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INITIAL RESULTS FROM CLEANING SMALL VERTEBRATE SKELETONS USING THE ENZYME TRYPSIN

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Abstract.—We conducted preliminary studies using the proteolytic enzyme trypsin to accelerate the maceration of a series of small bird skeletons (Aves: Apodidae) and four dolphin flippers (Mammalia: Delphinidae). Although the results from these studies can be considered tentative, they indicate that, under the proper conditions, trypsin speeds the maceration process. Trypsin-accelerated maceration introduces no detectable alteration in the amino acid composition of the cleaned bone as indicated by amino acid analysis, either in the hard, compact bone of bird skeletons, or in the more porous, cancellous bone of dolphins. In addition, amino-acid analysis of a specimen cleaned with a commercial enzyme-containing detergent indicated that about 75% of its bone protein had been destroyed and that oxidation of amino acids had occurred. Methionine sulfone (an amino-acid oxidation product) was identified in the amino-acid chromatogram, and its presence may be used to indicate whether commercial detergents used to clean bone also contain an oxidizing agent.

Many institutions that maintain osteological collections also maintain active colonies of dermestid beetles to clean specimens. The maintenance of these colonies, as well as the final steps for preparation of the skeletons after cleaning by dermestid beetle larvae, are time and labor intensive.

Maceration in water is a common practice that has been recommended and used for many years as a method of removing the remaining muscle from the skeletons of small, partially cleaned vertebrate specimens. The success of this method depends on the presence of exogenous protein-degrading enzymes. These enzymes are produced by airborne microorganisms that grow on the nutrients released into the water solution as a result of biological activity. Some of the drawbacks of this technique include the length of time necessary for complete dissolution of the tissue mass from the bone and the attendant potential for promoting protein hydrolysis. Also, some airborne microorganisms may produce collagenase (a collagen attacking enzyme) that can hydrolyze collagen-based connective tissue and bone protein.

To mitigate both the amount of time and labor used to prepare osteological material, protein-digesting enzymes have also been used for more than 90 yr to speed the maceration process (Kerchoff 1934, Luther 1949, Moser 1906). In particular, the proteolytic enzyme trypsin has been used in the Department of Vertebrate Zoology, United States National Museum of Natural History (USNM), Smithsonian Institution, for more than 20 yr.

Recently, a large number of uncataloged and partially cleaned avian osteological specimens, housed under a variety of conditions for up to 70 yr at the USNM were sorted and hand-cleaned in preparation for their addition to the permanent collections. During the hand-cleaning procedure, deterioration or softening of

bone was observed in a portion of the material. This deterioration was attributed to the previous use of trypsin as part of the preparation technique.

Noting this deterioration, we decided that efforts should be made to document osteological preparation methodologies that have been poorly recorded in the past, and that the use of proteinases as a method for preparing delicate or difficult to clean skeletal material required further examination. In addition, we wanted to address some of the long-term chemical effects (safety) of using the proteinase, trypsin, to accelerate maceration for cleaning research skeletal specimens.

We chose trypsin because: (1) it is active at a relatively neutral pH, (2) it is inexpensive, (3) it has a rapid hydrolysis rate, (4) it is active at a temperature not found in collection storage areas, yet easily attainable under laboratory conditions, and (5) it has been recommended and used in the past as a specimen preparation technique (Fraser and Freihofer 1971, Mahoney 1973, Taylor 1967).

The maximum hydrolytic activity and stability of trypsin occur at a pH between 7 and 9 and a temperature between 35°C and 50°C. There is a rapid fall in activity (stability) above 50°C, with deactivation occurring at about 70°C (Anonymous 1990). Consequently, an operational balance must be struck between the speed at which trypsin hydrolyzes proteins (faster at higher temperatures) and the length of time during which it retains its activity (longer at lower temperatures).

Materials and Methods

Experiments to examine the preferred conditions for trypsin maceration centered on Glossy Swiftlets, *Collocalia esculenta septentionalis* (Aves: Apodidae), collected by mist net on 11–12 March 1990 at Naguilian Church, Fuga Island, Babuyan Island Group, northern Philippines. Four pairs of previously macerated dolphin flippers (two *Tursiops truncatus* and two *Delphinus delphis*) were also analyzed to ascertain whether trypsin maceration could be used to clean cetacean (sea mammal) bones.

Sample Preparation

At the time of collection, the swiftlet specimens were weighed using a Pesola™ spring scale capable of measuring a maximum weight of 10 g. The specimens were tagged, skinned, sexed, eviscerated, and loosely bound with string before being air dried. Great care was exercised to ensure that no insect infestation occurred, and that no chemicals came in contact with the carcasses in the field or on their transport to the Museum.

In the museum, each dried carcass was weighed, without the string and tag, using a Fisher Model 300-D dual range capacity top-loading electronic balance in the 30 g mode with 0.001 g sensitivity. Carcass weights varied from 0.87–1.59 g, with a mean weight ($\bar{x}$) of 1.2 ± 0.12 g (SD). The total number of weighed specimens was 242. To limit variation in the size of individual samples, we selected only those carcasses that weighed within one standard deviation of the mean, or 1.08–1.32 g (171 specimens), to serve as the pool from which we drew specimens for the experiments.

The dolphin flippers were cleaned by hand prior to maceration to remove most of the skin, cartilage, and flesh.

Maceration Methods

Trypsin solutions were made by adding the purified enzyme (Fisher Scientific Co. T-360) to a 0.1-M potassium phosphate buffer (Gomori 1955) at pH 7.7.

Maceration experiments were conducted in a walk-in freezer that was converted to an environmental chamber by the installation of a Broan™ 500 watt (1,707 BTU/hr) fan-forced wall heater. Temperatures were monitored initially by a 7-day recording hygrothermograph and later optically monitored on a daily basis using a nonrecording wall-mounted thermometer. Temperatures were recorded in degrees Fahrenheit (but are reported here in degrees Celsius) and were found to fluctuate by 2°C ($\bar{x}$ ± 1°C) as the heating system cycled.

Ten series of 10 swiftlets each were macerated either in deionized water or in a buffered trypsin solution. We varied the amount of solution, the temperature, and the trypsin concentration.

Table 1. The weight in milligrams of bone samples taken for amino acid analysis.

	Sample number					
	1	2	3	4	5	Mean
Sd	7.01	5.39	12.99	13.83	15.89	11.02
S0	8.82	9.22	9.88	13.40	9.67	10.20
S1	9.93	10.19	8.22	9.94	9.62	9.58
S15	9.90	9.72	11.88	10.83	12.43	10.95
S20	10.79	10.67	9.72	12.16	—	10.98
D0	11.67	11.11	13.85	9.47	—	11.52
D10	9.12	11.08	11.57	12.27	—	11.01

Sd, dermestid-cleaned swiftlet; S0, water-macerated swiftlet; S1, swiftlet macerated using 1 g/L of trypsin; S15, swiftlet macerated using 15 g/L of trypsin; S20, swiftlet macerated using 20 g/L of trypsin; D0, dolphin macerated in water; D10, dolphin macerated in 10 g/L of trypsin.

Specimens were macerated at three temperatures: 35, 36, and 38°C. However, because of the constraints of time and sample size, the volumes of the solutions were not varied within a temperature regime, the volumes only varied between temperatures. Specimens macerated at 35°C were placed in 300 ml of solution. Trypsin concentrations were either 0 (deionized water only), 5, or 10 g/L. These tests were carried out from 28–64 days. Specimens macerated at 36°C were placed in 100 ml of solution. Trypsin concentrations were either 0, 1, 10, or 20 g/L. This test ended at 112 days if a trypsin solution was used and at 199 days if the specimens were in water. Specimens macerated at 38°C were placed in 200 ml of solution. Trypsin concentrations were 0, 10, and 15 g/L. These tests ended after 66 days.

The dolphin flippers were macerated at 38°C in 3-L flasks either in deionized water, or in a 10 g/L trypsin solution.

Bottles containing the maceration solution were manually agitated by shaking approximately every other day.

At the end of each maceration experiment (from 1–199 days, depending on maceration conditions), specimens were categorized as being either clean or not clean. For this study, no attempt was made to periodically quantify the degree of cleaning or to assign a rate at which the cleaning process proceeded. Specimens categorized as clean at the end of a given test were completely free of any adhering soft tissue. Specimens categorized as not clean included those that were unchanged, as well as those that may have had only a few tendons left attached to the sternum. Dermestid-cleaned skeletons and deionized water-macerated specimens were used for comparison.

Amino Acid Analysis

Ulnae or parts of ulnae weighing between 5.4–15.9 mg from 24 swiftlets were placed in 2-ml screw-cap, Teflon™-lined, glass vials for hydrolysis. These were ulnae that had been cleaned by dermestid beetles, macerated in deionized water, or macerated in various trypsin solutions. After maceration in water or a trypsin solution, samples of approximately 10 mg were taken from one edge of an ulna of the dolphin specimens and also were placed in 2-ml screw-cap hydrolysis vials. Table 1 contains a summary of the samples and their weights.

Approximately 0.5 ml of 6 N hydrochloric acid was added to each vial. Each sample then was flushed three times with dry nitrogen gas to exclude oxygen and heated at 150°C for 20 min to hydrolyze the proteins to their constituent amino acids. After hydrolysis, the hydrochloric acid was evaporated from the samples under a stream of nitrogen gas. One milliliter of dilute (0.02 N) hydrochloric acid then was added to the samples to redissolve the amino acids previously released by hydrolysis. These solutions were centrifuged to remove any remaining particulate matter.

The approximate amount of calcium present in each sample was estimated as being 75% of bone weight, and a relative part of the corresponding solution equal to or less than 0.1 mg of bone (25 μg bone protein) was taken for analysis. This small amount was taken to avoid subsequent deterioration of amino-acid analyzer column performance caused by calcium-resin binding. Amino-acid analyses were conducted on a high-performance liquid chromatograph similar to the one described by Benson and Hare (1975). Of the 18 amino acids commonly found in bone proteins (cf. Rose and Von Endt

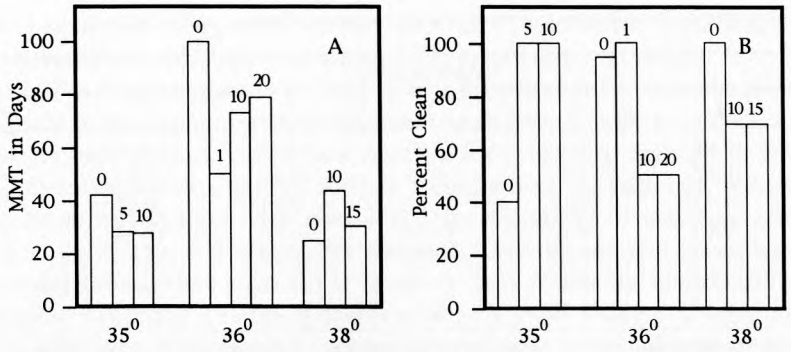

Figure 1. The mean maceration time in days (MMT: A) to achieve the mean degree of cleaning (B) in swiftlet ulnae at three temperatures. Trypsin concentrations (in g/L) are the numbers at the top of each bar.

1984), this analytical method is designed to determine the presence and amount of 15 of these (for details of the procedure, see Von Endt 1994). As used by us, this method does not record the presence of the amino acids hydroxyproline, proline, or hydroxylysine. Although indicative of bone collagen, these three amino acids are reasonably stable (compared to those such as serine and threonine), and we felt that their presence in our analyses would not have materially contributed to the question of protein deterioration by the trypsin cleaning method.

Results and Discussion

Maceration Experiments

The data for the swiftlets, grouped by temperature, are presented as mean maceration time per group in Figure 1A and as the percent cleaned in each group in Figure 1B.

For the specimens macerated at 35°C, the test ended at 64 days with all trypsin-solution treated specimens categorized as clean, but 40% of the water macerated specimens were categorized as not clean. Mean maceration time required to achieve these results per group varied from 28.4 days using 10 g/L trypsin, to 29.7 days using 5 g/L trypsin, to 41.6 days in water (Fig. 1).

For specimens macerated at 36°C, mean maceration time of the trypsin group at a concentration of 1 g/L was 48.9 days with all the specimens recorded as being clean. Mean maceration times of trypsin groups at concentrations of 10 g and 20 g/L were 72.0 and 79.7 days respectively, with 50% of the specimens categorized as clean at the end of 112 days. For the water-macerated group, mean maceration time was 100.9 days with one specimen considered not clean after 199 days (Fig. 1).

For specimens macerated at 38°C, mean maceration time ranged from 25.4 days for the water-only groups with all the specimens categorized as clean, to 43.4 days (10 g/L trypsin) with 70% of the specimens considered clean at the end of 66 days, to 35.9 days (15 g/L trypsin) with 70% of the specimens recorded as clean at the end of 66 days (although one specimen was clean at the end of 5 days) (Fig. 1).

The dolphin flippers in the 10 g/L trypsin solution were designated as clean at the end of 1 day; the water-macerated flippers also were clean, but at the end of 7 days.

The maceration data described above indicate that water (enzymes from airborne microorganisms) works to some degree, but it can take a significantly longer time than trypsin-enhanced maceration. At 35°C, the average maceration time for water cleaning was 41.6 days, but even at the end of 64 days, 40% of the specimens were still categorized as not clean. At 36°C, the mean maceration time for cleaning in water was 100.9 days, with 95% of the specimens considered clean at the end of 199 days. At 38°C, all specimens were categorized as clean in an average time of 25.4 days under the same conditions.

The trypsin-cleaning data are more variable and provide some apparent anomalies that require further study. At 35°C, all specimens categorized as clean in either 5 or 10 g/L trypsin solutions required a mean time of about 30 days; generally, about half the time it took in water under the same conditions. Interestingly, at 36°C, all the 1 g/L trypsin groups were categorized as clean after a short period ($\bar{x}$ = 49 days), about half the time for water at the equivalent temperature, whereas only 50% of the 10 and 20 g/L groups were considered cleaned at the end of 112 days. The latter results may represent the influence of the relatively small (100 ml) volume used in the experiments; a larger minimum volume may be required for enzyme maceration of these specimens. At a higher temperature, 38°C, the water-only maceration worked better than trypsin; the mean time for water was about 25 days for all the specimens to be categorized as clean, whereas 70% of the specimens in both the 10 and 15 g/L trypsin solutions were categorized as clean, in 43.4 and 35.9 days, respectively. This may be the result of the pure enzyme being slowly deactivated at this temperature, whereas the microorganisms (and their enzyme production) flourished in the deionized water.

