The Axenic Cultivation of *Rhabditis briggsae* Dougherty and Nigon, 1949 (Nematoda: Rhabditidae). II. Some Sources and Characteristics of "Factor Rb"

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It is obvious that success in the cultivation *in vitro* of parasitic helminths would open up important new experimental approaches in the fight against the diseases caused by them, but it is also obvious that such cultivation presents many difficulties. The study of free-living forms that can be grown axenically (i.e., free of other living forms) may well provide important clues to the problem of the growing of parasites free from their hosts. Moreover, such free-living forms constitute potentially valuable subjects for experiments on helminth metabolism, with such practical consequences as the testing of anthelminthics.

Thus, although the studies reported here have been carried out with the primary aim of working out the nutritional needs of the nematode, *Rhabditis briggsae*, for their implications in connection with possible biochemical genetic studies, the results are offered with the knowledge that they may prove interesting to investigators concerned with nematode metabolism from other standpoints.

The axenic cultivation of nematodes was first claimed in 1903 by Metcalf for a species identified by him as *Rhabditis brevispina*. Dougherty and Calhoun (1948) gave reasons for doubting the validity of his

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claims. In 1921 Zimmermann reported the successful axenic cultivation of the vinegar eel, now correctly known as *Turbatrix aceti*. In 1940 Glaser described the maintenance of an insect-parasitic form, *Neoaplectana glaseri*, in axenic culture for numerous generations, and two years later Glaser, McCoy, and Girth (1942) announced the successful cultivation of *N. chresima*. Recently Stoll (1948) reported the growth of *N. glaseri* on much simplified liquid media. *T. aceti* and the species of *Neoaplectana* can be grown on appropriate autoclaved media. However, recent work by the present author and his associates (Dougherty and Calhoun, 1948; Dougherty, 1950b; Dougherty, Raphael, and Alton, 1950) on species of the genus *Rhabditis* (*R. pellio* and *R. briggsae*) demonstrated that the latter forms require one or more heat-labile factors that behave like protein.

In addition to the successful growing of certain germ-free, free-living nematodes for numerous generations, some success has been achieved in the axenic cultivation of the free-living larval stages of certain nematodes of the suborder Strongylina, parasitic as adults. Thus Glaser and Stoll (1938a, b, c) reared the sheep stomach worm, *Haemonchus contortus*, and Weinstein (1949) the hookworms, *Ancylostoma duodenale* and *A. caninum*, and the trichostrongyle, *Nippostrongylus muris*, to the infective (third) larval stage under axenic conditions. The former workers also succeeded in getting the third (first parasitic) ecdysis *in vitro*, but only with larvae grown in fecal, hence nonsterile cultures (see particularly Stoll, 1940). These strongylines, like the rhabditids, appeared to require heat-labile factors. However, Lawrence (1948) reported obtaining third-stage larvae of the hookworm, *A. braziliense*, in heat-sterilized media containing, most importantly, kidney or veal preparations; but his results met with only limited success, only few larvae being obtained from inocula of many eggs. Possibly these larvae grew as the result of food substances stored in the egg plus limited ability to make required heat-labile factor(s).

Recent work with the soil-nematode, *Rhabditis briggsae*, has shown that it can be grown axenically at least through several generations and probably indefinitely on a complex ("complete") medium containing, in addition to known substances, fresh chick embryo juice (CEJ) as a source of the one or more heat-labile growth requirements (Dougherty, Raphael, and Alton, 1950); the possibility that CEJ also supplied other growth needs could not, however, be adequately investigated as long as it was not fractionated.

It was also observed that CEJ alone permitted the maturation, but not reproduction and subsequent growth of *R. briggsae*. This suggested that one or more of the known substances of the "complete" medium constituted reproductive factors not present in CEJ, at least in sufficient quantities, although it was also possible that one or more of these substances merely served to release inhibitory properties of the CEJ.

Subsequently to studies on *R. briggsae* so far reported (Dougherty, Raphael, and Alton, 1950) an effort was made to investigate other sources of the heat-labile factor or factors needed by this species and to characterize such requirements somewhat. It was proposed (Dougherty, 1950b) that a protein-like factor or factors involved be collectively designated "factor Rb" (after *Rhabditis briggsae*) until the chemical nature of the one or more constituents was determined with some precision. The results of recent studies, principally on factor Rb, are recorded here. A few of these observations are reported in abstract (Dougherty, 1950b).

