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The Dauerlarva, a Post-Embryonic Developmental Variant of the Nematode *Caenorhabditis elegans*

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In the postembryological development of the free-living nematode *Caenorhabditis elegans*, a morphologically recognizable, nongrowing stage, called the dauerlarva, may arise. Using synchronous populations and following growth and molting, it has been shown that the dauerlarva is formed by a facultative, reversible arrest at a specific point in the life cycle, the second of four cuticle molts, in response to external conditions.

At each molt a normal animal passes through "lethargus," a stage in which feeding and locomotion are transiently arrested. In the dauerlarva stage, feeding is arrested indefinitely and locomotion is markedly reduced. A simple quantitative assay, based on the exceptional resistance of dauerlarvae to sodium dodecyl sulfate (SDS), has been developed to study dauerlarva formation and its reversal. The SDS resistance of dauerlarvae requires both non-feeding and an especially impermeable cuticle. Dauerlarva formation can be efficiently induced by limiting the concentration of bacteria (the food supply), but not by complete starvation. Quantitative recovery to normal development can be induced by transfer to fresh medium with excess bacteria. Simpler stimuli can elicit recovery at slower rates, the principal factors besides nutrition being nutrition being optimal ionic and osmotic conditions and a noninhibitory concentration of animals. There are identifiable stages in recovery, beginning with a resumption of feeding. The cuticle, ultrastructurally very different from normal cuticle, is shed at the next molt, after which development appears normal. A temperature-sensitive mutant, which forms dauerlarvae at high temperature despite the presence of abundant food, is described, and the use of dauerlarvae for further mutant isolation is discussed.

INTRODUCTION

Many animals, faced with the onset of harsh environmental extremes, respond by producing developmental variants specialized for survival. These variants are of interest for at least two separate reasons. Firstly, because their formation generally requires time, it must be initiated by appropriate environmental cues which presage the harsh extremes. Such cues are frequently quite subtle, and it becomes intriguing to determine how they can trigger such pronounced changes. And secondly, because the variants undergo such profound changes, in which all organ systems are affected, they constitute a useful probe into the regulatory mechanisms governing an organism's behavior and development.

For the small soil nematode *Caenorhabditis elegans*, whose behavior and development are receiving much current attention (Brenner, 1974; Sulston and Brenner, 1974; Ward, 1973; Dusenbery, 1974; Dusenbery *et al.*, 1975; Byerly *et al.*, 1975; Ware *et al.*, 1975), the corresponding developmental variant is the dauerlarva, a juvenile stage specialized for survival under harsh conditions. This paper describes structural and behavioral characteristics of the dauerlarva, and establishes its position with respect to the normal developmental cycle. It also examines the environmental cues involved in dauerlarva formation and recovery, and describes the properties of a mutant defective in these processes. And finally it suggests how the dauerlarva can be used to study several interesting aspects of *C. elegans* behavior and development, notably feeding, molting, and chemotaxis.

MATERIALS AND METHODS

The nematode *Caenorhabditis elegans* var. Bristol, strain N2 was from the Cal-

tech stock collection, orginally obtained from S. Brenner. Its culture and handling using nutrient agar (NGMM) plates with bacteria (*E. coli* strain OP50) have been described elsewhere (Brenner, 1974; Dusenbery *et al.*, 1975). S-medium was as described by Sulston and Brenner (1974). Tryptone broth (0.8% Difco tryptone, 0.5% NaCl) and Hershey broth (0.8% Difco Nutrient broth, 0.5% Difco Peptone, 0.5% NaCl, 0.1% dextrose, pH 7.3) are standard bacteriological media. Nematode buffer (Bu/2) contained 0.35% Na_2HPO_4, 0.15% KH_2PO_4, 0.2% NaCl and had a pH of 7.0. Noble agar was obtained from Difco, propylene phenoxetol (PPX) from Nipa Laboratories, Ltd., London, and Temik, a cholinesterase poison, from Union Carbide.

Observations of behavior and morphology were made with a Wild M5 dissecting microscope, using infrared filtered transmitted light. Lengths were measured with an eyepiece graticule, using 1% PPX anesthesia to straighten and immobilize the animals. SDS-resistance was determined by treating animals with 1% sodium dodecyl sulfate (SDS), washing them twice with nematode buffer by sedimentation in a clinical centrifuge and applying them in a small volume to a local area on an NGMM plate with a bacterial lawn on another region to attract the live worms. Survivors were detected as animals which moved (spontaneously or when touched) 3–6 hr later, except where indicated otherwise. After SDS-treatment, dauerlarvae were physically separated from the dead animals when desired by centrifuging them into a solution of 9% (w/w) colloidal silica (Dupont Ludox, 150 μm particles) neutralized with H_3PO_4, made up in Bu/2. Thereafter they were thoroughly washed. Dauerlarvae were always used for experiments within 2 hr of preparation by SDS treatment and/or physical separation.

For synchronization, adults freed of small animals by differential settling were allowed to lay eggs for several hours on an NGMM plate with a bacterial lawn, and then carefully washed off with nematode buffer, taking care not to dislodge the eggs, which stuck to the surface. At approximately one-hour intervals the juveniles which hatched out since the last washing were similarly washed off, without disturbing the remaining eggs, and grown as a synchronous population. All culturing and other operations were at 22–23°C except as indicated.

Techniques for electron microscopy were as described by Ware *et al.* (1975).

RESULTS

Normal Development

Following embryogenesis, *C. elegans* hatches from the egg as a juvenile composed of about 600 cells. Subsequently it undergoes a several-hundredfold increase in mass with little change in form and very limited cell division except in the reproductive tissues. During this growth the animal moves and feeds (by pharyngeal pumping) almost continuously, except for a short period, called a lethargus, before each of the four molts. At the onset of each lethargus the pharyngeal pumping rate falls gradually over about 1 hr from the normal rate of 150–250 strokes/min to less then 1 stroke/min. It remains completely suppressed for the 1–2 hr lethargus period, and then returns abruptly to the normal rate over a few minutes. Separation and shedding of the old cuticle immediately follow the resumption of pumping. A second brief hiatus in pumping sometimes occurs just after shedding. General locomotion is also reduced during the lethargus period. The average time and size at which each lethargus occurs, as determined by pumping and activity measurements in 20°C-grown synchronous cultures, is shown in Fig. 3. The times are 13, 21.5, 29.5, and 41 hr after hatching, respectively, and the sizes are roughly 350, 470, 640, and 890 microns (μm) respectively.

Similar experiments, with qualitatively similar results, have been performed at growth temperatures of 22°, 23°, and 26°C;

although there is some (~40%) increase of developmental rate between 20° and 26°, the four molts occur at very nearly identical sizes in all cases.

Dauerlarva Properties

When adverse environmental conditions, not yet fully explored, interfere with normal development, juveniles of *C. elegans* can go into a dormant survival stage known as the dauerlarva (from the German, meaning "enduring larva"). A similar stage has been observed for the related nematode *Caenorhabditis briggsae* (Yarwood and Hansen, 1969) and other soil nematodes (Bird, 1971), and the so-called "infective larva" stage of parasitic nematodes appears analogous to all of these. Our results agree generally with those reported for *C. briggsae* dauerlarvae.

The dauerlarvae of *C. elegans* have several distinctive properties.

