

PROTOCOL FOR DETERMINING
CAUSE OF DEATH
IN
SEA TURTLES

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R. E. Wolke, DVM, PhD

Death in all living organisms is due to cessation of respiration in a sufficient number of cells to result in a cessation of respiration in the organism itself. The environmental conditions necessary to cause cellular respiratory failure are multiple and include such factors as trauma (plasmalemma rupture), bacterial toxins, poisons (cyanide), and metabolic failures (uremia). The degree to which cells and organs may be destroyed and still allow the organism to respire (live) is variable and based on empirical observation. Similarly, the levels of toxins and/or poisons present in the organism or the numbers of such structures as red blood cells compatible with life are also empirical observations determined for man and a number of domestic animals so that when cellular or tissue destruction or levels of toxins or red blood cells fall outside of normal ranges, because of past experience or experimentation, a cause of death can be suggested with some certainty.

Unfortunately, for the various species of sea turtles, such empirical observations and experimentation have not been made, except in a few instances. Therefore, until more data is collected (empirical observation/experimentation) determination of cause of death in these animals must depend upon those few observations made and inference from the phenomenon in other better studied species.

Recognizing these shortcomings, cause of death in sea turtles may be categorized as due to 1) infectious disease, 2) metabolic disease, and 3) physical disease. On the basis of present knowledge the signs of these conditions in sea turtles are basically the same as in other poikilotherms and higher vertebrates.

In all instances, as much historical background as possible must be collected. Frequently the lesions observed are non-specific, however, cause

of death can be determined with some certainty if circumstances surrounding the death are known.

There is a dearth of information regarding the diseases of sea turtles. In some instances, the role of the known diseases in producing death is uncertain. A protocol to determine the cause of death in sea turtles can only address known entities. Clearly, to speculate as to what might occur based upon observations in other better studied vertebrates may be stretching the logical approach of inference to an extreme.

This protocol is prepared under a contract which wishes the contractee to determine cause of death. The contractor states that the protocol should not address the diagnosis of disease since a disease may or may not lead to death. This statement is correct, a disease may or may not lead to death. Therefore, determination of cause of death must take into consideration whether or not a disease is present. One cannot separate them. Any ongoing deviation from the normal (disease) which destroys a sufficient number of cells necessary for life will result in death. To attempt to differentiate disease processes from determining the cause of death is artificial and naive. If an animal dies of pulmonary tuberculosis what is the cause of death, Mycobacteria or neuronal anoxia in the medulla oblongata? Every cause of death could undoubtedly be attributed to anoxia whether it was decapitation, drowning, hemorrhage, pneumonia or Spirorchidiasis. For this reason the protocol is directed towards disease phenomenon which result in cellular destruction not compatible with life.

I. To determine if a biological agent (virus, bacteria, fungus, protozoa, helminth) is a cause of death:

A. Recover pathogenic agent from blood or uncontaminated organ.

1. Definition of pathogenic agent. A pathogenic agent is one which is capable of causing disease or death in the host. Knowledge

of these agents comes about through observation and the fulfillment of Koch's Postulates. Few observations have been made in sea turtles and their protection prevents experimental production of disease to determine pathogenicity. For that reason, inference from known pathogens in other animals is necessary. Flow sheets and the isolation techniques which follow are based upon such inference:

a. Viral isolation

Method 1 - negative staining (1)

Method 2 - isolation using turtle tissue cultures (11)

b. Bacterial isolation

Flow sheet #1 - Identification of Potential Gram-negative Sea Turtle Pathogens.

Flow sheet #2 - Identification of Potential Gram-positive Sea Turtle Pathogens

c. Fungal isolation

Initial isolations - may be made on Sabouraud, cornmeal, or Lowenstein Jensen agar. Final identification requires the expertise of a mycologist.

d. Protozoal isolation

Isolation and examination of protozoa usually follow scrapings or wet mounts of lesions since protozoal culture is difficult and requires highly technical procedures. Immediate classification may be made on the basis of flagella (Flagellates), cilia (Ciliates), pseudopodia (Amoeba), or spores (Cnidosporidia). These characteristics are apparent on wet mounts and/or Giemsa stained air-dried slides.

