SOME ASPECTS OF THE PHYSIOLOGY AND

CULTIVATION OF TAPEWORMS

A Thesis

Presented to

the Faculty of Graduate Studies and Research

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

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September 1955

ACKNOWLEDGMENTS

Abstract

Investigations were carried out with the tapeworm Hymenolepis nana variety fraterna (Stiles) to determine optimum conditions for survival in vitro. The intestinal bacteria coexisting with the tapeworm in the rat ileum and contaminating cestode cultures were identified. The influence of a group of antiseptics and antibiotics on H. nana was determined in vitro. Penicillin and streptomycin appeared to be the least toxic to the cestodes while maintaining sterile or oligoseptic cultures. Optimum conditions were determined as to oxygen concentration, temperature, hydrogen ion concentration, osmotic pressure and inorganic ions. The vitamin requirements of H. nana in vitro were found to include ascorbic acid, riboflavin and biotin. The most satisfactory culture medium, in which H. nana may be maintained for periods of fifteen days, was composed of Tyrode's solution, rabbit serum, six hundred units of penicillin and 0.2 milligrams of streptomycin sulfate.

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INTRODUCTION

It is intended that this thesis shall present an account of what is known of the physiology and cultivation of cestodes with special reference to <u>Hymenolepis nana</u>, a parasite of the intestine of rats, mice and man . In past experimental work on cestode cultivation, survival times were so short as to indicate an abnormal physiological condition. The problem of cultivation of parasitic helminths demands the establishment and maintenance of an aseptic or an oligoseptic medium and the provision of the appropriate food requirements of the animal, Wardle (1934). Smyth (1947) pointed out that intestinal parasites are rapidly overcome in artificial media by the growth and development of microflora carried over from the host's gut, and successful cultivation depends therefore, on the attainment and maintenance of aseptic conditions. The first step in studying the normal physiology is to attempt to maintain the parasite alive in vitro for the longest possible time, Stephenson (1947).

Attempts at the cultivation of nematodes and trematodes have met with considerable success. Nematodes are usually very resistant to unfavorable environmental conditions and it is possible to keep some parasitic species alive and active in the laboratory for periods ranging from a maximum of about one month in the case of intestinal parasites of warm blooded animals, to several months in the case of some blood and

tissue inhabiting forms. However, the comparatively short life of the worms under experimental conditions and the changes which occur in their behavior, metabolism and chemical composition are reminders that we can hardly regard most of the specimens as normal and healthy, Hobson (1948). Glaser (1931) cultivated Neoplectana glaseri from the Japanese beetle throughout its life history. Brand and Simpson (1944) have kept the larvae of Eustrongylides ignotus alive in vitro for as long as two and one half years, and report the case of a single male individual which developed to the adult stage.

Trematode cultivation has been marked by considerable success in both the immature and adult stages. Stunkard (1940) cultivated the metacercariae of Plagitura parva to sexual maturity in aseptic media. Ferguson (1939, 1940) developed the metacercariae of Posthodiplostomum minimum into adult worms producing non viable eggs. Hunter (1952) reared Gynaecotyla aduca for periods up to eight days in diluted sea water and added nutrients.

Attempts at the cultivation of cestodes outside their hosts have met with much less success. Wardle (1933, 1934, 1937a, 1937b) has discussed the problem in full, and together with Green (1941), succeeded in maintaining Hymenolepis nana for periods up to twenty days. Chandler (1943) demonstrated worms of Hymenolepis diminuta are able to obtain all necessary nitrogenous material and vitamins directly from the host.

However, they require carbohydrate of particular kinds in the host's diet, Chandler, Read and Nicholas (1950). Smyth (1946, 1947) successfully cultured Schistocephalus solidus and Ligula intestinalis under aseptic conditions and (1953) attained fertilization of S. solidus in vitro.

In the present investigation, a wide range of antiseptics and antibiotics were tested in the hope of obtaining satisfactory aseptic or oligoseptic conditions. In addition, a number of physical and organic factors were examined in order to establish the optimum levels for survival and incorporate the information so obtained in any future attempts at cultivation. The factors so examined, included osmotic pressure, pH, temperature, oxygen and carbon dioxide concentration, inorganic ions and the most satisfactory vitamin level. Cestode metabolism is discussed only in so far as it appears to have direct bearing on the cultivation studies at hand.

Survival times were taken as the period extending from commencement of cultivation to death in vitro. Two criteria of culture death were employed, cessation of movement and the loss of the translucence of the protoplasm.

CHAPTER I

MATERIALS AND METHODS

In order to guarantee an edequate supply of living cestodes the year round, a small tapeworm inhabiting the rat intestines was deemed the most suitable. The tapeworm chosen, _Hymenolepis_ _nana_ var. _fraterna_ (Stiles) is marked by a life cycle requiring but a single host. Originally described by Grassi (1885) and Grassi and Rovelli (1892), the one host account of the life cycle was confirmed by Joyeux (1920). Woodland (1924) proved conclusively that direct infection can take place without the intervention of any intermediate host.

Infection was maintained in albino and hooded laboratory rats through the feeding of eggs obtained by the centrifugal faecal flotation method, Lane (1923), Faust (1939), or more successfully by the feeding of six to twelve ripe proglottides. Oral infection by means of an eye dropper was followed by a period of twenty-four hours of starvation in order to prevent the loss of introduced eggs by rapid peristaltic action. All infected rats were fed solely on bread. Infected rats were sacrificed after twenty days so that the tapeworms employed in in cultivation experiments would be of approximately uniform size. Nevertheless considerable variation in size and condition was observed due

in part to the crowding effect, Read (1951) and in part to
the nature of H. nana itself, Baylis (1924), Shorb (1933).
The number of worms ranged from one to seventy-five and the
average per infested rat was eleven worms. Of the seven
hundred rats infected in the course of the investigation,
slightly more than 60% were found to be infected on post
mortem.

The infected rats were autopsied and the worms removed
from the portion of the ileum immediately anterior to the
caecum. H. nana was not found other than in the terminal ten
centimeters of the small intestine.

The worms were removed by cautiously slitting the
length of the intestine and washing it in warm Tyrode's
solution, Parker (1938). Tyrode's solution was sterilized by
Seitz filtration and used throughout the cultivation and
physiology experiments in preference to physiological saline.
Tapeworms were cultured in Tyrode's solution together with
the nutrients under investigation. Where possible, twenty
cultures were prepared at each concentration and the results
set down in the form of tables or graphs.

After some nine months, the project was hampered by a
severe shortage of tapeworms. Both the percentage of rats
infected and the number of worms per rat fell to one quarter
of the level at the commencement of the problem, suggesting
either a marked decrease in the viability of the worm or a
sudden increase in the resistance of the strain of rats being
used.

CHAPTER II

MICROFLORA IN VIVO AND IN VITRO

In the course of the present investigation attempts were made to identify the enteric organisms adherent to the cestodes under study. Control experiments were based in part on the results of the study of contaminated cultures and in part on bacterial cultures prepared directly from ileal contents of laboratory rats. The early view that intestinal bacteria are entirely harmful through the production of toxic substances during putrefactive metabolism is now open to question. On the other hand, considerable evidence now exists in support of the value of the intestinal microflora in supplying certain nutritional needs, particularly some vitamins. Read (1950) remarked on the complexity of the microbial ecology of the intestine and pointed out how imperfectly the relationship of host gut and bacteria is now understood. Needless to say, even less is known of the interrelationships between intestinal helminths and intestinal bacteria. Becker (1926) found human Dibothriocephalus produced marked changes in bacterial distribution, particularly Escherichia coli. The effect of the microflora on intestinal helminths is even more profound. Mitchell and Isbell (1942) found thiamine, riboflavin, nicotinic acid, pyridoxine, biotin, inositol and pteroylglutamic acid

produced by intestinal synthesis and all present in appreciable amounts. A variety of bacteria appear to be involved in both vitamin synthesis and destruction, Benesch (1945) and Johansson and Sarles (1949).

Although the greatest degree of vitamin synthesis occurs in the rat caecum, Taylor et al. (1942), Day et al. (1943) and Mitchell and Isbell (1942), there is nevertheless an extensive microflora in the other portions of the digestive system. Contrary to accepted opinion, Porter and Rettger (1940) demonstrated both the stomach and small intestine of the albino rat contain appreciable numbers of viable bacteria and yeasts. Gall et al. (1948a, 1948b) demonstrated the probable part played by the bacteria of the small intestine of the mouse in vitamin synthesis. Parasites inhabiting the small intestine may thus have access to vitamins and other products of bacterial metabolism in useful amounts.

Long before the present investigation, it had become apparent to those attempting helminth cultivation, Wardle (1933, 1934, 1937), Smyth (1947), Hobson (1948) that the primary consideration was the elimination or retardation of contaminating microorganisms. The first stages of the present inquiry were thus directed at the isolation and identification of the microflora of the rat ileum and the bacterial contaminants of cestode cultures. The results of the identification tests were subsequently confirmed by the

Bacteriological Laboratory, Department of Health and Public
Welfare, Manitoba. The combined findings of the
Bacteriological Laboratory and the author were summarized in
Table I.

An examination of certain contaminated cultures
suggested the value of some bacteria in providing the
nutritional needs of cestodes in vitro. Attempts at isolat-
ing these beneficial contaminants in pure cultures were
unsuccessful. Similarly Ells and Read (1952) found Turbatrix
aceti grew more rapidly in a medium contaminated with
unidentified organism from 'mother of vinegar' than in the
same medium free of bacteria. Epps et al. (1950) isolated
both gram-positive cocci and gram-negative rods from the
intestinal tract of Ascaris lumbricoides; they did not
determine however whether these bacteria were incidental or
symbiotic. McCoy (1929(succeeded in growing hookworm larvae
on pure cultures of bacteria, notably Escherichia coli and
Bacillus subtilis. Not all bacteria tested were found suit-
able for the growth and development of the larvae.
Investigation into the value of bacterial metabolic products
to cestodes in vitro was hampered by the lack of any
bacteriostatic agent capable of reducing bacterial growth to
a low level and thereby permitting cestodes access to the
bulk of the nutrients in the medium. Further, the ileal
contents yielded so great a variety of bacteria that

TABLE I

IDENTIFICATION OF MICROORGANISMS IN VIVO AND IN VITRO

Source	Organisms identified
Contaminated cestode cultures	Chromobacterium (yellow pigment group)
	gram positive bacilli (subtilis group)
	Streptococcus
	achromogenic forms of Pseudomonas
Ileal contents of rats infected with cestodes	Chromobacterium (yellow pigment group)
	gram positive bacilli (subtilis group)
	Streptococcus faecalis
	Proteus
Ileal contents of rats not infected with cestodes	Chromobacterium (yellow pigment group)
	gram positive bacilli (subtilis group)
	Streptococcus faecalis
	Proteus
	coliform bacilli
	coagulase negative staphylococci
	diphtheroids

investigation into specific bacteriostatic agents was considered impractical and attention was directed instead to the milder antiseptics and broad spectrum antibiotics.

CHAPTER III

THE EFFECT OF ANTISEPTICS AND ANTIBIOTICS ON HELMINTHS

Wardle (1934) reviewed the few published observations concerning the effect of antiseptic substances upon the viability of cestodes. Coutelen (1927) noted the death of *Echinococcus granulosus* within three minutes when exposed to mercuric chloride (1:1000) and to formol (1:200). Clapham (1932) found individuals of *Hymenolepis murina* died within five minutes when exposed to concentrations between 1:1000 and 1:20,000 of resorcinol. Wardle (1934) tested the influence of a wide range of antiseptics upon larval specimens of *Nybelinea surmenicola*. Survival times in double strength Locke's solution were adversely affected by all the bactericides tested. The least toxic solutions were formol (.01 per cent), iodine (.0001 percent) and thymol (.01 per cent). Thymol alone was completely effective in controlling the growth of microorganisms. Wardle and Green (1941) investigated several organometallic bactericides of the type used in sterilizing mucous membranes. These bactericides together with resorcinol, phenol, trikresol, and chloramine T were tested upon *Moniezia expansa* in concentrations ranging from 1.0 per cent to 0.0001 percent. Silver protein and chloramine T (0.1 per cent) appeared to be the most

satisfactory. Fairbairn and Reesal (1950) developed an elaborate method for obtaining sterile <u>Ascaris lumbricoides</u>, using sodium sulfathiazole, 1:250; neutral acriflavine, 1:5000; azochloramide 1:5000; dihydrostreptomycin sulphate 40 mg., in saline solution. Similarly, considerable success has been obtained by Glaser and Stoll (1938a, 1938b) using Labarraque's solution (2.6 per cent sodium hypochlorite) for sterilizing and unsheathing <u>Haemonchus</u> larvae in vitro. Milton solution (1.01 per cent sodium hypochlorite) was found to be equally effective and somewhat less toxic, Glaser and Stoll (1940).

