COMMENT: This electronic version of the newsletter was created by scanning the original copy and editing as needed. Apologies for any scanning errors that have not ben corrected. Karsten E. Hartel, MCZ January 1994. hartel@mcz.harvard.edu

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POINT OF VIEW: A MESSAGE FROM THE PRESIDENT-- BRUCE B. COLLETTE, President, ASIH.- This issue marks the first year that this Newsletter has been in existence. The pages of this informal release have contained valuable and thought-provoking ideas that probably would not have been disseminated elsewhere.

This Newsletter can grow in value with response from you, the reader. A point of view section is available in each issue for presentation of short notes on personal ideas relating to curation of herpetological and ichthyological collections and, for rebuttal or comments on other articles that have appeared in the Newsletter.

Please send in your input or, at the least, let the committee know if you feel that the Newsletter is worthwhile.

PARAFORMALDEHYDE PROBLEMS - Use of paraformaldehyde in the field has several advantages over liquid formalin as previously stated in Copeia, 1962(1):209-210 and in A Report on Current Supplies and Practices Used in Curation of Ichthyological Collections (1979) prepared by this committee.

Some problems with paraformadehyde have been brought to our attention, namely, the rapid clearing of specimens following preservation. This problem was apparent following the addition of sodium carbonate with water and paraformaldehyde. Sodium carbonate was added as a catalyst to synergize the passage of paraformaldehyde into solution.

Based on experiences of four ASIH members, we suggest using sodium hydroxide pellets instead of sodium carbonate. Combining approximately 143g of paraformaldehyde + 7-8 NaOH pellets and one gallon of water + heat (boiling is OK) will yield one gallon of a 10% formalin solution. The NaOH method has been successful in the field.

Although the purity of locally-purchased formalin is often questionable, one should try to procure it as a backup to paraformaldehyde. -- WILLIAM G. SAUL, Academy of Natural Sciences, Philadelphia.

COMMENTS ON THE MAILING OF TYPE SPECIMENS -- KARSTEN E. HARTEL, Museum of Comparative Zoology, Harvard University.

This note is prompted by the loss of a series of paratypes that were shipped as a gift to the MCZ. Unfortunately, the package was mislabeled and unregistered, thus it is now untraceable.

The loan of primary types by mail is largely discouraged but there are variations in this general rule between institutions, within departments in institutions, and even between curators in departments. Secondary types are, however, commonly mailed for loans. It is not the object of this note to discuss the pros and cons of mailing primary or secondary types but to stress the

importance of the methods used in shipping types.

The primary concern is potential loss of specimens. It is imperative, therefore, that packages can be traced from the shipping location. This can be accomplished with the U.S. mails by either registering, insuring, or certifying the package. If a package is sent by a private carrier, such as United Parcel Service, there is automatic registration of the package upon acceptance by the carrier and a signature of the person receiving the material is required.

It really makes no difference which of the above methods is used, except that costs and services vary. The important thing is that the material is traceable if lost. Although types are under the care of a particular institution, they are philosophically the property of the scientific community and should be protected as such.

ALCOHOLS AND ALCIAN BLUE- see Dingerkus, G., 1981. The use of various alcohols for alcian blue in toto staining of cartilage. Stain. Tech., 56(2):128-129.

ON PRESERVATION OF COLOR AND COLOR PATTERNS --WILLIAM R. TAYLOR, National Museum of Natural History.

Although color and color patterns are among the most useful characteristics studied in systematic research, there is much variation in the methods employed for preserving them in liquids. Records are not retained of details of specimen preparation from formaldehyde fixation through subsequent alcohol preservation, thus limiting interpretation of satisfactory and unsatisfactory curatorial methods. In addition, colors and their patterns in life vary for many reasons such as individually, by season, sex, geographically, diurnally, etc. Thus, by random preservation methods we often compound the problems of systematic interpretation. Then too, many colors are changed or cannot be preserved in liquids by any known method, or if one color-producing mechanism is preserved, another may be extensively altered or destroyed by the preservative. Examples are colors produced by combinations of pigments and physical configurations, pigments and color producing materials generally beneath the integument such as blood and bile, and alcohol resistant-water soluble pigments (e.g. flavins) occurring with water stable but alcohol soluble pigments (e.g. carotenoids).

This article was prepared to: 1. Summarize previous suggestions for preserving color patterns of specimens in liquids; 2. discuss probable reasons for loss of color patterns, particularly melanin, in preservation; 3. suggest improvement of color preservation methods.

