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# Preparation of Nematodes for Microscopic Study—Perfusion by Vapor Phase in Killing and Fixing

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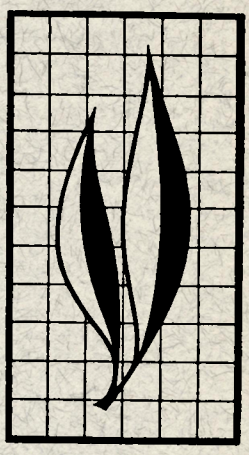

Procedures for immobilizing or killing and fixing nematodes preparatory to subsequent embedding and mounting procedures are discussed in detail. Techniques presented are offered as alternatives to the usual practice of heat killing.

The use of common laboratory gases for immobilization of nematodes to be used in studies other than permanent mounts is discussed. Inadvisability of subjecting immobilized rather than dead nematodes to further procedures designed for permanent mounting is thoroughly documented.

Superior specimens are obtained employing vapor-phase perfusion by formalin in combination with water, acetic acid, formic acid, propionic acid or hydrochloric acid for killing, rather than by using heat. The necessity of fixation subsequent to killing is discussed.

The reactions of nematodes manifested by changes of morphologic features as a result of gas immobilization, vapor-phase perfusion or heat, before and after processing into glycerin, are thoroughly documented in tabular form.

The final analysis lists sixty procedures found successful for processing nematodes into glycerin.

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# Preparation of Nematodes for Microscopic Study–Perfusion by Vapor Phase in Killing and Fixing[1]

## INTRODUCTION

THE FIRST STEP in the stabilization of a live, moving organism is to arrest its activity. Placement of the animal in the state of arrested activity (so-called narcotization) is distinct from killing. For special studies in physiology and ecology the animals must be revived. For profound microscopic examinations the animals must be killed; this can be done deliberately or inadvertently as the animals are processed. After death, animals must be stabilized ("hardened" or "fixed") in order to withstand the subsequent preexamination procedures of dehydration, embedding, etc. The initial steps of narcotization, killing, and stabilization are frequently combined in one or more ways and conducted in one operation. It is clear from references to cytological techniques (Lee, 1946; Gray, 1954; Goodey, 1957; and Baker, 1960)[2] that there are a bewildering number of techniques recommended for narcotization, killing, and fixing.

The present study investigates vapor-phase perfusion for killing and at least partially fixing nematodes preparatory to mounting. A limited number of the more common fixative agents were used to illustrate the possibilities of vapor-phase perfusion.

## MATERIALS AND METHODS

### NEMATODE SOURCES

Many species and genera in both Adenophorea and Secernentea were used, and these were obtained by wet screening of moist soil samples in a conventional fashion. Screened material, together with the associated debris, was collected and purified further by the usual Baermann funnel technique. Clean specimens obtained in this fashion were aliquoted among test treatments so that several hundred to several thousand nematodes were exposed to each treatment, depending upon the original number in the soil sample.

The use of all nematode groups would have been completely beyond the scope of this preliminary effort. Consequently it was assumed that results from using Adenophorea and Secernentea specimens found in soil samples as representative nematode samples would be indicative of the effects of the cytological techniques on other groups.

[1] Submitted for publication, September 15, 1964.
[2] See "Literature Cited" for citations referred to in text by author and date.

## GAS TREATMENTS

Nematodes in water were exposed to gaseous materials at ambient pressures by bubbling each gas vigorously through the suspension of animals. Methane (obtained from city gas assaying over 95 per cent methane) was scrubbed with sulfuric acid followed with a scrubbing of water when indicated. Nitrous oxide ($N_2O$) which was generated by dry distillation of ammonium nitrate was collected over water. Water was used to drive the nitrous oxide in the reservoir through the bubble tube into the suspension of nematodes. Carbon monoxide (CO) was generated by adding formic acid to concentrated sulfuric acid, and was scrubbed with buffer solution before being bubbled through the nematode suspension. Carbon dioxide ($CO_2$) was obtained from a dry ice generator; all other gases were obtained from commercially available high-pressure cylinders. Where indicated the nematode suspension was placed in a glass pressure vessel, the gas added at specified pressure and the inlet closed; the vessel was then placed on a shaker and agitated for a specified time. Hydrogen reduction catalysts, palladium on asbestos (PdA), platinic oxide ($PtO_2$) and palladium on carbon (PdC) were used at the rate of ½ per cent of the nematode suspension by weight.

## SOLUTION FIXATION

Solution fixatives used in these experiments were prepared as follows:

Formalin 2½ per cent v/v (formalin = 40 per cent aqueous formaldehyde).

F.A.A. (distilled water : 95 per cent ethanol : formalin : acetic acid :: 80 : 16 : 2.4 : 1.6).

T.A.F. (formalin: triethanolamine: distilled water :: 7 : 2 : 91)

Seinhorst's fixative, F.A. 4 : 10 (formalin: glacial acetic acid: distilled water :: 10 : 10 : 80).

Nematodes in a suspension to be treated by fixative were spun down in a centrifuge, supernatant removed and the fixative added.

## VAPOR-PHASE PERFUSION

Vapor-phase perfusion experiments were conducted in closed containers, namely, 2-ounce, wide mouth, screw-cap jars, 5 cm in diameter and 6½ cm in height. The nematode suspension was placed in a small cylindrical vial (10 mm in diameter by 7 mm in height) containing 0.5 ml of solution. The nematode-containing vial was normally suspended above 3 ml of fixative solution by a stainless steel screen support, or by glass beads. The fixative molecule would leave its reservoir, pass through the gas phase, enter the liquid of the nematode vial, and diffuse to the nematode. Fixative reservoir solutions were made from commercially available chemically pure preparations (40 per cent formalin, 88 per cent formic acid, 99 per cent acetic acid, 100 per cent propionic acid, 37 per cent HCl solution) in the proportions indicated. Since formaldehyde polymerizes in acid solutions each experiment was begun with a freshly-prepared reservoir solution. To insure an adequate rate of transfer of fixative, three or less nematode-containing vials per reservoir container were usually used.

## HEAT MODIFICATION

To test the use of heat ( hot solutions) for the modification of fixed or partially-fixed specimens, nematodes were centrifuged from the suspension, the supernatant poured off, and an excess of solution at 90°C added (Seinhorst, 1959). Occasionally the nematodes were centrifuged, the supernatant poured off and the animals (suspended in a drop of water) were picked up with a capillary pipette and delivered into an excess of 90°C solution.

## SPECIMEN MOUNTING

Specimens to be examined in aqueous media were prepared in the conventional fashion, or, more commonly, a

sample was pipetted onto a slide and the animals thereon examined with a water-immersion lens. Specimens to be examined in nonaqueous media were dehydrated with alcohol in an automatic solvent exchanger, or "autoexchanger" (Viglierchio and Maggenti, 1965). Following dehydration, the nematodes were centrifuged in 92 per cent (v/v) alcohol and all but 0.8 ml of the supernatant was removed and 0.8 ml of 10 per cent (v/v) ethanolic glycerin was then added; the resulting suspension was poured into a USDA BPI dish which, in turn, was placed in a Petri dish. The alcohol was allowed to evaporate overnight before the dish was placed in a desiccator over calcium chloride, and after 24 hours the nematode specimens were prepared in conventional anhydrous glycerin mounts. Each sample consisted of more than 20 specimens; these were normally examined in aqueous media. In nonaqueous mounts a representative selection of the kinds of animals found in the initial soil sample (usually 20 specimens) was used.

## TERMINOLOGY

The characteristics shown by nematode tissues and organs in these experiments are described here in broad terms — for example, "distortion," "clearing," "relaxation." Each characteristic in turn was more precisely delimited when necessary—for example, "muscular distortions," "internal organ distortions"; some of those effects more commonly observed are shown in figures 1 and 2. Additional characteristics not illustrated are explained below. The term "stock-like" refers to the similar appearance of treated specimens to those in the original sample. That is, in a fresh collection of nematodes from either soil or host material 5 to 10 per cent are undesirable animals; nematodes evaluable by simple water observation are dead, injured, parasitized or otherwise unhealthy. Such animals commonly give anomalous results; consequently, our evaluations were based on the majority of results observed. Clearing is a physical or chemical phenomenon in which there is either dissolution or other change in refractive index, resulting in the revelation or apparent disappearance of specific organs or structures. Coagulation consists of a congealing or stabilizing, or both, of internal organic materials, etc. in which there can be variation from an opaque gel, through intermediate granular precipitation, to acute shrinkage of body fluids from adjacent organs.

# OBSERVATIONS

## GAS IMMOBILIZATION

The three orders of physiological activity noted (nematodes unaffected, immobilized, or killed) were not correlated with gas types: inorganic, paraffin hydrocarbon, or a substituted paraffin hydrocarbon. Treatment of nematodes with the more common laboratory gases shows that deoxygenation, as achieved by bubbling an inert gas ($N_2$, He) through a suspension, is useless for immobilization or killing (table 1). Apparently, many nematodes can survive anaerobic conditions for at least short periods. When nematodes were not immobilized, increased clarity of internal organs sometimes resulted if gases were oil soluble—such gases, for example, as Freon 22, Freon 12 at 20 psi, propane, and propane butane, ethylene, hydrogen with a platinic oxide catalyst, and hydrogen with palladium on asbestos catalyst at 20 psi. Among the immobilizing gases only $CO_2$ (less than 8 hours) and butane had the effect of internal clearing. Nematodes could tolerate ethylene and propane at pressures of 1 to 2 atmospheres with little

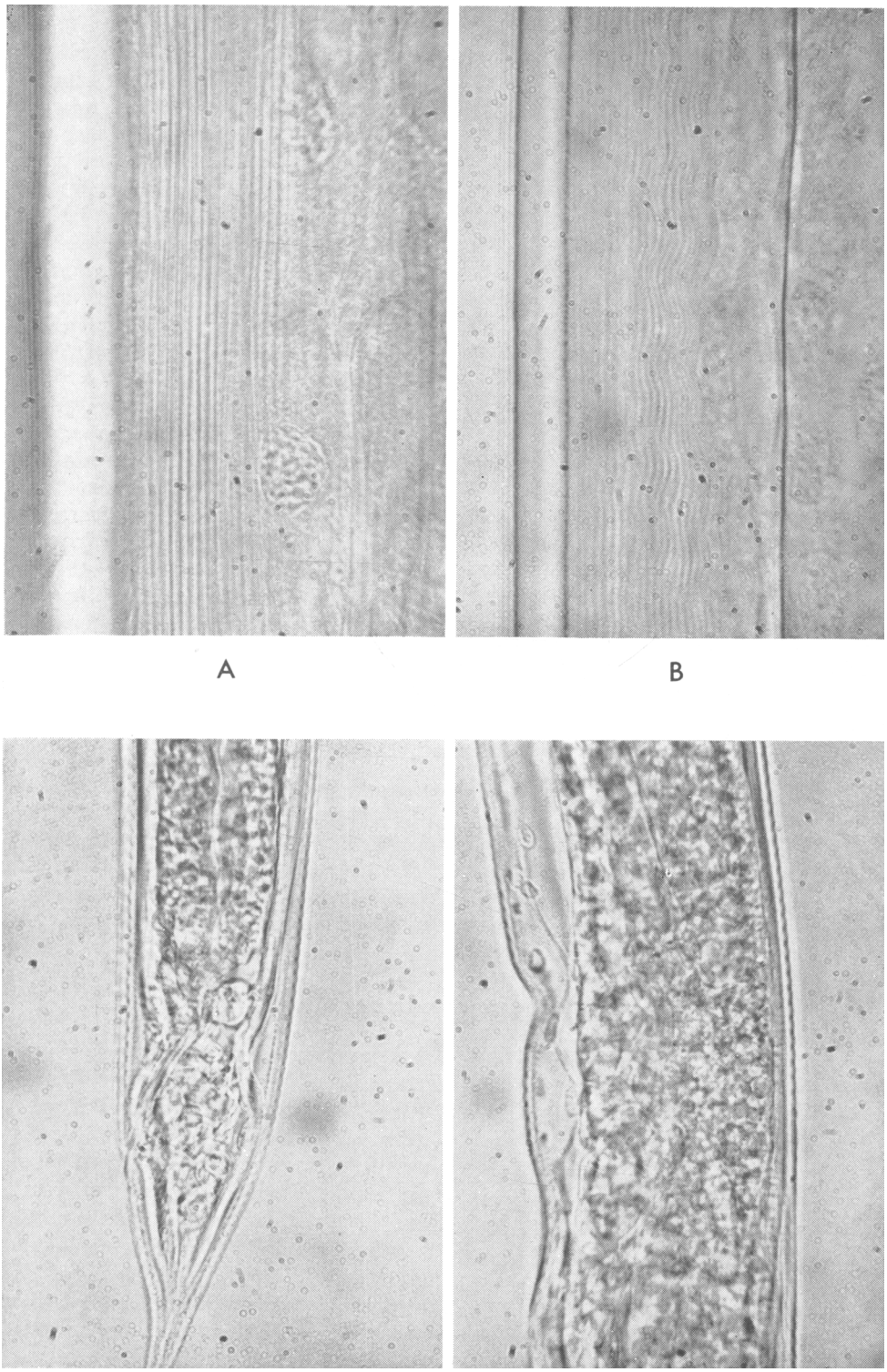


Fig. 1. Characteristic musculature and cuticle in the processing of nematode specimens: **A,** somatic musculature well preserved, fibers straight; **B,** somatic musculature poorly preserved, fibers wavy; **C,** somatic musculature pulled away from cuticle; **D,** cuticle swollen.

