) varied between 50-70% of the expected amount. The worms did not grow as fast as those of the same weights taken directly from fish. They suggested that this might be due to the absence of the precursors of certain metabolites in the medium or the masking of the outside

of microvilli by a deposition of a thin layer of material. In view of this observation it seemed reasonable to fortify the medium with a suitable supplement well known for its growth promoting properties. Chick embryo extract seemed to be a desirable supplement. It was prepared according to the technique described in Section I, stored frozen and used at 4%, 8% and 16% levels with the BSSG+HS+YE medium. A preliminary experiment was performed using 6 cultures for each group including the control to test if the addition of chick embryo extract would promote better growth than the original medium alone.

No definite conclusions can be drawn as the number of worms cultured was small, but the results (Table XII, see Appendix) suggest that none of the concentrations of CEE_{50} used improved the growth rate observed in unsupplemented medium. This experiment was repeated at a later date using 4% CEE_{50} and again no enhancement of growth during the 8-16th day of cultivation was obtained (Table XIII, see Appendix).

D. Discussion

Practically nothing is known about the growth rate of the plerocercoids of Schistocephalus in vivo. Most of the workers investigating growth of cestodes have studied adult worms in the definitive host (Penfold, Penfold and Phillips, 1937; Chandler, 1939; Wardle and Green, 1941; Archer and Hopkins, 1958; Hutchison, 1959; Roberts, 1961). The problem that confronts the investigator in this type of work is the selection of the basis of the measurement of growth, which can be measured in an organism in many ways. It can be ascertained by measuring the increase in length, breadth and thickness; increase in volume; increase in weight or increase in one of the constituent substances, such as protein. Measuring growth on the basis of change in length cannot be applied to worms like plerocercoids of Schistocephalus which vary in age, size and weight when obtained from natural infections and which, for the purpose of cultivation in vitro, have to be handled individually taking all aseptic precautions. Various aspects of growth of Schistocephalus can be studied if worms of known age can be made available, but this is not possible until details of the maintenance and breeding of copepods and their experimental infection are worked out

and a source of supply of different stages of the worm established in the laboratory.

The dry and fresh weights of the worms can be determined fairly accurately. Fresh plerocercoids obtained from sticklebacks can be weighed immediately to determine the wet weight, but they have some adherent surface moisture, the aseptic removal of which without possible damage to the tegument (cuticle) would not be practicable. The fresh weights of the same worms can be determined at the end of the culture period after lightly blotting them on a piece of filter paper, but for very accurate interpretation this involves the application of a suitable correction factor for the conversion of fresh weight into wet weight, or vice versa. This cannot be done very accurately as small worms adsorb a relatively larger quantity of surface moisture then large worms, due to having a higher surface area/volume ratio. Secondly, under some circumstances, measurement of growth rate on the basis of increase in fresh weight during a given period of time may not be accurate, as a plerocercoid may show an increased fresh weight due to imbibition of water from the culture medium. The rate of growth was therefore measured on the dry weight basis and expressed as percentage increase in the initial dry

weight of the plerocercoid. The graph plotted by McCaig and Hopkins (1965, Text-Fig. 2) was used to convert the initial wet weight into dry weight. Text-Fig. 5 of the same communication served as a guide to judge the normality of the worms as indicated by their percentage water content. Accordingly, results from cultured worms showing an abnormal dry weight/fresh weight ratio were discarded.

The growth rate of a single individual or a group of animals of the same age and size is usually measured under identical experimental conditions for different periods of time and represented on a size/time plot. This is not possible with plerocercoids of Schistocephalus on account of the difficulty in obtaining worms of the same age and size. Therefore, size has been used as a variable keeping the experimental time (8 days) constant. Since growth is essentially a multiplicative phenomenon, the results should have been plotted on a log. linear scale. As the specific growth rate of large plerocercoids was much less than that of small ones, this failed to give a straight line. However, it was found that if results were plotted on a log./log. grid, the points fell closer to a straight line. So the results shown in Figs. 1-5 are plotted in this manner. They reveal three interesting points at the first sight:

1. Growth rate decreases with increase in the weight of the plerocercoid.
2. Growth rate increases with increase in temperature from 4° to 23°C.
3. Above 7°C the points lie along two straight lines, one between 1 mg D.W. to 18-20 mg D.W. (approx. 5 mg to 60-67 mg F.W.) and the other for worms beyond this weight range.

The finding that the specific growth rate decreases rapidly with increase in size of plerocercoids has been observed previously by McCaig and Hopkins (1965). In a few other cases also, in which growth of a larval cestode has been studied (for example, larval Hydatigera taeniaeformis by Hutchison, 1958), it has been found that they conform to the pattern of initial rapid growth which decreases with increase in size. This is a normal phenomenon in nature and fits in well with the statement of Medawar (1945, p.167) that "living tissue progressively loses the power to reproduce itself at the rate at which it was formed." A worm of 2 mg initial D.W. increased in dry weight by 400% during 8 days in culture at 23°, whereas a worm of 32 mg D.W. increased by only 25-30% indicating that within these limits the growth rate on average is halved every time the weight is doubled.

A single regression line gave a moderately good fit at 4°C and 7°C, but it is very difficult to say whether this suggests a different growth response of the worms from that of those at higher temperatures or is due to errors in the accurate measurement of the very low rates of growth of large plerocercoids at these temperatures. The possibility of the latter seems more likely. At these temperatures, even worms as small as 2 mg D.W. do not show an increase of more than 40-50% in 8 days. The larger plerocercoids grew slower than the rate expected from an extrapolation of the growth rate plot of small worms (Figs. 3-5). The regression line in these plots passes through an inflection around 18-20 mg D.W. (60-67 mg F.W.), suggesting that some sort of definite but abrupt change occurs in the metabolism of the worms on reaching this size(developmental stage?).

The principle that growth rate decrease with increase in weight applies also to a single worm growing in culture from day 0 to day 8. The specific growth rate of any plerocercoid between day 0 and day 2 will be greater than that between day 4 and day 6. In fact, the specific growth rates shown in Figs. 1-5 represent the sum of various growth rates at all the weights through which a particular plerocercoid has grown during 8 days in culture. The rate plotted, therefore, is an average rate

approximating more closely to that of a plerocercoid whose weight is approximately equal to the mean between the initial and the final weight. A worm of 18 mg D.W. (approx. 60 mg F.W.) grows about 80% of its initial dry weight in 8 days (Fig. 5) and reaches a final dry weight of 32 mg (108 mg F.W.), but in fact, a growth of 80% would be that of nearly 25-26 mg D.W. worm if the growth rate was changing at a uniform rate. Therefore, although the line shows an inflection at 18-20 mg D.W. (60-67 mg F.W.), the change in growth rate may not occur until 24-26 mg D.W. (80-90 mg F.W.). A better way to confirm this interpretation is to plot the specific growth rate against the mean dry weight (Fig. 7). From this it appears that the growth rate decelerates at a faster rate when the plerocercoid reaches a size of 24 mg D.W. (80 mg F.W.). The question is now whether this change is a real physiological change in the plerocercoid or this happens due to unsuitable culture conditions. It may be argued that large plerocercoids produce more waste, use more metabolites from the medium, and change it rapidly enough to prevent growth. But even at lower temperatures where the metabolic rate is comparatively much slower, the change in growth rate occurs at the same approximate weight. This evidence suggests that at about 24-25 mg D.W. (80 mg F.W.), the

plerocercoid changes its behaviour. It is significant to note here the observations of McCaig and Hopkins (1965) who found that percentage glycogen content of Schisto-cephalus plerocercoids increased up to 80-90 mg F.W. but thereafter remained constant. They also observed a similar change in percentage water content which fell from about 80% in a 10 mg F.W. worm to about 69% in a 90 mg F.W. worm, and thereafter remained constant, or nearly so. The fact that all these three parameters, viz. percentage glycogen content, percentage water content, and growth rate alter at the same point clearly suggests that a definite physiological change occurs in the worms at about 80-90 mg F.W. comparable with the change that occurs between 5-10 mg F.W. when the young plerocercoid forms proglottids, and becomes infective to the final host, and that between 10-20 mg F.W. when the worms develop genital rudiments capable of maturing and producing eggs within 48 hours in a duckling (Hopkins and McCaig, 1963).

Another interesting feature that isindicated by Fig. 6 is that with the possible exception of large worms at low temperatures, the slopes of the regression lines do not differ much from one another. This suggests that a rise in temperature has the same effect on small as on large plerocercoids. However, the points near the end

of the lines (Figs. 1-5) are subject to the largest experimental errors. Those at the upper end are based on very small worms which are difficult to be weighed accurately and about which least is known regarding their normal dry weight contents. Similarly, the points towards the lower end are based on small differences between the observed final dry weight and the initial calculated dry weight of large plerocercoids. To minimise these possible errors, the growth rate at different temperatures was calculated for 10 mg D.W. worms, the point for which lies near the centre of the plots (Fig. 6).

The effect of temperature on the rate of growth of a 10 mg D.W. worm is best shown by plotting the log. of the growth rate against temperature (Fig. 8). This plot shows three phases in the response of Schistocephalus. In the first phase, between 4° and 23°, an increase in temperature increases the growth rate. Between 7° and 23°C, the rate of increase is nearly exponetial with a mean Q_{10} of 3.6 (Table 2). Below 7°C, the Q_{10} was found to be only 2.0, but it does not seem to be possible to attach any biological significance to this because growth rate is very low at 4°C and hence experimental errors are relatively large. The second phase in the growth response of Schistocephalus to temperature has the form of a plateau

or low curve which extends between 23° and 27°. In this region, growth is maximal. It is interesting to note here that in Britain, this temperature range is the maximum that Schistocephalus would face in the fish host under normal conditions. Phase three starts above 27°C when the growth rate starts to decline and by 30°C it has fallen by about 40%. At this point, before discussing the fall in growth rate at higher temperatures, it is worthwhile to consider the results obtained by Davies and Walkey (1966) on the metabolic rate of S. solidus, both plerocercoid and adult, as assessed by oxygen consumption, and compare them with the assessment made by the present work on the growth rates at various temperatures. Davies and Walkey measured the endogenous aerobic respiration using Warburg manometry. The plerocercoids were incubated at 10°, 20°, 30° and 40°C, and adults at 40°C in sterile, sugar-free Tyrode's solution (with phosphate buffer at pH 7.2) with atmospheric air as gas phase. The flasks were shaken at a rate of a hundred 5 cm strokes per minute. The duration of the experiment varied from 2 hours at high temperatures to 8 hours at 10°C. They made the following statements and observations:

1. In both summer and winter, "respiration rate of plerocercoids increases with temperature and is at a

maximum at 40°C. This is thought to be a pre-adaptation for respiratory function at the elevated body temperatures of the definitive host" (p.415).

2. Q_{10} of respiration rate was found to be as given below:

Temperature range	Summer	Winter
10-20°C	2.77	2.62
20-30°C	2.38	1.82
30-40°C	2.88	2.10

3. "Q_{10} of respiratory rate decreases at temperatures up to 30°C but then increases from 30-40°C, suggesting that at higher temperatures alternative enzyme pathways are utilized" (p,415).

4. "Respiration rate displays thermal acclimation on a seasonal basis; it is higher in winter than in summer and the Q_{10} is lower in winter than in summer" (p.415).

5. "The net result of acdimation in Schistocephalus is that a plerocercoid metabolizes at the same rate at 15°C in the summer as it does at 10°C in the winter. In the metabolic economy of the worm the low summer respiratory rate could be regarded as a compensatory process limiting the expenditure of energy at the higher environmental temperatures. Although the importance of

this to the parasite is less clear, its significance in the host/parasite relationship may lie in a relatively lower nutritional demand by the parasite on the host at the higher summer temperatures, i.e. at a time when the host itself is experiencing a higher metabolic demand" (p.423).

6. O_2 consumption per unit weight per hour is proportional to a negative power (-0.478 ± 0.009) of the dry body weight (meaning that small worms exhibit a higher respiratory rate than large worms) and this relationship is true for both plerocercoids and adults between 10°C and 40°C in both summer and winter.

A close examination of these observations made by Davies and Walkey raises some doubt as to their validity. According to these authors, Q_{10} of respiratory rate was 2.77 between 10° and 20°C, and 2.38 for 20-30°C. These figures indicate that metabolism increases nearly as rapidly between 20° and 30°C as it does between 10° and 20°C, whereas the figures calculated from the present work on the growth rate (Fig. 8) show a Q_{10} of 4.1 between 10° and 20°C and 0.8 between 20° and 30°C (Table 2). Secondly, the increased oxygen consumption between 20-30°C as shown by Davies and Walkey might be due to an increase in activity following the mechanical shock of removal from a fish and severe shaking during the period

of incubation in the Warburg flask. The same explanation may hold good for increased O_2 consumption between 10-20°C where again it may be said that much of the oxygen taken at these temperatures was used to support activity. The plerocercoids of Schistocephalus both in culture and in fish, appear to be inactive, only moving when agitated or subjected to a sudden change in temperature. Therefore, it seems doubtful that O_2 consumption in the Warburg flask bears much relationship to the O_2 uptake in vivo. Similarly, the claim that Schistocephalus shows acclimation, based on the observation that plerocercoids consume more oxygen at 10° and 20°C in winter than in summer, is not very sound. It may be argued that this result was due to the greater activity of the worms consequent upon a thermal shock on removal from a fish in winter (temperature appox. 3°C), whereas in summer, the worms had already an environmental temperature of 16°. This argument finds further support from another observation of Davies and Walkey that at 30°C (where plerocercoids would be subjected to a severe thermal shock in both summer and winter), there was no significant difference between oxygen uptake in winter or summer. Also, the explanation given for acclimation that the parasite has a relatively lower nutritional demand on the host at higher summer temperatures when

the latter itself is experiencing a higher metabolic demand, does not seem to be unequivocal. The reverse of this seems far more probable. In winter, many freshwater fish have an extremely low food turnover. The presence of a large burden of parasites having a mass equal to 40% of the body weight of the fish (Smyth, 1946) must be an ever open metabolic drain. It would be therefore of selective advantage to the parasite to minimise its demands on the host's poor metabolic economy in winter.

At high temperatures the amount of growth and degree of maturation depend upon two factors: temperature, and the size of the plerocercoid (Fig. 9, Table 3; Hopkins and Sinha, 1965). A rise of temperature to 33°C or over depresses the growth rate considerably but at the same time does not prevent weight increases occurring in small worms, whereas worms above 15 mg D.W. (53 mg F.W.) all lose weight. This loss in weight may be attributed to production of sperms and eggs and decrease in glycogen reserve which invariably occurs at high temperatures both in the presence and absence of glucose in the environment. It may also be said that weight loss occurs due to inadequate nutrition available to the worms in the medium. But this seems unlikely because the same medium supports considerable growth at

lower temperatures. It may again be argued that at higher temperatures, the metabolic rate of the worms is very high; larger worms produce more waste and make the medium too poor even to support their maintenance. However, loss of weight occurs even when medium is changed daily and all precautions are taken to prevent its becoming acid due to accumulation of wastes. Moreover, worms lose weight even in the intestine of a suitable host (duckling), about 10% in the first 48 hours and 20% in 96 hours (Hopkins and McCaig, 1963). Therefore, it seems reasonable to presume that loss in weight occurs not due to any nutritional deficiency in the medium, but due to sperm and egg production, greater loss in heavier worms being due to greater daily egg production (Parsons, 1966, private communication). Greater loss of weight by worms as the temperature rises from 33° to 40°C may be correlated with earlier egg production (Table 3, and Smyth 1952).

It is not possible to conclude from these studies that somatic growth ceases completely after maturation. It is quite likely that small amounts of growth which may occur, are masked and are in fact overbalanced by loss of eggs. Assuming that somatic growth does not stop completely, but is at a very low rate at high temperatures, an interesting physiological problem is posed. Does the

onset of maturation cause suppression of growth by a feedback mechanism, i.e. are there specific inhibitors of maturation and growth active below and above a certain temperature (Fig. 11a), or is cessation of growth due to the fact that at higher temperatures, the growth supporting enzyme systems are not functional (Fig. 11b)? The best way to solve this problem would be to suppress the genital development in worms at high temperatures and observe if any growth occurs. But in the absence of a suitable technique to do this, an indirect approach can be made to arrive at some conclusion.

Fig. 9 shows clearly that a rise in temperature above 27°C leads to a progressive fall in the growth rate of plerocercoids even as small as less than 3.5 mg D.W. (17 mg F.W.). The growth rate of a 2 mg D.W. (11 mg F.W.) worm at 40°C is only 5% of the rate at 27°C. Worms of these weights are too small to produce eggs but they possess clearly defined genital rudiments which respond to the rise in temperature (Hopkins and McCaig, 1963), and the cells associated with the genital rudiments may be triggering a growth suppressant system. A strong argument against this 'feedback' hypothesis is that the somatic growth rate of Schistocephalus plerocercoids ceases to rise after 23°C at which temperature

Figure 11

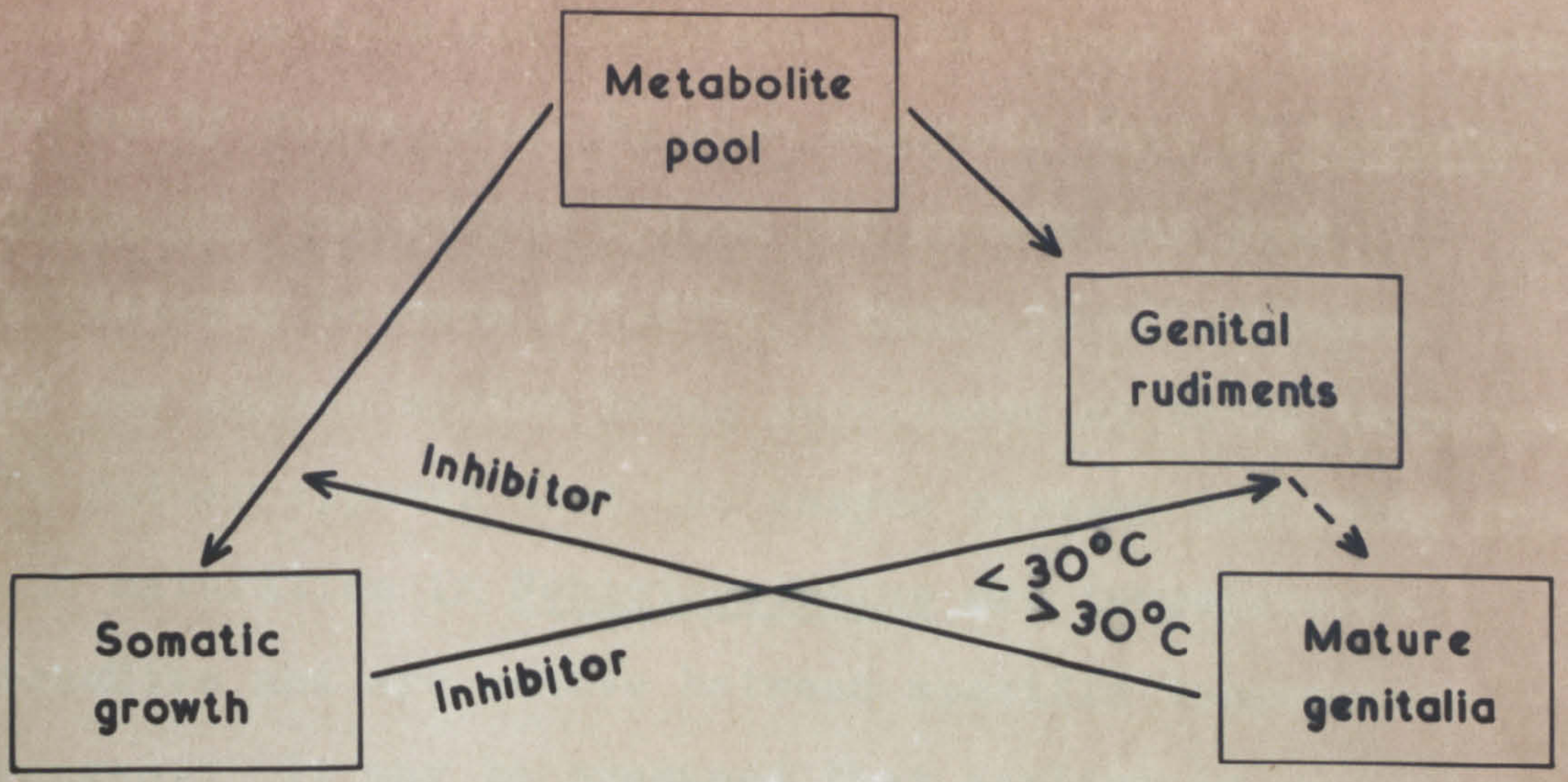
Two hypothetical control mechanisms to explain the observed pattern of morphogenesis in Schistocephalus.

A -'Feedback'mechanism

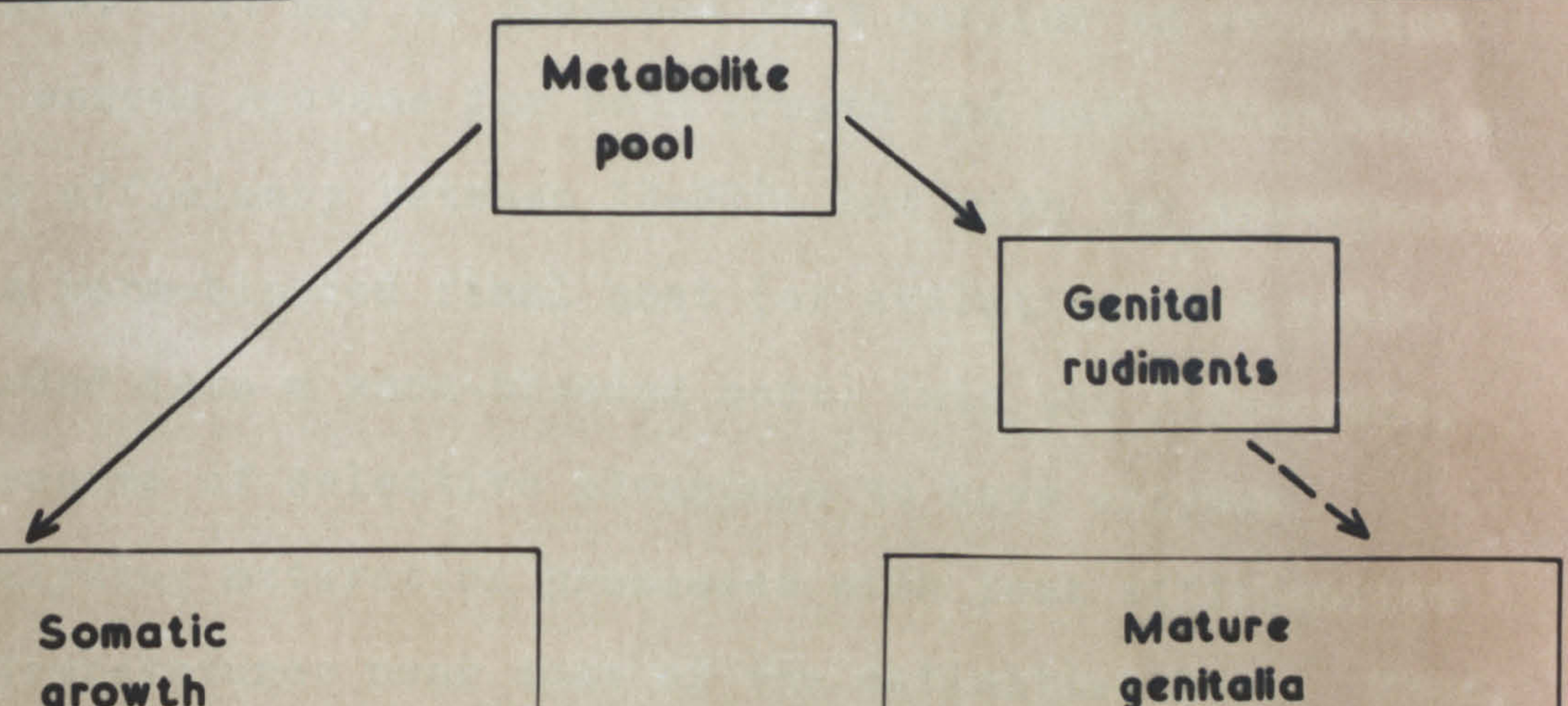
B - Non-function of growth-supporting enzyme systems at high temperatures.

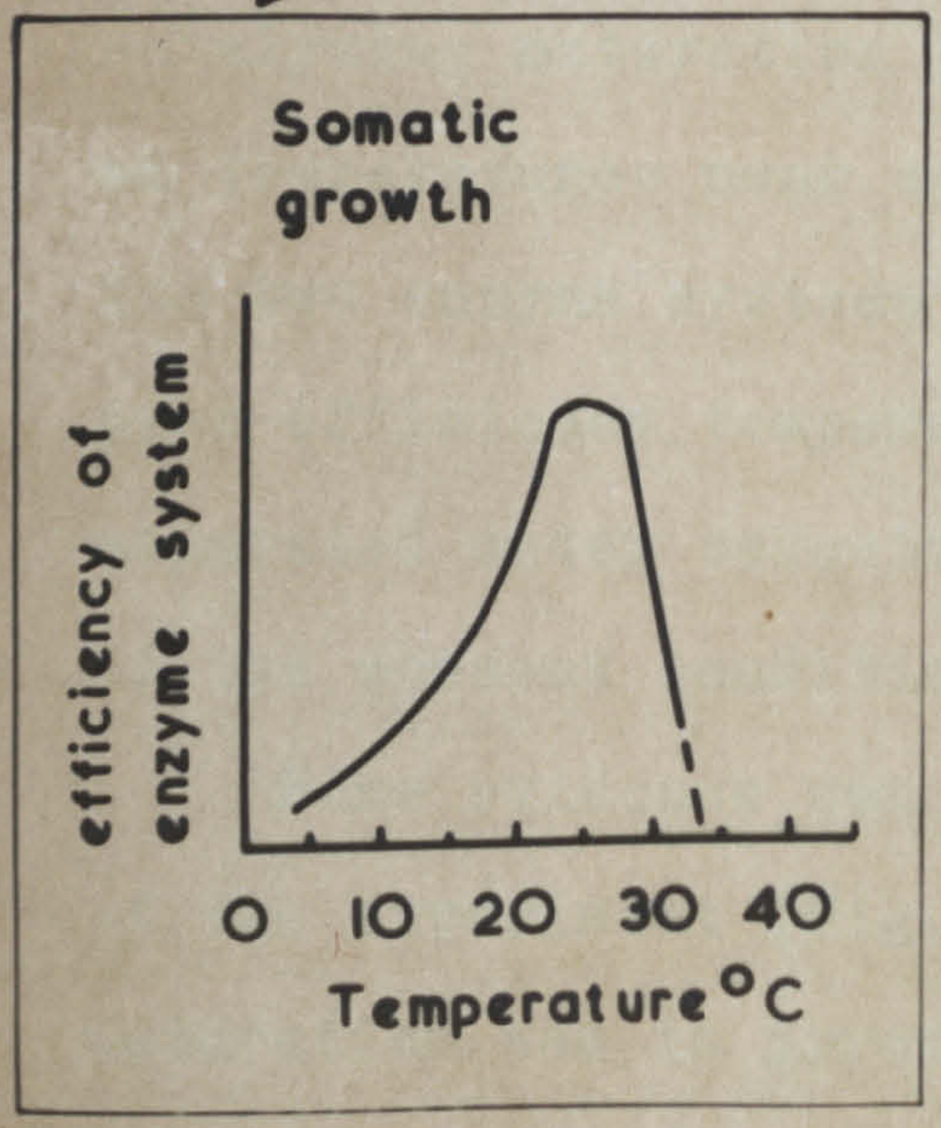
Figure 11.

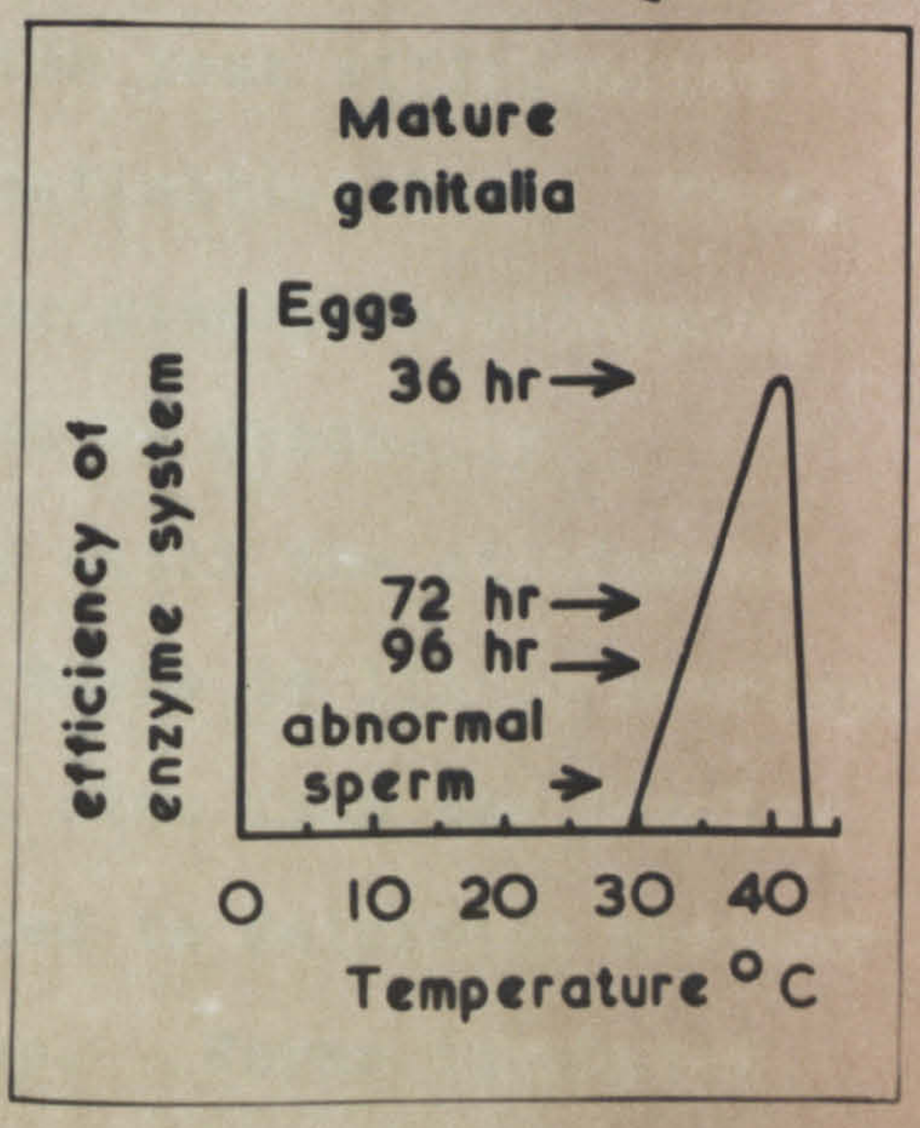
A


Metabolite pool
Genital rudiments
Inhibitor
< 30°C
> 30°C
Somatic growth
Inhibitor
Mature genitalia

B


Metabolite pool
Genital rudiments


Somatic growth
efficiency of enzyme system
0 10 20 30 40
Temperature °C


Mature genitalia
Eggs
36 hr
72 hr
96 hr
abnormal sperm
efficiency of enzyme system
0 10 20 30 40
Temperature °C

maturation does not commence. The shape of the growth velocity curve (Fig. 8) also suggests that in Schisto-cephalus, the enzyme system for growth has peak efficiency between 23°-27°C. There is no evidence of any enzyme system being suppressed at these temperatures. It rather appears that the enzyme systems catalysing growth and maturation in Schistocephalus are temperature-sensitive and are active between specific temperature ranges. It may be presumed that earlier in evolution the enzyme systems for both growth and maturation had a peak efficiency between 23-27°C, but with the incorporation of a warm-blooded final host (or evolution of a cold-blooded into a warm-blooded host) into the life-cycle, it became of selective advantage to have enzymes catalysing maturation processes with peak efficiencies at temperatures near that of the definitive host. Thus, the two enzyme systems which had originally overlapping peak efficiency temperatures came to be gradually separated under selection pressure until the attainment of the present condition in which there is little overlap.

Some evidence can be obtained also from the results of the experiment (Fig. 10) designed to determine whether maturation was in irreversible step. Worms incubated for 4 days at 35°C grew when they were returned to 23°C but only at 40-50% of the normal rate. It cannot be said

whether this low growth rate resulted because 'growth enzymes' had been damaged or degraded during the four days at 35°C,a loss which might have been made good, or because genetical information for growth had been partly destroyed. The evidence appears to be in favour of the hypothesis that genetical information for growth persists and can find expression again if the worm is returned to a low temperature. An ideal way of testing this hypothesis would be to first incubate plerocercoids at high temperatures and then return them to the body cavity of fish, using surgical transfer techniques of Bråten (1966) and record subsequent development over periods of weeks or months.

No experimental evidence could be obtained on the survival of the worms at zero and sub-zero temperatures or for a suitable minimum temperature at which plerocercoids can be stored in the laboratory. This was partly due to the non-availability of the worms at all times of the year and partly due to the long period of time involved in carrying out such experiments in order to be able to derive valid conclusions. However, it may be worthwhile to discuss this aspect on theoretical grounds and also in the light of the findings of other workers who have provided information on some other

pseudophyllidean cestodes. The biological temperature range for active life is between 0°-45° C. The upper temperature limit is unquestionably lethal to Schistocephalus but the lower limit could be safely assumed to be even below 0°C in view of the report of Wikgren and Nikander (1964) who found that plerocercoids of Diphyllobothrium latum tolerated cooling to -7° to -8°C and died at -9°C due to formation of ice crystals in the tissues and severe damage to the cuticle. Hilliard (1959) observed that plerocercoids of Diphyllobothrium sp. and Triaenophorus sp. survived at least 24 hours if their hosts were exposed to a temperature of -6° C. Scolari and Monzini (1954, quoted by Hilliard, 1959) found that 20 days would be require to kill the plerocercoids of Diphyllobothrium latum if the host, yellow perch (Perca fluviatilis) was exposed to a temperature of 0°C and at -5°C it would required only 72 hours. Since ice has lower specific gravity than water, and since water has a high specific heat and relatively low heat conductivity, aquatic animals in nature do not freeze so long as they remain in water. Therefore, in nature, Schistocephalus is probably not exposed to freezing or sub-freezing temperatures for long periods (fish protect themselves against freezing by sinking into deeper, warmer water) and it can be

extrapolated from the findings of the above authors that plerocercoids of Schistocephalus can be stored safely in a suitable medium for a considerable period at 1-2°C. However, this view is purely speculative, but it should not be difficult to test this assumption experimentally.

E. Summary

1. Plerocercoids of Schistocephalus solidus, recovered aseptically from the body cavity of 3-spined sticklebacks were cultured for 8 days in a medium composed of Hanks' balanced salt solution, glucose, yeast extract and horse serum.

2. The rate of growth of the plerocercoids was measured in culture at 4°, 7°, 12°, 17°, 23°, 27°, 30°, 33°, 35° and 40°C.

3. The growth rate increased with increase in temperature from 4° to 23°C, decreased with increase in the weight of the plerocercoid, and was maximal between 23° and 27°C.

4. Between 7° and 23°C, the increase in growth rate was nearly exponential with a mean Q_{10} of 3.6. Below 7°C, the Q_{10} was found to be only 2.0.

5. At temperatures above 27°C, after 8 days in culture, the rate of growth decreased. Small increase in weight was observed only in worms too small to mature. At 33°C, worms above 15 mg D.W., at 35°C above 6 mg D.W., and at 40°C, worms above 3.5 mg D.W. all lost weight. The larger the plerocercoid, and the higher the temperature up to 40°C, the greater was the percentage weight loss, due to earlier maturation and greater daily egg production.

6. The loss of weight after maturation appears to be due to egg production and not due to any nutritional deficiency of the medium.

7. Incubation of the plerocercoids for 4 days at 35°C, although induced maturation and suppressed somatic growth, did not destroy the ability of the worms to grow again when returned to 23°C.

8. Supplementation of the medium with chick embryo extract (CEE_{50}) did not make any significant improvement in the rate of growth of the worms.

9. Two hypotheses are propounded to explain the possible mechanisms involved in the suppression of growth after maturation. The evidence appears to be in favour of the existence of two distinct enzyme systems controlling growth and maturation, with peak efficiencies near 23° and 40°C respectively. Means of testing the validity of this hypothesis are suggested.

10. The effect of very low temperatures on the plerocercoids of Schistocephalus has been discussed in the light of the findings of various workers on some other pseudophyllidean worms and it is believed that it should be possible to store Schistocephalus plerocercoids in a suitable medium at 1-2°C.