Initial inferences drawn from these data indicate that slightly elevated temperatures (approximately 35–37°C, compared to a room temperature of about 21°C) and a larger volume of solution significantly enhance the maceration process. In general, the concentration of trypsin and the volume of solution (provided it is about 200 ml or more per specimen) seem to have less influence on the maceration rate or degree of success. However, it must be noted that the fastest time for cleaning a swiftlet was 5 days in a 15 g/L trypsin solution at 38°C. Also, in most cases, the use of trypsin reduced the maceration time by approximately 50%. The longest maceration time recorded was 199 days with water at 36°C, with even this length of time leaving one of the specimens categorized as not clean.

Two observations became apparent in the course of this study: (1) the growth of microorganisms in the maceration solutions was suppressed by the incorporation of trypsin, and (2) under the conditions of this study, trypsin exhibits a fairly short activity life (Anonymous 1990). These observations, although not completely unanticipated, are probably partially responsible for the observed anomalies in the survey results and must be incorporated in the experimental design of any future studies. The speed of enzyme action, enhanced by high temperature, must be balanced against enhancing the longevity (stability) of the enzyme using lower temperatures.

Amino Acid Analysis

Representative samples were subjected to amino-acid analysis to quantify the effect the maceration procedures had on the stability of bone protein. We also compared specimens that had been cleaned using dermestid beetles. The relative

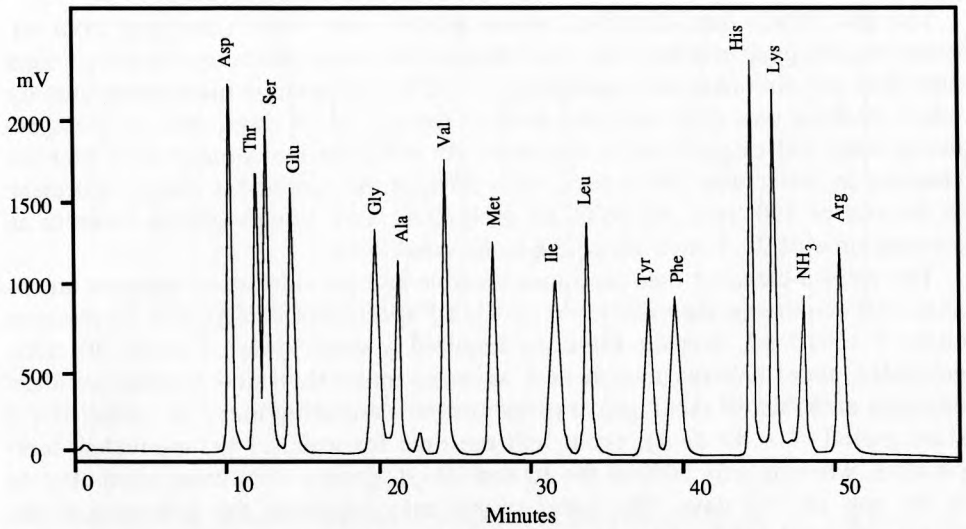

Figure 2. Analysis of a standard mixture of amino acids. Abbreviations are: aspartic acid (Asp), threonine (Thr), serine (Ser), glutamic acid (Glu), glycine (Gly), alanine (Ala), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), histidine (His), lysine (Lys), ammonia (NH_3), and arginine (Arg).

proportion of each amino acid was determined for each sample using the chromatograms produced by the amino-acid analyzer. Representative chromatograms are presented as Figure 2 (a standard mix of amino acids and ammonia that are used to identify and quantify the amounts of the amino acids in the specimen samples) and Figure 3 (a chromatogram of a swiftlet ulna cleaned by dermestid beetles). The data in Figures 2 and 3 indicate what is to be expected in an analysis of bone entering an osteological preparation laboratory, and these data serve as an internal standard. Figure 4 represents a sample from a swiftlet ulna cleaned in a concentrated (20 g/L) trypsin solution. As may be seen by comparing Figures 3 and 4, no detectable changes have occurred as a result of trypsin-accelerated maceration.

Furthermore, data from the analyzed specimens were quantified and are presented as the mean percent amino acid composition for each sample group in Table 2. Fifteen amino acids were found in the bone samples as expected. Ammonia (NH_3) in the chromatogram indicates amino acid deterioration introduced to some degree by the hydrolysis procedure and is a component of the buffers used to separate the amino acids on the analyzer. An abnormally high amount of ammonia in the macerated samples would indicate that bone deterioration had occurred. The distribution of amino acids within each sample was typical for collagen (Rose and Von Endt 1984), the major protein found in bone, and the variation in the amount of each amino acid among the samples was within acceptable limits of error. The amino acid glycine varied the most, 1.1%, with the rest of the amino acids varying less than 1% across the samples.

Of the 15 amino acids detected by the amino-acid analyzer, two (in addition to high levels of ammonia) can be particularly useful as indicators of bone protein deterioration. Ammonia was of interest because levels above the sum of that

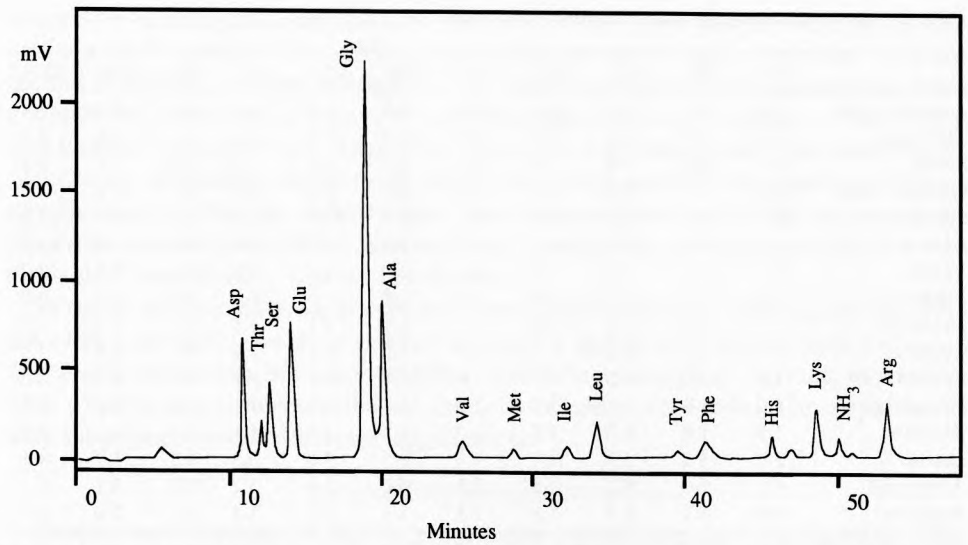

Figure 3. Analysis of a *Dermestes*-cleaned swiftlet ulna. Abbreviations are: aspartic acid (Asp), threonine (Thr), serine (Ser), glutamic acid (Glu), glycine (Gly), alanine (Ala), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), histidine (His), lysine (Lys), ammonia (NH_3), and arginine (Arg).

present in the buffers and expected from hydrolysis (>6%, Table 2, Figs. 2–4) would indicate clearly that the cleaning procedure had damaged the bone. The uniform (<0.9% variation) and low (3.8–6.0%) ammonia levels in all sample groups, including the dry, dermestid-cleaned control group, indicate that any de-

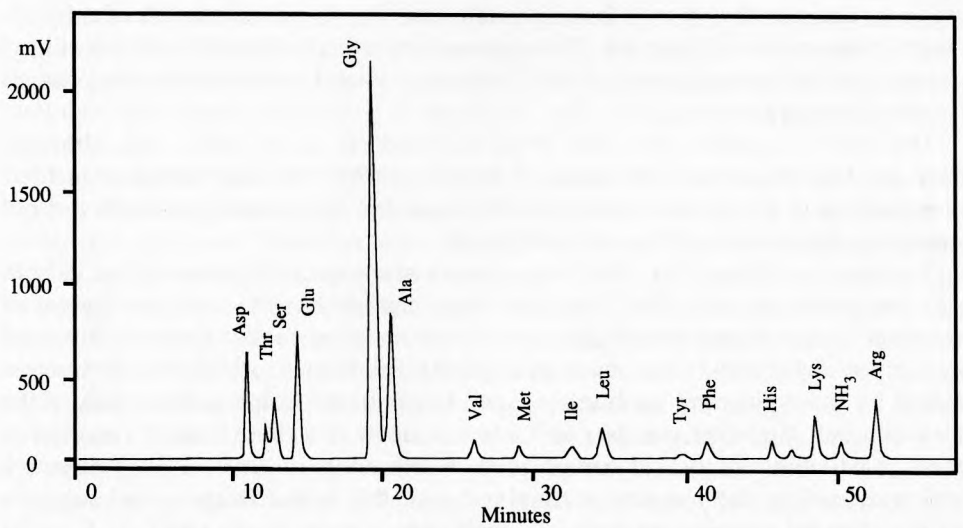

Figure 4. Analysis of a trypsin-treated swiftlet ulna. The pattern and amounts of amino acids are the same as the dermestid-cleaned control. Abbreviations are: aspartic acid (Asp), threonine (Thr), serine (Ser), glutamic acid (Glu), glycine (Gly), alanine (Ala), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), histidine (His), lysine (Lys), ammonia (NH_3), and arginine (Arg).

Table 2. Percent amino-acid composition of the ulnae.

	Sd	S0	S1	S15	S20	D0	D10	$\bar{x}$	SD	ENZ	COL[a]
Aspartic acid	6.6	6.2	5.9	6.1	6.2	6.1	6.2	6.2	0.2	8.5	7.1
Threonine	2.6	2.3	2.1	2.2	2.5	2.7	2.8	2.5	0.3	4.8	2.5
Serine	4.3	3.8	3.8	3.9	4.0	5.0	5.1	4.3	0.6	7.7	4.2
Glutamic acid	10.3	9.7	9.6	9.8	10.3	9.1	9.2	9.7	0.5	12.1	11.9
Glycine	35.1	36.7	38.2	38.4	36.9	36.2	37.1	36.9	1.1	35.4	25.3
Alanine	14.5	14.0	14.4	14.5	14.1	13.0	13.2	14.0	0.6	12.8	10.5
Valine	2.0	1.9	1.7	1.7	2.3	2.0	1.8	1.9	0.2	5.3	2.7
Methionine	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.0	—	0.8
Isoleucine	1.2	1.2	1.2	1.0	1.2	1.1	1.1	1.1	0.1	1.7	1.7
Leucine	4.1	3.4	3.2	3.2	3.7	3.2	3.1	3.4	0.4	4.4	3.9
Tyrosine	1.0	1.0	0.5	0.7	1.0	1.0	0.7	0.8	0.2	0.7	0.6
Phenylalanine	2.0	1.9	1.7	1.8	2.0	1.8	1.8	1.9	0.1	1.6	2.9
Histidine	1.5	1.9	1.7	1.8	2.0	1.8	1.8	1.8	0.2	—	1.0
Lysine	3.2	3.3	3.3	3.0	3.2	3.5	3.5	3.3	0.2	1.7	4.1
Ammonia[b]	4.1	6.0	5.7	4.8	3.8	6.0	5.8	5.2	0.9	3.1	—
Arginine	5.5	5.7	5.5	5.3	5.5	5.8	5.5	5.5	0.2	2.0	9.2

Sd, dermestid-cleaned swiftlet; S0, water-macerated swiftlet; S1, swiftlet macerated using 1 g/L of trypsin; S15, swiftlet macerated using 15 g/L of trypsin; S20, swiftlet macerated using 20 g/L of trypsin; D0, dolphin macerated in water; D10, dolphin macerated in 10 g/L of trypsin; ENZ, the Ossian Collection specimen.

[a] Collagen data adapted from Rose and Von Endt (1984) for comparative purposes.

[b] These concentrations have been adjusted to account for residual ammonia present in the analytical system buffers.

terioration of bone protein caused by the maceration-cleaning procedures was also very low, or nonexistent. Furthermore, of the naturally occurring amino acids found in bone, threonine (Thr) and serine (Ser) are two that are particularly sensitive to changes that disrupt bone protein, and they are therefore excellent indicators of bone protein integrity. Their presence at expected levels in all the sample groups used in the analyses similarly indicates minimal or no deterioration caused by the cleaning methods.

The other 13 amino acids that were analyzed are more stable, and, although they are less diagnostic indicators of bone integrity, minimal variation in their composition in all groups is another indication that the cleaning methods did not introduce deterioration (Figs. 3, 4, Table 2).

The species selected for these experiments represent extreme variation in both size and bone structure. The bird ulnae have the hard brittle structure typical of compact bone, whereas the dolphin ulnae were more cancellous (porous). Because of increased porosity, the surface area per unit volume available for destructive action by these cleaning methods is much larger in the dolphin ulnae than in the bird samples. However, the data in Table 2 indicate that there is little variation in their constituent amino-acid composition, which suggests little or no destructive action caused by the cleaning methods. Again, this is encouraging and suggests further that the cleaning methods are applicable across diverse taxa.

Limitations

Two limitations of these experiments are that the number of available swiftlets was insufficient to maintain valid sample sizes for the number of variables that

needed to be analyzed, and the time required to fully process all these specimens under a wide variety of conditions would necessitate the use of several environmental chambers, not the one which was currently available. Consequently, these experiments represent a "survey" of a few temperatures, volumes of solutions, and trypsin concentrations used to enhance the water maceration of osteological specimens. Although encouraging trends are discernable in the data, caution is advised in applying this information. More thorough testing of the temperatures, minimum maceration volume, and optimal trypsin concentration are planned, and those data may modify our survey results.

In future experiments we also plan to use a more finely graded series to describe the cleaning results, because the use of such a series here would have indicated that many specimens we categorized as not being clean had, in fact, more than 90% of adhering material removed from their bones. Moreover, these specimens may have been usable for some applications.