Fixation

The preservation of living state color patterns during formaldehyde solution fixation and storage has been only partly successful. Most techniques are based on reduction of oxidation by addition of an antioxidant to the aqueous solution. The antioxidants are moderately effective in preventing bleaching of the mostly yellow to red colors but seldom the violet to blue, and may aid in preserving the darker melanins.

Among the antioxidants known to me that have been suggested as useful with formaldehyde solutions is Butylated Hydroxytoluene (BHT

or Ionol) described by Waller and Eschmeyer (1965) and by White and Peters (1969). In addition to BHT, Toyama and Miyoshi (1963) found Butylated Hydroxyanisole (BHA) and Na-Erythrobate of some use in preventing color loss, but their addition of an "ultraviolet ray absorber" to the solutions does not appear to have been very beneficial. Sodium Ascorbate has also been described as an antioxidant (Yoshida, 1962). Many antioxidants are described in the chemical literature and some of the additional ones used in the food and brewing industries may be found useful in conserving color in preserved specimens.

Some problems with use of antioxidants in color preservation are: 1. The considerable acidity of many antioxidants, including Sodium Ascorbate and L-Ascorbic Acid. The effect of this acidity on bony structures, with or without acid formaldehyde, has not been described. 2. In a mimeographed "addendum" distributed with a separate of Fraser and Freihofer (1971), Dr. Fraser made the following statement: "The use of liquid ionol as a colour preservative during original fixation with 10% freshwater formalin affects the tissues as well. Using the technique described in this reprint, I had a 100% failure rate with ionol specimens. All specimens disintegrated within three days of being placed in the solution of KOH. Depigmentation proceeded very slowly. Ionol specimens are not recommended considering the effort needed to obtain good results."

Fraser's experience suggests to me that Ionol interferes with formaldehyde deactivation of collagenase rather than denaturization of connective tissue. Thus, addition of Ionol to formalin solutions may preclude adequate fixation of specimens for long time storage. 3. Antioxidants aid in preserving relatively few kinds of colors. 4. Many of the color pigments which are protected by antioxidants in aqueous solutions, particularly the caroteniods, are soluble in alcohol. 5. Antioxidants are often effective for a relatively short length of time. They may become neutralized, unstable, or used up, especially if the free air (head) space in a container is not eliminated. 6. The fixing solution should be adjusted as closely as possible toward pH 7.0. Concentrated acids may greatly alter color (Fox, 1976) and alkaline solutions dilute or fade the melanins (Fox, 1976; Taylor, 1977). Regardless of the presence or absence of added antioxidants, storing collections in a cool place will slow the rate of oxidation.

I have reported (1977) that glutaraldehyde is superior to formaldehyde as a fixative in preventing autolysis. When used alone, glutaraldehyde turns fish specimens yellow or brown, depending upon the pH of the solution. But, when formaldehyde and glutaraldehyde are combined in one fixing solution the undesirable colors are mostly avoided and specimens are nearly as well preserved as if glutaraldehyde alone had been used. See Taylor (1977) for comments concerning acidity, etc., and use of glutaraldehyde as a fixative).

Preservation

Studies of color patterns of museum specimens stored in alcohol are based largely upon the distribution of melanin pigments. The melanins are extremely stable and only radical chemical reactions degrade them. They are insoluble in most solvents (Fox, 1976; Nicolaus, 1968) and in neutral or weakly acidic aqueous solutions. However, they may be bleached or degraded by oxidizing agents such as hydrogen peroxide, chlorine, potassium permanganate, etc., and some melanins are soluble in dilute alkaline solutions. "Their stability is well illustrated by the discovery of melanin,

apparently unmodified, in a specimen of a fossil decapod 150 million years old" (Nicolaus, 1968).

If melanins are so stable and difficult to degrade, why do we find white or bleached specimens in collections? Why do we observe, in illustrated specimens, considerable fading after only a few months? These questions are aside from the fact that some specimens are naturally poorly pigmented and appear to lose their color pattern rapidly, yet others with dense concentrated pigment may retain a pattern for years.

The following is a listing of most of the postulated reasons for the loss of color patterns. It includes reactions which I think are important and taking place in collections along with some that are commonly believed to cause color pattern loss, but which I think are relatively insignificant. It is presented with the hope that it will stimulate thought and discussion, with experimentation, and result in better curatorial methods.