Continuing the work of Wardle and Green (1941) a number of recently developed antiseptics were tested together with those found most useful in the past. The antiseptics were prepared in dilutions of .1 per cent; .01 per cent; .001 per cent; .0001 per cent; .00001 per cent. Ten individual worms were washed in each concentration for three minutes and cultured in sterile Tyrode's solution. Of the antiseptics tested, zephiran chloride, a mild disinfectant, was found least toxic in a concentration of .0001 per cent, (Figure 2) while maintaining sterile conditions. Alkylamine hydro-chloride is both a disinfectant and wetting agent. Tapeworms were found to be adversely affected in all concentrations, with maximum longevity at .0001 per cent, (Figure 2). Metaphen (nitromersol) is a highly efficient antiseptic for

skin sterilization, but was found to be quite toxic to H. nana in all concentrations. A solution of .00001 per cent metaphen (Figure 1) was the least toxic but resulted in some contaminated cultures. Merthiolate (Figure 3), chloramine T, (Figure 1) and silver protein all appear to be relatively more toxic to H. nana than to contaminating bacteria. Thus worms died even when washed in solutions so dilute as to be no longer antibacterial.

A sedimentation method for removing bacteria was developed by Clevland (1928) for protozoa and adapted for trematode cercariae by Ferguson (1930, 1943) and for nematodes by Glaser and Stoll (1938a). Wardle and Green (1931) applied the method successfully to Hymenolepis nana and maintained sterile cultures for periods up to twenty days. The worms are removed from the host and allowed to fall ten times in succession through columns of sterile Tyrode's solution. The method was tested during the course of the present investigation but sterile worms rarely resulted. Investigation was directed instead to the effect of antibiotics and other bacteria inhibitors on tapeworms in vitro.

Antibiotics are chemical substances which have been produced by various species of microorganisms and which tend to suppress the growth of other microorganisms, Goodman and Gilman (1955). Penicillin was first described in 1942, but only in recent years have supplies of this and other anti-
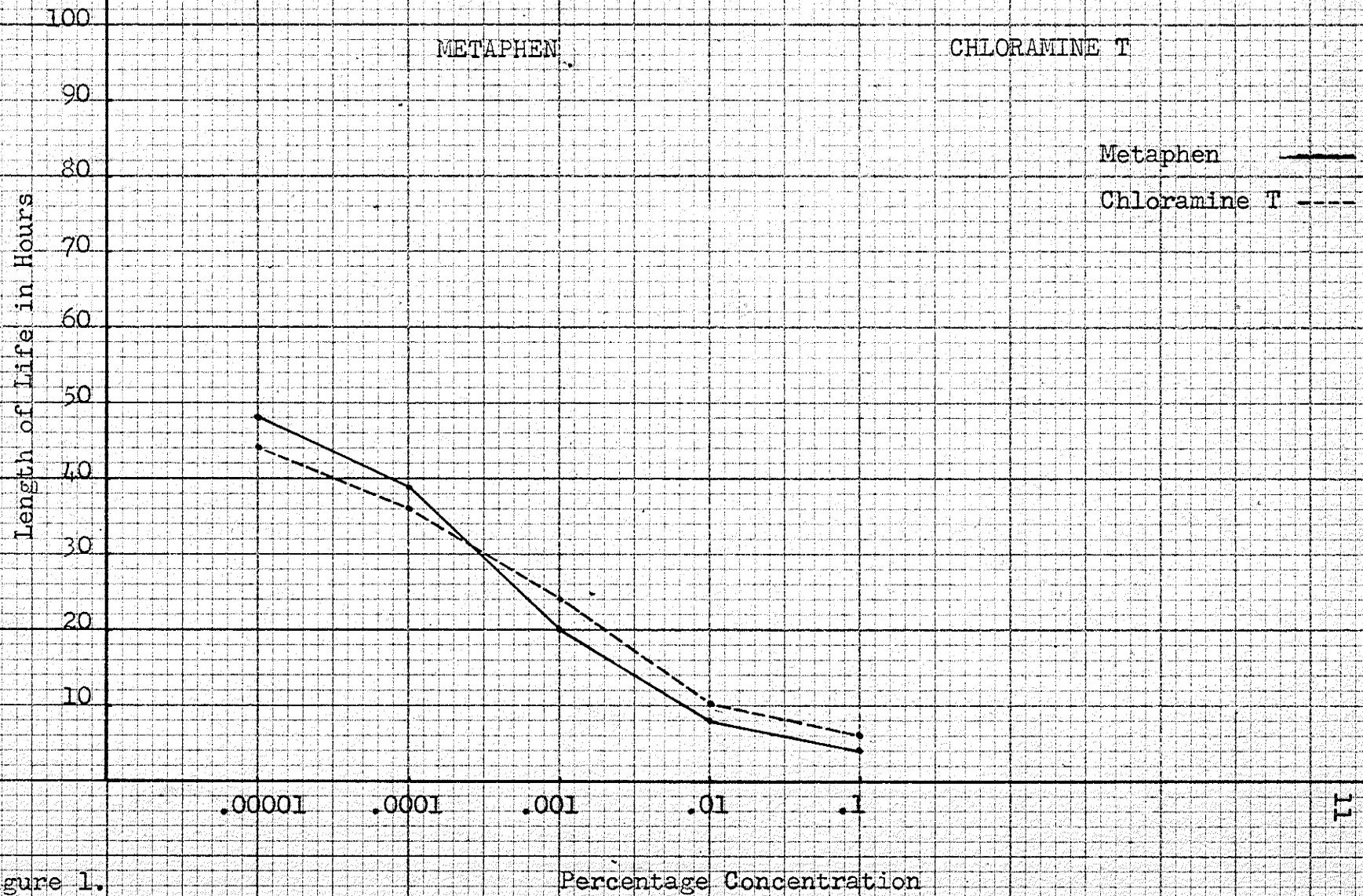
Figure 1.

biotics become available to parasitologists. Few published observations exist on the effect of antibiotics on helminth worms in vivo or in vitro. Spingarn and Edelman (1947) were able to prolong the viability of cultures of Endamoeba histolytica by the addition of streptomycin. Smyth (1950) used streptomycin (1:20,000) to inhibit the growth of micro- flora during the embryonation of Schistocephalus eggs. Using a similar method, Weinstein (1954) cultured Nippostrongylus muris and Necator americanus in various sterile media to which penicillin and streptomycin were added. Fairbairn (1950) improved the sterilization method of Fairbairn and Reesal(1950) by the omission of sulphatiazole and the addition of penicillin in the treatment of Ascaris. Epps et al. (1950) tested the effectiveness of penicillin, (1000 units per ml.); streptomycin, (500 ug per ml.); aureomycin, (200 ug per ml.); and bacitracin, (100 units per ml.). No single antibiotic removed all bacteria from Ascaris lumbricoides but effective sterilization of a significant number of worms could be obtained with combinations of antibiotics, notably penicillin, streptomycin and bacitracin. Ward (1951) found penicillin and streptomycin of some value in controlling microorganisms during in vitro experiments with Macracanthorhynchus.

High concentrations of some antibiotics appear to be toxic to helminths in situ. Wells (1951) found treatment with terramycin and aureomycin markedly reduced the number of

Aspicularis tetraptera in mice. Immature worms seem to be more susceptable than mature ones. Bacitracin is effective against young larvae but mature worms were not affected appreciably. Neomycin and dihydrostreptomycin apparently improve the environment so that treated animals retained a larger number of Aspicularis than did the control mice. Doys (1952) testing antibiotics and their effect on parasitism in swine found aureomycin had no effect on the larvae of Oesophagostomum longicaudum and Hyostronghlus rubidus. Hansen et al. (1954) concluded aureomycin in the diet of chickens reduced the number and size of Ascaridia galli in chickens. Delappe (1953) found penicillin and streptomycin, either singly or conjointly, facilitated the isolation and prolonged the survival of Histomonas meleagridis in vitro.

Penicillin is an antimicrobial agent prepared from cultures of Penicillium notatum. It is particularly effective in vitro against a number of pathogenic Gram-positive and Gram-negative cocci, such as staphylococci and streptococci. Some species of Gram-positive rods are affected, but penicillin appears to be ineffective against pathogenic Gram-negative bacilli. Of the four most useful natural penicillins (F, G, X, K), penicillin G is slightly superior.

The effect of penicillin G Potassium on Hymenolepis nana in Tyrode's solution is illustrated in Figure 4. Twenty cultures were prepared at each concentration (200 units, 400

units, 600 units, 800 units, 1000 units, 2000 units, 4000 units), using five milliliters of Tyrode's solution as the culture medium. As the curve indicates, H. nana appeared tolerant to a great variation in penicillin concentration. Maximum life in vitro was obtained at a concentration of six hundred units per culture, with worms living consistently to one hundred and ninety hours.

The effect of penicillin G Sodium on H. nana in vitro was illustrated in Figure 5. A wide range of tolerance was noted but the high survival times found with penicillin G Potassium could not be duplicated. Optimum longevity was noted at a concentration of six hundred units per culture, at which level H. nana survived in vitro for one hundred and twenty-nine hours.

Penicillin G procaine is a poorly soluble salt obtained from the interaction of penicillin G and procaine. Procaine penicillin maintains a uniform penicillin concentration over a prolonged period in vitro and for this reason was tested on H. nana. Ten cultures were prepared (Figure 6) at each concentration and the poor results obtained suggested further investigation was unwarranted. Survival times of seventy-three and seventy-five hours were observed at concentrations of two hundred and four hundred units respectively. The lower survival time in vitro is due in all probability to the presence of procaine and not to the maintained high level of penicillin in vitro.

PENICILLIN G POTASSIUM

Figure 4
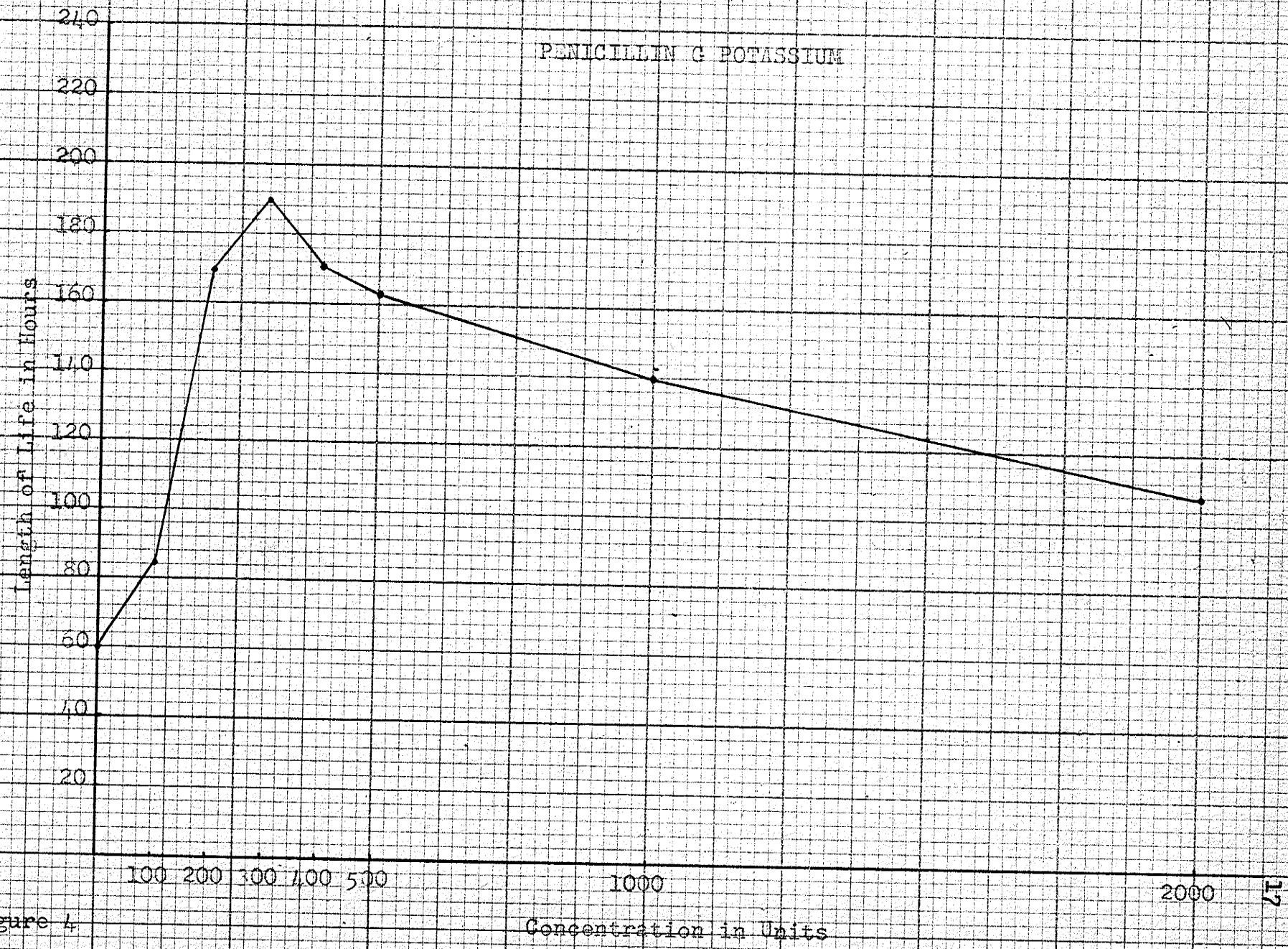
Length of Life in Hours — Concentration in Units

PENICILLIN G SODIUM

Length of Life in Hours (y-axis, marked 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240)

Concentration in Milligrams (x-axis, marked 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0)

Figure 5.