Further identification requires the expertise of a protozoologist.

e. Helminth isolation

Flow sheet #1 - Identification of Potential Helminth Sea Turtle Pathogens

Flow sheet #2 - Table III. Flow sheet Preparing Helminth Whole Mounts (30).

B. Presence of Lesion and/or Agent in Tissue

1. Gross lesions - Gross lesions (those apparent with the unaided eye) constitute changes in color, size, consistency, location or odor of an organ or groups of organs. They are indicative of tissue malfunction or destruction. Such areas are recorded, cultured and preserved for microscopic examination. The extent of such lesions are important in determining the cause of death. A focal 1 cm depression on the liver surface is of far less import than massive shrinkage and hardening of such an organ. The latter may result in complete cessation of hepatic function and lead to death, the former has little effect on hepatic function.

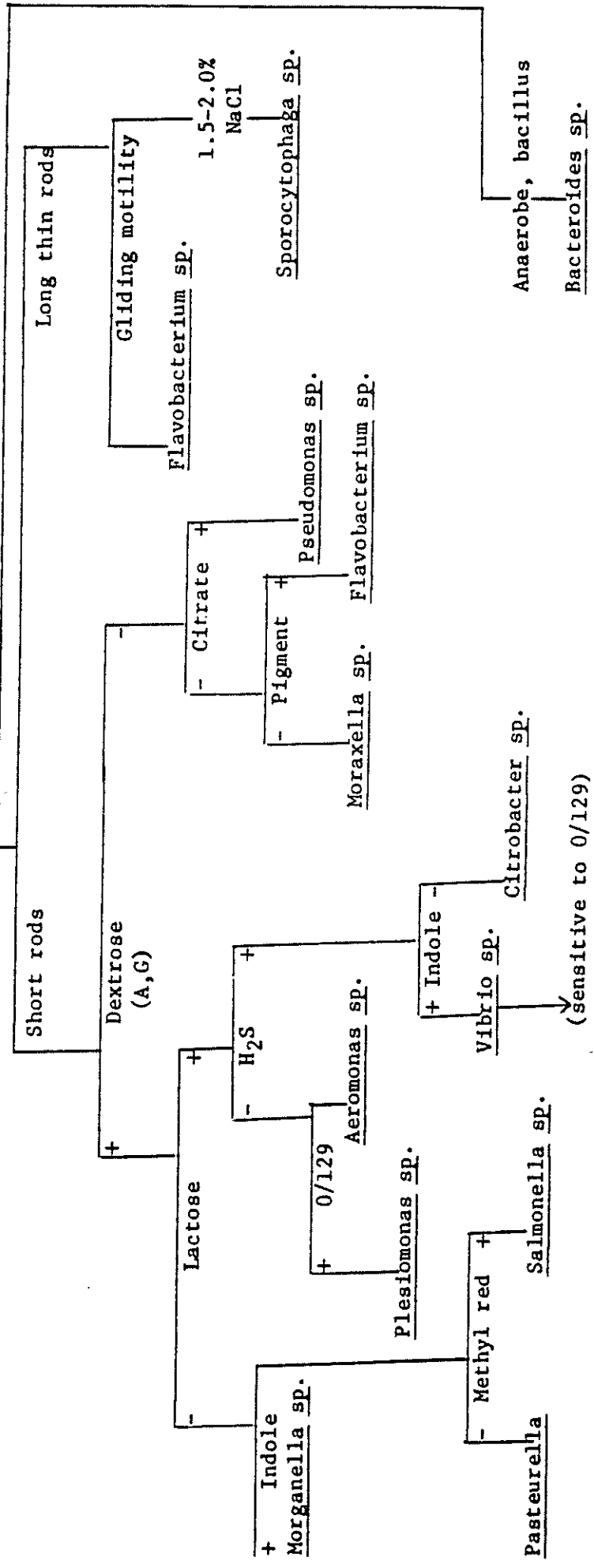
Recovery of a pathogenic agent from a lesion, especially if the culture is relatively pure, is circumstantial evidence of a cause and effect relationship. Positive evidence is dependent on microscopic identification.