Enzyme-containing Detergents

Shelton and Buckley (1990) criticized the use of enzymes for cleaning bone based on the observed deterioration of the Clair Ossian vertebrate collection, which had been cleaned using enzyme-containing detergents. Included in Table 2 are the results of an amino acid analysis of one of the Ossian-prepared specimens (Ossian 1970), *Pomoxis nigromaculatus* (Osteichthyes: Centrarchidae), labelled ENZ. The amino acids present in that sample exhibit a distinct bone-collagen distribution, and this distribution is comparable to the analyses of our material in Table 2. A distribution of about 35% glycine (Gly), 13% alanine (Ala) and about 10% each of aspartic acid (Asp) and glutamic acid (Glu) is the "signature" for collagen. Note that Table 2 does not indicate that the amino acids in the sample ENZ were observed to be at a level of about 25% of that observed for all other samples in this study. In other words, the bones of *P. nigromaculatus* described by Shelton and Buckley (1990) and analyzed by the authors were greatly deteriorated and had lost about three-quarters of their total (collagen) protein content; although the protein remaining in the bone still retained its collagen-like "signature."

Chaplin and Bucke (1990:140) provide a generic list of ingredients expected in enzyme detergents, which indicates that less than 1% of their composition is proteolytic enzymes. However, other listed components such as oxidizing agents (25%), surfactants (28%), and water softeners (38%), can easily account for the deterioration observed by Shelton and Buckley (1990). Oxidizing agents are known to remove the organic matrix of bone and compromise the integrity of bone by oxidizing and destroying both the proteins and their constituent amino acids. One indicator that this has actually occurred in the sample of *P. nigromaculatus* (Table 2, ENZ), is the absence of the amino acid methionine (Met) in the analysis. This is unusual, because all of the other samples analyzed for this study contain a small (about 1%) but detectable and quantifiable amount of Met (Figs. 2–4, Table 2). However, what did appear in the analysis of ENZ was a small (about 1%) peak identified as methionine sulfone (MES), an oxidation product of Met. The presence of MES indicates to us that oxidation has occurred in this sample (and probably the others reported by Shelton and Buckley 1990), and that the oxidizing agent in the enzyme-based detergent (probably a bleach) is

responsible for any observed bone deterioration. An amino acid analysis that includes the detection of MES may serve to indicate whether a bone sample has been treated with an oxidizing agent.

Surfactants and water softeners (many water softeners contain large amounts of chelating agents) can aid in the removal of destroyed proteins from the bone leaving only a part of its inorganic components, which are brittle and friable. Consequently, the conclusion that a proteolytic enzyme present in a commercial laundry detergent used to clean skeletal material was responsible for the damage observed (as reported by Shelton and Buckley 1990) is probably premature. However, based on the data and discussion presented above, we concur with Shelton and Buckley (1990) that the use of commercial detergents or presoakers (recommended by Ossian 1970 and Patterson and Brattstrom 1971) certainly should be discouraged. Further, enzyme mixes such as commercially available pancreatin (Egerton 1968) and papain (Luther 1949) also should be avoided, because enzyme mixes may contain other destructive agents.

One footnote of interest is that, though destructive when used to macerate bone, enzyme-containing detergents have been used with seeming success as a "signal enhancer" in DNA hybridization experiments (Johnson et al. 1993). Here, a laundry whitener/brightner was used to reduce nonspecific hybridization signals and improve the specificity of detection of mosquito species-specific DNA probes.

Other Preparation Methods

Preparation methods that utilize autoclaving (Brown and Twigg 1967) or boiling (Chapman and Chapman 1969, Storer 1988) are likely to hydrolyze proteins and enhance the migration of lipids and complex carbohydrates within the bone, as well as enhance the removal of protein fragments, lipids, and carbohydrates from bone to the surrounding solution. This potentially can cause damage to the bone by affecting its stability. Chelating agents such as ethylenediaminetetraacetic acid (EDTA), whose use is recommended by Hill (1975), react with the divalent calcium cation, destroying the bony inorganic matrix. Because calcium phosphate comprises about 75% of bone by weight, the use of chelating agents should be avoided. Soaking skeletal material in bases such as ammonium hydroxide solutions (Egerton 1968) promotes the hydrolysis of collagen, the principal organic component of bone, and could have potentially catastrophic results. The use of any oxidizing agent such as those found in some commercial detergents, and sodium hypochlorite bleach (Brown and Twigg 1967, Rhodin et al. 1976, Sanders 1953), will destroy the collagen matrix. In addition, the papain and pepsin methodologies proposed by Piechocki (1961) involve the use of acids that will rapidly dissolve the calcium phosphate portion of bone, as well as promote hydrolysis of bone collagen.

Enzymes effect a wide variety of chemical reactions. As biological catalysts they are not only efficient but because they are subject to control and reaction mechanisms themselves, they are very specific. The usefulness of purified enzymes for cleaning a variety of materials has gained acceptance by conservators of artistic and historic works (see Grattan et al. 1980, Hauser 1993, Morse 1992).

CONCLUSIONS

Commercial use of enzymes concurrent with research on their characterization and specificity has increased dramatically over the past decade (Chaplin and

Bucke 1990). New enzymes are being classified almost as fast as new uses for them are developed. The use of biological catalysts such as enzymes in the preparation and conservation of natural history objects has been overlooked to a large extent because of the prevalence of traditional approaches. Enzymes should be considered, however, because of their speed, ease of use, specificity, and efficacy.

Proteolytic enzymes have been used for many years to speed the laborious process of cleaning small vertebrate skeletons. In the past, the use of commercial detergent/enzyme combinations has often led to disastrous results.

We conducted preliminary studies using the purified proteolytic enzyme trypsin to speed the maceration of small bird skeletons and dolphin flippers. Our results indicate that, under the appropriate conditions (temperatures ranging from 35–38°C and an approximately neutral pH), the use of trypsin speeds the maceration process. It appears to be safe, because it introduces no detectable changes in the amino-acid composition of the bones used in these experiments. The use of trypsin appears to be preferable to some other skeleton-cleaning methods recommended in the literature.

However, amino-acid analysis of a fish skeleton cleaned with a commercial detergent that contained enzymes as one of its ingredients indicated that about 75% of its bone protein had been destroyed, and that oxidation of the amino-acid methionine had occurred to yield the oxidation product methionine sulfone. The presence of this compound in an amino-acid chromatogram may serve to indicate whether a commercial detergent used to clean bone also contained an oxidizing agent.

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CHANGES IN pH IN MUSEUM STORAGE FLUIDS, I–EFFECTS OF RESISTALL PAPER LABELS

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Abstract.—The impact of Resistall specimen tag paper on the pH of pure water and ethanol was tested. Resistall paper makes the pH of storage fluids more acidic. The acid from Resistall paper is highly mobile and will effect the pH of storage fluids in less than 4 days. The ratio of Resistall paper to quantity of storage fluid influences the rate of the lowering of the pH. Ethanol slows the process of increasing acidity, but the pattern of lowering the pH is the same for ethanol and pure water. There are some slight differences in how 28# and 36# Resistall effect pH, but the differences are not significant.

Traditionally, most natural history specimens have been obtained under field conditions, selected individuals have been tagged with specimen labels, and fixed by immersion or injection with formalin. Subsequently, the specimens have been washed with water and stored in sealed jars of 70% ethanol or 55% isopropanol (Cannell et al. 1988, Cato 1986, Hildebrand 1968, Phillips 1988, Simmons 1991, Smith 1965, Taylor 1977). Each of these processes and materials impacts the ultimate fluid environment in which the specimens are housed (Duckworth et al. 1993, Rose 1991, Simmons 1991). Because it is important to understand the potential effects that each of these materials and conditions may have on the pH level of solutions used in fluid-preserved natural history collections, we conducted a series of experiments to test the effects of cellulosic paper labels, jars, lids, environmental conditions, and preserved specimens upon the storage fluid (Simmons 1991, 1993, 1995, Taylor, 1977). The pH of the storage fluid is of concern to collection managers and research scientists, because decalcification of osteological tissues (Dingerkus 1982, Jones and Owen 1987, Quay 1974, Simmons 1987, 1991, Smith 1965, Von Endt and Hare 1997a, Zweifel 1966) and the deterioration of soft tissues and proteins (Stoddart 1989, Von Endt and Hare 1997b) occur over time when the pH of the storage solutions is excessively acidic or basic. The pH of the storage fluid ideally should be maintained between 6.5 and 8.0 (Dingerkus 1982, Hawks and Williams 1986, Jones and Owen 1987, Simmons 1995, Taylor 1977). Investigators should note that pH values above 8.0 are of concern as well as acidic values, because highly alkaline solutions can degrade proteins, resulting in clearing of the tissues of the specimens (Dingerkus 1982, Taylor 1977); however, our experiments did not focus on this phenomenon.

The purpose of the experiment reported herein was to test for changes in the pH of three solutions as a result of the introduction of Resistall paper specimens tags. Paper containing sizing, lignin, or organic acids, and other chemicals, which may serve as a catalyst for chemical reactions, is inherently unstable and may accelerate the deterioration of the label as well as the specimens (Hawks and Williams 1986, Kishinami 1989, Smith 1965). Hawks and Williams (1986) recommend 100% cotton stock paper with a neutral to mildly acidic pH (6.5–7.0)

for use in vertebrate collections. Resistall paper has been recommended for use in natural history collections for several decades (Jones and Owen 1987, Smith 1965, Williams 1990). Resistall is a long-fiber cotton paper that is stable in the solutions used in fluid-preserved collections. However, the problem with this paper is that it has a pH ranging from 4.5 to 5.2 (Williams 1990), decidedly not archival quality. The Resistall procedure incorporates a specially formulated wet-strength resin (melamine, $C_3H_6N_6$) into the cotton pulp used to make archival-quality paper. Melamine resins used in water-resistant coatings are usually a synthetic alkyd that is prepared by treating melamine with formaldehyde. Melamine is a trimer of cyanamide (CH_2N_2), which is a highly caustic compound prepared by carbonation of calcium cyanamide (Merck and Co. 1983, Brady and Clauser 1991). The paper pulp is treated with an acid that is used to force the melamine, remixed in an 8% alum solution known as paper-maker's acid, into a solution so that it will coat and harden every cotton fiber in the pulp, creating the paper's resistance to the solutions used in fluid-preserved collections and, in turn, lowering the paper's pH (Williams 1990).

It is important to have a basic knowledge of the pH scale and its underlying chemistry to understand how the pH of test solutions are changed (Joesten et al. 1991). The pH of a solution is the negative log of the hydrogen ion $[H^+]$ or hydronium ion $[H_3O^+]$ concentration. Pure water is neutral giving it a pH of 7 in which the number of H_3O^+ ions equals the number of hydroxide ions $[OH^-]$. Acids form H_3O^+ ions in water, thus having a pH of <7; bases form OH^- ions in water, thus having a pH of >7. When an acid reacts with a base, the acid supplies H^+ ions, which react with OH^- ions from the base to form water, H_2O. The reverse of this reaction, in which water breaks down, results in the production of a hydrogen ion and a hydroxide ion. Not all acids lose hydrogen ions as readily to water as do the strong acids. Some negative ions formed by the loss of the hydrogen ions in these weaker acids are capable of competing with water for the hydrogen ion being exchanged. The result of this competition is the establishment of an equilibrium in which the concentration of ions in solution remains unchanged even though reactions in both forward and reverse directions continue. In strong bases the reverse reaction is much stronger so that the negative ion formed by the base is a much stronger base than the water molecule.

Because it was the purpose of this experiment to test only the effects of the labels on the pH, all the tests used the same type of container and lid, and the solutions were all subject to the same environmental conditions. To clearly identify the effects of the labels on the pH of the solutions, specimens were not introduced as a factor in the experiments. All runs of this experiment incorporated controls. We are aware of the pH shift caused by ethanol solutions as described by Brokerhof (1997). We incorporated experiments using pure water to serve as a comparison to the ethanol solutions to assess the effects of this shift. Concentrations of the ethanol solutions were maintained as close to constant as possible to avoid shifting the properties of the ethanol (Waller and Strang 1996).

Materials and Methods

Resistall paper (manufacture's specifications–26#, 0.005 in. thick, and 36#, 0.006, presented in a letter from University Products, Inc., dated 4 April 1990, supplied in response to a request for information on Resistall), produced by Byron-Weston, was obtained from University Products. The paper

was cut into three different sizes (25 cm^2, 100 cm^2, 225 cm^2) and tested in three different solutions. Four ounce "composite test jars" were used in the experiment. These jars, consisting of flint glass, were purchased from the Berlin Packing Company. The lids (Berlin Packing Company) had a continuous-thread with a polyethylene foam liner (Simmons, 1995). The container interiors were rinsed three times with distilled water. The containers were stored in the dark, and the room temperature and relative humidity (RH) were monitored throughout the experiment.

The pH was monitored in three different fluids (pure water, 70% ethanol, and 70% ethanol after the paper was pretreated with buffered formalin). Each solution (100 ml) was added to the respective 4-oz containers, leaving a small air space at the top of each container comparable to that in the controls. All solutions had controls that consisted of the solution and no paper, and each solution had three replicate tests. Pure deionized water (Scientific Products: Reagent Grade Deionized Water, prepared at 18 megohm/cm specific resistance using reverse osmosis, mixed-bed deionization, activated carbon filtration, and final filtration to 0.2 microns) and 95% undenatured ethyl alcohol (manufactured by Midwest Grain Products Inc., Atchison, Kansas) were used for the experiment. The 70% ethanol solution was prepared using 95% undenatured ethyl alcohol and pure deionized water, measured with an alcohol hydrometer until the correct concentration was attained (all solutions were mixed in a temperature range of 71° to 74°F).

The third solution consisted of the same 70% ethanol with an added component that involved treating the paper with a 10% formalin fixative, using deionized water and formalin, buffered with 4 parts monobasic sodium phosphate to 6.5 parts dibasic sodium phosphate (Jones and Owen 1987). This treatment was intended to reflect the preparation protocol for vertebrate specimens. The Resistall 26# and 36# papers were treated separately. Each paper was first placed in the 10% formalin wash for 2 hr. The paper was removed from the wash and then placed in a wash of distilled water for 2 hr. During this period, the distilled-water wash was changed three times at 40-min intervals. The paper was then removed from the wash and allowed to dry for 2 hr, before it was introduced into the 70% ethanol.

