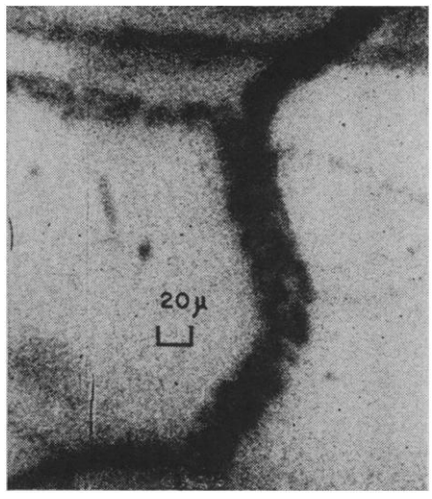

Fig. 3. Venule of bulbar conjunctiva of man. Magnification on original 16-mm film was × 20.

revealed large gaps of plasma between orderly files of erythrocytes. Similar studies in 13 patients, chosen because of profound erythrocyte aggregation of their blood in vitro (five patients with myeloma, five patients with severe coronary artery disease, and three patients with macroglobulinemia) revealed marked cell aggregation (clumps of 5 to 30 cells) in all vessels. Flow was considerably slower than in the normal, and in many vessels stasis was apparent. Vessel patterns demonstrated marked tortuosity and dilatation. These observations support the earlier recordings of conjunctival blood flow observed at lower magnifications (*1*). The observations of a cell-free layer of plasma at the wall, as noted in studies in vitro (*2*), were not confirmed by these studies in vivo. Red cells moved in a very heterogenous fashion without maintaining any uniform streamlines in both arterioles and venules.

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Culture of a Planktonic Calanoid Copepod through Multiple Generations

Abstract. Acartia tonsa *has been propagated through 12 filial generations during 1 year in small laboratory cultures. The mean generation time was 25 days at 17°C on a slightly suboptimum, mixed algal diet. Body size, reproductive capacity, and generation time were unchanged during the culture period.*

Copepods, particularly calanoid copepods, are the principal link between primary (photosynthetic) producers and carnivores in the sea. Their biology has been studied in the laboratory for several decades with the use of animals taken directly from nature. However, many aspects of their biology need to be studied under a degree of experimental control possible only with material of known uniform age and physiological status. For such studies substantial numbers of copepods must be raised in the laboratory under defined conditions.

The few instances where marine copepods have been bred and reared in the laboratory have involved noncalanoid species (*1*) and, in one instance, a nonplanktonic calanoid (*2*). Holoplanktonic marine calanoids seem to have been completely refractory to laboratory propagation for over 50 years, although several species, when brought into the laboratory after having mated in nature, will produce eggs that can be raised to the adult stage (*3*). Subsequent generations apparently have not been obtained in such cases.

From October 1963 to March 1964, starting with adults taken from the Chesapeake Bay, we successfully raised limited numbers of five consecutive generations of the holoplanktonic calanoid *Acartia tonsa* Dana (*4*). The animals were lost after an air-conditioning failure in our culture room, but we have now reestablished a culture of the same species from adults taken off the Virginia coast in late 1964. This new line has thus far been propagated into the 12th filial generation and continues to exhibit a vigorous and normal-appearing reproductive potential. Descriptions of our technique and the growth characteristics of the first ten generations are given in this report.

Stock animals are maintained and most experiments are carried out unaerated in various-sized pyrex crystallizing dishes covered with a flat piece of glass. Dishes that are 190 mm in diameter and contain 1500 ml of culture allow better survival than smaller dishes do, even when each size contains the same number of copepods per unit volume (*5*). Membrane-filtered sea water (salinity about 31 per mille) is used unsupplemented and usually unbuffered. We have buffered certain experiments with tris [tris (hydroxymethyl) amino methane] at 500 mg per liter brought to the *p*H of sea water with HCl. This system has no effect on the survival of nauplii or adults, and continued exposure likewise has no effect on hatching and maturation. Other systems and higher tris concentrations were not tested. All stocks and those experiments in which temperature and illumination are not variables are kept in a constant-temperature room at 17° ± 1°C under constant illumination in the range of 650 to 1300 lux (*6*).