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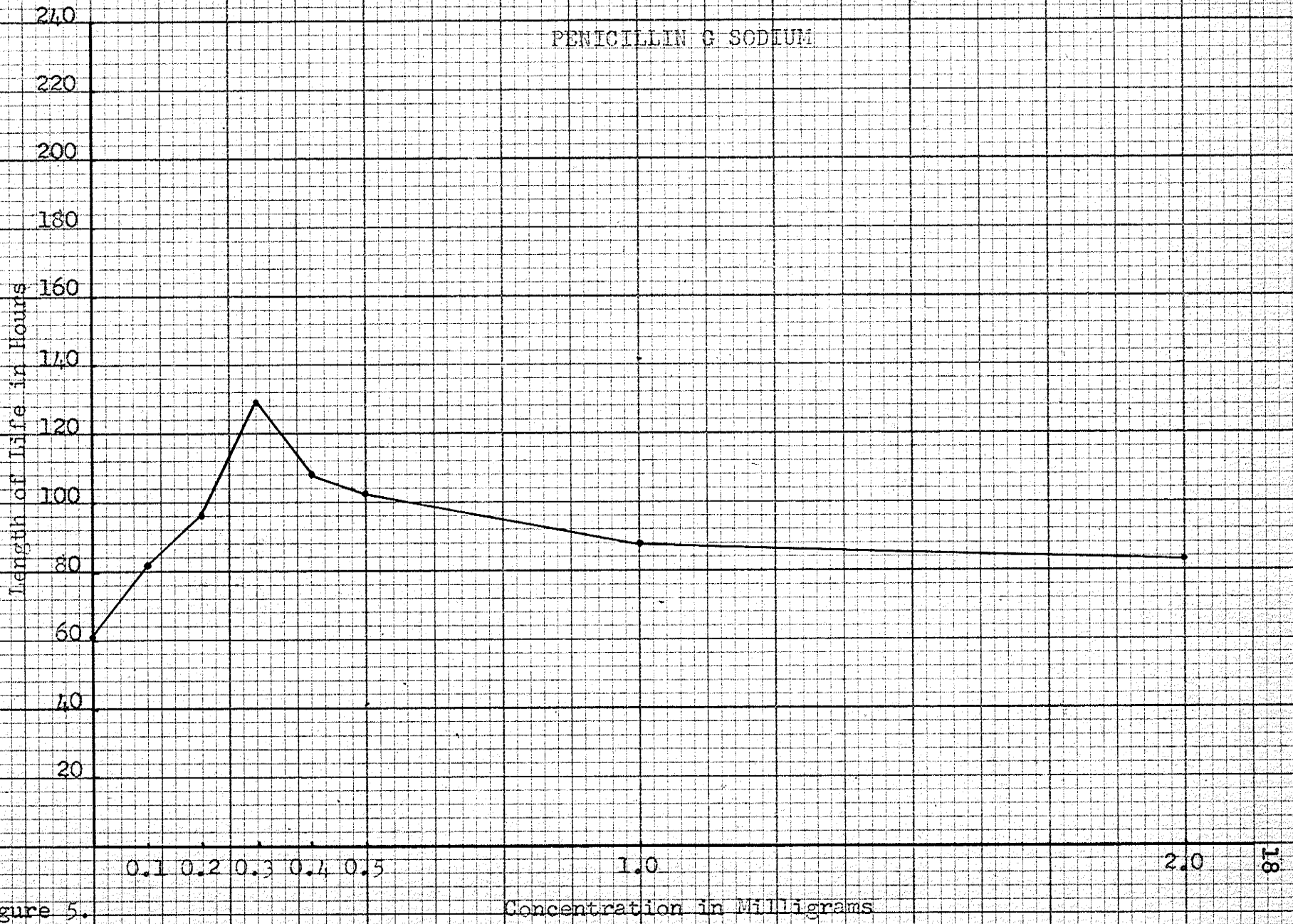
PENICILLIN G WITH PROCAINE

Length of Life in Hours
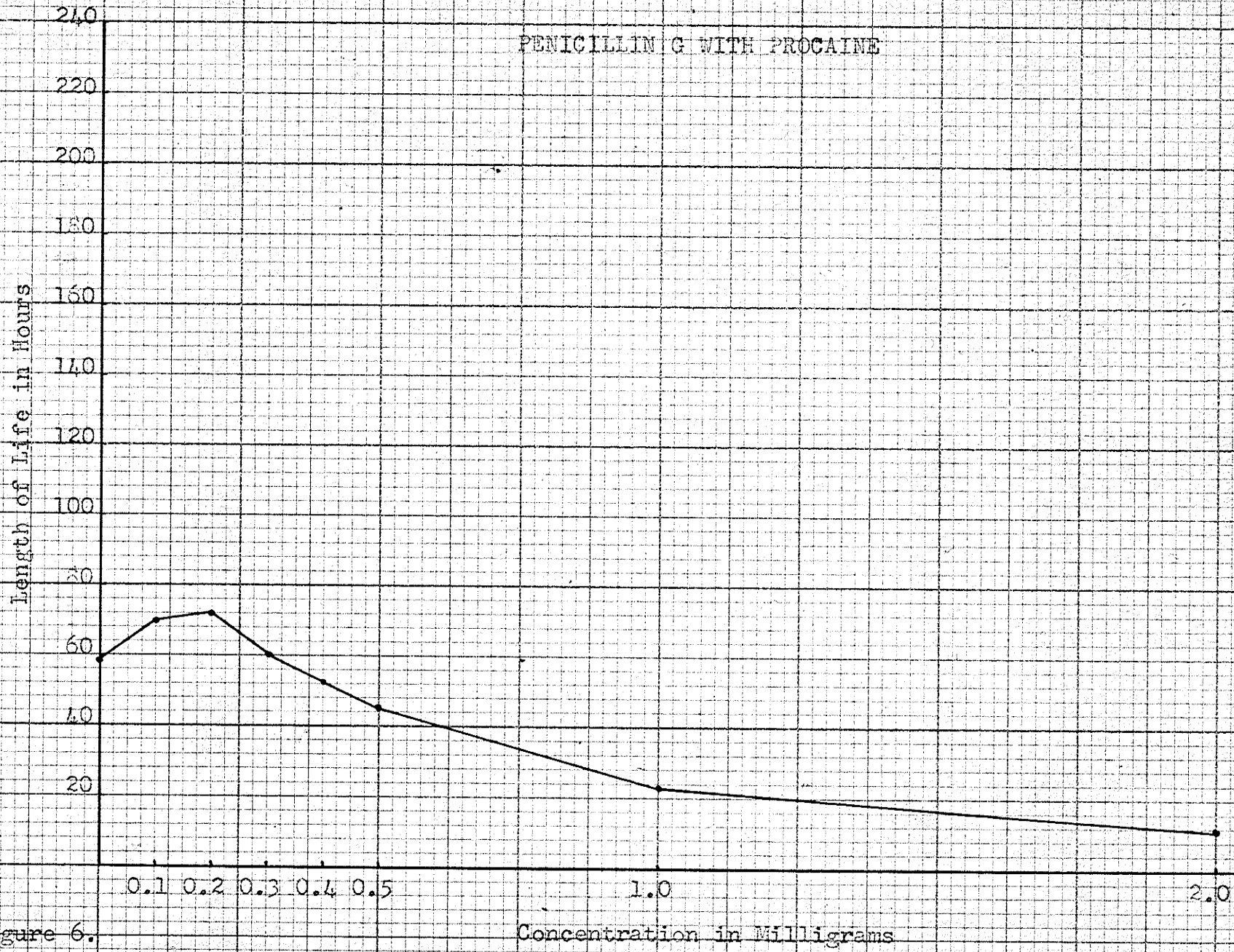
Concentration in Milligrams

Figure 6.

Streptomycin, (N-methyl-L-glucosaminidostreptosido-
streptidine), is an antibiotic prepared from the actinomycete
Streptomyces griseus. Streptomycin has a wide spectrum of
antibacterial activity and is particularly active against
gram-negative bacilli. It is moderately effective against
a variety of enteric organisms and common contaminants such
as Escherichia coli, Aerobacter aerogenes, Streptococcus
faecalis, Staphylococcus aureus, and Proteus vulgaris.
Streptomycin completely inhibits these organisms when present
in concentrations of ten to one hundred micrograms per milli-
liter, Goodman and Gilman (1955). Streptomycin was tested in
concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 1.0 and 2.0
milligrams per culture. Both streptomycin sulphate and di-
hydrostreptomycin were tried as antibacterial substances.
Preliminary tests indicated dihydrostreptomycin was consider-
ably more toxic and further use of it was postponed. Twenty
cultures were prepared at each concentration and the results
tabulated in Figure 7. Maximum longevity was obtained at a
concentration of 0.2 milligrams per five milliliter culture,
where H. nana could be maintained consistently in vitro for
one hundred and twenty hours. H. nana appeared exceedingly
tolerant to streptomycin, as ten times the optimum concentra-
tion, or 2.0 milligrams per culture, reduced the length of
life in vitro by only twenty-five per cent.

STREPTOMYCIN

Length of Life in Hours

Concentration in Milligrams

Figure 7.

The tetracyclines are a group of three derivatives of polycyclic naphthacenecarboxamide. The tetracyclines were found effective against a number of gram-negative and gram-positive cocci and bacilli. The spectrum of activity of these antibiotics overlaps that of penicillin and streptomycin and in addition they are effective against microorganisms unaffected by other agents, Goodman and Gilman (1955). All three antibiotics were tested in concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 1.0 and 2.0 milligrams per culture. Each culture contained five milliliters of Tyrode's solution and a single specimen of H. nana. Twenty cultures were prepared at each concentration.

Terramycin (oxytetracycline) is a product of the actinomycete Streptomyces rimosus. Used in the sterilization of H. nana in vitro (Figure 8), it proved extremely toxic in all concentrations. Maximum survival for tapeworms was found to be thirty-nine hours at a concentration of 0.1 milligrams per five milliliter culture.

Aureomycin (chlortetracycline) is obtained from the actinomycete Streptomyces aureofaciens. In vitro (Figure 9) aureomycin appeared less toxic than terramycin and the length of life of H. nana was extended by one quarter where 0.1 milligram was added per culture. Higher concentrations, however, were quite toxic and resulted in rapid death. Maximum longevity of seventy-seven hours was observed at the

TERRAMYCIN

Length of Life in Hours

240
220
200
180
160
140
120
100
80
60
40
20

0.1 0.2 0.3 0.4 0.5   1.0   2.0

Concentration in Milligrams

Figure 8.

23
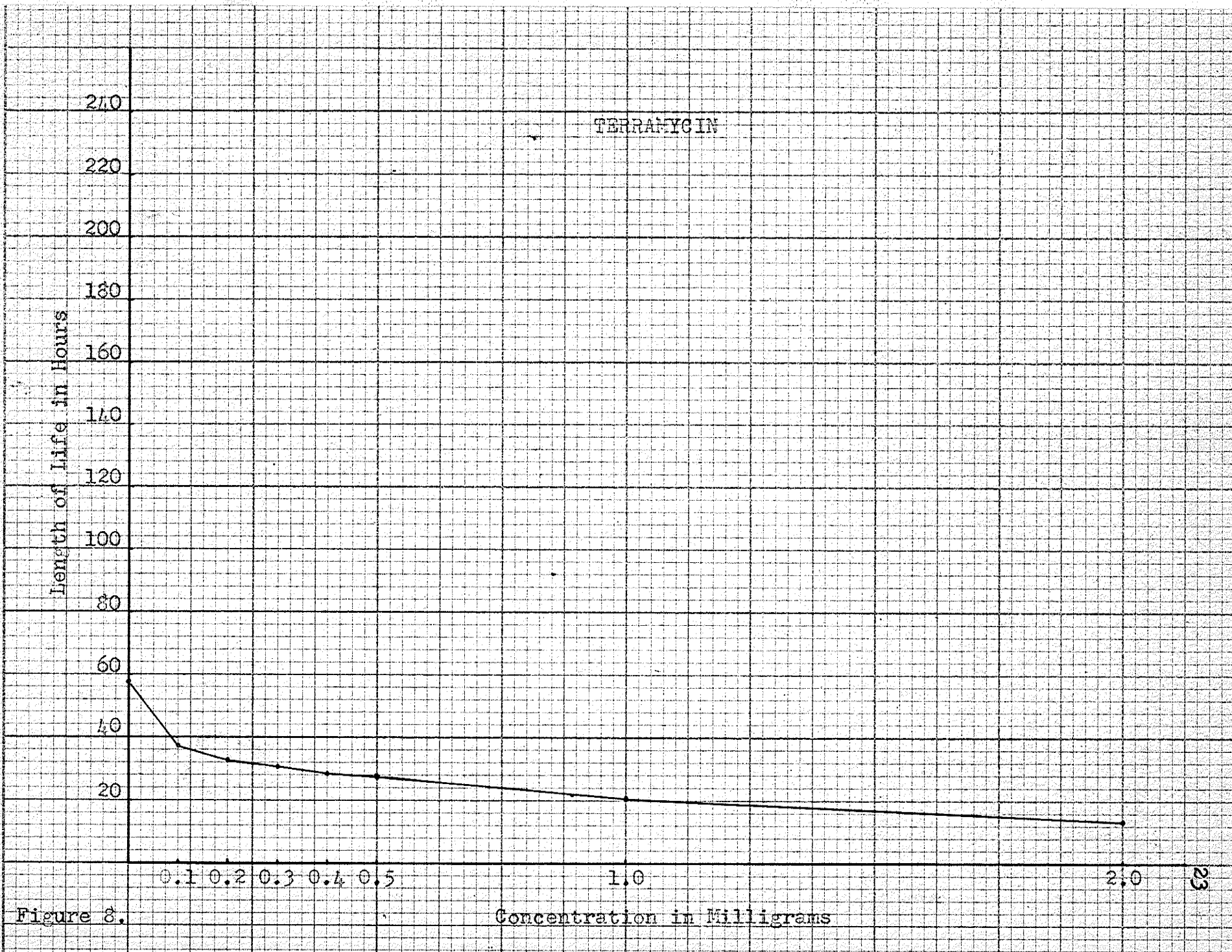
lowest concentration, 0.1 milligrams per culture.