Materials and Methods

The same strain of *R. briggsae* as studied hitherto, maintained in two-membered cultures with *Escherichia coli* and axenized (i.e., freed of the latter) as needed, was employed in the present investigations. In all cases tiny (mostly II stage) larvae of *R. briggsae* were used. These were routinely washed with about 10 ml of broth into a Petri plate from a slant containing them and axenized by the addition of a few milligrams of streptomycin to the broth, in which the worms were then maintained for one or more hours. A few milligrams of penicillin or aureomycin were also added as a precaution against possible contamination with bacteria not sensitive to streptomycin.

The use of *Neurospora* "growth tubes" was continued. As a rule 2 ml of final medium were employed per tube. Tubes were usually set up in triplicate, sometimes in duplicate only. Worms were generally inoculated directly from broth in which they were axenized. In such cases small amounts of streptomycin and penicillin (or aureomycin) were carried over into the "growth tubes," also a small amount of broth. No obvious difference between worms so treated and others washed with several centrifugations in distilled water was noted. The effects of various levels of streptomycin were not systematically investigated, however.

Various potential sources of factor Rb activity, in addition to fresh CEJ were tested as supplements to a synthetic ("basal") medium (modified Kidder-Dewey *Tetrahymena* medium (Dougherty, Raphael, and

Alton, 1950)) consisting of known ingredients—amino acids, vitamins, nucleotides (or their bases), energy sources, salts, and a detergent (Tween 85). In more recent work (described under *Results*) the "basal" medium was autoclaved liver homogenate with the precipitated part removed. The new sources tested included: 1) lyophilized commercial CEJ (Difco); 2) aqueous liver extract (LE) sterilized either (a) by a procedure involving the addition of toluene followed by lyophilization and, in some cases, by the use of antibiotics in the final medium or (b) by Seitz-filtration; and 3) human whole blood and plasma. In addition LE was fractionated into two parts: (a) that (fraction I) precipitating out up to 75% saturation with ammonium sulfate buffered to pH 7.0 and (b) the unprecipitated part (fraction II).

The idea of trying to sterilize LE by means of a toluene-lyophilization technique was suggested by the work of Railton, Cunningham, and Kirk (1941), who showed that certain protein solutions could be completely sterilized by mixing them with toluene and then lyophilizing the mixtures. In view of previous failure to obtain factor Rb activity for *R. briggsae* in Seitz-filtered LE (Dougherty, Raphael, and Alton, 1950), it was for a while felt that some such method was needed if liver was to be tested as a source of factor Rb. The LE was prepared by homogenizing a given weight of calf's liver with an equal weight of distilled water in a Waring Blendor. The resulting homogenate was treated by various methods of which the most recent and satisfactory is as follows:

The liver suspension is centrifuged for 2 or more hours at 20,000 G on a Sorvall high-speed centrifuge (a "Servall" model). The clear supernatant is sucked off in a Luer syringe provided with a long needle. This liquid can usually be sterilized by the toluene-lyophilization treatment alone, which consists of pouring the LE in 50–150 ml amounts into sterile lyophil flasks, adding 1–2 ml of toluene to each, shaking, freezing the mixture in a film on the inside of the flasks, and lyophilizing. Sterility is most easily maintained by using a lyophil apparatus in which the male part of the manifold-flask joint is on the flask. Flasks are sterilized and kept plugged until just before they are connected to the apparatus; plugs must be removed during lyophilization, however, to prevent melting the frozen solutions.

It was found that solutions with particulate matter were not sterilized satisfactorily by the toluene-lyophilization treatment. Thus, LE prepared by centrifugation of 4000 rpm on a conventional centrifuge was not successfully sterilized. However, the bacterial population was thereby drastically reduced, and it was possible in some cases to suppress bacterial growth completely with the small amount of antibiotics carried over with axenized larvae. In one instance out of several attempts it was

even possible with a pinch of streptomycin to suppress growth of bacteria in LE subjected only to Sorvall-centrifugation and then tested on *R. briggsae*.

Subsequently it was found possible to obtain LE with factor Rb activity even though sterilized by Seitz-filtration. This was possible provided the LE was centrifuged at 20,000 G before filtration; an arbitrary period of 2 hours of centrifugation was used. Two or more filtrations through a single Seitz pad were often necessary to ensure sterility. Better results were obtained with two pads used simultaneously. (Sintered glass filters permitted the passage of only a small amount of such LE before becoming clogged; this also proved active.)