Food organisms are added from pure cultures of various flagellates and diatoms at the time of each transfer to fresh medium and thereafter at 3-day intervals. Since the F_2 generation we have used a mixture of approximately equal parts of *Isochrysis galbana, Rhodomonas* sp., and an unidentified small diatom (5 to 6 μ). About 10,000 (total) cells per milliliter of copepod culture are added at the initial feeding and about 4000 cells per milliliter at each subsequent feeding. This allows a concentration of about 10,000 to 38,000 cells per milliliter to be maintained in the culture dishes at all times. The same feeding regimen is used for nauplii, copepodites, and adults.

Stock nauplii are not ordinarily removed from the parental dish until nauplius stage III or IV is reached. Offspring are then transferred into fresh medium to a final concentration of about 40 nauplii per liter. After producing-females are obtained, the concentration is further reduced to no more than five such animals in a 1500-ml culture in order to restrict production in any given dish and thereby prevent excessive concentrations of nauplii (*7*). Transfers are made with pipettes that have apertures of 1 to 3 mm in diameter, the size depending on the developmental stage of the animals involved. The animals are released very slowly beneath the surface of the medium. Great care is necessary in handling these animals, and significant differences in survival have been found when identical cultures were transferred

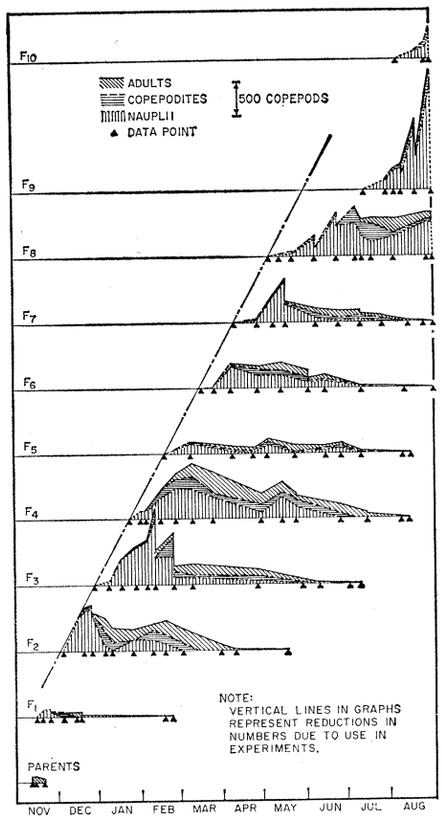

Fig. 1. Laboratory production of *Acartia tonsa* from 10 November 1964 through 1 September 1965.

by experienced and inexperienced operators.

The record of laboratory production is shown in Fig. 1. Each graph shows the actual numbers of living nauplii, copepodites, and adults counted on each of the dates indicated as data points. These counts were obtained during transfer of all the animals of the stated generation into fresh dishes. Numbers shown through the F_8 generation represent the maximum numbers that could be handled by a single person in addition to his other duties; they do not represent total production. Considerable mortality has resulted from overproduction in some dishes because transfers could not always be made at the proper time.

Of particular interest whenever a species is newly brought into culture is whether or not a reduced vitality occurs in later generations because of prolonged maintenance under the "unnatural" conditions of the laboratory. Some indicators of such an effect are the prolongation of maturation time, reduction in reproductive potential, and diminution in body size. Figure 1 shows that the total time of development has remained stable at around 25 days (dashed line through the origins of the graphs) except for generations F_1 to F_2 and F_8 to F_9, which are discussed below. Figure 1 also suggests that the reproductive potential has not diminished, although this cannot be determined with certainty since the numbers always represent less than total production. Average length of the cephalothorax of the female parents was 839 μ. For representative filial generations the average lengths in corresponding samples were 827 μ (F_3), 818 μ (F_5), 830 μ (F_7), and 849 μ (F_9). An analysis of variance shows ($P >> .05$) no significant change in adult size during nearly a year in laboratory culture.

The longer generation time between F_8 and F_9 resulted from the use of the earliest F_8 animals for experiments, before they matured. Stock dishes containing the first hatchings of F_9 were not touched, which resulted again in a generation time of 25 days for F_9 to F_{10}.