Morphology and ultrastructure. a) Most obviously, dauerlarvae are very thin, as shown in Fig. 1, with an axial ratio (length:width) of about 30:1, nearly double that of normal animals. b) They have an unusual internal appearance. There is no open space in the gut or pharynx where food would be in normal juveniles, and the gut cells have a characteristic dark appearance. c) Dauerlarvae have an increased specific gravity compared to normal juveniles, as measured in density gradients (R. C. Cassada, unpublished results). This density increase suggests that the thin shape is due to loss of a light component, e.g. water or perhaps lipid. d) Dauerlarvae have an altered cuticle, as seen in electron micrographs (Fig. 2). The outer cortex is somewhat thicker, and there is an additional striated underlayer, not seen in normal juveniles. This layer, which is also found in the infective larvae of parasitic species (cf. Bird, 1971), tapers down to a point and then vanishes at the lateral extremes of the animal, under the lateral alae (Fig. 2e). It appears to be replaced there by a fibrillar layer similar to that seen at the base of adult cuticle (Fig. 2c). e) Most, if not all dauerlarvae have a loose outer sheath when first formed. This is thought to be the retained second-stage cuticle as discussed below. It is readily lost with aging or upon washing and does not appear to be required to maintain the dauerlarva state. (Dauerlarvae without the outer sheath were used in most of the experiments described below.)

Resistance to harsh conditions. f) Dauerlarvae are less easily desiccated than normal juveniles, probably because they are less permeable to water (R. C. Cassada, unpublished results). g) They are also more resistant to inactivation by dilute acid, hypertonic glycerol or NaCl, guanidine hydrochloride, glutaraldehyde, and various detergents and anesthetics. Roughly speaking, for these treatments about an order of magnitude greater does is required to affect dauerlarvae, as compared to normal juveniles. h) Dauerlarvae survive preferentially in extremes of temperature outside the normal growth range of 15–25°C.

Behavior. i) Dauerlarvae have reduced activity similar to lethargus. Their pharyngeal pumping is completely suppressed and much of the time they lie motionless and straight, as if muscle tone were lost. However, they respond immediately to mechanical stimuli (touch, vibration of the growth plate) and while active they respond normally to chemical stimuli (independently observed by Ward, 1973). j) Dauerlarvae have the unique ability to stand on their tails on projections from the agar surface and, it aroused, to wave around in the air. Perhaps related to this, they are often found on inverted plates to have fallen from the agar surface into the drops of moisture which condense on the plate lids. These behaviors may serve a distribution function in nature, by allowing dauerlarvae to attach to appendages of any larger creature passing by.

An Assay for Dauerlarvae

Figure 4 shows the survival in 1% sodium dodecyl sulfate (SDS) of purified dauerlar-

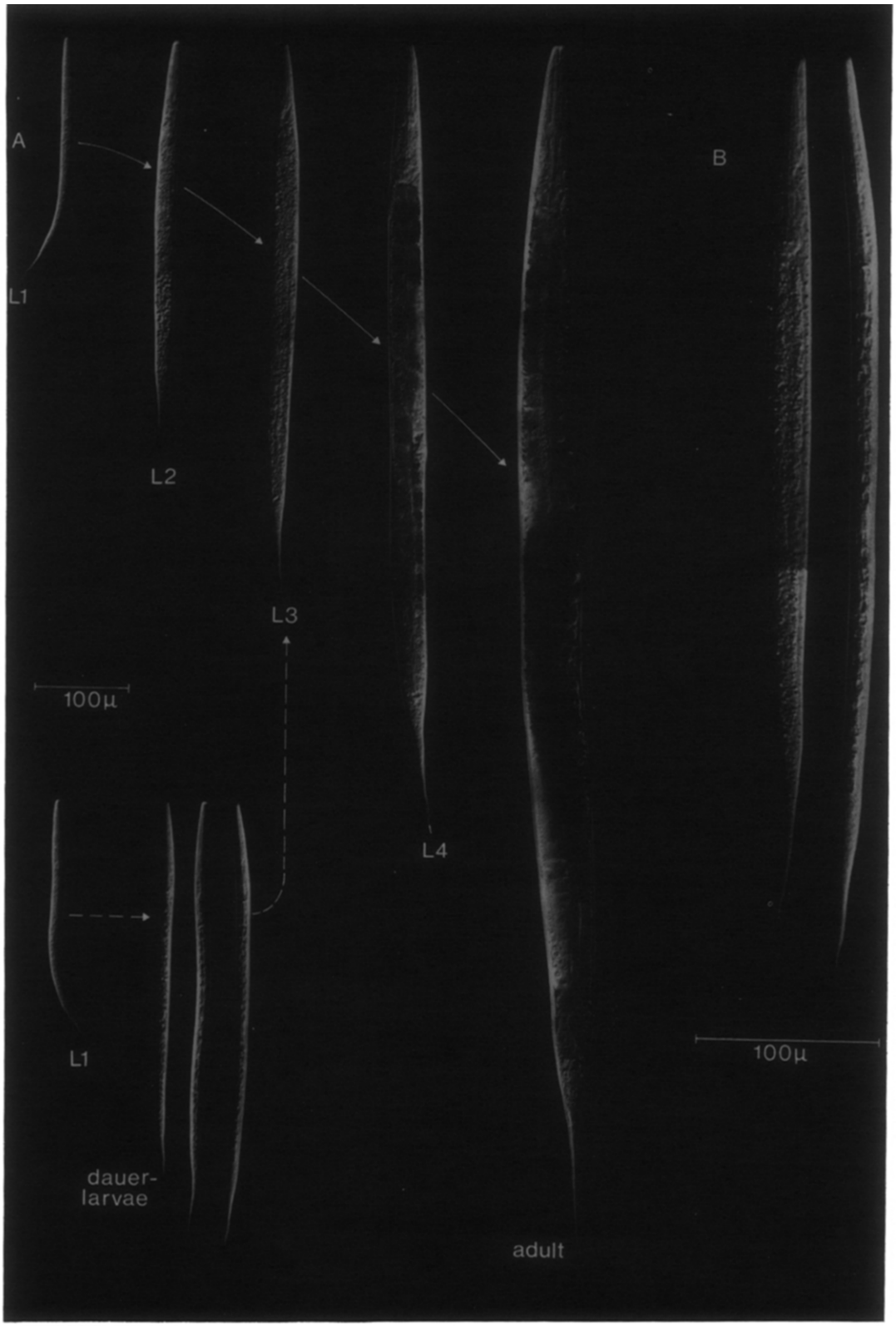

FIG. 1. Photomicrographs of *C. elegans* developmental stages: with Zeiss-Nomarski differential interference contrast. A. Normal juvenile stages L1–L4 and adult, together with dauerlarvae at same magnification. Direct developmental route depicted by solid arrows, dauerlarva route by dashed arrows. B. Normal second state (L2) juvenile and dauerlarva, at twice the magnification of part A. Note the thin, elongated dauerlarva shape. For these dauerlarvae the measured diameter was 14 μm, identical with that of L1 juveniles (14 μm) and much less than that of L2 juveniles (25 μm).