Achromycin (tetracycline) is a semisynthetic antibiotic produced by the hydrogenation of aureomycin. In vitro (Figure 10) achromycin proved to be markedly less toxic than the other tetracyclines. H. nana survived ninety-nine hours at a concentration of 0.3 milligrams per culture. Tapeworms under cultivation showed a wider range of tolerance than with either terramycin or aureomycin.

Chloromycetin (chloramphenicol) is a crystalline antibiotic obtained from Streptomyces venezuelae. It is marked by a moderately broad spectrum of antimicrobial activity, inhibiting many gram-negative bacteria, including E. coli, A. aerogenes and certain strains of Proteus. In vitro (Figure 11) chloromycetin prolonged the life of H. nana in low concentrations but proved toxic in higher concentrations. Maximum survival time was eighty four hours in cultures containing 0.2 milligrams per five milliliters of Tyrode's solution.

Figure 10.

ACHROMYCIN

Length of Life in Hours

Concentration in Milligrams

CHAPTER IV

AEROBIC AND ANAEROBIC CULTIVATION

Parasitologists have debated for many years the
nature of the metabolism of intestinal parasites. As early
as 1883, Tappeiner had analysed intestinal contents and
reported no oxygen present in the gut of sheep. Fries (1906)
noted 0.7 to 1.3 per cent of oxygen in human rectal gases.
Von Brand and Weise (1932) did a series of Van Slyke measure-
ments of the bile and gut contents of hogs, sheep, cattle,
and dogs and found the maximum content of bile to be only
0.084 per cent by volume. The gut was found to be absolutely
free of oxygen. Von Brand (1945) noted the probability of
higher oxygen tensions near the mucosa. Bergeim et al.
(1945) reported an oxygen tension of 8 to 30 mm. of Hg in
the paramucosal lumen, but suggested the oxidation-reduction
potential of this region may actually be close to zero.
Rogers (1949) measured the oxygen tension in the contents and
on the mucosa in rats and found the oxygen tension ranged
from 7.9 to 30.2 mm. of Hg. He noted that worms living near
the mucosa have access to larger amounts of oxygen than
analysis of the intestinal contents would suggest.

The utilization of oxygen by helminths has received
wide attention from experimental parasitologists. Alt and
Tischer (1931) recorded the consumption of oxygen by

Moniezia expansa under varying oxygen tensions and observed the same acid formation in the presence or absence of oxygen. Working with _Eustrongylides_, von Brand (1938) noted glycogen consumption under aerobic and anaerobic conditions indicated the metabolism resembled free living forms more than that of intestinal parasites. Anaerobic metabolism consumed three times as much glycogen as aerobic, suggesting much lower efficiency. These results confirmed von Brand's (1934) work on _Ascaris_, in which aerobic metabolism required one-quarter less glycogen than anaerobic. Rogers (1948) determined the oxygen uptake of a number of parasitic nematodes, including _Nippostrongylus muris_, _Ascaridia galli_ and _Nematodirus_. Ward (1952) measured glycogen storage in _Acanthocephala_ under aerobic and anaerobic conditions and noted higher glycogen levels in worms kept under aerobic conditions. Laser (1944) found in the oxidative metabolism of _Ascaris suis_, oxygen uptake is dependent on oxygen tension. Worms in an atmosphere of pure oxygen died fairly quickly, probably due to the formation of an excess of hydrogen peroxide. Continuing the work on oxygen utilization by _Eustrongylides ignotus_, von Brand and Simpson (1944) measured oxygen consumption and oxygen requirements. He found oxygen consumed greatly exceeded that required for the oxidation of the total carbohydrates consumed. In a following report, von Brand and Simpson (1945) reported _Eustrongylides_ are not damaged by low

oxygen tensions but do asphyxiate in the complete absence of oxygen. Average survival times under anaerobic conditions was eighteen days, under aerobic conditions ninety-eight days. Stephenson (1947) noted similar results with the adult liver fluke Fasciola hepatica in vitro. Aerobic cultures survived forty-five hours whereas under anaerobic conditions the flukes lived an average of thirty-seven hours. The results indicated a reduction in oxygen tension produced a reduction in survival times.

Hobson (1948) discussed the published observations on oxygen tension and the effect of aerobic and anaerobic conditions on nematode parasites. He concluded that in the majority of the small number of species examined to date oxygen under experimental conditions is more beneficial than its complete absence. The optimum oxygen tension has not yet been determined for any parasitic worm, so that the exact importance of oxygen is still unknown.

Smyth (1950) noted higher oxygen tensions adversely affected Schistocephalus solidus in vitro. A browning effect along the edges was noted together with the emission of strings of brown yolky material from the uterine pores instead of eggs. Read (1952) found previously starved Hymenolepis diminuta store glycogen if glucose is made available. He noted greater amounts of polysaccharides are stored aerobically than anaerobically, indicating a possible

aerobic metabolism. Fairbairn (1944) found Heterakis may make efficient use of metabolically produced carbon dioxide. Hunter and Vernberg (1955) determined the rate of oxygen consumption for the various stages in the life cycle of Gynaecotyla adunca and found a high of 0.290 microliters of oxygen per hour per cubic millimeter at 30.4° C.

The present investigation included a study of the effect of aerobic and anaerobic conditions on H. nana in vitro. Five cultures were prepared with sterile Tyrode's solution and aerated intermittently by a stream of warm, sterile air. Five cultures were prepared in a similar manner and oxygen slowly bubbled through the cultures at a uniform rate. Five cultures were prepared with sterile Tyrode's solution and nitrogen gas bubbled through the medium before and during the life of the cestode. Finally, five cultures were prepared with Tyrode's solution and a single H. nana worm and the entire culture was then placed in a chamber where alkaline pyrogallate solution removed all oxygen.

Aerobic cultures lived seventy-six hours, oxygenated cultures lived seventy-one hours, (Figure 12). No significance was attached to the differences in survival times of aerated and oxygenated cultures. Cultures prepared with nitrogen lived an average of twenty hours, whereas anaerobic cultures in alkaline pyrogallate solution survived only nine hours.

AEROBIC AND ANAEROBIC CULTURES

Length of Life in Hours

Aeration   Oxygenation   Nitrogenation   Alkaline-pyrogallate deoxygenation

AEROBIC   ANAEROBIC
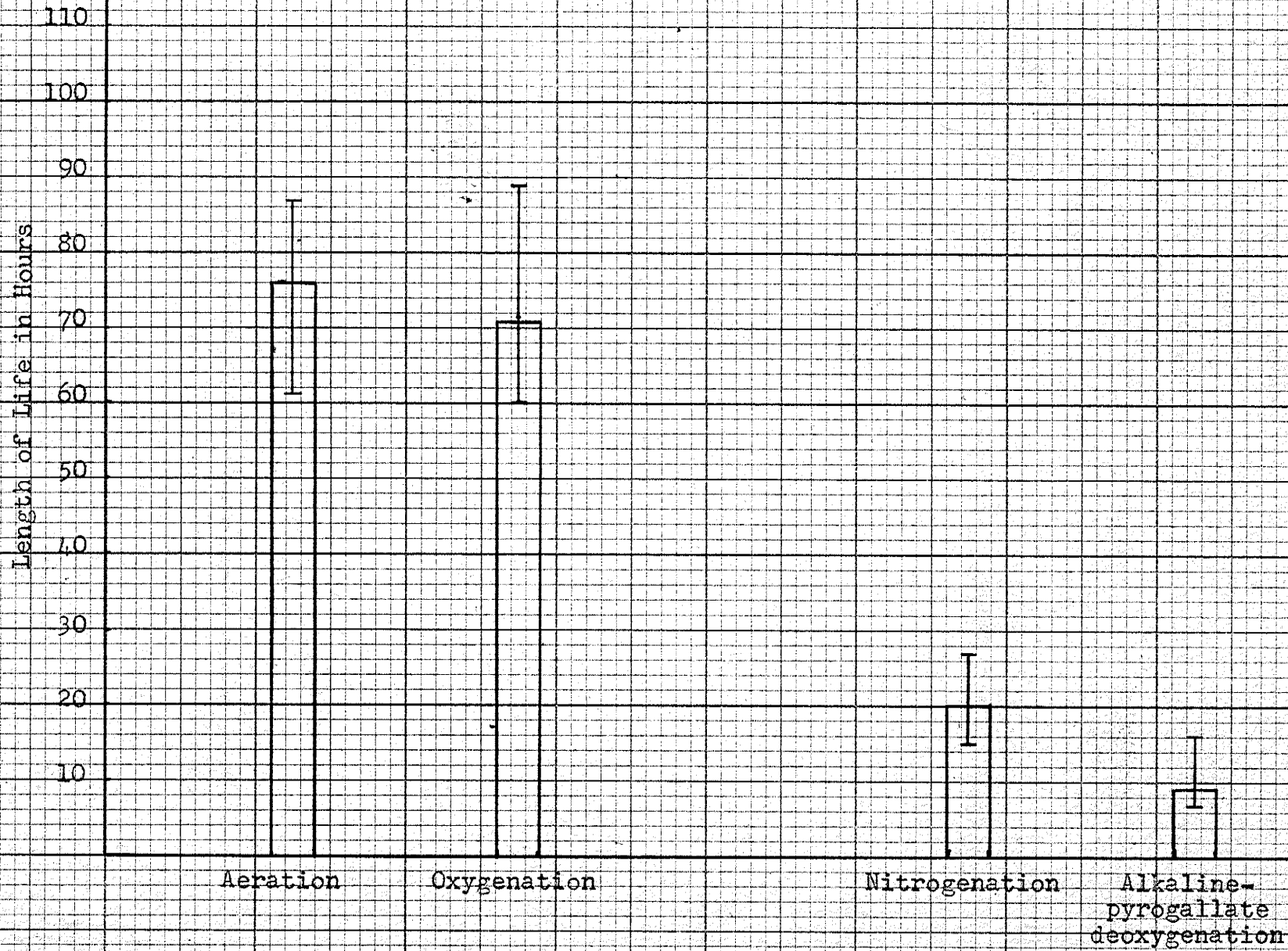
Figure 12.

The variation in survival times suggested a slight amount of oxygen present in nitrogen cultures, which prolonged life in vitro. The results showed $\underline{H}$. $\underline{nana}$ died rapidly in the total absence of oxygen and on the average lived four times as long in cultures where the oxygen tension is relatively high.

CHAPTER V

THE INFLUENCE OF TEMPERATURE, pH, OSMOTIC
PRESSURE AND INORGANIC IONS

Little information has been published to date on the
effect of temperature on parasitic helminths, in situ or in
vitro. Smyth (1946) observed Shistocephalus solidus survived
longer in vitro at temperatures below 40° C., but showed no
signs of undergoing spermatogenesis or oogenesis.
Plerocercoids cultured at 40° C., the body temperature of the
final host, developed in the normal manner and resulted in
oviposition. Von Brand and Simpson (1944) kept Eustrongylides
ignotus alive for two and one-half years at 20° C., much
longer than they would survive at 37° C., their normal
temperature. Von Brand and Simpson presented evidence to
show the metabolism differs at the two temperatures.
Stephenson (1947) observed Fasciola hepatica survived longest
at temperatures below the body temperature of the host. The
flukes in vitro survived considerably longer at 36° C. than
at the normal temperature of 38° C.

H. nana was cultured in Tyrode's solution at
temperatures of 15° C. to 48.5° C. Worms survived an average
of sixty hours at 38.5° C., the body temperature of the
laboratory rat. Survival times were shorter at all
temperatures above 38.5° C., although an increase of less

than 5° C. appears to have little effect, (Figure 13). At lower temperatures, the evidence is less conclusive. Tapeworms at 33.5° C. lived just as long as those cultured at 38.5° C. Several of the cultures prepared at 29.5° C. and 33.5° C. lived longer than sixty hours but the average of the five cultures prepared at each temperature did not exceed sixty hours. A more thorough investigation may well show H. nana will survive longer in vitro at lower than normal temperatures. H. nana was found markedly insensitive to slight temperature variation.