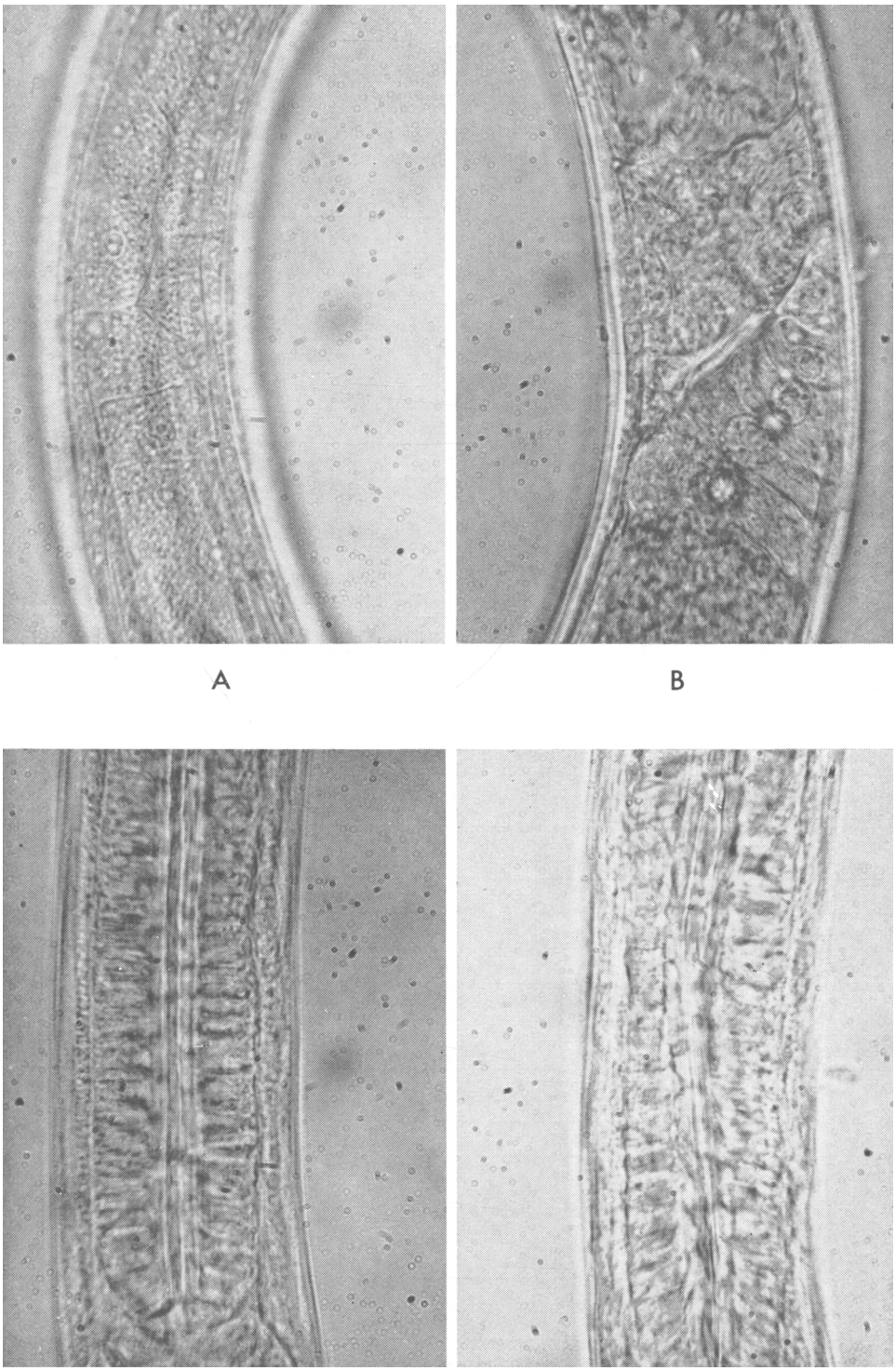


Fig. 2. Illustrations of range in definition of cells and organs. **A,** cells and nuclei of intestine well defined; **B,** cells and nuclei of gonads well defined; **C,** esophagus well defined; **D,** esophagus poorly defined.

TABLE 1

NEMATODE REACTION TO IMMOBILIZATION TREATMENTS BY EXPOSURES TO VARIOUS GASES. NEMATODES EXAMINED IN WATER.

| Gas used in treatment | Immobilization status (1 to 36 hours)* | | | | | | | Nematode reaction |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 4 | 8 | 16 | 24 | 36 | |
| $N_2$ | × | × | × | × | × | × | × | Stock-like appearance; not dead. |
| He | × | × | × | × | × | × | n.o. | Majority active, appearance stock-like; some appear dead. |
| $CO_2$ pH 4.5 | × | ○ | ○ | + | | | | Definition good; stock-like appearance. |
| $N_2O$ | × | × | × | × | × | × | × | Nematodes never inactive. |
| CO pH 6.8 | × | × | × | × | × | × | × | Nematodes never inactive. |
| $H_2$ | × | × | × | × | × | × | × | Nematodes never inactive. |
| $H_2$ PdA 20# | × | × | × | n.o. | n.o. | n.o. | n.o. | Nematodes cleared, but never inactive. |
| $H_2$ $PtO_2$# | × | × | × | n.o. | n.o. | n.o. | n.o. | Nematodes cleared, but never inactive. |
| $H_2$ PdC 20# | × | × | + | | | | | Nematodes dead; look very good except for adhering charcoal. |
| Methane unwashed | × | × | × | × | + | | | Initially look good; with time clearing extensive, vacuolation and distortion. |
| Methane washed | × | × | × | × | × | + | | 24 hrs. dead; appearance stock-like. Later cleared, vacuolated; esophageal distortion. |
| Propane | × | × | × | × | × | × | × | Stock-like appearance, but with some clearing. |
| Propane 20# | × | × | × | × | × | × | × | Active, stock-like appearance, slight clearing. |
| Butane | × | × | × | ○ | n.o. | n.o. | n.o. | Nematodes cleared. |
| Butane 20# | × | × | × | × | × | × | × | Nematodes active; stock-like appearance but cleared. |
| Ethylene | × | × | × | × | × | × | × | After 96 hours few dead and vacuolated; others remain active. |
| Ethylene 20# | × | × | × | × | × | × | × | Nematodes remain active but cleared. |
| Acetylene unwashed | × | × | + | | | | | Body contents vacuolated and coagulated. |
| Acetylene washed | × | × | ○ | n.o. | n.o. | n.o. | n.o. | Internally vacuolated. |
| Acetylene 20# | × | × | × | × | × | × | × | Vacuolated. After 3 days appear dead; coagulated and vacuolated. |
| Freon 12 | × | × | ○ | n.o. | n.o. | n.o. | n.o. | Stock-like appearance; look good. |
| Freon 12 20# | × | × | × | × | × | × | × | Active after 96 hours, stock-like. After three days appear coagulated. |
| Freon 22 | × | × | ○ | n.o. | n.o. | n.o. | n.o. | Appear stock-like. |
| Freon 22 20# | × | × | × | × | × | × | × | Killed at 67 hrs. but structures obscured, appear coagulated. |
| Methyl bromide | + | | | | | | | 15 min. dead. Post-uterine sac clear, Tylenchs take well; severe distortion and shrinkage with longer exposure. |
| Propylene oxide | × | + | | | | | | After 1 hr. dead, stock-like appearance. Globular accumulation and distortion with long exposure. |

* × = No immobilization; ○ = immobilization; + = dead animals; n.o. = no observation.

adverse effect. With butane, Freon 12, Freon 22, or acetylene, immobilization which occurred within 8 hours at 1 atmosphere did not occur within 36 hours at 2 atmospheres. Because the animals revived upon aeration this may be a transient physical boundary effect much like a permeability interruption. Prolonged exposure to most of the gases eventually resulted in killing, but most specimens were too distorted to be useful.

## GAS IMMOBILIZATION AND SOLUTION FIXATION

The effect of fixation on nematodes in varying degrees of immobilization was tested with gases which showed diverse results: propane, because no immobilization occurred but clearing was evident; carbon monoxide, because of no detectable effects; carbon dioxide and Freon 22, because of immobilization and improved clarity; acetylene (washed), because specimens were killed after 24 hours and their condition was good; the untreated, because the nematodes were neither immobilized nor dead (table 2).

Gas treatment did not improve nematode ability to withstand the distorting effects of solution fixation. Initial relaxation of specimens does not insure relaxation after fixation (carbon dioxide and Freon 22); data suggest that to obtain good results specimens should be dead prior to fixation (methane). Poorly-killed specimens probably cannot be improved by fixation (acetylene).

The unsatisfactory results obtained with this experiment indicated that a new approach to immobilization killing and fixation would be advisable.

## GAS IMMOBILIZATION AND VAPOR-PHASE PERFUSION FIXATION

Gas treatment preceding perfusion killing did not greatly improve the quality of the specimens observed in water. However, treatment with the vapors of

TABLE 2

NEMATODE REACTION TO TWO SOLUTION FIXATIVES FOLLOWING GAS IMMOBILIZATION. NEMATODES EXAMINED IN FIXATIVE SOLUTIONS.

| Gas used, and treatment time | Nematode reaction | |
|---|---|---|
| | Formalin (2½% solution) | F.A.A. |
| Untreated | Nematodes not relaxed; internal tissues coagulated as when heat killed. Esophagi and spear clear; some esophageal distortion and vacuolation. Nuclei not clear, internal organs in good relation. | Relaxation variable; generally good esophageal preservation. Definition poor. Dorylaims look good. Tylench cuticular parts prominent, large internal globules. In some (Cephalobs) esophagi separated from intestine. |
| Propane, 36 hrs. | Esophagi badly distorted, definition poor, not relaxed. General body shape maintained. | General body shape maintained, vacuolation in esophagi and intestine. Generally definition poor. |
| Methane, 24 hrs. | Body contents vacuolated, esophagi distorted; esophagi definition good. Nuclear definition poor; in some instances collapsed. | Definition of somatic musculature and esophagi poor. Large Dorylaims good. |
| Carbon dioxide, 2½ hrs. | Nematodes not relaxed; cellular definition good. Esophagi and stomatal structures well defined in Tylench. Body contents coagulated; esophagi sometimes distorted. | Internal contents shrunken, esophagi seldom well defined. Dorylaims good. Aphelench esophagi preserved but remainder of body structures poor. |
| Carbon monoxide, 6 hrs. | Nematodes not relaxed; coagulation of body cavity contents around esophagi; some vacuolation. | Internal organs not well preserved; some shrinkage. Dorylaims again best but not really good. |
| Freon 22, 4 hrs. | Esophageal distortion, intestinal shrinkage; some badly vacuolated and distorted. | Nematodes relaxed, appear better than formalin fixed but show distortions and some shrinkage. |
| Acetylene, 2½ hrs. | Relaxed nematodes show vacuolation and distortion. Remainder same appearance but not relaxed. | Nematodes relaxed but still poor. Internal structures better than with formalin alone. |

volatile fixative agents is superior to placing nematodes directly into the fixative solutions, whether or not the nematodes were alive or pre-killed by other means. Again, specimens pretreated with oil-soluble gases showed consistently better clarity (tables 3, 4).

