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POSTMORTEM FIELD PREPARATION OF BIRD AND MAMMAL CHROMOSOMES: AN EVALUATION INVOLVING THE POCKET GOPHER, *THOMOMYS BOTTAE*

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ABSTRACT—A simple and reliable method to prepare karyotypes from recently dead specimens was evaluated under field conditions. This postmortem method relies on a brief period of in vitro culturing of bone marrow cells from dead specimens. To assess the overall usefulness of this method for field studies, 87 pocket gophers (*Thomomys bottae*) were karyotyped with the postmortem method and their karyotypes were compared with those from 41 pocket gophers karyotyped with the conventional in vivo method. The quality and quantity of metaphase chromosomal spreads on preparations made with the postmortem method are not significantly different than those made with the in vivo technique. While the quality and quantity of metaphase cells decrease significantly as postmortem times increase, the mean quality is also not significantly different between postmortem chromosomal preparations obtained from the live- and dead-trapped specimens. Excellent karyotypes can be obtained from specimens with mean postmortem times of up to 2 h. The postmortem method is suitable for karyotyping birds and mammals and may also prove useful for karyotyping other vertebrates.

The conventional method of preparing vertebrate chromosomes for karyotypic analysis involves the treatment of dividing bone marrow cells using a modification of the colchicine–hypotonic citrate or vinblastine sulfate–potassium chloride technique (e.g., Ford and Hamerton, 1956; Patton, 1967; Lee, 1969; Baker, 1970; Baker et al., 1982). This technique arrests cell division at metaphase through the in vivo administration of a mitotic inhibitor and, thereby, facilitates a high mitotic index. Over the past two decades, the technique has been modified and used successfully for a wide variety of vertebrates. However, the technique has seen its greatest popularity in mammalian systematics inasmuch as it is a rapid and reliable method of obtaining chromosomal preparations for mammals in the field and in the laboratory.

While this technique has been used extensively, its reliance on in vivo treatment of animals prevents its even greater application. With the conventional protocol, live animals are injected intraperitoneally with a mitotic inhibitor (either colchicine or vinblastine sulfate) and sacrificed following an incubation period. In our experience with birds and mammals, three main problems arise from the required use of live animals: 1) many species are difficult to capture and maintain alive; 2) the handling and subduing of specimens during the intraperitoneal injection is tedious and, depending on the species, potentially dangerous; 3) the variable condition of the animal under the stress of captivity can result in the unreliable treatment of targeted bone marrow cells.

In this study, we present and evaluate a postmortem method for karyotyping vertebrates that may be used as an alternative to the conventional in vivo technique. This postmortem method is yet another modification of the conven-

tional protocol but relies instead on the in vitro culturing of bone marrow cells (see also Christidis, 1985). This postmortem method is rapid and reliable and is devoid of the aforementioned difficulties. We evaluate this postmortem method under field conditions in research involving the pocket gopher, *Thomomys bottae*.

MATERIALS AND METHODS—*Karyotyping Techniques*—The conventional in vivo technique (e.g., Baker et al., 1982) was used to karyotype a reference sample of pocket gophers. This technique involves the treatment of dividing cells using the vinblastine sulfate–potassium chloride sequence. Chromosomal preparations made with the conventional in vivo technique provide a baseline for comparison with postmortem (in vitro) karyotypic preparations.

A modification of Christidis' (1985) in vitro method for avian karyology was used to make metaphase chromosome preparations from bone marrow cells of postmortem specimens of pocket gophers. See Appendix 1 for details on the supplies and reagents as well as the protocol for the postmortem karyotypic method used in this study. Prior to collecting and karyotyping in the field, centrifuge tubes were filled with 3 ml of growth medium and 0.1 ml of 0.01% vinblastine sulfate. Centrifuge tubes were prepared under sterile conditions in the laboratory and stored at approximately 0 to 3°C in the field. One person (J. C. Hafner) performed the karyotyping chores routinely in the field.

Fieldwork—A reference sample of 41 specimens of *T. bottae* was collected and karyotyped using the conventional in vivo technique. Reference sample specimens were collected during July and August 1986 with Macabee traps. All Macabee traps used in this study were modified slightly to ensure that a high proportion of live animals were captured; the sharp, angled points of the jaws were cut off and filed to a blunt tip (generally, 40 to 50% of the animals captured at a locality were held alive in such modified traps).