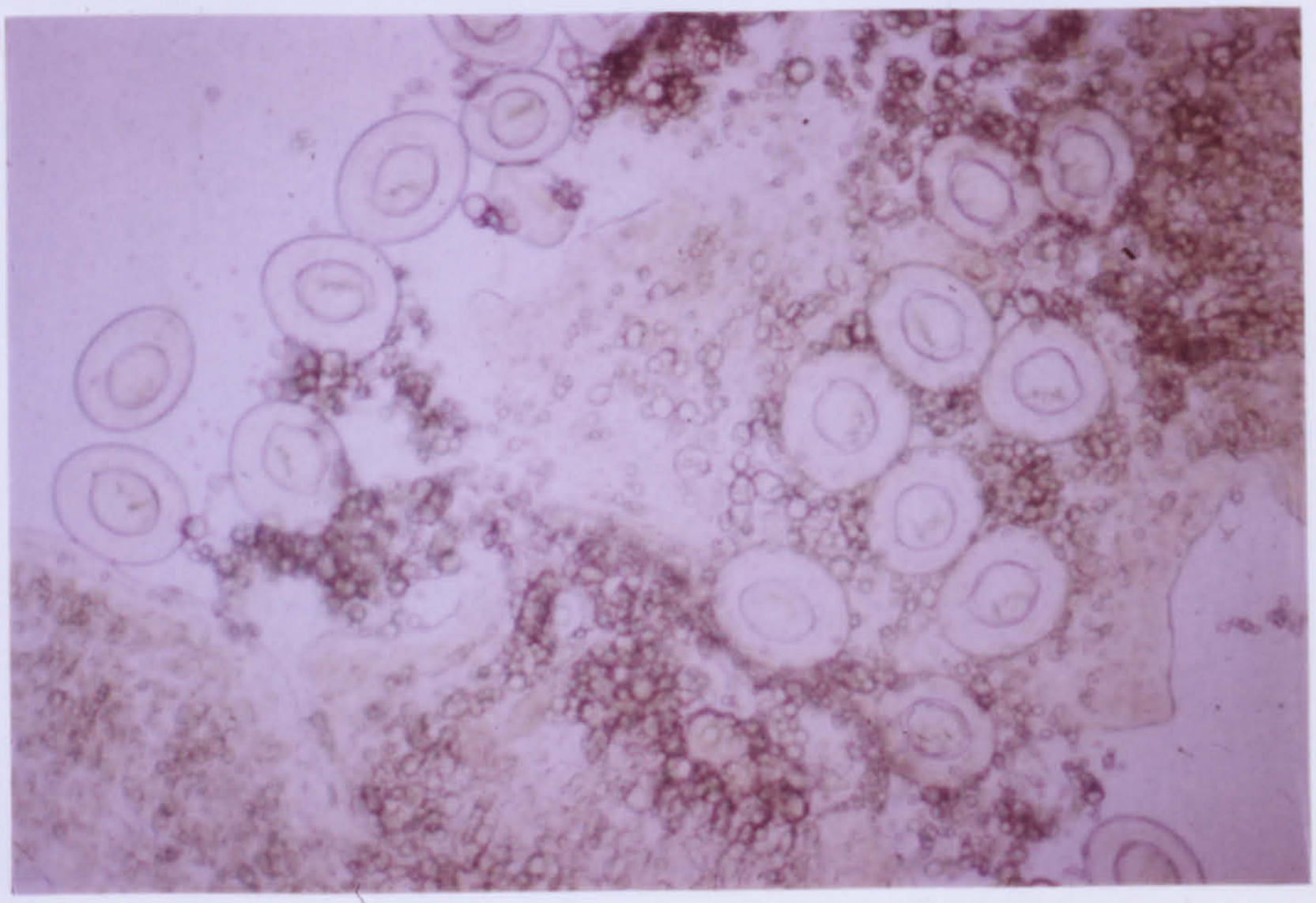
SECTION III. CULTIVATION OF *HYMENOLEPIS NANA IN VITRO*

Part of a gravid proglottid of *Hymenolepis nana* (12 days in culture) showing fully formed eggs:

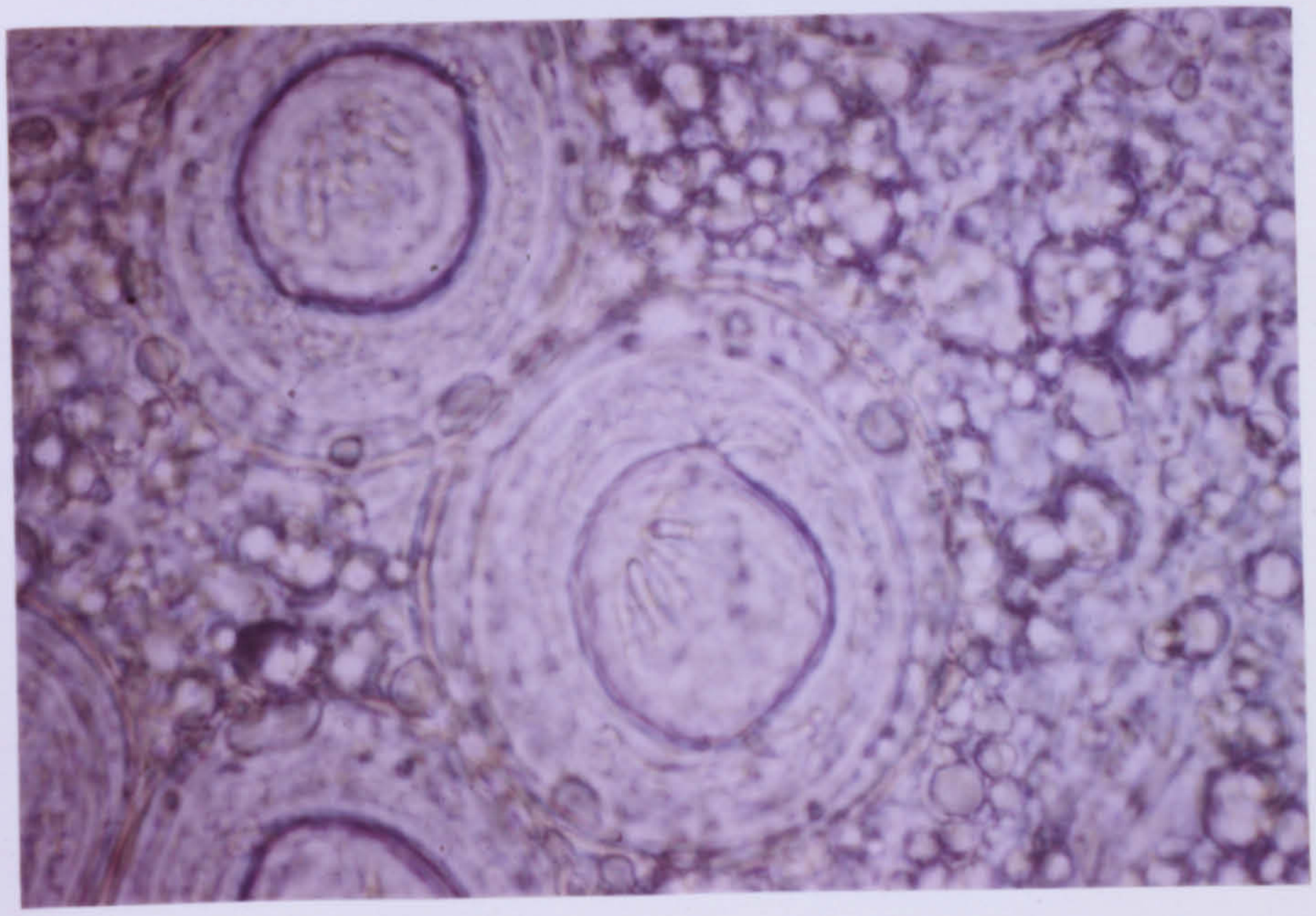
A. Low power view

B. High power view.

A



B



A. Introduction

As stated earlier (Section I, Introduction), work with Schistocephalus had to be discontinued on account of the difficulties in catching fish and obtaining a good supply of plerocercoids of suitable size at all times of the year. The dwarf tapeworm, Hymenolepis nana was chosen for studies on in vitro cultivation for the following reasons:

a) suitable small size of the adult worm
b) easy maintenance of the life-cycle of the worm in the laboratory using mice or hamsters and flour beetles
c) a short period (10-11 days) required for growth, maturation and egg production
d) thinness of the worm permitting easy observation of internal structures
e) itsimportance from the point of view of public health, as it infects human beings and has a world wide distribution
f) suitable for immunological studies because of the fact that the definitive host becomes resistant to further infection (Shorb, 1933; Hunninen, 1935; Hearin, 1941; Bailey, 1951; Heyneman, 1962, 1963).

So far, only three attempts have been made to culture Hymenolepis nana in vitro.

1. Green and Wardle (1941) made the first attempt with this cestode and tried to culture adults taken from the intestine of rats in the hope of establishing a nutrient medium in which the tapeworms would remain physiologically active for a period at least as long as the normal life period in the host. The mature cestodes recovered from rats were sterilised by allowing them to fall ten times through columns of sterile Tyrode's solution. They were then transferred to sterile media in petri dishes. The following results were obtained:

Medium	Results
1. Sterile Tyrode's solution	remained active for 9 days.
2. Baker's A medium*	remained active for 7 days, but the medium showed bacterial contamination and had to be changed every 2 days.
3. Mixture of Baker's A and Tyrode's solution in proportions of 1 : 3, 2 : 2 or 3 : 1	remained active for 3 days only.
4. Dilute solution of Baker's A in Tyrode's (10 drops of Baker's A in 5 ml of Tyrode's solution)	undulant activity maintained for 20 days.

* Baker's A medium, as given in the paper by Green and Wardle (1941), consisted of 0.675% Witte's peptone in BSS+ small quantities of cysteine HCl, hemin, insulin, thyroxine, glucose, vitamin A, ascorbic acid, and glutathione. Ten per cent ox serum was added just before use.

No growth or development was reported in any of these four media. The authors regarded the last medium (4) as quite satisfactory because it supported undulant activity of the worms for 20 days, a period nearly as long as that reported by Shorb (1933) for the normal life period of H. nana in vivo (11-16 days for prematuration and 11 days for post-maturation phases).

2. Taylor (1961) attempted to culture developing cysticercoids and excysted cysticercoids (juveniles) of both Hymenolepis diminuta and H. nana. She used small petri dishes or 15 ml roller tubes and a variety of media containing various combinations of a multitude of components viz: Ringer's or Gey's salt solution, medium 199, horse, calf, rat or human serum (both normal and that of a pregnant woman), glucose, amino acids mixture, bacterial metabolites, egg-yolk dialyzate, and extracts of chick embryo, mouse or rat intestine and tapeworm tissue. Unfortunately, all attempts were

unseccessful. The results obtained by her on the cultivation of H. nana are summarised below:

a) Cysticercoids cultured at 25°C in a medium containing a simple salt solution (dilute Ringer's solution, 1 : 1 with distilled water) and glucose survived for 5 days and were still infective to mice. They survived only for 3 days when rat or horse serum was added to a glucose-saline medium.

b) Early beetle stages could be maintained in vitro in normal condition for 7 days at 30°C, but no further development occurred.

c) The excysted cysticercoids (juveniles) remained alive and retained their infectivity for limited periods. The majority of media were inadequate to support any growth. Addition of serum to medium 199 prolonged the survival of the juveniles, which survived for 9 days in a mixture containing medium 199, amino acids and horse serum. They remained infective for 8 days when horse serum in the medium was replaced by calf serum.

d) The juveniles showed some growth if an extract of mouse intestine was added to the medium.

e) Media containing pregnant human serum, bacterial metabolites, and extract of H. diminuta were toxic.

f) Addition of chick embryo extract, normal human serum, horse serum ultrafiltrate, H. nana extract or rat's intestine extract did not produce any improvement in the growth and survival of the juveniles in culture.

g) A level of 400 units of Penicillin and 500 µg of Streptomycin (calculated from the figures given) in the medium was well tolerated by the parasites.

3. Berntzen (1962) made the first successful and dramatic breakthrough on the in vitro cultivation of cestodes by being able to culture H. nana from the excysted cysticercoid stage to the egg-bearing adult stage. He excysted the cysticercoids aseptically by using sterile pepsin-HCl and pancreatin-trypsin-bile salt solutions (see Section IV for details of excystation) and cultured them in an ingeniously designed continuous flow culture apparatus containing 100 ml of medium. Two media, designated 101 and 102 were used. The most consistent results and the highest percentage of recovered worms were obtained with medium 102 and a gas phase of 95% nitrogen + 5% CO_2 at 39°C. pH of the medium between 7 and 9 had no apparent effect on the growth and maturation of the worms. The

most interesting finding was that addition of antibiotics viz. crystalline Penicillin G, Streptomycin hydrochloride, Chloromycetin and Tetracycline inhibited the growth of the worms. He also compared the development and order of appearance of various organs and structures in cultured worms with that of worms recovered from experimentally infected mice and found that there was a difference of 1-2 days between the cultured and in vivo worms throughout the first 12 days of development. Worms in culture required 12 days to produce infective eggs whereas visibly normal and viable eggs were found in in vivo worms within 11 days after infection with cysticercoids. Eggs from cultured worms developed in beetles into normal cysticercoids with which mice could be infected.

This report of Berntzen was a significant contribution to the field of cestode cultivation. A few of his other studies (Berntzen, 1961; Berntzen and Mueller, 1964; Berntzen, 1965) also have added much to the present day knowledge of the cultivation of helminths in vitro. In view of his findings it was realised that several aspects of the biology and host-parasite relationships of H. nana could be studied if it were possible to exploit his techniques and reproduce the results obtained by him.

My work on the *in vitro* cultivation of *H. nana* (Results, Part I) was started with Berntzen's (1962) medium 102 and the continuous flow culture apparatus. It was not successful but it should be pointed out that some ambiguities exist concerning the actual composition of medium 102 which makes it impossible to equate the work with Berntzen's. Further attempts were made using various modifications of medium 102 but these also were not successful. It was decided therefore to make a fresh approach to the problem of the *in vitro* cultivation of *H. nana* and the experiments leading to the development of a successful medium in which freshly excysted cysticercoids of *H. nana* would grow to egg-bearing adults are described in Results, Part II.

B. Material and Methods

(1) Aseptic excystation of cysticercoids

Excystation of cysticercoids of H. nana was performed in a bench incubator at 37°C. All solutions and glassware used were sterile. The chemicals were supplied by the British Drug Houses. Three solutions were used for the excystation of the cysticercoids:

Hanks' BSS - See Section I for its formula, method of preparation and sterilisation.

Pepsin solution - 0.5g of pepsin (1:2500) was dissolved in 50 ml of Hanks' BSS and its pH adjusted to 1.7 by addition of 0.2N-HCl.

Trypsin-Bile salt solution - 0.25g of trypsin (83:1) and 0.15g of sodium tauroglycocholate were dissolved in 50 ml of Hanks' BSS. The pH of this solution was brought up to 7.0 with 0.2N-NaOH and then raised to 7.1-7.2 by addition of 1.4% $NaHCO_3$ solution.

The pepsin and trypsin-bile salt solutions were prepared once every 4-5 weeks and stored at 4°C. The ingredients were weighed in sterile aluminium foils and dissolved in sterile Hanks' BSS and pH of the solutions adjusted with sterile acid or alkali solutions. An aqueous solution of Crystamycin (Glaxo Ltd.) was added to these excysting fluids as well as Hanks' BSS to provide

Figure 12

Aseptic excystation of cysticercoids of H.nana in a bench incubator (37°C).

A - solid watch glass containing pepsin soln.

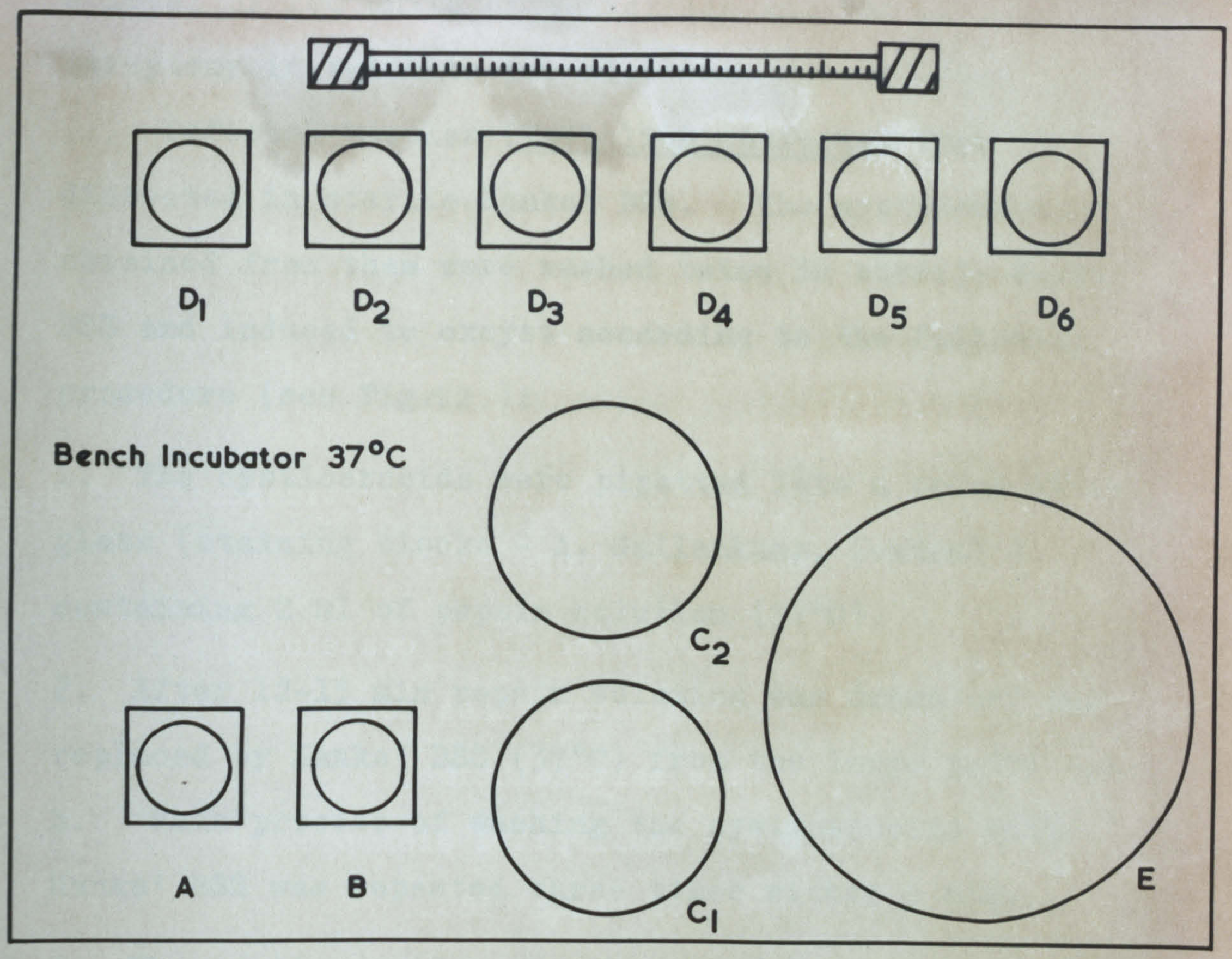
B - solid watch glass containing trypsin-bile salt solution.

C_1 - C_2 - small petri dishes containing 10-12 ml of Hanks' BSS.

D_1 - D_6 - solid watch glasses containing 1 ml of Hanks' BSS.

E - Large petri dish containing Hanks' BSS.

Figure 12.



a level of 100 units of penicillin and 100 μg of streptomycin sulphate per ml.

Infected beetles (Tribolium confusum) were dissected in sterile Hanks' BSS. The cysticercoids obtained from them were washed twice in sterile Hanks' BSS and induced to excyst according to the following procedure (see Fig.12):

1. The cysticercoids were pipetted into a solid watch glass (staining blocks - A. Gallenkamp, London) A containing 2 ml of pepsin solution (37°C).

2. After 12-15 min pepsin solution was drawn off and replaced by Hanks' BSS (37°C) from the large petri dish E. This process of washing the cysticercoids with Hanks' BSS was repeated three times within 5 min.

3. The cysticercoids were then transferred to another staining block B containing 2 ml of the trypsin-bile salt solution (37°C).

4. After 6 min when about 50% of the cysticercoids were found to have excysted (though not completely free from cyst tissue), half of the trypsin-bile salt solution was drawn off and an equal volume of Hanks' BSS (37°C) was added to the container B.

5. Two to three minutes later half the liquid in container B was again drawn off and replaced by an equal volume of

Hanks' BSS (37°C).

With a lot of 300-400 cysticercoids, 60-80% were found to excyst within 10-12 min of placing them in the trypsin-bile salt solution. The excysting cysticercoids were frequently examined under a dissecting microscope for their activity and appearance as prolonged exposure to the undiluted trypsin-bile salt solution caused damage to the worms, making them inactive and appear dark and wrinkled.

6. When about 60% or more of the worms were found to have excysted the trypsin-bile salt solution was removed from the container B and replaced by 3 ml of Hanks' BSS (37°C). This process of washing with Hanks' BSS was repeated three times.

7. The excysted worms were then transferred to a small petri dish (C_1) containing 10-12 ml of Hanks' BSS. From this container only good looking and active larvae were picked up individually and placed in another small petri dish (C_2) containing 10 ml of Hanks' BSS (37°C).

8. Worms, as many as desired, were transferred from the petri dish C_2 to each of several solid watch glasses (D_1-D_6) containing 1 ml of Hanks' BSS. The larvae soon settled down at the bottom of these containers. The Hanks' BSS was then drawn off from all these solid watch

glasses leaving the larvae in approximately 0.5 ml of Hanks' BSS in each.

These worms were then immediately inoculated into previously prepared culture tubes by means of a sterile Pasteur pipette.

(2) Culture vessels

In all experiments in which Berntzen's techniques were applied, a slightly modified form of his continuous flow culture apparatus was used. For other experiments, 125 x 25 mm roller tubes were used.

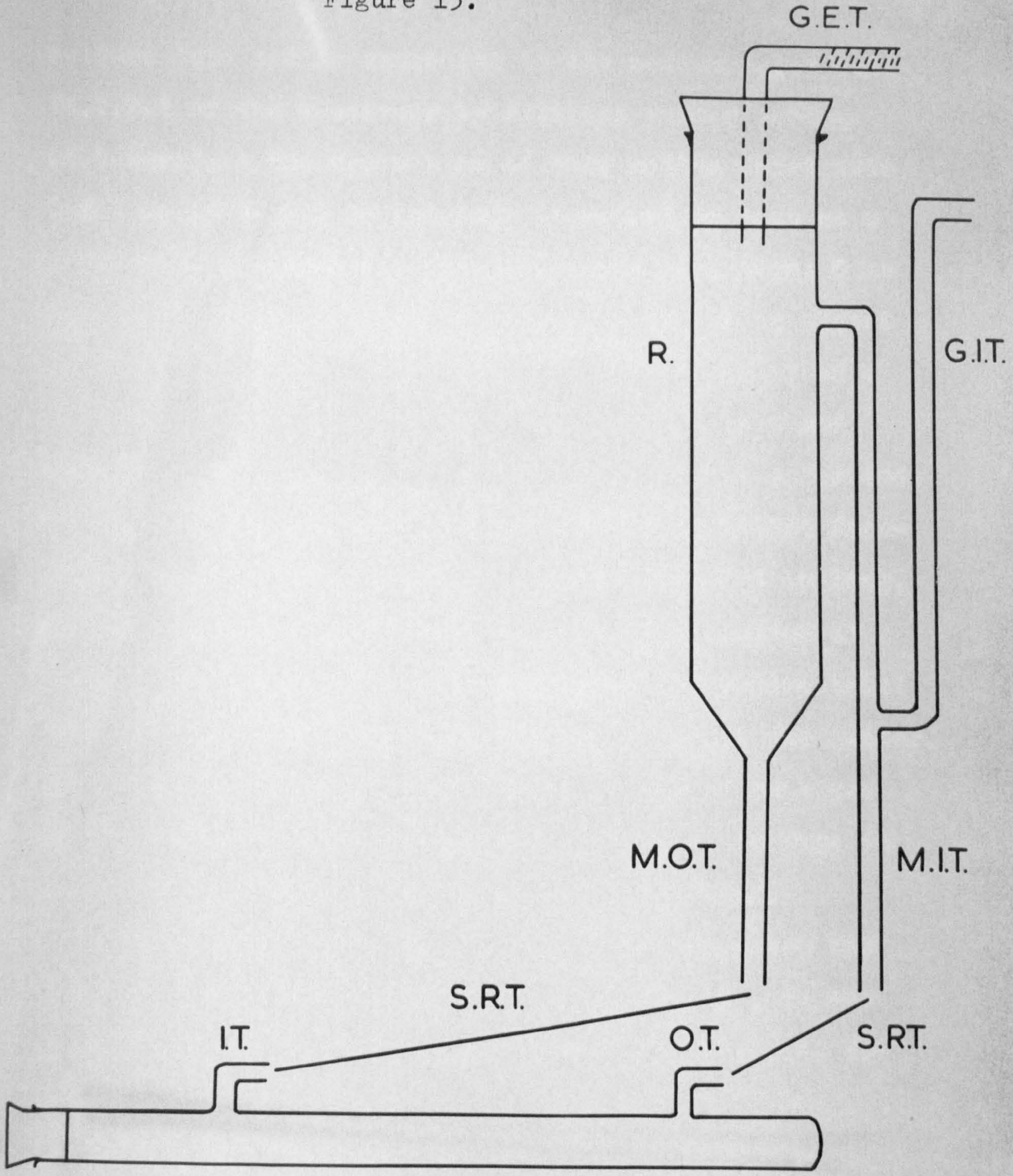
The continuous flow culture apparatus (Fig.13) is made of Pyrex brand glass and consists of 2 parts: a reservoir 'R' (17 cm long, 3.5 cm internal diameter) with a Y-shaped medium-gas inlet assembly, and a culture tube 'C.T.' (21 cm long, 1.2 cm internal diameter). The lower end of the reservoir leads into a medium outlet tube 'M.O.T.' (6 cm long, 0.4 cm internal diameter) which is connected to the inlet tube 'I.T.' of the culture tube by Escorubber translucent silicone rubber tubing 'S.R.T.' (4.5 mm bore). The inlet tube 'I.T.' is 3 cm long, 0.4 cm in internal diameter, bent in the middle at a right angle and is located at 5 cm from the open end of the culture tube. The medium outlet tube 'M.O.T.' having the same dimensions as that of the inlet tube is located 3.5 cm from the sealed end of the

Figure 13

Berntzen's Continuous flow culture apparatus
(modified)

C.T.	Culture tube
G.E.T.	Gas exhaust tube
G.I.T.	Gas inlet tube
I.T.	Inlet tube
M.I.T.	Medium inlet tube
M.O.T.	Medium outlet tube
O.T.	Outlet tube
R.	Reservoir
S.R.T.	Silicone rubber tubing

Figure 13.



CONTINUOUS FLOW APPARATUS

culture tube and is connected to the medium inlet tube 'M.I.T.' of the reservoir by silicone rubber tubing (4.5 bore). The upper 1 cm of this medium inlet tube (17.5 cm long, 0.4 cm internal diameter) is bent at a right angle to open into the reservoir at a point 5 cm from its mouth. The mouth of the reservoir is closed by an E33 Escorubber bung through which is inserted a gas exhaust tube 'G.E.T.' bent at a right angle and plugged with non-absorbent cotton wool at the outer end. The culture tube is closed by an E13 (Qual.TC156) Esco-rubber bung. The gas inlet tube 'G.I.T.' (16 cm long, 0.2 cm internal diameter) is bent at right angles at either end. Its lower end opens into the medium inlet tube at a point 11.5 cm from the upper end of the latter. The free end of the gas inlet tube is joined to a gas filter tube (for sterilising the gas) which in turn is connected by a rubber tubing to a gas cylinder.

The culture tube was lined with filter paper and when completely assembled as shown in Fig.13 , it was wrapped and sterilised by autoclaving at 15 p.s.i. for 20 min.

(3) Setting up of cultures

Continuous flow apparatus cultures - Each culture unit required 100 ml of medium. Before filling the culture

apparatus with the medium, the rubber tubing connecting the medium outlet tube ('M.O.T.' Fig.13) of the reservoir and the inlet tube (I.T.) of the culture tube was clipped tight. About 60 ml of medium was poured into the reservoir and the clip on the rubber tubing gradually loosened so as to allow the medium to flow slowly into the culture tube. While the culture tube was filling it was slightly slanted to allow air to escape through the medium outlet tube. The medium would slowly flow up through the outlet tube into the medium inlet tube of the reservoir. Finally, more medium was poured into the reservoir to bring its level just below the opening of the reservoir inlet tube. The opening of the reservoir was closed by a sterile bung and the culture apparatus was placed in an incubator and connected to the gas supply. The pressure and flow of gas was controlled in order to have a desired rate of flow of the medium in the culture apparatus. The incubator was so adjusted that the medium in the culture apparatus would have a temperature of 38.5°-39°C. The culture unit was placed in the incubator at least 2 hours before inoculation with excysted worms to achieve temperature and gas balance in the medium.

Just before inoculation of the culture tube with worms, the gas supply was closed, the rubber tubings

were clipped and the culture tube was tilted so as to have the stopper end up. The bung was carefully removed and freshly excysted worms were pipetted into the culture tube. The bung was replaced and the culture tube was returned to its original position. The clips were removed from the rubber tubings and the gas supply restarted and adjusted.

Medium replacement - The gas supply was closed and the culture unit was removed to a sterile cabinet. The exhaust stopper was removed from the reservoir and the medium was drawn off by means of a sterile pipette. New medium (previously gassed and heated to the same temperature as that of the culture) was poured to the original level and the exhaust stopper was replaced. The culture unit was then returned to the incubator and the gas supply started.

Roller tube cultures - 5 ml of medium was pipetted into each roller tube and gassed for about 40 sec. The gas was first allowed to pass through a gas filter tube and then through a sterile Pasteur pipette (plugged with cotton wool at the broader end) held above the medium. The roller tubes were immediately closed by E21 Escorubber bungs and placed in a roller drum, rotating once in 7 min. inside an incubator adjusted so

that the temperature of the medium would be 38± 0.5°C. This was done 2 hours before the inoculation of the cultures with the worms. Freshly excysted worms were pipetted into each roller tube which was then regassed for about 15-20 sec., sealed and returned to the roller drum inside the incubator.

Medium to be used for replacement was gassed after preparation and allowed to attain the temperature of the cultures. The old medium in each culture tube was drawn off and new medium added by means of a sterile pipette. The tubes were gassed for 15-20 sec., sealed and returned to the incubator.

Strict aseptic precautions were taken at all stages of the cultivation procedure. Inoculation of cultures, gassing, and medium replacement were all done inside a Hanovia cabinet fitted with a ultraviolet lamp. Antibiotics were not used in medium 102 or its modifications in view of the observations of Berntzen (1962) that "antibiotics, penicillin G (crystalline), streptomycin hydrochloride, chloromycetin, and tetracycline, when added to the culture medium, interfered with and inhibited growth of the worms." All media used in roller tube cultures contained Crystamycin (Glaxo Ltd.) so as to have 100 units of penicillin and 100 µg of streptomycin sulphate

per ml. Cultures contaminated with bacteria or fungi were discarded.

(4) Gas phase - In all cultures (continuous flow as well as roller tubes) a gas phase of 95% nitrogen and 5% CO_2 was used. Berntzen (1962) found only this gas mixture yielded positive results. Several other gases or gas mixtures (95% O_2 + 5% CO_2, 100% CO_2, 100% nitrogen, compressed air) used by him all gave negative results. The gas mixture of 95% nitrogen + 5% CO_2 was supplied by the British Oxygen Company.

(5) Medium 102

The following solutions were prepared using chemicals supplied by the British Drug Houses Ltd. The inorganic salts were 'ANALAR' grade chemicals. The solutions were dispensed in suitable aliquots into screw-top bottles and stored at -15°C.

Solution A - This was prepared in three parts:

Part I	$NaH_2PO_4.2H_2O$	1.14	
	KCl	2.24	
	$MgCl_2.6H_2O$	2.20	(stored in 16 ml aliquots)
	$MgSO_4.7H_2O$	2.50	
	deionised water	160 ml	
Part II	$CaCl_2$(dried)	0.50 g	(stored in 2 ml aliquots)
	deionised water	20 ml	

Part III	$NaHCO_3$	0.35 g
	de-ionised water	20 ml

(stored in 2 ml aliquots)

Parts I, II, and III were mixed in proportion of 16:2:2 to obtain Solution A.

Solution B - This was also prepared in three parts:

		g
Part I	L-arginine	0.700
	L-aspartic acid	0.500
	L-asparagine	0.500
	L-alanine	0.225
	B-alanine	0.200
	L-glutamic acid	0.700
	L-glutamine	0.700
	glycine	0.850
	L-histidine	2.500
	L-isoleucine	0.050
	L-leucine	0.075
	L-lysine HCl	0.625
	L-methionine	0.060
	L-proline	0.350
	L-phenylalanine	0.200
	DL-serine	1.100
	L-tryptophane	0.100
	L-threonine	0.200
	L-valine	0.100
	de-ionised water	250 ml

(stored in 12.5 ml aliquots)

Part II L-cystine (25 mg) was dissolved in 2.5 ml of N-HCl and the volume made up to 25 ml with de-ionised water. This solution was stored in 2.5 ml aliquots.

Part III 50 mg of L-tyrosine were dissolved in 25 ml of 0.1N-HCl. This solution was stored in 2.5 ml aliquots.

This is slightly different from the method used by Berntzen (1962), who dissolved L-cystine "in 1/N HCl" and L-tyrosine "in N/1 HCl." This presumably meant that he used normal HCl in both cases. Parts I, II and III were mixed in proportion of 25:2.5:2.5 to obtain Solution B.

Solution C

	g	
Sucrose	26.68	
Fructose	0.40	(stored in 20 ml aliquots)
Glucose	1.50	
deionised water	200 ml	

Solution D

Malic acid	0.700
alpha-ketoglutaric acid	0.450
Succinic acid	0.080
Fumaric acid	0.075
deionised water	100 ml

The solution of these acids was neutralised with N-KOH, volume made up to 200 ml with deionised water and stored in 20 ml aliquots.

Solution E

Solution E of Berntzen's medium 102 consists of separate solutions of each of the following vitamins in 25 ml of distilled water:

	mg
Thiamine HCL	2.5
Riboflavin	2.5
Ca pantothenate	2.5
Pyridoxine HCl	2.5
p-aminobenzoic acid	2.5
Folic acid	2.5
Niacin	2.5
i-inositol	2.5
Biotin	1.25
Choline chloride	25.00

Before use, 2ml of each vitamin stock solution are mixed together and 0.2 ml of the mixture is added to each 100 ml of medium (mixture of solutions A, B, C and D).

For use in the experiments reported here, the solution of the vitamins was prepared in three parts as given below (following Berntzen and Mueller, 1964):

Part I - 50 mg of choline chloride and 5 mg of each of the following vitamins were added to 25 ml of de-ionised water:- thiamine HCl, Ca pantothenate, pyridoxine HCl, p-aminobenzoic acid, niacin, and i-inositol.

Part II- 5 mg of folic acid and 2.5 mg of biotin were dissolved in 10 ml of de-ionised water to which a drop of N-KOH had been added.

Part III - 5 mg of riboflavin were dissolved in 15 ml of deionised water by heating to 50°C.

The three parts were mixed to have 50 ml of the vitamins solution which was stored in 2 ml aliquots. 0.02 ml of this solution was added to each 100 ml of medium. This amount provided the same level of each vitamin in the medium as 0.2 ml of the solution E of medium 102 (Berntzen, 1962).

Solution F - Agamma calf serum/Horse serum.

Berntzen used agamma calf serum at a concentration of 20% in the final medium, although in the description of the formula of medium 102 he wrote "Solution F - Agamma calf serum 20 ml." Due to the non-availability of this serum in Britain, horse serum was used in most of the experiments. Only 2 experiments were done using agamma calf serum. In all experiments serum was added to give a final concentration of 20%.

A quantity of the final medium sufficient for one culture was prepared by mixing the following:

Solution A	20 ml
Solution B	30 ml
Solution C	20 ml
Solution D	20 ml
Solution E	0.02 ml
Solution F	25 ml

This mixture was serially filtered using 1.2, 0.8, 0.45, and 0.22 μ millipore filters and then sterilised by passing it through a sterile 0.22 μ millipore filter. The pH of the medium was then adjusted to 7.3 to 7.5 by addition of 0.2N-KOH.

Filtration of the medium with serum was very difficult, hence in some experiments the mixture of solutions A, B, C and D was first sterilised by filtration and then 20% serum was added to this sterile mixture to complete the medium.