The shorter generation time for F_1 to F_2 is probably the result of subjecting the F_1 animals to more favorable nutritional conditions. During most of their development, these animals remained in the same vessel with their parents, and a considerable amount of natural food was carried over during transfer of the parents from a concentrated raw sample. In addition, cultured food was added regularly, so that the total concentration of food was much greater than for any subsequent generation. We have found that the nauplius development time, and consequently the total development time, can be shortened significantly from that shown in Fig. 1 by increasing the concentration of our standard food mixture without altering its quality (*8*). Heinle (*9*) has recently obtained short-term propagation of *A. tonsa* in sealed containers of raw water from the Patuxent River estuary and also has found a shorter total development time than we report in Fig. 1. Food concentration in his containers is, on the basis of counts we made on similar samples, initially two to four times the maximum concentration used in our cultures.

Thus our standard food mixture, used at a concentration ranging between 10,000 and 38,000 cells per milliliter, seems to be suboptimum, at least for nauplii. The 25-day development cycle is, however, roughly comparable to estimates for natural populations of *A. tonsa* made by Deevey (*10*) in Delaware Bay and Conover (*11*) in Long Island Sound, and by Raymont and Miller (*12*) for populations reared in large tanks of fertilized sea water. We have avoided using greater food concentrations in our stock cultures, since more frequent transfers would be required to prevent heavy accumulation of detritus. Eggs of *A. tonsa* settle to the bottom of the dishes, and we have observed that newly hatched nauplii tend to become fouled in such accumulations with a concomitant increase in mortality.

Most attempts to culture planktonic marine calanoids appear to have been made with pelagic species which seem to mate rarely or not at all under the laboratory conditions provided. *Acartia tonsa*, however, mates readily under a wide variety of conditions and seems to be a less-exacting species generally. We believe, however, that gentle, minimum handling, and perhaps the multiple diet, have also been major factors in the successful culture of this animal.

Acartia tonsa has a wide distribution and is often the dominant species in the summer zooplankton along the Atlantic Coast of North America. It has been studied extensively in the field and probably constitutes an important link between the phytoplankton and larger predators in its area. The relative ease with which it can be propagated in the laboratory now makes possible a wide range of genetic, developmental, biochemical, and physiological studies heretofore not practicable with specimens taken directly from nature.

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A. P. Orr, *Rapp. Procès-Verbaux Réunions Conseil Perm. Int. Explor. Mer* **153**, 92 (1962); M. Anraku, *J. Oceanogr. Soc. Japan* **20**, 221 (1964)] have confirmed this only for very small volumes. In spite of the increased mortality that corresponds to a decrease in dish size, we have found that the minimum development time for *A. tonsa* has remained unchanged in our 100-, 150-, and 190-mm dishes containing 200, 700, and 1500 ml of sea water, respectively. This suggests that a comparable nutritional status exists among these volumes, but leaves the cause of the increased mortality unexplained. We did not study development time in less than 200 ml.

6. The necessity for employing such a high degree of temperature control has not been established, but experience suggests that reasonable control over sudden increases of temperature is necessary. The temperature of 17°C was chosen because it is suitable for both the copepods and our various algal cultures, and, if temperature control is lost, it allows extra time for corrective action before lethal temperatures are reached. The range of illumination used appears to have no effect on either development or survival; investigations outside of this range are incomplete.
7. Although the concentration limit for satisfactory development with our standard feeding regimen has not been determined, cultures containing 100 or more nauplii per liter will usually show retarded or erratic development followed by high mortality. On one occasion, however, a natural population of *A. tonsa* eggs and nauplii matured and subsequently was maintained for at least a week at a concentration of more than 600 animals in 300 ml of sea water that contained a heavy dinoflagellate bloom. Adult mortality was low and nearly 100 percent mating occurred, but survival of the offspring was negligible.
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13. We thank Kathy Kirouac for technical assistance during the later phases of this work.

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Antagonism of Purified Asparaginase from Guinea Pig Serum toward Lymphoma

Abstract. *The tumor inhibitory activity of highly purified asparaginase from guinea pig serum toward the Gardner lymphosarcoma in C3H mice was compared with that of the serum itself. The purified enzyme, homogeneous by ultracentrifugation and immunoelectrophoresis, had activity comparable to that of the serum. The serum was also effective in mice which were made immunologically incompetent by radiation with cobalt-60.*

Kidd reported that normal guinea pig serum had inhibitory activity against certain transplantable lymphoma tumors in mice (*1*). Broome (*2*) suggested that the enzyme L-asparaginase (*3*) was the agent in the serum responsible for this effect. Evidence has been obtained that supports this suggestion (*4–7*).