Change in pH was monitored with a digital Orion, model 230A, pH meter and an Orion, model 9107, Low Maintenance Triode electrode, purchased from Scientific Products (Simmons and Waller 1994). Accuracy of the pH meter ranged ± 0.5. The pH of the solutions was tested on days 4, 8, 12, and 30. The pH meter was recalibrated, using fresh standard solutions of known pH values (4.0 and 7.0) after each run or every four jars. The pH standard solutions were prepared with pHydrion® buffer capsules, purchased from Scientific Products, and 100 ml of pure deionized water.

Statistical analyses were performed using the StatView® software package (Sager 1992). Specific analyses performed were simple regression analysis, paired *t*-test, and analysis of variance. Simple regression is used when the dependent variable (pH values) is modeled with one independent variable (time/days). The R^2 statistic, also called the coefficient of determination, given by the analysis is used to assess the quality of the regression analysis. It is the proportion of the dependent variable (change in pH) that is explained by the independent variable (time in days) with a maximum value of 1. The paired *t*-test and ANOVA give statistical significance of differences in group means. The pH readings were log transformed for analysis and then retransformed for presentation and interpretation.

RESULTS

The results of this experiment were submitted to regression analysis, *t*-tests, and analysis of variance. The results of these tests are shown in Figures 1–3 and Tables 1–3. Examination of Figures 1–3 shows the same pattern of change in the pH whether the fluid was pure water, ethanol, or if the Resistall was pretreated. In each fluid, the pH became lower, that is, more acid, with time. In each test the value for the control was far from the values for the test samples, so that there was no overlap of their 95% confidence limits. In water and ethanol with the untreated Resistall, the acidic effects of the Resistall appeared to be more pronounced than in the test of ethanol with Resistall that was pretreated with formalin. The pattern of variation between the 28# as compared with 36# Resistall showed little or no difference, as is true for the final pH values in each test.

The R^2 values for the regression analyses are shown in Table 1. The higher the

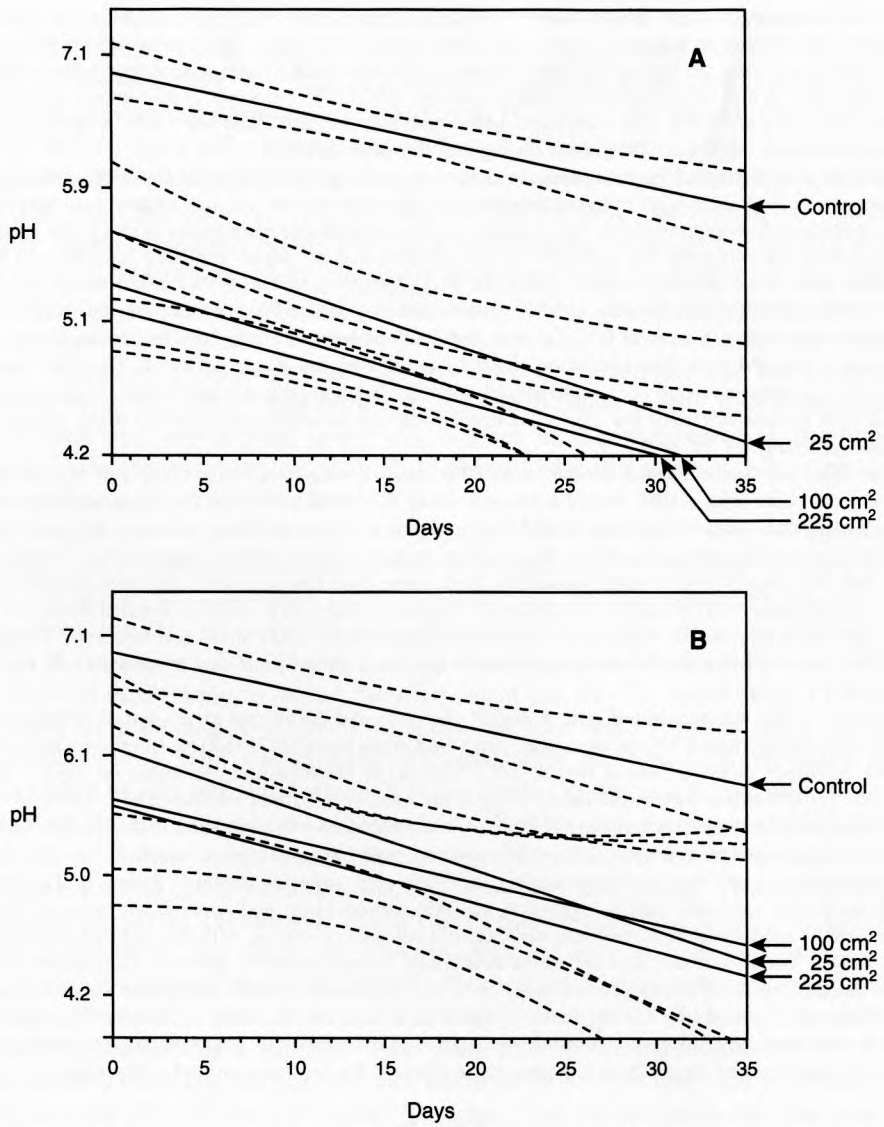

Figure 1. Changes in pH of pure water containing three sizes of 28# Resistall (A) and 36# Resistall (B) over a 30-day period. These changes can be compared with the control, which contained pure water but no Resistall paper.

R^2 values the more of the variation in the dependent variable (pH readings) is explained by the independent variable (days/time). R^2 values were generally above 0.500, which means that more than half of the variation in pH seen from day 4 until the end of the experiment could be explained by time. Particularly high values for R^2 were found for both 28# and 36# Resistall values in ethanol. Some of the lower R^2 are explained by the fact that the fluid was acidified very quickly prior to day 4, so most of the effects of the Resistall were already evident by that time.

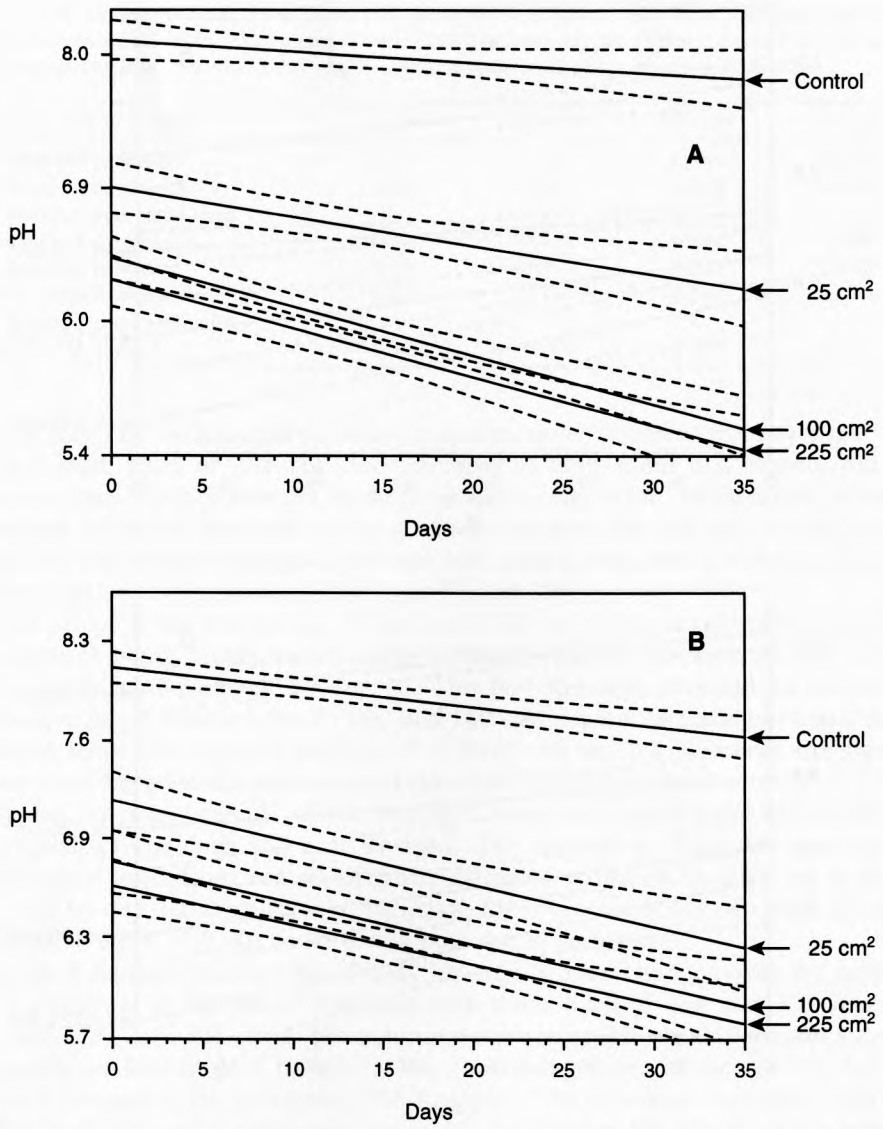

Figure 2. Changes in pH of 70% ethanol containing three sizes of 28# Resistall (A) and 36# Resistall (B) over a 30-day period. These changes can be compared with the control, which contained 70% ethanol but no Resistall paper.

Table 2 reports the results of pair-wise comparisons of the pH of three sizes of Resistall paper and the control in water, ethanol, and ethanol after pretreatment of the paper with formalin. These tests showed whether the differences observed on any particular day in Figures 1–3 were significant or not. The differences in the pH of the control in each experiment as compared to the pH of the fluids containing Resistall paper were all significant, most at $P < 0.001$ level.

One of the most interesting comparisons in Table 2 is between the pH of fluids containing 25 cm² and that containing 225 cm² of Resistall paper. With both 28#

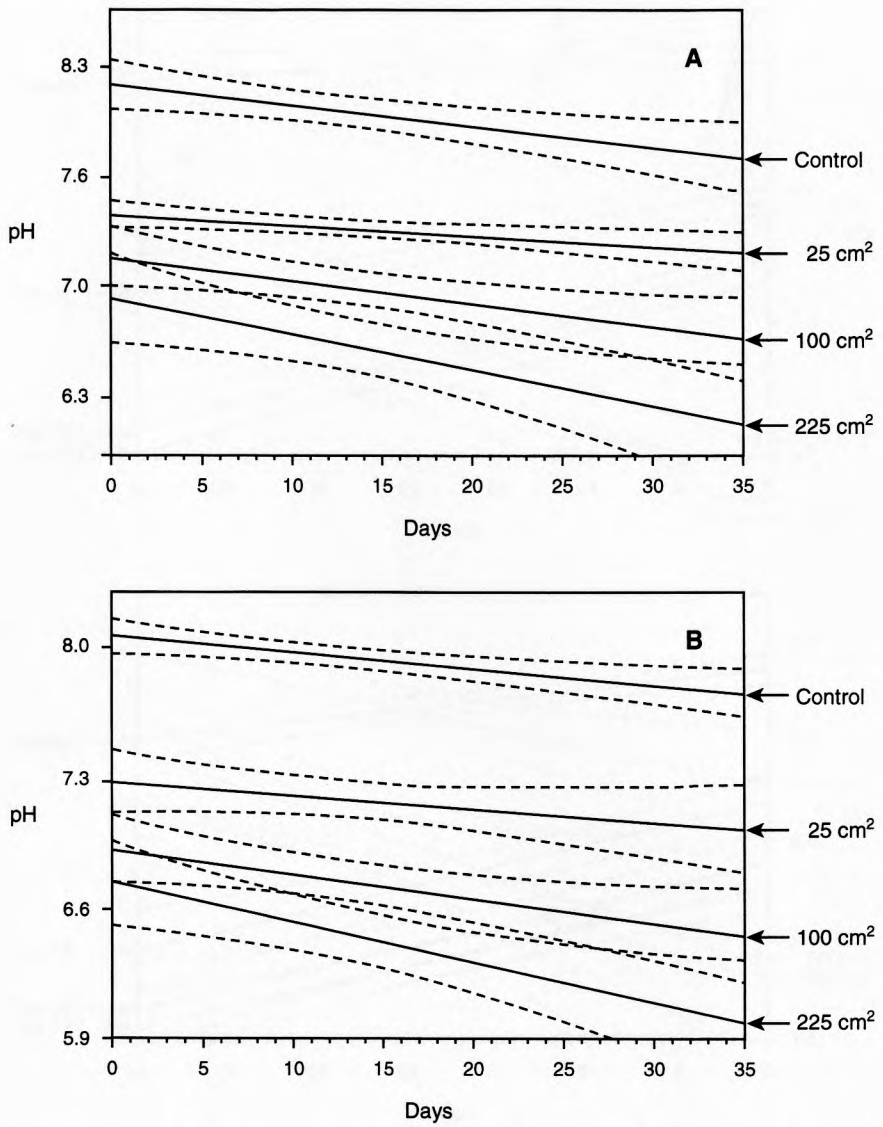

Figure 3. Changes in pH of 70% ethanol containing three sizes of 28# Resistall (A) and 36# Resistall (B), which had been pretreated with formalin, over a 30-day period. These changes can be compared with the control, which contained 70% ethanol but no Resistall paper.

and 36# Resistall in water, a similar pattern was seen. On day 4 the difference in the pH of the solutions containing the two different amounts of Resistall were highly significant, whereas by day 30 the difference was not significant. In the ethanol solution and the solution with ethanol and pretreated paper, there were significant differences in the pH of these solutions throughout the experiment and, with the exception of 36# Resistall in ethanol, the significance level increased from day 4 to day 30.

Comparing the pH of the solution containing 100 cm^2 with the solution con-

Table 1. R^2 values resulting from regression analyses of a control and three solutions containing different-sized pieces of Resistall paper (25 cm², 100 cm², and 225 cm²). Time (days) was used as the independent variable, and pH values of the solutions were used as the dependent variable.

Paper and treatment	Control	25 cm²	100 cm²	225 cm²
28# Resistall in water	0.743	0.512	0.569	0.632
28# Resistall in ethanol	0.609	0.232	0.865	0.831
28# Resistall pretreated with formalin in ethanol	0.500	0.479	0.484	0.486
36# Resistall in water	0.580	0.501	0.227	0.302
36# Resistall in ethanol	0.671	0.717	0.691	0.736
36# Resistall pretreated with formalin in ethanol	0.532	0.191	0.413	0.513

taining 225 cm² of Resistall revealed a pattern quite different from that just discussed. Both sizes of 28# and 36# Resistall in both water and ethanol did not have significantly different pH readings except on day 4 for 28# Resistall in water. However, when the Resistall was pretreated with formalin and then rinsed in pure water, another pattern emerged, with the two sizes having fluids with significantly different pH values, especially by days 12 and 30.