A second sample consisting of 87 specimens of *T. bottae* was collected with modified Macabee traps during July 1987. All of these specimens, whether live-trapped or dead-trapped individuals, were karyotyped using the postmortem method. During the field-collecting of these specimens, trap lines were run as frequently as possible to ensure accurate estimates of the times of death for those individuals killed in the traps. Detailed records of our trapping schedule were kept: initial trap-set time, trap-check times, capture time, time of death, time specimen was karyotyped. From these data, minimum, maximum, and mean times since death before karyotyping (postmortem times) were determined for each specimen. For example, a specimen collected dead at 1300 h from a trap previously set or checked at 1130 h and karyotyped at 1330 h would have a minimum postmortem time of 30 min, a maximum time of 120 min, and a mean time of 75 min. All animals trapped alive were held briefly in captivity and processed with other live-caught animals when convenient. Live specimens were sacrificed immediately before karyotyping and were karyotyped within 15 min. Accordingly, minimum, maximum, and mean postmortem times for all live-caught animals were recorded as 0, 15, and 7.5 min, respectively.

Laboratory Analyses—All specimens were karyotyped in the field, and microscope slides were later stained in the laboratory. One representative slide for each individual was scanned carefully (with a consistent search pattern) using a Zeiss Axioplan microscope. Each slide was assigned a score describing the overall quality of chromosomal spreads as well as a score relating the quantity of metaphase cells. The quality index included five scores: 0) few, if any, cells in mitosis, chromosomal spreads appear incomplete and individual elements not discernable; 1) individual chromosomes of metaphase cells discernable, elements poorly separated from one another and morphology indistinct (elements either over or under condensed); 2) chromosomes generally separated and morphology generally distinct, but quality of spreads variable and generally poor; 3) few overlapping elements and arm morphology visible; 4) very few, if any, overlapping chromosomes, well-stained elements (excellent contrast) and chromatids visible. The quantity index also included five scores: 0) no spreads in metaphase (mitotic cells, if present, have quality index score of 0); 1) 1 to 10 metaphase cells; 2) 11 to 20 spreads in metaphase; 3) 21 to 30 cells in metaphase; 4) >30 cell spreads in metaphase. One person (D. R. Sandquist) was responsible for scoring the chromosomal preparations.

Statistical Analyses—Mean postmortem times were transformed to logarithms (base 10) prior to statistical analysis. The regression subroutine of SPSS (Statistical Package for the Social Sciences; Nie et al., 1975) was used to analyze functional relationships between variables. Specifically, Quenouille's ordering test was used in lieu of parametric regression methodology to assess functional trends (see Sokal and Rohlf, 1981). This ordering test, easily performed as the Kendall rank-order test for association, was used to determine the presence of monotone increasing or decreasing trends. The

Kendall test was also used in this study, because our data contained many tied rankings, and Kendall's coefficient is known to produce a reliable test statistic in such situations (for review, see Sokal and Rohlf, 1981).

Mean index values between samples were compared using a subroutine of BMDP (Biomedical Computer Programs; Dixon et al., 1983) based on the Kruskal-Wallis test statistic, *H*. The Kruskal-Wallis test is a nonparametric analysis of variance and tests the significance between means. All statistical analyses were performed on a Prime 9955 computer at Occidental College.

Specimens Examined—All specimens of *T. bottae* used in this study are deposited as voucher material (skins plus skeletons) in the mammal collections at the Moore Laboratory of Zoology (MLZ). The reference sample was collected at 3.8 mi. S Cimarron, 6,500 ft., Colfax Co., New Mexico (n = 41; 24 males, 17 females; MLZ 1279–1285, 1290, 1291, 1296, 1298, 1302, 1304, 1306, 1308, 1310, 1312–1316, 1318, 1326, 1328–1331, 1340, 1343, 1350, 1351, 1355, 1357–1359, 1364, 1365, 1368, 1373–1375). The specimens karyotyped with the postmortem method were collected from 3.8 mi. S Cimarron, 6,500 ft., Colfax Co., New Mexico (n = 52; 30 males, 22 females; MLZ 1457–1508) and 2.8 mi. S, 2.0 mi. W Nogal, 7,300 ft., Lincoln Co., New Mexico (n = 35; 9 males, 26 females; MLZ 1511–1532, 1534–1541, 1543–1547).