We have recently obtained from guinea pig serum a preparation of L-asparaginase which is homogeneous in the ultracentrifuge under conditions of sedimentation equilibrium (*8*), shows the migration of a single homogeneous peak in an ultracentrifugal sedimentation velocity experiment (Fig. 1), gives a single band upon polyacrylamide gel electrophoresis (Fig. 2), and gives a single arc in immunoelectrophoresis (*9*). We now report the antagonistic effects of this highly purified preparation toward lymphoma and suggest that the evidence is conclusive that the enzyme L-asparaginase is responsible for the inhibitory activity of guinea pig serum.

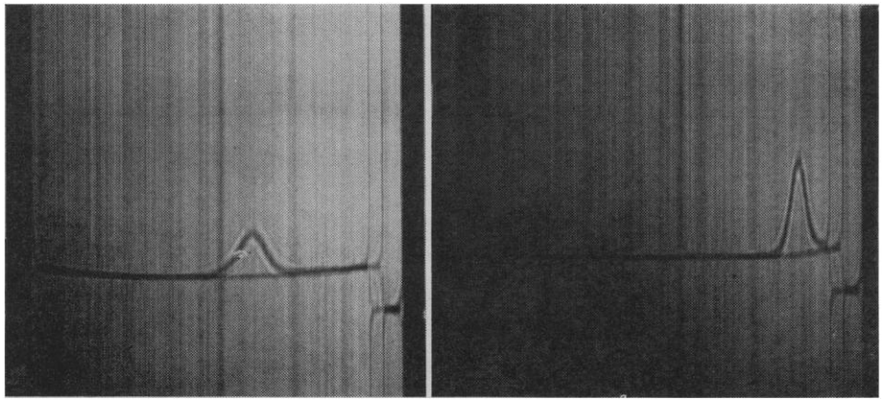

Fig. 1. Schlieren pattern of the descending boundary for guinea pig serum asparaginase in the double-sector cell after approximately 16 minutes (right) and 48 minutes (left) at 59,780 rev/min in the Beckman model E ultracentrifuge; 0.1*M* potassium phosphate buffer, *p*H 7.0, 25°C.

The inhibitory activity of the purified enzyme was tested as follows. Female C3H mice (Jackson Laboratories), weighing 18 to 25 g, were separated randomly into groups of ten animals each, and five were housed in a cage. Selection of treatments and the order of injections were also random. Each animal was given a single subcutaneous injection of 0.2 ml of a suspension of Gardner lymphosarcoma (6C3HED) cells in the right, lower ventral side. The suspension was prepared by briefly homogenizing 10 mg of tissue (wet weight) per milliliter of saline in a small Potter-Elvehjem homogenizer. The tissue was a 1-week-old tumor carried subcutaneously in C3H mice. Immediately after transplanation of the tumor, one group received an intraperitoneal injection of 0.5 ml of saline containing 1 mg of crystalline bovine serum albumin (BSA) per milliliter, another group 0.5 ml of guinea pig serum, and the third 0.3 ml of purified asparaginase solution to which was added BSA (1 mg/ml). Albumin is necessary to protect the enzyme against surface denaturation that occurs when the enzyme is highly purified and in dilute solution. After passage of enzyme solution through the needle used for injection, there was no loss of activity. One experimental group was given purified asparaginase (160 units, 75 μg), and the other was given 90 units in whole guinea pig serum. Under similar conditions, a second injection of 1.0 ml of serum given 12 hours after the first injection invariably protected the animals against appearance of the tumor for at least 30 days; and if more dilute suspensions of cells (2 to 5 mg/ml) were used in transplantation, this dose schedule prevented the appearance of tumors entirely. Tumors which appeared after a long latent period (30 days) remain sensitive to the serum, but Kidd (*10*) has shown that repeated low doses of guinea pig serum can produce sublines of tumors resistant to inhibition by guinea pig serum.

In control animals the tumor appeared within 4 to 6 days as a tiny, circumscribed elevation of the skin at the site of injection; it was revealed as a solid nodule by palpation. The tumors went into a "log" period of rapid growth in 12 to 14 days, and the animals died in 16 to 20 days.