Samples were taken from the animals described in table 3 and further processed into glycerin. In every case gas-treated specimens dehydrated in alcohol and processed into glycerin appeared no better than did untreated live animals similarly processed. Gas treatments did not improve the quality of specimens perfusion-killed, dehydrated and processed into glycerin. Evidently the vapor exchange of fixing agents was sufficient for immobilization. In vapor-phase perfusion, both killing and some degree of fixing are carried out in one procedure.

## VAPOR-PHASE PERFUSION, IMMOBILIZATION, OR KILLING

The vapor-phase perfusion technique for immobilization and killing allows considerable latitude in concentration of components and in exposure periods (see emphasized areas tables 5–9). It is relatively easy to get acceptable specimens in aqueous media, although each step in the system manifests its characteristic effect. In the formalin-water

TABLE 3

NEMATODE REACTION TO 1-HOUR FIXATION BY VAPOR-PHASE PERFUSION FOLLOWING GAS IMMOBILIZATION FOR VARIOUS PERIODS. NEMATODES EXAMINED IN WATER.

| Gases used and treatment time | Nematode reaction | | |
|---|---|---|---|
| | Formalin-vapor (40%, 1 hr.) | Formalin:acetic acid (1:1 solution, 1 hr.) | Formalin:formic acid (1:1 solution, 1 hr.) |
| Untreated | Nematodes not relaxed, but structures well defined. Esophageal lumen well defined, easily discernible in *Neotylenchus*. | Nematodes relaxed; definition good to poor. | Nematodes excellent and relaxed; some slight differences in definition. |
| Freon 22, 4 hrs. | Nematodes in general good, but some show constricted esophagi. Definition of internal organs good. | Nematodes better than untreated; internally structures better defined. | Nematodes very good in all respects except that in some of small Tylenchs the esophagus is poorly defined. |
| Acetylene, 2½ hrs. | In general, nematodes look good but internally structures obscured and difficult to discern. Nematodes relaxed. | Nematodes good in all respects. | Nematodes good but some evidence of vacuolation. |
| Carbon monoxide, 6 hrs. | Some nematodes show esophageal shrinkage; however, this is not consistent. Otherwise nematodes good. | Nematodes relaxed, look very good; internal definition very good. | Nematodes relaxed, in excellent condition. |
| Methane, 24 hrs. | Good definition but not relaxed. | Nematodes good, a few Dorylaims show esophageal distortion, nematodes relaxed. | Nematodes very good, definition good, nematodes relaxed. |
| Propane, 36 hrs. | Good definition but not relaxed. | Definition not as good as with formalin·formic acid; however still excellent nematodes, *Paratylenchus* excellent. | Nematodes excellent. |
| Carbon dioxide, 2½ hrs. | One Aphelench active; others immobilized, no relaxation. Nematode condition good with lumen of the esophagus well defined. | Nematodes relaxed; definition good to poor. | Nematodes excellent and relaxed; some slight difference in definition. |

TABLE 4

NEMATODE REACTION TO DEHYDRATION AND PROCESSING INTO GLYCERIN AFTER 1-HOUR VAPOR-PHASE PERFUSION FOLLOWING GAS IMMOBILIZATION. NEMATODES EXAMINED IN GLYCERIN.*

| Gas used and treatment time | Nematode reaction | | | |
|---|---|---|---|---|
| | Autoexchanger to 5% glycerin | Formalin vapor (40%, 1 hr.) | Formalin: acetic (1:1 solution, 1 hr.) | Formalin: formic (1:1 solution, 1 hr.) |
| Untreated | Poor nematodes, extreme distortions with shrinkage and pull-away of somatic musculature. | Shrinkage of somatic musculature, appears wavy. Some cuticular distortions associated with shrinkage of muscles. Esophagi very distinct. | Nematodes very good with only slight shrinkage of tissue from cuticle and internal organs. | Tissues generally good but many nematodes show distorted esophagi and pull-away of somatic muscle. Dorylaims less distorted. |
| Freon 22, 4 hrs. | A great deal of distortion, esophagi shrunken. | | Somatic muscle pulled away along body at intervals but the effect is never general. | Some apparent shrinkage of body contents; some esophageal distortion. Body alternately distended and collapsed, appearing wavy. |
| Acetylene, 2½ hrs. | Tissue shrinkage plus loss of definition with coagulation. | | Nematodes poor, severe tissue shrinkage and distortion. | Very bad tissue shrinkage but in general nematodes good with good nuclei and good definition. |
| Carbon monoxide, 6 hrs. | Very poor nematodes, little definition of structure, cell outlines usually good, somatic muscle destroyed, cuticle wavy. | | Nematodes good, body contents and nuclei good; some pull-away from cuticle evident. | Some nematodes show minor shrinkage but in general nematodes good with good nuclei and good definition. |
| Methane, 24 hrs. | Very poor. | | Nematodes greatly distorted, very poor. | Some nematodes very badly distorted; Dorylaims withstand treatment better but still poor. |
| Propane, 36 hrs. | Generally poor, reproductive cells quite distinct, esophagi distinct but with shrinkage of body contents. | | Dorylaims look very good. *Aphelenchoides* show body shrinkage and esophageal shrinkage. *Neotylenchus* not well defined but not shrunken. | Severe distortions associated with shrinkage and pull-away of organs. Some nematodes less severely affected, fair nematodes. |
| Carbon dioxide, 2½ hrs. | Cells distinct, some esophageal distortion. Generally organs obscure. | | Body contents maintain proper relations but definition poor. | In general nematodes fair, but often there is shrinkage of organs and tissues from body wall. Nuclei well defined, cuticle not affected. |

* Nematodes pretreated by formalin solution, vapor-phase perfusion, were not processed into glycerin because no differences could be noted between treated and untreated nematodes.

system, acceptable specimens can be obtained at all stages of the procedure save for those conditions utilizing higher formalin concentrations and the longer exposure periods (table 5). Formalin does not effect relaxation, in the sense that it does not bring about an apparent change in body configuration.

In the formalin-formic acid system exposure periods of less than 4 hours appear better. The clearing action of formic acid begins slowly but then proceeds rapidly and becomes so extensive after 4 hours as to make definition

TABLE 5

NEMATODE REACTION TO IMMOBILIZATION OR KILLING BY VARYING VAPOR-PHASE PERFUSION TREATMENTS (formalin-water system). NEMATODES EXAMINED IN WATER.

| Perfusion-treatment time | Nematode reaction | | | |
|---|---|---|---|---|
| | Formalin | Formalin: water (3:1 solution) | Formalin: water (1:1 solution) | Formalin: water (1:3 solution) |
| 1 hr. | Nematodes dead but not relaxed; organ definition good. | Nematodes not relaxed but internal definition good; organs in good condition. | Some nematodes still active; inactive ones look good. | Some nematodes still active; inactive ones look good. |
| 2 hrs. | Nematodes not fully relaxed; internal organs and reproductive system very good, nerve nuclei not prominent but organ preservation good. | Many nematodes not relaxed but tissues and organs well preserved. Nuclei well defined but not prominent feature. | Nematodes relaxed; look very good, nuclei well defined; no shrinkage of internal organs; somatic muscles well preserved. | A few nematodes not relaxed but in general excellent, nuclei and internal organs well defined. |
| 4 hrs. | Nematodes look good, definition good and preservation good but not relaxed. | Nematodes excellent but not relaxed. | Nematodes very good but not completely relaxed; *Tylencholaimellus* appears coagulated. | Nematodes in good condition, nuclei distinct; *Tylencholaimellus* appears vacuolated and coagulated. |
| 8 hrs. | Nematodes not relaxed; they look good, but there appears to be some loosening of the somatic musculature. | Nematodes not relaxed; in some nematodes somatic musculature pulled from body wall. Most in good condition. | Not all nematodes relaxed but definition good; no apparent distortions. | Not all nematodes relaxed; definition of internal organs excellent. |
| 24 hrs. | Nematodes not relaxed, but they show distinctness of structure; Secernentea show loosening of somatic musculature. | Nematodes not relaxed, but tissues and organs in proper relation; slightly obscure. | Nematodes are not relaxed, organs well preserved and well defined. | Organ definition very good but nematodes not relaxed. |

difficult. Specimens are relaxed and relatively undistorted (table 6).

All formalin-acetic acid combinations killed and relaxed nematodes regardless of the time intervals tested. It was not until specimens had been exposed for 24 hours to either pure acetic acid or any of these combinations that clearing became so excessive it was difficult to see internal organs. Two prominent features of this treatment are the clarity of the neurocyte nuclei and the clarity of the reproductive system and its nuclei (table 7).

The formalin-propionic acid system is similar to the formalin-formic acid one, although propionic acid appears gentler. Propionic acid, useless in the pure form at all exposure periods, modifies the formalin effect at high formalin concentrations over long exposure periods—this improves specimen quality; nuclear definition is excellent and it is possible to see the nuclei inside the esophageal cells. Clearing also proceeds less rapidly, perhaps because of lower volatility. As chain length of the saturated monocarboxylic acid modifier increases (formic, acetic, propionic acid), the exposure time and concentration in which desirable specimen effects are obtained are reduced (table 8).

The hydrochloric acid-water perfusion system is useless for water specimens. The ratio of 1:3 did not kill nematodes at any time in a 1- to 24-hour exposure; at the 1:1 ratio specimens were not killed until they had been exposed for a minimum of 4 hours. At

higher concentrations or longer exposure periods the destruction of internal organs (presumably through hydrolysis) begins at, or precedes, death (table 9). In the formalin-hydrochloric acid system the specimens are generally good. Only with a longer exposure period and a higher hydrochloric acid concentration (1:1 after 4 hours) was there incipient internal organ destruction, manifested by excessive clearing and shrinkage of somatic musculature from the body wall (table 9).