RESULTS—Eighty-three of 87 individuals (95.4%) were successfully karyotyped with the postmortem method. In general, metaphase karyotypic preparations were obtained from pocket gophers up to a mean postmortem time of 120 min. Poor preparations result after a mean postmortem time of 120 min, but our small sample size (two individuals) beyond this time point disallows an assessment of the full temporal range of the technique's utility. By comparison, 40 of 41 individuals (97.6%) karyotyped with the customary in vivo technique were successfully karyotyped.

In Vivo Versus In Vitro Method—The quality index provides one means of comparing the chromosomal preparations from the reference sample (the in vivo technique) with those preparations made with the postmortem technique. The quality index describes the general nature of the chromosomal spreads as well as the conformation, contrast, and clarity of the chromosomes on a microscope slide. The mean quality index for the reference sample is 2.83 (variance = 0.745), while the mean quality index for preparations made from live-trapped individuals (n = 34) karyotyped with the postmortem method is 2.68 (variance = 0.650). These means are not statistically different by the Kruskal-Wallis test (H = 0.58; P = 0.445). Even when the reference sample is compared with all live- and dead-trapped individuals (n = 87) karyotyped with the postmortem method (mean quality index = 2.47; variance = 1.089), the means are still not significantly different (H = 3.09; P = 0.079).

The overall condition of the chromosomal preparations can also be assessed by the quantity index. The quantity index simply relates the number of metaphase spreads observed per microscope slide; a superior preparation will have a high quantity index. The mean quantity index for the reference sample is 2.29 (variance = 1.212), and the mean quantity index for preparations made from live-trapped individuals karyotyped with the postmortem method is 2.50 (variance = 1.409). As is the case with the quality index, there is no statistical difference between the means for the quantity index for the two groups (H = 0.49; P = 0.485). Similarly, the mean for all live- and dead-trapped individuals karyotyped with the postmortem method (mean quantity index = 2.15; variance = 1.477) is not significantly different from that of the reference sample (H = 0.56; P = 0.455).