The media used in roller tube cultures are described at appropriate places. The methods of preparation and sterilisation of some ingredients such as Hanks' BSS, glucose, yeast extract, chick embryo extract and $NaHCO_3$ soln. have already been described in Section I.

C. Results

(Part I)

Cultivation of H. nana in medium 102 and continuous flow culture apparatus.

The results of ten experiments on the cultivation of H. nana using techniques employed by Berntzen (1962) are summarised in Table 4. Agamma calf serum, as used by Berntzen, could not be used throughout because of its non-availability in Britain. However, two experiments (Expts. 5 and 6, Table 4) with agamma calf serum did not indicate that it was any better than horse serum - an inexpensive product available easily all over the world. Supplementation of the medium 102 containing horse serum with chick embryo extract (CEE_{50}) or 1·0% Panmede (Expt. 8) or 0.5% Bacto Yeast extract (Expt. 10) or replacement of serum by chick embryo extract did not improve the results in any way. It rather appeared as if media containing chick embryo extract were toxic to the worms as indicated by their dark appearance and inactive condition in culture. Only on two occasions (Expts. 2 and 9) did worms show some growth with proglottid formation.

It would have been reasonable to attempt cultivation of H. nana in medium 101 (Berntzen, 1962), but the non-

TABLE 4. Cultivation of H.nana in medium 102 and its modifications using continuous flow apparatus (Berntzen,1962).

Expt.	No. of cultures	Culture No.	Medium 102 containing	pH	Worms inoculated	Day medium changed	Day culture opened	Worms recovered	observations
1	2	A	20% HS	7.5	21	-	3	17	Active, no proglottids, size 0.8-0.9 x 0.04-0.05 mm.
		B	,,	,,	17	-	3	13	,, ,, ,,
2	2	A	,,	,,	35	-	5	26	definite growth; 9 worms showing proglottids at the posterior end.
		B	,,	,,	20	-	2	-	Culture contaminated-discarded
3	2	A	,,	7.3	21	4	6	14	Worms active, but no growth
		B	,,	,,	12	4	6	3	,, ,, ,,
4	2	A	,,*	7.5	20	-	7	10-11	No growth, worms dead.
		B	,,	,,	19	-	7	,,	,, ,, ,,
5	2	A	20% HS	7.5	15	-	5	2	Worms inactive, no growth.
		B	20% ACS	,,	15	-	5	0	
6	2	A	20% HS*	7.4	16	6	9	9	Worms active, no growth.
		B	20% ACS*	,,	16	6	9	7	,, ,, ,,
7	2	A	20% HS	7.5	23	4	8	4	Worms dark, no growth.
		B	,,*	,,	26	4	8	5	No growth, 3 dead.
8	3	A	20% HS	7.5	30	3	6	21	Some growth, no proglottids.
		B	20% HS + 10% CEE_{50}	,,	31	3	6	11	Inactive, dark, no growth.
		C	20% HS + 1.0% P	,,	27	3	6	20	Active, but no growth.
9	3	A	20% HS	7.4	24	4	8	16	Active, 7 worms with indistinct proglottids.
		B	10% CEE_{50} (no serum)	,,	30	4	8	3	No growth.
		C	20% CEE_{50} (no serum)	,,	30	4	8	0	
10	2	A	20% HS	7.4	25	4	6	19	Some growth, no proglottids.
		B	20% HS + 0.5% YE	,,	35	4	6	25	,, ,, ,,

HS = horse serum ACS = agamma calf serum CEE_{50} = chick embryo extract P = 'Panmede' YE = Bacto yeast extract
* = added to the sterilized mixture of solutions A-E.

availability of 'trypticase' in this country, the omission of the name of the supplier of the yeast extract, the type of peptone used, and other ambiguous details concerning its preparation, made it difficult to use this medium.

The failure of the worms to grow in the media used in these experiments does not prove anything against medium 102 of Berntzen. Failure was probably due to not exactly duplicating the procedures he used (see Discussion). However, having failed to achieve any success with Berntzen's techniques, it was decided to make a fresh approach to the problem of cultivation of H. nana in vitro, using simple or complex media with natural or chemically defined components, or both. For the purpose of screening media and physical conditions, the use of a simple culture vessel such as a roller tube was felt essential. Although the continuous flow culture apparatus is an excellent culture vessel in several respects, several difficulties and disadvantages became apparent in use:

1. It is a special type of delicate and complex apparatus; its availability and maintenance would be difficult for average type laboratories.

2. Each culture unit requires about 100 ml of medium, thus

making the cultivation work quite expensive. It is therefore unsuitable for screening media.

3. It is difficult to run more than two cultures in one incubator. Experimentation on a large scale involving several cultures would either require a large number of incubators or a constant temperature room. If several gas cylinders and incubators are used in an experiment, results may vary as conditions may not be the same in each culture.

On the other hand, the advantages of using roller tubes are:

1. They are cheap, handy and easily prepared by any glass blower according to specifications.

2. Each tube requires a small volume (5-6 ml) of medium. Cultivation becomes economical even if ingredients of media are expensive.

3. 100-200 of such tubes can be placed on roller drums inside one incubator. Thus, a large number of media can be tested at one time and large numbers of cultures can be run even if only one incubator is available to the worker.

4. Continuous bubbling of gas is not required.

5. These tubes can be cleaned and sterilised in large

numbers whenever required.

The results obtained using roller tubes are described in Part II.

C. Results

(Part II)

Having decided to start the work on *in vitro* cultivation of *H. nana* afresh, the first problem was to develop a basal medium in which the freshly excysted juveniles would survive, grow, and possibly form proglottids. If this could be achieved, one could try to improve this medium by supplementing it with several other ingredients. It seemed reasonable to start along the lines of McCaig and Hopkins (1965) who developed a suitable medium for the plerocercoids of *Schistocephalus* and recorded dry weight increases up to 500% in 8 days in roller tube cultures.

(a) Early experiments on the development of a basal medium.

1. Glucose-saline, Glucose-saline + yeast extract, and Glucose-saline + yeast extract + Panmede.

(i) *Glucose-saline* (BSSG)

Hanks' BSS + 0.3% glucose (added as a 6.5% aqueous solution). Cultures were opened on days 2, 3 and 4. All worms were found dead and degenerating from their 'tail' ends on day 2. In one culture opened on day 4, only a single dead worm out of 21 inoculated was found. The final pH of the medium was between 7.2-7.5 It appeared as if glucose-saline medium was too poor to support survival

of the worms.

(ii) Glucose-saline + yeast extract (BSSG + YE).

Hanks' BSS + 0.3% glucose + 0.5% Bacto yeast extract (added as a 5% aqueous solution).

Cultures were opened on days 3, 5 and 9. On days 3 and 5, 50% or more of the worms were found alive and quite active, but apparently no growth had taken place. By day 9, all were found dead. The addition of yeast extract to glucose-saline seemed to produce a good effect.

(iii) Glucose-saline + yeast extract + Panmede (BSSG+YE+P).

Hanks' BSS + 0.3% glucose + 0.5% Bacto yeast extract + 0.5% Panmede (added as a 5% aqueous solution).

'Panmede' is an ox liver digest supplied by Paines and Byrne Ltd. Greenford, Middlesex. It has been successfully used in the preparation of culture media for protozoa, fungi, and bacteria and is said to be a source of all B-vitamins, trace elements of liver, and a balanced complement of liver amino-acids. It is readily soluble in water and used to replace peptone. It was felt that its addition to the BSSG+YE medium should have a beneficial effect. More than 30% of the worms were found alive in an active condition but without any appreciable growth up to day 5. On day 7, all were found dead. It was obvious that the addition of Panmede was of no advantage to the worms.

TABLE 5. Cultivation of H.nana in glucose-saline, glucose-saline + yeast extract, and glucose-saline + yeast extract + Panmede.

Medium	Culture No.	No. of worms	Day opened	No. alive	Observations
Glucose-saline (BSSG)	G 1	37	2	0	All dead
	G 2	32	3	0	All dead
	G 3	33	3	0	All dead
	G 4	21	4	0	Dead and autolysed
Glucose-saline + yeast extract (BSSG+YE)	Y 1	34	3	17	Active but not growing
	Y 2	33	5	22	,, ,, ,,
	Y 3	31	9	0	All dead
	Y 4	22	9	0	All dead
Glucose-saline + yeast extract + Panmede (BSSG+YE+P)	L 1	31	2	10	Active, no growth
	L 2	32	5	10	Active, no grpwth
	L 3	30	7	0	All dead
	L 4	30	7	0	All dead

Final pH of medium in cultures was between 7.2-7.5.

The observations on the worms cultured in these 3 media are summarised in Table 5. The addition of yeast extract to the glucose-saline medium resulted in the survival and maintenance of activity of the worms until day 5. It might be that the presence of yeast extract either produced a good effect or prevented the occurrence of the bad effect of the glucose-saline medium. It was therefore decided to use the BSSG+YE medium as a 'control' medium and observe the effect of supplementing it with chick embryo extract which is well known for its growth promoting properties and has been widely used by workers on tissue-culture and by those who have successfully cultured helminths *in vitro* (Dougherty *et al.* 1950; Nicholas, 1956; Weinstein and Jones, 1956; Nicholas *et al.* 1959; Berntzen, 1961).

2. Effect of adding chick embryo extract to BSSG+YE medium.

Chick embryo extract (CEE_{50}) was prepared as described in Section I and was used with BSSG+YE at four concentrations - 50%, 25%, 10% and 5%. Three cultures were set up in each medium including the control (BSSG+YE) and one culture tube of each medium was opened on day 4, 6 and 8. The results are recorded in Table 6.

50% CEE_{50}- Only 10% of the worms were found alive on day 4. They were in a bad condition and appeared dark and inactive. By day 6 all were dead.

25% CEE_{50}- About 30% of the worms were found alive on day 4 but they were inactive and abnormal in appearance. By day 6 all were dead.

10% CEE_{50}- About 90% of the worms were found alive in a very active condition on day 4. The same percentage survival was observed on day 6 but their appearance had deteriorated considerably. On day 8 only one out of 30 inoculated was found alive but in a miserable state.

5% CEE_{50}- On day 4, 33% of the worms were found alive. Out of these, only a few appeared normal; others were swollen and degenerating. On day 6, 17% survival was observed but worms were inactive and dark. By day 8, all were dead.

Control (BSSG+YE)- In the control cultures, 80% of the worms were found alive and in a very active and good condition up to day 6. About 10% of these had definitely grown but there was no sign of any proglottids. By day 8, although 24 out of 33 (72%) worms were alive, their appearance and activity had considerably deteriorated.

It may be noted here that although the medium in

TABLE 6. Cultivation of H. nana: Effect of adding chick embryo extract (CEE_{50}) to glucose-saline + yeast extract (BSSG+YE) medium.

Medium	Culture No.	No. of worms	Day Opened	No. alive	Observations
BSSG+YE (Control)	1	30	4	26	Active, good appearance, 2 worms growing
	2	36	6	27	3-4 worms with definite growth
	3	33	8	7	Poor condition, no further growth
BSSG+YE+ 50% CEE_{50}	1	30	4	3	3 worms, dark and swollen
	2	30	6	0	All dead
	3	31	8	0	All dead
BSSG+YE+ 25% CEE_{50}	1	31	4	9	Worms poor, no growth
	2	33	6	0	All dead
	3	30	8	0	All dead
BSSG+YE+ 10% CEE_{50}	1	31	4	27	Worms normal, healthy and active
	2	32	6	28	Worms active, but dark; little growth if any
	3	30	8	0	All dead
BSSG+YE+ 5% CEE_{50}	1	36	4	12	Some worms normal, others dead
	2	34	6	6	Dark, swollen and inactive
	3	32	8	0	All dead

Final pH of medium in cultures was between 7.2-7.4.

any of the culture tubes was never changed, the pH of the medium in each culture tube remained apparently unchanged and within the desired range. Out of the 4 different concentrations of CEE_{50} tried in this experiment, only 10% level of CEE_{50} in BSSG+YE seemed to show some good effect. However, the worms in this medium also were in no way better than those in the control medium, therefore the idea of using CEE_{50} as an additive was dropped.

3. Effect of adding preparations from rat's blood.

In spite of the advances in the knowledge of the nutrition of cells, tissues and organisms in cultures, the basis of most media has been some sort of biological fluid. The one that has been most commonly used is serum. Recently, Schiller (1965) has successfully grown Hymenolepis diminuta in a diphasic medium containing defibrinated rabbit blood. It was thought that a blood preparation from a natural host animal might have some specific essential substance(s) which is required for the growth and maturation of H. nana. Three preparations from rat's blood were therefore used to supplement the control medium BSSG+YE.

Defibrinated blood.

Rats were anaesthetised with 'trilene'(Trichloroethylene,

I.C.I.) vapour. Each anaesthetised rat was laid on its back, ventral surface swabbed with alcohol, and heart exposed using sterile instruments. Blood was collected from the heart by ventricular puncture by means of No. 1, 21G $1^1/2$" needles fitted to a Johnson's sterile, disposable, 5 ml luer syringe (Johnson's Ethical Plastics Ltd. Slough, Bucks), transferred immediately into a sterile screw-top universal container and stirred with a sterile glass rod. After a few minutes, the heavy clot adhering to the glass rod was discarded. Such bottles containing defibrinated blood were immediately stored at 4°.

Heparinised blood.

0.2 ml of 'Pularin' (Heparin injection containing 25,000 I.U. per ml, Evans Medical Ltd. Speke, Liverpool) was mixed with 25 ml sterile Hanks' BSS; 0.1 ml of this heparinised BSS was used in each universal container in which approximately 10 ml of blood was to be collected. Blood from rats was collected in the same way as described above.

Rat serum.

Blood was collected from rats and defibrinated as described above. It was then centrifuged in 10 ml centrifuge tubes for 20 minutes at 3,000 r.p.m. (approx. 2100g) at 4°C. The supernatant (serum) was aseptically

drawn off and stored at 4°C.

Each of these three blood preparations was tried at two levels - 25% and 10%, in the medium BSSG+YE. The observations on the effect of these on the survival and growth of H. nana are recorded in Table 7.

BSSG+YE+rat serum.

90% of the worms were alive on day 4 with excellent activity and appearance. They showed definite growth in size but no proglottid formation at either concentrations of serum. In both media, almost all were found dead on day 7 except a few which though alive were in a poor condition and without any further growth beyond that observed on day 4.

BSSG+YE+defibrinated blood.

In the medium with 25% defibrinated blood, no trace of the worms was found when a culture was opened on day 4. It was thought that the worms might have been overlooked in the dark and opaque medium. However, on day 6 also no trace of worms, dead or alive, was found. In one culture with 10% defibrinated blood, 8 worms out of 30 inoculated at the beginning were found to be in quite good and active condition on day 4 and appeared to have grown as much as they did in rat serum medium. On day 6 not a single living worm could be recovered.

BSSG+YE+heparinised blood.

It was very difficult to recover worms from a medium of this consistency. At 25% level, less than 20% of the worms were recovered alive on day 3 and were found to be inactive and dark in appearance. The final pH of the medium was 6.9. Only dead worms were found on day 5 and 6 at both concentrations of heparinised blood. The final pH of these cultures was in the range 6.7-6.8. Unfortunately, the medium was not examined microscopically for bacterial contamination and it was difficult to assess this by the consistency of the opaque medium.

BSSG+YE(control).

In the control cultures, 80% of the worms were alive on day 4. Growth, if any, was negligible. The worms were not good as compared with the serum medium. By day 7 all were dead.

It was felt that the use of defibrinated or heparinised blood in the medium made the visual examination of cultures and recovery of the worms very cumbersome. Hence, the use of these materials was abandoned. Although rat serum in the medium BSSG+YE showed its effect in the better survival, appearance, and growth of the worms than of those in control (BSSG+YE) culture, it was realised that the use of large quantities of rat serum for setting up cultures

TABLE 7. Cultivation of H.nana: Effect of adding preparations from rat blood to glucose-saline + yeast extract(BSSG+YE) medium.

Medium	Culture No.	No. of worms	Day opened	No. alive	Observations
BSSG+YE (control)	A 1	30	4	24	Active, but dark; no growth
	A 2	31	7	0	All dead
BSSG+YE+ 25% rat serum	B 1	34	4	31	Good and active; some growth
	B 2	33	7	5	21/26vdead; others in poor state
BSSG+YE+ 10% rat serum	C 1	31	4	27	Like those in Culture B 1
	C 2	30	7	3	26/29 dead; others dying
BSSG+YE+ 25% defib. blood	D 1	30	4	0	No trace of worms
	D 2	29	6	0	No trace of worms
BSSG+YE+ 10% defib. blood	E 1	30	4	8	Like those in culture B 1
	E 2	31	6	0	Only 2 dead worms recovered
BSSG+YE+ 25% heparin. blood	F 1	30	3[a]	5	Medium opaque; worm recovery difficult; worms dark and inactive
	F 2	30	6[a]	0	Only 2 dead worms found
BSSG+YE+ 10% heparin. blood	G 1	31	5[a]	0	10 deadworms found
	G 2	28	6[a]	0	3 dead worms found

a = final pH of medium 6.7-6.8; in others final pH of medium was between 7.2-7.5.

in future would be impracticable, especially when it is not available commercially and the yield per rat is very small. It was therefore considered reasonable to test the effects of serum from other animals.

4. Effect of some commercially available mammalian sera.

Horse, calf and rabbit serum were obtained from Burroughs Wellcome & Co. London, and were used at a concentration of 20% with BSSG+YE. Cultures in medium BSSG+YE+20% rat serum (prepared in laboratory as described previously) were used as 'controls'. One culture tube of each medium was opened on days 4, 6 and 8. The results are summarised in Table 8.

Rat serum (control) - Worms appeared normal and active and to have grown slightly in size when examined on day 4; none showed proglottids. Appearance and activity were maintained until day 6 with little growth beyond that observed on day 4. On day 8, the worms were inactive and none showed any sign of proglottid formation.

Rabbit serum No. 1 (pooled, natural clot, heated at 56°C.)- The worms looked as good as those in control culture on day 4. Over 20% showed the beginning of proglottid formation on day 6. In a culture opened on day 8, 4 out of 15 (27%) worms had distinct proglottids.

Calf serum No. 1 (natural clot, heated at 56°C) - The worms appeared in every respect as those in control culture on day 4. Beginning of proglottid formation was observed on day 6 and by day 8 growing worms had distinct proglottids.

Horse serum No. 2 (recalcified plasma, heated at 56°C)- Nearly 30% of the worms showed good growth and proglottids on day 4. On days 6 and 8, there appeared to be a significant increase in the percentage of segmented worms as well as in the length and number of proglottids of the worms as compared with that observed on day 4.

Horse serum No. 5 (natural clot, heated at 56°C) - Worms did not appear to be any better than those in the 'control' culture on day 4, but on day 6 about 40% of the worms were found to have grown very well and formed distinct proglottids. The culture opened on day 8 was oligoseptic but 3 worms out of 11 had grown very well and had a large number of proglottids.

The results of this experiment suggested that the worms would survive, grow and form proglottids if 20% horse serum or calf serum was added to the BSSG+YE medium.

5. Further observations on the effect of horse or calf serum.

The worms in the medium containing 20% horse serum

TABLE 8. Cultivation of H.nana: Effect of adding different sera at 20% level to glucose-saline + yeast extract (BSSG+YE) medium.

Medium	Culture No.	No. of worms	Day opened	A/R	Growth	with proglottids	Appearance and activity
Rat serum (control)	1	20	4	17/19	++	0	++
	2	21	6	14/14	+	0	++
	3	21	8	10/14	+	0	-
Rabbit serum No.1	1	26	4[b]	17/18	+	0	++
	2	21	6	13/13	++	3	++
	3	19	8	15/16	++	4	+
Calf serum No.1	1	20	4	15/18	+	0	++
	2	21	6	9/13	++	2	++
	3	24	8	12/17	+	2	++
Horse serum No.2 (3 months old)	1	23	4	18/18	++	5	++
	2	21	6[a]	7/8*	+	3	+
	3	25	8	15/24	++	7	++
Horse serum No.5	1	22	4	16/16	+	0	++
	2	21	6[b]	15/18	++	6	++
	3	22	8[a]	11/13*	+	3	+

a = final pH 6.8; b = final pH 7.6; in other cultures final pH was between 7.1-7.5.

++ Good; + moderate; - poor; * culture oligoseptic; A/R = number alive/recovered.

No. 2 showed the best growth, appearance and activity. The horse serum used in the experiment reported above was 3 months old. Sera have been known to show variations in their activity from batch to batch and on storage. It was therefore considered necessary to test horse serum No. 2 of a relatively recent batch and also to repeat the experiment with other sera to verify the results. Horse and calf serum were also obtained from the Virology Dept. of Glasgow University to test the effect of laboratory prepared and unheated sera. The medium prepared with the previously used horse serum No. 2 was used as a 'control' medium. Each kind of serum was used to give a concentration of 20% in the final medium. The results are summarised in Table 9. The following observations were made:

a) Horse serum (commercial or prepared in laboratory, heated or unheated) when added at 20% level to a medium consisting of Hanks' BSS + 0.3% glucose + 0.5% Bacto yeast extract would promote growth and proglottid formation in H. nana. Worms with proglottids were found in as early as 4-day-old cultures.

b) On a comparative basis, worms showed best growth, appearance and activity in the medium containing horse serum No. 2 or the unheated locally prepared horse serum.

TABLE 9. Cultivation of *H. nana* in BSSG+YE + 20% horse or calf serum.

Medium	Culture no.	No. of worms	Day Opened	A/R	Growth	With proglottids	Appearance & activity
Horse serum No. 2 (3 months old) (Control)	1	30	4	21/21	+	3	+
	2	23	5	20/20	++	6	++
	3	24	6	21/24	++	6	+
	4	22	8	14/16	++	8	+
Horse serum No. 2 (recent batch)	1	23	4	20/20	+	4	++
	2	25	5	20/20	++	8	++
	3	23	6	22/23	+	12	++
	4	22	8	17/18	+	11	+
Horse serum (unheated, local)	1	21	4[a]	10/14	+	2	++
	2	24	5[a]	13/19	++	7	++
	3	32	6[a]	27/31	+	10	++
	4	26	8*	14/17	+	6	+
Horse serum No. 5	1	19	4	10/13	+	2	++
	2	20	6	14/15	+	3	+
	3	21	8	20/20	+	4	+
Calf serum No. 1	1	20	4	12/13	-	0	++
	2	21	6	14/16	-	2	++
	3	27	8	22/26	-	6	+
Calf serum (unheated, local)	;	22	4	14/15	-	0	+
	2	20	6	16/16	+	3	++
	3	23	8	21/23	++	11	+

a = final pH 7.6-7.7; in other cultures final pH was between 7.4-7.5.

++ Good; + moderate; - poor; * culture contaminated; A/R= number alive/recovered.

c) In the calf serum medium, worms with proglottids were found slightly later (day 6).

d) Growth and proglottid formation occurred in all media within the pH range 7.3-7.6.

e) Worms tolerated the 100 units of penicillin and 100µg of streptomycin sulphate per ml of medium.

It was also noted that growth of the worms virtually stopped and their appearance and activity deteriorated after 6 days. This might be due to the following reasons:

a) exhaustion of certain essential metabolites present in only trace amounts in the medium

b) accumulation of waste products of the worms in the medium

c) inadequacy of the medium to satisfy the nutritional demands of the growing organisms

d) the worms consume all of some essential substance present in their body in the absence of a supply of that substance from the medium

e) some essential substance is leached out of the worm's body into the culture medium.

For the first two reasons one would expect a better growth of the worms if the medium is frequently changed. For others, it would be necessary to supplement the medium with some other ingredient(s) to raise its nutritional level.

It was decided to use horse serum No. 2 in the media

for all future experiments. This serum is supplied sterile, easily available, and is not expensive. The medium BSSG+HS+YE (Hanks' BSS+ 0.3% glucose + 0.5% Bacto yeast extract + 20% horse serum No. 2) appeared to be a useful basal medium for the study of the effects of the addition of other substances. Prior to this, it was necessary to know about the following problems:

(a) Is there an improvement in the survival and growth of the worms if the medium is frequently changed? If so, how often should the medium be changed?

(b) How to assess and measure growth? This would give an idea of growth rate and permit comparison of two or more media.

(c) Can the worms grow to sexually mature and egg-producing adults in the BSSG+HS+YE medium if cultures are run for 12 days or more with frequent changing of the medium?

Since the worms were too small for the determination of their fresh or dry weight, it was decided to measure their length at regular intervals. Due consideration was also given to the activity and appearance of the worms and to the degree of formation of proglottids. The best third (33%) out of the total number of worms recovered from each culture tube after a specified period

of cultivation were measured with the help of a calibrated ocular micrometer after relaxing the worms overnight in water at 4°C. The average length of 52 excysted juveniles after relaxation in a similar manner was found to be 0.43 mm (range 0.35-0.55 mm).

6. Effect of frequent changing of medium.

An experiment was designed to test the effect of medium changes on the growth of H. nana during a 12-day culture period. Each tube was inoculated with 20-25 freshly excysted juveniles.

Three groups of cultures were set up:

Group I - (Control) 12 cultures; medium not changed; 3 cultures opened on day 3, 6, 9 and 12.

Group II - 6 cultures; medium changed on day 6 and 9; 3 cultures opened on day 9 and 12.

Group III- 12 cultures; medium changed on day 3, 6, 9; 3 cultures opened on day 6, 9 and 12; remaining 3 cultures opened on day 15.

The growth of H. nana in these three groups of cultures during a 12-day cultivation period is shown in Fig. 14. Some worms in each culture were found to have a few proglottids on day 3. By day 9, worms in control cultures began to show deterioration in appearance and activity, whereas worms in those cultures in which medium had been

changed were healthy and showed better growth. The best growth (from 0.4 mm to 3 mm in 12 days) and activity were observed in cultures in which medium was changed every third day (Group III). None of the worms showed any sign of advanced genital development or maturation even on day 15.

Figure 14

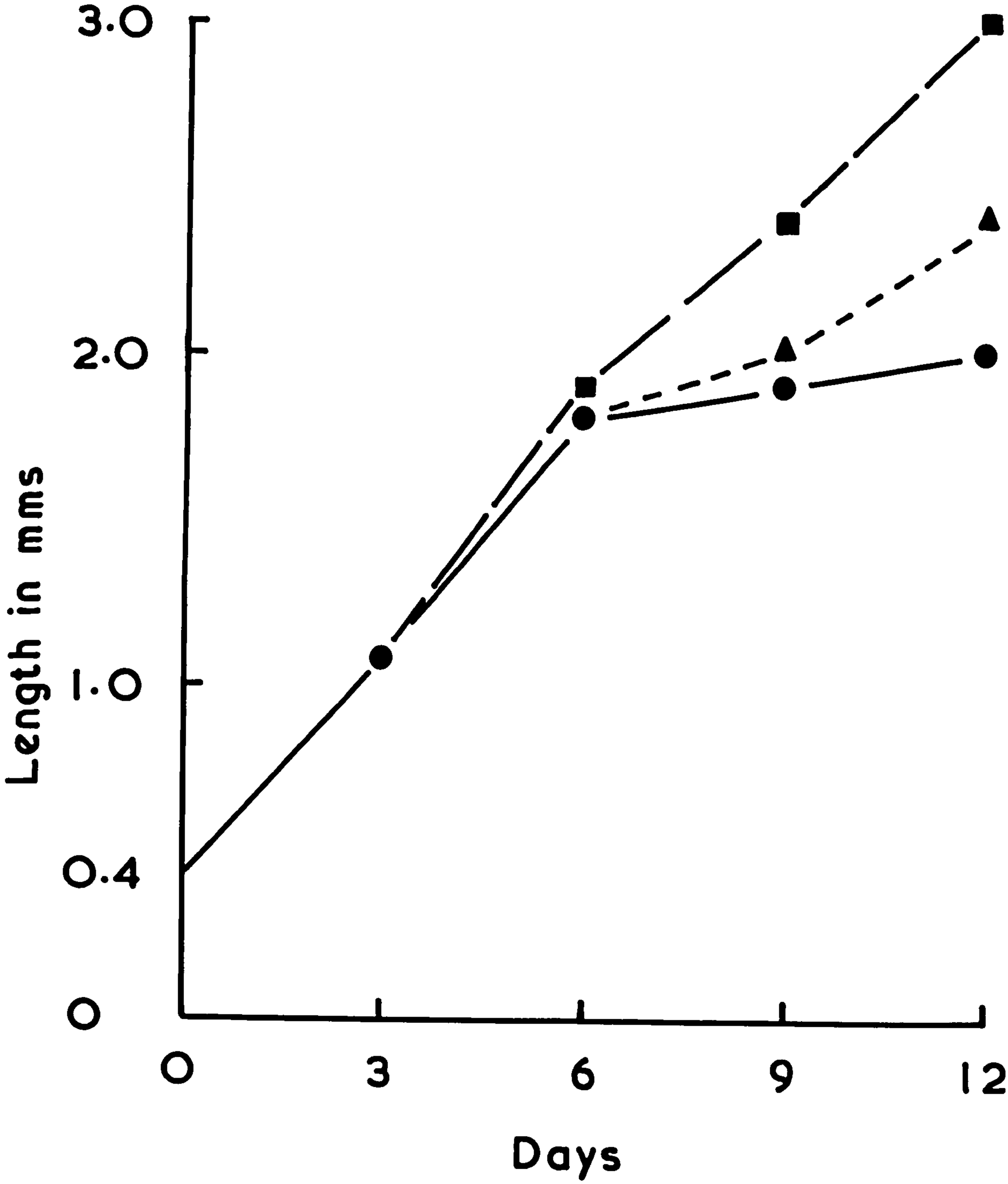
Effect of medium renewal on the growth of H. nana in 12 days in medium BSSG+HS+YE containing 0.5% "old" Difco yeast extract. Each point represents the mean length of 14-16 worms.

Squares - medium changed on days 3,6 and 9

Triangles - medium changed on days 6 and 9

Circles - medium not changed

Figure 14.



(b) <u>The yeast extract problem</u>

It was at this stage of the work that the entire content of the bottle of Bacto yeast extract (about 2-2^{1}/2 years old) which had been used in all these experiments was used up. Another bottle of a recent batch of the same yeast extract (hereafter referred to as "new" Bacto yeast extract) was obtained. The results of the incorporation of this "new" yeast extract at 0.5% level into the medium (Hanks' BSS+ 0.3% glucose + 20% horse serum + 0.5% yeast extract) were very disappointing. The worms did not show any growth even after 6 days in culture and their survival could be ascertained only by occasional movements of their scolices. There could be two possibilities; either the "new" yeast extract was ineffective and toxic or there was something wrong with the other ingredients of the medium. A few other preliminary experiments gave further support to the feeling that the "new" yeast extract was toxic. This presented a serious problem and several experiments were performed with yeast extracts from various sources. The results of these experiments are described below:

(1) <u>Effect of "new" Bacto yeast extract</u>.

All solutions were prepared fresh and a fresh bottle of horse serum obtained. Two groups of cultures, each

TABLE 10. The difference in effect of two batches of Bacto yeast extract on the growth of H.nana in the medium BSSG+HS+YE (Hanks' BSS+0.3% glucose+20% horse serum+0.5% yeast extract).

Medium with	Culture no.	Day opened	A/R	With proglot.	Appearance & activity	l (mm)	L (mm)
0.5% Bacto yeast extract ('old')	1	4	14/14	5	++	1.52 (5)	
	2	4	18/19	6	++	1.58 (6)	1.58 (17)
	3	4	17/18	6	++	1.65 (6)	
0.5% Bacto yeast extract ('new')	1	4	16/17	0	-	0.53 (5)	
	2	4	13/17	0	-	0.59 (4)	0.54 (14)
	3	4	14/16	0	-	0.52 (5)	

++ Good; + moderate; - poor or none; A/R = number alive/recovered;

l = average length of 33% worms in a culture; L = mean of average lengths of worms from all tubes.

Figures in brackets indicate the number of worms measured.

consisting of three tubes were set up. All culture conditions and medium ingredients in the two sets of cultures were the same except that the medium in one group contained 0.5% "old" yeast extract (used and found good in all previous experiments), whereas that in the other group contained 0.5% "new" yeast extract. About 20 worms (from the same lot of excysted worms) were placed in each culture. The tubes were opened on day 4; the results are shown in Table 10.

The results clearly indicated that the inactivity and inhibition of the growth of worms was due to the "new" yeast extract. It was toxic at this concentration and presumably lacked something the "old" yeast extract possessed. It was, therefore, decided to try yeast extracts from various manufacturers in order to find out one that would give results approximating to those obtained with the "old" yeast extract.

(2) Effect of different yeast preparations.

The following yeast preparations were obtained and used as a 5% autoclaved aqueous solution to give an concentration of 0.5% in the final medium (BSSG+HS+YE).

'Oxoid' yeast extract (code L21, Oxo Ltd. London)

Bacto Autolysed yeast (Difco Labs. Detroit, U.S.A.)

TC Yeastolate (Difco Labs.)

Bacto yeast extract (Difco Labs.)

Cultures were set up in media using 4 types of yeast preparations. Each culture tube contained 20-22 worms. The Bacto yeast extract was the one which had been used in the previous experiment and found to be toxic. Cultures in the medium containing this yeast extract served as 'controls.'

Autolysed yeast was only partially soluble in water. A suspension of 5g of autolysed yeast in 100 ml of de-ionised water was heated to boiling point, cooled and filtered. The filtrate was autoclaved at 10 p.s.i. for 10 mins; 10 ml of this was used in 100 ml of the final medium.

The effects of these yeast preparations on the growth of H. nana during a 6-day culture period are shown in Fig. 15 and Table 11.

In Bacto yeast extract and TC Yeastolate media, worms were miserable and inactive; no growth had occurred. In Bacto autolysed yeast medium a good number of worms in each culture was found dead by day 6. Those which were alive had not grown at all and were inactive, showing only occasional movement of the scolex. The worms showed definite growth (but no proglottids) and good appearance and activity in the 'Oxoid' yeast extract medium.

It was felt that these yeast preparations might have

TABLE 11. Cultivation of H. nana: a comparison of 'Oxoid' yeast extract, 'Difco' autolysed yeast, 'Difco' Yeastolate, and 'Difco' Bacto yeast extract at 0.5% level in BSS+ 0.3% glucose+ 20% horse serum.

Explanation of symbols as in Table 10.

Yeast extract in medium	Day opened	No. of cultures	A/R	Growth	Appearance & activity	L(mm)	
'Oxoid' yeast extract	3	3	48/49	+	++	0.78 (15)	
	6	3	50/53	+	++	1.20 (16)	
'Difco' Bacto yeast extract	3	3	46/54	-	-	0.46 (16)	
	6	3	47/61	-	-	0.52 (16)	Toxic
'Difco' autolysed yeast	3	3	51/61	-	+	0.49 (17)	
	6	3	35/54	-	-	0.49	Toxic
				Worms not measured; no growth.			
'Difco' yeasto-late.	3	3	46/57	-	-	0.48 (15)	
	6	3	39/60	-	-	0.48	Toxic
				Worms not measured; no growth.			

Final pH of medium in cultures between 7.2-7.4.

Worms without proglottids in all media.

Figure 15

Effect of different yeast preparations at 0.5% level in BSSG+20% horse serum on the growth of H. nana. Each point represents the mean length of 15-17 worms.

Solid triangles - 'Oxoid' yeast extract
Crosses - Autolysed yeast (Difco)
Closed circles - TC Yeastolate (Difco)
Open circles - Bacto yeast extract ("new")

Figure 16

Effect of different yeast preparations at 0.2% level in BSSG+20% horse serum. Each point represents the mean length of 10-15 worms.

Solid triangles - 0.5% 'Oxoid' yeast extract
Solid squares - 0.2% 'Oxoid' yeast extract
Crosses - 0.2% Autolysed yeast
Closed circles - TC Yeastolate
Open circles - "new" Bacto yeast extract

Figure 17

Comparison of the effects of different batches of 'Oxoid' and 'Difco' yeast extracts. Each point shows the mean length of 20-25 worms. The points representing growth of H.nana in BSSG+HS+YE containing the original (old) Difco yeast extract is taken from Fig. 14 to illustrate the difference.

Figure 15.