Although it is possible to immobilize animals with gases or kill them in a variety of ways to provide good specimens for water observations, these are not suitable criteria for predicting success with permanent mounts. Generally, only in those treatments in which appreciable fixation could have occurred were specimens able to withstand dehydration with alcohol and infiltration with glycerin; when these specimens were further processed into glycerin the effects of the preceding steps were manifest, and it was immediately evident that for obtaining satisfactory specimens the range of concentrations and time intervals should be reduced (tables

TABLE 6

NEMATODE REACTION TO IMMOBILIZATION OR KILLING BY VARYING VAPOR-PHASE PERFUSION TREATMENTS (formalin-formic acid system). NEMATODES EXAMINED IN WATER.

| Perfusion-treatment time | Nematode reaction | | | |
|---|---|---|---|---|
| | Formalin: formic acid (3:1 solution) | Formalin: formic acid (1:1 solution) | Formalin: formic acid (1:3 solution) | Formid acid |
| 1 hr. | Nematodes dead and relaxed; internal organs in good condition. | Nematodes dead and relaxed; generally internal organs good, but some obscurity. | Nematodes dead and relaxed; internal organs well preserved but definition poor. | Nematodes dead; smaller species very obscure in internal structures, poor definition. |
| 2 hrs. | Nematodes relaxed; internal tissues and organs well preserved but definition poor; esophagi obscure. | Nematodes relaxed; nuclei prominent; *Neotylenchus* esophagi obscure but in large species as *Rhabditis*, *Cephalobus*, *Dorylaimus* esophagi very good. | Nematodes relaxed; esophagi of Tylenchs obscured by surrounding tissues. Neurocytes and nuclei of internal body organs well defined. | Somatic musculature and esophageal definition poor; excessive clearing. Nuclei distinct; other internal organs well preserved. |
| 4 hrs. | Nematodes relaxed; good preservation, nuclei distinct, definition of larger species very good; some obscurity in smaller species. | Somatic muscles clearing, excellent definition in larger species; nuclei very distinct. | Nematodes show more clearing so that in smaller species internal organs difficult to differentiate. Nuclei very prominent. Large species still very good. | Clearing so extensive that internal organs no longer distinguishable; nuclei no longer visible. |
| 8 hrs. | Somatic muscles extremely clear, other internal organs difficult to distinguish; no apparent distortions; nuclei very prominent. | Nematodes generally good but clearing extensive so definition poor; somatic muscles not visible. | Nematodes in good condition but extremely clear. | Clearing so extensive that nematodes appear ghost-like. |
| 24 hrs. | Internal organs maintain proper relations; nuclei very prominent; too much internal clearing. Dorylaims very good except for esophageal definition. *Aphelenchus* shows pull-away of tissues from body wall. | Internal tissues and organs maintain proper relations but too much clearing; somatic musculature no longer visible. | Nematodes too clear, cannot see somatic musculature. Internal tissues and organs seem to maintain proper relationship. | Extreme clearing with internal organ destruction; somatic musculature pulled away from body wall. |

TABLE 7

NEMATODE REACTION TO IMMOBILIZATION OR KILLING BY VARYING VAPOR-PHASE PERFUSION TREATMENTS (formalin-acetic acid system). NEMATODES EXAMINED IN WATER.

| Perfusion-treatment time | Nematode reaction | | | |
|---|---|---|---|---|
| | Formalin: acetic acid (3:1 solution) | Formalin: acetic acid (1:1 solution) | Formalin: acetic acid (1:3 solution) | Acetic acid |
| 1 hr. | Nematodes dead but not all relaxed. Internal tissues well preserved, definition fair. | Nematodes dead; relaxed. Organ preservation good, definition good. | Nematodes dead; relaxed; preservation good, definition fair. | Nematodes dead, relaxed; internal organs well preserved, definition fair. |
| 2 hrs. | Nematodes relaxed; organs well preserved, nuclei prominent, fair definition of esophageal outline including *Neotylenchus*. | Nematodes relaxed, organs well preserved, nuclei prominent; somatic muscles cleared. Esophagi obscured by nuclei of neurocytes. | Somatic musculature just about disappeared; nuclei still prominent. | Body contents pulled away from cuticle. Nuclei still very prominent; esophagi not affected by shrinkage of contents of body. |
| 4 hrs. | Definition of internal organs very good; nuclei prominent. *Neotylenchus* esophageal outline and nuclei well defined. | Nematodes clearing; nuclei still prominent; reproductive system in excellent condition. | Internal organs well preserved but poor definition due to clearing. | Somatic muscles not visible; cells of reproductive system separating; appears to be a general breakdown of internal organs and tissues. |
| 8 hrs. | Internal organs in good condition but definition poor. | Somatic musculature definition good; nuclei very prominent. | Somatic musculature cleared; nuclei and esophagi well defined. | Internal organs not subject to distortions but so clear that almost invisible. |
| 24 hrs. | Nematodes relaxed; internal tissues difficult to see because of clearing. No distortions; nuclei prominent. | Somatic musculature cleared; nuclei very prominent. Internal definition good. | Somatic musculature almost completely cleared; nuclei extremely prominent. | Nematodes very ghost-like; yet no apparent internal distortions. |

10–14). Perfusion with water-diluted formalin requires that nematodes be exposed at least 24 hours at all concentrations; apparently, lesser concentrations and exposure periods do not sufficiently preserve or fix the tissues for further treatment (table 10). Because of reaction to perfusion with formalin-formic acid vapors, specimens must be exposed for at least 24 hours. Disadvantages associated with formalin-formic acid vapors can be avoided by decreasing the concentration of formic acid in the various solutions, or by decreasing time of exposure (table 11). The only useful characteristic of formalin-acetic acid was evident at a 24-hour exposure of the 1:1 ratio; here, clarity of nuclei and cuticular structure was outstanding (table 12). Formalin-propionic vapors have a wider range of usefulness than formalin-formic vapors, but pure propionic acid cannot be utilized as a vapor to kill nematodes which are to be processed further (table 13). Specimens sustained processing to glycerin even though they were not killed by exposure to vapor of diluted hydrochloric acid (table 14). In the formalin-hydrochloric acid system, the better specimens (when processed into glycerin) were obtained with longer exposure periods (table 14).

## VAPOR-PHASE PERFUSION WITH TRI-COMPONENT SYSTEMS

Since the bi-compound vapor-phase perfusion systems described produced variable manifestations of morphological characteristics it was decided to add another component to selected systems. It appears, however, that additional

components serve no useful purpose since specimens from these experiments were graded only as fair (table 15).

## FIXATION AS A FUNCTION OF KILLING

Because specimens were usually in good condition only in vapor treatments in which appreciable fixation could have occurred, it was necessary to study fixation after killing. Of the seven fixatives tested, four are commonly used in nematology and three were selected from vapor exchanges previously found successful (table 16). The Seinhorst (1962) method of killing was used, as it is the most successful of heat-killing methods.

Formalin-hydrochloric acid perfusion is suitable for killing Tylenchs when followed by fixation in formalin vapor, solutions of F.A.A., 2½ per cent formalin, or F.A. 4:10. Formalin-acetic acid killings require fixative solutions rather than vapor if specimens are to withstand further treatment. Formalin-propionic acid provides good specimens if wavy somatic musculature is unimportant. Formalin-water perfusion killing followed by any method of fixation generally appears to affect musculature when specimens are processed into glycerin. Using Seinhorst's method of killing, the best specimens were obtained by subsequent treatment with formalin vapor for 24 hours or F.A.A.

TABLE 8

NEMATODE REACTION TO IMMOBILIZATION OR KILLING BY VARYING VAPOR-PHASE PERFUSION TREATMENTS (formalin-propionic acid system). NEMATODES EXAMINED IN WATER.

| Perfusion-treatment time | Nematode reaction | | | |
|---|---|---|---|---|
| | Formalin: propionic acid (3:1 solution) | Formalin: propionic acid (1:1 solution) | Formalin: propionic acid (1:3 solution) | Propionic acid |
| 1 hr. | Nematodes dead but not relaxed; internal organs well preserved. | Nematodes dead; not relaxed. Preservation good but definition of internal organs poor. | Nematodes dead; internal organs well preserved, definition fair. | Nematodes dead; relaxed. Shrinkage of cuticle and intestine. |
| 2 hrs. | Nematodes dead but not relaxed; some distortion of esophagi in shape and position; esophageal nuclei very distinct. | Nematodes look good; internal organs well preserved, cells distinct, nuclei prominent especially those of the esophagi. Some nematodes slight pull-away of somatic musculature. | Nematodes very good; esophagi and nuclei well defined. | Cuticle swollen, internal organs and tissues broken down; nuclei and esophagi still all right; internal destruction severe. |
| 4 hrs. | Nematodes not relaxed; nuclei distinct, cellular outlines distinct; some esophageal distortion. | Nematodes relaxed; no esophageal distortion; nuclei prominent; internal organs somewhat obscure. | No nematode distortion; internal globule formations. Nuclei very prominent. | Somatic muscles pulled from cuticle, nuclei prominent; esophagi obscure. Some show complete internal breakdown. *Paratylenchus* very good. |
| 8 hrs. | Nematodes look excellent; relaxed, internal organs well defined with prominent nuclei. | Nematodes in good condition; organs and nuclei very prominent. | Nematodes in good condition; definition excellent. | Nematodes extremely clear, very ghost-like. |
| 24 hrs. | Coagulation of body fluids, organs well defined. Cuticle distorted and wavy. Nuclei prominent. | Some coagulation of body fluids, organs well defined. Clearing extreme but nuclei prominent. | Clearing extreme, nuclei still prominent; some esophageal distortion. | Organs and cellular outlines disappearing, nuclei very prominent within esophagi. |

TABLE 9

NEMATODE REACTION TO IMMOBILIZATION OR KILLING BY VARYING VAPOR-PHASE PERFUSION TREATMENTS (water-hydrochloric acid and formalin hydrochloric acid systems). NEMATODES EXAMINED IN WATER.

| Perfusion-treatment time | Nematode reaction | | | |
|---|---|---|---|---|
| | Hydrochloric acid: water (1:3 solution) | Hydrochloric acid: water (1:1 solution) | Formalin: hydrochloric acid (1:1 solution) | Formalin: hydrochloric acid (3:1 solution) |
| 1 hr. | Nematodes alive, appear unaffected. | Approximately 10 per cent unaffected by treatment; still very active. | Nematodes relaxed; no distortions, some clearing. | Definite internal clearing; nematodes relaxed and in good condition. |
| 2 hrs. | Nematodes alive. | Somatic muscles in those dead pulled away from cuticle; about 5 per cent unaffected and active. Esophagi and nuclei prominent. | Internally organs appear well preserved; some show destruction of somatic musculature; clearing extensive. | Slight pull-away of somatic musculature; esophagi well defined.. |
| 4 hrs. | Nematodes alive. | All nematodes dead; destruction of internal organs and pull-away of somatic musculature from cuticle very pronounced. | Clearing good so internal organs well defined; nuclei prominent; clearing appears to be associated with internal tissue breakdown. | Nematodes well preserved and well cleared. |
| 8 hrs. | Nematodes alive. | Nematodes very poor; internal structures completely destroyed. | Nematodes relaxed; internal organ definition good; some shrinkage of somatic muscle from body wall, especially in intestinal region. | Nematodes relaxed; definition good; excellent, except body fluids slightly coagulated. |
| 24 hrs. | Nematodes alive. | Complete internal destruction; inner layer of cuticle is separated from outer layer. | Nematodes ghost-like from excessive clearing. | Definition of internal organs and nuclear differentiation good, no distortions. |

## GLYCERIN EXCHANGE AS A FUNCTION OF FIXATIVE

Formalin-water at a ratio of 1:1, with an exposure of 24 hours, was selected as one method of killing (table 17). After killing, nematodes were treated by the same seven fixative agents used in the previous experiment and then were processed into glycerin by five different methods. The best method for processing nematodes into glycerin involves an initial slow dehydration of the nematodes through absolute alcohol as when using the autoexchanger (Viglierchio and Maggenti, 1965). Those nematodes in absolute alcohol should then be further processed through glycerin by slow exchange (automatic exchanger) utilizing a 50–50 glycerin-alcohol solution. This improves definition; any initial distortion present after killing and fixation remains unchanged.