The pH of water containing 25 cm² and 100 cm² of Resistall paper exhibited a pattern of being highly significantly different early in the tests, but the differences were not significant by day 30. The 28# Resistall revealed an increasing difference in pH between the 25 cm² and 100 cm² sizes with the significance level between these two sizes of paper at $P < 0.001$ on day 30. However, the significance level between the two sizes of the 36# Resistall differed at the $P < 0.001$ until day 30, when the pH of the two tests were not significantly different. The pretreated 25 cm² and 100 cm² Resistall 28# showed a steady increase in the significance level from not significantly different on day 4 to differing at the $P < 0.001$ by day 30. With the 36# Resistall, the two tests of the two sizes of paper differed at the $P < 0.001$ level throughout the experiment.

Table 3 presents the results of tests for significance changes over the term of the experiment in the pH of solutions with three sizes of 28# and 36# Resistall (25 cm², 100 cm², 225 cm²). The solutions containing 25 cm² of Resistall showed a significant lowering of the pH levels (becoming more acidic) except for the ethanol containing the pretreated 36# Resistall. The solutions containing 100 cm² of Resistall showed significantly lower pH levels over the length of the experiment, except the pure water containing the 36# Resistall, which showed no significant changes in the pH. The results of the ethanol containing the 100 cm² of Resistall revealed a highly significant lowering of pH values. The pH levels of the solutions containing 225 cm² of Resistall followed the same pattern, a lowering of pH value, displayed by the solutions containing 100 cm² of Resistall; a deviation occurred only in the pretreated 36# Resistall in ethanol in which the significance level was $P < 0.01$ rather than $P < 0.05$. However, the ethanol containing all three sizes of the untreated 36# Resistall showed a highly significant lowering of the pH.

Discussion

Because the primary purpose of preserving specimens in formalin is to be able to study the gross anatomy and hard tissues of organisms, anything that subse-

Table 2. Paired comparisons (using *t*-test) for pH of solutions containing three sizes of Resistall paper and the control on days 4, 8, 12, and 30 of the experiment. Three replicates were run for each.

Paper, treatments, and days	Control vs. 25 cm²	Control vs. 100 cm²	Control vs. 225 cm²	25 cm² vs. 100 cm²	25 cm² vs. 225 cm²	100 cm² vs. 225 cm²
28# Resistall in water						
Day 4	***	***	***	***	***	**
Day 8	***	***	***	**	**	ns
Day 12	***	***	***	ns	*	ns
Day 30	**	***	***	ns	ns	ns
28# Resistall in ethanol						
Day 4	***	***	***	*	*	ns
Day 8	**	***	***	*	**	ns
Day 12	***	***	***	***	**	ns
Day 30	***	***	***	***	***	ns
28# Resistall pretreated with formalin in ethanol						
Day 4	***	**	***	ns	**	ns
Day 8	**	**	*	*	ns	ns
Day 12	***	***	***	**	***	**
Day 30	***	***	***	***	***	***
36# Resistall in water						
Day 4	**	**	***	ns	***	ns
Day 8	***	***	***	***	*	ns
Day 12	***	***	***	**	ns	ns
Day 30	***	*	*	ns	ns	ns
36# Resistall in ethanol						
Day 4	***	***	***	**	**	ns
Day 8	***	***	***	**	***	ns
Day 12	***	***	***	**	***	ns
Day 30	***	***	***	ns	*	ns
36# Resistall pretreated with formalin in ethanol						
Day 4	***	***	***	**	***	*
Day 8	**	***	***	**	**	ns
Day 12	***	***	***	**	***	**
Day 30	***	***	***	**	***	*

ns, not significant; *, significant at $P < 0.05$; **, significant at $P < 0.01$; ***, significant at $P < 0.001$.

quently hinders these studies is a major concern to investigators. Certainly, one of the primary concerns is the fluid environment in which the organisms are preserved and then stored. Many factors can affect this environment, making it less than suitable for the long-term stability of the preserved material (Simmons 1995). The pH of the fluid medium is one of the factors that may affect the long-term storage of specimens, because an acidic medium may result in the decalcification of osteological tissues and the deterioration of soft tissues, and a basic pH may result in the clearing of tissues. In a series of papers, we will investigate the agents that may effectively change the pH of the fluid storage medium. In

Table 3. Results of analyses of variance of changes in pH of solutions containing three sizes of Resistall paper over the 30 days of the experiments (readings on days 4, 8, 12, 30). Three replicates were run for each.

Paper and fluid types	25 cm^2	100 cm^2	225^2 cm
28# Resistall in water	**	**	**
28# Resistall in ethanol	*	***	***
28# Resistall pretreated with formalin in ethanol	*	*	*
36# Resistall in water	*	ns	ns
36# Resistall in ethanol	***	***	***
36# Resistall pretreated with formalin in ethanol	ns	*	**

ns, not significant; *, significant at $P < 0.05$; **, significant at $P < 0.01$; ***, significant at $P < 0.001$.

this paper, we are exploring the results of the effects that Resistall paper, used for specimen labels, has upon the pH of pure water and ethanol.

Resistall paper of 28# and 36# significantly lowered the pH of pure water and ethanol; that is, they were made more acidic. The acids remaining in the paper from the melamine process (Williams 1990) are highly mobile, with the test solutions all differing significantly from the controls at least at the $P < 0.01$ level by day 4 (Table 2, Figs. 1–3). Comparison of Figures 1, 2 reveals that the acid released in pure water and 70% ethanol solutions had similar effects on the pH of the storage fluids. This is important because, as Brokerhof (1997) pointed out, there is a shift factor involved in measuring the pH of ethanol solutions. Evidence of this shift is present in our data set, because the pH reading for the ethanol solutions are higher, that is, more basic, than the pure water readings. Nevertheless for our results, this shift is not an issue, because it is clear that the pH readings of pure water and ethanol followed the same pattern of variation with the addition of Resistall paper. Both became more acidic at approximately the same rates.

The relatively high R^2 values shown in Table 1 indicate that the acidic levels continued, in most cases, to build throughout the experiment. The lower R^2 values seen in Table 1 for 100 cm^2 and 225 cm^2 of 36# Resistall in pure water undoubtedly indicate that these relatively large-sized pieces of paper had already significantly lowered the pH of the solution by day 4; subsequent impact on the solutions was therefore relatively low. The low R^2 value for the 25 cm^2 of 28# Resistall in ethanol is more difficult to explain; however, inspection of Figure 2A reveals that the pH in this test changed only slightly over the term of the experiment. The results of this experiment do support the hypothesis that time was an important factor in determining the amount of increase in acidity of the storage fluid.

The comparisons of the impact of the three sizes of Resistall are shown in Table 2. The most striking differences were seen in the comparison of 25 cm^2 and 225 cm^2 of Resistall. In both tests using pure water, the differences in pH caused by these two sizes were highly significant on day 4, but were not significant by day 30. We believe that this is the result of the large pieces of Resistall quickly shedding a large amount of acid into the water, but as the experiment progressed the acid in the 225 cm^2 piece of Resistall was near an equilibrium

level with the acid in the water, resulting in a small change in the pH. The 25 cm^2 piece, because of its smaller size, added acid at a slower rate, but continued to shed acid until later in the experiment while it was approaching the equilibrium pH of the 225 cm^2 piece. Ethanol seemed to slow the release of acid, so that an equilibrium state was not reached by the 225 cm^2 piece of Resistall by day 30; therefore, the differences in pH between these two sizes of Resistall were significant throughout the experiment, although the significance level for the 36# Resistall dropped on day 30.

The results of the comparison of the effects of the 25 cm^2 and 100 cm^2 pieces of Resistall on the pH of the storage fluid showed a similar pattern when comparing the 25 cm^2 and 225 cm^2 pieces. An unusual result occurred with the 36# Resistall in ethanol in which the results of this test resembled the 36# Resistall in pure water. The difference between the two pieces was lowered to the point that it was not significant on day 30, in contrast to the pattern of increasing differences between the two samples in other ethanol comparisons. Currently, we have no explanation for this unusual result.

Generally, there was no statistical difference in the rate at which the 100 cm^2 and 225 cm^2 samples lower the pH. Another interesting result occurred in the experiment using paper that had been pretreated with formalin, washed with pure water, and finally placed in ethanol. The two pieces do show a difference in the rate of lowering of the pH following this pretreatment. In this case, the difference in the pH values generally increased throughout the experiment. We believe that this is evidence that some of the acid was removed in the pretreatment process and this affected the smaller pieces of Resistall more than the larger ones.

Table 3 presents the differences in the pH of the storage fluid containing three sizes of Resistall paper over the length of the experiment. In all cases except three, there was a significant lowering of the pH of the storage fluid. The exceptions for 100 cm^2 and 225 cm^2 of 36# Resistall in pure water was a result of the highly mobile acid in the paper having lowered the pH significantly by day 4 and then probably being near an equilibrium state (see also Figs. 1B, 2B). We believe that the nonsignificant result for the 25 cm^2 of 36# Resistall in ethanol after pretreatment was caused by the pretreatment process. Enough of the acid in the paper was removed in the washing process so that the pH of the storage fluid was not significantly lowered, as shown in Figure 3B, where the regression line for 25 cm^2 is almost straight across the graph. The effect of the different sizes of Resistall paper is clear in the test where the 100 cm^2 size is significant at the $P < 0.05$ level and the 225 cm^2 size is significant at the $P < 0.01$ level. The differences in the slope of the regression lines in Figure 3B also support this conclusion. The patterns of differences for the remaining tests were almost identical, with the only exception being the 25 cm^2 of 28# Resistall in ethanol.

We also examined Table 3 for differences related to the effects of 28# as compared to 36# Resistall. There are three cases where there is a difference in this pattern. For 25 cm^2 of 28# Resistall in ethanol, the difference over the period of the test was significant at the $P < 0.01$ level, whereas with the 36# Resistall the significance was at the $P < 0.001$ level. In this case, the 36# Resistall made the storage fluid more acidic than the 28#. The results for 100 cm^2 and 225 cm^2 of Resistall in pure water were more difficult to interpret. It must be remembered that the nonsignificant results for 36# Resistall was believed to be the result of

the immediate lowering of the pH of the water before day 4. With this in mind, it would appear that the differences in the effects of the 28# and 36# Resistall should be interpreted as resulting from the slower acidification of the storage fluid by the 28# Resistall.

The following conclusions can be drawn from this set of experiments:

(1) Resistall paper tags lower the pH of the storage fluids in which they are used (make them more acidic).
(2) The acid in Resistall paper is highly mobile and will impact the pH of storage fluids in less than 4 days.
(3) Resistall paper will continue to lower the pH of storage fluids for at least 30 days and beyond, but will do so at a decreasing rate.
(4) The size of Resistall paper tags will affect the rate at which the pH is lowered.
(5) The ratio of the amount of Resistall paper to storage fluid must be lower than our smallest piece (25 cm^2 to 100 ml of storage fluid or 250 mm^2 per ml) to prevent significant impact on the pH of the storage fluid.
(6) Ethanol appears to slow down the process of increasing acidity; both ethanol and pure water follow an identical pattern, which displays a lowering in pH values.
(7) Following a process that simulates the field procedure for fixation, followed by rinsing in water, removes some of the acid from the Resistall tags and thereby decreases its effect on the pH of the storage fluid. It should be emphasized that the rinsing process uses fresh water to remove excess fixative. Specimens should not be soaked for a long period as was recommended in the past (Smith 1965). A long period of "soaking of specimens in water following formalin fixation is undesirable for the best preservation. It removes many of the cross-link bonds, thus reversing and materially destroying many of the valuable effects of formalin fixation, leaving specimens in a condition to combine with other substances or undergo autolysis" (Taylor 1977, see also Cannell et al. 1988, Simmons 1991).
(8) Only slight differences were noted in the effects of 28# Resistall as opposed to 36# Resistall on the pH of the storage fluid. There was some indication of a slightly slower rate of lowering of pH by 28# Resistall, as well as a slight indication of less overall lowering of pH by 28# Resistall. However, we conclude that when considering pH, 28# and 36# Resistall may be used interchangeably.

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EVALUATION OF TEMPERATURE REGIMES FOR THE CONTROL OF INSECT PESTS OF MUSEUM COLLECTIONS

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Abstract.—Inherent dangers associated with the use of chemicals to protect museum collections against pest attack have promoted investigation into alternative control methods. One such method is the use of thermal control. Two species of insects (Coleoptera: Dermestidae) known to attack and damage museum specimens and materials were exposed to high and low temperature regimes for varying time periods. The results show that certain time and temperature combinations were successful in controlling all stages of both species tested. Based on these results, a lethal boundary limit is proposed and the feasibility of using the technique in museum applications is discussed.

The use of chemicals to protect museum collections against damage by pests has a long tradition. Although much of this tradition has evolved through trial and error, the quality and integrity of collections has, in general, remained intact. This is witnessed by the considerable quantity of collections, many dating back several hundred years, stored in museums throughout the world. However, concern over the health and safety implications of the use of chemicals in the museum environment, effectiveness, and potential adverse effects on museum specimens and associated materials has led to a reappraisal of collection protection policies (Dawson and Strang 1992, Irwin 1987, Linnie 1996, Peltz and Rossol 1983). This has focussed attention on alternative methods of controlling and eradicating pests from museums. The use of high and low temperature regimes (Strang 1992), gamma, infrared and microwave radiation (Brower and Tilton 1971, Hall 1981, Kirkpatrick et al. 1973), and controlled atmospheres (Burke 1996, Navarro 1978) have each received attention as potential alternatives to conventional control methods. Of these, exposure to high and low temperature regimes and, in particular, sub-zero temperatures have generated the most interest among museum workers. Although the technology required to kill insects by manipulating the ambient temperature exists in the form of conventional ovens and freezers, further information on the precise exposure rates and temperature requirements for controlling various insect species and their life stages is required for the application of the technique to museum collections (Strang 1992).