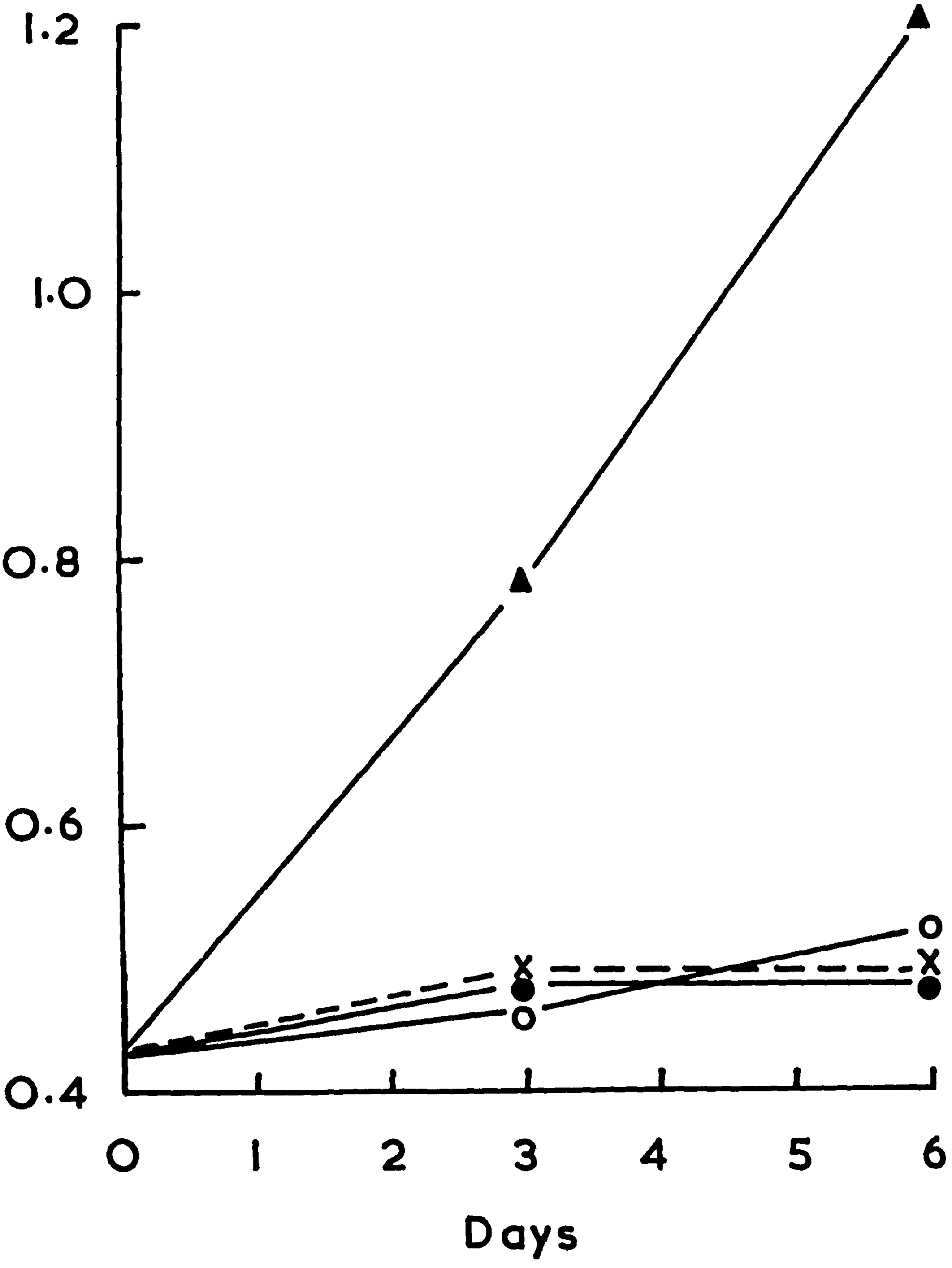


Figure 16.

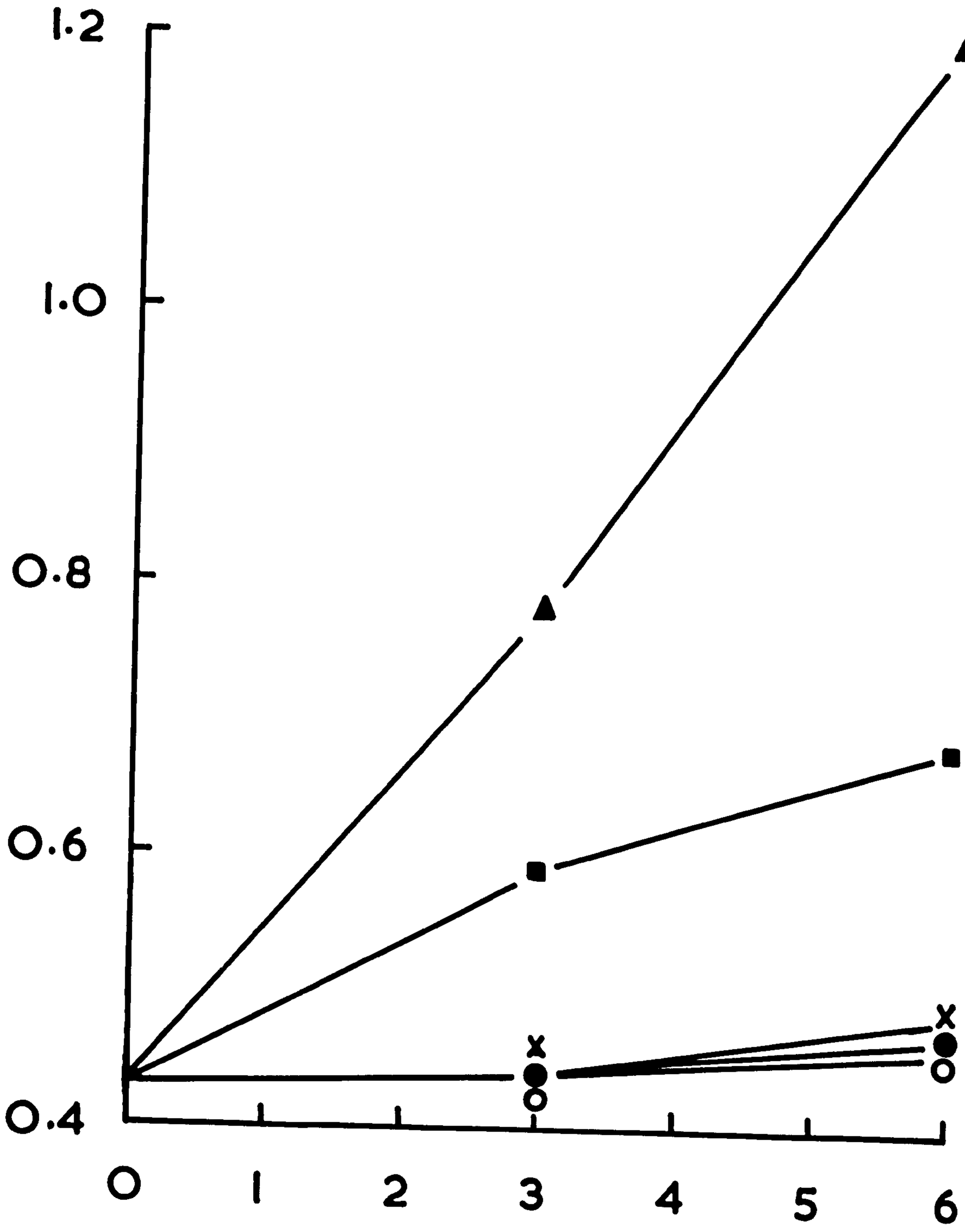
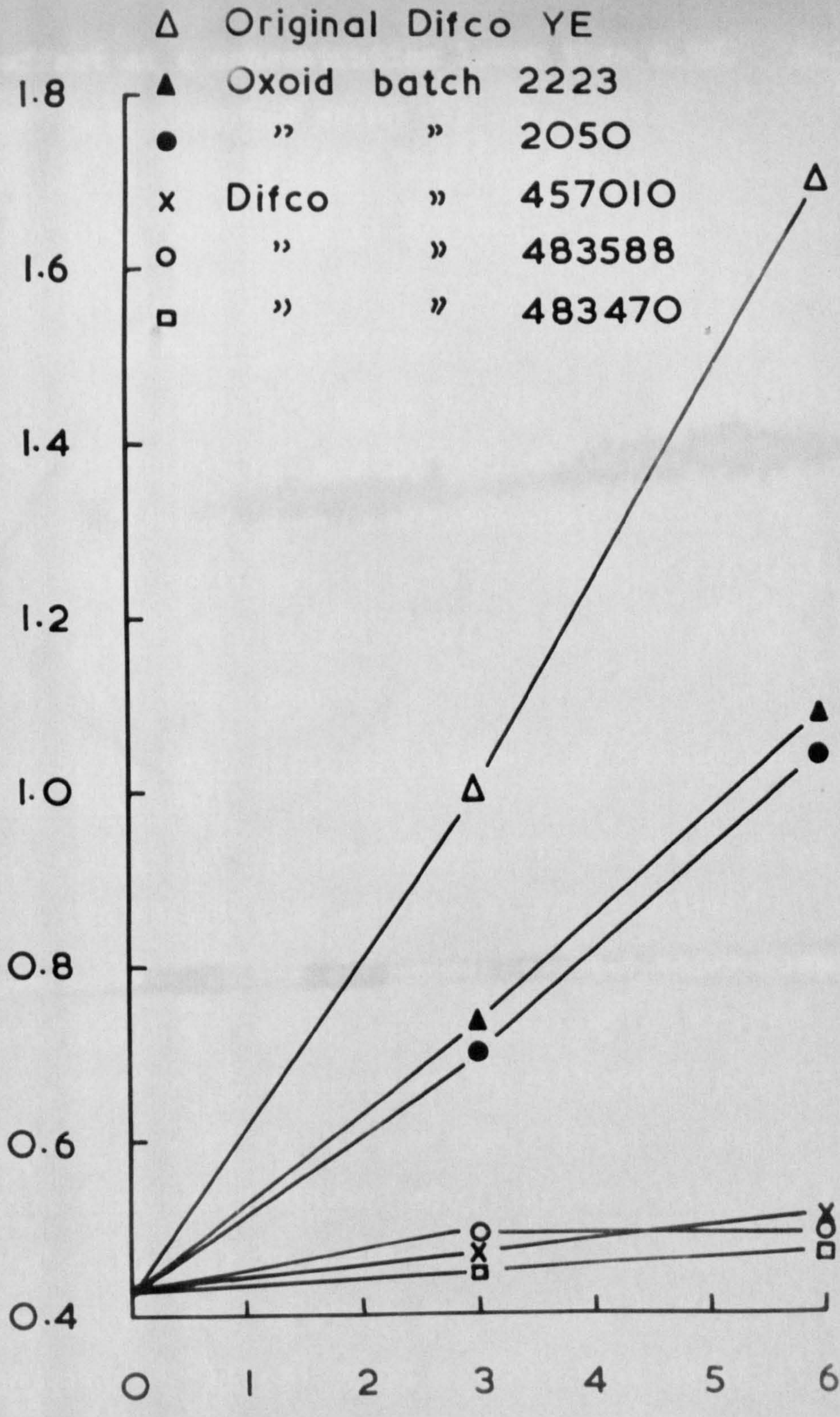


Figure 17.


Δ Original Difco YE
▲ Oxoid batch 2223
● " " 2050
x Difco " 457010
o " " 483588
□ " " 483470
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
length in mms.
0 1 2 3 4 5 6
Days

a good effect at a concentration lower than 0.5%. An experiment was performed using the same 4 yeast preparations at 0.2% level. The worms in the medium containing 0.5% 'Oxoid' yeast extract served as 'Controls.' Cultures, each containing 20-22 worms, were run for 6 days. The worms were examined and measured on days 3 and 6. The results on growth are shown inFig. 16. Bacto yeast extract, autolysed yeast and TC Yeastolate all proved toxic and inhibitory at this concentration also. The worms in control cultures, though not forming proglottids by day 6, showed much better growth than those in the 0.2% 'Oxoid' yeast extract medium.

(3) <u>Experiment with different batches of 'Oxoid' and 'Difco' yeast extracts</u>.

The results of the two experiments reported above suggested the suitability of 'OXoid' yeast extract at 0.5% level. However, it was also realised that there might be a batch-to-batch variation in the activity of natural products and some other batch of 'Oxoid' yeast extract might not be so good as the one under trial. Similarly, some other batches of 'Difco' yeast extract might be so good as the 'old' Bacto yeast extract. An experiment was done, therefore, in which two batches of 'Oxoid' and three batches of 'Difco' yeast extract were tried at 0.5% level. Ten cultures were set up (with 20-25 worms in each

tube) for each type of medium. Five cultures of each were opened on days 3 and 6. The results are shown in Fig. 17, from which it would appear that practically no growth occurred in media containing any of the 3 batches of 'Difco' yeast extract, whereas worms showed definite growth in the media containing 'Oxoid' yeast extract and there did not appear to be any significant difference in the extent of growth in the 2 batches of 'Oxoid' yeast extract used. Although the worms did not develop proglottids even in 6 days in the 'Oxoid' yeast extract media, they were active and looked in good condition.
In Bacto yeast extract media, apart from the fact that no growth occurred, the worms were dark and almost motionless, suggesting that all the 3 batches of 'Difco' yeast extract were toxic.

(4) Further observations on the effect of 'Oxoid' and 'Difco' yeast extract in the medium BSSG+HS+YE: growth of Schistocephalus plerocercoids.

Before deciding to reject 'Difco' yeast extract, one confirmatory test was done. The 'Old' Bacto yeast extract which gave good results with H. nana was the same which had been used in the medium for experiments with Schistocephalus (see Section II). The question was whether the recent batches of 'Difco' yeast extract were

bad for H. nana only, or were they equally unsuitable for Schistocephalus. If the 'new' Difco yeast extract proved for Schistocephalus as good as the 'old' yeast extract, it would mean that the 'old' yeast extract contained some substances which were essential for the growth of H. nana but not for Schistocephalus.

The growth factors in yeast extract are mostly thermostable but it is possible that some essential substance is destroyed when the yeast extract is autoclaved for sterilisation. It was decided, therefore, to test the effect of 'Oxoid' yeast extract, sterilised both by autoclaving and filtration, and compare results with those obtained in 'old' yeast extract (Fig. 5, see Section II).

The effect of autoclaved 'new' Difco yeast extract, and autoclaved and filter-sterilised 'Oxoid' yeast extract (all at a concentration of 0.5% in BSSG+HS+YE) on the growth of Schistocephalus plerocercoids in 8 days at 23°C, are shown in Figs. 18-20. The lines are shown together in Fig. 21. The regression lines representing growth of the plerocercoids in BSSG+HS+YE medium containing old yeast extract has been taken from Fig. 5 (Section II). The inadequacy of the 'new' Difco yeast extract is clearly marked when specific growth rates of small worms are considered. A 2 mg D.W. worm which increased in dry weight

Figures 18-21

Growth of plerocercoids of Schistocephalus solidus in BSSG+HS+YE medium: effect of 'Oxoid' and 'Difco' yeast extracts at 0.5% level.

Figure 18 -Autoclaved 'Difco' yeast extract ("new") ;

Figure 19 -Autoclaved 'Oxoid' yeast extract;

Figure 20 -'Oxoid' yeast extract sterilized by filtration;

Figure 21 -Composite diagram of data on Figures 18-20.

A. Autoclaved "old" Difco yeast extract; (taken from Figure 5)

B. Filter-sterilized 'Oxoid' yeast extract;

C. Autoclaved 'Oxoid' yeast extract;

D. Autoclaved 'Difco' yeast extract ("new").

Figure 18.

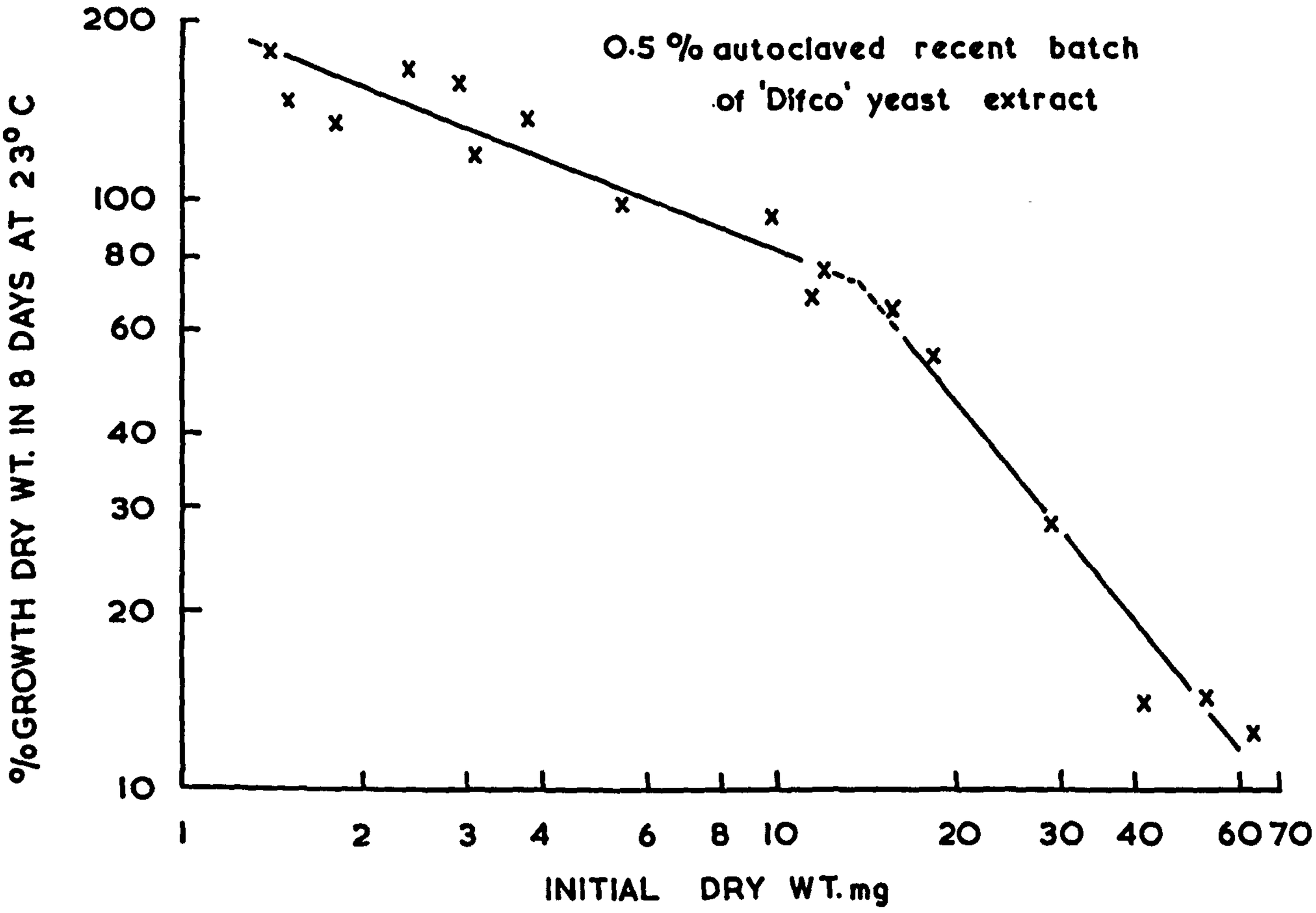

0.5 % autoclaved recent batch of 'Difco' yeast extract
%GROWTH DRY WT. IN 8 DAYS AT 23° C
200
100
80
60
40
30
20
10
1
2
3
4
6
8
10
20
30
40
60
70
INITIAL DRY WT. mg

Figure 19.

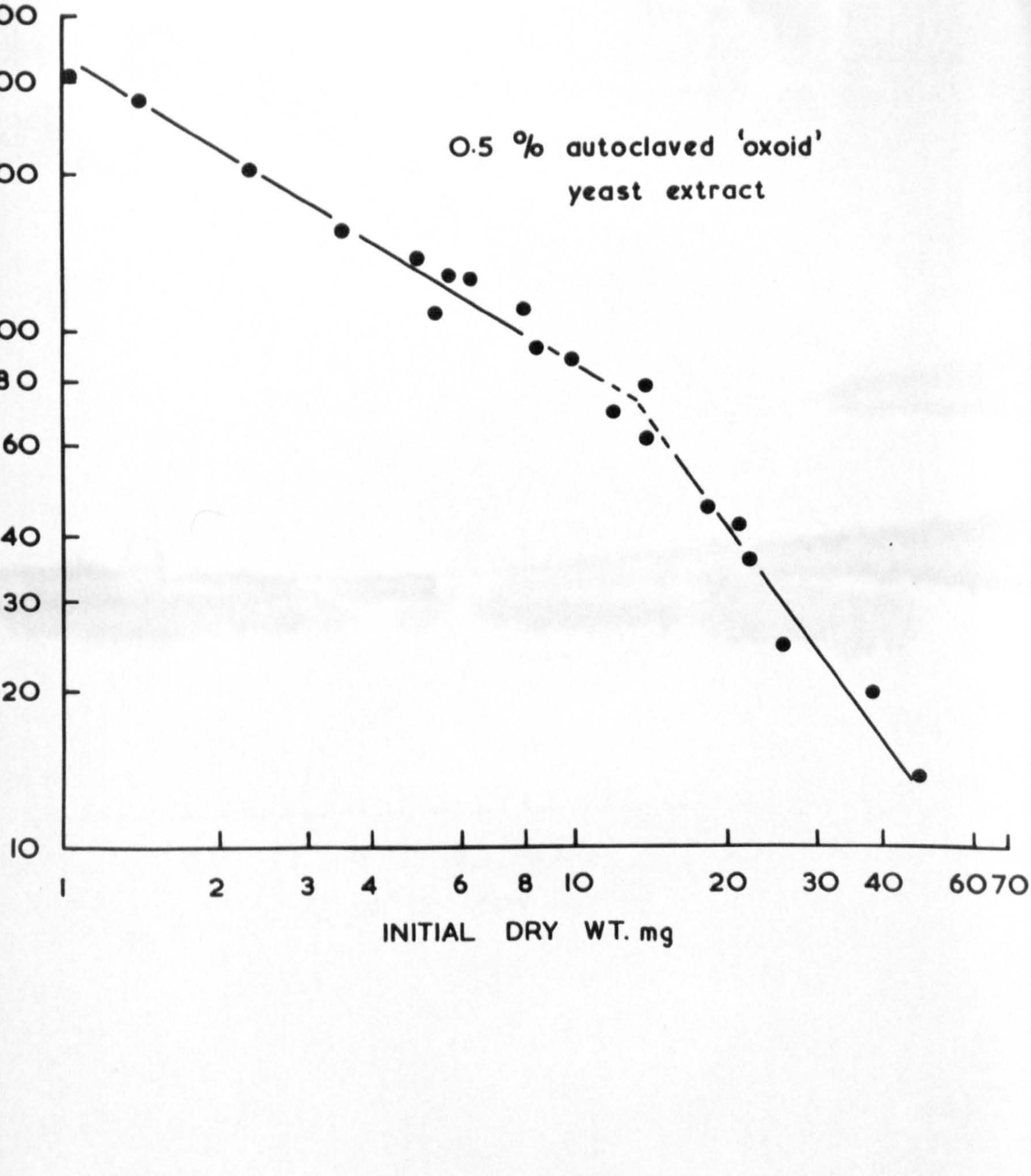

0.5 % autoclaved 'oxoid'
yeast extract
% GROWTH DRY WT. IN 8 DAYS AT 23°C
400
300
200
100
80
60
40
30
20
10
1
2
3
4
6
8
10
20
30
40
60
70
INITIAL DRY WT. mg

Figure 20.

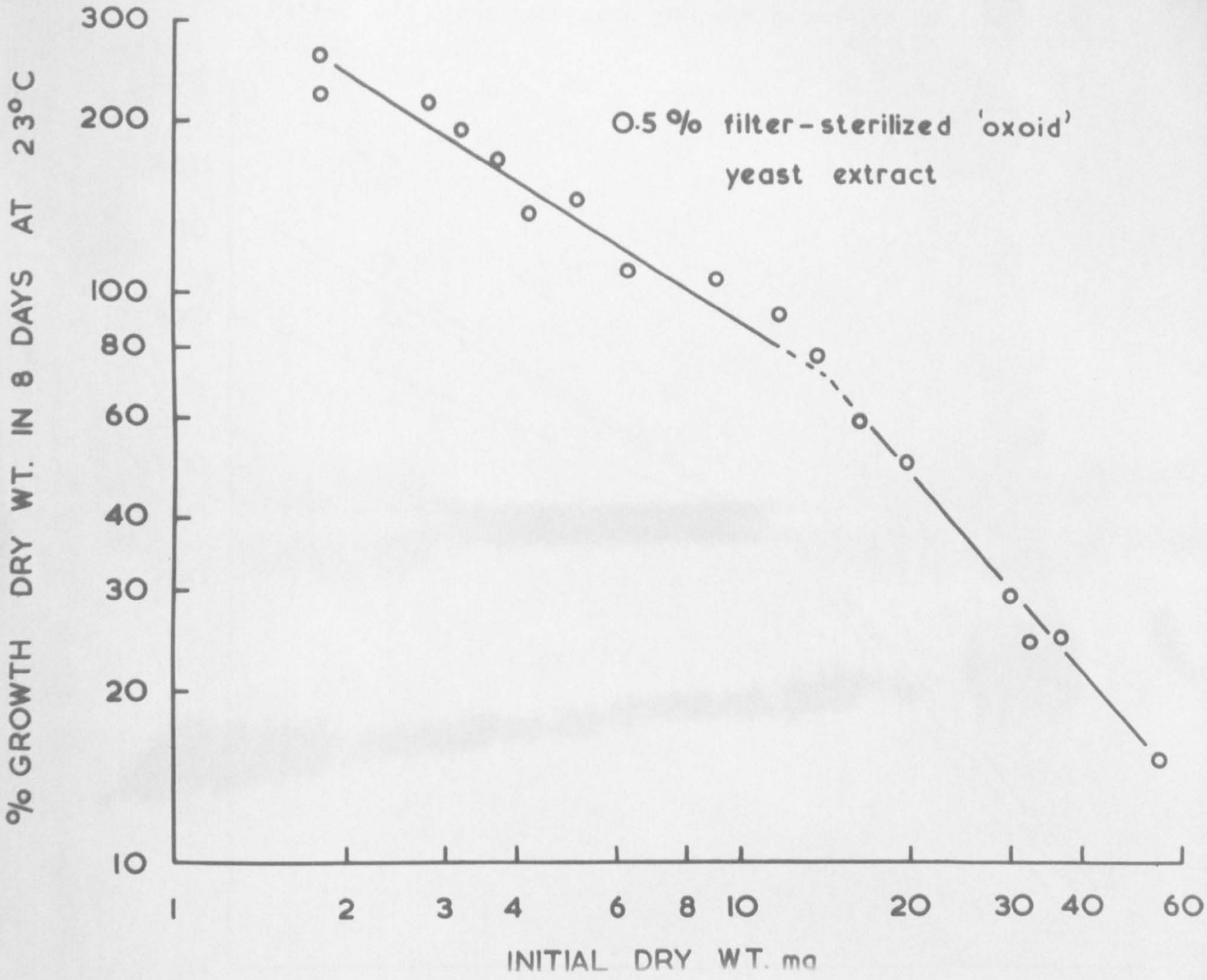
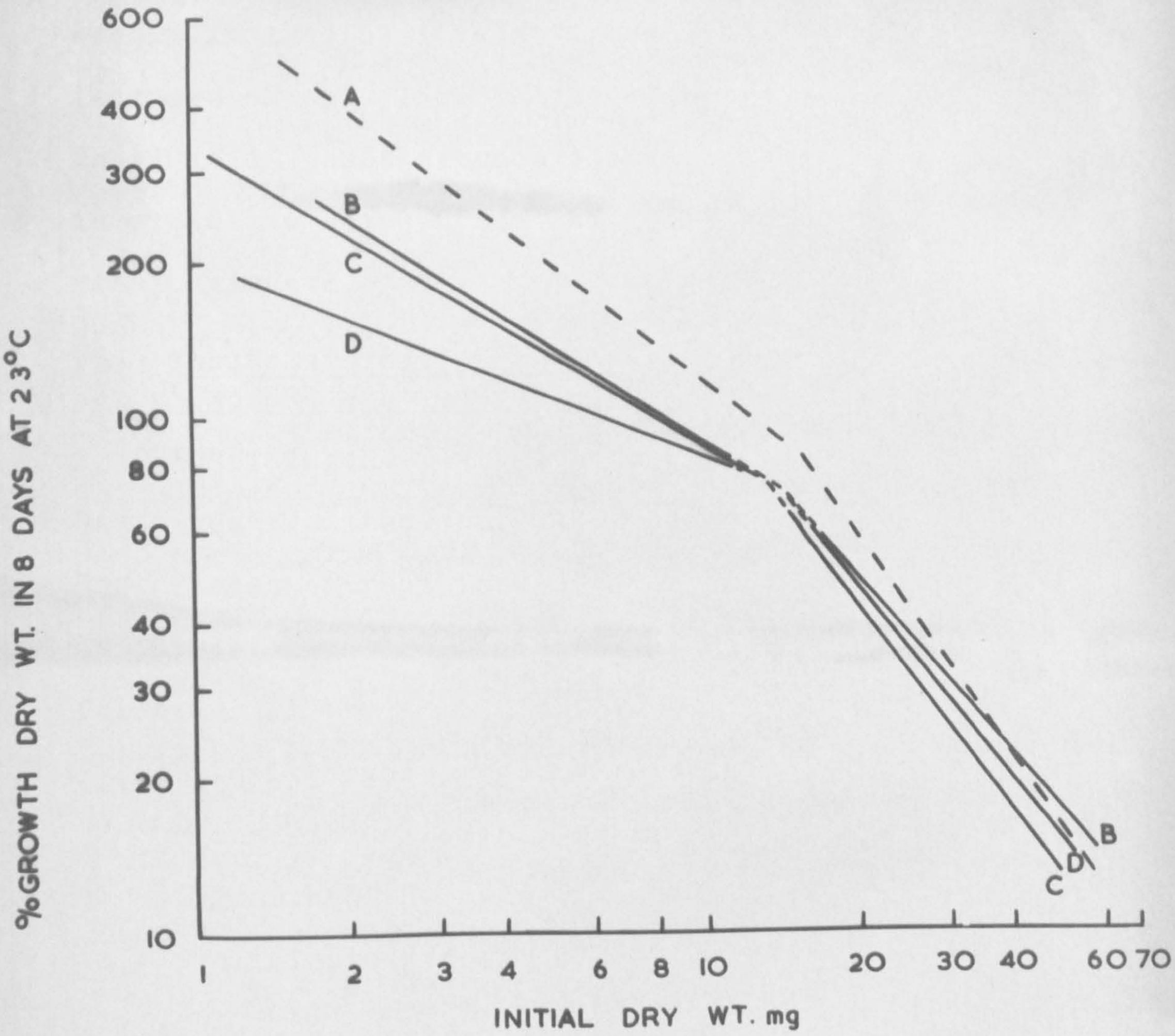

0.5 % filter-sterilized 'oxoid' yeast extract
% GROWTH DRY WT. IN 8 DAYS AT 23°C
300
200
100
80
60
40
30
20
10
1
2
3
4
6
8
10
20
30
40
60
INITIAL DRY WT. mg

Figure 21.


Effect of yeast extracts on the growth of Schistocephalus
600
400
300
200
100
80
60
40
30
20
10
%GROWTH DRY WT. IN 8 DAYS AT 23°C
A
B
C
D
1
2
3
4
6
8
10
20
30
40
60
70
INITIAL DRY WT. mg

in 8 days to the extent of 400% of its initial dry weight in the 'old' yeast extract medium, showed an increase of only 150% in the 'new' yeast extract medium. The 'Oxoid' yeast extract seemed to have an effect intermediate between that of 'old' and 'new' Bacto yeast extracts.

(5) <u>The determination of the right concentration of 'Oxoid' yeast extract in the medium BSSG+HS+YE</u>.

'Oxoid' yeast extract proved better than 'new' Bacto yeast extract but much less effective than 'old' Bacto yeast extract for the growth of <u>H. nana</u> (Fig. 17). At this stage it seemed necessary to investigate the following:

(a) Is it necessary to have yeast extract in a medium containing BSS+glucose+20% serum? How far is growth of <u>H. nana</u> affected if yeast extract is eliminated from the medium BSSG+HS+YE?

(b) If yeast extract is necessary, at what concentration should it be used in the medium?

'Oxoid' yeast extract was used in the medium BSSG+HS+YE to give a final concentration of 0.0, 0.2, 0.5, 0.75 and 1.0%. Cultures in the medium containing 0.5% yeast extract served as 'controls'. Four cultures, each with 20-22 worms, were set up in each medium. Two cultures of

each group were examined on days 3 and 6. The results are shown in Table 12 and Fig. 22.

In the absence of yeast extract, the worms on day 3 appeared healthy and were active but had not grown. By day 6, more than 25% of the worms in each culture were dead, and those which were alive, were swollen, inactive and filled with a dark granular material. There was very poor growth at 0.2% level. At 0.75% and 1.0% concentrations worms remained active and in good condition, and showed definite growth during the 6-day culture period, but the worms were in no way better than those in the control cultures (0.5% level). In fact, the control worms showed better growth. However, none of the worms formed proglottids in 6 days at any of the five concentrations of yeast extract.

Another important point was revealed by this experiment was that the medium containing 20% serum but no yeast extract was inadequate to support growth of the worms.

TABLE 12. Cultivation of H. nana: Effect of 'Oxoid' yeast extract at different concentrations in medium containing Hanks' BSS+0.3% glucose + 20% horse serum.

Explanation of symbols as in Table 10.

Conc.of yeast extract	Day opened	No.of cultures	A/R	Growth	Appear-ance and activity	Av. length (mm)	Remarks
0.0%	3	2	33/36	-	+	0.45(11)	Poor
	6	2	29/41	-	+	0.49(9)	Poor
0.2%	3	2	31/32	+	+	0.59(10)	Poor
	6	2	32/33	+	+	0.68(10)	
0.5% (control)	3	2	39/39	++	++	0.81(13)	Good
	6	2	27/31	++	++	1.43(9)	
0.75%	3	2	34/35	+	++	0.64(11)	Not better than control.
	6	2	28/30	++	++	1.29(9)	
1.0%	3	2	39/41	+	++	0.64(13)	Not better than control.
	6	2	23/25	++	++	1.22(9)	

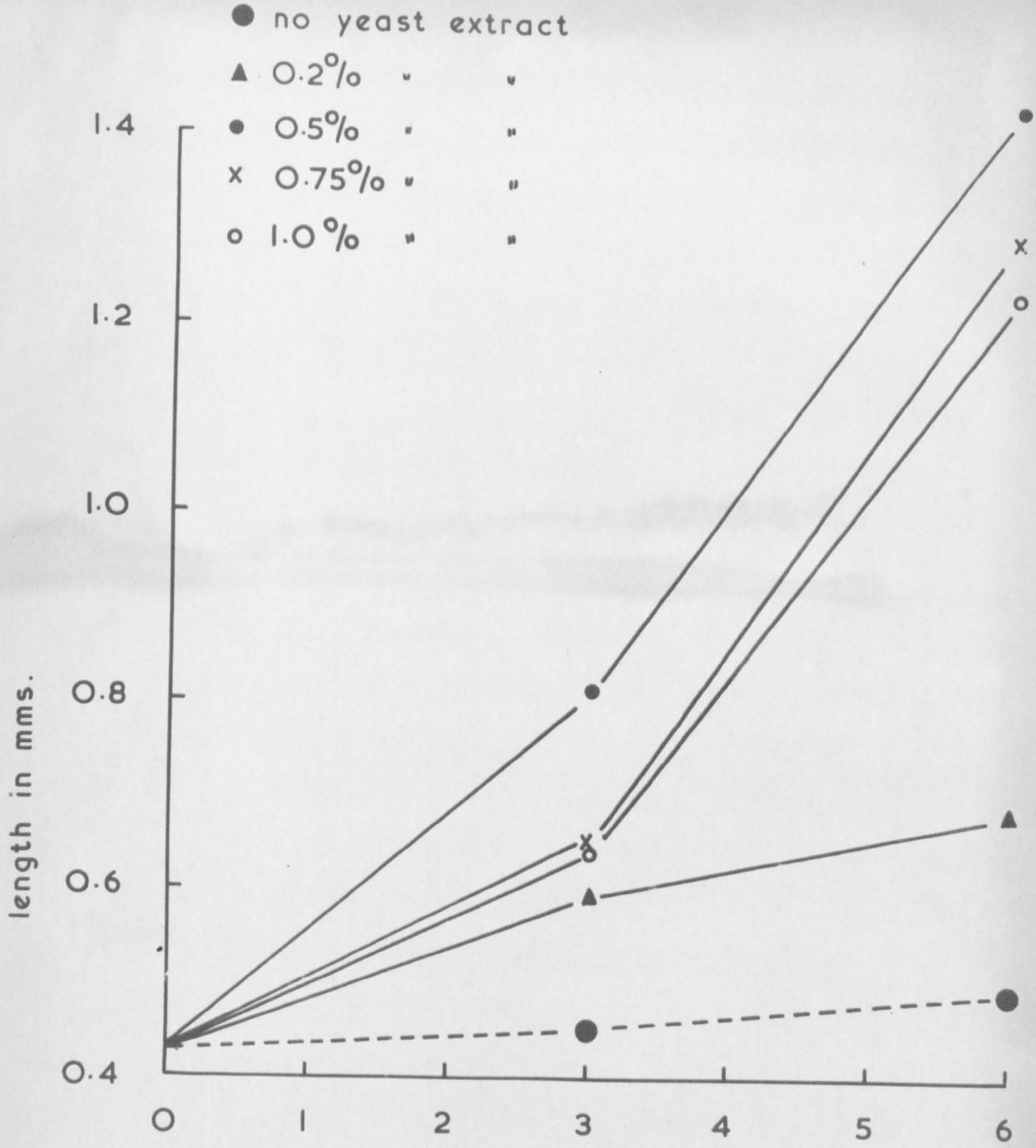
Final pH of medium in all cultures between 7.2-7.4.