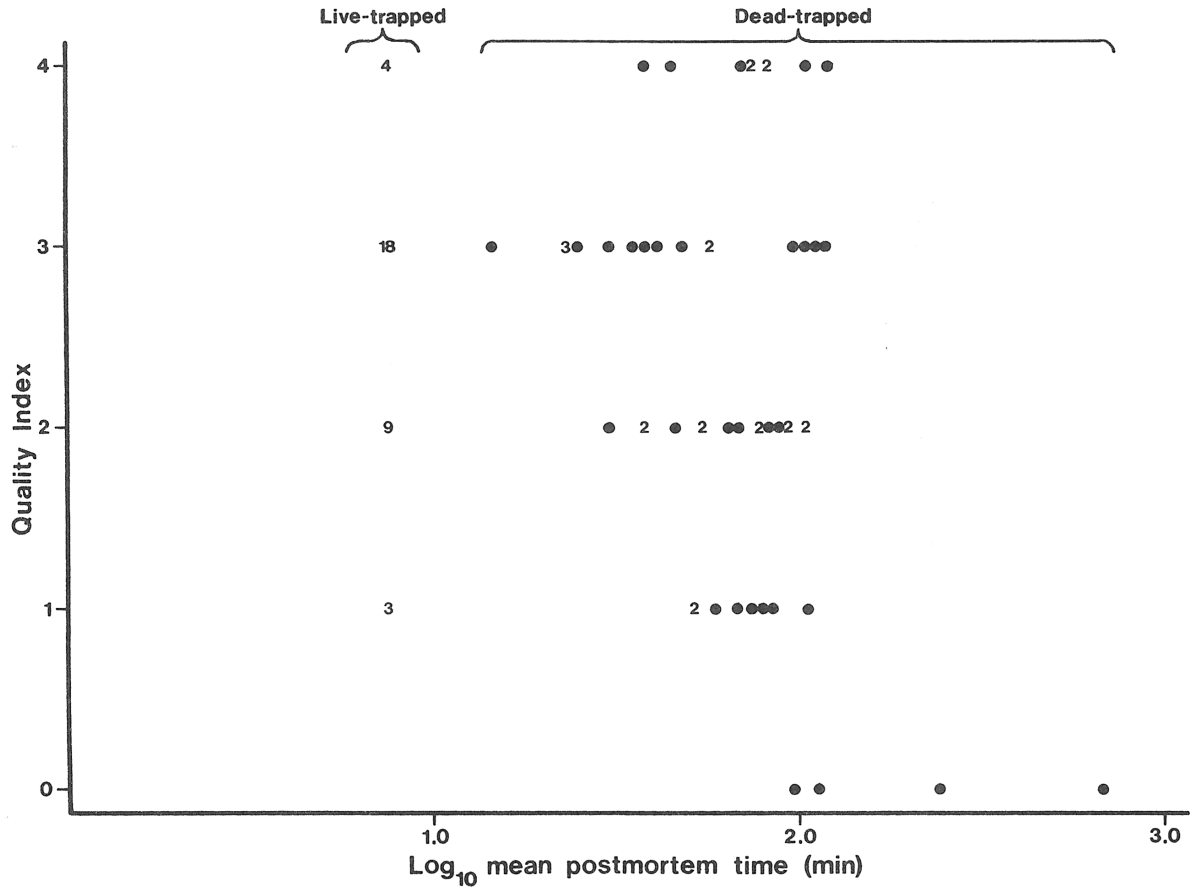

FIG. 1—Functional relationship between quality index and $\log_{10}$ mean postmortem time. Sample sizes are indicated directly on the plot; dots represent a single observation.

Postmortem Method—The overall quality of the chromosomal preparations obtained by using the postmortem karotyping method seems to vary both among and within postmortem time periods. The relationship between the quality of the chromosomal preparations (quality index) and $\log_{10}$ mean postmortem time is shown in Fig. 1. The data show that there is a significant trend between quality and time ($r_K = -0.1745$; $P = 0.021$). While the association is weak, the quality index decreases slightly as the mean postmortem time increases (Fig. 1).

In addition to the general trend over time, the quality index also varies among individuals within postmortem time periods (see Fig. 1). The quality index scores for the 34 live-trapped individuals (see Fig. 1) vary from a low score of 1 to a high score of 4; mean quality index = 2.68 for live-trapped specimens. In comparison, the mean quality index for the 53 dead-trapped specimens is 2.34 and the range is 0 to 4. The mean quality index scores for the live- and dead-trapped individuals are not significantly different ($H = 1.75$; $P = 0.186$).

Figure 2 depicts the relationship between the quantity index and $\log_{10}$ mean postmortem time. As is the case with the quality index, a significant monotone decreasing trend occurs between the quantity index and time ($r_K = -0.2871$; $P < 0.001$), although the association is weak. For example, the mean postmortem time for the live-trapped pocket gophers is only 7.5 min, but the quantity index values for these individuals vary greatly (see Fig. 2; range of 1 to 4). Also, the