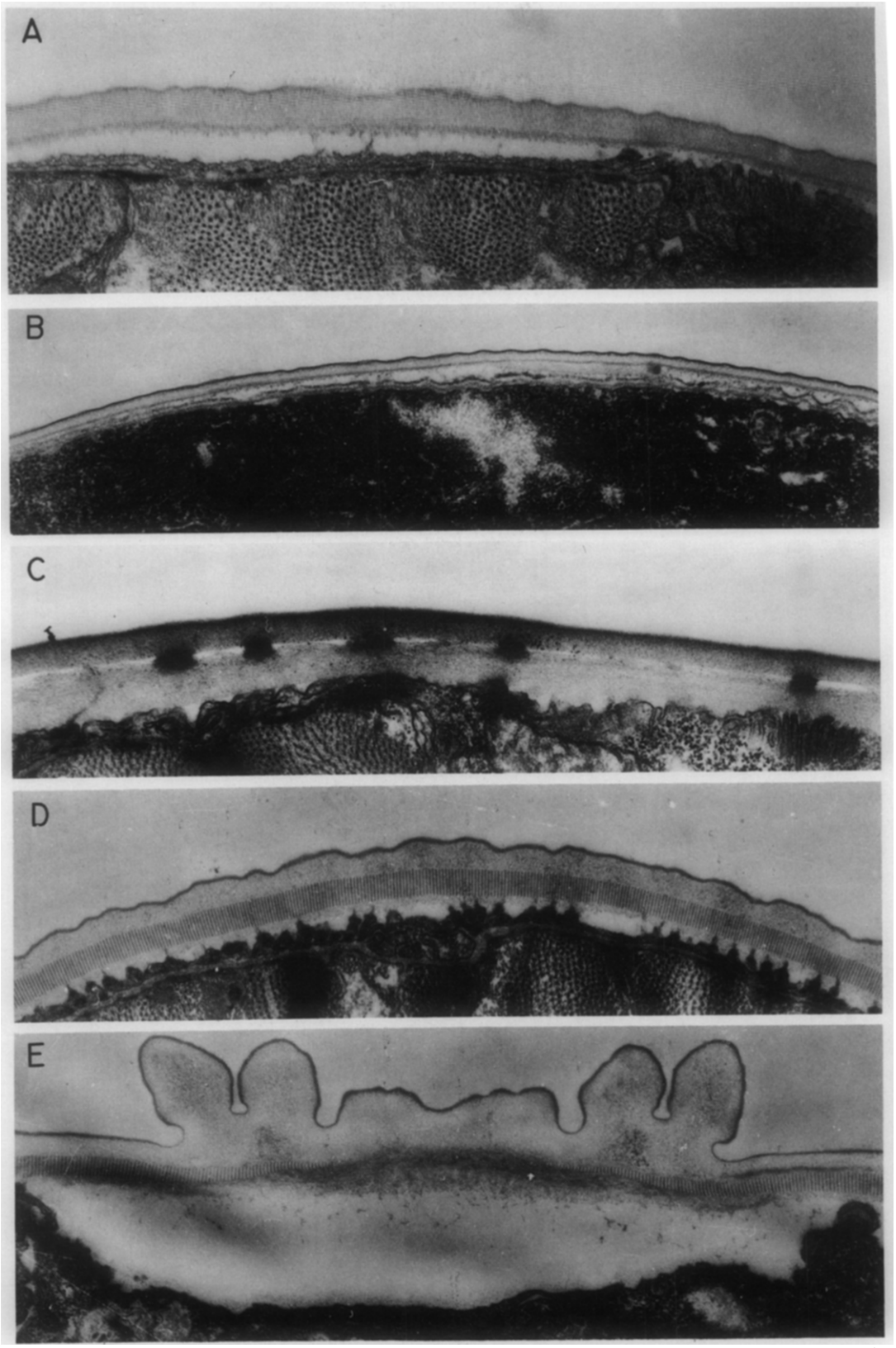

FIG. 2. Electron micrographs of cuticles of *C. elegans* developmental stages. A. Normal third-stage juvenile (L3). B. Normal juvenile at third molt (L3–L4). C. Adult. D. Dauerlarva. E. Dauerlarva at lateral line. Note striated layer in dauerlarva, tapering down at lateral line. All pictures are from 800 Å-thick transverse sections, at a nominal magnification of 21,000× (uncalibrated).

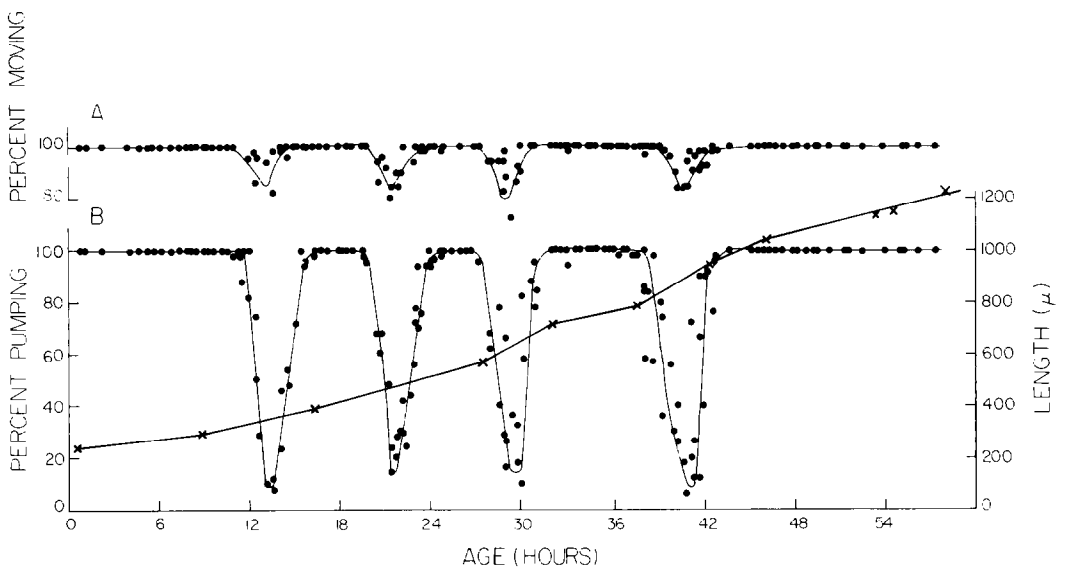

FIG. 3. Lethargus periods in normal development. Synchronous populations of several hundred animals hatched at time zero ± 40 min were grown at 20° with *E. coli* on NGMM plates. At frequent intervals, 50 animals were observed at 20°C for 5 sec each, using infrared-filtered "cool" light and taking precautions to minimize mechanical stimulation. The percentage which pumped and the percentage which moved any other part of the body during observation were separately recorded and plotted. At greater intervals a portion of the agar was removed, and 50 animals eluted from it were measured after PPX-anesthesia to determine an average length.

vae, normal adults, normal juveniles and just-laid eggs. Within a few minutes the normal worms dissolve, leaving ghosts and less than 0.1% survivors, whereas the dauerlarvae are stable for several hours. Although the eggs also survive a short SDS-treatment, they are easily distinguishable from dauerlarvae, and if a longer exposure is used, they also become SDS-sensitive, just before hatching.

When mixed populations containing dauerlarvae are treated with 1% SDS, all the survivors look distinctly like dauerlarvae, and virtually all visually identified dauerlarvae prove to be SDS-resistant when tested. Because of the dramatic difference in survival and the simplicity of the technique, SDS-resistance was chosen as an operational test for distinguishing dauerlarvae in the studies reported below. As will be shown, the SDS-resistance of dauerlarvae depends both on their lack of pumping and on their special cuticle.

Dauerlarva Formation

Several normal, uncrowded growing cultures containing a total of 10^4 animals were scanned for SDS-resistant dauerlarvae; none were found, although occasional SDS survivors arose either from internally hatched worms or from eggs. We conclude that dauerlarva formation is not an obligatory part of the *C. elegans* life cycle in an optimum environment. For parasitic nematodes, by contrast, the analogous "infective larva" stage *is* generally considered obligatory (Bird, 1971).

