A TISSUE CULTURE TECHNIQUE FOR THE STUDY OF AVIAN CHROMOSOMES

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ALTHOUGH birds are without doubt one of the best known and most studied groups in the animal kingdom, their karyology is very poorly understood, perhaps the least known among the vertebrates. One of the main reasons for this is the paucity of tissues available for chromosome studies and the lack of suitable simple techniques for preparing them.

For a long time the only technique available was the same used in plant cytology, sectioned preparations of directly fixed tissues with high mitotic frequency. The results obtained were by no means satisfactory, especially for the study of chromosome morphology. About 1950 sectioning was replaced by the squash technique, which with the addition of colchicine and hypotonic pretreatment, produced much better preparations. The squash technique is especially convenient where laboratory facilities are limited. In 1958 the air-drying technique was introduced (Rothfels and Siminovitch, 1958). This technique requires more laboratory facilities including a centrifuge, but gives uniform flattening of the cells in division and is more suitable for photomicrographic analysis.

Three main sources of cells are available for chromosome study: direct preparation of tissues with relatively high mitotic frequency (testes, spleen, bone marrow, feather-pulp, embryonic tissues), leukocyte culture, and culture of other tissues. In the first group the best results are obtained from testes and embryonic tissues. The successful use of the spleen (Ohno et al., 1964), bone marrow (Tjio and Whang, 1963), and feather-pulp (Sandnes, 1963; Krishan et al., 1965) has also been reported. But in all these tissues the mitotic frequency is considerably lower than in cultured cells and the results more often than not are unsatisfactory.

Culture of leukocytes is widely used in human karyological research, and it has been used successfully in birds (Newcomer and Donelly, 1963; Krishan et al., 1965; Hungerford, 1965). However, all our attempts with avian leukocyte cultures have failed to produce satisfactory results, and others too, report failure of this technique with bird leukocytes (Vegni-Talluri and Vegni, 1965; Fechheimer and Jaffe, 1966).

Culture of tissue other than leukocytes is also widely and successfully used in the chromosome study of both humans and other animal groups. Although tissue culture requires special laboratory facilities and is more time-consuming than the direct fixation techniques, it has many advantages. The results obtained are so superior to the direct fixing techniques, especially in mitotic frequency and chromosome spread and definition, that

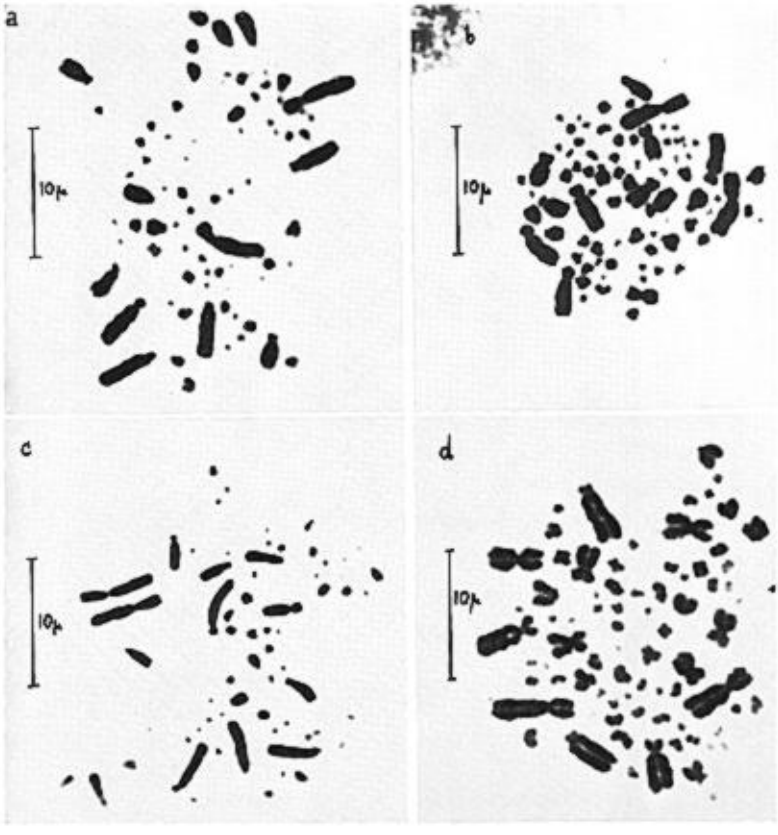

Figure 1. Metaphase plates from cultured lung and kidney cells (Giemsa stain). Key: a, Common Crow (*Corvus brachyrhynchos*); b, Slate-colored Junco (*Junco hyemalis*); c, Blue Jay (*Cyanocitta cristata*); d, Brown Thrasher (*Toxostoma rufum*).

the effort is well repaid. Once growth is obtained, it is possible to accumulate a large number of metaphases by replanting the original explants. This is a great advantage, especially when one works with smaller birds and rare specimens. In addition, culturing makes it possible to use colchicine without having live birds in the laboratory, an important consideration because it is often impossible to obtain certain desired specimens, except by shooting.