2. Histopathologic lesions - Histopathologic lesions are morphologic lesions at the microscopic level characterized by cellular degeneration or necrosis, abnormal pigment or mineral deposition, abnormalities of growth or inflammation. The patterns of degen-

Identification of Potential Gram-negative

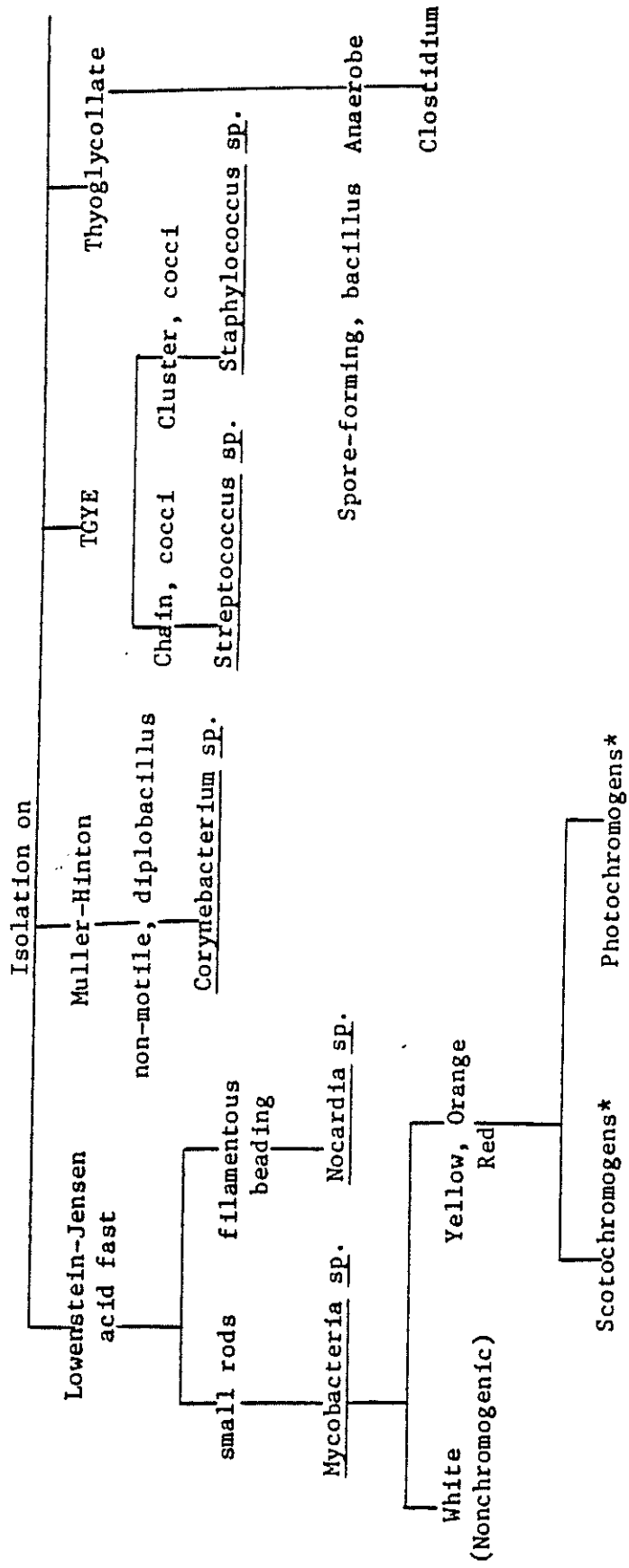