When the environment is less than optimum, as on old exhausted culture plates, *C. elegans* dauerlarvae can accumulate in sizable numbers. To obtain populations of dauerlarvae for further study, the following systematic method was used. From a densely grown plate with many worms and many ($\sim 10^4$) eggs, the worms were eluted just as the bacterial lawn was seen to be

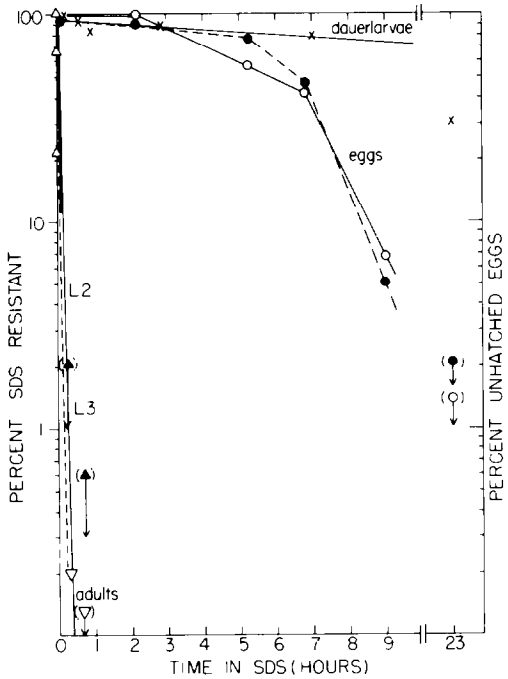

FIG. 4. Survival of various developmental stages in 1% SDS. Second-stage juveniles (L2; Δ), third-stage juveniles (L3; ▲) and adults (▽) were obtained by synchronous growth from eggs. Dauerlarvae were purified from an exhausted plate by a brief (3 min) treatment in 1% SDS. These were separately incubated in 1% SDS for the indicated time, diluted 50-fold in Bu/2 to stop the incubation, washed and plated as described in the methods section, and examined for survivors. Where no survivors were found, a bracketed point indicates the upper limit of the surviving percentage. Eggs: eggs laid over a 3.5 hr interval at 22° were used. One portion of several hundred eggs was allowed to hatch without treatment to determine the percentage of unhatched eggs as a function of time (●). Other portions containing an average of 70 eggs each were treated with 1% SDS for the indicated times, washed, allowed to hatch and scored for surviving animals one day later. This survival is then expressed as a percentage of the untreated control, since after the SDS treatment the killed eggs and dead hatched juveniles are very difficult to see.

exhausted, leaving the eggs adhering to the agar surface. These crowded eggs appeared to be retarded in hatching, but in 2–4 days, after quantitative hatching, a fairly synchronous population was obtained in which a high but variable proportion of the animals had become dauerlarvae. The kinetics of dauerlarva formation in one such preparation is shown in Fig. 5. Dauerlarvae were harvested from these plates at 4–5 days, when yields were generally best and when there were no adults or eggs to interfere with the SDS-resistance assay.

Dauerlarvae could also be formed in liquid medium, by limiting the bacterial food supply, as shown in Fig. 6. However, dauerlarvae could not be formed by transferring normal juveniles of any age abruptly to bacteria-free conditions, nor by transferring them to a medium which prevented pumping but did not kill (Bu/2 containing 1 mg/ml Temik, a cholinesterase poison); the transferred juveniles gradually decreased in size but they did not become thin or acquire other dauerlarvae properties. These experiments suggest that starvation must be gradual to produce dauerlarvae, but they do not show how gradual, nor when in development the starvation must be applied to be effective.

As shown in Fig. 7, dauerlarvae have an average length of 495 ± 60 μm, very close to the length of normal juveniles in the second lethargus period. Taken together with their initial possession of a sheath, their suppressed pumping, and their general inactivity, this suggests that they are arrested in a state corresponding to the second lethargus, as is reported to be the case for *C. briggsae* (Yarwood and Hansen, 1969).

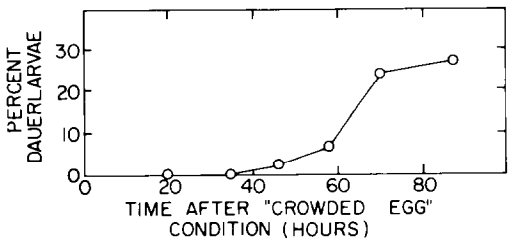

FIG. 5. Formation of dauerlarvae on an exhausted plate. A 22° growth plate with many worms and many eggs was washed free of worms just as the bacterial lawn was exhausted, leaving essentially only eggs. At the indicated times thereafter, sections of the agar were removed, and the animals washed from them were assayed for resistance to a 30-min treatment with 1% SDS. In other experiments, up to 80% dauerlarvae have been obtained from plates examined at 80–100 hr.

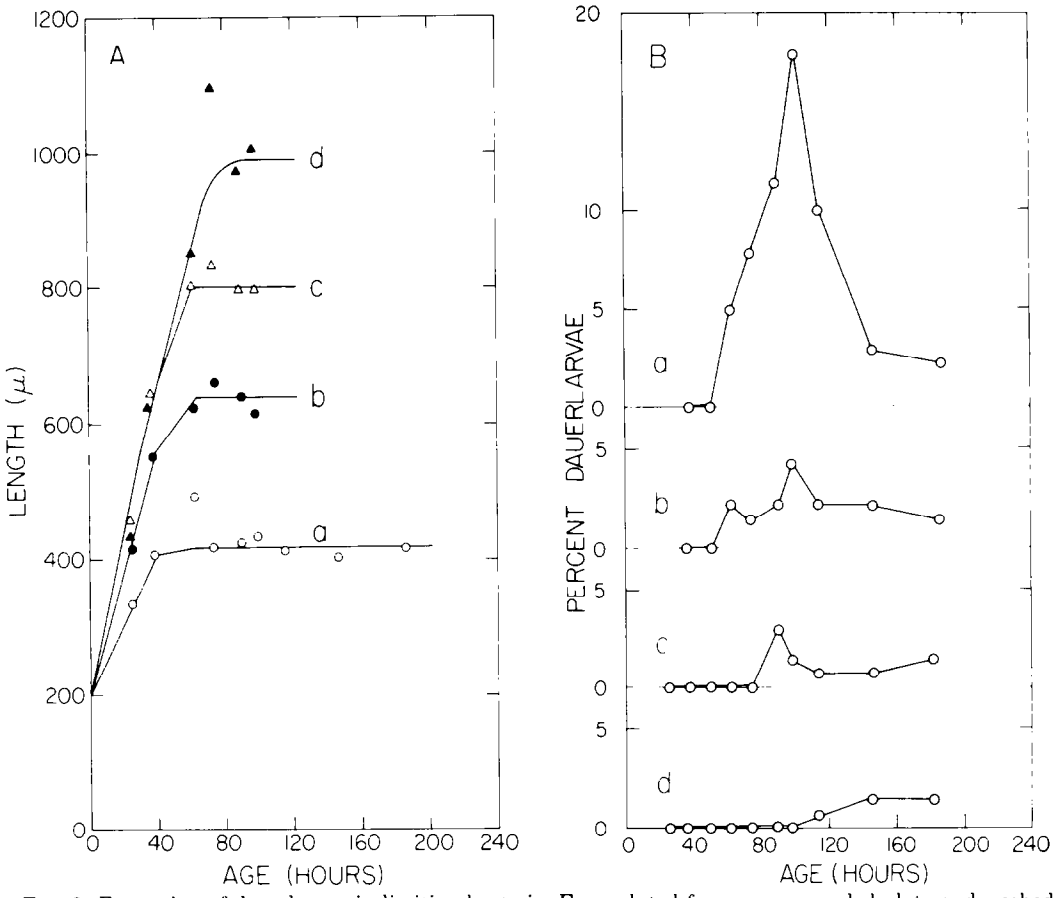

FIG. 6. Formation of dauerlarvae in limiting bacteria. Eggs, eluted from an uncrowded plate and washed free of bacteria, were divided into groups of about 250 and incubated at 21°C in 5-ml vials containing 0.2 ml of tryptone-grown *E. coli*, washed free of nutrients, at one of several bacterial concentrations, a (○) 3×10^8/ml; b (●) 1×10^9 ml; c (△) 3×10^9/ml; d (▲) 6×10^9/ml. The depth (0.2 cm) allowed good aeration. At the indicated times, vials were opened and their contents either measured for length after PPX anesthesia (part A) or assayed for resistance to 30 min of 1% SDS (part B). Average lengths are for at least 30 animals, and SDS-resistant percentages for at least 140 animals. Dauerlarvae from all populations were of the characteristic dauerlarva size (see Fig. 5).