Protein fractionation of LE was suggested by the work of Johnson and Tatum (1945) on a heat-labile factor or factors required by *Paramecium multimicronucleatum* and present in pressed yeast juice. The most recent method of fractionation used follows:

> To LE (after high-speed centrifugation) a saturated solution of ammonium sulfate prepared at 20°C and buffered at pH 7.0 is added dropwise until 75% saturation is reached; this is carried out at 4°C. The resulting suspension is then centrifuged on the Sorvall centrifuge, and the precipitate dissolved in 1/15 *M* phosphate buffer (pH 7.0) by adding the latter a little at a time and each time mixing it into the pasty precipitate thoroughly. The resulting solution is then centrifuged at 20,000 G for 15 minutes and the sediment discarded. The supernatant is reprecipitated, the precipitate redissolved in buffer, and the resulting solution recentrifuged as before. After the new supernatant is decanted, it is dialyzed in a Visking tubing against buffer with several changes and occasional stirring over a 30-hour period.

(It was previously observed that factor Rb was not inactivated by dialysis, for it was demonstrated that a small amount of CEJ dialyzed for 1 week against several changes of sterile distilled water retained activity in the non-diffusible portion.)

The supernatant from the first centrifugation was saved, and it too was dialyzed.

In this way a protein fraction (I) precipitating out up to 75% saturation with ammonium sulfate was separated from a non-precipitating fraction (II). These were sterilized both by the toluene-lyophilization procedure and by Seitz-filtration.

The protein contents of LE preparations were tested by precipitation of aliquots with 1.0 *N* trichloroacetic acid, drying, and weighing; this permitted the preparation of comparable dilutions.

Human whole blood was obtained from the author by aseptic vene-

puncture, once with an oxalate anticoagulant and once with heparin. Plasma was separated in the former case by centrifugation.

Results

With Chick Embryo Juice

A few new properties of whole CEJ were observed or tested. Most of the juice used in the experiments reported here was originally prepared for tissue culture work and had been standing for varying periods in the ice box. It was found that whereas all of such CEJ, when used together with "basal" medium, supported growth of *R. briggsae* to large adults, some batches did not support reproduction. Moreover, alone it did not support maturation, unlike the fresh CEJ of previous studies. Preliminary studies with various components of the Kidder-Dewey (K-D) medium tested as separate supplements to this CEJ showed that only when the vitamin group was included were uniformly large adults produced. The CEJ used in these last tests did not support reproduction.

Lyophilized CEJ (Difco), supplementing the K-D medium, was found to support maturation and reproduction with subsequent growth of *R. briggsae* quite as well as did fresh CEJ in earlier studies (Dougherty, Raphael, and Alton, 1950).

With Liver Extract

LE sterilized by toluene plus lyophilization and supplementing the Kidder-Dewey (K-D) "basal" medium supported growth and reproduction of *R. briggsae,* and massive cultures were obtained. However, maturation was markedly slower than in the presence of CEJ, probably taking 10–14 days, and significant reproduction did not begin until 2 weeks or more had passed. A single tube of basal medium with LE treated only by high-speed centrifugation (at 20,000 G) and rendered successfully germ free with a bacteriological loopful of streptomycin (in addition to the antibiotics introduced with the axenized larvae) showed no obvious differences in the growth and reproductive pattern of the worms raised in it from that of worms raised on basal medium with LE sterilized by toluene treatment plus lyophilization.

LE subjected to high-speed centrifugation in an angular centrifuge ("Spinco" model) at approximately 100,000 G for 2 hours showed normal activity in the supernatant.

When, after high-speed centrifugation, LE was sterilized by Seitz-filtration with the use of pressure to push the fluid through rather than

suction to pull it, the preparation showed activity similar to that of LE sterilized by the toluene-lyophilization procedure. It had a pH of approximately 6.5. LE so prepared and supplementing the clear supernatant from autoclaved liver homogenate (ALH), which also had a pH of about 6.5, supported maturation and reproduction with subsequent

TABLE I

Growth of Rhabditis briggsae *on Basal Media Supplemented with Various Sources of Factor Rb*

Source[a]	Protein content (mg/ml)	Basal Media[b]					
		K-D		ALH		None	
		Maturation	Reproduction[c] with subsequent growth	Maturation	Reproduction[c] with subsequent growth	Maturation	Reproduction[c] with subsequent growth
None		−	−	−	−		
CEJ (fresh or lyophilized)		+	++			+	−
CEJ (old)		+	±			−	−
LE, whole	50	+	++	+	++	+	−
	25	+	++	+	++		
	12.5	+	−	+	+		
	6	−	−	+	±		
fraction I	12.5			+	++	+	−
	6	+	−	+	++		
fraction II	10	−	−				
Human whole blood and plasma		+	−			+	−

[a] CEJ = chick embryo juice; LE = liver extract; fraction I = LE fraction precipitating out from 0–75% saturation with $(NH_4)_2SO_4$, dissolved in buffer, and dialyzed; II = that above 75%.