## HEAT-KILLING MODIFICATIONS

Since the Seinhorst method is superior to other recommended hot-water treatments, additives other than acetic acid were tried—but none was as good, or better, than those used in the Seinhorst method (table 18). In the heated-drop technique it is difficult to distinguish killing, immobilization with killing, and overcooking of specimens; additionally, unkilled or overcooked animals do not process satisfactorily. The system combining equal amounts of boiling water and water with nematodes at ambient temperature is handicapped by variation in temperatures caused by

TABLE 10

NEMATODE REACTION TO DEHYDRATION AND PROCESSING INTO GLYCERIN AFTER VARIOUS VAPOR-PHASE PERFUSION TREATMENTS (formalin-water system). NEMATODES EXAMINED IN GLYCERIN.

| Perfusion-treatment time | Nematode reaction | | | |
|---|---|---|---|---|
| | Formalin | Formalin: water (3:1 solution) | Formalin: water (1:1 solution) | Formalin: water (1:3 solution) |
| 1 hr. | Somatic musculature shrunken and wavy; definition poor. | Very poor; muscles pulled away from cuticle and wavy. Appear similar to over-heated nematodes. | Large nematodes very good; small nematodes have wavy somatic musculature. Generally good. | Distorted somatic musculature; otherwise good. |
| 2 hrs. | Internally coagulated; definition poor; somatic musculature wavy. | Reproductive system, esophagi and body cavity muscles well defined. Somatic muscles wavy; some shrinkage of body fluids in base region of esophagi. | Somatic musculature wavy; otherwise, specimens good with prominent nuclei. | Somatic musculature wavy; body contents coagulated around esophagi, good specimens. |
| 4 hrs. | Internally coagulated, somatic musculature wavy. | Coagulation of body fluids in esophageal region; esophagi well defined. Cells, nuclei, and organs well defined. Some small nematodes show esophageal distortion. | Large nematodes very good, good definition. Small nematodes have wavy somatic musculature, esophagi usually distorted. | Large nematodes very good; small nematodes with wavy somatic musculature. |
| 8 hrs. | Nematodes coagulated; somatic musculature wavy. | Generally good; some show wavy somatic musculature; some coagulation in esophageal region. | Wavy somatic musculature; coagulation around esophagi; reproductive system well defined. Generally poor. | Somatic musculature wavy; some Dorylaims excellent. |
| 24 hrs. | Definition good; no distortion of somatic musculature. A few Aphelenchs show pull-away of somatic muscles. | Nematodes in good condition; very good esophagi; some coagulation in base region of esophagi; definition fair. Somatic musculature very good. | Very good, some coagulation around esophagi; esophagi prominent. Somatic musculature very good. | Cellular preservation good, nuclei prominent, some coagulation around esophagi and intestines definition good. Somatic musculature very good. |

different vessels, and by variability in ambient water temperatures. In contrast, the Seinhorst method permits all steps to be reproduced and kills all specimens.

Specimen condition was inferior to previous perfusion experiments; all treatments manifested the disadvantages of hot water: internal coagulation with poor definition and distortion of somatic musculature. The addition of formic acid, propionic acid and hydrochloric acid to hot water was detrimental to specimen quality.

## SEINHORST KILLING, FIXATION AND PROCESSING INTO GLYCERIN

Various methods of fixation or processing of nematodes into glycerin were tested for use with the Seinhorst method of killing. Nematode reactions to alternative modes of dehydration and processing into glycerin following different fixation treatments after heat-killing in water solutions are shown below (nematodes were examined in glycerin):

I. Live nematodes to exchanger—to 5% glycerin in abs. EtOH:
Very poor; internal distortions and pull away of somatic musculature.

II. $H_2O$ at 90°C with 0.5% acetic acid (14.5 cc of heated solution added to nematodes in 0.5 cc of water).

A. Exchanger—to 5% glycerin in abs. EtOH:
Variable reaction; some nematodes show esophageal distortions; some somatic musculature pull-away; some very good.

B. 2½% formalin 24 hours—to exchanger—to 5% glycerin in abs. EtOH:
In general, internal organs well preserved; nuclear and cell definition good; some evidence of collapse.

C. F.A.A. 24 hours—to exchanger—to 5% glycerin in abs. EtOH:
Good, but some show pull-away or wavy somatic musculature.

D. TAF 24 hours—to exchanger—to 5% glycerin in abs. EtOH:
Very good condition; a few nematodes show slight pull-away of somatic musculature.

E. F.A. 4:10 24 hours—to 5% glycerin in methanol at 50°C:
Poor condition; somatic musculature pulled away; in some nematodes cuticle swollen; definition poor.

F. F.A. 4:10 24 hours—to exchanger—to 5% glycerin in abs. EtOH at 40°C for 3 hours:
Definition poor; coagulation of body fluids in esophageal and intestinal region. Some pull-away of musculature.

G. F.A. 4:10 24 hours—to exchanger—to 5% glycerin in abs. EtOH:
Poor; no definition; many distortions; esophagi pulled from intestine; esophagi distorted; stomas collapsed.

H. F:P:H: (2:1:1) 2 hours—to exchanger—to 5% glycerin in abs. EtOH:
Definition good; *Paratylenchus* spear distorted; *Aphelenchus* internally badly distorted; some evidence of somatic muscle distortions (wavy); other nematodes little evidence of any pull-away.

I. F:P:H: (2:1:1) 4 hours—to exchanger—to 5% glycerin in abs. EtOH:
Appear to be very good; some show wavy somatic musculature and fair definition.

J. F:P:H: (2:1:1) 8 hours—to exchanger—to 5% glycerin in abs. EtOH:
Good condition; poor definition of nuclei and cuticular parts; large nematodes show some distortions of somatic musclature.

K. F:P:H: (2:1:1) 8 hours—to exchanger—to 5% glycerin in abs. EtOH at 40°C for 3 hours:
Wavy somatic musculature but not pulled away; definition poor; cuticular parts difficult to see.

L. F:P:H (2:1:1) 8 hours—to 5% glycerin in methanol at 50°C:
Body fluids coagulated; organ definition good; wavy somatic musculature; in general good.

III. $H_2O$ at 90°C with 0.5% acetic acid (nematodes in 0.5 cc of water added to 14.5 cc of heated solution).

A. Exchanger—to 5% glycerin in abs. EtOH:
Stoma collapsed in open stoma forms as *Cephalobus* and *Rhabditis;* in general fair; some esophageal distortions.

B. F.A. 4:10 24 hours—to 5% glycerin in methanol at 50°C:
Esophagi distorted, nuclear definition poor; wavy somatic musculature; swollen cuticle in some nematodes.

No differences were noted in specimen reaction whether the 90°C—0.5 per cent aqueous acetic acid solution was added to nematodes (Seinhorst, 1962), or whether nematodes were added to the hot aqueous acetic acid solution. If nematode specimens are to be further processed (for example, by dehydration and infiltration with glycerin) they should be stabilized; any fixative listed other than Seinhorst fixative F.A. 4 : 10 is preferred. Enforced glycerin infiltration with heated methanol or ethanol, as suggested in the Seinhorst (1959) rapid methods, is inadvisable. Slow evaporation of alcohol from 24 to 48 hours, concomitant with slow infiltration of glycerin, is more likely to produce good specimens. Thus, it is apparent that the Seinhorst fixative is not the best for the Seinhorst method of killing.

## EVALUATION OF STEPWISE OPERATION EFFECTS ON SPECIMEN QUALITY

To study the effects of killing, fixing, dehydration, and processing into glycerin on specimens during the preparation of permanent mounts, microscopic observations of a number of specimens were made between steps (tables 19–22). Four methods of perfusion killing were used: formalin-water, 3:1; formalin-HCl, 3:1; formalin-propionic acid, 3:1; and formalin-formic acid, 3:1; all specimens were in good condition except those exposed to formalin-formic acid combinations.

Specimens killed by diluted formalin in a vapor phase tolerated these fixatives with no adverse effects.

After alcoholic dehydration all chemically-fixed specimens looked good except those treated with F.A.A., or $H_2O$ at 90°C then to F.A. 4:10, or T.A.F. then to $H_2O$ at 90°C. Specimens subsequently processed through glycerin before reexamination in glycerin revealed additional adverse effects in the case of F.A. 4:10, F.A.A., $H_2O$ at 90°C then to F.A.A. and formalin $H_2O$ at 90°C. Some good specimens in alcohol became poor in glycerin, some poor specimens became worse, and some remained unchanged. However, it was noted that the majority of treatments resulted in good glycerin preparations.

TABLE 11

NEMATODE REACTION TO DEHYDRATION AND PROCESSING INTO GLYCERIN AFTER VARIOUS VAPOR-PHASE PERFUSION TREATMENTS (formalin-formic acid system). NEMATODES EXAMINED IN GLYCERIN.

| Prefusion-treatment time | Nematode reaction | | | |
|---|---|---|---|---|
| | Formalin: formic acid (3:1 solution) | Formalin: formic acid (1:1 solution) | Formalin: formic acid (1:3 solution) | Formic acid |
| 1 hr. | Nuclei well defined. Cephalobs show shrinkage from cuticle; Dorylaims shrink from anterior end; Tylenchs have separation of esophagus from intestine. | Very poor; somatic muscle pulled away from cuticle; intestine collapsed; nuclei prominent. | Shrinkage of somatic musculature from body wall; Cephalobs show greatest amount of shrinkage. | Very poor, excessive shrinkage of internal organs and pull-away of somatic musculature. |
| 2 hrs. | In general, fair; some internal distortion of organs and esophagi. Some pull-away of somatic musculature from cuticle. | Nematodes show shrinkage of internal organs, pull-away of somatic muscles and esophageal distortions. | Somatic muscles cleared; nuclei prominent; internal shrinkage. | Very poor, excessive shrinkage of internal organs and pull-away of somatic musculature. |
| 4 hrs. | Very poor, shrinkage of internal organs, somatic muscles pulled away from cuticle; esophageal distortion and definition very poor. | Somatic muscle pulled away; very poor. | Internal shrinkage and pull-away of somatic musculature. | Poor in all respects. |
| 8 hrs. | Very poor; complete internal shrinkage and pull-away from cuticle. | Wavy somatic musculature. Some clearing but most nematodes look coagulated. | Shrinkage and pull-away from cuticle severe; reproductive system, nuclei and esophagi prominent. | Poor; Cephalobs show internal disruption, shrinkage and vacuolation. |
| 24 hrs. | Some nematodes excellent; some internal shrinkage and pull-away of somatic musculature. | Some nematodes show esophageal intestinal separation, clearing of somatic muscles, in general in good condition, nuclei prominent. | Some pull-away evident. Esophagi good; nuclei not well defined. | Nematodes ghost-like; anteriorly body contents pulled away from cuticle. |

TABLE 12

NEMATODE REACTION TO DEHYDRATION AND PROCESSING INTO GLYCERIN AFTER VARIOUS VAPOR-PHASE PERFUSION TREATMENTS (formalin-acetic acid system). NEMATODES EXAMINED IN GLYCERIN.

| Perfusion-treatment time | Nematode reaction | | | |
|---|---|---|---|---|
| | Formalin: acetic acid (3:1 solution) | Formalin: acetic acid (1:1 solution) | Formalin: acetic acid (1:3 solution) | Acetic acid |
| 1 hr. | Generally poor; wavy somatic musculature; esophagi and other internal organs distorted. Cells of reproductive system and other internal organs well defined. | Cuticle intermittently swollen; intestine collapsed; nuclei and reproductive system prominent. Somatic muscle reaction variable, some very good, some wavy. | Somatic musculature pulled away; nuclei prominent. | Very poor, swollen and distorted, but reproductive system cells well defined. |
| 2 hrs. | Wavy somatic musculature; intestine shrunken; cuticle intermittently swollen; nuclei well defined. | Definition poor; cuticle intermittently swollen. | Esophagi distorted; intestine collapsed or pulled from esophagi; very poor. | Very poor. |
| 4 hrs. | Definition and internal preservation good, except for swelling of cuticle in anterior region. | Small nematodes very poor, but nuclei prominent. | Poor; but interesting for definition of spear guiding apparatus. | Extreme shrinkage of body fluids and distortion of internal organs, somatic muscle good, definition of nuclei and cuticular structures good. |
| 8 hrs. | Wavy somatic musculature in anterior region; slight cuticular swelling in anterior region. | Cuticle swollen with internal organs and somatic musculature pulled away. Reproductive system well defined. | Definition good; coagulation of body fluids; cells of reproductive system dissociated. | Very poor, complete pull-away of internal structures and fluids. |
| 24 hrs. | General preservation good; wavy somatic musculature; intestinal shrinkage in a few. | Very good. Some coagulation around esophagi bases; nuclei very well defined. | Nuclei prominent; poor because of internal coagulation and shrinkage of body fluids. | Nematodes completely destroyed, only cuticular shell left. |

Specimens killed with formalin-formic acid vapor generally could not tolerate any fixatives except T.A.F. then to $H_2O$ at 90°C. Since T.A.F. showed distortion the heating step may have partially corrected the abuse; however, further processing showed distortions. This killing procedure is apparently valueless.