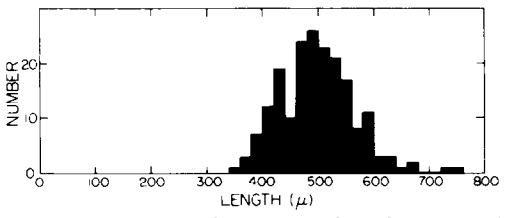

FIG. 7. Size distribution of dauerlarvae. 183 dauerlarvae, obtained from a plate like that of Fig. 3 by 5-min SDS treatment and density separation, were measured after PPX anesthesia.

Support for this idea comes from the recovery properties of normal dauerlarvae and from the properties of a conditional dauerlarva-forming mutant, as described below.

Dauerlarva Recovery

When dauerlarvae are transferred to a fresh plate with bacteria, a sequence of events occurs by which they return to the normal developmental program. Animals which have not yet lost their outer sheaths shed them either during washing, or soon after transfer, or when pumping resumes. A small minority retains the sheath and sheds it with the next cuticle at the following molt. Transferred animals generally begin pharyngeal pumping after a 1–2 hr lag, although a small minority requires 3–4 hr and a few others take less than 20 min.

The lag is not due to SDS treatment, since dauerlarvae picked solely by appearance show the same lag. After the lag, pumping is initially intermittent and increases in rate gradually to 150–200 strokes/min, taking 1–2 hr to reach maximal rate. Qualitatively, the events of pumping recovery are similar to those after a normal lethargus, but somewhat slower. After recovery, pumping proceeds continuously for several hours without any sign of growth. Thereafter, the worms swell up to normal juvenile diameter, but there is still almost no increase in length until they molt, at 14 ± 3 hr after transfer.

During the initial stages of recovery, dauerlarvae populations lose their resistance to SDS. As shown in Fig. 8a, if animals at a given recovery time are treated with SDS for varying durations, exponential killing is observed, at a rate characteristic of the recovery time. As shown in Fig. 8b, this rate increases fairly sharply at about 2 hr, just when pumping resumes for most of the population. When slightly recovered dauerlarvae are transferred to agar plates containing 1% SDS, they survive until they initiate a pumping sequence (usually 10 strokes or more), whereupon they die within the next minute, presumably killed by internal detergent action of SDS. Different individuals wait quite different times before initiating pumping, so that the population as a whole is only gradually killed, and the killing rates of Fig. 8b can thus be interpreted as measuring the probability that a (lethal) pumping event will occur during the SDS treatment. Interestingly, the continuing change in this probability with time requires continued exposure to favorable conditions, and is not simply triggered by

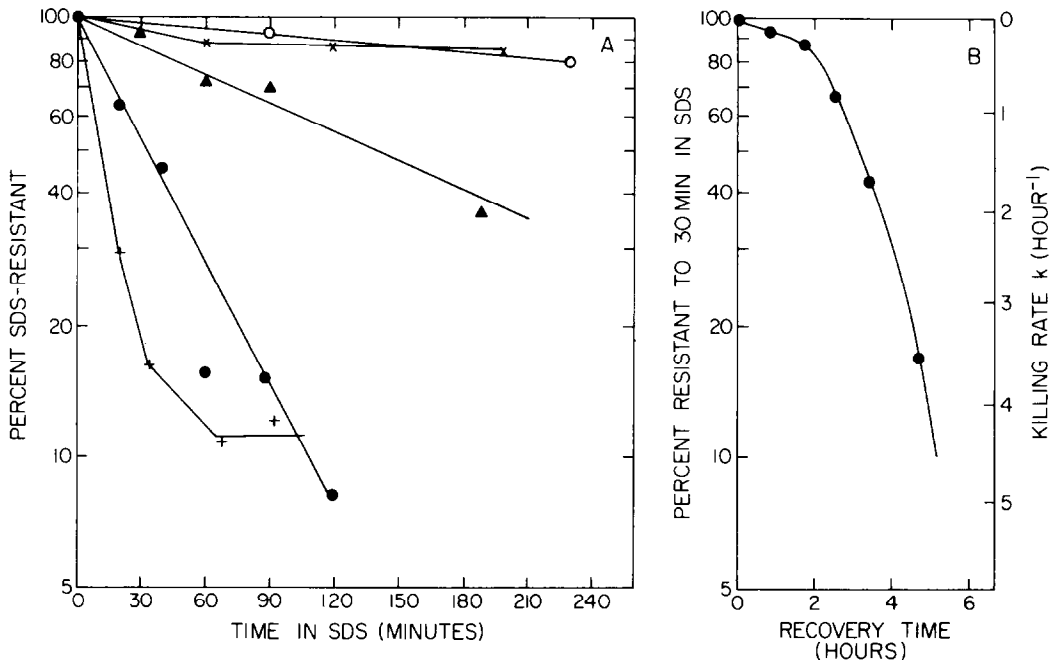

FIG. 8. Survival of recovering dauerlarvae in 1% SDS. Purified dauerlarvae were allowed to recover for intervals of 0 (○), 50 (×), 100 (▲), 150 (●), and 200 (+) min at 22°C on NGMM plates with *E. coli*. At these times animals were eluted and incubated in 1% SDS to obtain survival curves. At the indicated times (part A) aliquots of approximately 100 animals were removed, washed free of SDS, and transferred to fresh plates where survivors (viable growing animals) were scored 24 hr later. For part B, resistant percentages at 30 min SDS treatment, determined by interpolation from curves similar to those of part A but based on survival after 4–6 hr of SDS-treatment, are plotted against recovery time. The inferred killing rate k assumes an exponential killing so the surviving fraction S/S_o is e^{-kt}.

an initial exposure; if partially recovered animals are transferred back to starvation conditions, kept for as much as 6 hr there, and then assayed for SDS-resistance, their killing rate increases only slightly during the starvation period, as shown in Table 1. On the other hand, transfer back to starvation conditions does not eliminate the increase in killing probability already acquired by partial recovery.

Consistent with the notion that internal pumping of SDS is responsible for killing recovering dauerlarvae, the SDS-resistance which these animals lose during a short recovery period can be nearly completely restored if pumping is subsequently prevented by anesthesia during the SDS treatment, as shown in Fig. 9. However, similar anesthesia does not confer SDS-resistance on normal second or third stage juveniles, suggesting that the special dauerlarva cuticle is also important for SDS-resistance. Indeed when the special cuticle is shed, and replaced by a normal juvenile cuticle (without striations), at about 13–16 hr of recovery (Fig. 9), anesthesia becomes markedly less effective in protecting recovering dauerlarvae against SDS. Thus the SDS-resistance of dauerlarvae depends both on their lack of pumping and on their special cuticle.