[b] K-D = Kidder-Dewey *Tetrahymena* medium; ALH = clear supernatant from autoclaved liver homogenate. (pH = 6.5.)

[c] ++ = production of massive culture; + = limited reproduction.

growth at levels down to 12.5 mg of protein (by dry wt) per ml of final medium. At lower levels (6 mg/ml) growth was slow and significant reproduction did not take place. As the criterion of maturation the production of egg-containing adults was used.

At least part, possibly all, of the growth-promoting activity of LE was shown to reside in the fraction (I) precipitated out up to 75% saturation with ammonium sulfate. This part was destroyed by autoclaving. Twice-precipitated fraction I by itself supported maturation of partly

grown larvae, but not significant reproduction. Massive cultures were obtained with fraction I plus ALH.

LE and especially fraction I supplementing the ALH "basal" medium supported maturation and reproduction with subsequent growth better than when supplementing the K-D "basal" medium (with pH adjusted to 6.5). In the latter case, fraction I protein showed a greater tendency to precipitate out.

By contrast with fraction I the fraction (II) of LE left after precipitation up to 75% saturation with ammonium sulfate was inactive. In fact it appeared toxic, for most larvae introduced into it not only did not grow, but were dead in a few days whereas control larvae in "basal" media alone, although not growing significantly, survived for several weeks. Only toluene-lyophil-sterilized fraction II was tested.

Numerous runs with LE and its fractions were carried out. Studies with LE were rendered difficult because of the gradual denaturation and hence precipitation of protein. This resulted in a medium with an opaque, flocculent precipitate that impeded visual inspection. For this reason maturation and reproduction time were only estimated. With fraction I, however, less precipitation was encountered.

With Human Blood and Plasma

Human whole blood and plasma, supplementing K-D medium, supported growth of *R. briggsae*, and large adults were produced in media in which the whole blood constituted only 1/8 by volume of the total. At 1/20 of the total, blood supported development of mature, but rather smaller worms. Blood dilutions were made with distilled water; only undiluted plasma was tested. In the clear plasma medium and in that containing 1/8 by volume of whole blood it was possible to observe that a few II generation larvae were produced, but these died without growing appreciably.

The foregoing investigations, with their quantitative data, are summarized in Table I together with a few data from an earlier study (Dougherty, Raphael, and Alton, 1950), included for comparative purposes.

Discussion

Factor Rb, the heat-labile,[3] protein-like requirement of *R. briggsae*, has been shown to occur in fresh liver and in lyophilized CEJ, as well as in fresh CEJ, and to a certain extent at least, in blood.

[3] The heat-lability was sharply emphasized by the fact that a number of batches

The studies reported here provide certain significant information about this factor. The most important thing is the demonstration that in LE, factor Rb activity follows the protein precipitating from 0 to 75% saturation with ammonium sulfate. The failure of factor Rb to diffuse through a dialysis membrane has shown that it is not a small molecule. The fact has been demonstrated that it resists the chemical effects of toluene and physical effects of lyophilization, hence also of freezing; it has in addition been observed to survive repeated ordinary thawings.

In a previous paper (Dougherty, Raphael, and Alton, 1950) failure to grow *R. briggsae* on Seitz-filtered LE was reported despite preliminary success described in a still earlier paper (Dougherty and Calhoun, 1948). It is now possible to offer an explanation for this discrepancy. The earliest attempts were made with LE prepared by homogenation of liver and filtration of this homogenate first through filter-mesh and then through a Seitz-filter under considerable pressure (2-3 atmospheres). Usually the product was Seitz-filtered a second time. The later attempts were made with LE Seitz-filtered by suction rather than pressure. In either case, with pressure or suction, the initial Seitz-filtration was very slow. Although quantitative estimations of protein were made in none of these preparations, it seems reasonable to assume that the earliest procedure resulted in LE with considerably higher protein content; this is born out by the fact that the typical flocculent precipitate of LE on standing in the "complete" medium was much more abundant in the earliest trials. By contrast with the results so far described, LE prepared by high-speed centrifugation passes relatively rapidly through a Seitz-filter. The protein content is nevertheless reduced from approximately 100 mg to 50 mg (by dry weight)/ml. Thus the explanation for failure of *R. briggsae* to grow on certain LE preparations almost certainly lies in the fact that these were too poor in protein content, hence in factor Rb, to support growth.