Thermal regulation for the control of insect pests has been used by the food processing and food storage industry for most of the last century (Mullen and Arbogast 1984), although Leechman (1931) appears to have been the first to apply the technique to pests of museums. However, adoption of thermal regulation methods of pest control by museums has not been widely reported. This has been largely because of concerns over potential damage to museum objects arising from exposure to raised and/or lowered temperatures. Hall (1981) questioned the effects of raised temperature regimes on seed viability, whereas others expressed concern over the potential structural damage to plants (Philbrick 1984) and changes in the mechanical properties (strength, elasticity) of certain materials (Florian 1987). Other workers (Ketcham-Trosak 1984, Mullen and Arbogast 1984) have ques-

tioned the suitability of low temperature regimes in museum applications because of potential freeze-resistance and freeze-tolerance in certain insect species and the lack of available data on insect susceptibility to heat and cold (see review by Strang 1992). Insufficient information available on precise temperature levels and exposure durations required for the range of pests likely to be encountered also contributes to the cautionary approach adopted by museums. Despite this, there appears to be general interest in alternative methods of control and, as regulations on the use of chemicals become increasingly stringent, the indications are that nonchemical techniques will in time become more prevalent in museums. Against this background, there is a need to investigate the feasibility of using modified temperature regimes as a pest-control measure in museums. In this study the effectiveness of raised temperature and low temperature environments in controlling recognised insect pests of museum collections is investigated. Based on the results obtained, an evaluation is made on their feasibility as potential pest-control methods in museums.

Exposure to Low Temperature Regimes

Insects are poikilotherms, that is, their body temperature closely follows that of their environment. In general, as an insect's body temperature is lowered its activity level decreases until it eventually comes to rest and shows no activity. A further decrease in temperature may result in death. This thermal death point varies with each species and is dependent on the life stage of the organism (Salt 1961) and the actual temperature and duration of exposure to that particular temperature (Asahina 1966). Until recently, the lethal effects of cold were less well studied than those of heat, largely because of the difficulties experienced in determining the actual point of death. This is because many organisms, including insects, become quiescent at low temperatures and do not appear to respond to physical stimuli thereby making it difficult to confirm a moribund condition.

Freezing occurs either by the growth of a crystal from a particle or structure that organises water molecules to form an ice nucleus (inoculative nucleation) or by the chance and spontaneous aggregation of water molecules to form a nucleus (spontaneous nucleation). Nucleation is the key process in ice-crystal formation, and freezing can be avoided by inhibiting these processes. The freezing point of a fluid is defined as the temperature at and below which a seed ice crystal will grow. However, many fluids may be cooled well below this temperature, because spontaneous nucleation does not occur in them, a phenomenon known as supercooling (Cossins and Bowler 1987). Most insects have the ability to become supercooled. Even in those insects that do not possess antifreeze substances such as glycerol, the supercooling point very frequently occurs in the vicinity of −20°C (Salt 1961). Supercooling is probably the chief factor in cold resistance (Somme 1964). As the temperature of the insect is lowered, the water in the insect's body becomes supercooled, but ice does not form in the tissues until the temperature falls to a critical point (−10°C to −15°C). At this point, the temperature suddenly increases to approximately −1.5°C through the release of latent heat. Following this increase, the temperature proceeds to fall once more and freezing of the tissue begins. Supercooling is not necessarily dangerous to insects and many species regularly manage to overwinter successfully in a supercooled state.

In insects, various mechanisms have evolved to ensure survival in a variable

and potentially hostile thermal environment. Such mechanisms can be used to categorise insects into three different groups depending on their resistance to cold. They may be categorised as freeze-tolerant, freeze-sensitive, or freeze-resistant (Zachariassen 1985). Freeze-tolerant insects are capable of withstanding the formation of ice in the body fluids at temperatures equal to or below the supercooling capacity of the body fluid. Freeze-sensitive insects, however, lack tolerance to the formation of ice crystals in the body fluids, whereas freeze-resistant insects use avoidance mechanisms such as acclimation, dehydration, and the production of alcohols to prevent freezing. The latter mechanism is also found in other terrestrial arthropods where the production of the so-called "antifreezes" such as glycerol, sorbitol, and mannitol are used to to prevent freezing of their blood.

Freezing of the body fluids involves potentially lethal effects. Metabolic pathways are generally slowed, potentially leading to an imbalance in the overall cellular metabolism. This may lead to the osmotic swelling of intracellular and/or intra-intestinal tissues, eventually resulting in rupture. Also, the growth of ice crystals into the cell may cause damage to the cellular structure. Extracellular freezing may cause cells to shrink, leading to intolerable osmotic stress across cell membranes. Freezing will also increase the concentration of inorganic salts to levels that may irreversibly change the structure and function of the host enzymes. There are accounts of insects of concern to museums that survive freezing temperatures (Ketcham-Trosak 1984, Mullen and Arbogast 1984, Strang 1992). Failure to control the Herbarium beetle, *Lasioderma serricorne* (Coleoptera: Anobiidae) after 24 hr exposure to −30°C was reported by Brokerhof (1989), but this was attributed to the insulation of the material surrounding the insects that prevented an even distribution of temperature. Florian (1986) stated that control failures will also occur if a temperature regime of −20°C for 48 hr is not maintained. Salt (1961) stated that pests which do not normally encounter cold temperatures may not be capable of tolerating freezing temperatures and that hibernating insects improve their tolerance to low temperatures by acclimation, or "cold-hardening." Wigglesworth (1972) reported that following repeated freeze/thaw cycles the supercooling ability is eliminated and freezing occurs when a freezing temperature is reached.

Some of the literature relating to thermal-mortality limits for museum-insect pests is based on generalised experience by museum workers rather than scientific investigation and controlled experiments. Leechman (1931), in an early reference to low temperatures for the control of museum pests, recommended exposure to −18°C for 24 hr. Dawson and Strang (1992) recommended maintaining textiles, animal skins, and similar artifacts at temperatures of ~4°C to discourage insect infestation. Crisafulli (1980) reported that freezing at temperatures between −18°C to −40°C for 2–7 days was effective in killing insect pests in herbarium material, whereas Florian (1986) recommended −20°C for 48 hr as a guideline for killing insect pests of museums. Strang (1992) provided a comprehensive review of the literature on lethal temperatures for museum pests with lethal boundary temperature limits.

Exposure to High Temperature Regimes

Thermal injury and death in insects arises from the failure of one or more homeostatic systems. Although it is not known with certainty which of these

systems is the most susceptible, it is believed that the thermal liability of cells is probably a function of two groups of macromolecules: the proteins and the membrane lipids (Love 1966). The denaturation of proteins exposed to high temperatures resulting in the inactivation of important enzymes is well known, and although in certain cases protein inactivation is reversible (provided that correct conditions of pH and ionic strength are maintained) heat injury and probable death are the likely result of extreme heat exposure (Pain 1987). Reported changes in the state of membrane fluidity following extreme heat exposure arise from studies based on mammalian cell cultures (Cossins and Bowler 1987). Precisely which aspect of membrane function fails as a result of increased molecular motion has not been shown, although permeability characteristics across cell membranes would appear to be affected.

In a museum context, the main consideration when using raised temperature environments as a method of treating known or suspected insect infestations is whether the maximum temperature and exposure period used will kill the target pest without causing an adverse effect on the actual specimen(s) and associated material being treated. Reports of failure to control museum insect pests using high temperature exposures rarely appear in the literature (Strang 1992). In cases where failure has occurred, there is little supporting evidence of posttreatment investigation of the methods used or the precise techniques involved. Strang (1992) proposed lethal boundary temperature limits for museum insect pests. These are based on achieving 100% mortality levels and are drawn from a review of the published literature from museum sources and entomological journals. Strang (1992) reports that for the Hide beetle, *Dermestes maculatus* (Coleoptera: Dermestidae), 100% mortality was achieved at −23°C and also at 60°C, whereas for the Varied carpet beetle, *Anthrenus verbasci* (Coleoptera: Dermestidae), temperatures of −20°C and above 40°C were required. Armes (1985) found that a 2-hr exposure to 40°C was lethal to all stages in the life cycle of *Anthrenus sarnicus* (Coleoptera: Dermestidae), whereas Busvine (1966) found that a 30-min exposure between 43°C and 45°C was lethal to larvae of clothes moths (*Tineola* spp.) (Lepidoptera: Tineidae). Armes (1985) also reported that insect pests found in boxed entomological specimens at the British Museum of Natural History (London) were killed by exposure to 50°C for 24 hr. Watling (1989) recommended using a temperature regime between 40–42°C in a drying cabinet to kill mycophagous insects in herbarium material.

Other than concerns about potential effects on seed viability and the alteration of physical characteristics of certain materials, there is little reference to the use of raised temperatures as a pest control measure in museums, although there are many references on this technique in the food-storage industry. Few museums have reported using the technique and with little published relevant experimental work on the subject, this will continue to be the case. However, in situations where conventional methods of treating pest infestations are not feasible, exposure to raised temperature regimes may have some usefulness. In this study, time/temperature mortality relationships for all stages in the life cycle of two species of insects considered important pests of museums is investigated. Based on the results, lethal boundary limits are proposed as a guideline for the eradication of insect pests from museum specimens and artifacts and as a potential "quarantine" treatment for incoming material.

Materials and Methods

The test insects used were the Hide beetle, *Dermestes maculatus* and the Varied carpet beetle, *Anthrenus verbasci* (Coleoptera; Dermestidae). Both are recognised as pests of museums (Beauchamp et al. 1981) and have been recorded as "serious" pests of museums by respondents to surveys on pest control (Linnie 1987, 1994).

Each species was reared on a diet consisting of dried fish meal supplemented with cholesterol and yeast. Selection of individual stages for actual testing was as follows: for *D. maculatus,* adult beetles, viable pupae, and mature larvae of known age (~30 days) and similar size (10–12 mm) were randomly selected from stock cultures. Eggs (0–3-day old) were collected by first isolating mature adult beetles from stock cultures. Adults were provided with a small amount of freshly ground media and the contents examined on a daily basis for eggs. *Anthrenus verbasci* eggs (0–3-day old) were collected in a similar manner. Nonquiescent adults, pupae (~5–10-day old), and mature larvae (~5 mm long) were randomly selected from stock cultures. All stages were held at 20°C with a small amount of food prior to the commencement of experimental conditions. Exposure of test insects to temperatures of 50°C, 45°C, 40°C, 35°C, and −10°C was performed in a Gallenkamph™ cooled incubator. For exposure to the temperature of −20°C an Esta™ freezing cabinet was used. Exposure to temperatures between 15°C and 35°C was not undertaken, because this is within the normal viability range for these species. Exposure to temperatures below 15°C and above −10°C was also excluded, because trial experiments indicated that there was no significant increase in mortality levels when compared with control samples.

Insects were contained in 9.0-cm-diameter plastic petri dishes with lids throughout the experimental period and were only introduced to the incubator or freezing unit when it was established that the relevant operational temperature had stabilised. The temperature throughout each experiment was monitored using a Squirrel™ data logger with temperature probe. After the exposure period had been completed, insects were removed and held at 20°C for monitoring and subsequent determination of recovery or mortality levels. Recovered larvae and adult stages capable of controlled, coordinated behaviour were recorded daily. Emerging larvae from the test eggs were also counted daily. Observation and counting of all stages continued until either recovery or death was determined. For adult beetles the lack of movement and failure to respond to gentle prodding have been considered a moribund condition (Boles et al. 1974). However, such a condition could also indicate paralysis (O'Brien 1960). In this study the loss of the righting posture and absence of spontaneous movement or an irreversible, uncontrolled, lethargic condition was used to determine death in larval and adult stages. Such criteria cannot be used for egg and pupal stages, therefore the cessation of normal development followed by obvious physical deterioration such as discolouration and shrinkage was considered an indication of death. Control batches for each life-cycle stage and species were held at 20°C in parallel with the test samples and throughout the subsequent monitoring period. Each test was repeated once and mean values calculated. For *D. maculatus*, 40 individuals were used for each test. Eggs and larvae of *A. verbasci* were also used in groups of 40, but because of the slow generation time exhibited by this species, pupal and adult stages were not as readily available and so these were used in groups of 20.

Results

The destructive effects of exposure to extreme temperature regimes are time dependent and it is therefore not possible to quote a single lethal temperature for an animal without stating the exposure period. The most usual method of defining the lethal conditions of temperature and time for a group of animals is to determine the combination of temperature and exposure time that kills a given percentage of the group. In this study, this was done by subjecting the target insects to a range of potentially lethal and sublethal temperature ranges over different exposure periods.

The experimental data for each time exposure and temperature combination performed are presented in Table 1. The data are also presented in plots of temperature against time to show 100% mortality levels for both species. In Figures 1, 2 the solid lines represent the approximate limits for the proposed

lethal time/temperature boundaries for all life stages of the species tested. For exposure to high temperatures, all stages failed to survive above this threshold at the combinations shown, whereas for low temperature exposures, all stages failed to survive below the boundary line shown. This information is intended as a general guideline for the determination of thermal mortality boundaries for these pests. Such a model would be beneficial to museum workers faced with treating suspected infestations of these pests, because it would be vital to know the required temperature and exposure combination to achieve complete extermination. However, it should be noted that in a museum context, account must be made to allow for the thermal insulation of objects that would necessitate increasing the exposure time required (see review by Strang 1992).

Exposure to Low Temperature Regimes

Eggs of both *D. maculatus* and *A. verbasci* showed a high level of susceptibility to exposure at −10°C. Six hours of exposure proved lethal to eggs of *D. maculatus*, whereas *A. verbasci* eggs failed to survive 3 hr of exposure. Larval stages for both species were more tolerant however, with 90% of *A. verbasci* and 65% of *D. maculatus* surviving 24 hr of exposure. Pupal stages of *D. maculatus* were largely unaffected by exposure to −10°C, although survival rates of *A. verbasci* pupae declined significantly after 12 hr of exposure. Subsequent monitoring of recovered immature stages of both species showed that normal development rates continued without adverse effect.

Adult stages of *D. maculatus* and *A. verbasci* were however, highly susceptible to exposure at −10°C. After 3 hr of exposure, all adults of *D. maculatus*, although still alive, appeared lethargic and uncoordinated, and although mobility increased significantly within 24 hr, all adults failed to survive beyond 2 wk following exposure. Of the 20% of *D. maculatus* adults that appeared to survive 6 hr of exposure, no adults subsequently survived to 3 wk post-exposure. Adults of *A. verbasci* were also highly susceptible to exposure to −10°C. There were no adult recoveries recorded after 3 hr of exposure. This was consistent for exposure periods monitored over a 24-hr period. Egg, larval, pupal, and adult stages of *D. maculatus* and *A. verbasci* were highly susceptible to an exposure temperature of −20°C. All stages were exposed to −20°C for periods ranging from 3–24 hr. After exposure all stages were monitored over a 3-mo period to determine recovery rates. There were no recoveries observed for any stages of either species.