The first postrecovery molt, in which the dauerlarva loses its special cuticle, is preceded by a normal lethargus. As in other lethargi, pumping is suppressed, and since recovery is fairly synchronous, the lethargus can be revealed by monitoring the fraction of pumping animals, as shown in Fig. 10. If the special dauerlarve cuticle remains an effective barrier to SDS during some or all of this lethargus, previously SDS-sensitive animals should become temporarily SDS-resistant when pumping is suppressed at the onset of lethargus. An indication that this happens is given by the

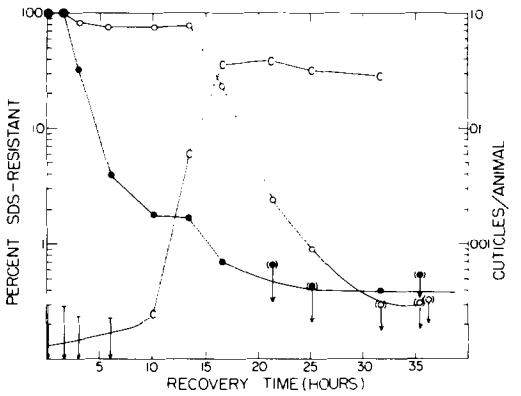

FIG. 9. Effects of anesthesia on SDS-resistance of recovering dauerlarvae. Purified dauerlarvae were transferred to an NGMM plate with an *E. coli* lawn and allowed to recover for varying times. Approximately 200 partially recovered animals were then tested for resistance to 30 min of 1% SDS (●), or first anesthetized for 30 min with 1% PPX and then diluted with an equal volume of 2% SDS to determine the resistance of preanesthetized animals to 30 min of 1% SDS (○). Treatment with 1% PPX alone for 1 hr did not reduce the viability of dauerlarvae or of 24-hr recovered animals. The appearance of shed cuticles on the original recovery plate (—c—) was followed by direct observation.

TABLE 1

INTERRUPTION OF DAUERLARVA RECOVERY BY STARVATION[a]

Recovery time	Subsequent starvation time	Killing rate, k (hr^{-1})		
		Before starvation	After starvation	
			Observed	Expected if recovery continued
150 min	390 min	1.61 ± 0.38	2.41 ± 0.52	7.5 ± 1.0
170 min	180 min	1.81 ± 0.35	3.12 ± 0.53	5.5 ± 0.7

[a] Experimental conditions as in Fig. 6. One set of aliquots was assayed immediately for killing as a function of time in 1% SDS, and a second set was held in H_2O for the indicated time and then assayed similarly. The killing rate was estimated in each case by fitting the data to an exponential survival curve (surviving fraction $S/S_0 = e^{-kt}$).

plateau at 13 hr in the no-anesthetic survival curve of Fig. 9, and in other experiments an actual increase in survival has been observed at this same time.

Once the special dauerlarva cuticle is shed, recovering animals undergo rapid longitudinal growth, as if the dauerlarva cuticle restricted growth in a way that normal juvenile cuticle does not. The growth profile is shown in Fig. 11. At about 20 hr, recovered dauerlarvae are indistinguishable from normal fourth stage juveniles and have apparently fully rejoined the normal development sequence. As in normal development, these fourth stage juveniles undergo one furhter lethargus and molt (Fig. 10) and shortly thereafter become sexually mature. The fact that dauerlarvae undergo two postrecovery molts supports the inference made earlier that most if not all of them are arrested in a state corresponding to the second-molt lethargus. If any rare individuals had been arrested later, for example at the third lethargus, they should have achieved sexual maturity before the majority reached the last lethargus, but in fact no such cases were observed among approximately 1000 animals examined.

Recovered dauerlarva populations are not enriched for males. A population of crowded eggs from a male -enriched culture was divided into two portions; one was allowed to proceed through normal development and the other was allowed to form dauerlarvae. The dauerlarvae were then selected by SDS treatment, transferred to fresh plates, and allowed to recover to form adults. The percentage of males among these adults (6%) was the same as for the adults of the directly developing portions, showing that males pass through the dauerlarva state with the same efficiency as hermaphrodites. From casual observation, however, we note that *C. elegans* males, like those of *C. briggsae* (Yarwood and Hansen, 1969) appear capable of maturing under conditions where most hermaphrodites cannot, and we propose therefore that this increased maturational capacity of males must apply to some stage after the decision to form dauerlarvae has passed.

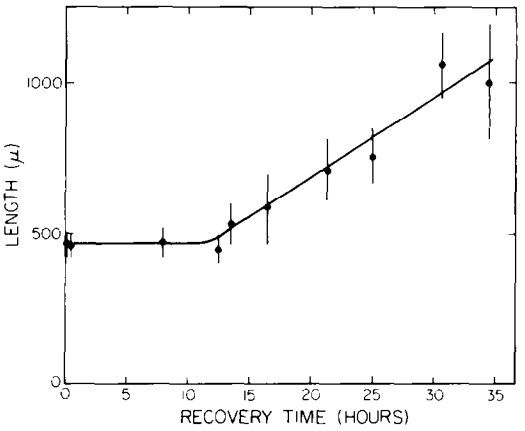

FIG. 11. Growth of recovering dauerlarvae. Aliquots of samples from the experiment of Fig. 7 were measured after anesthesia with 1% PPX. Each point represents the mean and standard deviation of a sample of at least 40 animals.

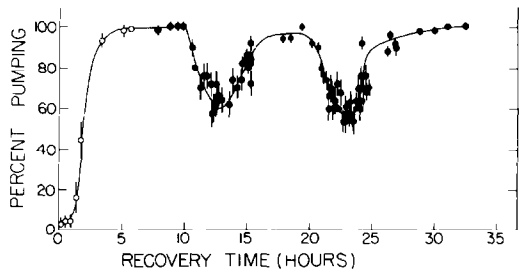

FIG. 10. Lethargus periods in dauerlarvae recovery. Purified dauerlarvae were transfered to an NGMM plate with *E. coli* and observed at intervals thereafter for pharyngeal pumping, as in Fig. 1. Each of the two observed lethargus periods was followed closely by an observed molt, and virtually all animals developed (>95%). Points from two experiments (○ and ●) are plotted.

Conditions for Dauerlarva Recovery

In liquid cultures, recovery can be induced by fairly low concentrations of bacteria, as shown in Fig. 12. Interestingly, the threshold concentration for effective recovery, approximately 10^8/ml, is near the concentration of 3×10^8/ml which caused a significant, although transient accumulation of dauerlarvae when fed to newly hatched juveniles (Fig. 6). On plates, the

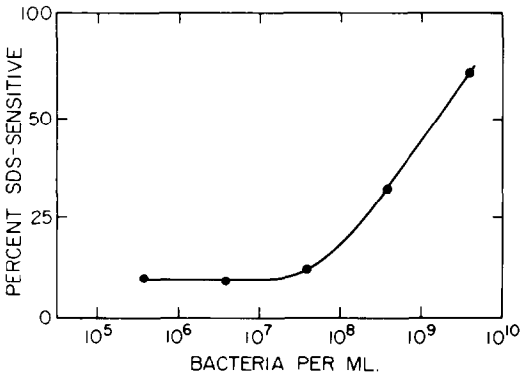

FIG. 12. Recovery of dauerlarvae in various concentrations of bacteria. Samples of approximately 100 purified dauerlarvae were incubated with the indicated concentrations of *E. coli* bacteria in 1 ml of Bu/2 for 7 hr, then washed, treated for 15 min with 1% SDS, washed again, and scored for sensitivity (loss of movement) 3–6 hr later. Sensitivity, not resistance, is plotted. Bacterial concentrations were determined with a Petroff-Hauser microscope slide.

concentration of bacteria in the lawn ($\sim 10^{12}$/ml) is considerably higher than is possible in liquid, presumably stimulating even more efficient recovery.

As Table 2 shows, plate conditions also apparently offer additional advantages for recovery over liquid culture conditions, even in the absence of bacteria; recovery on plates at 7 hr is more complete than recovery, under otherwise identical conditions, in liquid at 27 hr. Table 2 also shows the importance of crowding; recovery is much less effective at several thousand worms per ml than at several hundred. For these reasons, subsequent testing of other effects on recovery was carried out on plates, with animals at a few hundred per ml or less.