Other important new information on the nutritional requirements of *R. briggsae* derives from the observation that at the same pH a medium combining LE fraction I and ALH supports much better growth than does one combining the former and the K-D components. It is not possible at this time to do more than speculate on why this is so. ALH may

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of LE prepared during an early phase of the studies with it were discovered to be inactive, and upon careful checking this was found to be due to what had been considered minor heating during centrifugation for an hour on a conventional-type centrifuge. Probably the LE went up to above 50°C.

have additional important growth factors, or it may merely be a more favorable balance of constituents. In any event, ALH provides a better basis from which to work out nonprotein requirements of *R. briggsae.* Studies on vitamin supplementation of CEJ give evidence that one or more known vitamins are needed by *R. briggsae.* In view of the accumulating information on the comparative nutritional needs of animal organisms of diverse groups this is not surprising, for most of the B vitamins appear to be universal animal requirements. Vitamin supplementation of CEJ suggests that loss of its growth-promoting activity on standing at ice-box temperatures must be at least partly related to degradation of vitamin content. ALH plus fraction I support growth as well as media in which factor Rb activity is provided by fresh CEJ, and is, moreover, much more practical.

It seems obvious from a number of observations that the most critical phase of *R. briggsae* growth is that of the early larva. Media that provide for the initial development of large adults from small larvae (probably of the second or even third larval stage) may be quite inadequate for the production of a viable subsequent generation. This has been best noted in blood-/or plasma- supplemented cultures, in which, by reason of the clearness of the medium, limited reproduction has been observed with ease, but in which the few larvae produced have failed to grow or survive long. Moreover, in certain cultures on chick embryo carried out as already described (Dougherty, 1950a), an observation not previously recorded is that great numbers of eggs have been seen to accumulate without hatching, and these, if several days or more of age, have proved non-viable on being transferred to slants with *E. coli.* Whether such effects are due to dietary deficiencies or to inhibitory agencies cannot be said at this time.

In nutritional requirements *R. briggsae,* as well as *R. pellio* previously studied (Dougherty and Calhoun, 1948), has now been shown to resemble the free-living larvae of certain nematodes that are parasitic as adults. Most recently, Weinstein (1949) carried out such investigations, studying the hookworms, *Ancylostoma caninum* and *A. duodenale,* and the trichostrongyle, *Nippostrongylus muris;* these forms are members of the suborder Strongylina and belong with the rhabditids (suborder Rhabditina) in the order Rhabditida. Like the latter the strongylines can be grown through free-living stages on CEJ (or rat LE). This points to the likelihood that factor Rb may be a fairly wide requirement among nematodes. It is interesting to note that Weinstein observed that rat LE may

"at times contain a factor which acts as a powerful growth inhibitor" for his forms. Perhaps this inhibition is related to the toxic properties of fraction II.

Factor Rb shows obvious similarities to the pressed yeast juice factor required by *Paramecium multimicronucleatum* (Johnson and Tatum, 1945); the latter also is heat-labile, non-diffusible in dialysis, and precipitated out at 75% saturation with ammonium sulfate. Such heat-labile requirements are probably quite widespread among animal organisms. Whether in such cases the intact protein serves as a metabolite, or whether some substituent part actually is the requirement remains to be determined. It is even possible that factor Rb and similar substances are only necessary in axenic cultivation to prepare the substrate (as by enzyme action) or to act in some physicochemical way for the organism.

Immediate efforts are now being directed on the one hand to further fractionation of LE and on the other hand to the definition of the essential components of ALH. Further fractionation of LE has already been accomplished and will be reported in a subsequent publication.

Summary

1. A heat-labile, proteinlike substance (or group of substances) required by the nematode *Rhabditis briggsae* for growth and reproduction under axenic conditions is here shown to be non-dialyzable and to be precipitated by ammonium sulfate "salting-out." This has been called factor Rb.

2. It is provided in chick embryo juice (CEJ), either fresh or lyophilized, in aqueous liver extract (LE) sterilized either by a procedure involving mixing with toluene followed by lyophilization or by ultra-filtration, and at least partly in human whole blood and plasma. The best sources are fresh or lyophilized CEJ and a LE fraction.