Exposure to High Temperature Regimes

All stages of both *D. maculatus* and *A. verbasci* were extremely susceptible to the test temperature of 50°C. For *D. maculatus*, 100% mortality for egg, larval, pupal, and adult stages was reached after 3 hr of exposure. Egg and larval stages of *A. verbasci* were also killed after 3 hr of exposure, although pupal and adult stages required 6 hr of exposure to achieve complete mortality.

At the test temperature of 45°C, high recovery levels were observed for all stages of *D. maculatus* during the first 3 hr of exposure. Larval, pupal, and adult stages failed to recover after 9 hr of exposure, and although 40% of eggs appeared to hatch normally, emergent larvae subsequently failed to survive. All stages of *A. verbasci* were more susceptible to 45°C than *D. maculatus*. Pupae and adults failed to survive beyond 3 hr of exposure, and although 85% of larvae did survive,

Table 1. Mortality of *Dermestes maculatus* and *Anthrenus verbasci* following exposure to various temperature regimes.

	% Mortality of *D. maculatus* at 50°C								% Mortality of *D. maculatus* at 45°C							
Exposure (hr)	3	6	9	12	15	18	21	24	3	6	9	12	15	18	21	24
Eggs	100	100	100	100	100	100	100	100	25	60	60	100	100	100	100	100
Larvae	100	100	100	100	100	100	100	100	0	0	100	100	100	100	100	100
Pupae	100	100	100	100	100	100	100	100	10	40	100	100	100	100	100	100
Adults	100	100	100	100	100	100	100	100	5	15	100	100	100	100	100	100
	% Mortality of *D. maculatus* at 40°C								% Mortality of *D. maculatus* at 35°C							
Exposure (hr)	3	6	9	12	15	18	21	24	3	6	9	12	15	18	21	24
Eggs	5	10	10	20	15	30	60	100	0	0	0	5	0	0	0	10
Larvae	0	0	5	0	0	0	0	5	0	0	5	0	0	5	0	0
Pupae	5	30	25	30	55	65	70	70	0	0	5	0	0	5	0	10
Adults	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	% Mortality of *D. maculatus* at −10°C								% Mortality of *D. maculatus* at −20°C							
Exposure (hr)	3	6	9	12	15	18	21	24	3	6	9	12	15	18	21	24
Eggs	60	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Larvae	25	10	15	25	25	30	40	35	100	100	100	100	100	100	100	100
Pupae	15	25	20	10	20	10	15	15	100	100	100	100	100	100	100	100
Adults	0	80	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	% Mortality of *A. verbasci* at 50°C								% Mortality of *A. verbasci* at 45°C							
Exposure (hr)	3	6	9	12	15	18	21	24	3	6	9	12	15	18	21	24
Eggs	100	100	100	100	100	100	100	100	80	100	100	100	100	100	100	100
Larvae	100	100	100	100	100	100	100	100	5	15	100	100	100	100	100	100
Pupae	85	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Adults	85	100	100	100	100	100	100	100	90	100	100	100	100	100	100	100

Table 1. Continued.

	% Mortality of *A. verbasci* at 40°C								% Mortality of *A. verbasci* at 35°C							
Exposure (hr)	3	6	9	12	15	18	21	24	3	6	9	12	15	18	21	24
Eggs	30	65	90	100	100	100	100	100	50	55	40	50	60	40	45	50
Larvae	5	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0
Pupae	5	10	40	50	45	55	50	60	0	0	5	0	10	5	0	5
Adults	0	5	35	75	95	100	100	100	0	0	0	10	0	5	10	20
	% Mortality of *A. verbasci* at −10°C								% Mortality of *A. verbasci* at −20°C							
Exposure (hr)	3	6	9	12	15	18	21	24	3	6	9	12	15	18	21	24
Eggs	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Larvae	0	0	0	0	0	5	0	10	100	100	100	100	100	100	100	100
Pupae	25	10	15	45	80	65	70	80	100	100	100	100	100	100	100	100
Adults	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	% Mortality of *A. verbasci* at 20°C								% Mortality of *D. maculatus* at 20°C							
Control samples (hr)	3	6	9	12	15	18	21	24	3	6	9	12	15	18	21	24
Eggs	<1	0	<1	0	<1	0	2	1	<1	2	1	<1	0	1	0	<1
Larvae	<1	1	0	1	0	1	0	0	0	<1	0	0	0	0	<1	0
Pupae	0	<1	0	0	1	0	<1	0	0	0	0	0	0	<1	0	<1
Adults	0	0	<1	0	0	0	<1	<1	1	0	1	1	0	0	0	0

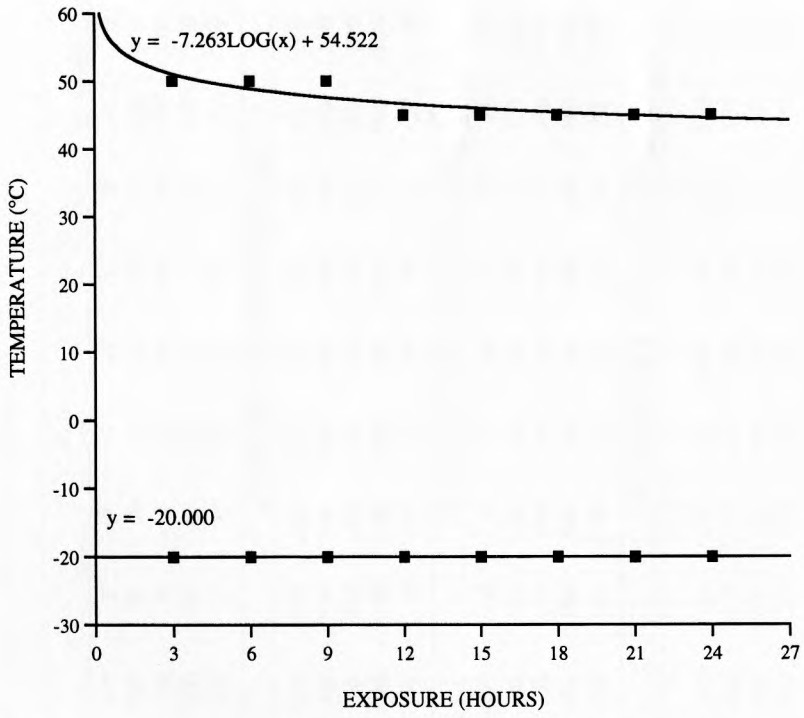

Figure 1. Time/temperature relationships for 100% mortality in *Dermestes maculatus*.

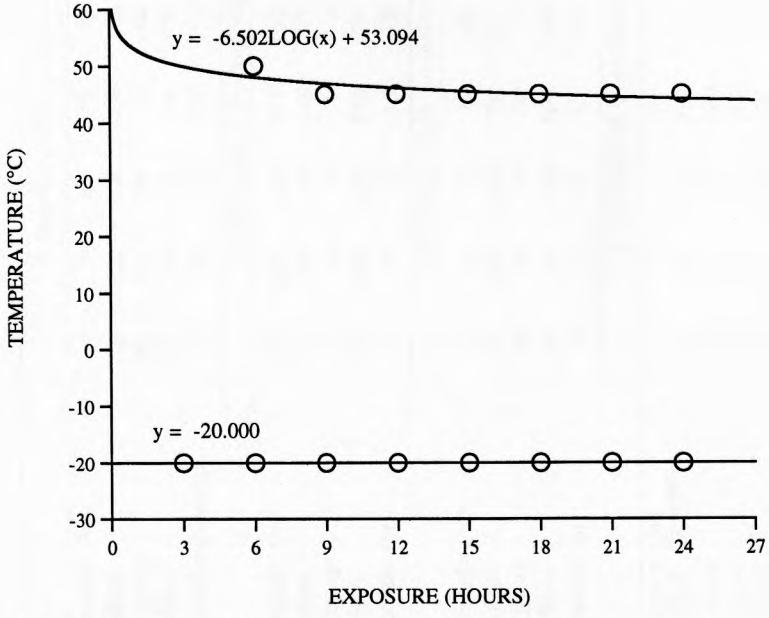

Figure 2. Time/temperature relationships for 100% mortality in *Anthrenus verbasci*.

100% mortality for all stages, including larvae, was recorded after 9 hr of exposure. Adults of *D. maculatus* were highly tolerant to the test temperature of 40°C. No mortalities were recorded after 24 hr of exposure. Mortalities at the pupal stage reached 55% after 15 hr of exposure, eventually reaching 70% after 21 hr. Larvae appeared unaffected at this temperature with virtually no mortalities achieved up to and including 24 hr of exposure.

The egg stage however, was not as tolerant. Hatching gradually declined as exposures increased with no eggs surviving after 24 hr of exposure. The larval and pupal stages of *A. verbasci* showed similar mortality levels to *D. maculatus* at 40°C. These stages exhibited normal movement and behaviour patterns after the exposure period. Recovered pupae subsequently matured into adults, and average egg production and emergence rates for the species were observed. However, adults of *A. verbasci* did not survive beyond 18 hr of exposure, and mortality markedly increased after 6 hrs of exposure. Eggs of *A. verbasci* were less tolerant to 40°C than were eggs of *D. maculatus*. High mortality levels were recorded after 6 hr of exposure.

At the test temperature of 35°C, *D. maculatus* and *A. verbasci* (with the exception of *A. verbasci* eggs) showed little adverse reaction to exposure periods of up to 24 hr. Normal development continued after termination of experiments and all stages progressed without noticeable adverse effects. Further observation of the recovered immature stages showed that on maturation to adults, females produced eggs which later produced active larvae. This was not unexpected. Although the optimum development temperature for *D. maculatus* and *A. verbasci* is 20°C and 23°C respectively (Hinton 1945), both these species have sustained normal development rates when held at 30°C. There was, however, a significant mortality rate for the egg stage of *A. verbasci* when exposed to 35°C. Fifty percent of eggs failed to hatch after 3 hr of exposure. This mortality rate was reasonably consistent over exposure periods of up to 24 hr.

Discussion

In general, insects may be considered sensitive to cold shock, provided they are cooled rapidly enough to a sufficiently low temperature. Typically, viability is dependent on the rate of cooling, with greater mortality observed following "rapid" rather than slow cooling. Injury and subsequent death increased as the period of isothermal incubation at the reduced temperature increased. This is an important consideration in a museum context because the insulating properties of bulk materials containing suspected insect infestations are likely to slow down the rate of freezing. Exposure times would need to be increased to compensate for this factor. The ability of museum insect pests to adapt to, or survive temperatures recommended for low temperature control has raised concern among some workers. However, Strang (1992) in a review of literature relating to insect thermal mortality found no evidence of museum insect pests exhibiting a high degree of either freeze-tolerant or freeze-avoidance behaviour that would prevent their death below −20°C, with the exception of *Camponotus herculeanus* (Hymenoptera: Formicidae) (Somme 1964). Similarly, there are no data available on the ability of museum pests to adapt to temperatures higher than 50–60°C. (Evans 1986). Failure to achieve desired mortality levels has been attributed to the insulating effects of artifacts, the mechanical performance of heating and cooling

systems (Brokerhof 1989, Strang 1992), and inadequate temperature monitoring (Florian 1990).

There is little doubt that prolonged exposure to high temperatures will have a lethal effect on insects. Difficulties arise in attempting to assess damage to specimens and materials caused by the heating process. It is acknowledged that heat is an accelerating process that will increase oxidation, thereby increasing ageing. Whether such effects will be within acceptable limits is difficult to evaluate. Wood, for example, may split or warp enabling easier access for future attacks by pests. Adhesives binding materials together may also be vulnerable, so it is important to be aware of the properties of the adhesive in relation to flow temperature and potential strength implications. The regulation of relative humidity as the exposure temperature increases is also important to avoid excessive drying and overheating of objects. The removal of representative fragments of individual specimens for molecular and DNA analyses has been used as a vital taxonomic tool, but how this practice is affected by prior exposure of the material to a raised temperature regime has not yet been ascertained. A review of the relevant literature indicates that little information is available on the harmful effects, if any, of extreme temperatures on museum specimens and materials. Florian (1990) reported that damage to museum specimens and materials following exposure of up to −20°C should not occur if they are adsorbent and free of moisture. Exposure to sub-zero temperatures rather than raised temperatures appears to be favoured by museums and recommendations exist for the treatment of material prior to and after treatment (Berkouwer 1994, Florian 1987, 1990, Hillyer and Blyth 1992). Several museums now use freezing as their principal method of eliminating pests from materials and as a "quarantine" tool for incoming material (Anonymous 1980, Crisafulli 1980, Florian 1986). No deleterious effects have been reported by these workers.

Results of this study indicate that a time/temperature/mortality relationship of 50°C for 6 hr was required to achieve 100% mortality for all stages in the life cycles of both *D. maculatus* and *A. verbasci*. Exposure to a temperature of −20°C for 6 hr also resulted in 100% mortality for all stages in both species. However, before these results can be adopted as a method of eradicating pests from museum objects, several factors should be considered. Museum specimens vary considerably in size and composition. Large and/or dense specimens and materials may insulate inherent pests from the desired treatment temperature and should therefore be allowed to equilibrate to the operating temperature before the actual exposure time commences. The precise build-up time required will depend on the particular objects involved and also the efficiency of the temperature control unit.

For exposure to sub-zero temperatures, the bulk of the material for treatment relative to the capacity of the freezing unit is also important. Mullen and Arbogast (1979) found that freezing units filled to capacity with commodities previously held at 25°C showed an initial elevation in temperature that increased the time required for the commodities to be chilled throughout. This differential can be corrected by determining the time necessary for the temperature of the objects under treatment to reach equilibrium with the temperature of the freezing unit and then adding on the exposure time required.