No formation of proglottids by worms at any concentration of yeast extract.

Figure 22

Growth of H.nana at different levels of 'Oxoid' yeast extract in medium BSSG+HS+YE. Each point represents the mean length of 9-13 worms.

Figure 22.


● no yeast extract
▲ 0.2% " "
● 0.5% " "
x 0.75% " "
o 1.0% " "
1.4
1.2
1.0
0.8
0.6
0.4
length in mms.
0
1
2
3
4
5
6
Days

(c) Attempts to improve the basal medium.

Experiments were performed to find out if better results on the growth of H. nana could be obtained by reducing or raising the concentration of serum and glucose and by using some other balanced salt solution instead of Hanks' BSS.

(1) Level of horse serum in BSSG+HS+YE.

Horse serum was used in the medium BSSG+HS+YE at 5 levels - 0, 10, 20, 30 and 50%. Five groups of 10 cultures each were set up. Each tube was inoculated with 18-20 worms. Five cultures of each group were examined on day 3, and five on day 6. Worms in the medium containing 20% serum served as controls. The results are shown in Fig. 23.

In the absence of serum, worms appeared inactive, swollen and dark when examined on day 3. All were found dead on day 6. When serum was present in the medium at any level between 10-50%, worms showed definite growth, and good appearance and activity. Growth at 10 and 20% serum levels was poorer than that at 30% and 50% levels. There did not seem to be any marked difference in the growth of the worms in media containing 30% and 50% serum. Although worms did not form proglottids in any medium, they grew better and slightly longer at 30% serum level. As

a result of this experiment 30% horse serum was used in all media thereafter.

(2) Level of glucose in BSSG+HS+YE.

Media were prepared containing Hanks' BSS, 0.5% 'Oxoid' yeast extract, 30% horse serum, and varying concentrations of glucose. This was added as a 6.5% solution to give a final concentration of 0.0, 0.1, 0.3, 0.6 and 1.0%. Ten cultures, each with 18-20 worms, were set up in each medium. Worms in the medium containing 0.3% glucose served as 'controls'. Cultures were run for 6 days. Five tubes were opened on day 3 and five on day 6. The growth of H. nana at different concentrations of glucose in the medium BSSG+HS+YE is shown in Fig. 24.

The elimination of glucose from the medium did not result in a marked inhibition of growth of the worms during a 6-day culture period. Presumably, the worms utilised glucose present in the serum and yeast extract. No marked difference in growth was observed at 0.1% and 0.3% glucose levels and increasing the level of glucose beyong 0.3% to 0.6% or 1.0% did not bring any improvement in the growth of the worms. Since in all previous experiments, glucose had been used to give a final concentration of 0.3%, it was decided not to alter glucose level in the medium in future experiments.

Figure 23

Growth of H.nana in medium BSSG+HS+YE containing different levels of horse serum. Each point represents the mean length of 24-27 worms.

Figure 24

Growth of H.nana in medium BSSG+HS+YE containing different levels of glucose. Each point represents the mean length of 25-27 worms.

Figure 25

Growth of H.nana in medium BSSG+HS+YE containing different balanced salt solutions. Each point represents the mean length of 24-30 worms.

Crosses - Hanks' BSS

Closed circles - Krebs' BSS

Open circles - Earle's BSS

Figure 23.

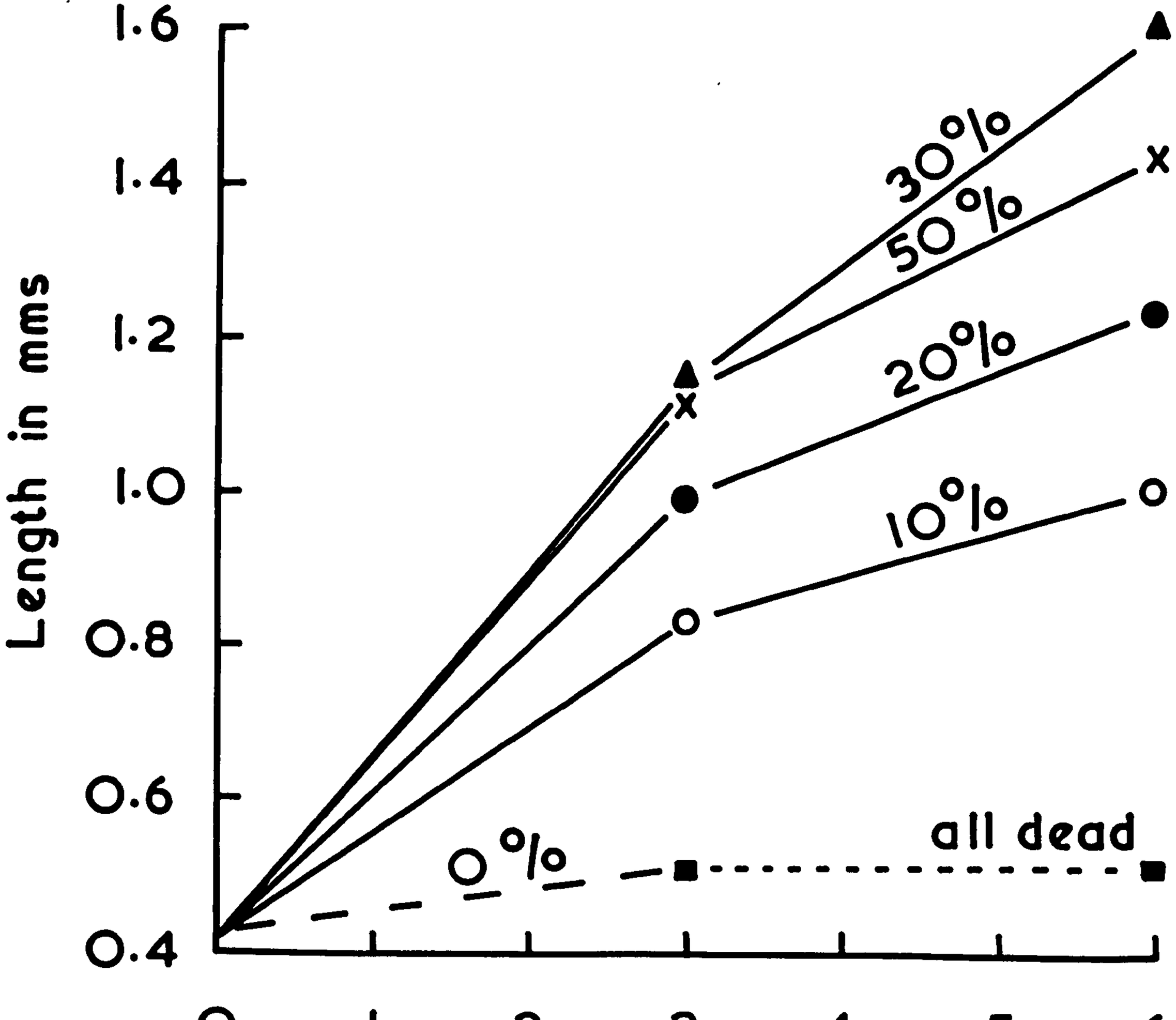

1.6
1.4
1.2
1.0
0.8
0.6
0.4
Length in mms
30%
50%
20%
10%
0%
all dead
0
1
2
3
4
5
6
Days

Figure 24.

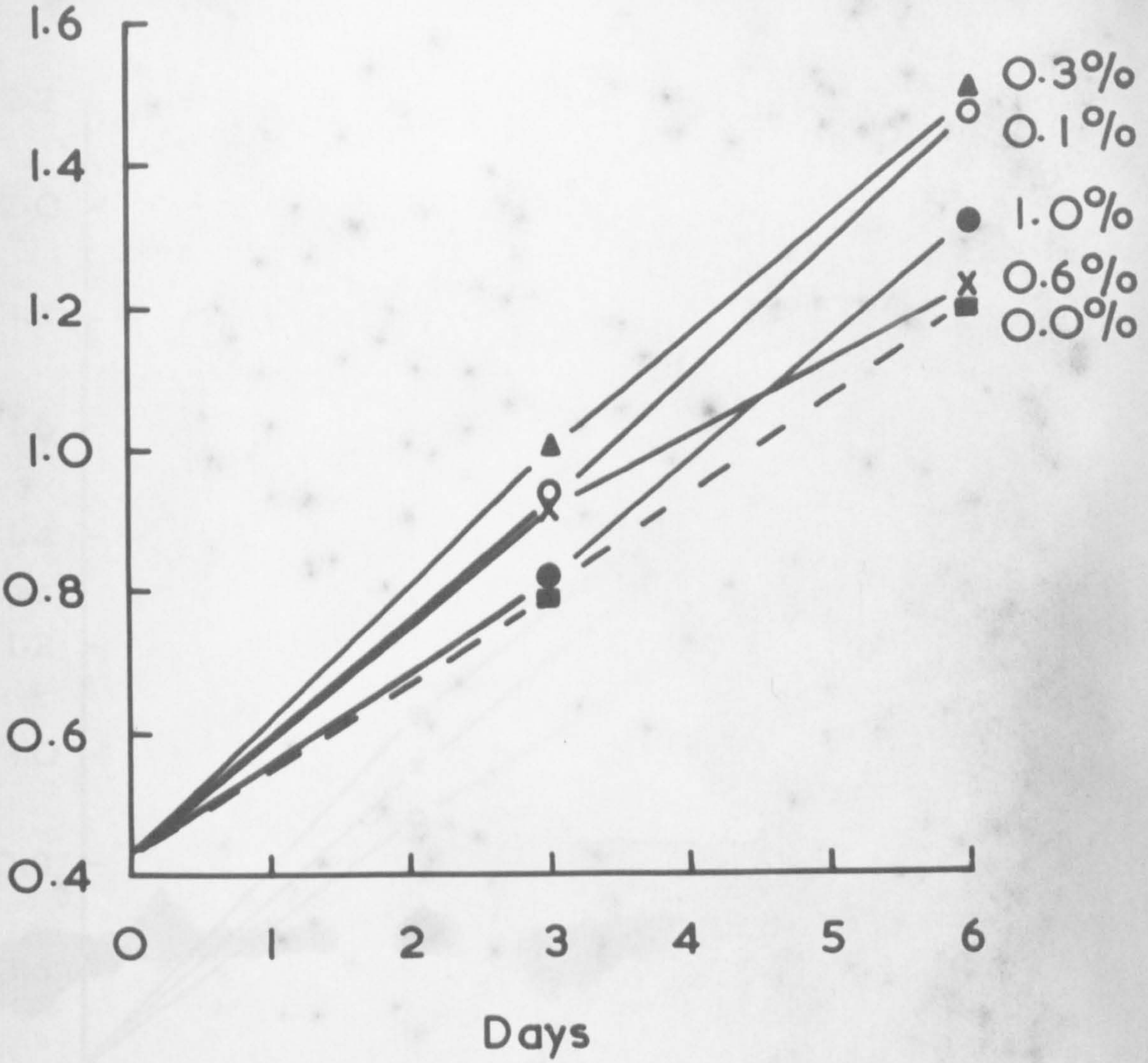
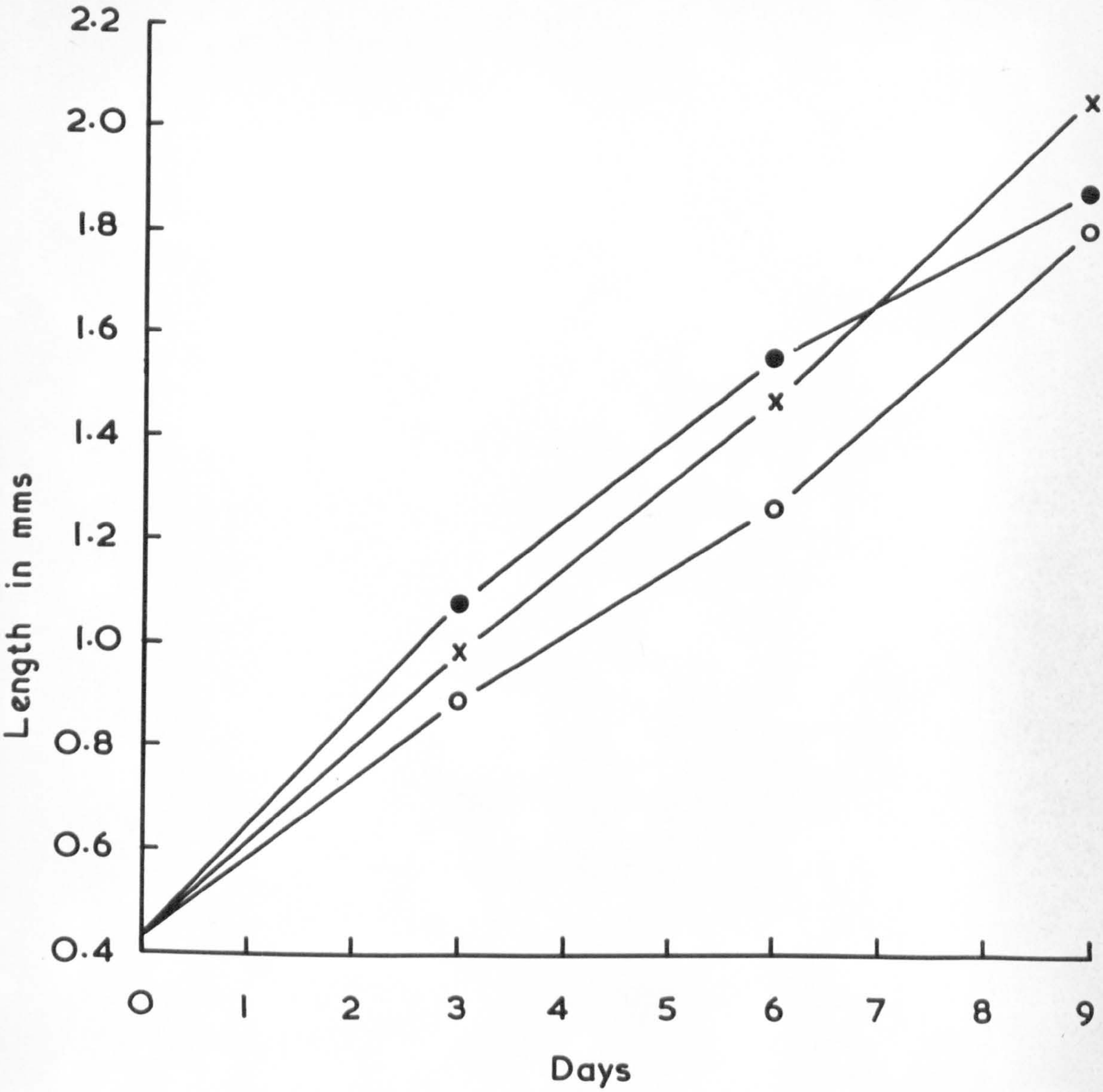


Figure 25.


2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
Length in mms
0
1
2
3
4
5
6
7
8
9
Days
x
x
x
o
o
o

(3) Medium BSSG+HS+YE containing Earle's or Krebs' BSS.

Earle's BSS is one of the commonest balanced salt solutions and has been designed to equilibrate with a gas phase containing a high CO_2 tension. Krebs' BSS, though generally used in metabolic studies, has not been employed in works on tissue-culture and helminth cultivation. Its importance lies in the inclusion of Krebs' cycle intermediates.

Earle's BSS was prepared by dissolving the contents (8.7g) of a sachet ('Oxoid' BR 21) in a litre of deionised water, and sterilised by autoclaving at 15 p.s.i. for 15 mins. Krebs' BSS (medium III) was prepared according to a slight modification of the formula quoted by Paul (1960, p.82). The following solutions were prepared and all except solution 7 were sterilised by autoclaving at 15 p.s.i. for 15 mins. Solution 7 was sterilised by filtration. These solutions were mixed in proportions indicated against each:

1.	0.9% NaCl	95 parts
2.	1.15% KCl	4 parts
3.	1.2% $CaCl_2$	3 parts
4.	3.82% $MgSO_4.7H_2O$	1 part
5.	2.11% KH_2PO_4	1 part
6.	Sodium phosphate buffer (100 parts of 1.42% $Na_2H.PO_4$ + 25 parts of 1.56% $NaH_2PO_4.2H_2O$)	

7.	Sodium pyruvate	0.352g	
	sodium fumarate	0.483g	15 parts
	sodium L-glutamate	0.338g	
	deionised water	75ml	

The original formula contains an amount of 5.4% glucose and 1.3% $NaHCO_3$. These two ingredients were omitted from Kreb's BSS and as usual added separately while preparing the final medium. Glucose and $NaHCO_3$ were added as 6.5% and 1.4% solutions respectively.

The final medium was prepared by mixing the following:

BSS	50 ml
6.5% glucose	5 ml
5.0% 'Oxoid' yeast extract	10 ml
horse serum	30 ml
1.4% $NaHCO_3$	5 ml

Three media were prepared using Hanks', Earle's and Kreb's BSS. Forty-five cultures were set up, 15 tubes for each medium and 18-22 worms in each tube. Cultures were run for 9 days. Worms were examined and measured on days 3, 6 and 9. The results are shown in Table 13 and Fig. 25.

In all the three media, worms with well-formed proglottids were observed on day 9. In media containing Hanks' or Kreb's BSS, segmented worms were found on day 6.

TABLE 13. Effect of different balanced salt solutions on the growth of H. nana in the medium BSSG+HS+YE.

BSS in the medium	Day opened	No. of cultures	A/R	% with proglottids	average length (mm)
Hanks' (control)	3	5	81/85	0	0.98 (27)
	6	5	73/78	4.1	1.48 (24)
	9	5	80/82	17.5	2.06 (26)
Krebs'	3	5	82/83	0	1.08 (27)
	6	5	80/83	3.8	1.55 (26)
	9	5	75/84	6.6	1.87 (24)
Earle's	3	5	90/91	0	0.89 (29)
	6	5	92/93	0	1.26 (30)
	9	5	85/88	8.2	1.79 (27)

A/R = no. alive/recovered; figures in brackets indicate the number of worms measured.

With regard to the uniformity and extent of growth and percentage of worms with proglottids, the medium with Earle's BSS seemed least satisfactory. Worms showed relatively better activity and appearance and more uniform growth in the medium with Hanks' BSS than that with Kreb's BSS. As a result of this experiment it was decided to continue with Hanks' BSS for all future work.

(4) Supplementation of the medium BSSG+HS+YE with protein hydrolysates and an amino acid mixture.

The following solutions were prepared and used with the basal medium consisting of Hanks' BSS+0.3% glucose+ 0.5% 'Oxoid' yeast extract+ 30% horse serum.

(a) 'Difco' Proteose Peptone - A 5% aqueous solution was prepared, sterilised by autoclaving at 10 p.s.i. for 15 mins. and used to give a final concentration of 0.3%.

(b) Casein Hydrolysate (BDH, not vitamin-free) - Prepared, sterilised and used as Proteose peptone.

(c) Amino acids mixture - Solution B of medium 102 (Bertzen, 1962) was prepared as described earlier in this Section (see Material and Methods). This solution was sterilised by filtration using GS 0.22μ millipore filter. Thirty millilitres of this amino acids mixture was used for the preparation of 100 ml of the final medium.

Figure 26

Growth of H.nana in medium BSSG+HS+YE supplemented with proteose peptone, casein hydrolysate, or an amino acids mixture. Each point represents the mean length of 27-29 worms.

BSSG+HS+YE = Hanks' BSS + 0.3% glucose + 0.5% 'Oxoid' yeast extract (autoclaved) + 30% horse serum.

Amino acids mixture = Solution B of medium 102 (Berntzen,1962).

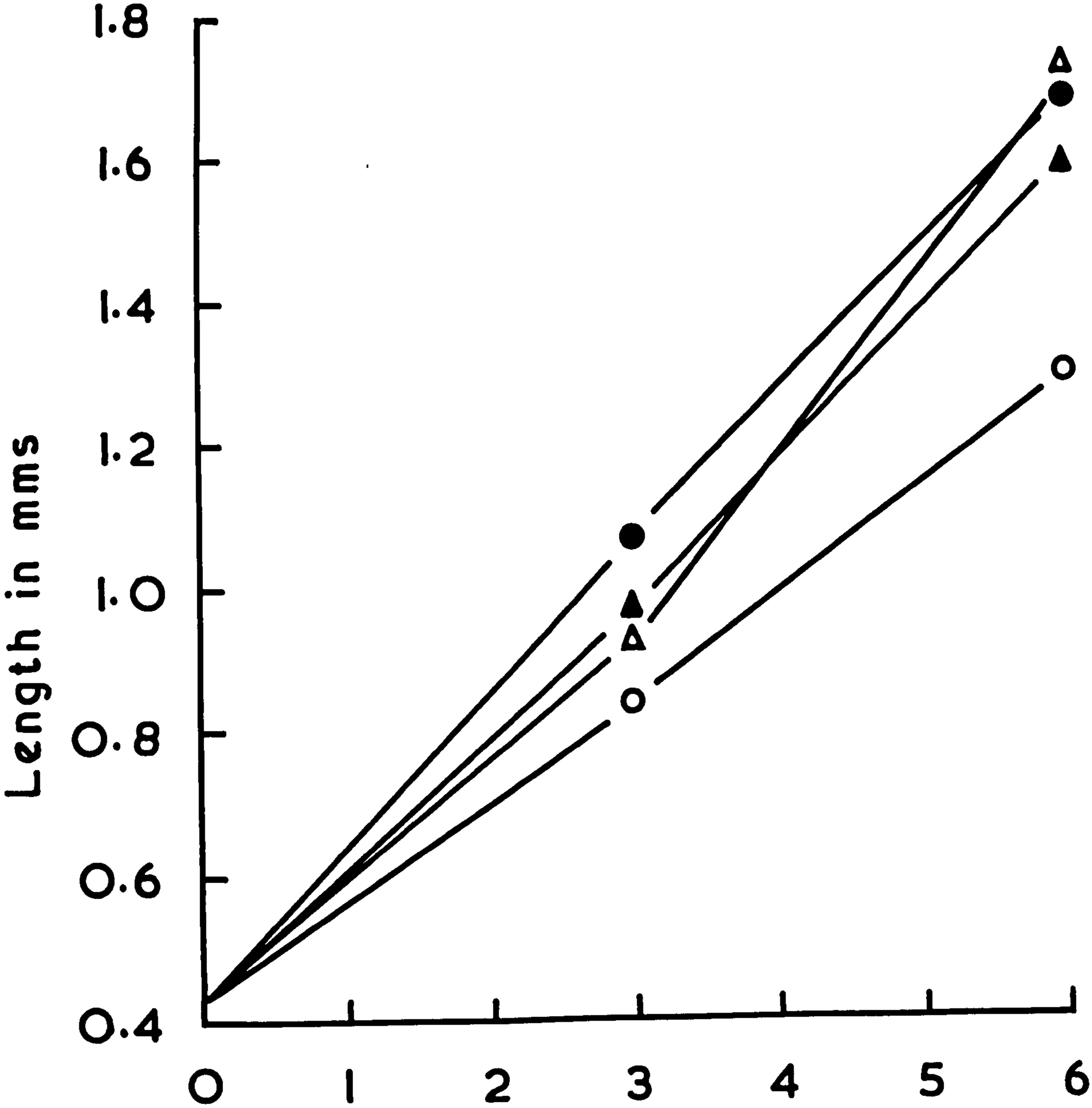
Solid triangles - BSSG+HS+YE

Open triangles - BSSG+HS+YE + amino acids mixture

Closed circles - BSSG+HS+YE +0.3% proteose peptone

Open circles - BSSG+HS+YE +0.3% casein hydrolysate

Figure 26.



Ten cultures were set up in each medium including the 'control' (BSSG+HS+YE). Each tube was inoculated with 18-22 worms. Cultures were run for 6 days. Five cultures of each medium were examined on days 3 and 6. The results are shown in Fig. 26.

The addition of casein hydrolysate was of no advantage. Worms in media containing proteose peptone and amino acids mixture were very healthy and active and about 10-12% of them were found to have proglottids on day 6. The worms in the control cultures also grew to the same extent in 6 days as those in media containing proteose peptone or amino acids mixture, but only 5% showed proglottids on day 6. It appeared as if the addition of these supplements had not a very marked effect on the growth of worms. It was obvious that the basal medium even after supplementation with the above-noted ingredients was inadequate to meet the demands of the worms.

Realising later that the amino acid solution was considerably hypotonic, it was interesting to note that the worms survived well in the medium containing 30% of the amino acids mixture by volume and appeared apparently unaffected by the low osmotic pressure.

(5) <u>Trial of some other yeast extracts and effect of yeast extract sterilised by filtration</u>.

Although the growth factors in yeast extract are

mostly known to be thermostable, it was felt that *H. nana* might have a requirement for a trace substance(s) that is destroyed by autoclaving the yeast extract. It seemed worthwhile, therefore, to compare the effects of autoclaved and filter-sterilised yeast extract. Two other yeast preparations, namely Yeast Extract Powder and Yeast Hydrolysate (supplied by Nutritional Biochemicals Corporation, Cleveland, U.S.A.) were also obtained, and tried as autoclaved and filter-sterilised preparations. A 5% aqueous solution of each type of yeast extract was prepared by heating to 70°C and sterilised by serial filtration using 1.2μ, 0.8μ, 0.45μ and 0.22μ millipore filters. These yeast extracts were used to give a final level of 0.5%. Seven groups of cultures were set up. Cultures in media containing 'Oxoid' yeast extract were run for 9 days; others were terminated on day 6. Each tube contained 18-22 worms, 5 cultures of each medium were examined on days 3 and 6, and those in 'Oxoid' yeast extract medium were examined also on day 9. The cultures in autoclaved 'Oxoid' yeast extract medium served as 'controls'. The results are shown in Table 14 and Fig. 27, a glance at which reveals the markedly good effect of filtered 'Oxoid' yeast extract on the growth of *H. nana*. Thirty-two percent of the worms in this medium

TABLE 14. Cultivation of H. nana: Effects of different types of autoclaved and filtered yeast extracts.

Yeast extract	Day opened	No. of cultures	A/R	% with proglottids	L(mm)
'Oxoid' Y.E. (Autoclaved) (control)	3	5	91/91	0	1.08 (29)
	6	5	84/87	3.5	1.60 (28)
	9	5	80/92	19.0	2.00 (27)
'Oxoid' Y.E. (filtered)	3	5	90/91	0	1.19 (29)
	6	5	87/87	32.1	1.98 (28)
	9	5	77/84	35.0	2.71 (26)
'NBC' Yeast Hydrolysate (autoclaved)	3	5	79/85	0	0.71 (26)
	6	5	90/98	0	1.13 (30)
'NBC' Yeast hydrolysate (filtered)	3	5	77/83	0	0.72 (25)
	6	5	74/84	0	1.13 (25)
'NBC' yeast extract (autoclaved)	3	5	77/78	0	0.96 (26)
	6	5	84/86	0	1.45 (27)
'NBC' yeast extract (filtered)	3	5	84/85	0	1.16 (28)
	6	5	83/84	7.2	1.59 (27)
'New' Bacto yeast extract (filtered)	3	5	96/98	0	no growth
	6	5	worms dead or dying		no growth

A/R = number alive/recovered; figures in brackets indicate the number of worms measured; L = average length.

Figure 27

Growth of H.nana in medium BSSG+HS+YE : effect of different types of yeast extracts,used as autoclaved and filter-sterilized preparations (0.5% level). Each point represents the mean length of 25-30 worms.

Open triangles - Filtered 'Oxoid' yeast extract

Solid triangles - Autoclaved 'Oxoid' yeast ext.

Crosses - Filtered 'NBC' yeast extract

Solid rectangles - Autoclaved 'NBC' yeast ext.

Open circles - Filtered 'NBC' yeast hydrolysate.

Closed circles - Autoclaved 'NBC' yeast hydrolysate.

- - - - - - Filtered "new" Bacto yeast extract.

Figure 28

Growth of H.nana in medium BSSG+HS+YE : effect of autoclaved and filter-sterilized 'Oxoid' yeast extract at 0.5% level (medium changed every 3rd day). Each point represents the mean length of 24-29 worms.

Closed circles - Autoclaved

Open circles - Filter-sterilized

Figure 27.

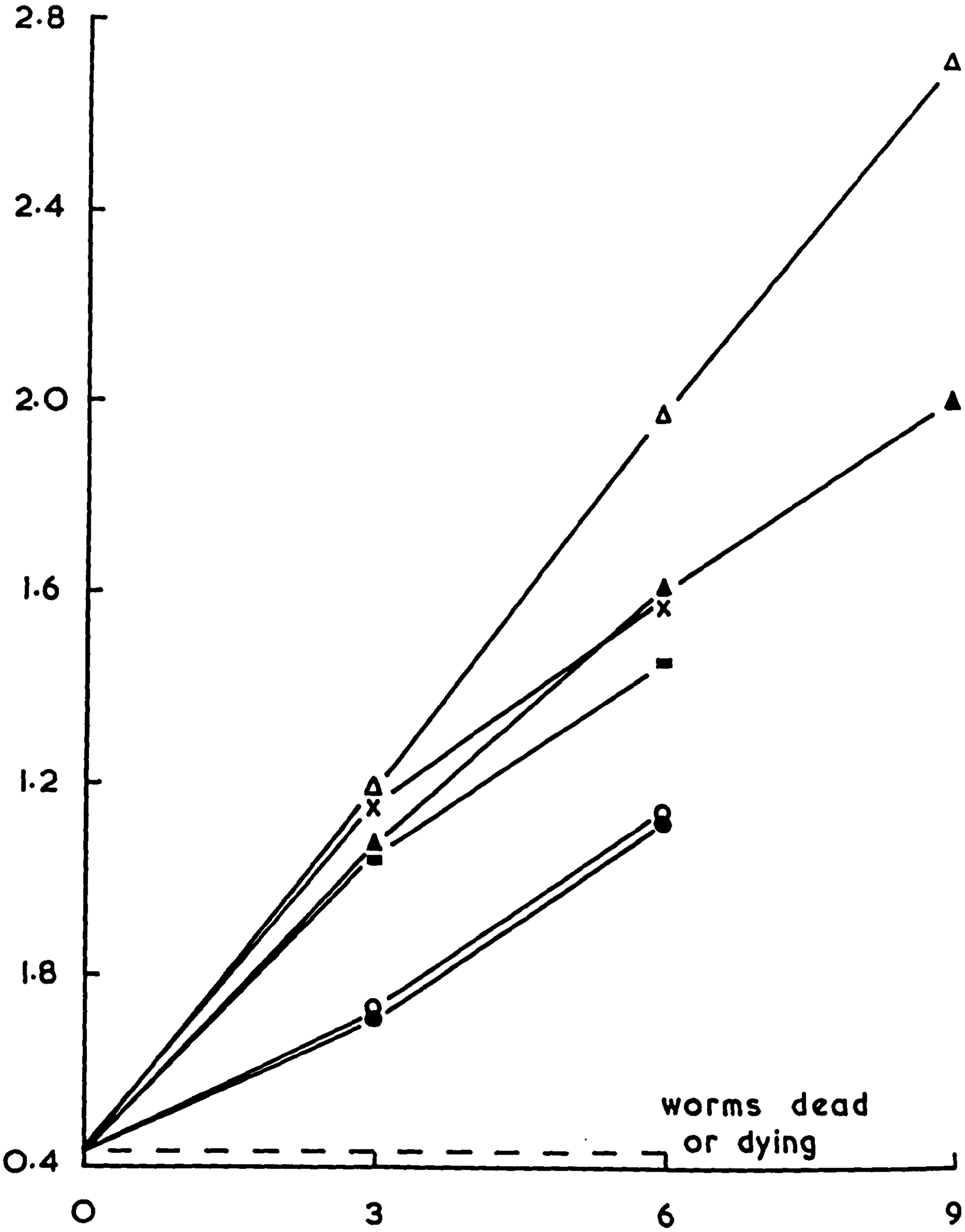
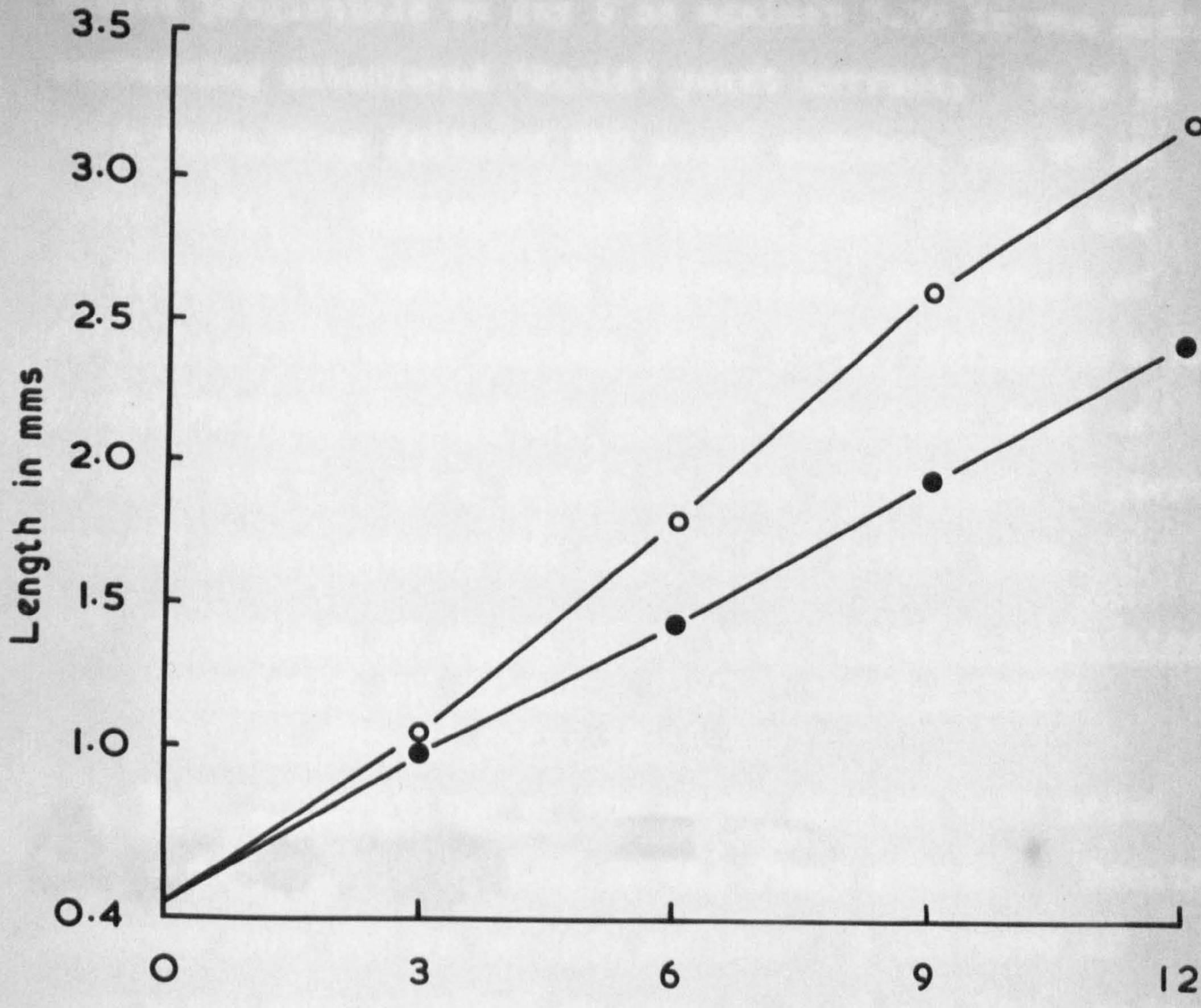


Figure 28.



showed proglottids on day 6 as against 3.5% in the medium containing autoclaved 'Oxoid' yeast extract; 7.2% in that containing filtered NBC yeast extract, and 0% in other media. Filtered 'new' Bacto yeast extract was as toxic as the autoclaved preparation, the effect of which has been shown earlier (Fig. 15-17, Tables 10 & 11). Worms, without showing any growth, were found dead or dying on day 6. As a result of this experiment, filtered 'Oxoid' yeast extract was used in all future work.