Sea Turtle Pathogens

Isolation on TCYE Agar

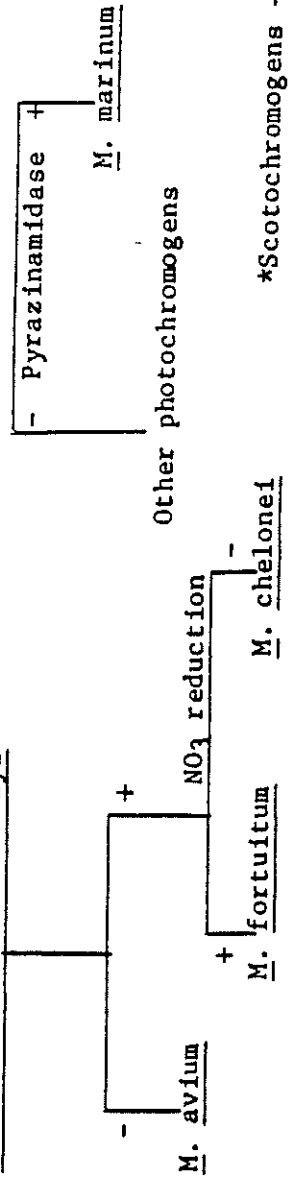


Identification of Potential Gram-positive

Sea Turtle Pathogens



Growth in less than 7 days

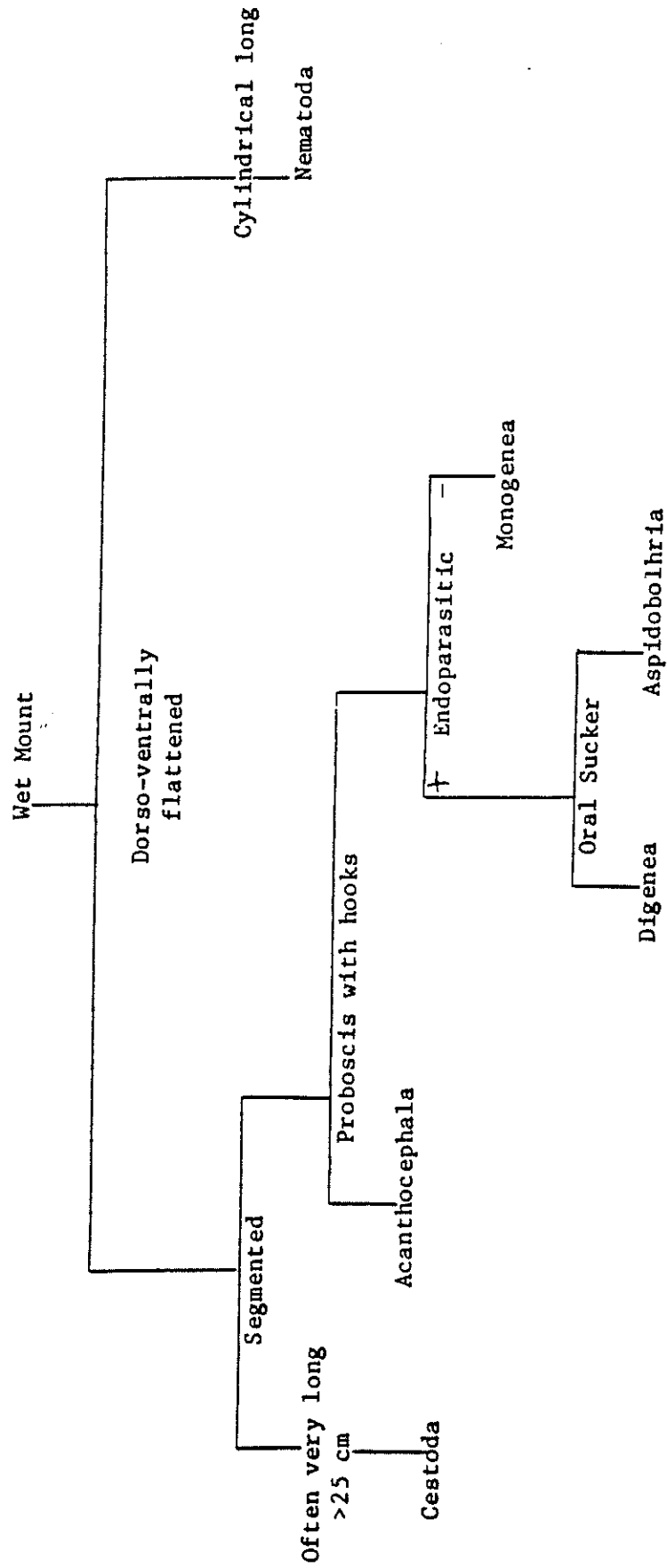


*Scotochromogens - pigmented in dark

*Photochromogens - non-pigmented in dark, pigmented in light.

