The Sterilization and Preliminary Attempts in the Axenic Cultivation of the Black Planarian, *Dugesia dorotocephala.*

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Introduction

Research on regeneration has been principally on worms, chiefly planarians, on hydra and on the limbs and tails of urodeles. Fresh-water planarians possess the remarkable, almost unlimited, power to regenerate the body after injury. This phenomenon has been thoroughly demonstrated and studied with a variety of techniques by many investigators. Brøndsted (1) has recently published an excellent review and evaluation on several phases of planarian regeneration. However, a close examination of the literature on this field of study leads one to conclude that little insight into the physiological or chemical control of the process of regeneration has been gained. Consequently the interest in planarians as experimental material has lagged in recent years. There is, then, a definite need for biochemical studies on regeneration and related growth phenomena in planaria by the use of more modern techniques under rigorously controlled conditions.

It is the purpose of this paper to report our preliminary success toward establishing planarians in an axenic medium which will support growth of the whole worms and the regenerating parts thereof. To our knowledge this is the first report on the sterilization and aseptic handling of planaria or, for that matter, of any platyhelminth. Thus, the initial investigations have been directed toward the selection of the most suited materials and techniques for the work.

Materials and Methods

It would appear that the fresh-water organism, *Dugesia dorotocephala*, is quite suited for these investigations by virtue of its large size, its capacity to reproduce both sexually and asexually, and its high regenerative ability. This species was used throughout these experiments. Other species may show some advantages over *D. dorotocephala* for certain aspects of the work, and we are now examining *D. tigrina* with this point in mind (see below). *D. dorotocephala*, however, appears to be much more easily maintained under laboratory conditions and tolerates a wider temperature range than *D. tigrina*.

Stock cultures of the worms are maintained in fresh, boiled spring water at several different temperatures ranging from 4° to 30° C. with the lower part of the range being more suitable. As is well known, only a feeding or two per week of fresh beef liver is required to maintain a normal strain. Fewer feedings are required at the lower temperatures. The culture is easily built up merely by cutting the worms into pieces. Also, Child (3) reports that fission can be induced or prevented in *D. dorotocephala*, but we have not observed asexual reproduction by the method of fission.

We were fortunate in obtaining the sexual production of egg cap-
sules or cocoons, and at a time other than they are normally laid in a
natural habitat. According to Kenk (13), the periods of cocoon laying
are in the spring and summer months, and thus correspond in nature with
external factors, chiefly temperature. Kenk (12) believes there is an
inherent cyclic reproductive rhythum in *D. tigrina*, but Hyman (10)
found that the same species can, when fully grown, be caused at will to
develop a copulatory apparatus and lay viable eggs by exposure to low
temperature followed by a rise in temperature. Some time must elapse
before another sexual period can be induced. Apparently, by this same
method, we induced the one-hundred percent production of cocoons in
D. dorotocephala. We received a shipment of planaria in January, kept
them in the cold for a short while longer, and then, upon placing them at
a warmer temperature the worms began laying cocoons. Several indi-
viduals of the culture laid more than one cocoon. Experiments designed
to induce premature sexuality and egg production in both *D. doroto-
cephala* and *D. tigrina* under controlled temperature conditions are now
in progress.

This technique is quite important, for it has allowed the development
of not one but two successful methods of obtaining sterile material: first,
the treatment of a cut portion of an adult worm; and secondly, the
sterilization of the more easily handled cocoons. The sterilization was
accomplished, in both methods, by a procedure of aseptic washings and
treatment with antibiotics for varying periods of time.

Two primary obstacles which are encountered in attempts to free an
organism such as a planarian from all of its associated forms are: one,
eliminating the microflora from its extensive gastrovascular cavity; and
two, eliminating the organisms on the surface, as the animal is covered at
all times with a sticky mucus which affords an excellent asylum for
bacteria and other microorganisms. Our initial approach was designed
to minimize the difficulties. We first thoroughly washed the adult worm
in sterile spring water, to which had been added a little agar to effect
a more viscus solution for a purely mechanical action. An anterior cut
of the worm was then made in the region of the auricles, and treatment
was continued on the head portion only, thereby eliminating virtually all
of the "gut" along with its microfloral content. It should also be re-
called that the head is the region of the highest metabolic activity, and
therefore, it was reasnoed that this portion, as apposed to a more
posterior segment, would be more favorable to work with from the point
of view of better viability and faster growth rate. The heads were washed
a few more times and placed into various concentrations of different
antibiotics. The sterilization of the cocoons also involved a pre-washing
step through several changes of the sterile spring-water-agar solution,
and of spring water alone, prior to placing them into the antibiotic
solution. Both the heads and the cocoons were allowed to remain in the
antibiotic solution for varying periods of time, after which they were
again aseptically washed and carefully transferred to a sterile medium.
The washings and treatments with antibiotics were performed at room
temperature (23-27° C.) in sterilized depression slides inside of sterilized