Nematodes killed by formalin-propionic vapors, and examined in water or fixative or in ethyl alcohol, revealed excellent specimens very similar to those killed by formalin-HCl vapors. When the same specimens were viewed in glycerin, however, distortions were much more apparent. In all cases pull-away of somatic musculature was noted, although nuclear definition remained excellent.

Specimens killed with formalin-HCl vapors appeared to be in excellent condition in all fixative agents, except for treatments with water at 90°C, or F.A.A. then to $H_2O$ at 90°C. When specimens had been both processed and viewed in alcohol, distortions not noted in water or fixative became apparent. When specimens were killed with formalin-HCl vapors, subsequent fixation in F.A.A. maintained specimens in excellent condition; when specimens were treated with hot water prior to or after F.A.A. they did not maintain their original appearance. Specimens apparently in excellent condition in aqueous

media and alcohol (2.5 per cent formalin; $H_2O$ at 90°C then to 2.5 per cent formalin; 2.5 per cent formalin then to $H_2O$ at 90°C) were adversely affected by processing into glycerin. Nearly half the treatments resulted in good glycerin specimen preparations. Distortions apparent in ethyl alcohol were usually compounded when specimens were mounted in glycerin.

## DISCUSSION

The literature on cytological techniques (Lee, 1946; Gray, 1954; and Baker, 1960) suggests that there is no technique for preserving all tissues in a whole mount eternally and in a lifelike condition; there are differences in the physico-chemical properties of the tissues in multi-tissue specimens which prevent any one procedure from preserving each tissue at optimum quality. A totomount evaluation often is a compromise between acceptable qualities of many tissues in contrast to the exceptional quality of one.

In preparation of nematodes for microscopic study, quality of the final product is largely determined by the harshest step to which specimens are

TABLE 13

NEMATODE REACTION TO DEHYDRATION AND PROCESSING INTO GLYCERIN AFTER VARIOUS VAPOR-PHASE PERFUSION TREATMENTS (formalin-propionic acid system). NEMATODES EXAMINED IN GLYCERIN.

| Perfusion-treatment time | Nematode reaction | | | |
|---|---|---|---|---|
| | Formalin: propionic acid (3:1 solution) | Formalin: propionic acid (1:1 solution) | Formalin: propionic acid (1:3 solution) | Propionic acid |
| 1 hr. | Some coagulation of body fluids; definition of internal organs good; cuticle sometimes wavy as if there has been partial collapse and recovery. | Cleared, cellular detail good; body fluid coagulated in some nematodes. | Somatic musculature pulled away, sometimes wavy. Reproductive system good. | Nuclei prominent; esophageal distortion and coagulation of body fluids; reproductive system poor. |
| 2 hrs. | Somatic muscles sometimes pulled away, muscles sometimes wavy; many nematodes good. | Coagulation of body fluids in some nematodes, some waviness of somatic musculature; in general fair. Nuclei well defined. | Coagulation of body fluids; well defined nuclei. Some nematodes with wavy somatic musculature. In general, fair. | Somatic musculature pulled away from cuticle; nuclei of Secernentea very prominent. In general, poor. |
| 4 hrs. | Large nematodes: wavy somatic musculature; internal structures look good.<br>Small nematodes: in general very good; some wavy somatic musculature. Cephalobs very good, nuclei very prominent. | Wavy somatic musculature; other tissues and internal organs good; nuclei well defined; intestinal shrinkage in Cephalobs. | Most nematodes very good; some show somatic muscle pulled away but generally nuclei, cells and cuticular parts very prominent. | In general, internal tissues and organs shrunken or pulled from cuticle; however, nuclei, and cuticular parts prominent. |
| 8 hrs. | Internal organs and nuclei very prominent; good. | Internal coagulation of body fluids and shrinkage of intestine, but nuclei prominent. Wherever shrinkage and coagulation not present specimens excellent. | Nuclei and reproductive system very good; somatic musculature cleared; sometimes pulled away. | Internal organs and nuclei well defined. Intestinal shrinkage and somatic musculature pulled away. |
| 24 hrs. | Nematodes in good condition, definition excellent, nuclei well defined. | Small nematodes excellent; in large nematodes somatic muscles sometimes pulled away. | Nuclei good; somatic muscles good; some nematodes show wavy distortion of cuticle. | Shrinkage from cuticle almost complete, but no appearance of breakdown of internal organs. |

TABLE 14

NEMATODE REACTION TO DEHYDRATION AND PROCESSING INTO GLYCERIN AFTER VARIOUS VAPOR-PHASE PERFUSION TREATMENTS (hydrochloric acid-water and formalin-hydrochloric acid systems). NEMATODES EXAMINED IN GLYCERIN.

| Perfusion-treatment time | Nematode reaction | | | |
|---|---|---|---|---|
| | Hydrochloric acid: water (1:3 solution) | Hydrochloric acid: water (1:1 solution) | Formalin: hydrochloric acid (1:1 solution) | Formalin: hydrochloric acid (3:1 solution) |
| 1 hr. | Most nematodes quite good; a few show collapse of cuticle but internally good. | Most distortions cuticular rather than internal. If not intermittently collapsed would be good. | Some intestinal shrinkage; some somatic muscles pulled away; reproductive system and nuclei well defined. | Somatic muscles shrunken and pulled away from cuticle, otherwise, good. |
| 2 hrs. | Esophageal preservation very good, somatic muscles well preserved; remainder of body poorly defined; Cephalobs have poor reproductive systems and esophagi. | Nuclei very prominent; somatic musculature pulled away in some nematodes otherwise good. Cephalobs as a group very poor. | Tissues pulled away and distorted. | Somatic musculature pulled away and wavy; internal structures well defined; nuclei prominent. |
| 4 hrs. | Large nematodes in good condition, small nematodes show loosening of cuticle at anterior end; wavy somatic musculature. | Esophagi generally separated from intestine; somatic muscle extremely cleared; large nematodes good, cuticular structures well defined. | Few nematodes with intestinal shrinkage; nuclei, reproductive system and cuticle very good. | Large nematodes have somatic muscles pulled away; some coagulation. Small nematodes show internal shrinkage; nuclei well defined. |
| 8 hrs. | Somatic muscle pulled away from cuticle; esophagi distorted; reproductive system well defined, nuclei poor. | Internal tissues and organs just about completely destroyed. | Few nematodes with intestinal shrinkage; somatic muscles good; generally good but lack definition. | Generally good; somatic muscle not always defined on small nematodes. |
| 24 hrs. | Too poor to process. | Too poor to process. | Good but lack nuclear definition. | Good; nuclei prominent, esophagi and reproductive system good, some nematodes show separation of body fluids at base of esophagi. |

subjected. To proceed with caution through all but one step violates the essential principle of gradual, gentle exchanges. Permanent mounting media cannot improve poor specimens (except for refractive-index manifestations), but they can destroy good specimens. Such gentle exchanges are emphasized by Gray (1964) in his criticism of a 20–30 percent series in alcohol exchanges: "This series is not reasonable, for there is a much greater and more violent diffusion current when a specimen is passed from water to 30 per cent alcohol than there is when a specimen is passed from 70 per cent to 90 per cent alcohol." Heat can speed exchange rate by increasing diffusing rates, but temperatures should not exceed 30–40°C and exposure time should be short (2–4 hours). Gentle exchanges maintain good specimens and often make it possible to improve them by increased definition.

Defining a "good specimen" is a subjective evaluation which is influenced by the requirements of the observer. "Relaxation" in nematodes is such an evaluation. During heat-killing the serpentine configuration of a live nematode is altered, its dead body usually changing from a nearly linear to a crescent or acute helix shape; unfortunately, characteristic external body configuration may be obtained while internal configu-

ration is completely distorted. (On the other hand nematodes can be immobilized without internal distortion or external change in body configuration.) A relaxation procedure in which a nematode assumes a tight helical configuration can be advantageous for purposes of preliminary tentative identification, but can be disadvantageous in a morphological study with serial sections. Moreover, relaxation can interfere with identification; if the nematode assumes a tight coil, "relaxed," taxonomic characters may be hidden by superimposed coils.

Clearing implies an increase in transparency by substituting solvents having refractive indices comparable to tissues for the highly refractive elements of tissues. Since internal characters have different clearing rates it is possible to improve definition of tissues and organs by the clearing of globules and inclusions. As clearing progresses the definition of internal characters is lessened; and excessive clearing results in a complete loss of definition. This can often be reversed to some degree by subsequent re-infiltration with solutions of suitable refractive index. It was often noted that poor or fair definition of noncuticular parts of a fixed animal were improved by infiltration with glycerin. On the other hand, cuticular parts (spear, cephalic framework spicules, etc.) which are well defined in formalin or T.A.F. are poorly defined or invisible in glycerin.

Slight internal distortions frequently can be tolerated when only one organ or tissue is affected. Wavy somatic musculature, often the only form of distortion, apparently does not affect other internal organs. If a reproductive system is to be studied, a procedure which preserves it but is detrimental to esophageal preservation is more desirable than one which attempts total preservation at some expense to all organs.

One of the most difficult forms of distortion to evaluate is total body shrinkage or swelling, especially when internal distortions are not evident. In fact, it may sometimes be impossible to determine the extent of shrinkage or swelling in the final state because the animal could not be measured properly in the living state. By putting specimens of the same species through two different processing procedures it is possible to effect a 10 per cent difference in size without noticeable internal distortion.

TABLE 15

NEMATODE REACTION TO DEHYDRATION AND PROCESSING TO GLYCERIN AFTER VARIOUS TRI-COMPONENT VAPOR-PHASE PERFUSION TREATMENTS. NEMATODES EXAMINED IN GLYCERIN.

| Perfusion-treatment time | Nematode reactions | | | |
|---|---|---|---|---|
| | Propionic acid Hydrochloric acid Water (1:1:1 solution) | Propionic acid Hydrochloric acid Water (1:1:2 solution) | Formalin Propionic acid Hydrochloric acid (1:1:1 solution) | Formalin Propionic acid Hydrochloric acid (2:1:1 solution) |
| 2 hrs. | Somatic musculature pulled away; definition poor. | Esophagi often distorted; some nematodes collapsed; definition fair. | Somatic musculature pulled away; definition good. | Definition poor; somatic collapse and intestine shrinkage but no pull-away of somatic musculature. |
| 4 hrs. | Few internal distortions; definition very poor. | Some nematodes collapsed; esophagi distorted and definition poor. | Coagulation of body fluids; wavy somatic musculature. | Definition fair; some somatic collapse and internal shrinkage in anterior region; noted especially in *Rhabditis*. |
| 8 hrs. | Internal organ shrinkage; definition poor. | Poor nematodes; collapsed. | Some nematodes collapsed, definition poor. | Nuclei and definition good; in some pull-away or wavy somatic musculature. |

TABLE 16

NEMATODE REACTION TO DEHYDRATION AND PROCESSING TO GLYCERIN AFTER STABILIZATION BY DIFFERENT FIXATION TREATMENTS FOLLOWING ALTERNATIVE METHODS OF KILLING. NEMATODES EXAMINED IN GLYCERIN.