Because of their relatively low capital cost, domestic freezing units are often used by museums. Many models designed for domestic use are capable of main-

taining a temperature of −30°C. Shchepanek (1996) demonstrated that domestic freezers are adequate for achieving and maintaining temperatures required to eradicate insect pests. Some modifications related to the time allowed for cooling and warming and the packaging of specimens are recommended to ensure complete success of the low temperature technique. Despite these claims, there remains the possibility that such freezing units may not maintain desired temperatures throughout the exposure period. If this is the case, continuous monitoring of the temperature can be provided by the use of temperature recorders or data loggers designed for the purpose. Pinzl (1993) recommends mechanical modifications to freezing units to achieve lower and constant temperatures.

To prevent dessication of materials, condensation, and the escape of live insects, it is vital that all items for freezing are packed and sealed within a combined vapour and insect barrier. Strang (1996) recommends the use of low density polyethylene (LDPE) film with folds and seams heat sealed to maintain the moisture equilibrium.

Summary

Eggs, larval, pupal, and adult stages of two species of insects known to infest and damage museum collections were exposed to a range of temperature regimes to determine time/temperature/mortality relationships. The results show that although there was some variation in susceptibility between the two species evaluated and also between the different life-cycle stages, exposure to a temperature regime of −20°C for 6 hr was sufficient to achieve 100% mortality throughout all stages of both species. The treatment of bulk material, or material likely to insulate insects against extreme temperature regimes, may require extended exposure periods to ensure that acceptable mortality levels are achieved. An operating temperature of −20°C can be reached and maintained using widely available domestic freezers. Many models are now capable of reaching temperatures of −25–30°C. The choice of unit will depend on the manufacturer's specification in relation to operating temperatures, cabinet capacity, and insulation properties. For the treatment of bulk materials, large exhibits, and entire collections, cold-storage units and blast freezers may be appropriate and can be hired commercially (Berkouwer 1994, Hillyer and Blyth 1992).

Although a temperature/exposure regime of 50°C for 6 hr was found to be effective in killing all life-cycle stages of both insect species tested, the inadequate amount of information available on the potential adverse effects of raised temperature on museum materials raises doubts regarding its suitability. For this reason, exposure to sub-zero temperature regimes appears, at least in the short term, more feasible for museum applications. Based on these results a lethal boundary model is proposed for the eradication of insect infestations from museum collections and also as a potential quarantine method for incoming material.

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SCORE! A METHOD FOR CONSTRUCTING IMPROVED POLYETHYLENE LINERS FOR SPECIMEN TRAYS

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Abstract.—Many specimens are stored in standard-sized, open specimen trays with insufficient padding, potentially resulting in physical damage to specimens. Polyethylene foam tray liners are quick, convenient, and cost-effective solutions for mitigating the risk to specimens and their labels. The method described here (scoring and friction-fitting the liner to a standard-sized tray) results in removable liners with four padded sides and a square bottom, eliminates the mess of glue and the risk of burns from hot-glue applicators, and eliminates seams along the bottom of the tray liner where small fragments could disappear.

Many natural history collection specimens, such as fossil vertebrates, invertebrates, rocks, and minerals, are stored in standard-sized, open specimen trays with insufficient padding. Often, more than one object is stored in a single tray. Although this type of storage provides easy access, it can also result in serious physical damage to the specimens. Insufficiently padded objects can abrade one another or the bottom and sides of storage containers whenever they are handled. Specimen labels can also be damaged by abrasion.

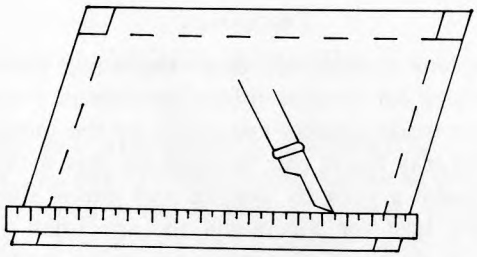
Permanent, archival-quality storage upgrades made from polyethylene foam can mitigate the risk of physical damage to specimens and their labels. A quick, convenient, and cost-effective technique for treating specimens that are stored in standard-sized specimen trays is the manufacture of polyethylene foam tray liners. By scoring and folding the foam as described below, liners that cover the bottom and sides of specimen trays can be made from a single piece of foam. This method has several important advantages. First, it eliminates seams along the bottom of the tray liner where small fragments could disappear. Second, when properly fitted, tray liners made by this method are held in place by friction and require no additional adhesive. This saves time, reduces expenses for both labor and materials, and eliminates the mess of excess glue and the risk of burns from hot-glue applicators. Moreover, glueless liners can be removed or replaced and are completely interchangeable between specimen trays of the same size. Finally, specimen labels can be inserted upright between the liner and the tray. This separates labels from direct contact with the specimens, provides a secure, standard location for labels, and eliminates the need to handle specimens to access the label data. The polyethylene foam liner provides the requisite padding for the specimen and a separate space for documentation while retaining the convenience of the specimen tray.

MATERIALS

You will need standard-sized specimen trays, polyethylene foam sheeting (¼-in. thick), a scalpel or pen knife, a ruler, and a template (made as described below).

METHODS

Calculate the size of ¼-in. polyethylene sheet required and cut foam.—(1) Measure both the inside length and the inside width of the specimen tray, (2) subtract ½ in. (twice the thickness of the poly-



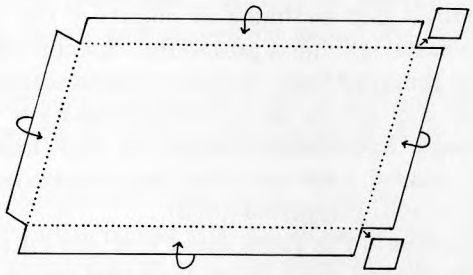


Figure 1 (top). After cutting a sheet of ¼-in. polyethylene foam to the appropriate dimensions, score all four sides.
Figure 2 (bottom). Flip the liner over, cut out all four corners completely, and fold up the sides before inserting the liner into the tray.

ethylene sheet) from both measurements to accommodate the fold on both sides of the tray, (3) measure the inside height of the specimen tray, double it, and add that quantity to the figures for both length and width, and (4) cut a piece of ¼-in. polyethylene foam to these dimensions.

Scoring the template.—Using the scalpel and ruler (or template) deeply score all four sides of the foam in the same pattern as shown in Figure 1. Be careful not to cut all the way through the foam. A template can be made from plexiglass to facilitate scoring. The template should be as long as the longest side of the polyethylene sheet, and as wide as the height of the standard-size specimen tray, less ~¼ in. (the thickness of the polyethylene sheet). For scoring, the template should be lined up along the edges of the polyethylene sheet, and the score should be made along the inside edge of the template (see Fig. 1).

Cutting and forming the liner.—Cut out the four corners completely and save them. Flip the liner over, fold up all four sides, and slip it into the specimen tray (see Fig. 2). The four sides should meet tightly in the corners of the specimen tray, and there should be a smooth crease, but no seam, along the bottom of the tray liner. If the foam is difficult to fold, then the score is too shallow. If the liner does not fit snugly or if it bulges or warps, the initial measurements or the width or placing of the template may have to be adjusted to make a tray liner of the perfect size.

Specimens that do not fit securely into a foam-lined tray can be accommodated by using the extra foam squares cut from the four corners as inserts. Or thicker inserts can be custom cut (see Waller 1992) from blocks of polyethylene foam. Inserts can be wedged or glued into place to prevent specimens from tumbling. Tray liners can also be adapted for specimens with multiple elements by subdividing the interior of the tray with strips or blocks of polyethylene foam appropriately sized and arranged.

Discussion

Custom-fit polyethylene mounts are time consuming and costly to construct, and they are unnecessary for smaller robust specimens. Polyethylene foam tray liners, however, can be made quickly and easily by the method described above. With practice, superior tray liners can be made by this method in less than half the time it takes to make a liner by cutting and gluing individual pieces. This method seems to work best for specimens and specimen trays of intermediate size. Very large, fragile, or heavy specimens are better suited for custom-fit supports, and the smallest specimen trays (less than ~1 in.) are difficult to fit with a foam liner using this technique. Best results were achieved on specimen trays ranging in size from 8 × 12 in. to 1 × 2 in.

Polyethylene foam tray liners offer several advantages over the drawer liners described by Fitzgerald (1992). First, experience in the geology collections at the Field Museum has shown that treatment of objects at the tray level, rather than the drawer level, increases specimen portability. Specimens stored in polyethylene-lined trays can be removed from drawers without directly handling the specimen. In this way, the tray serves as a secure housing for the specimen outside of its permanent storage. Specimens arranged in trays can also be more easily reorganized. Finally, padded trays are more appropriate housing for specimens that have a tendency to roll (Fitzgerald 1992).

The tray liners described in this paper differ from the liners described by Waller (1992) in having four padded sides rather than two and a square bottom rather than a rounded one. These differences should be considered when choosing between the two methods. The cautions noted by Waller (1992) should be consulted, because they are all relevant to this method as well.

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BOOK REVIEW

COLLECTION BUILDING IN ICHTHYOLOGY AND HERPETOLOGY, 1997, T.W. Pietsch and W.D. Anderson, Jr., eds. (Printed for the American Society of Ichthyologists and Herpetologists by Allen Press, Lawrence, Kansas, USA, 593 pp. $50 US + shipping and handling.) The history and scientific value of zoological collections receive relatively little attention. This deficiency is odd considering that collections not only are fundamental to systematic studies, but also that data derived from collections have served to provide the foundations for investigations in ecology, evolution, and conservation biology. The goals of the editors and authors of the ASIH volume were to provide a resource that includes a history of ichthyological and herpetological collections, focusing on individuals and institutions, as well as to identify prospects and challenges facing the future of biological collections.

Following an introductory chapter, the book is divided into seven parts related to collection development in various geographic regions. A brief summary of each follows.

The first section, *Collection Building in the Old World,* begins with a chapter on the Swedish Museum of Natural History including the influence of Linnaeus. Subsequent chapters cover Cuvier and Valenciennes and collections in France, the Leiden Museum in the Netherlands, Steindachner and the Natural History Museum in Vienna, ichthyologists of England, Krefft and the ichthyology collection in Hamburg and, finally, a detailed account of collections and museums in South Africa. Although most chapters focus on one or two highly influential persons, detailed accounts also are given of the histories of particular museums and associated curators and scientists.

Collection Building in the Western Pacific is the second section and includes discussions of institutions in Japan, Australia, and New Zealand. A great amount of detail is given to museums in particular regions, as well as to the individuals responsible for building collections.

The third section is the most extensive and consists of ten chapters on David Starr Jordan, his students, and the influence of the Stanford School. The information presented shows the truly amazing influence that David Starr Jordan and his colleagues and students have had on ichthyology and herpetology. This section is an excellent history of much of North American ichthyology and herpetology.

Collections in the southern United States are reviewed in the fourth section. The first chapter focuses on Alexander Garden who was the principal figure in the introduction of New World taxa into the Linnaean system of nomenclature. Chapters that follow cover the early collecting efforts of William Bartram, John Edwards Holbrook, Lewis Reeve Gibbes, and Silas Sterns. Considerable attention is given to the collections at the University of Alabama and Tulane University, and to collections in Mississippi.

The fifth section covers collections in the midwestern United States and Canada. The first two chapters are devoted to herpetology in the Chicago area. The first focuses on the Field Museum of Natural History and the second concentrates on the efforts of Howard K. Gloyd at the Chicago Academy of Sciences. The

remaining chapters describe the work of Stephen A. Forbes and Philip W. Smith at the Illinois Natural History Survey, Milton B. Trautman and his work on the fishes of Ohio, Vadim D. Vladykov and his study of lampreys, and finally the history and scientists of the Royal Ontario Museum.

Section six is comprised of one chapter on collection building in the Neotropics. The chapter discusses the collection and the scientists at the Museu de Zoologia da Universidade de São Paulo, Brazil.

The final section covers small collections and the philosophy of collection building. The University of New Mexico's Museum of Southwestern Biology is used as an example of the positive aspects, as well as the problems, associated with maintaining a small regional collection. The last chapter is an overview of collection building in herpetology related to Louis Agassiz and his lineage of students. It serves as a good conclusion to the book by comprehensively examining historical trends in collection building and identifying some of the challenges of maintaining collections in the future.

Overall, the history of ichthyology receives much more attention than does the history of herpetology. However, the early overlap of the two fields is diligently reviewed making this book worthwhile to anyone interested in the history of the biology of cold-blooded vertebrates. The editors believe, and rightly so, that this volume will contribute to a better appreciation and understanding of the part that collections have played and continue to play in the acquisition of our knowledge of natural history and in the conservation of biological diversity. Those involved in the study of fishes, amphibians, or reptiles, regardless of the discipline, owe it to themselves to read this book and gain a better understanding of the history of their science.—*Jason H. Knouft and Lawrence M. Page, Illinois Natural History Survey, 607 E. Peabody Drive, Champaign, Illinois 61820, USA.*

Museum Collection Resources Display Available for Loan

The Resources Subcommittee of the Conservation Committee (SPNHC) maintains a display of supplies and materials that are preferred by many museums for the storage and perservation of natural history collections. Examples of items included in the display are: materials used in the construction of storage containers and specimen supports; equipment for monitoring storage environments (e.g., Humidity, temperature, insects); and a variety of containers for the storage of collections and documentation. Some of the products are discipline-specific (e.g., pH neutral glassine for interleaving between herbarium sheets) but most can be used in multidisciplinary collections (e.g., ethafoam™ for lining shelves; Tyvek™ tape for box and tray construction). The exhibit is available for loan to interested parties for meetings, conferences, and other museum-related activities. Shipping costs to and from the requested venue are the responsibility of the borrower. There is no loan fee but SPNHC invites borrowers to make a voluntary contribution to cover the costs of routine maintenance. For additional information, or to borrow the display, contact:

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Jones, E.M. and R.D. Owen. 1987. Fluid preservation of specimens. Pp. 51–64 in *Mammal Collection Management* (H.H. Genoways, C. Jones, and O.L. Rossolimo, eds.). Texas Tech University Press, Lubbock, Texas. 219 pp.

Thomson, G. 1986. *The Museum Environment,* 2nd ed. Butterworths, London, England. 293 pp.

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Submission Procedure

Manuscripts intended for *Collection Forum* should be submitted in triplicate (original and two copies) to the Managing Editor. Letters to the Editor and correspondence relating to manuscripts should be directed to the Managing Editor. Books for review should be sent to the Associate Editor for Book Reviews.

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