petri dishes. Culturing was carried out in test tubes, different kinds of flasks, and depression slides. Low magnification observations could readily be made in some instances. No fixing or staining of the material has as yet been done.

Results

In the initial experiments, on the cut head portions, it was necessary to determine what the most effective antibiotics were, and the optimal concentrations of them, which would effect the desirable bactericidal action and yet remain non-toxic to the planarian tissues. Fourteen heads were treated and, of these, only three survived the testing because of antibiotic toxicities, contaminations, and the unfavorable effects of certain media. A similar survey treatment was carried out on the cocoons once they were obtained. Those antibiotics tried were; penicillin, dihydro-streptomycin, achromycin, and sulfadiazine. Potassium penicillin-G at a concentration of 2,500 to 5,000 units/ml and dihydrostreptomycin sulphate at a concentration of 25 to 50 mg/ml, used both singly and, more effectively, in combination were effective in producing sterile material. The length of time that the material may be allowed to remain in the antibiotic solution depends, of course, upon the concentration of the antibiotic and upon the type of material used. The cut head portions were, as expected, more susceptible to antibiotic toxicity than were the cocoons. The heads exposed to the penicillin-streptomycin solution for periods of 18 hours or less showed the best survival, while the cocoons left in the same concentration for periods of 48 hours, were found to hatch viable worms.

In these studies it has become quite obvious that the cocoons offer the most favorable starting material in obtaining sterile planarians. When the production of the cocoon or egg capsule is completed it contains several eggs, usually from 2 to 20, and hundreds of yolk cells. The cocoons which we have observed from *D. dorotocephala* are spherical to ellipsoidal in shape and about one millimeter in diameter. When first laid the cocoon is soft and bright orange in color. After a few hours, the surface hardens and the color changes to red, and finally to dark brown. At normal temperatures the egg capsule will hatch in from one to two weeks. They may be stored at lower temperatures to delay hatching for longer periods of time. Thus, in comparison to a cut portion of an adult worm, the cocoon may be subjected to rather rigorous treatment and more versatile handling.

We have treated approximately 50 cocoons and placed them into a variety of different media, obtaining from each cocoon one to seven minute, non pigmented, worms. About 80 percent of the cocoons hatched sterile worms after the antibiotic treatment. Sterility was checked in all cases by noting contamination of the nutrient medium into which they were placed, and by periodic checking for contamination by several different bacteriological procedures such as plating-out, nutrient broth, thioglycollate medium, etc.

The growth and length of life of the sterile worms varied greatly depending upon which medium they were placed in. Although several

different media have been tested with both hatched worms and the head pieces, little can be said about which is the best at the present time. The media used were composed of different combinations of the following; an autoclaved, aqueous extract of fresh beef liver; Difco liver extract; heat-killed baker's yeast; various preparations of yeast extracts; Difco proteose-peptone; egg yolk; and egg white. We have also tried variations of the rather complex *Paramecium* axenic medium (19), and we have even introduced sterile planarians into an axenic culture of paramecia. Several different concentrations of these various nutrients were tested. In all cases the dilutions were made with spring water.

Two of the sterile head pieces lived for two weeks in a dilute liver solution, and showed good regeneration before being lost in manipulation. More than seventy-five sterile-hatched planarians were maintained from periods of a few days to as long as six weeks. Some lived for a few weeks in sterile spring water alone, but those cultured in liver extracts, yeast extracts, and the egg yolk medium showed the greatest increase in size and the most extensive pigmentation. The liver and yeast extracts were heat sterilized, while the egg yolk was removed aseptically from a fresh egg and diluted with spring water. A detailed description of various specific media tested has been purposely omitted because the results on the growth of the worms is still quite preliminary. The worms that had survived for several weeks died near the end of the school year when the temperature in the laboratory was high. Thus, there is some reason for believing that unfavorable temperature conditions may have caused the termination of the first experiment. Current experiments are being carried out in an air conditioned laboratory.