| Fixative treatment and treatment time | Nematode reaction | | | | |
|---|---|---|---|---|---|
| | Formalin: HCL (3:1 solution, 3 hrs.) | Formalin: acetic acid (1:1 solution, 1 hr.) | Formalin: propionic acid (3:1 solution, 8 hrs.) | Formalin: water (1:1 solution, 24 hrs.) | Water at 90°C + 0.5% acetic acid |
| Formalin vapor, 24 hrs. | Some nematodes look good; majority show shrinkage of somatic musculature from cuticle, esophagi generally good, Tylenchs good. | Some shrinkage of somatic muscle, especially *Tripyla;* nuclei well defined. In other specimens get poor esophageal definition. | Very good, show only slight amount of somatic muscle shrinkage; *Tripyla* very good. | Wavy somatic musculature; poor definition; internally cells appear well preserved. | Good without noticeable distortions. |
| Acetic acid vapor, 2 hrs. | Somatic muscle pulled away; look fair, no severe distortions. | Somatic muscle pulled away very severely; swelling of cuticle and shrinkage of internal organs; very poor. | Tylench good, *Tripyla* shows collapse; internally tissues well preserved and well defined. | Definition fair, shrinkage slight. | Somatic muscle slightly pulled away; esophagi well defined; good except slight muscle distortion. |
| Propionic acid vapor, 1 hr. | Shrinkage and pull-away of somatic muscles. Nuclei very well defined. | Somatic muscle pulled away; esophagi distorted and internal organs obscure. | All nematodes but *Tripyla* look good; *Tripyla* shows somatic muscle pull-away; internal organs and nuclei well defined. | Some esophageal distortion and shrinkage; somatic muscle wavy; definition fair, appear coagulated. | Good except for slight pull-away of somatic muscle, which appears wavy. |
| F.A.A., 36 hrs. | Tylenchs stand treatment very well; remainder of nematodes poor or fair. | Tylenchs very good, some poor; some excellent except for slight shrinkage of somatic musculature. | Somatic muscles pulled away, otherwise would be good. | Only slight shrinkage of organs, but not good. | Very good, almost no internal shrinkage. |
| 2½% formalin, 36 hrs. | Variable reaction. Some Tylenchs good, but Adenophorea esophageal distortions and pull-away of somatic muscle; nuclei well defined. | Some Tylenchs excellent; Adenophorea shrunken, some collapse and pull-away of somatic musculature, too much distortion to be good. | Somatic muscles pulled away; tissues and nuclei well defined but not good. | Show pull-away of somatic muscle; shrinkage and coagulation. | Good except for slight pull-away of somatic musculature. |
| T.A.F., 36 hrs. | In general fair, some pull-away. | Somatic muscle slightly wavy with fiber separation, cuticle of some swollen. Aphelenchs very good. | Somatic muscle pulled away, otherwise good. | Somatic musculature wavy and pulled away from cuticle in some places, otherwise slight shrinkage and good definition. | Slight shrinkage of somatic muscle, otherwise good. |
| F.A. 4:10, 36 hrs. | Good except somatic muscle pulled away in *Tripyla*, and shrinkage in Rhabdits; Tylenchs and Aphelenchs look very good. | Tylenchs, Aphelenchs and Dorylaims look very good but other genera show swelling of cuticle and somatic muscle pull-away. | Somatic muscle pulled away, otherwise good. | Generally appear quite good; little internal shrinkage; some appear coagulated. | Internal shrinkage and wavy somatic musculature. |

TABLE 17

NEMATODE REACTION TO DEHYDRATION AND DIFFERENT MODES OF PROCESSING TO GLYCERIN AFTER STABILIZATION BY DIFFERENT FIXATION TREATMENTS (Nematodes killed by formalin-water vapor-phase perfusion system). NEMATODES EXAMINED IN GLYCERIN.

| Fixative treatment, and treatment time | Nematode reaction | | | | |
|---|---|---|---|---|---|
| | 100% ETOH by exchanger to 5% glycerin in 100% ETOH | 100% ETOH by exchanger at 40°C to 5% glycerin in 100% ETOH | 100% ETOH + 1% Acetic acid by exchanger to 5% glycerin in 100% ETOH | 100% ETOH by exchanger to 50–50 glycerin + ETOH by exchanger | 6% glycerin in 100% ETOH by exchanger |
| Formalin vapor, 24 hrs. | Wavy somatic musculature; poor definition; internally cells appear well preserved. | Internal cells and nuclei well defined; wavy somatic musculature; intestine shrunk or collapsed. | Internal cells and nuclei prominent; body fluids vacuolated; somatic muscle wavy, intestine shrunk or collapsed. | Pull-away of somatic muscle and waviness; no internal distortions evident; nuclei and cells pronounced with good definition but some vacuolation. | Somatic muscle wavy and pulled away in some areas. No internal distortions noted and nuclei and cells prominent. |
| Acetic acid vapor, 2 hrs. | Definition fair, shrinkage slight. | In general have coagulated appearance. Dorylaims very good. | Pull-away of somatic muscle from cuticle; definition not good. | Some pull-away of somatic musculature but definitely less than other treatments; no internal shrinkage, definition good. | Collapsed; internally coagulated; cells of intestine very prominent. |
| Propianic acid vapor, 1 hr. | Some esophageal distortion and shrinkage; somatic muscle wavy; definition fair, appear coagulated. | Esophageal distortions; wavy somatic musculature; internally coagulated but definition good. | Very poor in all respects. | Definition good; somatic muscles wavy; some esophageal distortions, Dorylaims very good. | Collapsed and with distorted esophagi. |
| F.A.A., 36 hrs. | Only slight shrinkage of organs, but not good. | Poor, internal shrinkage of organs from one another; definition poor. | Poor, internal shrinkage of organs from one another, definition poor. | No internal distortions but appear coagulated internally, therefore, definition fair. | Collapsed; internal shrinkage and organ distortions. |
| 2½% formalin, 36 hrs. | Show pull-away of somatic muscle; shrinkage and coagulation. | Dorylaims, Mononchs, good except for slightly wavy somatic musculature; others coagulated. Definition fair. | Some esophageal distortions with Rhabdits but no other internal distortions noted, definition fair. | Somatic musculature wavy; some nematodes collapsed internally coagulated. Definition fair. | Most nematodes collapsed except for Dorylaims which are very good. |
| T.A.F., 3½ hrs. | Somatic musculature wavy and pulled away from cuticle in some places, otherwise slight shrinkage and good definition. | Somatic musculature wavy; definition good; some pull-away. In *Tripyla* the esophagus is contracted laterally. | Somatic musculature wavy; some nematodes collapsed; definition good; in some nematodes muscles pulled away. | Definition good; internal organs in very good condition; only *Tripyla* appears coagulated inside. | Somatic musculature wavy; some nematodes collapsed; clarity good. |
| F.A. 4:10, 3½ hrs. | Generally appear quite good; little internal shrinkage; some appear coagulated. | Good but definition poor due to coagulation. | If definition were better would be good. | Coagulation of body fluids; definition fair. | Good except for some vacuolation and wavy somatic musculature. |

TABLE 18

NEMATODE REACTION TO DEHYDRATION AND PROCESSING TO GLYCERIN AFTER FIXATION (nematodes heat-killed in aqueous solutions). NEMATODES EXAMINED IN GLYCERIN.

| Treatments at 90°C | Nematode reactions | |
|---|---|---|
| | F.A.A. | F.A. 4:10 |
| Water | Generally good; with smaller nematodes there is vacuolation around esophagi; some esophagi distorted, as if contracted; somatic musculature wavy. | Generally good, but pull-away of somatic musculature and vacuolation of smaller nematodes. |
| Aqueous acetic acid, 0.5 % | Generally very good; some intestinal shrinkage, some vacuolation. | In general, good; some pull-away of somatic musculature. |
| Aqueous formalin, 0.5% | Some waviness of somatic musculature; good nuclear definition; some good, some bad. | Some waviness of somatic musculature and evidence of shrinkage in posterior intestine as well as pull-away anteriorly and posteriorly. |
| Aqueous formic acid, 0.5% | Somatic musculature poorly preserved; pull-away and shrinkage very evident; esophagi and nuclei well defined. | Poor somatic musculature; pull-away and internal shrinkage very evident. |
| Aqueous propionic acid, 0.5% | Very poor; shrinkage; pull-away of somatic musculature. | Distortions; pull-away of muscle and shrinkage of internal organs, very poor. |
| Aqueous hydrochloric acid, 0.5% | Very poor; collapse; shrinkage of internal organs, somatic musculature distorted. | Very poor. |

Such distortion may not be important to a morphologic study of a particular organ system, but is is extremely important taxonomically. Accordingly, more than one procedure should be employed, whether a study is taxonomic, morphologic or histologic.

The suitability of any specimen is determined by the use to which it will be put. Often internal distortion or complete internal destruction has no effect upon the taxonomic value of the specimen—this is true of morphological studies of gross form, or of taxonomic studies based on cuticular parts, surface manifestations, setal patterns, or secondary sexual characters. Methods which can produce such results are advantageous for the identification of animal-parasitic nematodes by eggs, or plant-parasitic nematodes such as *Meloidogyne* spp. by perineal pattern, or *Heterodera* spp. by cuticular reticulation, or insects by copulatory organs (or setal pattern in the case of thrips).

Hot-water killing stabilizes organ tissues and allows a wider selection of fixative treatments, but this is also the method's greatest disadvantage. Stabilization by heat causes permanent effects that resist specimen improvement by subsequent chemical treatments. Without heat-killing, procedural modifications designed to enhance desired specimen features (such as improved definition, or clearing, or both) can be introduced; with heat-killing no known technique will improve vacuolation or coagulation.

The principal usefulness of gas treatments is their so-called narcotization effect. With gas it is possible to immobilize a nematode for one experimental purpose and then revive it for another. Narcotization experiments should be short termed (Lee, 1946 and Gray, 1954). Long-term narcotization begins with reversible physiological changes which are followed by irreversible physiological changes that result in morphological changes. In such slow immobilization or killing, morphological manifestations appear which are not evident in quicker kills.

A limited number of components was used in our vapor-phase perfusion tests; any of a number of other components could be as good, or better. Because more successful methods have been moderately rapid, yet relatively gentle, the autoexchanger offers a completely new approach through the use of aqueous non-gaseous solutions for killing and fixation. As a result of the present study there are now multiple methods for killing or fixing, or both, of nema-

TABLE 19

EVALUATION OF NEMATODES STABILIZED IN DIFFERENT FIXATIVES AFTER KILLING BY VAPOR-PHASE PERFUSION (formalin:water; 3:1 solution, 24 hours). NEMATODES EXAMINED AT THE END OF EACH STEP.

| Fixative treatments | Nematode reaction | | |
|---|---|---|---|
| | Water or fixative | Ethyl alcohol | Glycerin |
| $H_2O$ | Nematodes in excellent condition. | Nematodes in good condition. | Nematodes in fair condition. |
| $H_2O$ at 90°C | All nematodes look good; one Rhabdit shows some shrinkage from head end. | Nematodes in good condition. | Nematodes in fair condition. |
| F.A. 4:10 | Nematodes in excellent condition; no shrinkage or distortion. | Nematodes in good condition. | Some shrinkage around reproductive system; Cephalobs with wavy somatic musculature. |
| TAF | Nematodes in excellent condition; no shrinkage or distortions. | Nematodes in good condition. | Very little distortion; clearing evident, almost too much; shrinkage of eggs, otherwise good. |
| F.A.A. | Nematodes in excellent condition; no shrinkage or distortions. | Some internal shrinkage noticeable; this includes some musculature pull-away from cuticle. | Generally poor; nuclei well defined; somatic musculature distorted. |
| Formalin, 2½% | Nematodes not relaxed; some esophageal distortion, otherwise very good. | Nematodes in good condition. | In some nematodes shrinkage around esophagus and eggs; cuticle waved as if partially collapsed; wavy somatic musculature. |
| $H_2O$ at 90°C to F.A. 4:10 | Nematodes excellent; no shrinkage or distortions. | Some nematodes show slight internal shrinkage. | Some distortion of somatic musculature but in general specimens quite good. |
| $H_2O$ at 90°C to TAF | Nematodes excellent; no shrinkage or distortions. | Nematodes in good condition. | Some nematodes become too clear, organs poorly defined; very little distortion but eggs of Secernentea shrunken. |
| $H_2O$ at 90°C to F.A.A. | Nematodes excellent; no shrinkage or distortions. | Good; no indication of shrinkage. | Some distortion and shrinkage; in some, pull-away of somatic musculature from cuticle. |
| $H_2O$ at 90°C to 2½% formalin | Nematodes excellent; no shrinkage or distortions. | Nematodes in good condition. | Very little somatic muscle distortion; in Aphelenchs and Rhabdits some shrinkage; internally, generally good. |
| F.A. 4:10 to $H_2O$ at 90°C | Some nematodes appear to show internal shrinkage. | In general, nematodes look good. | Good; some appear coagulated in esophageal region, especially Tylenchs. |
| TAF to $H_2O$ at 90°C | Nematodes excellent; no shrinkage or distortions. | Show evidence of collapse. | Generally good; some pull-away of somatic musculature from cuticle in Rhabdits; some waviness of somatic musculature. |
| F.A.A. to $H_2O$ at 90°C | Nematodes excellent; no shrinkage or distortions. | Nematodes in good condition. | Shrinkage of esophagi; variable waviness of somatic musculature; generally good. |
| 2½% formalin to $H_2O$ at 90°C | Nematodes excellent; no shrinkage or distortions. | Nematodes in good condition. | Shrinkage, distortions, and coagulation evident; pull-away of somatic musculature in some. |