(6) Comparison of growth of H. nana in media containing autoclaved and filtered 'Oxoid' yeast extract (Medium changed every third day).

Both preparations of the yeast extract were added to a concentration of 0.5% in the final medium. Twenty cultures were set up in each medium; 5 cultures of each group were examined on days 3, 6, 9 and 12; medium was changed on days 3, 6 and 9. Results are shown in Table 15 and Fig. 28. A greater percentage of worms formed proglottids and growth was better in the medium containing filtered 'Oxoid' yeast extract. However, none of the worms showed advanced genital development even in this medium in 12 days.

TABLE 15. Growth of H. nana in medium BSSG+HS+YE containing autoclaved or filtered 'Oxoid' yeast extract (medium changed every third day).

Yeast extract	Day opened	No. of cultures	A/R	% with proglottids	L(mm)
Autoclaved (control)	3	5	80/81	0	0.98 (26)
	6	5	81/83	4.9	1.44 (26)
	9	5	84/88	11.9	1.95 (27)
	12	5	77/77	25.9	2.42 (24)
Filtered	3	5	83/83	0	1.05 (27)
	6	5	90/90	20.0	1.80 (29)
	9	5	80/82	26.3	2.63 (24)
	12	5	79/80	34.1	3.22 (25)

A/R = number alive/recovered; figures in brackets indicate number of worms measured; L = average length.

(d) Supplementation of the basal medium with Liver Extract

Basal medium = Hanks' BSS + 0.3% glucose + 0.5% filtered 'oxoid' yeast extract + 30% horse serum.

The idea that liver extract would be a very suitable and effective supplement arose from the following reasons:

(a) Liver extract has been incorporated into many media successfully used for the cultivation of helminths by workers, e.g. Weinstein & Jones, 1956; Nicholas, 1956; Dougherty et al, 1959; Nicholas et al, 1959; Silverman, 1959; Sayre et al, 1963; Stoll, 1953, 1959; etc.

(b) Liver has a high protein content (190 mg/g of fresh rat liver) (Spector, 1956).

(c) Even autoclaved liver extract has been reported to have considerable growth promoting properties (Nicholas et al, 1959; Williams, 1961).

(d) Filtered extracts of liver are translucent and worms can easily be examined and recovered from media.

(1) Rat Liver Extracts

Three types of extracts were prepared using rat liver.

Autoclaved aqueous rat liver (ALE)

50 g of fresh rat liver were allowed to autolyse for 8 hours at room temperature in a crystallising dish.

The liver tissue was then homogenised with 100 ml of deionised water at room temperature using an MSE over-head drive blender. The homogenate was allowed to stand overnight at 4°C in a screw-top bottle. It was then autoclaved at 10 p.s.i. for 12 min and allowed to cool. The bottle was shaken to break the protein clot. The autoclaved homogenate was then filtered through a Whatman No. 1 filter paper under negative pressure (suction). The yellowish serum-like filtrate was then sterilised by passing through a sterile GS 0.22 μ millipore filter. This sterile extract (ALE) was stored at -15° in 5 ml and 10 ml aliquots.

Autoclaved buffer-prepared rat liver extract (BLE)

The method of its preparation was a modification of the technique described by Nicholas et al (1959), 50 g of fresh rat liver were allowed to autolyse in a refrigerator (4°C) for 4 days. The liver tissue was then homogenised with 50 ml of M/15 potassium phosphate buffer (pH 6.8) in a chilled container surrounded by ice. The homogenate was autoclaved at 10 p.s.i. for 12 min and allowed to cool. The autoclaved homogenate was squeezed through a piece of muslin and then filtered through a Whatman No. 1 filter paper under negative pressure(suction). The filtrate was sterilized by passing through a sterile GS 0.22 μ millipore filter and stored at -15°Cin 5 ml and 10 ml aliquots.

Unheated rat liver extract (RLE_{20})

This liver extract was prepared by a minor modification of the technique described by Stoll (1953). Fresh rat liver (50 g) was allowed to autolyse in a refrigerator (4°) for 2 days. The liver tissue was homogenised with 200 ml of deionised water in a chilled homogenising flask surrounded by crushed ice. The pH of the brei (pH 6.5) was adjusted to 4.0-4.1 with N-HCl. The homogenate was centrifuged for 20 min. at 2000 r.p.m. (932 g) at 4°C. The supernatant (liver extract) was collected and serially filtered using 1.2 μ, 0.8 μ, 0.45 μ and 0.22 μ millipore filters. The extract was finally sterilised by passing through a sterile 0.22 μ millipore filter. Filtration was very difficult and there was a considerable loss of the extract at each step. The reddish, serum-like extract was stored at 4°C in 5 ml and 10 ml aliquots.

4 groups of cultures were set up in the following media:-

Group I Basal medium (Control)
Group II Basal + 10% ALE
Group III Basal + 10% BLE
Group IV Basal + 10% RLE_{20}

Each group had 20 cultures and each culture tube was inoculated with 18-22 excysted juveniles. Cultures were run for 12 days. Medium was changed every 3rd day. 5 cultures of each group were examined on days 3, 6, 9, and 12. The results are recorded in Table 16 and a comparative picture of growth in these 4 media, based on average length, is presented in Fig. 29.

The addition of autoclaved liver extracts (ALE and BLE) resulted in little improvement in the growth of the worms as compared with that produced by the addition of unheated rat liver extract (RLE_{20}). In the latter medium active sperms were seen in the mature proglottids of the worms on day 9 and 12% of the worms showed visibly normal eggs while in others there were only few (2-8) eggs present in a few proglottids behind the middle region of the strobila. The most interesting observation was the irregular coating of the culture tubes with a colourless precipitate a mass of which was also present at the bottom of the tube covered with the fluid medium.

To confirm the good effect of the unheated rat liver extract, RLE_{20} was prepared again and 2 groups of cultures were set up with 5 tubes in the basal medium (Hanks' + 0.3% glucose + 0.5% filtered 'oxoid' yeast extract + 30% horse serum = BSSG+HS+YE) and 5 in the RLE_{20} (Basal + 10% RLE_{20}). Cultures were examined on day 12.

TABLE 16. Growth and development of H.nana in basal medium supplemented with different types of rat liver extract.

Medium	Day opened	% with proglottids	L (mm)	Observations
Basal	3	0	1.07 (27)	proglottids small and narrow; worms showing deterioration and full of dark granular material after day 9; size range on day 12, 1.9-5.7 mm.
	6	24.6	1.62 (27)	
	9	34.5	2.70 (28)	
	12	-	2.96 (24)	
Basal + 10% ALE	3	0	1.03 (26)	As above; size range on day 12, 2.2-4.8 mm.
	6	22.5	1.43 (25)	
	9	35.0	2.40 (25)	
	12	-	3.32 (25)	
Basal + 10% BLE	3	0	1.10 (30)	appearance, activity, and growth of worms better than controls; size range on day 12, 2.2-5.3 mm; no genital development.
	6	25.3	2.18 (25)	
	9	38.4	2.86 (25)	
	12	-	3.32 (26)	
Basal + 10% RLE_{20}	3	31.8	1.26 (29)	very good growth; proglottids very prominent; seminal vesicles prominent and sperms present on day 9; 20% of worms with mature genitalia and 12% with fully formed eggs on day 12; size range on day 12, 2.2-17.2 mm.
	6	38.7	2.36 (27)	
	9	-	3.70 (27)	
	12	-	6.20 (24)	

L = average length of 33% of worms; figures in brackets indicate the number of worms measured from 5 tubes; 18-20 excysted cysticercoids in each culture; medium changed every 3rd day.

Figure 29

Growth of H.nana in medium BSSG+HS+YE (Hanks' BSS + 0.3% glucose + 0.5% filtered 'Oxoid' yeast extract + 30% horse serum) supplemented with 10% rat liver extract.

Solid triangles - BSSG+HS+YE (=Basal)

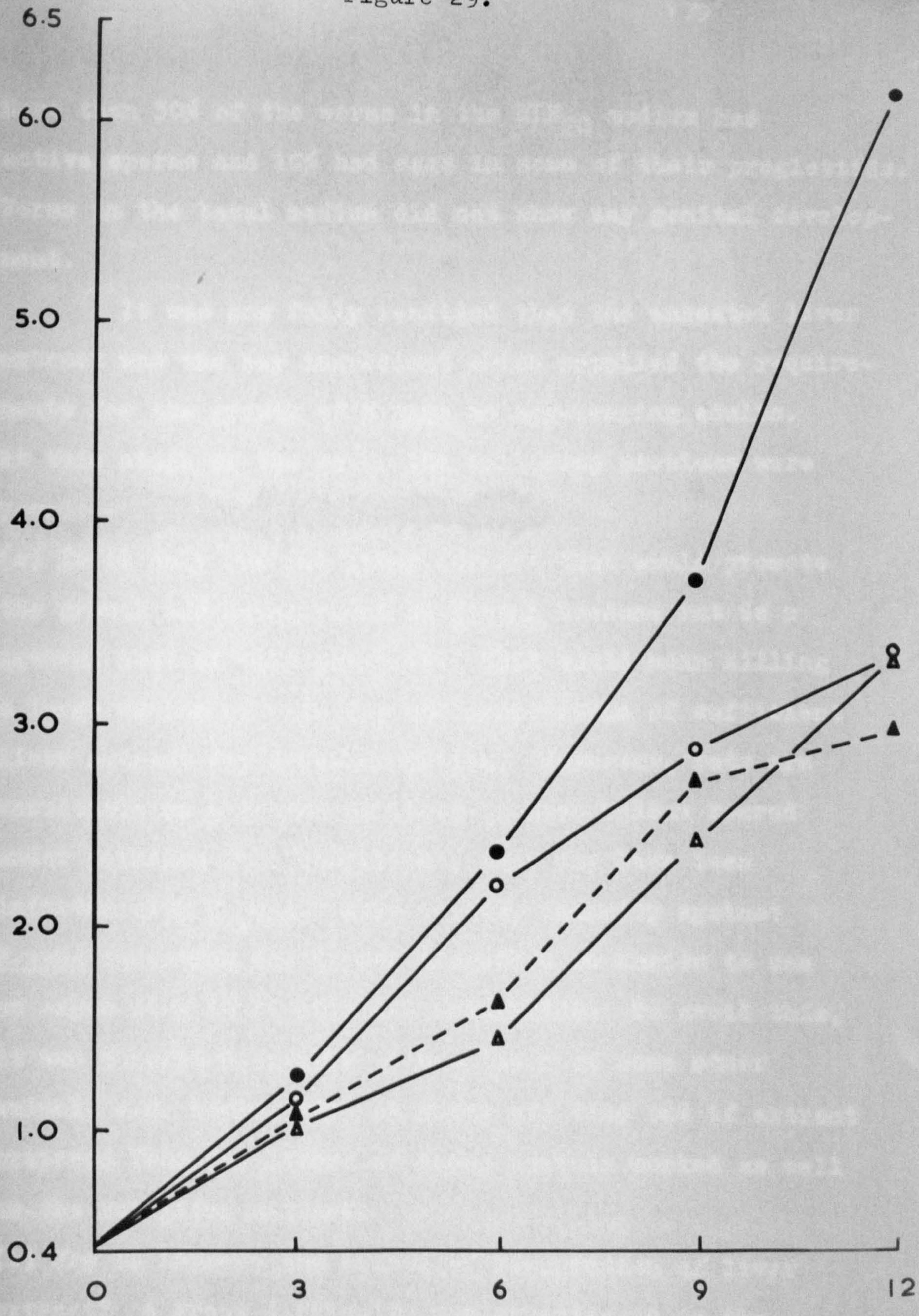
Open triangles - Basal + 10% autoclaved aqueous rat liver ext. (ALE).

Open circles - Basal + 10% autoclaved rat liver extract prepared in M/15 Sorensen's phosphate buffer (BLE).

Closed circles - Basal + 10% unheated rat liver extract (RLE_{20}).

Each point represents the mean length of 24-30 worms.

Figure 29.



More than 30% of the worms in the RLE_{20} medium had active sperms and 10% had eggs in some of their proglottids. Worms in the basal medium did not show any genital development.

It was now fully realised that the unheated liver extract was the key ingredient which supplied the requirements for the growth of H.nana to egg-bearing adults. At this stage of the work, the following problems were posed:--

(a) Is it only the rat liver extract that would promote the growth and development of H.nana because rat is a natural host of this parasite? This would mean killing a large number of rats for the preparation of sufficient liver extract for experimentation. Moreover, rats are unsuitable as a source of liver because unless reared under pathogen-free conditions, they may have been infected with H.nana. Rats develop immunity against H.nana infection and it may therefore be unwise to use an organ rich in circulating antibodies. To avoid this, it was thought desirable to use liver of some other animal, e.g. horse, pig, or sheep and find out if extract prepared from the livers of these animals worked as well as the rat liver extract.

(b) How to improve the method of preparation of liver extract? A great difficulty was experienced in the filtration of the liver extract for sterilization.

The method in use required much time and many expensive millipore filters.

In order to solve these problems, it was decided to use sheep liver extract prepared after a high speed centrifugation of the homogenate.

(2) Sheep Liver Extract (SLE_{20})

Fresh sheep liver was obtained from a local abbatoir and brought to the laboratory in an iced container. It was immediately chopped into small pieces and stored at $4^{o}C$. After 2 days, 150 g of this liver tissue was homogenised with 600 ml of deionised water and its pH altered to 4.0 with N HCl. The homogenate was divided into two equal portions. One portion was centrifuged at 30.000 r.p.m. for 15 minutes in a 'Spinco' (Beckman) ultra centrifuge at $4^{o}C$. The supernatant was sterilized without any difficulty by passing first through a HA 0.45 and then through a sterile GS. 0.22µ millipore filter. This liver extract (SLE_{20} - A) was stored at $4^{o}C$.

The other portion of the homogenate was centrifuged as usual at 2000 r.p.m. (932 g) for 20 minutes and the supernatant was sterilized with great difficulty by serial filtration through millipore filters of

graded porosities. This sheep liver extract (SLE_{20} - B) was also stored at $4^{o}C$.

Rat liver extract was also prepared as SLE_{20} - B and stored at $4^{o}C$.

Three groups of 8 cultures each were set up. Each kind of liver extract was used to give a concentration of 10% in the basal medium. Four cultures of each medium were opened on days 10 and 14. Worms in the RLE_{20} medium served as controls, The following criteria were used for the assessment of the effects of the three kinds of liver extracts:

(a) Percentage of worms with proglottids on day 6.

(b) Percentage of worms showing mature genitalia (presence of sperms) and average length of the best 33% of worms on day 10.

(c) Percentage of worms showing mature genitalia, and fully formed eggs, and average length of the best 33% of worms on day 14.

The following conclusions were reached from the results shown in Table 17.

(i) Sheep liver extract is as good as the rat liver extract.

(ii) Sheep liver extract prepared by high speed

TABLE 17. Growth and development of H.nana in BSSG+HS+YE+10% sheep liver extract.

Medium	Day 6	Day 10		Day 14		
	% worms with proglottids	% mature	L (mm)	% mature	% with eggs	L (mm)
RLE_{20} (control)	35	23	4.1 (21)	33	13	7.2 (22)
SLE_{20}-A	41	26	4.5 (21)	35	11	6.9 (21)
SLE_{20}-B	37	19	3.7 (22)	39	16	8.4 (22)

8 cultures in each medium; 4 tubes opened on day 10 and 4 on day 14; each culture was inoculated with 18-20 excysted worms; figures in brackets indicate the number of worms measured; L = average length of worms from 4 tubes (33% of worms measured).

centrifugation of the liver homogenate retains its growth promoting properties and is easily sterilized by filtration.

Experiments with liver extract and "SLE_{20} Medium"

Once known that sheep liver extract could be used for a successful *in vitro* cultivation of *H.nana* from cysticercoid stage to egg-bearing adult stage, a standard method for its preparation was worked out. The method now in use is based on the technique described by Stoll (1959) and is as follows:

(i) Lamb or sheep liver is brought from slaughter house in an iced container, quickly chopped into small pieces, and stored at -15°C.

(ii) When required, it is thawed at 4°C for 24-48 hours. One part by weight of this liver is homogenised with 4 parts by volume of deionised water at 4°C using a MSE overhead drive blender. The homogenising flask is surrounded by crushed ice. In practice, homogenisation is done in small lots; 20-25 g of liver are homogenised with 20-25 ml of water for 1½ min.

(iii) The brei (pH 6.3 - 6.6), after adjusting its pH to 4.0 with N-HCl (approximately 2.5 ml N-HCl required

for 100 ml of brei), is squeezed through muslin and then centrifuged at 6,000 r.p.m. (5420 g) for one hour at $4^{o}C$.

(iv) The supernatant (LE_{20}) is collected and filtered through a HA 0.45μ millipore filter (with a millipore AP20 microfibre glass disc prefilter) and finally sterilised by passing through a sterile GS 0.22μ filter.

(v) This liver extract is dispensed in 5 ml and 10 ml aliquots into screw-top containers and stored at $-15^{o}C$.

Before arriving at the decision to store the liver extract at $-15^{o}C$, it was routinely stored at $4^{o}C$. It was observed that within 2 weeks of storage, a fine brownish precipitate appeared in the liver extract. The formation of a precipitate in the liver extract has also been recorded by Stoll (1959) and Nicholas et al (1959). These workers used to resuspend the precipitate before adding the liver extract to a medium. In view of the importance of the liver extract for the growth and maturation of H.nana in vitro, it was necessary to investigate the following problems:

(a) Does the liver extract retain its activity for long period at 4℃ ? If not, what is the best way of storing it? An answer to this would enable a worker to prepare a large amount of the extract at a time and use it for long periods.

(b) What is the suitable concentration at which LE_{20} should be used with BSSG+HS+YE? It is quite likely that a concentration of less than 10% may be sufficient or a concentration or more than 10% would promote better growth of the worms.

(c) What is the effect of temperature on the activity of LE_{20}? Is it activated if it is heated at a specified temperature for a specified period of time? This was of interest since Sayre et al (1963) reported that their basal medium supplemented with liver extract prepared from the homogenate heated at 53℃ for exactly 6 minutes supported growth and reproduction of Caenorhabditis briggsae at the maximal rate.

(d) Is it essential to resuspend the precipitate formed in the liver extract on storage at 4°C ? Will H.nana grow if this precipitate is eliminated and only the supernatant is used?

(e) Is this liver extract needed as a whole or is only a small fraction essential? Is it possible to fractionate the liver extract? If this could be done, it would be of great interest to students of parasite biochemistry to attempt its chemical analysis and find out in strict/

/chemical terms the nutritional requirements of H.nana.

(f) How often should one prepare and change the medium? In the initial work the medium had been prepared on the day of use, but this practice was not considered good, as medium prepared each time would not be quite the same.

All experiments reported henceforward in this thesis were designed to investigate these problems. The "control" medium (designated and hereinafter referred to as "SLE_{20} medium") which supported growth and development of H.nana from cysticercoid to egg-bearing adult consisted of the following ingredients mixed in the order given below:

Hanks' BSS	40.0 ml
Crystamycin solution	0.1 ml
* 6.5% glucose	5.0 ml
5.0% filtered 'oxoid' yeast extract	10.0 ml
Sheep liver extract (SLE_{20})	10.0 ml
Horse serum No.2 (Burroughs Wellcome, London)	30.0 ml
0.2N-HCl	1.6 ml
1.4% $NaHCO_3$ solution	5.0 ml
	=100 ml (approx.)

*Five ml of 6.5% glucose solution in 100 ml of medium would give a concentration of 0.325%. For convenience this is /

/mentioned in the text as 0.3% glucose in the medium.

This medium was pipetted into culture tubes, gassed and sealed as described earlier (see'Materials and Methods') and placed in the roller drum. Within 2 hours a flocculent precipitate appeared in the medium. It was observed that good growth of worms occurred only when this precipitate was present in the medium. Liver extract was prepared fresh for each experiment and used within a week of storing at 4°C until experimental evidence was obtained that storing the liver extract at -15°C for long periods did not cause deterioration.

(1) Concentration of SLE_{20} in the medium

Sheep liver extract was prepared and used at 3 levels in the SLE_{20} medium. The results are shown in Table 18. The following observations were made in this experiment:-

(a) At 5% level, growth was very poor. Worms did not mature by day 10. Although a small percentage of worms became mature (showed sperms) on day 14, none was found to have any eggs.

(b) Growth, maturation, and egg-production occurred equally well at 10% and 20% levels of liver extract.

As a result of this experiment, 10% liver extract was used in the SLE_{20} medium in all future experiments.

TABLE 18. Growth of H.nana at different concentrations of sheep liver extract in the medium (BSSG+HS+YE+SLE_{20}).

Concentration of SLE_{20}	Day 6	Day 10			Day 14		
	% worms with proglottids	% with genitalia	% mature	L (mm)	% mature	% with eggs	L (mm)
5%	25.3	32.3	0.0	3.7(21)	9.3	0.0	4.0 (21)
10%	35.7	42.6	16.3	4.8(20)	34.8	12.1	10.6 (21)
20%	34.5	33.3	10.0	3.2(20)	36.6	16.6	11.6 (19)

8 cultures in each medium; 4 cultures of each group were examined on days 10 and 14.

L = average length of 33% worms from 4 tubes; figures in brackets indicate the number of worms measured.

(2) Effect of liver extract stored at 4°C and -15°C for various periods of time and the importance of the precipitate formed in the liver extract on storing at 4°C.

As stated earlier, it was necessary to know the conditions under which liver extract should be stored without deterioration of activity. It was also important to know what effect it would have on the growth and development of H.nana if the precipitate that appeared in the liver extract within 2 weeks of storage at 4°C, was eliminated. Liver extract stored for 3, 6, and 10 weeks at 4°C was used for this experiment. The medium with the liver extract in which the precipitate was resuspended before use served as 'control' for the other medium in which only 10% of the clear supernatant was used. The medium containing fresh (stored at 4°C for less than a week) liver extract served as a 'control' for all sets of cultures in an experiment. The effects of long term refrigeration or deep freexing on the activity of liver extract, as assessed by growth and development of H.nana in media containing such extracts, are shown in Table 19 and 20.

TABLE 19. Growth of H.nana in the medium containing 10% SLE_{20} stored at -15° or 4°C for varying periods.

Expt.	SLE_{20}	Day 6 % with proglottids	Day 10 % with genitalia	Day 10 % mature	Day 10 L(mm)	Day 14 % mature	Day 14 % with eggs	Day 14 L (mm)
1	Fresh (at 4°) (control)	34.5	39.3	13.7	4.0(17)	38.4	19.2	11.4(18)
	3 weeks at 4° (with ppt.)	36.0	35.7	14.3	3.6(21)	34.9	14.0	8.7(20)
	3 weeks at 4° (without ppt.)	25.8	10.2	0.0	2.5(22)	0.0	0.0	2.9(20)
	Fresh (at -15°)	38.1	44.6	21.6	6.6(21)	35.0	19.3	13.4(19)
2	Fresh (at 4°)	30.5	34.4	19.6	4.5(20)	42.3	16.9	12.9(20)
	6 weeks at 4° (with ppt.)	37.0	39.3	13.1	3.1(20)	23.8	7.9	6.4(20)
	6 weeks at 4° (without ppt.)	13.1	12.7	0.0	2.2(18)	0.0	0.0	3.2(19)
	10 weeks at 4°	24.8	40.9	6.5	2.6(20)	21.8	7.2	4.7(18)
	6 weeks at -15°	38.2	52.5	28.8	4.4(20)	57.6	20.3	10.5(19)

8 cultures in each medium; 4 cultures of each group examined on days 10 and 14; L = average length of 33% of worms from 4 tubes; figures in brackets indicate the number of worms measured.

TABLE 20. Effect of temperature on the activity of liver extract.

Expt.	SLE_{20}	Day 6	Day 10			Day 14		
		% with proglottids	% with genitalia	% mature	L (mm)	% mature	% with eggs	L (mm)
1	Fresh (at 4°)	36.3	40.7	25.9	4.4(19)	37.2	15.2	10.1(19)
	at 22°for 48 hrs, then stored at 4°.	29.0	44.6	17.8	4.8(18)	23.5	9.8	7.2(17)
	at 53°for 6 min, then stored at -15°.	34.3	48.3	28.3	3.7(20)	50.7	14.2	10.4(21)
	at 56° for 30 min, then stored at 4°.	28.2	34.9	14.3	3.0(20)	31.2	9.3	7.5(22)
2	Fresh (at -15°) (control)	43.2				49.2	19.7	9.2(23)
	at -15° for 16 weeks	44.8				43.0	20.8	9.9(23)
	at 53° for 6 min, then stored at -15°.	42.0				42.4	16.4	8.1(24)
	at 70° for 20 min, then stored at -15°.	34.4				34.8	13.9	7.3(28)
	autoclaved at 15 p.s.i. for 15 min, then stored at -15°.	33.3				20.5	4.1	4.4(23)
3	Fresh (at -15°) (control)	52.5				52.7	24.3	11.2(25)
	at 70° for 20 min, then stored at -15°.	33.5				29.3	12.0	6.8(19)
	autoclaved at 15 p.s.i. for 15 min, then stored at -15°.	30.6				19.3	5.2	4.6(23)

8 cultures in each medium in Expt.1 and 5 cultures in each medium in Expts. 2 and 3;
L = average length of 33% of worms; figures in brackets indicate number of worms measured.

The following results were obtained from 3 experiments (Expts. 1 and 2, Table 19; Expt. 2 Table 20):-

(a) Liver extract stored at -15°C for as long as 16 weeks was found as good as fresh liver extract. In fact, there is some evidence that liver extract stored at -15°C works better than that stored at 4°C. In all the 3 experiments mentioned above, some worms cultured in media containg deep frozen liver extract started depositing eggs in the culture medium on day 10. Beetles (Tribolium confusum) were successfully infected by feeding them gravid proglottids obtained from these worms and the cysticercoids obtained from these beetles were excysted in the usual way. The larvae (juveniles) were visibly normal and very active.

(b) Liver extract stored at 4°C for 6 weeks and over was found to be poor. Growth and percentage of worms maturing and producing eggs in media containing these liver extracts was very low.

(c) There was an indication that there was practically no loss of activity of the liver extract up to 3 weeks at 4°C.

(d) The medium containing liver extract from which precipitate was eliminated was no better than a medium without any liver extract at all. Worms did not mature and growth was very poor in such media. The presence of a precipitate characteristic of the normal culture medium was never observed in media containing only the fluid supernate of the liver extract.

(3) Effect of temperature on liver extract

Liver extract was prepared fresh and a portion of it was dispensed in 5 ml and 10 ml aliquots and stored at 4°C and -15°C. The remaining portion was divided into several lots and heated at different temperatures for specified periods of time as given below, cooled and stored at 4°C or -15°C.

(a) held at 22°C in an incubator for 48 hours in 5 ml aliquots and then stored at 4°C.

(b) a 40 ml portion heated in a water bath at 53°C for exactly 6 minutes, colled to below 10°C in a refrigerator, dispensed in 5 ml aliquots, and stored at -15°C.

(c) a 40 ml portion heated in a water bath at 56°C for 30 minutes and stored at 4°C in 5 ml aliquots.

(d) a 40 ml portion heated in a water bath at 70°C for 20 minutes, cooled to below 10°C, and then stored

in 5 ml aliquots at -15°C.

(e) 5 ml aliquots were autoclaved at 15 p.s.i. for 15 minutes, cooled, and stored at -15°C.

The results of three experiments performed to study the effect of differently heated liver extract on the growth and development of H.nana are shown in Table 20. The following cnnclusions were reached from these experiments:-

(a) Liver extract did not undergo deterioration if heated at 53°C for 6 minutes, nor was there any evidence that it was in any way activated by this treatment.

(b) Heating the liver extract at 22°C for 48 hours, at 56°C for 30 minutes, and at 70°C for 20 minutes appeared to diminish its activity. The percentage of worms maturing and producing eggs by day 14 was low in media containing these liver extracts.

(c) There was a great deterioration but not complete destruction of the growth promoting properties of the liver extract on autoclaving. The percentage of worms maturing and producing eggs was considerably lower and growth poorer in media containing autoclaved SLE_{20}.

(4) Attempts at fractionation of liver extract

The idea behind this type of experiment was to see if the incorporation of the whole liver extract into the medium was essential or whether one or more fractions contained all those substances required by H.nana for growth and development. Fractionation by Sephadex (Pharmacia, Uppsala, Sweden) gel filtration was preferred to 'salting out' which, it was thought, would complicate the experiments in several ways. Sephadex gel available in the form of insoluble dry powder composed of macroscopic beads of several mesh grades, is a non-ionic material and provides an excellent tool for molecular sieving. Using proper type of gels and 'markers', molecules of different molecular weights can be separated. For a preliminary test, it was decided to fractionate the liver extract using Sephadex G-100 gel (approximate exclusion limit, molecular weight 100,000) and 'blue dextran' and phenol red as 'markers'. 'Blue dextran 2000' is readily soluble in water and salt solutions, and because of its high molecular weight (2,000,000), it is excluded from all types of sephadex columns. In a preliminary test, it was observed that a fine precipitate was formed when SLE_{20} was mixed with Hanks' BSS resulting in a very slow and incomplete elution.

It was decided therefore, to elute liver extract with water, recombine various fractions, sterilize by serial filtration and add a calculated amount of x10 Hanks' BSS so as to have SLE_{20} in Hanks' in a proportion of 1:4 (the proportion in the final medium). Now, before different fractions of liver extract could be tested separately, it was important to ascertain the following:-

(a) Whether liver extract previously mixed with Hanks' BSS and stored at 4°C will retain its activity,

(b) whether a filtered mixture of SLE_{20} and Hanks' BSS is as good as unfiltered but sterile mixture,

(c) whether blue dextran is without any effect on the worms in culture so that it can be safely used as a 'marker' during fractionation of the liver extract,

(d) if recombined fractions of liver extract after sephadex treatment would work as well as the normal untreated liver extract.

A 2.5% aqueous solution of blue dextran was autoclaved at 15 p.s.i. for 15 minutes and stored at 4°C. A calculated amount of this solution was used to give 1 mg of blue dextran per ml of liver extract.

Liver extract for use in this experiment was treated in the following ways:-

(i) normal SLE_{20} prepared fresh and stored at 4°C.

(ii) SLE_{20} + Hanks' BSS (1:4) mixture sterilized by filtration.

20 ml of SLE_{20} and 80 ml of Hanks' BSS were mixed and sterilized with great difficulty by serial filtration using millipore filters of graded porosities (1.2μ-0.22μ). The clear sterile mixture was stored at 4°C in 12,5 ml aliquots. This amount was used for preparing 25 ml of the final medium required for setting up 5 cultures.

(iii) SLE_{20} + Hanks' BSS (1:4) mixed aseptically and stored in 12.5 ml amounts at 4°C.

(iv) SLE_{20} + Hanks' BSS + blue dextran mixture sterilized by filtration.

20 ml of SLE_{20} + 0,8 ml of 2.5% blue dextran + 80 ml of Hanks' BSS were mixed together, sterilized by serial filtration and stored at 4°C in 12.5 ml amounts.

(v) SLE_{20}+ 0.8 ml blue dextran solution + 80 ml Hanks' BSS, mixed aseptically and the mixture stored at 4°C in 12.5 ml amounts.

(vi) SLE_{20} eluted with deionised water through a sephadex column, fractions recombined and sterilized by filtration and stored after addition of a calculated amount of x10 Hanks' BSS.

40 ml of SLE_{20} + 1.6 ml of blue dextran solution were eluted with 112 ml of deionised water using sephadex G-100 column. The water used for this purpose contained 1 ml of 0.2% phenol red solution per 100 ml. The total volume of 152 ml collected after elution was serially filtered for sterilization and ultimately 114 ml of the sterile mixture was obtained. Since the original volume (152 ml) contained 40 ml of the liver extract, 114 ml would contain 30 ml, which in the final medium would be mixed with 120 ml of Hanks' BSS giving a total of 150 ml. This was done by adding x10 Hanks' BSS in the following manner:

eluted and sterilized material	114 ml
x10 Hanks' BSS	12 ml
sterile deionised water	24 ml
	150 ml

This solution was stored at 4°C in 12.5 ml aliquots.

6 groups of cultures with 4 tubes each were set up. The final medium was prepared on the day of use by adding 0.3% glucose, 0.5% filtered 'oxoid' yeast

extract and 30% horse serum to each of the 6 preparations described above. Cultures were run for 14 days. Medium was changed in each group on days, 3, 6, 9, 11, and 13. The results of this experiment are shown in Table 21.

Although the results with regard to growth, maturation, and egg production were not very good in the control medium (group A), presumably due to some damage done to the worms during excystation or the use of a bad batch of serum, the following interesting points are clearly indicated:-

(i) Liver extract loses its properties when it is filtered after mixing with some other ingredient. Filtration removes a fine suspension of particulate matter which gradually settles down to form a precipitate when SLE_{20} is mixed with Hanks' BSS or water. Worms do not grow in media containing filtered liver extract.

(ii) The presence of Blue dextran does not have any ill effect on the worms. However, it is difficult to filter a solution containing blue dextran through millipore filters even under a pressure of 16 p.s.i. Filters become clogged with a blue particulate matter. This is further indicated by the loss of the intensity of blue colour in the filtrate.

TABLE 21. Efficiency of liver extract serially filtered in combination with Hanks' BSS or after Sephadex column fractionation.

Culture group	Medium containing	Day 6	Day 10	Day 14		
		% with proglottids		% mature	% with eggs	L (mm)
A	SLE_{20} (control)	36.1	-	26.3	8.7	10.3(18)
B	SLE_{20}+Hanks' (filtered)	0.0	no proglottid formation, growth negligible, cultures discarded			
C	SLE_{20}+Hanks' (unfiltered)	35.3	-	25.0	7.8	9.6(18)
D	SLE_{20}+Hanks'+BD (filtered)	0.0	as in B; cultured discarded			
E	SLE_{20}+Hanks'+BD (unfiltered)	38.4	-	23.5	6.8	9.0(16)
F	SLE_{20}+Hanks'+BD (SLE_{20}+BD Sephadex treated and filtered)	0.0	as in B; cultures discarded			

4 cultures in each medium; 14-16 worms in each tube; L = average length of 33% of worms; figures in brackets indicate the number of worms measured.

(iii) Liver extract can be mixed with Hanks' BSS and stored at 4° and can be used at any time for the preparation of the final medium during a 14-day experimental period (no evidence for longer periods).

(iv) Liver extract reconstituted by recombining the fractions obtained after elution through a sephadex column is ineffective. It is difficult to say that the failure of worms to grow in a medium containing such liver extract is due to the fractionation of the liver extract or to its subsequent filtration for sterilization. The possibility of the latter seems more likely.

(5) How often to prepare and change medium

Up till now the medium of the cultures, during a 14-day culture period, was changed on days 3, 6, 9, 11, and 13. Medium was every time prepared fresh on the day of use. It was wished to discover whether one could prepare the medium in large amount, store it, and use it for the whole experiment. It was also wished to know whether the medium could be left for longer periods without renewal. The results of an experiment designed to obtain information

TABLE 22. Effect of freshly prepared and stored(4°C) media and frequency of changing medium on the growth and development of H.nana in vitro(SLE$_{20}$medium).

Group	Day medium prepared	Day medium changed	No. of cultures	Day 6 % with proglottids	Day 14 % mature	Day 14 % with eggs	Day 14 L (mm)
A (control)	0,3,6,9,11,13	3,6,9,11,13	5	43.2	49.2	19.7	9.2(26)
B	0	3,6,9,11,13	5	52.8	53.7	30.0	13.6(27)
C	0,4,8,12	4,8,12	5	44.8	23.0	7.6	6.0(26)
D	0,6,11	3,6,9,11,13	5	47.9	45.3	18.7	10.0(21)
E	0,5,9,12	5,9,12	5	34.6	42.2	12.6	8.7(24)

Medium to be stored was dispensed in suitable aliquots, gassed with 95% N_2+5% CO_2, and stored at 4°C; L = average length of 33% of worms; figures in brackets indicate the number of worms measured.

on these problems is recorded in Table 22. The most interesting finding is that one can prepare the medium in large amounts, dispense it in suitable aliquots, and store it at 4°C after gassing with 95% N_2 + 5% CO_2. The results, though not conclusive, also indicate that the practice of changing the medium on days 3, 6, 9, 11 and 13 is better than that of changing at longer intervals.

D. Discussion

Attempts to grow H.nana in medium 102 using Berntzen's techniques failed. The worms survived in this medium but no growth occurred. In the early stage of the work when horse serum was used as solution F of medium 102, it was felt that agamma calf serum, as used by Berntzen (1962) was probably essential, but later work revealed that the medium containing agamma calf serum was in no way better than that containing horse serum (Table 4). This raised a number of problems and it was difficult to know the point in the technique, which involved many steps at which the mistake was occurring. It must be pointed out that it was impossible to imitate Berntzen's technique in entirety from his paper (Berntzen, 1962). The following difficulties were experienced in the preparation of medium 102:-

(a) In solution A, only $CaCl_2$ has been recommended by Berntzen to be prepared separately, but it was observed that when $NaHCO_3$ was added to the other ingredients of solution A and stored at $4^{o}C$, a precipitate appeared in the solution. For this reason $NaHCO_3$ solution had had to be prepared separately and added at the time of preparing the final medium.