Identification of Potential Helminth

Sea Turtle Pathogens



eration, necrosis and inflammation are often diagnostic in that they conform to the kinds of changes known to occur in the presence of the agent previously isolated or in that they suggest further examination of special methods for a particular organism which might result in such a pattern.

For instance, focal granulomata are often the result of mycotic infections. Even if the fungus were not isolated its presence within the granulomata would indicate a cause and effect relationship. If in turn the destruction (via the necrosis and granulomata) is sufficient enough and wide-spread the determination can be made that the cause of death was due to the destructive influence of the fungus, i.e. pneumonia, granulomatous, mycotic; cause of death pulmonary failure.

Each individual case must be weighed on its own merits. The pathologist must consider pathogen virulence, host susceptibility, and degree and type of damage suffered by the host. No simple 'flow-sheet protocol' can be designed to determine the cause of death for any organism.

C. Patterns of Inflammation

1. Viral infections - In general viral infections in vertebrates are responsible for focal coagulation necrosis and generalized lymphocytic response. In some instances, especially in the herpes group, intranuclear and intracytoplasmic inclusions are present. Multinucleated giant cells may also be present. An acute infection, therefore, characterized by necrosis, lymphocytes, and inclusion bodies is indicative of a viral infection. In sea turtles, the reported viral infection is Gray-patch disease caused by an Herpes virus.

a. Gray-patch disease (11-13,16,17,21,36)

Gross -- spreading irregular gray patches or gray, sharply-circumscribed papules. These lesions may be found on head, neck, and flippers.

Microscopic -- epidermal hyperkeratosis and acanthosis followed by necrosis. Basophilic intranuclear inclusions.

2. Bacterial infections (33,49) -- The host response to bacterial pathogens is extremely variable and cannot be generalized. Gram-negative organisms tend to acute infections (vascular, non-proliferative, necrotic) while gram-positive organisms are often chronic (proliferative, granulomatous). Further, many bacterial organisms are responsible for specific exudates, i.e. Streptococcus sp. and purulence, Pasturella sp. and fibrin. Supportive responses are certainly suggestive of pyogenic organisms (Streptococcus sp., Staphylococcus sp., Psuedomonas sp.).

a. Clostridia sp. - Insidious Hatchling Disease (25,42)

Gross -- no signs; acute mortality.

Microscopic -- no signs; (J. K. Leong, 1982, personal communication)

b. Bacteroides sp. (47)

Gross -- necrotic, spreading, non-walled skin lesions;

plus → suppurative lesions on tongue, cloaca, skin.

Microscopic -- not reported.

c. Salmonella sp.

Gross -- lower intestine, acute mucosal congestion and desquamation. ← peeling

Microscopic -- coagulation necrosis and loss of superficial colonic epithelium. Mixed inflammatory influx.
 Remarks -- of unknown significance in general population - one recorded case in captive sea turtles (20).

d. Mycobacterium sp. (2,25)

Gross -- raised, circumscribed, white to yellow nodules (0.5 to 2 mm) on surface or within parenchyma of any organ especially lung and liver. Smooth, firm cut surface.
 Microscopic -- classical granulomata, characterized by Langhans' giant cells, reticuloendothelial cell influx, caseous necrosis (soft tubercles) and frequent encapsulation. Acid fast tissue or impression smears are positive.

Remarks -- a known cause of death in severely infected animals.

3. Fungal infections - In general fungal infections result in a proliferative chronic response, most frequently as focal granulomata, however, a diffuse granulomatous response may be present. Fungal infections are being found with increasing frequency in both captive and wild sea turtles. The identification of the organism is dependent on isolation. Many of the organisms resemble one another in tissue section. Grouped as to agents found in individual disease outbreaks.

a. Cladosporium sp., Sporotrichium sp., Paecilomyces sp. (14, 15,18,25).

Gross -- multiple, random, focal pulmonary or hepatic nodules.

Microscopic -- focal granulomata containing hyphae and caseous centers. Hyphae branching and septate. Mixed inflammatory reaction and giant cells.