1. A small portion of the loss of pigment intensity may be the result of shrinkage of chromatophores and thus the crowding and condensation of their pigment granules during transfer of specimens from formalin into alcohol.

2. Bagnara and Hadley (1973) and Nicolaus (1968) indicated that phaeomelanins are soluble in dilute alkali solutions, distinguishing them from the eumelanins. The former are described as components of hair and feathers, whereas the eumelanins are widely distributed in vertebrates and invertebrates. I have reported (1977) loss of color pattern in fishes stored in alkaline formalin. I cannot state whether this observation is of a breakdown of melanin (eumelanin?) or a separation of the pigment from a protein. On re-examination of these same specimens that have now been stored in borax-buffered fixatives for over six years I found that the dark pigment is almost gone in specimens stored in solutions having pH readings 8.6 - 9.0, and much of it has faded in specimens in solutions initially having readings of pH 8.0 - 8.5. All specimens in the near neutral or unbuffered formalin solutions, both acidic, retain dark, distinct pigment patterns. Based on memory, these dark patterns have not faded greatly from the original. This also suggests that oxygen bleaching has been minimal. Why so little bleaching of melanin considering that oxygen is so effective in destroying carotenoids in formalin solutions? Is the melanin cross-linked by reaction with formaldehyde and not oxidizable or has the oxygen become depleted in the solution by combination with the formaldehyde and formation of formic acid?

Regardless of the answers, these observation indicate that melanophores are destroyed in alkaline solutions. Thus, random use of basic natural waters for dilution of alcohols might account for some loss of pigment by creating a preservative with alkaline activities. As an example of what might happen, sodium borate mixed with water results in a pH reading of about 9.0 but when mixed with ethanol, the pH reading becomes 10.5 to 11.0. To avoid solubilization of pigment by alkalis in alcohol, use only distilled water for dilution.

The whitish or silvery pigment, guanine, generally insoluble in both water and alcohol is not readily destroyed by other agents. This pigment is soluble in strong alkaline solutions.

3. Light has been widely blamed for fading of specimens in collections. I feel it has been overemphasized in the case of

melanin and is no more important than other forms of electromagnetic radiation and of considerably less significance than heat. The general concept of the effect of light is that it causes a photochemical reaction. I am of the opinion that photooxidation is virtually impossible with artificial light when specimens are stored as they are today. In order to have photooxidation, either the pigment molecule or the oxidizing molecule or an intermediate activator must be sensitized by light. Oxygen is activated by light waves shorter than 200 m, well into the far ultraviolet. This radiation is not emitted by the ordinary fluorescent or incandescent lamps and would hardly be expected to pass through glass. Moreover, the melanin molecule is one of the most stable of pigments in the presence of light. It is distributed in many organs and organisms to provide protection from the damaging effect of light, especially ultraviolet light. The preservative (alcohol and water) is not known to be light sensitive. Thus, I fail to see how the pigment loss in stored preserved specimens can be attributed to photochemical activity. I believe that light as a factor in producing faded specimens in collections has been overemphasized.

4. Bagnara and Hadley (1973) considered the disappearance of melanin in the living system and suggested that in addition to molting, etc., there is a possibility of enzymatic degradation. They stated: "If such enzymatic breakdown occurs, it is possible that it relates to a destruction of the protein component of melanoprotein rather than to the eumelanin polymer itself." There is thus no clear evidence that melanin whitening is in part enzymatic, and a reason for enzymatic degradation is not evident to me. Still, our methods of fixation with formaldehyde and storage in alcohols (especially dilute alcohols) would not necessarily denature the enzyme system if it exists

5. Oxygen. The melanin of specimens can rapidly be made invisible, if not completely dissolved, by the action of hydrogen peroxide (H202), or bleaching may be accomplished more slowly in an aqueous medium ln direct sunlight (or in the presence of x-radiation) by the action of ozone (03). The loss of color in stored specimens in not unlike that produced by ozone or peroxide but is much slower. The essential materials for oxidation, oxygen and water, are present in ethyl alcohol collections (Figure 1).

Water is involved in the oxidation reaction as indicated by the comment of Swan (1974): "It seems that atmospheric oxygen is unable to diffuse into the radical-containing regions of the [melanin] polymer." Furthermore, although often ignored in the chemical literature, water (or some other transfer material) is a necessary part of most oxidation reactions at low temperatures. Thus, melanin, like many other substances including iron, would not be expected to oxidize in a dry atmosphere; -oxidation reactions must include water and oxygen plus energy.