TABLE 20

EVALUATION OF NEMATODES STABILIZED IN DIFFERENT FIXATIVES AFTER KILLING BY VAPOR-PHASE PERFUSION (formalin:formic acid; 3:1 solution, 24 hours). NEMATODES EXAMINED AT END OF EACH STEP.

| Fixative treatments | Nematode reaction | | |
|---|---|---|---|
| | Water or fixative | Ethyl alcohol | Glycerin |
| $H_2O$ | Pull away of somatic musculature noted at anterior end more than posterior end. | Nematodes in fair condition. | Some nematodes excellent; some internal shrinkage and pull-away of somatic musculature. |
| $H_2O$ at 90°C | Appear to be good; no shrinkage anteriorly. | Nematodes in good condition. | Nematodes in fair condition. |
| F.A. 4:10 | Internal distortions and pull-away of somatic musculature. | Cuticle swollen; pull-away of somatic musculature; internal organ definition poor. | Somatic musculature pull-away; some distortions of internal organs. |
| TAF | Internal distortions and pull-away of somatic musculature. | Some distortions of esophagi and intestine. Pull-away of somatic musculature. | Some good, some poor. |
| F.A.A. | Internal distortion and pull-away of somatic musculature. | Some poor, some good. | Poor. |
| Formalin, 2½% | Internal distortion and pull-away of somatic musculature. | Some nematodes seem all right; others show distortions and pull-away of somatic musculature. | Fair, somatic musculature pull-away, nuclei prominent, definition good. |
| $H_2O$ at 90°C to F.A. 4:10 | Pull-away of somatic musculature noted more at anterior end. | Variable; some show shrinkage of internal organs. | Very poor. |
| $H_2O$ at 90°C to TAF | Internally organs distorted; somatic musculature pull-away more anteriorly. | Appear all right except for pull-away of somatic musculature. | Very poor. |
| $H_2O$ at 90°C to F.A.A. | Esophagi distorted; somatic musculature pull-away anteriorly. | Appear all right except for pull-away of somatic musculature. | Very poor. |
| $H_2O$ at 90°C to 2½% formalin | Internal shrinkage and distortion of organs; somatic musculature pull-away. | Cuticle swollen and somatic musculature pull-away. | Very poor. |
| F.A. 4:10 to $H_2O$ at 90°C | Some internal shrinkage. | Seem all right. Some esophageal distortions. | Worthless. |
| TAF to $H_2O$ at 90°C | Nematodes in good condition. | Some internal distortion as shrinkage of intestine; otherwise all right. | Poor; internal organs with fair definition. |
| F.A.A. to $H_2O$ at 90°C | Internal shrinkage, distortion and swelling of cuticle. | Variable reaction; internal shrinkage, distortion and vacuolation; some good. | Poor; definition fair. |
| 2½% formalin to $H_2O$ at 90°C | Internal shrinkage and distortion; some swelling of cuticle. | Some shrinkage of esophagi and reproductive system. | Very poor. |

TABLE 21

EVALUATION OF NEMATODES STABILIZED IN DIFFERENT FIXATIVES AFTER KILLING BY VAPOR-PHASE PERFUSION (formalin:propionic acid; 3:1 solution, 24 hours). NEMATODES EXAMINED AT END OF EACH STEP.

| Fixative treatments | Nematode reaction | | |
|---|---|---|---|
| | Water or fixative | Ethyl alcohol | Glycerin |
| $H_2O$ | Nematodes in very good condition. | Nematodes in good condition. | Nematodes in good condition; nuclei well defined; definition excellent. |
| $H_2O$ at 90°C | All good but Mononchs show wavy somatic muscles. | Nematodes in good condition. | Nematodes in fair condition. |
| F.A. 4:10 | Nematodes in very good condition. | Nematodes in good condition. | Somatic musculature pull-away; internally very good; nuclei prominent. |
| TAF | Nematodes in very good condition. | Nematodes in good condition. | Somatic musculature pull-away; internal organs and nuclei very prominent. |
| F.A.A. | Some nematodes show pull-away of somatic musculature; may be due to swelling of cuticle rather than shrinkage. | Nematodes in good condition. Some pull-away of somatic musculature. | Somatic musculature pull-away; some distortion of internal organs but nuclei prominent. |
| Formalin, 2½% | Most excellent; some show slight pull-away of somatic musculature. | Generally good but some distortions of cuticle approaching collapse. | Some pull-away of somatic musculature; otherwise, excellent. |
| $H_2O$ at 90°C to F.A. 4:10 | Slight pull-away of somatic musculature; otherwise excellent internally. | Good. Some pull-away of somatic musculature. | Somatic musculature pull-away; internal organs distorted; nuclei prominent; definition good. |
| $H_2O$ at 90°C to TAF | Somatic musculature pull-away; otherwise excellent internally. | Some collapse evident; generally good. | Some pull-away of somatic musculature; internally, organs very good and nuclei prominent. |
| $H_2O$ at 90°C to F.A.A. | Some swelling of cuticle; generally good. | Nematodes in good condition. | Some nematodes very poor; others definition of organs and nuclei good. |
| $H_2O$ at 90°C to 2½% formalin | Nematodes in very good condition. | Nematodes in good condition. | Somatic musculature pull-away from cuticle; internal organs, nuclei, and esophagi very good. |
| F.A. 4:10 to $H_2O$ at 90°C | Nematodes in good condition. | Nematodes in good condition. | Somatic musculature distorted; nuclei and reproductive system good. |
| TAF to $H_2O$ at 90°C | Nematodes in good condition. | Nematodes in very good condition. | Good; definition excellent; a few show pull-away of somatic muscles. |
| F.A.A. to $H_2O$ at 90°C | Some pull-away of somatic musculature but otherwise good. | Some pull-away of somatic musculature and partial collapse. | Cuticular distortions and pull-away of somatic musculature; definition very good. |
| 2½% formalin to $H_2O$ at 90°C | Nematodes in good condition. | Nematodes in good condition. | Secernentea take treatment very well; others show pull-away of somatic musculature; internally very good. |

TABLE 22

EVALUATION OF NEMATODES STABILIZED IN DIFFERENT FIXATIVES AFTER KILLING BY VAPOR-PHASE PERFUSION (formalin:hydrochloric acid; 3:1 solution, 24 hours). NEMATODES EXAMINED AT END OF EACH STEP.

| Fixative treatments | Nematode reaction | | |
|---|---|---|---|
| | Water or fixative | Ethyl alcohol | Glycerin |
| $H_2O$ | Most nematodes very good; some show shrinkage at anterior end. | Nematodes in good condition. | Good; nuclei prominent; esophagi and reproductive system good; some coagulation at base of esophagus. |
| $H_2O$ at 90°C | Nematodes same as unheated but shrinkage when present more severe. | Nematodes in fair condition. | Nematodes in fair condition. |
| F.A. 4:10 | Nematodes in good condition. | Somatic musculature pulled away from cuticle. | Very good; some coagulation of body fluids. |
| TAF | Nematodes in good condition. | Monochs take treatment well; some have somatic musculature pulled away. | Very good; almost too clear. |
| F.A.A. | Nematodes in good condition. | Nematodes in good condition. | Adenophorea very good specimens; Secernentea variable reaction; Rhabdits poor. |
| Formalin, 2½% | Nematodes in good condition. | Nematodes in good condition but some shrinkage around eggs. | Very poor; too much internal distortion, clearing and separation. |
| $H_2O$ at 90°C to F.A. 4:10 | Nematodes in good condition. | Nematodes in good condition. | Somatic musculature pull-away in many nematodes, otherwise would be good. |
| $H_2O$ at 90°C to TAF | Nematodes in good condition. | Some pull-away of somatic musculature, but internal organs good. | Somatic musculature pull-away in some nematodes; internally very good. |
| $H_2O$ at 90°C to F.A.A. | Nematodes in good condition. | Pull-away of somatic musculature and distortions of esophagi. | Very poor; pull-away of somatic musculature; distorted esophagi and coagulation of body fluids. |
| $H_2O$ at 90°C to 2½% formalin | Nematodes in good condition. | Nematodes in good condition. | Very poor; coagulated; somatic musculature pull-away. |
| F.A. 4:10 to $H_2O$ at 90°C | Nematodes in good condition. | Some pull-away from cuticle anteriorly, otherwise good. | Too clear; somatic musculature pull-away; distorted internally. |
| TAF to $H_2O$ at 90°C | Nematodes in good condition. | Some pull-away; vacuolation. | Good; a little too clear. |
| F.A.A. to $H_2O$ at 90°C | Some vacuolation and shrinkage of body fluids from base of esophagi. | Generally all right; some pull-away of somatic musculature; some vacuolation and shrinkage anteriorly. | Poor; too much internal distortion and pull-away. *Criconema* somatic muscles very good. |
| 2½% formalin to $H_2O$ at 90°C | Nematodes in good condition. | Good; some pull-away of somatic musculature. | Very poor, coagulated, distorted and somatic musculature pull-away. |

todes; however, new and completely different methods are still desirable. When different procedures are combined with use of the autoexchanger for multiple processing to embedding it will be possible to study nematodes prepared in various ways, thereby eliminating procedural artifacts.

As the complexity of operations increases additional restrictions are added; the number of methods by which a specimen may be suitably stabilized to yield an acceptable specimen will be reduced. A distorted specimen is rarely improved by techniques used after it becomes distorted; attempts to make such improvements are inadvisable, as latent distortion is liable

to become manifest even after superficial improvement has been shown. There are a number of methods by which a nematode may be processed into glycerin to yield a good specimen; some are indicated below:

I. Killing:
- A. Seinhorst method (90°C water + 0.5 per cent acetic acid)
- B. Vapor-phase perfusion:
  1. Formalin-water, 3:1, 24 hours
  2. Formalin-propionic acid, 3:1, 24 hours
  3. Formalin-HCl, 3:1, 24 hours

II. Fixation:
- A. F.A.A. (Seinhorst method only)
- B. 2½ per cent formalin (only with perfusion, formalin-water or formalin-propionic acid)
- C. T.A.F., all methods of killing
- D. T.A.F. to 90°C $H_2O$, all methods of killing
- E. F.A. 4:10 (all except perfusion formalin-water)
- F. 90°C $H_2O$ to F.A. 4:10 (only with perfusion formalin-water)

III. Processing into Glycerin:
- A. Dehydrate gradually to absolute EtOH (5.0 per cent increments or automatic exchanger, Viglierchio and Maggenti, 1965)
  1. Infiltrate with 5.0 per cent glycerin in absolute EtOH
  2. Infiltrate with 5.0 per cent glycerin in absolute EtOH at 40°C
  3. Infiltrate (by exchanger) 50-50 solution of glycerin and absolute EtOH
  4. Infiltrate (by exchanger) 50-50 solution of glycerin and absolute EtOH at 40°C

Any number of other methods could have been selected, depending upon the purposes and needs of the investigation. The use of a particular system does not assure success with any nematode; nematodes differ markedly in tissue structure, permeability and other physical-chemical properties, and these differences can be manifested adversely in any tissue-processing. The probability of success with an unknown animal is likely to be greater if the procedure used has been satisfactory for a wide variety of nematodes. There is no assurance that a procedure which leads to a good specimen in glycerin, for example, will necessarily lead to a good specimen in paraffin, although the probability of success is likely to be greater.

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