The vertebrate gut has been investigated by physiologists and parasitologists alike, but little information has been published on the physiological interrelationships between host and parasite. Abrahamson and Miller (1925) determined the hydrogen ion concentration of the gastro-intestinal tract of the albino rat. They found a stomach pH of 3.8, upper small intestine 5.8 and lower small intestine 6.0. Redman et al. (1927) found somewhat less acid values, stomach pH 4.0, duodenum 5.5, jejunum 6.0 and ileum 6.8. Rats fed on a low fat rachitogenic diet developed an alkaline reaction throughout the intestinal tract. Thus worms such as H. nana would probably experience a pH of about 6.8 or neutrality in the terminal portion of the rat ileum.

Grayzel and Miller (1927) found the hydrogen ion concentration of the intestinal contents of dogs very nearly
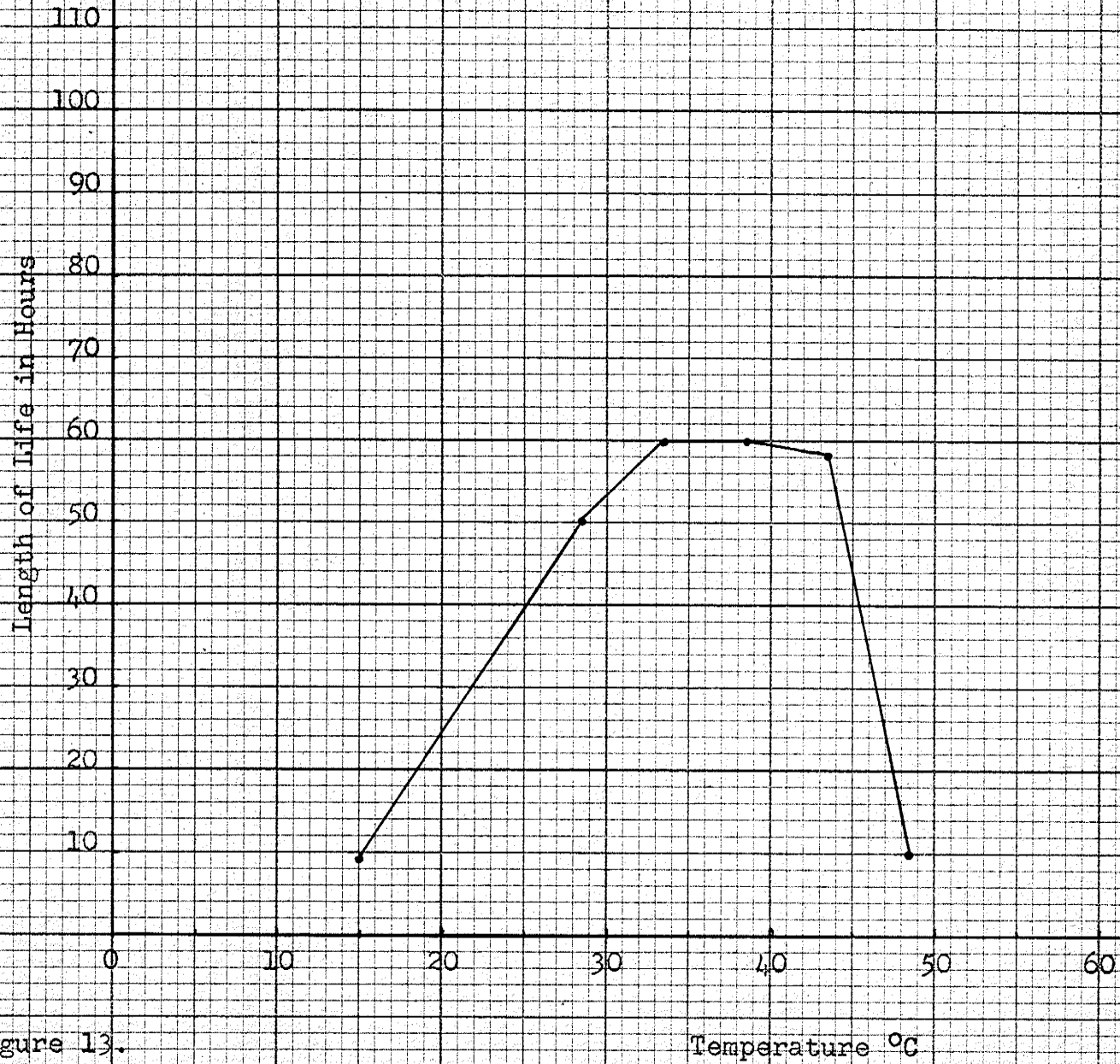
Figure 13.

the same as rats (Abrahamson and Miller). Mann and Ballman
(1930) found considerable variation in the dogs, duodenum pH
5.5 to 7.6, jejunum 7.0 to 7.6 and ileum 7.0 to 8.0.
Determinations were made with a quinhydrone gold electrode
and were probably more accurate than earlier colorimetric
tests.

Kofoid et al. (1932) measured the pH of the intestinal
contents and scrapings from the gut walls. Using a quinhydrone
electrode they demonstrated considerable difference exists
between the hydrogen ion concentration of the ileum contents
(7.51) and that of the ileum wall (6.98). Kofoid et al.
(1933) correlated the distribution of protozoa in the rat gut
with the pH of the intestinal wall and contents. They observed
individual species were limited to certain regions of the gut
where the pH was most suitable. Ball (1935) confirmed the work
of previous investigators by measurement of the hydrogen ion
concentration by means of a glass electrode. They found a pH
of 6.85 for the ileum wall of the rat and 7.45 for the ileum
contents. Robinson (1935) experimenting with rats, filled
the intestine with isotonic calcium chloride from the bile
duct to the ileocecal valve and tied it off into segments.
Each segment assumed a pH characteristic of that section, with
the pH increasing from the bile duct to the caecum. Robinson
concluded that each portion of the gut tends to establish in
its contents its own characteristic reaction and that the

intestinal wall is the source of the controlling mechanism.
He also suggested that there is a zone at the surface of the
mucosa where the pH is stabilized and not necessarily
identical with the pH of the intestinal contents in that
region. Thus parasites close to the mucosa would experience
less variable conditions than those living free in the
intestinal contents. Ball (1939) using a capillary glass
electrode determined the pH of the digestive tract of living
rats. The values obtained compared closely with the
determinations made previously on freshly killed animals.
The ileum wall, site of attachment for H. nana, was found to
vary from 5.98 to 7.89, with an average value of 7.32. Thus
a parasite inhabiting the ileum would be subjected to a con-
siderable variation in hydrogen ion concentration. Robinson
et al. (1943) corroborated the earlier work and investigated
the factors producing the characteristic pH. They found the
progressive increase in pH in the small intestine is due to
a decrease in carbon dioxide tension and an increase in
bicarbonate concentration of the intestinal secretion.

Ackert et al. (1940) investigated parasitism in
chickens infected with Ascaridia lineata. The pH of the
worm habitat ranged from 6.6 to 7.4 but variations in pH
appeared to have no effect on the number and size of the
worms. Von Brand (1943) demonstrated the effect of pH on
oxygen consumption in larval Eustrongylides. Von Brand and

Simpson (1944) investigated the influence of pH upon the
survival of Eustrongylides. The worms survived longest at
pH values near neutrality, but also showed extreme resistance
to hydrogen ion concentrations ranging between 1.9 and 9.4.
Changes occurred in the sterile cultures tending to bring the
pH levels to about pH 5. Stephanson (1947) noted Fasciola
hepatica survived longest at a pH of 8.2 to 8.6, a
comparatively narrow range of optimum conditions. Smyth (1950)
found a rapid fall in pH in Schistocephaul solidus cultures
due to the production of carbon dioxide and acidic metabolic
waste products.

The present investigation included determination of
the importance of hydrogen ion concentration to the survival
of H. nana in vitro. The pH was established by means of
Sorensen's phosphate buffer and five cultures prepared in
Tyrode's solution at each pH level. H. nana appeared to
favor a pH ranging between 6.5 and 7.5, with higher or lower
hydrogen ion levels becoming increasingly toxic (Figure 14).

The effect or osmotic pressure on helminths in vivo
and in vitro has not been determined. Gilman and Cowgill
(1931, 1933) found the osmotic pressure of the gastric
juice to be very close to that of the blood. Panikkar and
Sproston (1941) determined the osmotic relations of some
metazoan parasites. Stephenson (1942) investigated the
effects of variations in osmotic pressure on a soil nematode.

Figure 14.

pH of Cultures

The nematodes were cultured in a 30 mM NaCl solution. Stephenson concluded the species normally lives in an environment which is hypotonic to the body fluids and maintains its internal osmotic pressure by an active method of osmotic regulation. He also noted the main aqueous exchanges occur through the cuticle, which does not act as an impermeable outer covering insulating the animal from its environment.

The importance of osmotic pressure to cestodes has received scant attention. Wardle (1932) noted the internal osmotic pressure of _Dibothriocephalus latus_ was three to four atmospheres, lower than the six atmospheres of the muscle juice of the pike. Schopfer (1932) found the internal osmotic pressure in _Moniezia expansa_ was eight atmospheres, less than the two atmospheres of the gut contents. He also found the osmotic pressure for _Eubothrium crassum_ was twelve atmospheres, twice that of the host. Smyth (1946) studied the effect of osmotic pressure on _Schistocephalus solidus_ and noted optimum level in vitro ranged between 0.72 and 0.78 per cent sodium chloride. Reviewing the literature on nematode cultivation, Hobson (1948) concluded parasitic nematodes are not very sensitive to changes in osmotic pressure and that species inhabiting the gut must be subjected to considerable variation.

H. nana was tested in a series of saline solutions ranging from 0.5 to 1.0 per cent sodium chloride. The worm itself was too delicate to employ the method of Smyth (1946),

who measured changes in weight. The criteria used in the
case of H. nana were changes in volume, changes in the rate
of contraction of the worm and the relative longevity.
H. nana survived the longest and most satisfactorily in
saline solutions of 0.75 to 0.85 per cent sodium chloride.

The influence of inorganic ions on parasitic helminths
has received slight attention from parasitologists. Wardle
(1933) studied the effect of saline solutions on the
plerocercoids of D. latus, and found molar concentrations of
0.05 sodium chloride and 0.2 calcium chloride resulted in
the greatest survival times. Wardle (1934) repeated the
experiment on Nybelinia surmenicola and obtained maximum
survival time in one-tenth molar sodium chloride and sodium
bicarbonate.

Stephenson (1944) investigated the rates of penetration
of inorganic substances for Rhabditis terrestris and noted the
following order of penetration: sodium chloride > calcium
chloride > magnesium chloride, potassium chloride. Smyth
(1946, 1950) determined the influence of a variety of saline
solutions on S. solidus and concluded three quarters Locke
solution best satisfied the requirements of Schistocephalus
in vitro.

Ackert and Gaafar (1949, 1953) determined the effect
of calcium, manganese and phosphorus on Ascaridia galli.
Worms from chickens fed on a diet deficient in calcium or

phosphorus were smaller and less numerous than those of control animals, indicating both calcium and phosphorus are essential for normal nematode growth.

Varying concentrations of inorganic ions were tested on H. nana in vitro, using Tyrode's solution as the basic saline. Sodium, potassium, calcium, magnesium, bicarbonate, chloride and phosphate ion concentrations were each increased and decreased in turn by one-quarter. Of the ions tested, only increased bicarbonate and phosphate increased slightly survival time in vitro. Sodium, potassium, calcium, magnesium, and chloride ion appeared to be present in near optimum concentrations in regular Tyrode's solution and an increase or decrease in the ion concentration resulted in reduced survival time.

CHAPTER VI

THE IMPORTANCE OF VITAMINS TO PARASITIC WORMS

The importance of vitamins in helminth nutrition has received considerable attention from parasitologists, particularly from the pioneers in vitamin-parasite investigations, Chandler and Ackert. Zimmerman et al. (1926) studied vitamin B as a factor in the resistance of chickens to _Ascaridia perspicillum_. The worms from chickens on a vitamin B deficiency diet were larger and more numerous than those from chickens on a balanced diet. Ackert et al. (1931a) found similar results with _Ascaridia lineata_ and diets deficient in vitamin A. Significantly larger numbers of _A. lineata_ remained in chickens on vitamin A deficiency diets. Ackert and Nolf (1931b) confirmed the effect of vitamin B deficiency on _A. lineata_ and attributed the larger numbers of worms to the greatly weakened peristalsis aiding the worms in remaining in the intestine. They also noted somewhat larger _A. lineata_ were found in those chickens whose diet contained yeast, suggesting yeast contains a factor favourable to growth of the worms. Beach and Ackert (1932) were unable to demonstrate any growth factor present in yeast and required by _A. lineata_.