Remarks -- responsible for swimming abnormalities, cachexia and mortality in captive 5-month-old C. mydas. The author has seen similar fungal infections in the lungs of wild L. kempi suffering from hypothermia.

b. Scolecobasidium sp. (side swimming pneumomycosis)

Gross -- no lesions reported - fair assumption similar to previous fungal complex.

Microscopic -- as above (25).

Remarks -- captive L. kempi and C. caretta, results in mortality via pneumonitis.

4. Protozoal infections - The host response to protozoa, like bacteria is too variable to generalize. The response will depend on location of the organism (intracellular, extracellular, on surface of body) and upon the chronicity of the infection. Manifestations of the diseases caused vary from acute necrosis (amoebiasis) to granulomata (microsporidia).

a. Caryospora sp. (Coccidiosis) (24,35).

Gross -- cachexia and caseous fecal impactions. Whole length of gut involved distal to bile duct, especially posterior third. Lumen contains blood and tissue debris.

Microscopic -- ulceration and necrosis of mucosa. Epithelial hyperplasia at edges of ulceration. Oocysts are elongate ($\pm 35\mu\text{m}$), sporocysts contain 8 sporozoites. Fecal smears contain masses of oocysts.

Remarks -- affects young captive animals.

b. Entamoeba sp. (6)

Gross -- acute, hemorrhagic colitis and focal abscessation of the liver.

Microscopic -- no histopathological lesions described. Undoubtedly acute supportive focal hepatitis and acute necrotizing colitis.

Remarks -- animals affected had 100% mortality, had been kept in fresh water. Organism will not live in salt water.

5. Helminth infections - This group of organisms includes Nematodes, Acanthocephalans, Cestodes, and Trematodes which in the vast majority of cases cause greatest damage as larval forms. In most instances, the host response is granulomatous with encapsulation and an eosinophilia. This is especially true for degenerating forms, either adult or larval. Often the relationship between host and histozoic parasite has been long standing and the host response is minimal resulting in space occupying cysts with little or no inflammatory response. Adult parasites occupying the gut lumen, while pathogenic, are seldom a cause of death unless they result in massive blood loss or serve as a vehicle for secondary bacterial invasion.

a. Anisakis sp. (3)

Gross -- hemorrhagic focal ulcerations at the pyloro-duodenal junction. Larva coiled beneath gastric serosa surface and Glisson's capsule.

Microscopic -- hemorrhagic ulcerative verminous gastritis and enteritis. Nematodes in submucosa and below serosa eliciting a lymphocytic and eosinophilic response. Moderate fibrous encapsulation.

Remarks -- not associated with mortality but a potential cause of death.

- b. Hapalotrema sp., Carettacola sp., Learedius sp., Monticellius sp. (Spirorchidiasis) (10,26-29,31,34,39,41)

Gross -- cachexia, anemia (pale membranes, watery blood) and enteritis of the hind gut characterized by raised linear lesions greenish-black, dry and friable.

Microscopic -- granulomatous gastritis, enteritis, hepatitis, pneumonitis and nephritis. Acute and chronic vasculites accompanied metastasis of eggs. Hepatic hemosiderosis.

Eggs yellow-brown, often elongate with hooked terminal processes or ovoid with a single sharp terminal process.

Formalin fixed eggs vary in length from 135 to 280 μ m. Present in fecal smears or flotations.

Remarks -- evidence has been presented that spirorchidiasis is prevalent in sub-adult loggerhead sea turtles, is responsible for extensive lesions and may be responsible for significant debilitation and mortality (48).

- II. To determine if a physical agent (trauma, hypo- or hyperthermia, suffocation or chemical) is a cause of death.

A. Obvious Disruption of Tissue

1. Trauma is defined as a wound or injury but in a more specific sense refers to physical description of tissue secondary to a blow. In sea turtles this form of trauma most often follows gunshot wounds, lacerations from boat propellers, shark attacks, and weakened animals being thrown against rocks by wave action. In the vast majority of instances hemorrhage occurs secondary to the tissue disruption and the affected animals die of exsanguination. The lesions are easily seen grossly and the degree

of damage can be rapidly evaluated. Signs of anemia are frequently present. When animals are shot by a rifle or pistol through the carapace, the projectile may be present in the body cavity otherwise a perforating wound is present.