(b) In the preparation of solution B, Berntzen writes "L-cystine (dissolved in 1/N HCl), 2.5" and "L-tyrosine (dissolve in N/1 HCl), 5.0". It was difficult to follow

from this the normality of HCl Berntzen used to dissolve these amino-acids.

(c) KOH was used to neutralise the organic acids in solution D and also to adjust the pH of the final medium. The molarity of this KOH solution was not stated. A very strong as well as a very dilute solution of this alkali would affect the osmotic pressure of the final medium to a considerable extent.

Apart from the ambiguities mentioned above, several other points had not been made clear in his paper, for example:

(a) Details of the method of excystation (no mention of the strength of the excysting solutions) which, it is now realised, is critical for subsequent growth and survival of the worms <u>in vitro</u> is not given.

(b) Necessity of the use of agamma calf serum - this implies that normal horse or calf serum (commonly used in media for tissue-culture and helminth cultivation) with a high gamma globulin content, was not suitable. However, no evidence is presented to support this.

(c) Inhibition of the growth of worms in medium containing antibiotics. Antibiotics have been used in all media developed so far for the successful cultivation of many helminth species. Reid and Boles (1949) used various commercially available antibiotics to prevent bacterial

growth in media devised for the in vitro cultivation of Hymenolepis diminuta and H.nana. They found a combination of 1852 units of penicillin, 1000 µg of streptomycin, and 800 µg of chloromycetin per ml of media (Locke's solution + 19 amino-acids and 23 other constituents) to be very effective in preventing bacterial contamination introduced by equipment, other solutions, and cestodes. Aureomycin (500 µg/ml) in combination with penicillin was found to be toxic. Taylor (1961) found that 400 units of penicillin and 500 µg of streptomycin per ml of media were well tolerated by H.diminuta and H.nana in culture. As reported in this work also, H.nana has been successfully cultivated from cysticercoid stage to egg-producing adult in SLE_{20} medium which contains 100 units of penicillin and 100 µg of streptomycin per ml. Cultivationists would appreciate how difficult and risky it would be not to use antibiotics in media if cultures are to be run for long periods with frequent medium renewal.

(d) Omission of NaCl from medium 102 and compensation of osmotic pressure by addition of high amount of sucrose (2.668 g/90 ml of final medium without serum) - Berntzen states "If NaCl was introduced in amounts of 5 to 7 g/l and the sucrose of solution c reduced accordingly, only negative results were obtained. However, if 4 g or less NaCl were substituted, the results were the same as if only sucrose had been used". This would mean that an increase of 1-3 g/l

of NaCl, above the 4 g/l level, was toxic. The toxicity could be due to Na^+, Cl^-, or to the osmotic effect (Hopkins, 1967). NaCl is the commonest electrolyte of the biological environment and it is difficult to see why it should be entirely omitted.

(e) The reason for using Krebs' cycle primers (solution D) under anerobic conditions (gas phase 95% N_2 + 5% CO_2 is not stated.

Hopkins (1967) has critically reviewed Berntzen's work and has pointed out several other peculiarities. For instance, solution A of medium 102 contributes only 11 mM Na^+ and 61 mM Cl^- to the final medium whereas a common BSS such as Hanks' contains 142 mM Na^+ and 146 mM Cl^-. He has also quoted Code et al (1966) to point out that the equilibrium concentration of these ions, particularly Cl^-, in the ileum is lower than that in serum (152 mM Na^+, 81 mM Cl^- in ileum of dog, and 136 mM Na^+, 104 mM Cl^- in man). In view of the ambiguities mentioned above and also realising that trying to solve these problems would not be easier than starting the whole thing anew, attention was directed to roller tube culture system which were more suitable for rapid screening of media and easier for observation of the growth and development of worms without terminating cultures.

Cultivation of progenetic larvae like plerocercoids of Schistocephalus to maturity does not require media of a

high nutritional level since such larvae are already well advanced in their development when removed from the intermediate host and their sexual differentiation is not accompanied by increase in mass. But cultivation of undifferentiated larvae such as cysticercoids of H.nana presents many difficulties because considerable tissue synthesis and differentiation must occur before sexual maturity. This necessitates apart from the provision of a suitable physical environment, the provision of a highly nutrient medium to meet the demands of the growing organisms. This demand may involve the supply of protein(s) and/or amino-acids and other growth factors in a form readily assimilable by the worms growing in culture (Smyth, 1959).

The physical parameters important from the point of view of cultivation in vitro are osmotic pressure, temperature, pH, and the gas phase. Of these the first three offer little difficulty. Helminths are fairly resistant to small changes in osmotic pressure. The osmotic pressure of the paramucosol lumen is known to be close to that of blood serum. Follansbee (1945) has reported the osmotic pressure of rat's intestinal content to be close to that of blood. Therefore H.nana should be in osmotic balance with a medium having $\Delta = 0.56^{o}C$. The body temperatures of the natural hosts of H.nana (mouse 35.2 - 37.8; rat 35.8 - $37.6^{o}C$) (Spector, 1956) are known, but since Berntzen (1962) found that

percentage of positive cultures and recovered worms was higher at 39°C than at 37°C, the medium temperature was maintained at 38±0.5°C — a temperature intermediate between the body temperatures of the natural hosts and the one reported by Berntzen as more suitable. The pH was adjusted in such a way that the medium after equilibration with the gas would have a pH of 7.2±0.2 approximating the average pH of the lower ileum or rat or man (Wiseman, 1964. p 218). The nature of the gas phase is a very important factor and a mixture of 95% N_2 + 5% CO_2 was used in all media as a result of several considerations:

(a) Berntzen (1962) obtained positive results for growth and develppment of H.nana only when a mixture of 95% N_2 and 5% CO_2 was used. His worms died within 48 hours under a gas phase of 95% O_2 + 5% CO_2, "compressed air", or 100 CO_2. The use of 100% N_2 resulted in the survival of the worms for three days without any growth. His experiments led him to conclude that a high O_2 tension in the gas phase was inhibitory and that prescence of CO_2 was essential. Schiller (1965) successfully cultured H.diminuta from cysticercoid to egg-producing adult in a diphasic medium with a gas phase of 97% N_2 + 3% CO_2. Taylor (1961) used a variety of media for growing H.diminuta and H.nana but failed. This failure might have been due to the fact that she used air as gas phase.

(b) Read and Simmons (1963) have pointed out that CO_2 is

present in a high concentration in the intestinal lumen, and intestinal helminths may depend on this gas for a number of physiological processes. Intestinal juice pouring into the intestinal lumen has a H_2CO_3-HCO_3 buffering system and the average CO_2 tension of the human jejunal juice is 100 mm Hg (= 13% CO_2 in gas phase) (Read, 1950).

(c) The presence of CO_2 in the gas phase, in association with $NaHCO_3$ in the medium provides CO_2- HCO_3 buffering system, characteristic of biological habitats.

(d) Fairbairn et al (1961) have reported that CO_2 stimulates glucose utilization and is essential for glycogenesis in H.diminuta. Schwade (quoted by Read and Simmons, 1963) also observed that H.diminuta showed vigorous glycogenesis in HCO_3- CO_2 buffers.

(e) That CO_2 is essential for cellular growth and cellular respiration is inhibited in its absence has been unanimously accepted by workers in the field of tissue-culture. It has been suggested that CO_2 is required for purine and pyrimidine synthesis (Paul, 1965).

(f) The works of Campbell (1960) and Fairbairn et al (1961) have indicated that there may be some mechanism of CO_2 fixation in H.diminuta. Hopkins (1967) has pointed out two possible pathways for CO_2 fixation in tapeworms excreting large quantities of succinate (as found in H.diminuta) and has presented arguments on theoretical grounds in favour of

using 5-10% CO_2 in the gas phase for cultivation of cestodes in vitro.

Anaerobiosis in hymenolepids is suggested by the work of Read (1951) who demonstrated in H.diminuta several enzymes associated with phosphorylative glycolysis. The data suggested that H.diminuta has enzyme systems for glucose degradation by Embden-Meyerhof sequence. However, although it is usually accepted that the intestine is essentially an anaerobic habitat, there is a significant diffusion of oxygen from the mucosa into the lumen. This oxygen is rapidly used by intestinal microflora resulting in a steep gradient from the mucosa to the centre of the lumen. Rogers (1949, quoted by von Brand, 1952, p 179) found the oxygen tension near the mucosa of the rat's intestine to be 8-30 mm Hg (= 1-4% in the gas phase). Recently, Crompton et al (1965) have determined ~~by means of~~ the oxygen tension in duck's intestine and found it to be up to 25 mm Hg in the vicinity of the villi and 0.5 mm Hg in the lumen. These observations as well as the occurrence of mitochondria in the cestode tegument and underlying tissues indicate the possibility that tapeworms, believed to inhabit the 'paramucosal lumen' (Read, 1950) in the hosts' intestine make use of the available oxygen. Recently Smyth (1967) has reported his successful cultivation of Echinococcus granulosus from protoscolex to sexually mature adult in a diphasic medium with a gas

phase of 8% O_2 + 5% CO_2 in nitrogen. It would be interesting to see the effect of low concentrations of oxygen in the N_2 + CO_2 mixture on the growth, development, and egg production of H.nana in vitro.

Cultivation of H.nana was started in glucose-saline (BSSG) medium in which the worms died in less than two days (Table 5). This was not surprising since Smyth (1947b) had observed that plerocercoids of Diphyllobothrium sp. also had a mean survival period of two days in glucose-saline (¾ Locke's solution + 0.1-0.5% glucose). The addition of 0.5% Bacto Yeast extract to BSSG prolonged the survival of worms to 6-8 days (Table 6). The beneficial effects of yeast extract in a culture medium has also been reported by a number of workers. Wyllie et al (1960) found that a high proportion of the strigeid trematodes, Diplostomum phoxini, developed follicular testes and some showed active sperms when yeast extract was added to a basal medium (albumen + gluco saline). McCaig and Hopkins (1965) found that addition of yeast extract to glucose-saline medium prevented weight loss and supported a low level of growth of the plerocercoids of Schistocephalus. Yeast extract is known to contain a high proportion (45%) of amino acids as well as B vitamins, carbohydrates and peptones. Recently Balamuth and Kawakami (1963) have been able to replace yeast extract by a combination of 5 nucleotides, a B vitamins mixture, choline, inositol

and acetate, in Diamond's (1960) medium (for Entamoeba invadens) in which the source of the amino acids was 'Tripticase'. It is probable therefore, that the beneficial effect of yeast extract was due to its contribution of amino acids, B vitamins, and nucleotides.

The incorporation of chick embryo extract (CEE_{50}) into the glucose-saline + yeast extract (BSSG + YE) medium proved ineffective and toxic (Table 6). This was surprising especially in view of several reports in the literature on tissue-culture and helminth cultivation on the growth-promoting properties of chick embryo extract. The inactivity of the CEE_{50} might be due to the loss of some heat-liable growth-promoting factor(s) during its preparation. The preparation and extraction, except centrifugation of the homogenate, were done at room temperature (see Section I). Weinstein and Jones (1956) prepared CEE_{50} using chilled Tyrode's solution and frozen embryos and thereafter extracting for one hour at $5^{o}C$ before centrifuging at $0^{o}C$. This CEE_{50} alone could support growth and development of Nippostrongylus muris (brasiliensis) from infective larval stage to a stage comparable to that in the lungs of a rat. When added to a mixture of caseinate, liver filtrate and rat serum, this CEE_{50} promoted the development of N. muris (brasiliensis)to sexual maturity. Mueller (1959) also used a 'cold' CEE_{50} in a medium consisting of Mixture 199 + calf

serum for the successful cultivation of Spirometra mansonoides from procercoid to plerocercoid stage. On the other hand, the addition of CEE_{50} (extracted for 3 hours at room temperature before centrifugation) to a mixture of medium 199 + serum + amino acids solution had no effect on the growth and survival of Hymenolepis nana and H. diminuta (Taylor, 1961). In the light of these findings, it should be worthwhile to test the effect of a cold preparation of chick embryo extract in the BSSG + YE or BSSG + HS + YE (glucose-saline + yeast extract + horse serum) medium.

The addition of serum to BSSG + YE medium had a marked effect. With horse serum, worms were found to have proglottids on day 4, whereas with calf serum, worms showed proglottids on day 6. The medium containing horse serum but no yeast extract was inadequate to support any growth of the worms. The combination of serum and yeast extract had a synergistic effect (Fig. 23) similar to that observed by Wyllie et al (1960) on Diplostomum phoxini, and McCaig and Hopkins (1965) on plerocercoids of Schistocephalus, proving that serum and yeast extract contributed different ranges of essential metabolites.

In the course of attempts to develop a suitable medium for the cultivation of H.nana, it was observed that yeast extracts obtained from different sources differed considerably in their properties. There was also evidence for batch-to-

batch variation in the yeast extract from the same source of supply. An old batch (2-3 years) of Bacto yeast extract (Difco) gave the best results; the worms developed proglottids on day 4 in the medium containing it. Recent batches of the same yeast extract were found toxic. 'Oxoid' yeast extract was found relatively more suitable than that from other sources. Filter sterilised yeast extract was more effective than the autoclaved preparation suggesting that H.nana required from yeast extract some substance(s) which was destroyed by autoclaving (Table 14, Fig. 27).

Experiments designed to investigate the effects of different types of balanced salt solutions and varying concentrations of glucose, yeast extract, and horse serum in the medium led to the development of a basal medium (BSSG + HS + YE) consisting of Hanks' BSS + 0.3% glucose + 0.5% filtered 'oxoid' yeast extract + 30 % horse serum (Tables 12-14, Figures 22-25,27). The renewal of the medium every third day resulted in a marked improvement in the appearance, growth, and activity of the worms(Table 15, Fig. 28).

Supplementation of the basal medium with Proteose peptone (Difco) or casien hydrolysate, or an amino acids mixture did not improve the growth of the worms (Fig. 26). The addition of autoclaved rat liver extracts (water or buffer-prepared) did not promote genital development of the worms. The incorporation of 10% unheated rat liver extract (RLE_{20})

into the basal medium produced a marked change in the cultures; worms showed excellent growth and were found to have fully formed and normal eggs in some of their proglottids when examined on day 12 (Table 16, Fig. 29). The experiment was repeated with another batch of similarly prepared RLE_{20} and results confirmed. Later experiments, for reasons stated earlier (see Results - Part II), led to the use of sheep liver extract (SLE_{20}), improvement in the method of its preparation, its storage in the deep freeze ($-15^{o}C$), and the development of the "SLE_{20} medium". In this medium (SLE_{20} medium = Hanks' BSS + antibiotics + 0.3% glucose + 0.5% filtered yeast extract + 30% horse serum; pH 7.2±0.2 adjusted with 0.2 N NaOH and 1.4% $NaHCO_3$; temperature 38±0.5^{o}C) prepared once at the beginning of the experiment, stored at $4^{o}C$ after gassing with 95% N_2 + 5% CO_2, and renewed on days 3,6,9,11, and 13, during a 14 day cultivation period, worms showed active sperms as early as on day 8, some started depositing eggs on day 10, and by day 12, about 20-30% of the worms showed a series of gravid proglottids with fully formed eggs which developed into normal cysticercoids in beetles (Tribolium confusum).

Experimentation with the liver extract (SLE_{20}) permitted some very interesting observations. Within 2 hours of placing the roller tubes in the incubator, a flocculent

precipitate appeared in the SLE_{20} medium. A thin irregular coating was found also on the inner wall of the culture tubes. It was observed that good growth of worms leading to sexual maturity and egg production occurred only when this protein precipitate was present in the tubes. A precipitate appeared also in the liver extract within two weeks of its storage at $4^{o}C$. When this precipitate was eliminated and only 10% supernatant was used, the liver extract failed to promote growth and maturation of the worms (Table 19).

There was a progressive deterioration of the activity of the liver extract after storage for 6 weeks at $4^{o}C$. No loss of activity was detected on storage at $4^{o}C$ up to 3 weeks (Table 19). On the other hand, liver extract retained its full activity for long periods (experimental evidence for as long as 16 weeks, Table 20) when stored at $-15^{o}C$. Liver extracts stored at 4^{o} for 8 weeks and over had a pH of 4.6-4.8 (original pH 4.0-4.1), whereas practically no change in pH ($\leqslant$ 0.1 units), was detected in extract stored at $-15^{o}C$. Careful experimentation would be necessary before one can show correlation between the loss of activity of liver extract and rise in pH but it would be interesting to see if liver extract prepared at pH 4.5 and over was completely ineffective or relatively less active than one known to be active when prepared at pH 4.0-4.1.

There appeared to be some loss of activity when liver

extract was heated at 22°C for 48 hours, at 56° for 30 mins. and at 70° for 20 mins. No deterioration was detected after heating at 53°C for 6 mins., nor was there any indication of activation by this treatment as reported by Sayre et al (1963). The percentage of worms maturing and producing eggs was considerably lower and growth poorer in medium containing autoclaved SLE_{20}, suggesting that autoclaving caused considerable loss but not complete destruction of the activity of the liver extract (Table 20). This was interesting since autoclaved rat liver extracts prepared in water or Sørensen's phosphate buffer, and in which pH was not adjusted to 4.0-4.1 had been found totally ineffective (Table 16, Fig. 29). Dougherty and Calhoun (1948) also reported that water-prepared liver extracts were unaffected by repeated freezing and thawing, only slightly impaired by heating at 55°C for 30 min and completely destroyed by autoclaving at 15 p.s.i, for 15 minutes. It is believed that the inactivity of such liver extracts is due to the removal of the clotted mass of protein during preparation.

A fine suspension of particulate matter which gradually settled down to form a precipitate, was also observed on mixing fresh or frozen liver extract with Hanks' BSS or water. Filtration of this mixture as well as of that after elution with Sephadex column resulted in total loss of activity of the liver extract.

All these observations draw attention to the importance of liver extract as a whole and especially its protein precipitate. A considerable body of information on the properties of unheated, heated, and autoclaved liver extracts and its protein components has been provided by Dougherty and his co-workers (1948 et seq.) in connection with their investigations on the cultivation of the rhabditid nematode, Caenorhabditis briggsae. The heat-labile protein-linked growth factor in liver extract responsible for the growth and maturation of C.briggsae has been termed by Dougherty (1950, 1951) "factor Rb" which has now been isolated from liver and found to be a globulin yielding 12 amino acids on hydrolysis (Sayre et al, 1961; Buecher et al, 1966). It has been found by Buecher et al (1966) that this proteinaceous growth factor completely loses its activity when it is filtered in combination with a basal medium. Hansen(1967) has stated that the effectiveness of this protein (growth factor) appears to depend on interaction with the other components of the medium. These findings are in close agreement with those reported earlier in this work, that is, the appearance of a precipitate in the medium on addition of liver extract and loss of activity of liver extract after filtration in combination with Hanks' BSS or water.

Nicholas et al (1959) reported that Stoll's raw liver extract (it may be recalled that SLE_{20} was prepared according to

Stoll's technique) also contained 'factor Rb' and worked well with Caenorhabditis briggasae. One is therefore tempted to think that the growth factor in SLE_{20} required by H.nana is 'factor Rb' (the globulin isolated by Sayre et al, 1961) or some closely related globulin. It would be profitable to obtain this globulin from Dr E.L. Hansen (Clinical Pharmacology Research Institute, Berkeley, California 94704) and find out if it can replace SLE_{20}. Alternatively, a suitable method for the fractionation of SLE_{20} should be worked out and the nature of the active fraction determined by biochemical techniques.

The important thing to know is whether H.nana requires SLE_{20} as a whole or only its protein portion. Experiments suggest that H.nana does not grow if the precipitate is eliminated from the liver extract or if a precipitate does not appear in the culture medium. Hence it appears that the protein precipitate is essential. The appearance of the precipitate makes the medium diphasic comparable with one devised by Smyth (1967) for the strobilation and maturation of the protoscolices of Echinococcus granulosus. As suggested by Smyth (1967), this protein substrate may be nutritive, or stimulatory operating through some neurosecretory mechanism, or both. If it is nutritive, it is difficult to see how H.nana makes use of this protein without digesting it. This may be true for E.granulosus in which the scolex

secretes proteases (Smyth et al, 1966) which may be digesting the nutritive coagulated serum base and making nutriment available to the worm. Sensory receptors on the rostellar tip of E.granulosus may be responsible for the stimulation phenomenon, but to date, there is no evidence of such secretions or receptors in H.nana. Smyth (1967) has also suggested that H.nana does not require a solid nutritive base since it can develop to maturity in vitro in liquid media Berntzen, 1962). It may be pointed out that Berntzen's (1962) culture system cannot be termed monophasic since he provided a filter paper lining in his culture tubes and observed that the worms attached their scolices to the filter paper and flattened their strobilae against its surface. It is not unlikely that the worms benefited from the presence of a fine particulate protein precipitate which may have settled on the filter paper.

In tissue-culture the need of a small amount of serum is supposed to be a need for serum proteins which, it is believed, promote attachment, growth, and differentiation of cells and make up for deficiencies in media which are quantitatively and qualitatively minor (Waymouth, 1965). Some workers believe that proteins are careers of minor nutrients of low molecular weight. Experiments with isotopically labelled proteins have made it clear that the function of protein in cell,cultures is not to provide amino acids, but

the available evidence suggests "that growth promotion is due, not to protein as such, but to relatively small molecular weight components which are either liberated from or produced from protein in the course of its proteolytic degradation" (Eagle,1960, quoted by Eagle & Levintow, 1965).

It is also quite likely that liver extract, apart from providing unidentified nutrient factor(s), also corrects the imbalance of other necessary nutrients, or facilitates the assimilation of other nutrients by providing an absorbing surface and hence a source of concentrated nutriment. Whatever factor or factors it is that are provided by the liver extract, the establishment of the SLE_{20} medium and a simple technique for the cultivation of H.nana in vitro permits an experimental approach to the investigation of several physiological problems, for example,

(1) effect of oxygen tension in the gas phase,

(2) cultivation of H.nana in media containing extracts of liver and intestinal mucosa from immune mice or rats to show whether immunity developed by the definitive host against further infection is local or humoral,

(3) the mechanism of passage of nutrients.

Some other areas of future investigation are also quite apparent. The extent to which liver extract can dispense with other undefined components of the medium should be examined. Will H.nana grow if the precipitate is enclosed in a semi-

permeable tubing or if it is replaced by a non-nutritive (agar) base? A detailed study of the liver extract and the nature of its action of its protein precipitate will lead to rewarding results. Through such studies, new biochemical facts of far-reaching importance, about the growth requirements of cestodes may prove accessible.

E. Summary

1. Attempts to grow Hymenolepis nana using Berntzen's medium 102 and the continuous flow cultures apparatus failed. The ambiguities which made the exact duplication of Berntzen's technique difficult are pointed out.

2. A fresh approach to growing H.nana in vitro led to the development of a basal medium (BSSG + HS + YE) consisting of Hanks' BSS + glucose + yeast extract + horse serum. In this medium at 38± 0.5°C, and with a gas phase of 95% N_2 + 5% CO_2, worms strobilated and grew to about 3 mm in length in 12 days, but no genitalia were formed.

3. Great variation was found between yeast extracts obtained from different sources. Filter-sterilized 'Oxoid' yeast extract was found more effective than the autoclaved preparation.

4. Supplementation of the basal medium with proteose peptone, casein hydrolysate, amino acids mixture, or autoclaved water or buffer-prepared rat liver extracts failed to promote genital development of the worms.

5. The addition of 10% unheated rat liver extract (RLE_{20}), prepared according to Stoll's technique, supported growth and development of H.nana from cysticercoid stage to egg-bearing adult stage in 12 days.

6. Sheep liver extract (SLE_{20}) was found to be as good as rat liver extract.

7. Liver extract stored at -15°C retains its activity for long periods, whereas that stored at 4°C deteriorates after 3 weeks.

8. Liver extracts also loses its growth promoting properties when a precipitate which appears in it after storage at 4°C is eliminated or when it is filtered in combination with Hanks' BSS or water.

9. Liver extract is unaffected by heating at 53° for 6 min. There is some loss of activity if SLE_{20} is heated at 22°C at 48 hours, at 56° for 30 min and at 70° for 20 min. A considerable loss but not complete destruction occurs when liver extract is autoclaved at 15 p.s.i. for 15 min.

10. The <u>in vitro</u> technique for the cultivation of <u>H.nana</u> from cysticercoids to egg-producing adult involves artificial excystation of cysticercoids and their cultivation in roller tubes at 38±0.5°C at 5ml of "SLE_{20} medium" consisting of Hanks' BSS + antibiotics (100 units of penicillin and 100 g of streptomycin per ml) + 0.3% glucose + 0.5% filtered 'Oxoid' yeast extract + 10% unheated sheep liver extract + 30% horse serum, at pH 7.2± 0.2, and with a gas phase of 95% N_2 + 5% CO_2. The medium is prepared at the atart of an experiment, dispensed in suitable aliquots in screw-top bottles, gassed with 95% N_2 + 5% CO_2, and stored at 4°C. It is used for renewal on days 3,6,9,11 and 13 during a 14 day

culture period. Under these culture conditions, sperms are observed in worms as early as on day 8, and in very good cultures, worms start depositing eggs in the medium on day 10. By day 12, 20-30% of the worms show a series of gravid proglottids with fully formed eggs with which beetles (Tribolium confusum) can be infected. The cysticercoids obtained from such infected beetles are normal.

11. The importance of the use of CO_2 in the gas phase and also that ofthe protein precipitate appearing in the culture medium or in the liver extract on storage at $4^{o}C$, is discussed.

12. It is believed that the technique would permit investigation of several problems in tapeworm physiology. Some of these problems requiring immediate attention are suggested.

SECTION IV. EXCYSTATION OF CYSTICERCOIDS OF *HYMENOLEPIS NANA* IN VITRO

A. Introduction

The first requirement for the *in vitro* cultivation of *Hymenolopis nana* from the larval cysticercoid stage to egg-bearing adult is the successful excystation of the encysted larva *in vitro*. These cysticercoids, experimentally maintained in beetles, represent the infective stage in the life-cycle of the tapeworm and their establishment in the ileum of the definitive host is preceded by their ingestion, passage through the strongly acid stomach where the cyst wall is acted upon by gastric juice containing pepsin and HCl, and then their passage through the duodenum where the pH is near neutrality and where they are bathed in pancreatic juice and bile. Advantage is taken of this knowledge and cysticercoids are induced to excyst *in vitro* with the help of artificial excysting solutions under conditions known to exist in the alimentary tract. Several studies on the evagination or excystment of tapeworm larvae [Edgar, 1941; De Waele, 1934 (quoted by Read & Simmons, 1963); Read, 1955 (quoted by Read & Simmons, 1963); Rothman, 1959;

Sawada, 1959; Rycke & Grembergen, 1965,1966] have proved in cestodes maturing in warm-blooded hosts that intestinal secretions play an important role in these processes. At the same time, these studies have indicated that the larval forms react differently to the physico-chemical factors and these differential reactions are important determinants of whether or not a given host-parasite combination may be affected.

Rothman (1959) made a comparative study of the excystment of five species of cyclophyllidean tapeworms including Hymenolepis diminuta and H.nana. However, he could not pay special attention to the excystment of H.nana in such a large project of study. The potencies of enzymes which he used in his experiments were also not stated. Rothman made his observations on 10-15 larvae contained in 30-50 µl of excysting solutions and placed on a coverslip inverted on a welled slide. This technique was considered unsuitable for the aseptic excystation of a

large number of cysticercoids for subsequent *in vitro* cultivation. Further, he did not take into account the normal appearance and activity of the larvae after excystment.

Berntzen (1961) obtained excystment of the cysticercoids of *H. diminuta* in Tyrode's solution at 37°C at pH 7.8 and could induce rapid excystation by raising the pH from 7.8 to 8.0-9.0 by addition of 0.1N-NaOH or N-$NaHCO_3$. A preliminary test to test the suitability of this simple technique on the excystation of *H.diminuta* and *H.nana* failed. Berntzen (1962) used "pepsin-HCl solution" and "pancreatin-trypsin-bile salt solution" for the excystment of *H.nana* cysticercoids which were subsequently grown to egg-bearing adults *in vitro*. It may be recalled that attempts to reproduce his technique and results failed (see Section III). It could have been due to the fact that his method of excystation was not used as details of the concentrations of various ingredients in the excysting solutions were not stated.

In view of these considerations as well as of the report by Smyth and Haslewood (1963) that several bile salts have a lytic effect on the protoscolices of Echinococcus granulosus, it was decided to undertake a detailed study of excystment of H.nana in vitro so as to be able to establish a satisfactory method for obtaining active larvae for cultivation.

B. Material and Methods

The cysticercoids of H.nana were reared in the flour beetle, Tribolium confusum. The infected beetles were maintained at 25°C. The cysticercoids obtained from them were washed twice in Hanks' BSS and then treated with various excysting solutions. Excystation was performed in a bench incubator at 37°C. The chemicals used in this study were obtained from the British Drug Houses Ltd.

The effects of pepsin (1:2500), trypsin (83:1). pancreatin, sodium tauroglycocholate, sodium taurocholate, and cholic acid on the excystment of H.nana cysticercoids were studied. All solutions were prepared fresh on the day of use. Pepsin solution was prepared with Hanks' BSS and its pH was adjusted with 0.2N-HCl in such a way that the final solution would contain 1.0g pepsin/100 ml and would have a pH of 1.7. Trypsin, pancreatin, and bile-salt solutions were prepared separately as one per cent solution in Hanks' BSS , and their pH was adjusted to 7.0 with

0.2N-NaOH and then raised to 7.1-7.2 with 1.4% $NaHCO_3$ solution. Solutions of these substances of desired lower concentrations were obtained by further dilution with Hanks' BSS.

A one per cent solution of cholic acid (solubility in water 0.28g/l at 15°C) was prepared in the following way:

0.5g of cholic acid was weighed out and placed in a bottle. It was dissolved in a minimum of 0.2N-NaOH which was added drop by drop until the powder dissolved. The volume of the solution was then made up to 40 ml by addition of Hanks' BSS. The pH of the solution was brought down to 7.2 by addition of 0.2N-HCl and the volume was made up to 50 ml by addition of Hanks' BSS. A precipitate appeared in the form of flakes with the addition of acid in the solution but it dissolved on shaking.

Observations were made on 25-40 cysticercoids placed in 1.5-2.0 ml of the various test solutions contained in solid watch glasses.

The effect of a particular treatment was assessed by the time required for 90% excystation of the cysticercoids. The larvae were considered excysted when they were completely out of the cyst but not necessarily free from a small bit of cyst material adhering at the posterior end.

C. Results

(1) Effect of pepsin

After ingestion by the definitive host, the cysticercoid arrives in the stomach where gastric juice containing pepsin and HCl is secreted. The effect of pepsin-HCl on the excystment of cysticercoids was therefore, studied. Since it was shown by Rothman (1959) that pepsin had a maximum effect at a pH below 2.0, and a rapidly decreasing effect above pH 2.0, the pH of the pepsin solution was adjusted to 1.7. The rate of excystation in a trypsin-bile salt solution (o.5% trypsin + 0.3% sodium tauroglycocholate in Hanks' BSS, pH 7. 1-7.2, 37°C) gave a measure of the effect of pre-treatment with pepsin at different concentrations for different lengths of time. The cysticercoids were washed thrice in Hanks' BSS after pepsin treatment and then transferred to the trypsin-bile salt solution.

The results are recorded in Tables 23 and 24.

Table 23. The effect of pretreatment of cysticercoids of H.nana with 1% pepsin (pH 1.7, 37°C) on the subsequent excystation in trypsin-bile salt solution (0.5% trypsin + 0.3% sodium tauroglycocholate in Hanks' BSS, pH 7.1-7.2, 37°C).

Period of pepsin pretreatment (min)	0	15	20	30	45	60
Time (min) required for 90% excystation	45	10-12	8	6	6	6

Table 24. Excystation of H.nana cysticercoids in trypsin-bile salt solution (0.5% trypsin + 0.3% sodium tauroglycocholate in Hanks' BSS, pH 7.1-7.2, 37°C) after pretreatment for 30 min in different concentrations of pepsin (pH 1.7, 37°C).

Pepsin concentration	1%	0.5%	0.3%	0.1%
Time (min) required for 90% excystation	7	7	7	13

Observations

1. The cercomer portion of the cysticercoid was digested by pepsin pretreatment, and it broke off when the treated cysticercoids were washed in Hanks' BSS.
2. Pretreatment with pepsin enhanced considerably the excystment of cysticercoids in trypsin-bile salt solution.
3. There is a maximum time of pretreatment (20-30 min) beyond which further pepsin treatment had no further enhancing effects.
4. Exposure to pepsin solution for more than 30 min was deleterious. Some worms excysted in the pepsin solution and died.

(2) Effect of HCl

In view of the fact that HCl is a normal secretion of vertebrate stomach and an ingredient of the gastric juice and also that pretreatment with a pepsin-HCl solution at pH 1.7 (37°C) caused a rapid subsequent excystment in trypsin-bile salt solution, it seemd desirable to determine the effect of HCl alone. Since pepsin solution was dissolved in Hanks' BSS, it was also wished to know if Hanks' BSS had any effect.

Cysticercoids obtained from the same lot of infected beetles were divided into 4 groups of 30 each and allowed to excyst in a trypsin-bile salt solution (0.5% trypsin + 0.3% sodium tauroglycocholate in Hanks' BSS) after various types of pretreatment. Percentage of worms excysted at different periods of time after each type of pretreatment is recorded in

Table 25.

Group I. pretreated in 1% pepsin solution (pepsin-HCl, pH 1.7, 37°C)

Group II. pretreated in Hanks' BSS-HCl (pH 1.7, 37°C).

Group III. pretreated in Hanks' BSS (pH 7.1,37°C)

Group IV. larvae without any pretreatment (transferred straight to trypsin-bile salt solution).

Table 25. Effect of pretreatment with pepsin-HCl, Hanks' BSS-HCl, and Hanks' BSS on subsequent excystation of H.nana cysticercoids in trypsin-bile salt solution.

	% excysted at:							
Time (min)	5	8	10	15	20	30	40	45
Group I	66	94	100	-	-	-	-	-
Group II	x	x	33	60	100	-	-	-
Group III	x	x	x		23	80	90	100
Group IV	x	x	x	x	x	10	82	90

Observations

1. HCl, apart from providing a low pH to the pepsin solution, affects the cysts causing their rapid digestion in the trypsin-bile salt solution.
2. Pretreatment in Hanks' BSS alone (pH 7.1, 37°C) did not enhance the rate of excystment in trypsin-bile salt solution.
3. Excystation occurred in trypsin-bile salt solution without any pretreatment but it took a long time.
4. Pretreatment with pepsin-HCl resulted in a very rapid excystment in trypsin-bile salt solution.

3) Effect of trypsin, pancreatin, and bile salts, alone or in combination, with or without pretreatment.

Since excystation could occur in a trypsin-bile salt solution even without pepsin pretreatment, it was of interest to know whether either or the two, trypsin or bile salt, alone would induce excystment or whether both were essential. Was there any qualitative and quantative effect of the bile salts? It was also realised that when partly digested cysts reached the duodenum of the definitive host, they would be subjected to the action of bile and pancreatic juice, containing many other enzymes,

as well as trypsin. Treatment with pancreatin solution would therefore, be a more natural way of artificial excystation than with trypsin alone. The results showing the effects of trypsin, pancreatin, and 3 bile salts, namely, sodium tauroglycocholate, sodium taurocholate, and cholic acid are shown in Tables 26-31. The time recorded in each case is the time when 90% excystation was reached.

A preliminary test with sodium deoxycholate revealed it to be highly toxic to H.nana. At all concentrations of trypsin and sodium deoxycholate each between 0.2 and 0.5%, the worms excysted immediately and died almost instantaneously and completely dissolved. In 0.1% trypsin and all concentrations of sodium deoxycholate (0.1-0.5%) the majority of larvae died within the cyst before excystation. They soon became active and tended to force their way out but cyst dissolution was not rapid at 0.1% concentration of trypsin and they died within 15 min while trapped in the cyst.

Observations

In general, the results were in close agreement with those reported by Rothman (1959).

TABLE 26. Excystation of cysticercoids of H.nana in different combinations of trypsin and sodium tauroglycocholate (pH 7.1-7.2) after 30 min pretreatment with 1% pepsin solution (pH 1.7, 37°C).