Hager (1940) noted a diet deficient in vitamins B_1 and G resulted in a decrease in egg production. This was subsequently shown to be the result of G complex deficiency

rather than B_1. Watt et al. (1943) and Watt (1944) found
a diet deficient in either thiamine (vitamin B_2) markedly
reduces the resistance of rats to infection with
Nippostrongylus muris. Chandler (1943) and Addis and
Chandler (1944) found the average number of Hymenolepis
diminuta per rat was reduced by a diet deficient in either
fat-soluble vitamins or the G complex. Chandler suggested
the lack of the fat-soluble vitamins may be due to altered
bile secretion, preventing proper cyst evagination. The lack
of some factor associated with the G complex in the diet re-
sulted in a marked stunting in worm growth. Addis and
Chandler (1946) concluded tapeworms are independent of
vitamin B_1 in the diet of the host for normal establishment
and growth, independent of vitamins A, D, and E for growth
but dependent on fat soluble vitamins for normal establishment.
Tapeworms were found to be also dependent on some factor or factors
found in brewer's yeast for normal establishment and growth.

Smyth et al. (1944) investigated the distribution of
vitamin C in Nycototherus cordiformis, Opisthoglyphe ranae and
Toxocara canis. They found such large concentrations of
vitamin C in the gut cells of Toxocara, as to imply that the
vitamin is obtained from the food of the host. In Opisthioglyphe
the vitamin was found concentrated on the walls of the excretory
system and immediately below the epidermis. El-Kordy and
Latif (1946) found thiamine, nicotinic acid and ascorbic acid

in hydatid fluid. Chance and Dirnhuber (1949) investigated the water-soluble vitamins of some parasitic worms and summarized the available information. A portion of this work has been condensed and entered as Table II.

Larsh (1948) noted vitamins A, C and D had a marked effect in preventing alcoholic debilitation of mice infected with Hymenolepis. Chandler et al. (1950) injected radioactive thiamine parenterally into rats infected with H. diminuta. They found the thiamine in the tapeworms and in the intestinal mucosa of the hosts had the same specific activity, indicating the worms obtained thiamine directly from the host.

Sadun et al. (1949) found A. galli required relatively large amounts of a substance present in liver extract. They suggested this unknown substance may be vitamin B_{12}, the animal protein factor. Maldonado and Asenjo (1953) demonstrated neither vitamin B_{12} or pteroylglutamic acid were important in the infection or development of Nippostongylus muris in rats. Brody (1954) confirmed the work of Sadun (1949) and showed a deficiency of vitamin B_{12} and pteroylglutamic acid resulted in increased worm numbers and worm length in chickens infected with A. galli.

Read (1950) pointed out the difficulty of separating host and parasite physiology in experiments on vitamin requirements. The effect of vitamins on host immunity must not be confused with the vitamin needs of the intestinal

TABLE II

THE B - VITAMINS OF PARASITIC WORMS

Content in µg/g. dry weight of whole worm

Species	Aneurin	Nicotinic Acid	Pantothenic Acid	Pyridoxin	Riboflavin
Moneizia benedeni	8.6	190	10.0	7.1	no assay
Fasciola hepatica	16.0	293	9.0	29.0	3.25
Ascaris lumbricoides	10.0	220	11.2	20.0	14.0
Nippostrongylus muris	20.0	119	19.0	13.0	84.0

parasite. For this reason the vitamin requirements of H. nana were determined as far as possible in vitro rather than in situ. In all, six water-soluble vitamins were tested, including: thiamine, nicotinic acid, ascorbic acid, riboflavin, biotin and inositol. The vitamins were tested in cultures prepared with five milliliters of Tyrode's solution and a single H. nana. Five cultures were prepared at each concentration tested. The limited supply of tapeworms available did not permit an investigation of the fat-soluble vitamins.

Thiamine hydrochloride was tested (Figure 15) in concentrations of 0.5 to 10.0 milligrams per culture. No significant change in survival time was observed. Nicotinic acid (Figure 16) was tested in the same concentrations and no significant change in the survival time of H. nana was seen. Ascorbic acid was tested in concentrations of 0.5 to 10.0 milligrams per culture. H. nana lived approximately seventy-five per cent longer in cultures containing 0.5 to 5.0 milligrams per culture, (Figure 17) than in cultures without ascorbic acid present. Riboflavin was tested (Figure 18) in concentrations of 0.01 to 0.20 milligrams per culture. An increase of approximately eighty per cent in survival time was observed in cultures containing 0.01 to 0.10 milligrams per culture. Biotin was tested (Figure 19) in concentrations of 1.0 to 20.0 Gamma. Maximum survival time was obtained at a concentration of 10.0 Gamma, with

culture survival time more than double that of cultures
without biotin. Inositol was tested (Figure 20) in
concentrations of 0.5 to 10.0 milligrams per culture. No
significant change in survival time was observed at any
concentration.

THIAMINE HYDROCHLORIDE
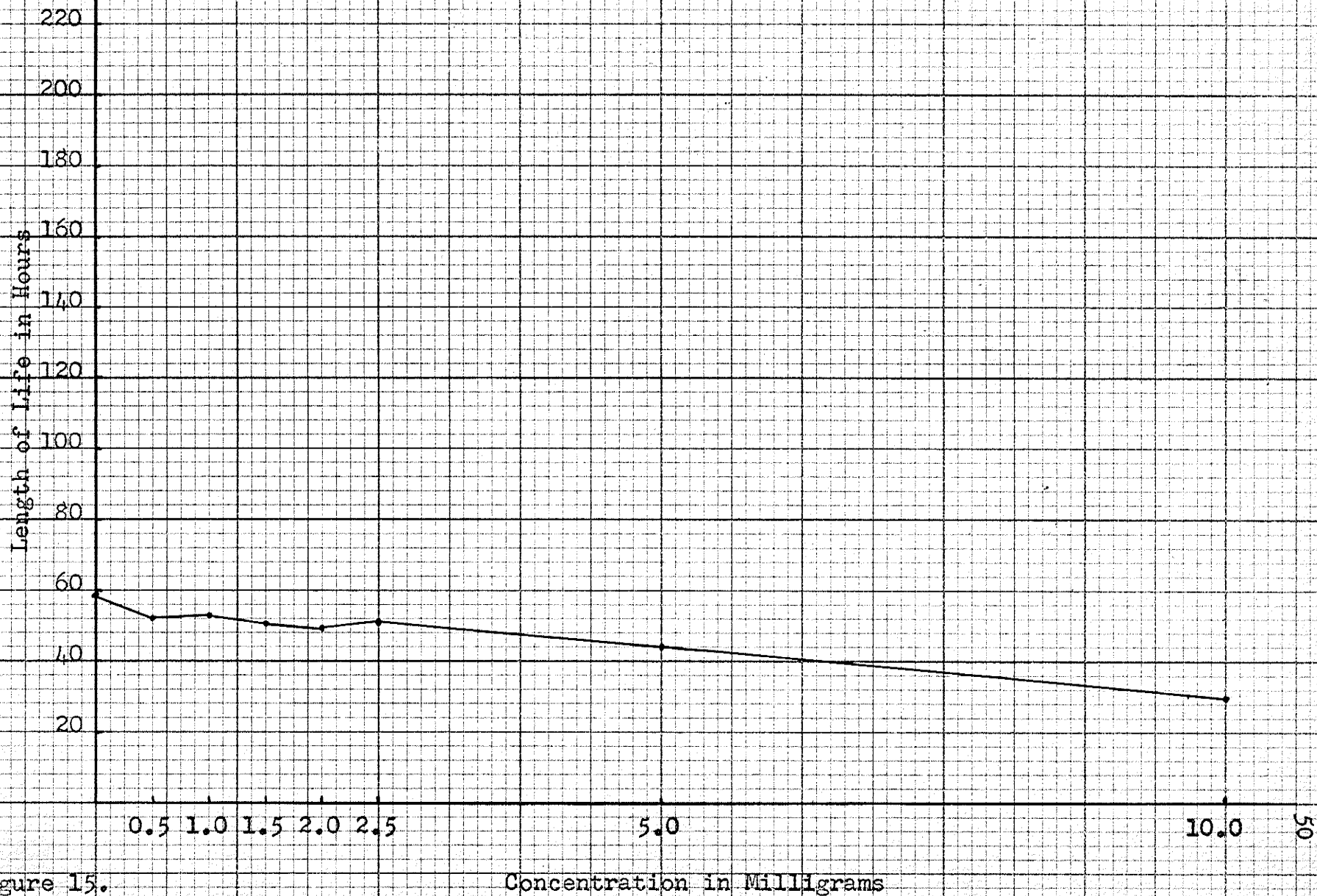
Length of Life in Hours

Concentration in Milligrams

Figure 15.

NICOTINIC ACID

Figure 16.

Concentration in Milligrams

Figure 17.
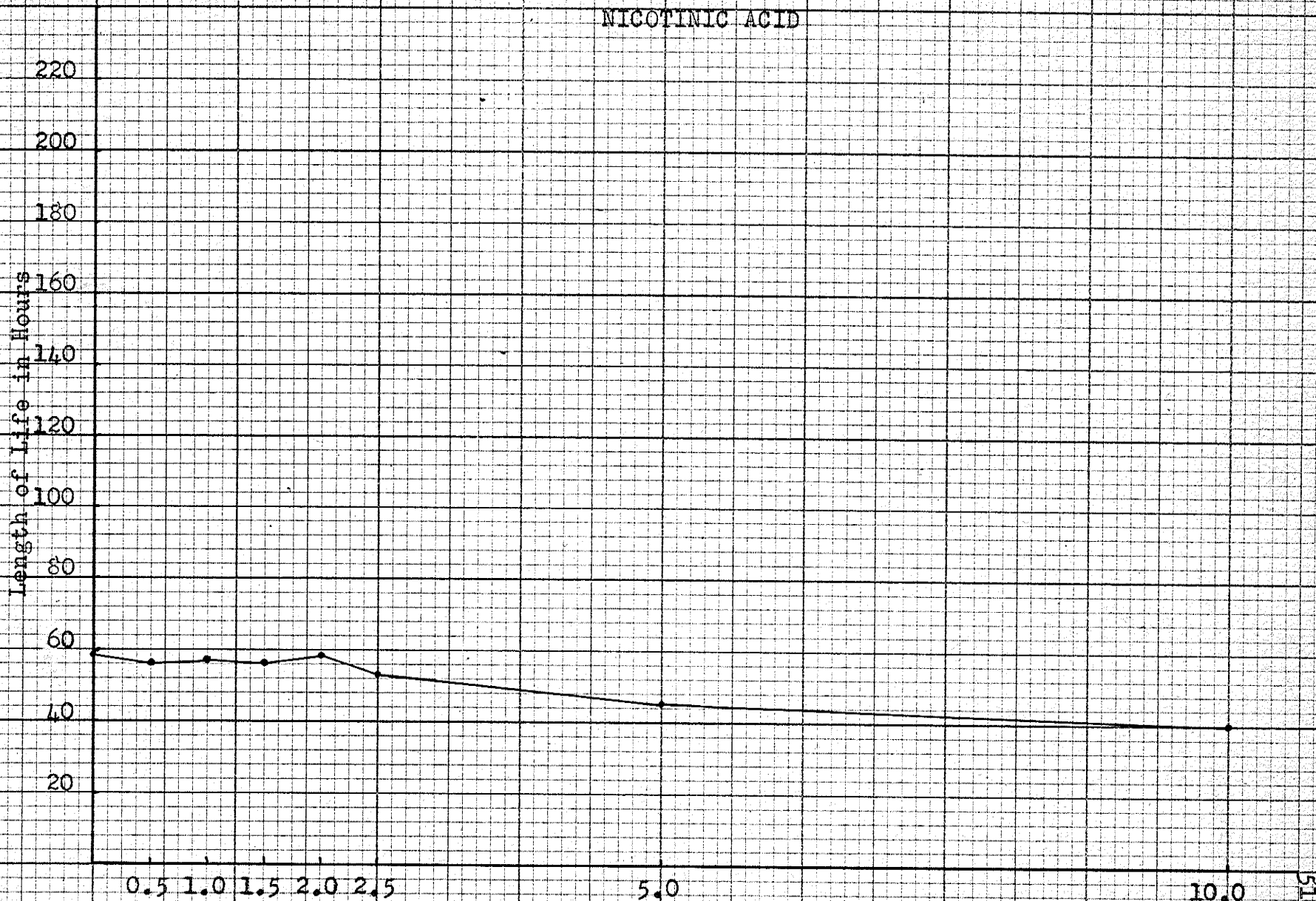
ASCORBIC ACID
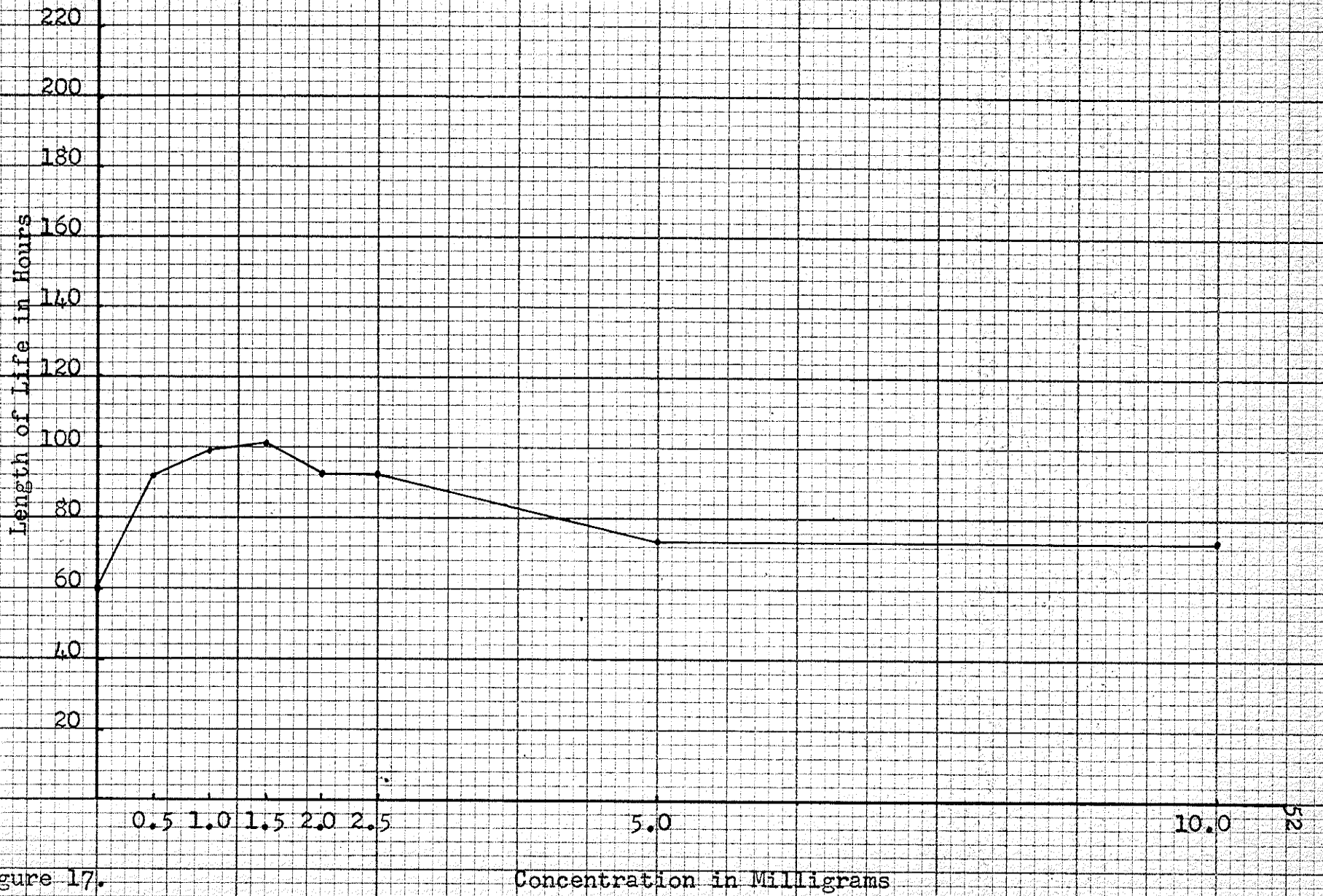
Length of Life in Hours