B. Absence of a Biological Agent in Culture and in Tissue Section

1. The presence or absence of a biological agent, specifically a bacteria, within a wound resulting from trauma or chemical insult is certainly dependent on the rapidity of death and post-mortem examination. Culture may in fact be positive but for contaminants, and microscopic examination will reveal little or no inflammatory response. Further, the organism will usually be at the point of trauma and will not be recoverable from organs removed from the site. Determinations as to the cause of death must depend upon the degree of damage, freshness of cadaver, agents isolated, and locations of isolation. For instance, if a Proteus sp. is recovered only from the track of a bullet passing through the medulla oblongata in an animal dead 24 hours, one could hardly attribute the cause of death to a Proteus sp. encephalitis or septicemia. Rather it should be considered due to respiratory collapse and anoxia secondary to massive destruction of the medulla.

C. Presence of Tissue of Chemicals in Excess of Known Normal Amounts

1. This is self-evident. Unfortunately, we have to date very few determinations of the chemical content of sea turtle organs (7, 44). Until such data is collected one must make reference to other vertebrates.

D. Lesions Compatible with Hypo- or Hyperthermia, Electromagnetic Radiation, Suffocation (drowning), or Chemical Destruction

1. Patterns of inflammation and tissue destruction have been described for other vertebrates for various physical insults. The kidney lesions associated with mercury poisoning in homeotherms are well documented. It is probable that such lesions would occur in sea turtles were they exposed to excessive amounts of this agent. Confirmation would await reproduction of the disease in the laboratory or recovery of wild turtles with high levels of mercury in their tissue coupled with similar kidney lesions.
2. Patterns of inflammation which indicate that cause of death is related to a physical agent include:

- a. Hypothermia (4,22,23,32,37)

Gross -- at present, no gross lesions can be associated with the diagnosis of hypothermia in marine turtles. Foam was found in the respiratory tract of 13 of 22 marine turtles in which a possible diagnosis of hypothermia was made. The presence of foam may indicate, although it is not certain, drowning as a result of hypothermia.

Microscopic -- liver: fatty metamorphosis. Kidney: vacuolated tubular epithelium, hyaline droplet degeneration of tubular epithelium, tubular casts (cellular or proteinaceous). Nephrosistubular, gomerular congestion, swollen Bowman's capsule with debris often present; nephrocalcinosis may be present however, its association with hypothermia is not clear at this time.

Remarks -- marine turtles strand in the Cape Cod Bay area

during November and December when water temperatures range from 2.3 - 11.0°C. Dead turtles washed ashore when water temperatures were 2.3 - 9.5°C. Live turtles washed ashore when water temperatures were 9.5 - 11°C. One turtle was recovered alive when the water temperature was 4°C, but died in captivity 9 days later.

b. Suffocation, drowning

Gross -- until definitive experiments are conducted (and permitted by the Federal Government) the signs associated with suffocation/drowning cannot be stated with certainty. See remarks.

Microscopic -- see remarks.

Remarks -- studies and observations made by the prosector in the year following the necropsies reported herein have raised the question whether turtles held forcibly below the surface of the water for protracted periods of time will drown in the sense that suffocation will occur due to the inhalation of water. The prosector has observed turtles present at the necropsy table with three general appearances to the airways and lung:

1. Pink, dry, spongy, essentially flattened lobes with dry unobstructed airways.
2. Red, wet, swollen lobes from which fluid runs readily when the lobes are placed on the table. The airways are wet and often contain a pink to white tenaceous (persistent) foam.
3. Pink, dry, spongy, hyperinflated lobes with dry unobstructed airways. Lungs return to state #1 when glottis is opened manually.

In the first instance the lungs are normal. In the second the signs are similar to drowning in homeotherms, although we cannot be certain that some of the signs may occur due to submergence after death.

This finding (#3) indicates that the turtle is able to overcome the carbon dioxide reflex of non-aquatic animals. The finding is compatible with the known fact that turtles can remain submerged for many hours and reach high levels of cerebral anoxia without inhaling water or seeking the surface for air.