The effectiveness of a number of commonly used laboratory media in inducing recovery is shown in Fig. 13. The richer media are more effective, as has also been noted in liquid culture studies of recovery, but since no feeding occurs before recovery, it seems unlikely that this difference is nutritional. Even distilled water (solidified with Noble agar) promotes some recovery, suggesting that an important part of recovery might simply be removal from an

TABLE 2

EFFECTS OF MEDIUM PHASE AND CROWDING ON DAUERLARVA RECOVERY[a]

Medium	Animals/ml	Recovery time (*hr*)	Percent Recovery
Bu/2-Noble agar	30	7	89
Bu/2-Noble agar	300	7	85
Bu/2-Noble agar	3000	7	29
Bu/2-Liquid	45	27	88
Bu/2-Liquid	420	27	45
Bu/2-Liquid	3800	27	7

[a] Purified, well-washed dauerlarvae were incubated on agar or in thin layers of liquid for the times indicated and then tested for resistance to treatment with 1% SDS for 70 min. Shorter recovery times in liquid gave much less loss of resistance.

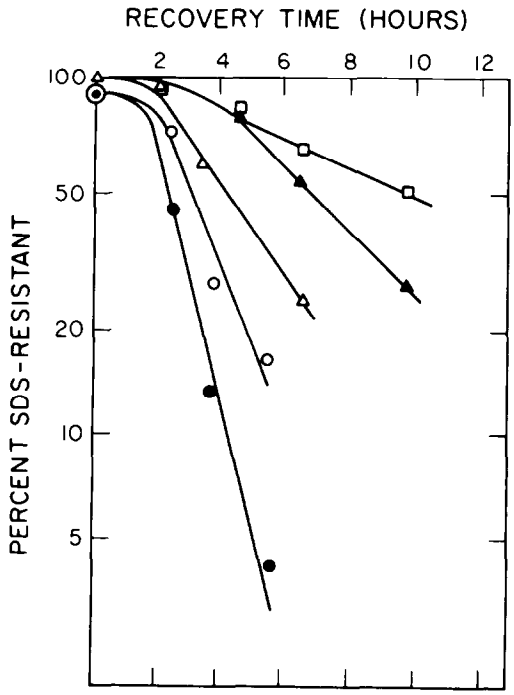

FIG. 13. Recovery of dauerlarvae on various agar media. Samples of approximately 200 purified dauerlarvae were thoroughly washed and transferred to plates made either in standard fashion (NGMM, with bacteria, (●); NGMM, without bacteria (○)) or by solidifying various media with 2% Noble agar (Bu/2, (△); distilled H_2O, pH 5 (▲); 0.05 *M* Tris-HCl, pH 7.4 (□)). At intervals thereafter, aliquots were removed, treated for 30 min with 1% SDS, washed, and examined for survivors.

inhibitor. Preliminary experiments with exhausted liquid culture medium (Cassada, R. C., and Johnson, C. D., unpublished results) suggest that there is indeed such an inhibitor, which is soluble, heat stable, and prevents recovery in tenfold dilution.

As Fig. 14a shows, recovery also varies with NaCl concentration, reaching an optimum at about 0.15 *M*. Similar results have been obtained with other salts, but not with equal osmolarities of glucose (Fig. 14b) suggesting a general ionic but not an osmotic requirement. Above the ionic optimum, recovery may be inhibited for osmotic reasons, as suggested by the effects of glucose (Fig. 14b). Whether recovery is assayed near the NaCl optimum or well below, it is quite insensitive to pH over a range from pH 6–8, as shown in Fig. 15.

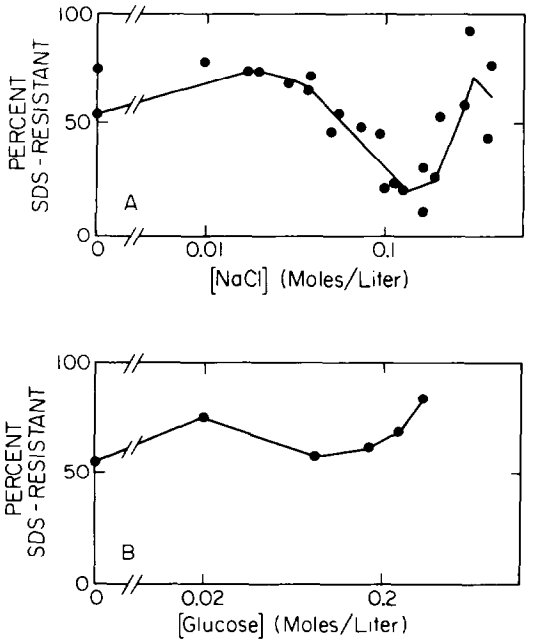

FIG. 14. Recovery of dauerlarvae on plates with NaCl or glucose. Approximately 150 purified dauerlarvae were allowed to recover for 7–7.5 hr on each of several plates containing 5 ml of 2% Noble agar, 0.01 *M* sodium phosphate buffer, pH 7.0, and the indicated concentrations of NaCl (A) or glucose (B). They were then eluted, exposed for 65–70 min to 1% SDS, washed, and examined for survivors. In all cases, viability was 100% with no SDS treatment. Concentration scales are logarithmic.

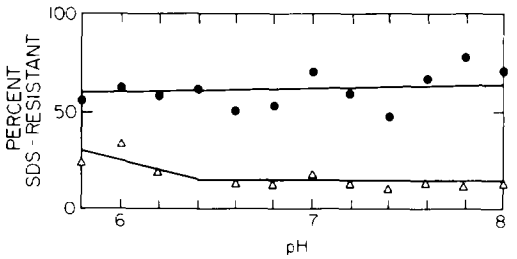

FIG. 15. Recovery of dauerlarvae on plates at various pH's. Approximately 150 purified dauerlarvae were allowed to recover for 7–7.5 hr on each of several plates containing 5 ml of 2% Noble agar, buffered to the indicated pH's with appropriate mixtures of Na_2HPO_4 and NaH_2PO_4, as follows: 0.05 *M* buffer, no NaCl (●); 0.01 *M* buffer, 0.1 *M* NaCl (△). They were then eluted, exposed for 60–65 min to 1% SDS, washed and examined for survivors.

Clearly dauerlarva recovery is a complex process, requiring for its maximal rate the removal of an inhibitor, appropriate ionic conditions, and, probably, additional factors present in bacteria. It is unclear to what extent sensory responses may be involved in these requirements.

A Mutant of Dauerlarva Formation

The unique SDS-resistance of dauerlarvae permits a selection for several potential interesting classes of mutants. For instance, because dauerlarvae arise at such very low frequencies under normal growth, a mutant which forms them at higher frequency should be quite easily selected from a mutagenized population where is is initially present as a very small minority. Similarly, since recovery is normally accomplished by nearly all dauerlarvae upon transfer to fresh plates, it should be quite easy to select mutants defective in any of the several processes (detection of the recovery stimulus, pumping, digestion, molting) required for full recovery. Of course, since absolutely defective mutants of these sorts would probably be lethal, it is necessary to isolate conditional, reversible defectives, e.g., temperature-sensitives. For this purpose, the 15–25°C growth range of *C. elegans* is adequate, and *C. elegans* offers in addition a self-fertilizing, hermaphroditic mode of reproduction very conducive

to the isolation of mutants, as has been explained fully elsewhere (Brenner, 1974; Duesenbery, *et al.*, 1975).

Using this rationale, we have isolated two classes of temperature-sensitive mutants, those which enter the dauerlarva state aberrantly, and those which fail to leave it normally. A full description of these mutants will be published elsewhere, but the properties of one particularly interesting mutant are described here because they shed further light on the process of dauerlarva formation. When grown at 16°C, this mutant forms very few (<1%) dauerlarvae, but when transferred to 25°C just after hatching and allowed to grow up, it forms almost exclusively (>99%) dauerlarvae, despite the presence of abundant food. The 25°C development of such a transferred population is shown in Fig. 16. The pumping behavior strongly confirms the conclusion, arrived at earlier, that dauerlarvae are arrested at a state analogous to the second lethargus. Interestingly, full SDS-resistance does not arise until some time after most animals fail to show pumping in the test interval; whether this indicates a gradual further decline of an already low pumping probability or, instead, the time required to complete the specialized cuticle, is not yet known. Since the first 25°C molt of the mutant occurs at a fairly normal time and length, and since juveniles of the mutant shifted to 25°C after the second molt grow up to become fertile adults, the mutant defect seems unlikely to be one of complete general starvation (as for example, a complete defect of pumping or of intestinal absorption). However, the defect might be a partial one of this kind or, alternatively, might involve processes more specific to dauerlarva formation. Whatever the nature of the defect, temperature shift experiments (not shown) indicate that a decision is made between dauerlarva and normal development at a time shortly after the first molt.