Concentration in Milligrams

RIBOFLAVIN
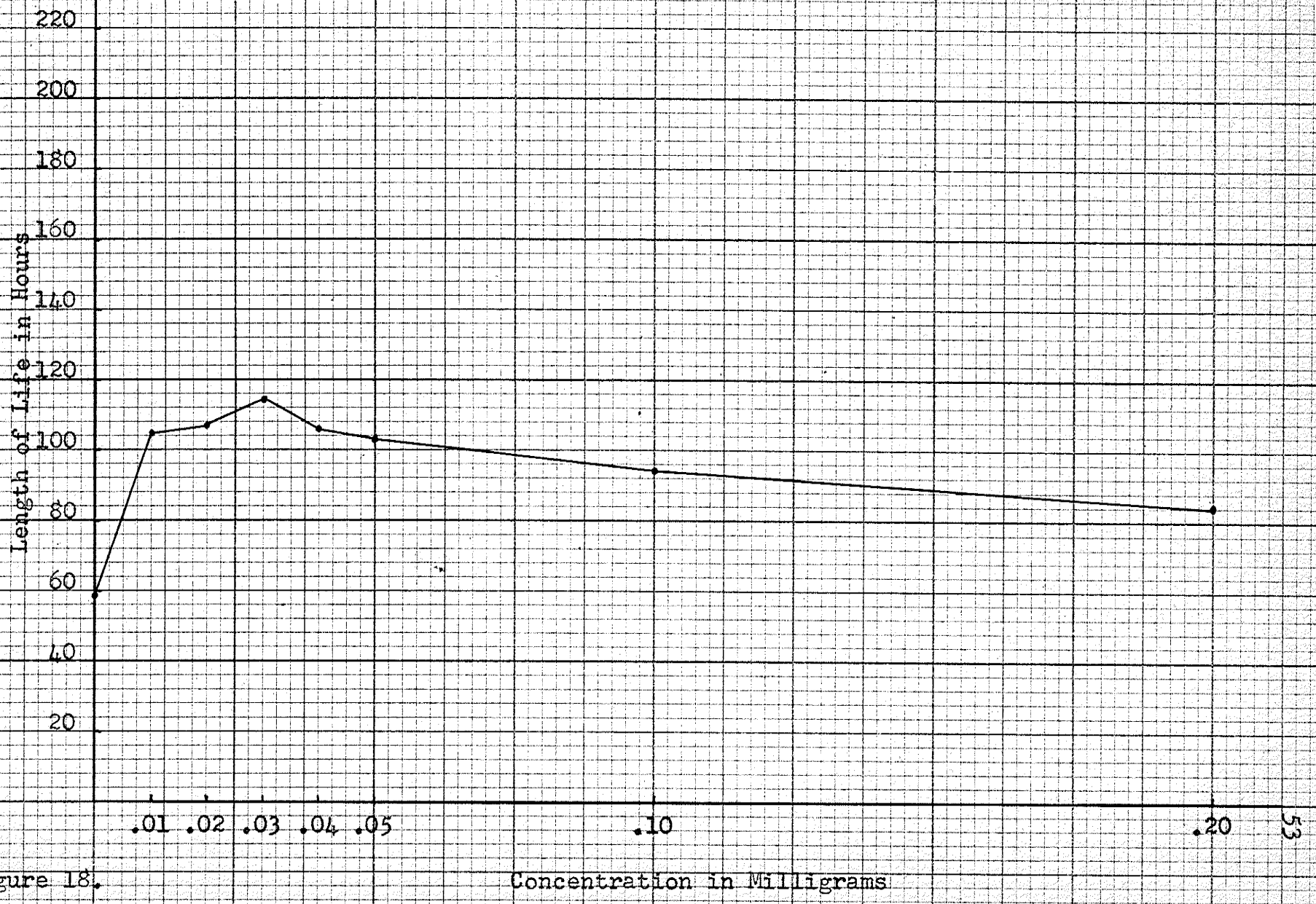
Figure 18.

Concentration in Milligrams

53

BIOTIN
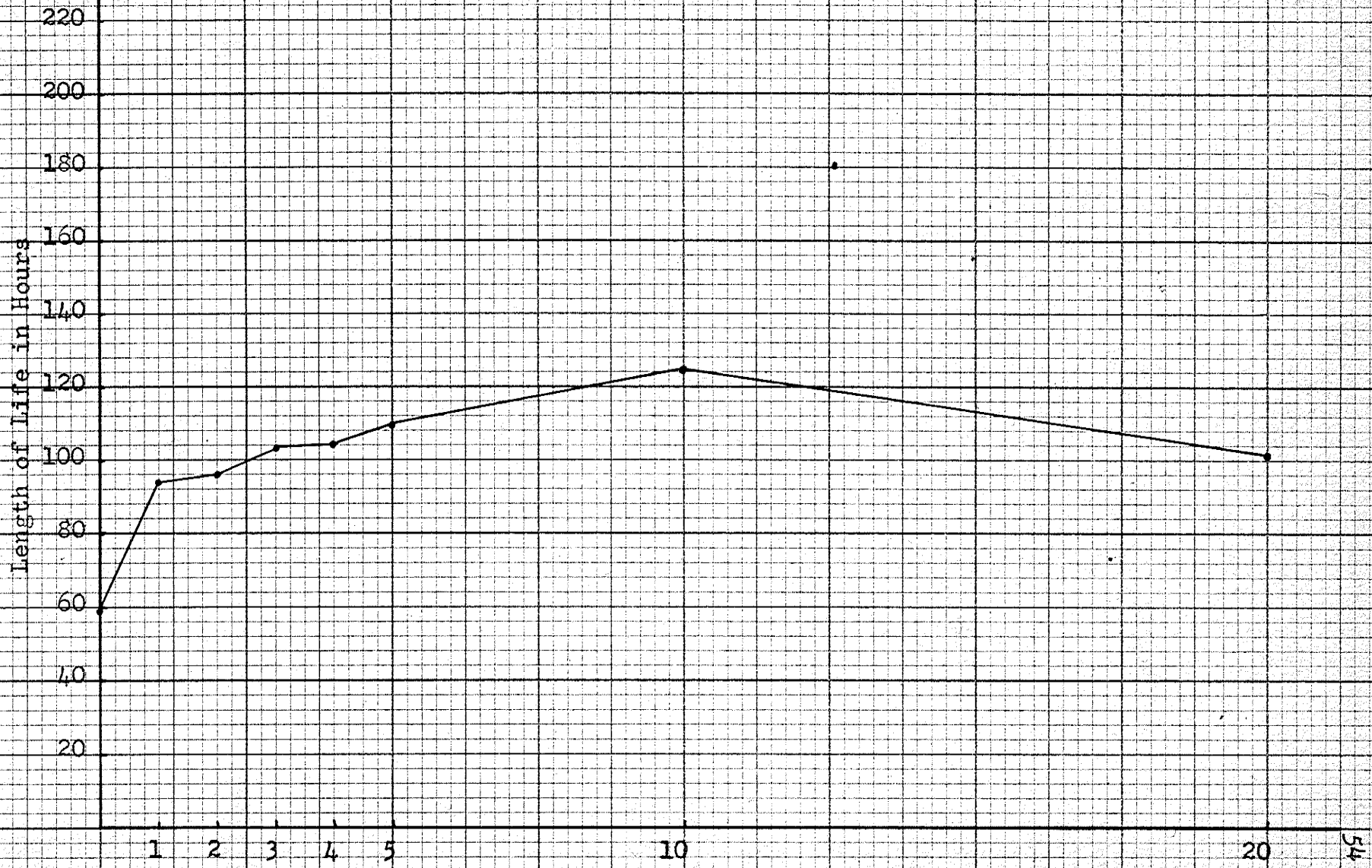
Length of Life in Hours

Concentration in Gamma

Figure 19.

54

INOSITOL

Length of Life in Hours

Concentration in Milligrams

0.5 1.0 1.5 2.0 2.5   5.0   10.0

Figure 20.

55

CHAPTER VII

HELMINTH METABOLISM

The early investigations into helminth metabolism consisted largely of the identification of the polysaccharide glycogen and speculation as to the nature of its breakdown. Weinland (1900, 1901) noted particularly the production of some of the lower fatty acids by helminths in saline media. He attempted to show these acids were the result of anaerobic breakdown of glycogen. Weinland and von Brand (1926) emphasized how small parasites may live a much more aerobic existence than larger ones due to the diffusion of oxygen through parasitic tissues. They noted also the predominantly anaerobic metabolism of Fasciola and other large species. Von Brand (1929) stressed the fact that elemental oxygen was not available to intestinal parasites as it was to free-living animals. He noted the excretion products of Moneizia included lactic, succinic and other higher fatty acids, indicating these substances could not be made use of in an anaerobic existence. Vogel and von Brand (1933) found the developmental stages of Fasciola hepatica do not contain fat globules in their excretory system, a feature characteristic of anaerobic glycogen metabolism in the adult fluke. They concluded the metabolism of immature stages is quite different from that of the adult. Wardle (1934) called

attention to the fact that no accurate information was available concerning the food requirements of a cestode, but that it was generally assumed foodstuffs entered the worm by diffusion through the body surface.

Von Brand (1937a, 1937b) measured the rate of glycogen consumption by Ascaris lumbricoides and noted the low level of resynthesis. He found the glycogen rebuilt is only one-tenth to one-twentieth of that consumed, a marked difference from the muscle of vertebrates where four-fifths to five-sixths is resynthesized. Von Brand and Otto (1938) studied the carbohydrate metabolism of the hookworm, Ancylostoma caninum. They found the oxidative metabolism and absence of the periodic feeding habit resulted in a very low glycogen content.

Reid (1942) found Raillietina, the fowl cestode, consumed ninety-four per cent of stored glycogen during twenty-four hours of host starvation. A marked fluctuation in the glycogen level occurred with the normal feeding habits of the fowl. Reid (1945) noted a similar glycogen depletion in A. galli, resulting in expulsion of the worms after prolonged host starvation.