These observations then raise a second question; is it possible for turtles to maintain the glottal lock until levels of oxygen drop so low that death ensues via suffocation without the pulmonary intervention of water? If this is the case, forcible submergence could result in death without drowning and the signs presently thought to be due to drowning might be due to passive entry of water into the respiratory system following death. The resolution of this question depends on carefully controlled experiments with both live and dead animals. In all animals, including man, it is difficult to determine if the victim has drowned. There are not pathognomonic or certain signs which point to drowning as the cause of death (5,43). This fact was apparent in the turtles examined during this study. However, it was possible to compare turtles known not to have drowned with those thought to have drowned, and definite differences were apparent. Unfortunately, it is not certain

that these differences were due to drowning alone. This is due to two factors: 1) the compared turtles were not in the same states of preservation and 2) there exists no experimental evidence that the so-called signs of drowning could not have arisen after a turtle had died of other causes and been submerged for some period of time. Compared to controls (turtles known to have died by other than drowning) probable drowned turtles had inflated, wet lungs with a tenacious white froth in the bronchi and trachea. On occasion the bronchi and trachea contained sand and debris such as vomitus.

While these signs are certainly suggestive of drowning, just as they are in man, two problems arise which might mask or produce them. Firstly, the problem of postmortem changes. In almost every animal dead for any period or frozen, a liter or two of serosanguinous fluid was found in the peritoneal-pleural cavity. When the animal is placed in dorsal recumbance this fluid comes in contact with the lungs and may cause the lungs to become quite wet especially if the plura is accidentally cut at necropsy. As postmortem decay proceeds, the lungs tend to become emphysematous and moist and the tenacious foam tends to disappear. The situation thus arises in which postmortem changes can both mimic and mask the signs of drowning.

Secondly, is the question of fluid finding its way to the lungs after a turtle has died of other causes and remained submerged for some time. It is known that humans submerged after death will in 24 to 48 hours begin to develop signs

of drowning (5). The use of drowning tests such as the presence of diatoms in the distal bronchi might be applicable to turtles and should be conducted in further examinations (5,43).

In this study four (4) turtles had signs of drowning. Two were possible cases, while two were quite probable when compared to another turtle (#5) which died of other causes. A good deal of research is needed to resolve the question of drowning deaths as it appears that the present state of the art is quite subjective.

However, if one finds signs in a fresh animal indicative of drowning, one can, after further examination, postulate whether that drowning came about due to the weakened condition of the animal or due to some unforeseen circumstance such as the intervention of man. It is not likely that a perfectly healthy animal would suddenly drown unless forced to remain submerged or traumatized.

In this light, the study revealed that at least 50 percent of the animals examined had serious lesions or disease processes which could lead to debilitation, weakness, and death. It seems probable that such animals would be far more likely to drown or to be drowned. Further it seems likely that stranded sick animals are probably only a small proportion of those dying at sea and never reaching shore. Disease processes observed which could result in debilitation were: anemia (verminous) and acute enteritis.

III. To Determine if a Metabolic Disease is a Cause of Death

- A. Absence of trauma, chemical or biological agent on culture or within tissue.
- B. Presence of a lesion compatible with metabolic disease in other vertebrates, or if known or reproduced in sea turtles.
 - 1. The author knows of no metabolic diseases which have been reported in wild sea turtles and which in turn have been incriminated as a cause of death. This fact simply reflects the lack of information regarding these animals.

a. Nephrocalcinosis

Gross -- no lesions in mild to moderate cases except a gritty consistency when cut. In advanced cases focal white areas would be present and sand-like particles would be present when the organ was pressed between the fingers. Microscopic -- accumulations of an amorphous to laminated basophilic material is present in tubular luminae. This calcific material results in pressure necrosis of underlying tubular epithelium. When epithelium is destroyed a chronic interstitial inflammatory response is present peri-tubularly.

Remarks -- this condition is known most vertebrate kidneys. Its cause is unknown in the turtle and the fish. It has been attributed to upsets in calcium and vitamin D metabolism. The author has observed it with frequency in sub-adult turtles suffering from hypothermia. As an entity if severe enough it could cause kidney destruction leading to excessive ammonia levels and death.

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