3. Factor Rb activity follows that part (fraction I) of LE precipitated out up to 75% saturation with ammonium sulfate buffered at pH 7.0. It is not contained in the unprecipitated part (fraction II).

4. Fraction I supplementing the clear amber fluid (ALH) produced by autoclaving liver homogenate (i.e., poured off from the heat-precipitated protein) provides a much better medium than one comprised of fraction I supplementing the Kidder-Dewey *Tetrahymena* (K-D) medium. It compares favorably with media consistng of CEJ and the K-D ingredients.

5. The relation of factor Rb to heat-labile requirements in other nematodes and in *Paramecium multimicronucleatum* is briefly discussed.

6. Studies are now under way on the further fractionation of LE and the definition of the essential ingredients in ALH.

References

DOUGHERTY, E. C. 1950a. Sterile pieces of chick embryo as a medium for the indefinite axenic cultivation of *Rhabditis briggsae* Dougherty and Nigon, 1949 (Nematoda: Rhabditidae). *Science* **111**, 258.

DOUGHERTY, E. C. 1950b. Some sources and characteristics of the heat-labile nutritional requirement(s) of the nematode, *Rhabditis briggsae*. *Anat. Rec.* **108**, 514.

DOUGHERTY, E. C., AND CALHOUN, H. G. 1948. Experiences in culturing *Rhabditis pellio* (Schneider, 1866) Bütschli, 1873 (Nematoda: Rhabditidae), and related soil nematodes. *Proc. Helminthol. Soc. Wash.* **15**, 55–68.

DOUGHERTY, E. C., RAPHAEL, J. C., JR., AND ALTON, C. H. 1950. The axenic cultivation of *Rhabditis briggsae* Dougherty and Nigon, 1949 (Nematoda: Rhabditidae). I. Experiments with chick embryo juice and chemically defined media. *Proc. Helminthol. Soc. Wash.* **17**, 1–10.

GLASER, R. W. 1940. The bacteria-free culture of a nematode parasite. *Proc. Soc. Exper. Biol. Med.* **43**, 512–514.

GLASER, R. W., MCCOY, E. E., AND GIRTH, H. B. 1942. The biology and culture of *Neoaplectana chresima*, a new nematode parasitic in insects. *J. Parasitol.* **28**, 123–126.

GLASER, R. W., AND STOLL, N. R. 1938a. Development under sterile conditions of the sheep stomach worm *Haemonchus contortus* (Nematoda). *Science* **87**, 259–260.

GLASER, R. W., AND STOLL, N. R. 1938b. Sterile culture of the free-living stages of the sheep stomach worm, *Haemonchus contortus*. *Parasitology* **30**, 324–332.

GLASER, R. W., AND STOLL, N. R. 1938c. Sterile culture of the free-living and parasitic larval stages of *Haemonchus contortus*. *J. Parasitol.* **24** (suppl.), 16.

JOHNSON, W. H., AND TATUM, E. L. 1945. The heat-labile growth factor for *Paramecium* in pressed yeast juice. *Arch. Biochem.* **8**, 163–168.

LAWRENCE, J. J. 1948. The cultivation of the free-living stages of the hookworm, *Ancylostoma braziliense* de Faria, under aseptic conditions. *Austral J. Exper. Biol. Med. Sci.* **26**, 1–8.

METCALF, H. 1903. Cultural studies of a nematode associated with plant decay. *Tr. Am. Micr. Soc.* **24**, 89–102.

RAILTON, I. R., CUNNINGHAM, B., AND KIRK, P. S. 1941. The preparation of sterile proteins in the "lyophiled" state. *Science* **94**, 469–470.

STOLL, N. R. 1940. *In vitro* conditions favoring ecdysis at the end of the first parasitic stage of *Haemonchus contortus* (Nematoda). *Growth* **4**, 383–406.

STOLL, N. R. 1948. Axenic cultures of *Neoaplectana glaseri* Steiner in fluid media. *J. Parasitol.* **34** (suppl.), 12.

WEINSTEIN, P. P. 1949. The cultivation of the free-living stages of parasitic nematodes in the absence of living bacteria. *J. Parasitol.* **35** (suppl.), 14.

ZIMMERMANN, A. 1921. Recherches expérimentales sur l'élevage aseptique de l'anguillule du vinaigre (*Anguillula oxophila* Schneider). *Rev. Suisse Zool.* **28**, 357–380.