Baldwin and King (1942) prepared glycogen from A. lumbricoides and found it to be a typical polysaccharide with a chain length of twelve to thirteen units and no demonstrable immunological properties. Von Brand and Simpson

(1945) studied the metabolism of larval Eustrongylides.
They found nutritive substances in vitro were consumed only
under aerobic conditions, anaerobic worms were unable to
utilize peptone or sugar. Glycogen consumption under
anaerobic conditions was about three times that found under
aerobic conditions. Reid (1945) found the glycogen content
of H. diminuta fluctuated with the feeding of the host, as
had been found with Raillietina by Reid (1942). H. diminuta
appeared to utilize sodium lactate, indicating some aerobic
metabolism may be carried on by this cestode.

Axman (1947) noted proportionately greater amounts of
glycogen stored in large trematodes than small ones and con-
firmed some of von Brand's earlier findings on aerobic
metabolism in small parasites. Rogers and Lazarus (1949)
identified phosphatase activity in A. lumbricoides and
examined acid-soluble phosphorus compounds in different
tissues. They noted also energy was transferred by means of
high energy phosphate bonds and carbohydrate fermentation in
nematode parasites was similar to mammalian muscle as far as
lactate formation. Read (1951a) found anaerobic glycogen in
H. diminuta proceeds by processes similar to that in
vertebrate tissue.

Read (1951b, 1952, 1953) investigated cestode
metabolism of carbohydrates and furnished evidence to show
the first steps of carbohydrate utilization in H. diminuta

are essentially the same as in vertebrate muscle. He was
able to demonstrate the presence of phosphorylase,
phosphohexomutase, hexokinase, aldolase, phosphoglyceraldehyde
dehydrogenase and lactic oxidase, essentially components of
the Embden-Meyerhof cycle of glycolytic reactions. Read
suggested the endoparasitic way of life, eliminating the need
for bursts of energy, had resulted in the loss of the
phosphagen energy storage system. Read (1952) demonstrated
the presence of cytochrome exidase and succinic dehydrogenase
in H. diminuta. Read (1953) demonstrated fumarase,
α-glycerophosphoric dehydrogenase, and pyridine-nucleotide
enzymes catalizing the oxidation of malic, L-glutamic and
α-glycerophosphoric acids.

    Daugherty (1952a) stressed the critical role of
transamination reactions in nitrogen metabolism, particularly
oxidative deamination and amino acid synthesis. The
transaminase systems in Fasciola hepatica were tested on
aspartic acid, isoleucine, leucine and valine with markedly
high activity. Somewhat lesser activity was noted in the
case of arginine, alanine, phenylalanine, tyrosine,
methionine and proline. Daugherty (1952b) using paper
chromatography, isolated the same amino acids from H. diminuta
as were found in F. hepatica, plus significant amounts of
taurine. Daugherty (1953a) described for the first time the
utilization of ammonia by H. diminuta. The ammonia was

formed into amino nitrogen and was associated with alanine
and glutamic acid. Daugherty suggested ammonia fixation was
an important mechanism in protein synthesis in H. diminuta.
Daugherty (1953b) described the metabolism of sulfur amino
acids in H. diminuta. He noted radioactive methionine was
changed rapidly to taurine via cystine and that the high
taurine content of cestodes is not necessarily due to bile
constituents. Aldrich, Chandler and Daugherty (1954)
identified eighteen amino acids from the tissues of
H. diminuta. They also found host castration reduced
transaminase activity in H. diminuta, resulting in an
increased fat deposition in the worm.

CHAPTER VIII

THE CULTIVATION OF PARASITIC WORMS

The term 'axenic' was proposed by Baker and Ferguson (1942) to describe an organism free of all demonstrable life apart from that of its own protoplasm. Axenic is used in the following cultivation discussion in preference to the rather misleading term, sterile.

Nematode cultivation has met with a considerable degree of success, due largely to the rather less delicate nature of the worms. Glaser and Stoll (1938) cultured *Haemonchus contortus* in sterile media into the third and fourth larval stages. The medium consisted of a 0.5 per cent agar in Ringer's solution containing sheep liver extract, heat killed ground yeast, sheep blood and sheep kidney. Glaser et al. (1942) obtained similar success with *Neoplectana chresima*, a nematode parasitic in insects. Ackert et al. (1938) cultured larval *Ascaridia lineata* in a medium of chicken eggs, dextrose and agar. Ackert et al. (1939) obtained successful growth of *A. lineata* in a simple medium of dextrose, sodium chloride and corn meal agar. Von Brand and Simpson (1942) maintained the larvae of *Eustrongylides* for periods up to three hundred and forty-six days in saline yeast extract of glucose. Von Brand and Simpson (1944) cultured larval *Eustrongylides* at 20° C. in a medium of 1.0

per cent Bacto-Proteose Peptone, 0.85 per cent sodium
chloride and 0.5 per cent glucose. Hobson (1948) summarized
the information on cultivation and suggested the absence of
a 'growth factor' as the cause of poor nematode development
in vitro. Stoll (1953) successfully cultured Neoaplectana
glaseri through more than one generation in acid veal or beef
heart infusion broth supplemented with a raw liver extract.
Shaking the cultures in the dark resulted in greater yields.
Pitts and Ball (1953) obtained some success, with
A. lumbricoides surviving up to thirty-nine days. The medium
contained yeast extract, peptone, glucose, human serum and
Fenwick's solution.

Experimenting with the trematode Posthodiplostomum
minimum, Ferguson (1940) cultured sterile metacercariae to
adults in a medium of chicken serum, yeast extract and
diluted Tyrode's solution. Ferguson (1943) cultivated
cercariae of Diplostomum flexicaudum aseptically in a variety
of media, but the resulting metacercariae were non-infective.
Hunter et al. (1952) obtained seventy to ninety per cent
encystment of Gynaecotyla aduca in a dilute saline containing
pepsin and hydrochloric acid.

The cultivation of tapeworms did not receive serious
consideration until the investigations of Cook and Sharman
(1930) and Wardle (1932, 1933, 1934). Previous to 1930
experimentation was limited to maintaining various cestodes

in simple saline solutions. Cook and Sharman noted the effect of acids and bases in Ringer's solution on the tapeworm _Moniezia trigonophora_. Maximum survival in vitro was obtained using M/10,000 sodium hydroxide and M/10,000 hydrochloric acid. Wardle (1932) investigated the influence of various salines upon the survival of _D. latum_. Maximum survival times at 38° C. were obtained with 0.05 M sodium chloride and 0.2 M calcium chloride. Wardle (1934) maintained _Nybelinia surmenicola_ for periods up to four hundred and fifty hours. Most satisfactory media were serum saline gel, Locke bouillon, and Locke glucose. No increase in size or significant maturation was observed in any culture medium. Reference may be made to Wardle (1937) for a summary of experiments in cestode cultivation.

Markov (1938, 1939) cultured the plerocercoid larvae of _D. latum_ sixty days in fish extract and fifty-six days in glucose plus vitamins. Wardle and Green (1941) cultured the plerocercoids of _D. latum_ on various nutrient agar media. Where agar plus hog serum was used, considerable growth resulted although survival time did not exceed five days. _H. nana_ was cultured for periods up to twenty days in a mixture of the tissue culture medium, Baker A and Tyrode's solution.

Smyth (1946) removed _Schistocephalus solidus_ aseptically from the body cavity of the three-spined

stickleback <u>Gasterosteus</u> <u>aculteatus</u>, thus avoiding the need
for sterilizing the plerocercoid before culturing in sterile
media. Successful results were obtained with peptone-broth,
in which plerocercoids lived up to three hundred days.
Similar success was obtained, Smyth (1947a), culturing
<u>Ligula</u> <u>intestinalis</u> to maturity in peptone-broth. Smyth
(1947b) cultured unidentified larval Diphyllobothriidae for
ten days in peptone-broth plus ten per cent horse serum. The
plerocercoids showed no development and no loss in total
glycogen during cultivation. Smyth (1948) successfully
cultured fragments of plerocercoids of <u>S</u>. <u>solidus</u> and <u>L</u>.
<u>intestinalis</u>. All fragments developed and produced eggs
although there was no tissue regeneration in anterior or
posterior regions. Smyth (1949) noted a small percentage of
embryonated eggs were produced in cultures of <u>L</u>. <u>intestinalis</u>
and <u>S</u>. <u>solidus</u>, providing the pH was carefully controlled by
strongly buffered media. Previously eggs produced in vitro
had not shown embryonation. Smyth (1950) found embryonated
eggs produced in vitro contained normal, miniature, or
abnormal coracidia. Miniature coracidia could be made to
hatch artificially, but abnormal coracidia in which the
embryophore failed to develop could not be matured. Smyth
(1952) noted the importance of temperature in the development
of <u>S</u>. <u>solidus</u> in vitro. Adequate nutrients in vitro could
not cause the plerocercoids to develop unless accompanied by

a rise in temperature to 40° C., the normal body temperature of the adult host. Successful fertilization of S. solidus in vitro was obtained by Smyth (1953) through the use of semi-permeable cellulose tubing. This method provided the necessary compression during maturation and permitted the diffusion of toxic metabolic waste products. Smyth (1954) obtained a high degree of fertility (seventy-seven per cent), by continuous shaking of the plerocercoids of S. solidus inside cellulose tubing suspended in a tube of horse serum. A constant temperature of 40° C. was maintained for forty-eight hours to bring about maturation.

The present investigation included the culturing of H. nana in a wide range of semi-liquid and liquid media.

The semi-liquid media included: aqueous agar, saline nutrient agar, aqueous gelatine, saline nutrient gelatine, aqueous anaerobic agar, aqueous chocolate agar, and artificial blood clots. Tapeworms responded unfavorably to these media and survived less than thirty-six hours. An alternative technique was devised in which a more concentrated, semi-solid layer of one of the above nutrients was poured into the lower portion of the culture flasks. A liquid layer of sterile Tyrode's solution was added, plus a single tapeworm. The worms in these cultures did not attach themselves to the semi-solid gelatin as hoped, but rather burrowed into it and promptly died.

In terms of longevity in vitro, liquid media were much more successful. The most suitable media tested and maximum survival time in hours were listed in Table III. Liquid media containing Kracke's blood medium, Witte's peptone, proteoses, tryptose phosphate or muscle infusion proved of no value in culturing H. nana.

TABLE III

THE MAXIMUM SURVIVAL TIME IN NUTRIENT MEDIA

Nutrient media	Survival time in hours
Tyrode's solution plus rabbit serum, 600 units penicillin and 0.2 milligrams streptomycin	366
Tyrode's solution plus rabbit serum, 600 units penicillin	295
Tyrode's solution and rat blood serum	145
Tyrode's solution plus Baker's medium A	130
Tyrode's solution	89
Vogelaar and Erlichman's solution	45

CHAPTER IX

SUMMARY AND CONCLUSIONS

Summary. The microorganisms coexisting with H. nana in the rat gut and in vitro are known, together with the most useful antibiotics for combating them.

The optimum hydrogen ion concentration, temperature, osmotic pressure and inorganic ion levels are established.

The influence of aerobic and anaerobic conditions are discussed as well as the relative value of water-soluble vitamins.

A comparative study of various liquid and semi-solid culture media indicates Tyrode's solution plus rabbit serum, 600 units penicillin and 0.2 milligrams streptomycin are best suited to the cultivation of H. nana.

Conclusions.

1. The organisms found in the ileum of the laboratory rat and most commonly contaminating cestode cultures are: Chromobacterium, gram positive bacilli, coagulase negative Staphylococcus, Streptococcus faecalis, Proteus, coliform bacilli, and diphtheroids.

2. All antiseptics appear to be more toxic to H. nana than to the contaminating bacteria. Zephiran chloride and alkylamine hydrochloride are the least toxic.

3. A group of the more common antibiotics are non-toxic to H. nana in vitro, while retarding the growth of microorganisms. Penicillin G potassium is the most useful, yielding consistent survival times of one hundred and ninety hours in cultures containing six hundred units per five milliliters of culture medium. Terramycin and aureomycin are relatively toxic in all concentrations. Achromycin, chloromycetin and streptomycin have a relatively low toxicity in dilute solutions.

4. Aerobic cultures live three to four times as long as anaerobic ones. H. nana appears unable to utilize glucose under anaerobic conditions.

5. H. nana is markedly insensitive to small variations in temperature.

6. H. nana favors a comparatively narrow range of hydrogen ion concentration and survives longest at a pH ranging between 6.5 and 7.5.

7. The vitamin needs of H. nana appear to include ascorbic acid, riboflavin and biotin. Thiamine hydrochloride, nicotinic acid and inositol do not appreciably increase or decrease the survival time in vitro.

8. The recent contributions to cestode metabolism are discussed, with special reference to cestode enzymes acting on proteins and carbohydrates.

9. Axenic cultures of H. nana may be maintained consistently for periods of up to fifteen days in a medium Tyrode's solution and rabbit serum, plus six hundred units of penicillin G potassium and 0.2 milligrams of streptomycin sulfate.

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