DISCUSSION

As described above, the dauerlarva of *C. elegans* appears arrested in a state analogous to the second premolt lethargus, with a special impermeable cuticle that, together with suppression of feeding (i.e., pharyngeal pumping), confers resistance to a number of denaturants, including SDS. SDS-resistance serves as the basis for a dauerlarva assay and has also been used to select mutants, one of which spontaneously forms dauerlarvae at 25°C but is normal at 16°C.

The lethargus to which the dauerlarva state appears analogous is poorly understood. It has been noted in several other nematode species (see Rogers and Somerville, 1969) including the closely related species *Caenorhabditis briggsae* (Hansen *et al.*, 1964). In at least three species, lethargus is a period of increased oxygen uptake (Hopkins, 1955; Wieser and Kanwisher, 1960; Van Gundy and Stolzy,

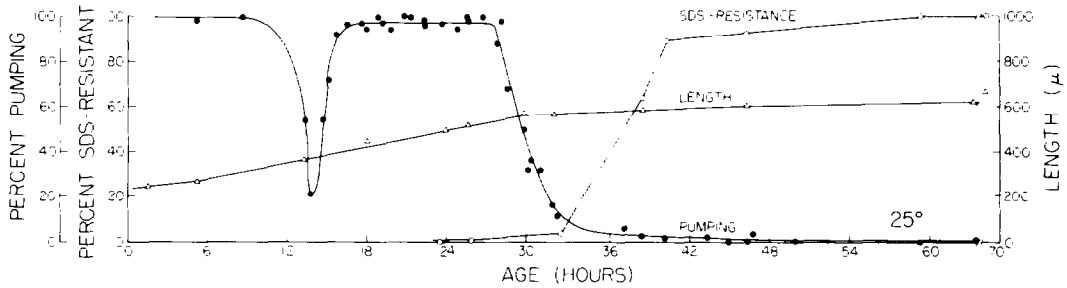

FIG. 16. Development at 25° of the mutant RC32. Mutant juveniles hatching over a 2-hr interval at the permissive temperature (16°C) were transferred to 25°C NGMM plates seeded with bacteria, and at intervals thereafter pumping and length were monitored as in Fig. 1. For SDS-resistance, aliquots of approximately 130 animals were withdrawn, washed, exposed for 35 min to 1% SDS, rewashed, and examined for survivors.

1963), suggesting increased metabolic (perhaps synthetic) activity. Although locomotor inactivity is characteristic of *C. elegans* in lethargus, this seems unimportant for molting, since slight disturbances lead immediately to a resumption of activity. The lack of pumping during lethargus, also observed in other species (Hansen *et al.*, 1964; Bailey, 1967) has been thought to prevent swallowing the shed pharyngeal lining. It seems equally likely to us that it might allow a build-up of internal pressure to facilitate molting; in support of this possibility, we note that *C. elegans* undergoes a definite change of shape during molting (Byerly, Cassada and Russell, in preparation). Although the mechanism of pumping suppression is not known, we know that the pharyngeal nervous system is almost completely isolated from the central nervous system, being joined only by two nerve fibers (Ware *et al.*, 1975).

Whether lethargus and subsequent molting are controlled in *C. elegans* by neurosecretory events is unknown. In *Phocanema decipiens*, Davey and Kan (1967, 1968) have noted that animals undergoing the fourth molt *in vitro* show changes both in the level of stainable "neurosecretory" material in nerve (?) cells of anterior dorsal and ventral ganglia, and in the level (in the excretory cell) of stainable leucine aminopeptidase, an enzyme implicated in molting in another species (Rogers, 1965, 1970). From these observations and from the ability of anterior extracts from appropriate stages of *P. decipiens* to stimulate the appearance of leucine aminopeptidase in isolated excretory cells, they have concluded that molting is under neurosecretory control. For *C. elegans*, we can only say that there are at least two cells in the vicinity of the nerve ring which, by electron microscopy, have many irregular, variably staining large vesicles suggestive of neurosecretory function (Ware *et al.*, 1975).

Besides a modified lethargus, the dauerlarva has a special cuticle and other morphological changes, all analogous to the properties of dauerlarvae from other free-living species and of infective larvae from animal parasitic species (see Bird, 1971) and all apparently reflecting a role of survival without growth.

The special dauerlarva cuticle layer (Fig. 2) is interesting because of the extremely regular spacing of its striations. These striations have also been observed in other species (see Bird, 1971) and in *Heterodera rostochiensis* Wisse and Daems (1968) have shown by tilting-stage electron microscopy that they are composed of a series of rods arranged in a rectangular lattice. Lee (1966) has suggested from the regular spacing that the whole striation layer might be a paracrystalline protein array protecting the animal from environmental fluctuations. In *C. elegans*, the dauerlarva cuticle is indeed protective, as shown above, and the striations are indeed shed together with the protective cuticle at the first postrecovery molt (our unpublished observations) suggesting that the striations may confer the special protective propteries. Conceivably the striations could play a role in two other unusual features of the dauerlarva cuticle, its apparent inability to grow longitudinally and its abnormal site of cleavage during molting, somewhat posterior to the usual larval site (our unpublished observations, and see Yarwood and Hansen, 1969). Nothing is known of the composition of the striations, but the gradual transition between the striations and a loosely oriented fibrillar layer under the lateral alae suggests that both might be composed of similar materials, differently organized at the supramolecular level. Interestingly, similar striations have been seen in the adult males of at least one species (Bird, 1971), but not in *C. elegans* males (D. Hall, personal communication).

The successive events in entry into the dauerlarva state have not been well studied previously because of the difficulty in obtaining synchronous populations which

form dauerlarvae efficiently. The recognition that gradual starvation is important should help here, but more importantly, the mutant described above provides such populations with ease. Accordingly, it should now be possible to determine accurately the temporal relations between the cessation of pumping, the depression of locomotor activity, and the deposition of the special cuticular striations.

However, the mutant does not initially provide much advantage for studying the environmental cues which lead to dauerlarva formation, since it effectively bypasses these cues. For *C. elegans*, these cues are still somewhat unclear. Gradual starvation is important, but there may be other factors, in particular crowding and/or the presence of excretory products.

Recovery from the dauerlarva state has been easier to study in synchronous populations, and we have shown that it proceeds in an ordered sequence, beginning with a resumption of pumping, then radial expansion, then molting and rapid growth to rejoin the normal developmental sequence. The cues for carrying out this process seem very likely to be chemical in nature, since purely chemical differences can make profound differences in recovery rates, and it seems likely that chemical detectors are involved. The nature and location of these detectors is unknown, but they must be either protected against externally applied SDS or else resistant to its action, for SDS-treated dauerlarvae recover with apparently normal kinetics. Existing mutants with chemotaxis defects (Dusenbery *et al.*, 1975) should afford a route for exploring the nature of these detectors more fully.

The unique SDS-resistance of dauerlarvae and the ordered events of the recovery process allow conditional mutants of various types to be efficiently selected. These mutants can be useful not only for exploring the dauerlarva state (as has been shown above) but also for examining normal feeding and molting. Several mutants have already been isolated and others are being sought be a variety of related selection procedures.

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