Discussion

The flatworms have been neglected biochemically in spite of the great amount of research which has demonstrated their remarkable powers of regeneration. The prospects of obtaining planarians in pure culture, described in this paper, might pave the way for a more penetrating analysis of regeneration and the regulation of metazoan growth than has hitherto been possible. The most significant contribution of this work is the demonstration of the ease of obtaining sterile material via the treatment with antibiotics. The sterilization of planarians or of tissue therefrom has been attempted by others. Murray (15), in her doctorate work under Child, re-examined some of the earlier unsuccessful attempts to use such bactericidal agents as mercuric chloride, mercurochrome, and hydrochloric acid (which causes the copious secretion of mucus by the worms and which may be followed by aseptic washings). She also reported on her use of ultra-violet radiation to effect sterility. This appeared the most promising of all the treatments. In the use of U. V. radiations for sterilization purposes one would, of course, need to take into account its mutagenic effects on the planarian tissue. Dougherty and co-workers (9) have used similar techniques in rendering two representatives of rhabditoid nematodes aseptic. They accomplished this by a 24-hour treatment of gravid females with a mixture of penicillin and streptomycin, followed by sterile water rinses and a 1-hour exposure

to an aqueous solution of merthiolate. Further treatment involved the viable larvae only. Axenic cultures of these worms were maintained for many transplants on a complex medium of nutrient agar supplemented with Seitz-filtered liver extract on which fractionation was later carried out (8).

The fact that we have been able to culture sterile planaria for a period of six weeks in a heat sterilized media is very encouraging. However, we are now examining filter sterilized liver extracts in the hope that it may support better growth. Once a workable culture method is perfected, an attempt will be made to chemically define the medium. It should be obvious that a completely defined medium, although desirable, need not exist in order to begin some very exacting biochemical studies; but a sterile or axenic medium is most essential.

Some work on the inhibition and acceleration of growth and regeneration in planarians by specific compounds such as carcinogenic hydrocarbons (16, 17), sulfhydryl groups (5), and amino acids (14) has been reported. These were carried out in uncontrolled media and have thus lead to contradictory results. Never-the-less such findings would be worth re-examinating under controlled conditions. Brøndsted (2) has recently reported that ribonucleic acid accelerates the rate of regeneration in starved planarians. This correlates with the finding by Clement (4) that ribonucleic acid disappears during organogenesis of the regenerating organisms. Brøndsted (1) also reports that B-vitamins, especially riboflavin, accelerates regeneration. And Weimer et. al. (20) record an acceleration of eye formation in *Phagocata gracilis* by thyroxin.

One other series of observations seems worthy of consideration in a discussion of planarian nutrition. Wulzen and Bahrs (21) reported the development of a specific disease in planaria fed on the tissues of guinea-pigs and rabbits which lacked greens in their diet. When greens were included in the diet of the rodents, the planarians thrived on their tissues. When the guinea-pigs were maintained on the deficient diet for months, a general muscle stiffness developed, followed by extensive calciferous deposits and finally death. Van Wagtentonk and Wulzen (18) succeeded in isolating a sterol from plant sources with antistiffness activity which was later identified as stigmasterol (11). In the course of this work the assay shifted entirely to mammalian organisms, and the direct need for the "antistiffness factor", or stigmasterol, as an essential metabolite for planaria has never been investigated. In the development of an axenic medium for *Paramecium aurela*, Conner, van Wagtendonk, and Miller (7) found that this organism also required a plant factor. The "antistiffness factor" could replace the "P. aurelia plant factor", and it was subsequently shown that stigmasterol was one of the most active of the sterols tested (6). Other unrelated organisms have now been shown to require sterols for growth, and from the above discussion it would seem probable that planaria might fall into this pattern.

These interesting but isolated observations might be brought together and re-examined in an axenic culture of planaria with the possibility of obtaining specific knowledge of the chemical requirements for planarian regeneration.

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