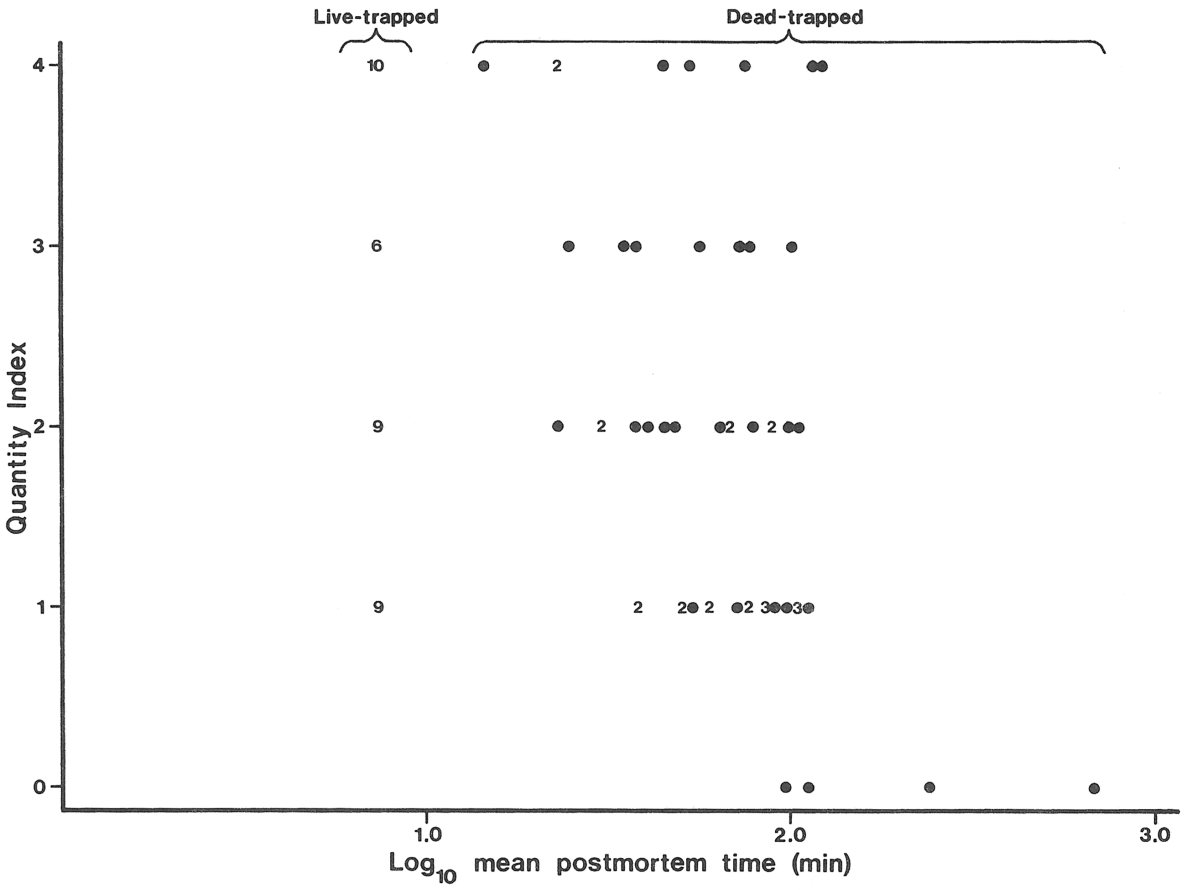

FIG. 2—Relationship between quantity index and $\log_{10}$ mean postmortem time. Dots represent a sample size of one; samples greater than one are indicated on the plot.

quantity index is correlated significantly with the quality index ($r_K = 0.4233$; $P < 0.001$).

The mean quantity index is 2.50 for the live-trapped individuals, whereas the mean quantity index for the dead-trapped individuals is 1.90. These means are significantly different by the Kruskal-Wallis test ($H = 4.44$; $P = 0.035$). However, if the two dead-trapped individuals with mean postmortem times of over 120 min are omitted from the analysis (see Fig. 2), the means for the quantity index are not significantly different between the live- and dead-trapped groups ($H = 3.47$; $P = 0.063$).

DISCUSSION—The postmortem procedure for karyotyping relies on a brief period of in vitro culturing of bone marrow cells in a growth medium. The technique, originally described by Christidis (1985) for karyotyping birds, is a simple modification of the traditional procedure that was popularized in mammalogy by Patton (1967) and Baker (1970). In addition to numerous minor changes in materials and procedures (see Appendix 1), our method differs from Christidis' (1985) technique by three principal modifications: 1) we follow Baker et al. (1982) in using a different mitotic inhibitor in a higher concentration for standard karyotypes (0.1 ml of 0.01% vinblastine sulfate in 3 ml of growth medium versus 0.1 ml of 0.001% colchicine in 5 ml of growth suspension); 2) we incubate the mitotic cells in the growth medium over twice as long (90 min as opposed to 37 to 42 min); 3) we incubate the cells in hypotonic solution for

a shorter period (15 min and not 20 min). Of the three differences, the increased incubation time in the growth suspension seems to be the most substantive change. It has been our experience that shorter incubation periods (approximately 40 min) result in early metaphase (elongate) chromosomes and, generally, a lower quantity of metaphase spreads than is deemed suitable for standard karyotypes; this seems particularly true for birds.

Data pertaining to the postmortem karyotypic preparations indicate that the means and ranges for the quality and quantity index scores are very similar for the live- and dead-trapped specimens. The quality of the karyotypic preparations from dead-trapped specimens is as good as that obtainable from the live-trapped specimens. It appears, however, that with increasing postmortem times, there is a decreasing quantity of metaphase cells as cell death continues to occur. This attrition results in the statistical difference in mean quantity index between the live- and dead-trapped specimens. It is also noteworthy that karyotypes with good (3) to excellent (4) index scores are obtainable from specimens with mean postmortem times as high as 120 min (see Figs. 1 and 2).