Figures 1 to 3 [not reproduced in this electronic version] are presented to illustrate that oxygen i very soluble in methanol, ethanol, and isopropanol, and that the volume of oxygen soluble in the three alcohols is very similar at identical pressures and temperatures. Much more oxygen dissolves into absolute alcohol than into water.

Figure 1 illustrates the solubility of oxygen in different ratios of ethyl alcohol:water solutions. Kretschmer, Nowakowska and Wiebe (1946), although not presenting data, seem to agree (with Fig. 1) that the major depression in solubility of both nitrogen and oxygen in alcohols occur between O and 50% alcohol. Regardless of that

depression, there appears to be sufficient dissolved oxygen at these percentages for extensive bleaching of specimens.

If pigment loss was due to oxygen alone, it would be expected that specimens retained in strong alcohols would be paler than those stored in dilute, 30% or 40% alcohols. To the contrary, my observations suggest that, on the average, specimens stored in presumed 40% isopropanol are paler than similar specimens stored for many years in stronger ethanol. Complicating these observations is the extent of time specimens have been left to soak or wash in water, the amount of handling in air, and the actual strength of the storage alcohol solutions. I feel it logical that soaking or washing preserved specimens in water, or placing them in water for observation contributes to their loss of pigment. Although alcohol undoubtedly inhibits the oxidation reaction, the reaction theoretically continues slowly at most strengths, most rapidly in those solutions containing more than 50% water, and is progressively diminished as concentrations of alcohol in the solution increase.

There has been much work on the inhibition of oxidation by various solvents, but I have not found alcohol discussed. In general, but not universally, the greater the proportion of most other solvents to water, the greater the inhibition of oxidation. In nearly all studies of solutions in which the ratio of the solvents to water is less than 1:1, sizable or extensive oxidation has been shown.

A simple experiment suggests the relevance of these concepts. Iron nails were added to distilled water-ethyl alcohol solutions of 0%, 15%, 35%, 55%, and 75% alcohol. Within a week, extensive deposits of iron hydroxide had formed in the first three solutions, but none was noted in the other two. In four months a slight yellow color and a very thin deposit of rust was noted in the 55% solution, but not in the 75% solution. A 55% solution kept in darkness was slightly better oxidized than the one in direct light.

I thus believe that oxidation of melanin probably is absent in absolute alcohol, that the reaction is fastest in water and dilute alcohols in which the volume of water exceeds the alcohol volume, and that pigment destruction is slow, grading to almost zero as the concentration of alcohol is increased.

Thus it is concluded that to best preserve color patterns, specimens should not be stored in dilute alcohol solutions. Further, washing or soaking specimens in water is harmful as it bleaches specimens and defeats the purpose of the fixation process. I suggest that an experiment should be performed to learn if it would also be useful to exhaust the oxygen in alcohols by displacement with inert nitrogen for the protective storage of very valuable specimens.

Furthermore, it would probably be wise to reduce as much of the head space (free air) in containers as possible, avoid shaking or agitation of the alcohol solution, not replace stained alcohol unless the percentage is low, be certain that the alcohol percentage is high (i.e. see Curation Newsletter 2), and avoid placing specimens in areas of extensive heating or intensive wide spectrum light.

Summary

This essay is presented to review some of the factors bearing on color preservation in liquids, and to present suggestions for improvement. I hope it stimulates thought, discussion and experimentation, with improvement of curation methods. Many

present curation practices and concepts when applied to color preservation are of dubious value and some are probably harmful. In short:

1. Antioxidants may be of temporary value for preservation of some color patterns, but good knowledge of their effect on specimens to be retained permanently-in collection is lacking. Before using antioxidants, consider their effect on long-term storage and what will be done with the specimens.

2. Avoid placing specimens in alkaline fixatives and preservatives for best pigment retention. It is best to use distilled water whenever possible for dilution of alcohol and formaldehyde solutions. Alcohol solutions do not need adjustment, but formaldehyde solutions should be buffered to pH 6.3 to 7.0.

3. It is illogical to fix specimens in a formalin solution and then remove most of the crosslinkage by washing the specimens in water. Specimens should not be washed in water for best preservation but should be placed directly into alcohol. Much bleaching of color patterns takes place in the water as well as in dilute alcohols.

4. Keep collections cool as possible. They should not be heated.

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