Trypsin conc.(%)	Concentration of sodium tauroglycocholate (%)					
	0.5	0.4	0.3	0.2	0.1	0.0
	Time(min) required for 90% excystation					
0.5	4	4-5	7	8	25	40
0.4	4	4-5	7-8	9	26	42
0.3	5	5-6	8-9	9	26	52
0.2	6	5-6	10-11	12	28	76
0.1	6	7	16	16	35	115
0.0	15	23	35	42	-	0

TABLE 27. Effects of trypsin and sodium tauroglycocholate without pepsin pretreatment.

Trypsin conc.(%)	Concentration of sodium tauroglycocholate (%)				
	0.5	0.4	0.3	0.1	0.0
	Time (min) required for 90% excystation				
0.5	30-32	30-32	39-43	80-85	40% after 2 hrs.
0.4	30-32	33	45	80-85	40% after 2 hrs.
0.3	39	41-42	65-70	90-95	30% after 2 hrs.
0.2	55	59	80	105	25% after 2 hrs.
0.1	85	88-90	124	-	16% after 2 hrs.
0.0	only activation; no excystation				

TABLE 28. Effect of pancreatin and sodium tauroglycocholate at different concentrations after 30 min pre-treatment in 1% pepsin solution (pH 1.7, 37°C).

Pancreatin conc. (%)	Concentration of sodium tauroglycocholate (%) 0.5	0.3	0.2	0.1	0.0
	Time (min) required for 90% excystation				
0.5	5	5	8-10	28-30	none after one hour
0.3	10	15	15-16	35	-
0.1	16-18	18	20	42	-

No excystment was observed in pancreatin solution alone up to 60 min even after pepsin pretreatment for 30 min. Without pepsin pretreatment, worms were only activated within the cyst in all combinations of pancreatin and tauroglycocholate.

TABLE 29. Effect of trypsin and sodium taurocholate after 30 min pepsin pretreatment.

Trypsin conc. (%)	Concentration of sodium taurocholate (%) 0.5	0.3	0.2	0.1
	Time (min) required for 90% excystation			
0.5	6	7-8	15	25
0.3	6	8-10	18-20	32
0.1	15	16-18	24	35

TABLE 30. Effect of trypsin and cholic acid (pH 7.1-7.2, 37°C) after 30 min pretreatment with 1% pepsin solution (pH 1.7,37°C).

Trypsin conc. (%)	Concentration of cholic acid (%) 0.5	0.3	0.2	0.1
	Time (min) required for 90% excystation			
0.5	5	6	6-7	8
0.3	6-7	8	18-20	22-25
0.2	6-7	8-9	18-20	22-25
0.0	13-14	19-20	38-40	0

TABLE 31. Effect of trypsin and cholic acid without pepsin pretreatment.

Trypsin conc (%)	Concentration of cholic acid (%) 0.5	0.3	0.2	0.1
	Time (min) required for 90% excystation			
0.5	16	18	28-30	40
0.3	21	25-27	32-35	45
0.2	30-35	45-50	54-55	62
0.0	no excystation up to 1.1/2 hours.			

Effect of bile salts

1. Larvae were only activated if treated with bile salt alone; no excystment occurred.
2. Excystment occurred readily in a bile salt solution if the cysticercoids were pretreated with pepsin solution. However, pretreated larvae appeared to struggle hard to make their way out of the cyst in a bile salt solution and they did not appear to be in a good condition after excystation. Moreover, in the absence of trypsin, the larvae could never free themselves from adherent cyst material.

Effect of Trypsin

1. Trypsin alone had little effect on the cysticercoids.
2. Excystation did occur in trypsin solution alone if the cysts were pretreated with pepsin solution, but it took a long time and the larvae after emergence were blackish and not very active.
3. A few larvae excysted in trypsin solution after a long time even without any pepsin pretreatment but they appeared dark after excystation and did not stay alive.

Effect of trypsin or pancreatin + bile salts

I. The addition of a bile salt to trypsin solution had a

profound synergistic effect resulting in a rapid excystation of cysticercoids especially after pepsin pretreatment.

2. The rate of excystment increased with higher concentration of trypsin and bile salt up to a particular limit. But at high concentrations (for example, 0.5% of each), worms soon became dark and wrinkled, if they were not washed immediately two or three times with Hanks' BSS after excystation.
3. The addition of bile salt to trypsin solution had a marked effect on the digestion of the cyst. This quick effect was not observed with either substance if used alone.
4. Pancreatin at the same concentration as trypsin appeared to have a milder effect on excysted worms. Excysted worms stayed active for a relatively longer period in pancreatin + sodium tauroglycocholate solution.
5. Prolonged expsoure of the excysted worms in all combinations of trypsin and different bile salts made the worms dark and wrinkled. The worms remained normal and active for several hours if they were washed immediately after excystation in two to three changes of Hanks' BSS.

Discussion

The fact that physical and chemical factors induce the evagination and excystation of tapeworm larvae in the intestine of the definitive host, has been emphasised by several workers. Read (1955; quoted by Read & Simmons, 1963) studied the mechanism of excystation of cysticercoids of Hymenolepis diminuta and suggested 5 physiological effects of gut secretions, namely, the "priming effect" by pepsin, "irritability effect" and "protein alteration effect" by bile salts, "proteolytic effect" of trypsin, and a "temperature effect", since a temperature corresponding to that of the host was required for excystment. Rothman (1959) studied the excystment of three species of Hymenolepis (H.diminuta, H.nana and H.citelli) and observed that all of these required the presence of a proteolytic enzyme to dissolve their cyst and bile salts for excystment. The rate of excystment was enhanced considerably if the larvae were pretreated in a pepsin-HCl solution before their treatment with a trypsin-bile salt solution. The present study has confirmed these findings. Since pepsin solution consisted of three ingredients, pepsin, Hanks' BSS and HCl, it seemed

desirable to know what effect Hanks' BSS and HCl had on excystment of H.nana. Rothman (1959) studied the effect of HCl (pH 1.5, 37°C) and Krebs-Ringer-phosphate (KRP, pH 6.7, 37°C) on subsequent excystment of H.citelli and H.diminuta in a trypsin-bile salt solution. In both species, pretreatment in HCl resulted in a rapid excystment. Only H.citelli cysticercoids excysted readily in trypsin-bile salt solution after KRP pretreatment; H. diminuta cysticercoids were unaffected. A similar study with H.nana (Table, 25) showed that cysticercoids of H.nana were like those of H.diminuta excysting readily in trypsin-sodium tauroglycocholate solution after pretreatment in HCl (pH 1.7, 37°C) for 30 min and remaining unaffected by a pretreatment in Hanks' BSS (pH 7.1, 37°C) for 30 min.

Exposure to pepsin solution (pH 7.1,) for more than 30 min had a deleterious effect. Some larvae excysted and died. This indicates that an animal with highly acid conditions in the stomach and a long gastric evacuation time cannot be a potential host of H.nana.

Excystation occurred in trypsin or bile salt solution alone if the larvae were pretreated in pepsin-HCl solutinn.

Trypsin and bile salt together had a synergistic effect. The dissolution of the cyst was very rapid indicating that the bile salt produced some change in the cyst material which could then be acted upon by trypsin.

There did not seem to be any significant difference in the stimulating effects of sodium taurocholate, sodium tauroglycocholate and cholic acid after pepsin pretreatment. Without pretreatment, exystation was relatively quicker in trypsin + cholic acid than in trypsin + tauroglycocholate. Sodium deoxycholate was found to be highly toxic to the larvae. Prolonged exposure to trypsin + any of the other bile salts was deleterious to the excysted worms; they became dark and inactive if they were not washed with Hanks' BSS immediately after excystment. It may be pointed out that trypsin and bile salt used in this study were not biochemically pure. Smyth and Haslewood (1963) discovered the presence of deoxycholate as an impurity in several commercial samples of sodium taurocholate and glycocholate. Smyth (1962c) reported that sodium tauroglycocholate caused

lysis of the cuticle of the protoscolices of Echinococcus granulosus and accumulation of lipids within their cells. Smyth and Haslewood (1963) have further pointed out that salts manufactured from crude ox bile (for example, sodium tauroglycocholate) contain many other kinds of compounds. In view of these reports, it appears that the bad effect of the commercial bile salts on excysted larvae maybe due to the presence of traces of deoxycholic acid and its salts and probably some other toxic substances. The toxicity of deoxychoclate to H.nana larvae accounts for the complete absence of this tapeworm from rabbit and hare in which deoxycholic acid makes up the bulk of the bile acids, and lends further support to the view of Smyth and Haslewood (1963) that "the nature of bile in a host may act as a powerful selective agent in determining host specificity in intestinal parasites".

It has been experienced in cultivation of H.nana that worms which are damaged during excystation do not grow, but swell up and ultimately die in culture.

It is therefore, important to work with chemically pure substances and determine their effects on excystment and excysted larvae. The present study has suggested that use of crystalline trypsin and crystalline cholic acid at right concentrations should result in efficient excystment without damage to the larva.

Summary

1. The effects of pepsin, HCl, Hanks' BSS, trypsin, pancreatin, sodium tauroglycocholate, sodium taurocholate, cholic acid and sodium deoxycholate on the excystation of the cysticercoids of Hymenolepis nana was studied.
2. Pretreatment in a pepsin-HCl solution caused a rapid excystment of the larvae in a trypsin-bile salt solution. Exposure of cysticercoids to pepsin solution for more than 30 min was deleterious.
3. HCl (pH 1.7, 37°C) affected the cysts and caused their rapid digestion in the trypsin-bile salt solution.
4. Pretreatment in Hanks' BSS (pH 7.1, 37°C) did not enhance subsequent excystment in a trypsin-bile salt solution.
5. Larvae were only activated if treated with bile salt solution alone; no excystment occurred.
6. Excystment occurred readily in a bile salt solution if the cysticercoids were pretreated in pepsin HCl solution.
7. Trypsin alone had little effect on cysticercoids. Excystation occurred in trypsin solution alone if cysticercoids were pretreated with pepsin-HCl solution,

but it took a long time and the larvae after emergence were blackish and inactive.

8. The addition of a bile salt to a trypsin solution had a a synergistic effect resulting in quick digestion of the cyst and excystation of larvae especially after pepsin pretreatment. The rate of excystation appeared to depend upon the concentration of trypsin and bile salt up to a particular limit.

9. Pancreatin solution had a milder effect than a trypsin solution of the same concentration. Worms stayed active after excystment for a relatively longer period in pancreatin + sodium tauroglycocholate than in trypsin + sodium tauroglycocholate.

10. Prolonged exposure to trypsin-bile salt solution after excystment was deleterious to the larvae. They stayed active and normal for long periods if they were washed two or three times with Hanks' BSS after excystment.

11. Sodium deoxycholate was highly toxic.

12. It is believed that the commercial bile salts are biochemically impure and contain traces of deoxycholic acid and its salts, and this may be the cause of the deleterious effect of bile salts on larvae after excystment. The use of crystalline trypsin and crystalline cholic acid for safe and efficient <u>in vitro</u> excystation of the cysticercoids is suggested.

APPENDIX

TABLE I. Growth of Schistocephalus plerocercoids in BSSG+HS+YE in 8 days at 4°C.

Initial		Fresh wt.		Dry wt.		
wet wt.	dry wt.	day 8	% change	day 8	% change	% D.W./F.W.
6.2	1.1	8.4	35.4	1.6	45.5	19.0
9.2	1.7	12.8	39.1	2.3	35.3	17.9
9.3	1.7	12.1	30.1	2.2	29.4	18.1
10.8	2.0	14.0	29.6	2.8	40.0	20.0
12.6	2.3	15.8	25.3	3.1	34.7	19.6
13.4	2.6	17.9	33.5	3.4	30.7	19.0
13.7	2.6	16.6	21.1	3.3	26.9	19.8
17.5	3.5	20.9	19.4	4.2	20.0	20.0
25.0	5.7	29.0	16.0	6.8	19.3	23.4
28.9	6.9	31.8	10.0	8.0	15.9	25.1
38.9	10.2	42.9	10.2	11.7	14.7	27.2
40.0	10.5	43.4	8.5	11.7	11.4	26.2
50.4	14.1	54.2	7.5	15.8	12.0	29.1
59.6	17.4	64.7	8.5	18.5	6.3	28.5
65.2	19.3	71.3	9.3	21.5	11.3	30.1
78.3	23.7	82.5	5.3	25.8	8.8	31.2
83.2	25.3	88.5	6.3	27.0	6.7	30.5
90.1	27.5	98.2	8.9	30.1	9.4	30.6
93.8	28.8	96.7	3.0	29.7	4.1	30.7
102.8	31.6	107.2	4.2	32.9	3.9	29.3
111.0	34.4	118.3	6.5	36.4	5.8	30.7
136.1	42.3	142.2	4.4	44.4	5.4	31.2

Initial dry wt. calculated (see Text)
All weights shown in mgs.

TABLE II. Growth of Schistocephalus plerocercoids in BSSG+HS+YE in 8 days at 7°C.

Initial		Fresh wt.		Dry wt.		
wet wt.	dry wt.	day 8	% change	day 8	% change	% D.W./F.W.
10.2	1.9	14.6	43.1	2.9	52.6	19.8
11.0	2.0	17.2	56.3	3.1	55.0	18.0
11.5	2.1	16.3	41.7	3.0	42.8	18.4
12.1	2.3	17.5	44.6	3.5	52.1	20.0
12.9	2.4	17.3	34.1	3.2	33.3	18.4
15.4	3.0	18.0	16.8	3.7	23.3	20.5
15.5	3.0	18.2	17.4	3.8	26.6	20.8
16.7	3.3	21.3	27.5	4.3	30.3	20.1
20.2	4.3	22.9	13.4	5.2	20.9	22.7
22.6	4.9	27.5	21.6	6.4	30.6	23.2
24.6	5.6	29.2	18.6	6.7	19.6	22.9
32.2	7.9	38.4	19.2	9.2	16.4	23.9
35.3	9.0	42.7	20.9	10.8	20.0	25.2
49.7	13.8	55.1	10.8	15.4	11.5	27.9
66.3	19.7	72.6	9.5	22.1	12.1	30.4
70.6	21.1	75.8	7.3	22.6	7.1	29.8
81.4	24.7	88.0	8.1	26.3	6.4	29.8
82.4	25.1	96.4	17.0	26.9	7.1	27.9
84.9	25.8	90.6	6.7	28.2	9.3	31.1
88.0	26.9	95.7	8.7	28.9	7.0	30.1
93.0	28.5	102.0	9.6	31.0	8.7	30.3
94.3	29.0	101.2	7.3	30.9	6.5	30.5
96.7	29.7	103.2	6.7	31.5	6.0	30.5
127.5	39.6	137.1	7.5	42.1	6.3	30.7
145.4	45.4	157.0	7.9	48.4	6.6	30.8

Initial dry wt. calculated (see Text)

All weights shown in mgs.

TABLE III. Growth of Schistocephalus plerocercoids in BSSG+HS+YE in 8 days at 12°C.

Initial		Fresh wt.		Dry wt.		
wet wt.	dry wt.	day 8	% change	day 8	% change	% D.W./F.W.
7.5	1.4	13.2	76.0	2.6	85.7	19.6
8.1	1.5	12.0	48.1	2.4	60.0	20.0
9.3	1.7	15.3	39.7	3.0	76.4	19.6
10.0	1.9	15.7	57.0	3.1	63.1	19.7
13.6	2.5	19.5	43.7	4.1	64.0	21.0
15.0	2.8	21.3	42.0	4.1	46.4	19.2
18.5	3.8	25.9	40.0	5.6	47.3	21.6
20.8	4.4	26.6	27.8	6.2	40.0	23.3
25.0	5.7	34.7	38.8	8.2	43.8	23.6
35.0	8.9	43.5	24.2	11.4	28.0	26.2
36.5	9.4	46.9	27.1	12.3	30.8	26.2
38.8	10.1	52.5	35.3	13.0	28.7	24.7
43.4	11.7	53.7	23.7	15.0	28.2	27.9
49.8	13.9	61.9	24.2	17.4	25.1	28.1
50.8	14.3	58.6	15.3	17.3	20.9	29.5
53.8	15.3	69.0	28.2	19.6	28.1	28.4
56.2	16.2	63.7	13.3	19.2	18.5	30.1
57.2	16.5	68.2	19.2	20.1	21.8	29.4
72.1	21.6	87.5	21.3	24.6	13.8	28.1
73.0	21.9	82.2	12.6	24.5	11.8	29.8
83.3	25.4	94.2	13.0	28.7	12.9	30.4
94.1	28.8	107.6	14.3	32.7	13.5	30.4
95.0	29.2	105.0	10.5	32.1	9.9	30.5
144.7	45.1	154.7	6.9	48.3	7.09	31.2
150.9	47.0	173.7	15.1	50.9	8.2	29.3
190.1	59.9	208.6	9.7	63.7	6.3	30.5

Initial dry wt. calculated (see Text)

All weights shown in mgs.

TABLE IV. Growth of Schistocephalus plerocercoids in BSSG+HS+YE in 8 days at 17°C.

Initial		Fresh wt.		Dry wt.		
wet wt.	dry wt.	day 8	% change	day 8	% change	% D.W./F.W.
8.0	1.4	18.5	131.2	3.7	164.2	20.0
8.1	1.5	20.3	150.6	3.9	160.0	19.2
8.8	1.6	26.2	197.7	5.4	237.5	20.6
11.9	2.2	27.0	126.8	5.8	163.6	21.4
12.4	2.3	28.3	128.2	6.2	169.5	21.9
13.0	2.4	24.3	86.9	5.4	125.0	22.2
13.4	2.5	29.1	117.1	6.7	168.0	23.0
18.2	3.7	34.1	87.3	7.7	108.1	22.5
19.0	3.9	38.4	102.1	9.2	135.8	23.9
20.0	4.2	34.2	71.0	7.6	80.9	22.2
21.2	4.6	39.3	85.3	9.1	97.8	23.1
29.8	7.2	49.1	64.7	12.6	75.0	25.6
56.0	16.1	75.1	34.1	21.8	35.4	29.0
63.3	18.6	94.2	48.8	27.4	47.3	29.0
71.3	21.3	98.7	38.4	29.5	38.5	29.8
82.1	25.0	112.4	36.9	33.2	32.8	29.5
87.5	26.7	113.6	29.8	34.1	27.7	30.0
90.0	27.5	117.0	30.0	34.0	23.6	29.0
106.0	32.7	137.3	29.5	40.2	22.9	29.2
106.5	32.9	141.1	32.4	41.6	26.4	29.4
143.5	44.6	171.4	19.0	50.8	13.9	29.6
146.7	45.7	168.4	14.7	51.0	11.5	30.2
182.6	57.3	211.7	15.9	63.4	10.6	29.9

Initial dry wt. calculated (see Text)

All weights shown in mgs.

TABLE V. Growth of Schistocephalus plerocercoids in BSSG+HS+YE in 8 days at 23°C.

Initial		Fresh wt.		Dry wt.		
wet wt.	dry wt.	day 8	% change	day 8	% change	% D.W./F.W.
9.0	1.6	37.4	315.5	8.7	443.7	23.2
9.8	1.8	34.4	251.0	7.7	327.7	22.3
10.0	1.8	40.7	307.0	8.9	394.4	21.8
10.9	2.0	36.3	233.0	8.7	335.0	24.0
11.5	2.1	45.2	293.0	10.0	376.1	22.1
14.1	2.7	54.1	283.6	11.7	333.3	21.6
16.1	3.1	57.7	260.6	12.5	303.2	21.6
17.2	3.4	50.2	191.8	12.0	253.0	23.9
18.8	3.9	65.5	248.4	14.8	279.4	22.5
19.8	4.2	66.0	233.3	16.0	280.9	24.2
20.0	4.2	46.7	133.5	12.5	197.6	26.7
24.8	5.6	69.6	180.6	17.0	203.5	24.4
25.2	5.7	63.0	150.0	17.1	200.0	27.1
26.1	6.0	72.0	175.8	18.1	201.6	25.1
26.7	6.2	77.5	190.2	19.1	208.0	24.6
27.5	6.5	75.1	173.0	18.7	187.6	24.9
28.0	6.6	62.9	124.6	15.8	139.3	25.1
31.7	7.8	82.4	159.9	20.7	165.3	25.1
33.6	8.4	82.5	145.5	23.8	183.3	28.8
33.8	8.5	65.3	93.1	17.4	104.7	26.6
37.2	9.6	85.7	130.3	23.4	143.7	27.3
37.5	9.6	82.2	119.2	21.6	125.0	26.2
37.9	9.8	70.8	86.8	18.4	87.7	25.9
39.0	10.2	83.3	113.5	21.5	110.7	25.8
41.4	11.0	81.0	95.6	23.6	114.5	29.1
42.9	11.5	83.0	93.4	22.6	96.5	27.2
45.0	12.2	81.7	81.5	21.0	72.1	25.7
52.4	14.8	100.4	93.3	26.1	76.3	25.9
53.1	15.0	103.7	95.2	29.6	97.3	28.5
58.8	17.1	112.0	90.4	30.0	75.4	26.7
59.0	17.1	104.0	76.2	29.0	69.5	27.8
62.3	18.3	103.4	65.9	28.2	54.0	27.2

TABLE V. (contd.)

Initial		Fresh wt.		Dry wt.		
wet wt.	dry wt.	day 8	% change	day 8	% change	% D.W./F.W.
71.2	21.3	124.5	74.8	33.5	57.2	26.9
76.0	23.0	113.2	48.9	34.0	47.8	30.0
81.3	24.7	126.4	55.4	37.2	50.6	29.4
95.7	29.5	148.8	55.4	41.9	42.0	28.1
106.6	32.9	159.1	49.2	44.0	33.7	27.6
107.5	33.2	152.6	41.9	41.4	24.6	27.1
108.4	33.5	162.7	50.0	44.7	33.4	27.4
113.0	35.0	138.9	22.9	41.4	18.2	29.8
122.2	37.8	161.3	31.9	47.5	25.6	29.4
130.0	40.4	164.7	26.6	48.1	19.0	29.2
190.9	60.1	242.5	27.0	68.9	14.6	28.4
122.5	37.9	150.7	23.0	45.5	20.0	30.1

Initial dry wt. calculated (see Text)

All weights shown in mgs.

TABLE VI. Growth of Schistocephalus plerocercoids in BSSG+HS+YE in 8 days at 27°C.

Initial		Fresh wt.		Dry wt.		
wet wt.	dry wt.	day 8	% change	day 8	% change	% D.W./F.W.
9.4	1.7	39.1	315.9	8.7	411.7	22.2
10.9	2.0	53.8	393.5	11.3	465.0	21.0
11.0	2.0	46.6	323.6	10.3	415.0	22.1
20.6	4.4	65.0	215.5	16.1	263.6	24.7
21.4	4.6	64.9	203.2	15.7	241.3	24.1
31.8	7.8	81.6	156.6	21.3	173.0	26.1
44.2	11.9	86.5	95.7	23.3	95.7	26.9
67.0	19.8	116.2	73.4	32.4	63.6	27.8
86.9	26.5	125.6	44.5	35.3	33.2	28.1
87.2	26.6	137.0	57.1	37.0	39.0	27.0
91.6	28.1	139.7	52.5	39.8	41.6	28.4
93.0	28.5	121.9	31.0	35.0	22.9	28.7
97.2	29.9	145.9	50.1	40.1	34.1	27.4
123.0	38.1	158.5	28.8	45.7	19.9	28.8
128.0	39.6	163.6	27.8	46.5	17.4	28.4
132.5	41.2	166.4	25.5	46.5	12.8	27.9
136.8	42.6	173.3	26.6	49.8	16.9	28.7

Initial dry wt. calculated (see Text)
All weights shown in mgs.

TABLE VII. Growth of Schistocephalus plerocercoids in BSSG+HS+YE in 8 days at 30°C.

Initial		Fresh wt.		Dry wt.		
wet wt.	dry wt.	day 8	% change	day 8	% change	% D.W./F.W.
9.8	1.8	43.5	343.8	9.9	450.0	22.8
14.3	2.7	50.5	253.1	11.2	314.8	22.1
17.2	3.4	49.8	189.5	11.0	223.5	23.4
22.5	4.9	49.8	121.3	13.8	181.6	27.7
27.0	6.3	54.3	101.1	13.3	111.1	24.4
33.8	8.5	65.6	94.0	15.8	85.8	24.0
37.4	9.7	54.1	44.6	15.1	55.6	27.9
44.1	11.9	71.3	61.6	20.1	68.9	28.1
54.7	15.6	79.7	45.7	22.6	44.8	28.3
62.2	18.3	86.5	39.0	23.8	30.0	27.5
78.2	23.6	104.3	33.3	28.5	20.7	27.3
89.0	27.2	113.8	27.8	31.7	16.5	27.8
90.0	27.5	106.5	18.3	30.9	12.6	29.0
94.7	29.1	120.0	41.6	33.0	13.4	27.5
115.6	35.8	134.1	16.0	40.1	12.0	29.9

Initial dry wt. calculated (see Text)

All weights shown in mgs.

TABLE VIII. Growth of Schistocephalus plerocercoids in BSSG+HS+YE in 8 days at 33°C.

Initial		Fresh wt.		Dry wt.		
wet wt.	dry wt.	day 8	% change	day 8	% change	%D.W./F.W.
11.4	2.1	34.5	202.6	6.9	228.5	20.0
18.2	3.7	36.8	102.1	8.8	137.8	23.9
21.6	4.7	35.7	65.2	8.2	74.4	22.9
36.2	9.3	50.4	39.2	12.0	29.0	23.8
39.9	10.5	42.5	6.5	12.4	18.0	29.1
40.3	10.6	44.0	9.1	12.8	20.7	29.0
50.2	14.0	59.5	18.5	15.8	12.8	26.5
51.1	14.3	45.2	- 11.5	11.3	- 20.9	25.0
51.4	14.4	61.7	20.0	16.7	15.9	27.0
54.0	15.4	62.3	15.3	16.8	9.0	26.9
56.9	16.4	53.9	- 5.2	14.6	-10.9	27.0
57.3	16.5	46.5	- 18.8	12.4	-24.8	26.6
59.1	17.2	45.4	- 23.1	13.1	-23.8	28.8
78.8	23.8	60.8	- 22.8	16.2	-31.9	26.6
90.4	27.6	83.5	- 7.6	22.0	-20.2	26.3
91.7	28.1	84.0	- 8.3	22.0	-21.7	26.1
88.2	26.9	71.8	- 18.5	22.0	-18.2	30.6
106.5	32.9	100.2	- 5.9	27.7	-15.8	27.6
119.3	36.9	118.6	- 8.9	32.5	-11.9	29.9
124.2	38.5	110.0	- 11.4	27.9	-27.0	25.3

Initial dry wt. calculated (see Text)

All weights shown in mgs.

TABLE IX. Growth of Schistocephalus plerocercoids in BSSG+HS+YE in 8 days at 35°C.

Initial		Fresh wt.		Dry wt.		
wet wt.	dry wt.	day 8	% change	day 8	% change	%D.W./F.W.
7.8	1.4	15.2	94.8	3.0	114.2	19.7
8.0	1.5	15.5	93.7	3.1	106.6	20.0
9.0	1.7	15.8	75.5	3.3	94.1	20.8
9.2	1.7	14.0	55.5	2.9	70.5	20.7
12.0	2.2	16.1	34.1	3.3	50.0	20.4
14.0	2.6	18.2	30.0	3.8	46.1	20.8
17.9	3.6	20.9	16.7	4.5	25.0	21.5
21.0	4.5	23.6	12.3	5.3	17.7	22.4
22.4	5.0	23.1	3.1	5.1	2.0	22.0
22.5	5.0	23.3	3.5	5.5	10.0	23.6
23.0	5.1	24.2	5.2	5.2	1.9	21.4
28.5	6.8	25.8	- 9.4	6.6	- 2.9	25.5
32.3	8.0	28.3	-12.3	7.0	-12.5	24.7
34.8	8.8	28.4	-18.3	7.3	-17.0	25.7
58.0	16.8	46.3	-20.1	12.6	-25.0	27.2
60.2	17.6	46.7	-22.4	12.8	-27.2	27.4
63.7	18.9	41.4	-35.0	11.0	-41.7	26.5
70.5	21.1	47.6	-32.4	13.9	-34.1	29.2
87.6	26.8	60.2	-31.2	16.9	-36.9	28.0
125.4	38.8	85.1	-32.1	20.0	-48.4	23.5
134.5	41.8	84.4	-37.2	21.3	-49.0	25.2
143.8	44.8	99.0	-31.1	24.5	-45.3	24.7

Initial dry wt. calculated (see Text)

All weights shown in mgs.

TABLE X. Growth of Schistocephalus plerocercoids in BSSG+HS+YE in 8 days at 40°C.

Initial		Fresh wt.		Dry wt.		
wet wt.	dry wt.	day 8	% change	day 8	% change	% D.W./F.W.
8.3	1.15	10.6	27.7	2.0	33.3	18.8
11.3	2.1	13.5	19.4	2.5	19.0	18.5
13.2	2.4	14.3	8.3	2.7	12.5	18.8
18.3	3.7	12.1	- 33.8	2.6	- 29.7	21.4
19.9	4.2	17.3	- 8.0	3.4	-19.0	19.6
21.1	4.5	17.4	- 17.5	3.6	- 20.0	20.6
22.0	4.7	17.0	- 22.7	3.6	-23.4	21.1
33.0	8.2	21.9	- 33.6	5.9	- 28.0	26.9
41.0	10.9	29.2	- 28.7	6.3	- 42.2	21.5
45.4	12.3	25.6	- 43.6	7.0	- 43.0	27.3
50.0	14.0	35.4	- 29.2	8.3	- 40.7	23.4
50.9	14.3	34.7	- 31.8	8.5	- 40.5	24.4
61.6	18.0	39.3	- 36.2	9.9	- 45.0	25.1
84.6	25.8	49.3	- 41.7	10.9	- 57.7	22.1
113.2	35.0	55.0	- 51.4	12.2	- 65.1	22.1
122.1	37.8	77.5	- 36.5	17.5	- 53.7	22.5
142.7	44.5	70.1	- 50.8	15.9	- 64.2	22.6

Initial dry wt. calculated (see Text)

All weights shown in mgs.

TABLE XI. Growth of Schistocephalus plerocercoids in BSSG+HS+YE at 23°C after incubation at 35°C.

Initial		Fresh wt.			Dry wt.		
				% change		%	
wet wt.	dry wt.	day 4	day 8	day 5-8	day 8	change	% D.W./F.W.
Group I. Plerocercoids at 35°C for 8 days							
7.8	1.4	12.0	15.2	+26.7	3.0	114.2	19.7
9.0	1.7	12.8	15.8	+23.4	3.3	94.1	20.8
14.0	2.6	16.4	18.2	+11.0	3.8	46.1	20.8
22.4	5.0	20.8	23.1	+11.1	5.1	2.0	22.0
28.5	6.8	26.7	25.8	-3.4	6.6	-2.9	25.5
32.3	8.0	31.9	28.3	-11.0	7.0	-12.5	24.7
34.8	8.8	31.8	28.4	- 10.7	7.3	-17.0	25.7
58.0	16.8	53.5	46.3	-13.5	12.6	-25.0	27.2
60.2	17.6	54.2	46.7	-13.8	12.8	-27.2	27.4
63.7	18.9	44.2	41.4	- 6.3	11.0	-41.7	26.5
70.5	21.1	56.6	47.6	-15.9	13.9	-34.1	29.2
87.6	26.8	66.9	60.2	-10.0	16.9	-36.9	28.0
Group II. Plerocercoids at 23°C for 8 days							
11.5	2.1	27.1	47.2	74.2	10.0	376.1	21.1
16.1	3.1	33.7	57.7	71.2	12.5	303.2	21.6
18.8	3.9	40.5	65.5	61.7	14.8	279.4	22.5
19.8	4.2	41.1	66.0	60.6	16.0	280.9	24.2
24.8	5.6	50.8	69.6	37.0	17.0	203.5	24.4
26.7	6.2	54.5	77.5	42.2	19.1	208.0	24.6
31.7	7.8	56.7	82.4	45.3	20.7	165.3	25.1
37.2	9.6	70.5	85.7	21.6	23.4	143.7	27.3
39.0	10.2	65.1	83.3	28.0	21.5	110.7	25.8
71.2	21.3	104.0	124.5	19.7	33.5	57.2	26.9

(Table cotinued)

TABLE XI.(contd).

Initial		Fresh wt.			Dry wt.		
				% change		%	
wet wt.	dry wt.	day 4	day 8	day 5-8	day 8	change	%D.W./F.W.
			Group III. Plerocercoids at 23°C after 4 days at 35°C.				
9.0	1.7	12.9	22.6	75.2	5.0	194.1	22.1
10.1	1.9	13.6	22.0	61.8	4.2	121.0	19.0
15.9	3.1	21.2	28.6	34.9	6.2	100.0	21.6
34.4	8.7	30.1	39.0	29.6	10.3	18.3	26.4
35.6	9.1	28.5	40.1	40.7	10.8	18.6	26.9
37.8	9.8	35.7	42.2	18.2	11.3	15.3	26.7
49.7	13.9	47.0	56.7	20.6	15.2	9.3	26.8
52.5	14.9	44.1	54.2	22.9	13.7	- 8.0	25.2
57.1	16.5	49.2	63.8	29.7	16.9	2.4	26.4
60.2	17.6	46.3	56.9	22.9	15.0	-14.7	26.3
66.4	19.7	60.0	67.2	12.0	17.8	- 9.6	26.4
78.2	23.7	64.0	71.8	12.2	18.7	- 21.0	26.0
121.4	37.6	89.9	97.6	8.6	26.5	- 29.5	27.1

Initial dry wt. calculated (see Text)

All weights shown in mgs.

TABLE XII. Growth of Schistocephalus plerocercoids in BSSG+HS+YE supplemented with chick embryo extract (CEE_{50}), in 8 days at 23°C.

Initial wet wt.	Initial dry wt.	Fresh wt. day 8	Fresh wt. % change	Dry wt. day 8	Dry wt. % change	% D.W./F.W.
			BSSG+HS+YE (control)			
17.2	3.4	50.2	191.8	12.0	253.0	23.9
20.0	4.2	61.7	208.5	12.5	197.6	20.2
25.2	5.7	68.0	169.8	17.1	200.0	25.1
33.8	8.5	70.3	107.9	17.4	104.7	24.7
57.0	16.4	109.2	91.5	29.0	76.8	26.5
82.5	25.2	113.9	38.0	32.3	28.1	28.3
			BSSG+HS+YE+4% CEE_{50}			
14.5	2.7	54.6	376.5	12.1	348.1	22.0
25.5	5.8	70.9	178.0	15.9	174.1	22.4
40.6	10.7	84.7	108.6	22.8	113.0	26.9
50.8	14.2	87.3	71.8	23.8	67.6	27.2
86.5	26.5	130.1	50.3	38.6	45.6	29.6
105.0	32.4	139.3	32.6	40.6	25.3	29.1
			BSSG+HS+YE+8% CEE_{50}			
16.6	3.3	45.9	176.5	10.8	227.2	23.5
26.4	6.1	56.3	113.2	14.8	142.6	26.2
30.4	7.4	61.6	102.6	16.2	118.9	26.2
33.5	8.4	70.9	111.6	18.9	125.0	26.6
39.6	10.4	66.2	67.1	17.9	72.1	27.0
50.8	14.3	83.8	64.9	23.0	60.8	27.4
			BSSG+HS+YE+16% CEE_{50}			
6.1	1.1	26.5	334.4	4.5	309.0	16.9
23.4	5.2	52.6	124.7	13.8	165.3	26.2
29.6	7.1	69.3	134.1	18.3	157.7	26.4
32.4	8.0	59.5	83.6	15.0	87.5	25.2
68.9	20.5	101.2	46.8	27.9	36.0	27.5
70.4	21.0	110.1	56.3	30.4	44.7	27.6

Initial dry wt. calculated (see Text)
All weights shown in mgs.

TABLE XIII. Growth of Schistocephalus plerocercoids in in BSSG+HS+YE and BSSG+HS+YE+4% CEE_{50} between day 8 and 16 at 23°C.

Initial		Fresh wt.		Fresh wt.		
wet wt.	dry wt.	day 8	% change	day 16	% change day 8-16	% change expected
			BSSG+HS+YE (control)			
10.5	1.9	51.9	394.2	73.7	42.0	80
15.8	3.1	61.2	287.3	84.2	37.5	70
32.9	8.2	66.0	100.6	87.6	32.7	70
78.9	23.9	118.9	52.0	138.5	16.4	25
123.2	38.2	160.7	30.4	166.8	3.8	12
			BSSG+HS+YE+4% CEE_{50}			
15.6	3.0	64.5	313.4	85.9	33.1	70
26.2	6.0	102.2	290.7	122.0	19.3	30
54.0	15.4	95.5	76.8	118.3	23.8	40
86.5	26.5	130.1	50.3	140.8	8.2	20
105.0	32.4	139.3	32.6	145.0	4.0	15

Initial dry wt. calculated (see Text)

All weights shown in mgs.

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