This study demonstrates the usefulness of the postmortem procedure for karyotyping mammals under field conditions. The overall quality of chromosomal preparations made with the postmortem karyotyping method is not significantly different from that obtained using the conventional in vivo technique. The postmortem method is simple to use in the field; it does not involve any untoward procedures and can be accommodated into the field routine. As such, this method avoids the difficulties associated with the in vivo technique (e.g., improper injecting technique, poor physical condition of animal) that can result in unreliable treatment of targeted bone marrow cells. Perhaps most important, the method allows one to maximize the overall utility of the specimens collected; essentially every individual taken, whether dead or alive, can be karyotyped successfully.

We have used the postmortem method described here to karyotype other species of rodents as well as passerine birds (see also Christidis, 1985); this method may be used successfully to karyotype other species of vertebrates as well. Indeed, the postmortem procedure may be particularly appropriate in situations where it is more expeditious to shoot or kill-trap specimens rather than to capture specimens alive. For further discussion of advantages of the postmortem method and its application to chromosomal banding studies see Christidis (1985).

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APPENDIX 1

Method for preparing bird and mammal karyotypes.

Reagents and Equipment—The following list includes supplies and equipment used in the field and lab for karyotyping:

1. mitotic inhibitor: 0.01% Velban (sterile vinblastine sulfate, USP), Eli Lilly and Company;
2. growth medium: Eagles medium (Minimum Essential Medium [Eagle], with Hanks' salts and HEPES Buffer), GIBCO Laboratories;
3. 15-ml centrifuge tubes: sterile and disposable (polypropylene) tubes with plug seal cap, Corning Laboratory Sciences Company;
4. hypotonic solution: 0.075 M potassium chloride;
5. Carnoy's fixative: one part glacial acetic acid and three parts absolute methanol (must be made fresh before use);
6. Giemsa blood stain: one part stock Giemsa with eight parts hot tap (or distilled) water (must be made fresh before use);
7. dehydration solutions: two baths of acetone, one bath of acetone and xylol (1:1), and two baths of xylol;
8. miscellaneous lab equipment, glassware and supplies: Pasteur pipettes (6 inch), rubber latex bulbs, 1-cc insulin syringes, Coplan staining jars, microscope slides (1.0 mm), cover slips (24 by 50, No. 1 thickness), Permount slide mounting medium, gasoline power generator, clinical centrifuge, centrifuge tube racks, 100-ml graduated cylinder, beakers, microscope slide boxes.

Protocol—The following methods were used to karyotype freshly killed bird and mammal specimens.

1. Immediately excise the midsection of the femur (for mammals) or the tibiotarsus (for birds) from the postmortem specimen and flush out the bone marrow into a 15-ml centrifuge tube with an insulin syringe filled with a solution of growth medium and the mitotic inhibitor.
2. Aspirate bone marrow to suspend cells using Pasteur pipette. If necessary, use tip of pipette to break bone marrow apart thoroughly and achieve a homogeneous cell suspension.
3. Cap centrifuge tube and incubate at 37°C for 90 min (place centrifuge tube in pants at waistline or in chest pocket under vest or coat).
4. Centrifuge for 3 min at approximately 3,000 rpm.
5. Carefully pour off and discard the supernate (leaving cell button) and add approximately 3 ml of hypotonic solution.
6. Resuspend cells in hypotonic solution by aspirating cell button with pipette.
7. Recap centrifuge tube and incubate at 37°C (near body) for 15 min.

8. Centrifuge for 3 min at approximately 3,000 rpm.
9. First wash: carefully pour off and discard supernate (leaving cell button), add approximately 3 ml of fixative solution, and resuspend cells in fixative.
10. Centrifuge for 3 min at approximately 3,000 rpm.
11. Second wash: pour off and discard the supernatant fixative, add approximately 2 ml of new fixative, and resuspend cells.
12. Aspirate entire cell suspension in pipette and filter through cheesecloth into the original centrifuge tube. If necessary, add more fixative to bring volume of suspension to approximately 3 ml.
13. Centrifuge for 3 min at approximately 3,000 rpm.
14. Third (concentrating) wash: pour off and discard supernatant fixative, add approximately 1 ml of fresh fixative, and resuspend cells. The solution should be visibly cloudy and without extraneous debris.
15. Pipette three to five drops of cell suspension onto microscope slides and either air dry or dry by the Scherz (1962) ignition method.
16. Stain in Giemsa for 15 min.
17. Pass slides through dehydration baths of acetone and xylol. To prevent excessive destaining, allow slides to remain in acetone baths only a few seconds each. Take slides directly from xylol and immediately mount with three to four drops of Permount using a large cover glass.