The tissue culture technique we have been using is a modification of Harnden's (1960) method for human skin cultures. We find this technique much simpler and more convenient than the two tissue culture techniques previously reported for birds (Rothfels et al., 1963; Vegni-Talluri and Vegni, 1965).

Cultures of both lung and kidney tissue have yielded equally satisfactory results. Both can be obtained sterilely even under field conditions, and adequate amounts are present even in small birds. We obtained tissues from the birds caught in mist nets and brought alive to the laboratory and from birds shot in the field. In the latter case the pieces of lung and kidney, removed under sterile conditions, were put in a plastic container with mixture No. 199 (Morgan et al., 1950) with 20 per cent fetal calf serum and kept at field or room temperature until cultured. The longest interval between taking the tissue from the bird shot in the field and culturing was 24 hours, and in that instance the cells grew very well. This interval possibly could be longer, because human skin usually grows well even after several days post mortem, provided the skin has been cooled and is not grossly contaminated.

To help prevent contamination penicillin and streptomycin as well as "Fungizone" (amphotericin B) should be added to the medium No. 199.

The culture procedure is as follows:

1. Place the tissue in a sterile petri dish containing mixture No. 199 and slice it with sharp sterile scalpels into 1–2 mm pieces. Avoid crushing.

2. Into one side of another sterile petri dish add about 0.5 ml of chick plasma and into the other side 0.5 ml of chick embryo extract (CEE), so that they form two separate pools. Using a Pasteur pipette coat the flat side of several sterile Leighton tubes (one for each 4–5 sliced pieces of tissue) with a very thin layer of chick plasma.

3. Again with the use of a Pasteur pipette transfer the explants (one set of 4–5 pieces at a time) first into the chick plasma in the Petri dish and then into the CEE.

4. Place the explants on the flat side of the Leighton tubes that have previously been coated with chick plasma. Four to five pieces per tube is probably an optimal number. They should be evenly spaced. Leave tubes on a slant with stoppered end down (use silicone rubber nontoxic stoppers) and allow them to stand for a couple of hours so that the CEE-chick plasma clot will form and stick to the tube. If the explant fails to stick, try leaving it for a longer period of time before adding the nutrient medium.

5. Carefully add 0.5 ml per tube of nutrient medium (60 per cent of mixture No. 199: 20 per cent CEE: 20 per cent fetal calf serum, with 0.5 ml of antibiotics to 50 ml of nutrient) without disturbing the clots. Incubate in the thermostat at 37°–39°C.

6. Check the growth under the microscope and change the medium every other day.

7. In about a week or ten days the explants can be replanted and the original growth saved. All tubes may be combined when harvesting. Sufficient growth usually is present after another 5–7 days.

8. For replanting remove the explants with a Pasteur pipette and prepare a fresh CEE-chick plasma clot and proceed as in steps 3–5.

9. Fresh nutrient should be added 20 hours before harvesting and colchicine may be added 2 hours before harvesting.

10. To harvest, pour off the medium, wash twice with Ca^{++} Mg^{++} free Hanks solution, and reincubate with trypsin (1 ml per tube) to detach the cells from the glass.

11. Transfer the cell suspension to a centrifuge tube and centrifuge 5 min at 500 rpm.

12. Remove the supernatant and add 0.7 per cent Na-citrate for hypotonic treatment. To prevent formation of a fibrin clot 0.1 ml of "Varidase" (streptokinase-streptodornase mixture) per 2 ml of hypotonic solution may be added at this time. Resuspend the cells by careful pipetting and incubate 10 min at 37°–39°C.

13. Centrifuge 5 min at 500 rpm.

14. Remove supernatant and fix with cold methanol-acetic acid (3:1), breaking up the cell button to insure thorough mixing with the fixative.

15. Centrifuge 5 min at 500 rpm.

16. Remove supernatant and resuspend cells in fresh fixative.

17. Let stand in refrigerator for at least 15 min (can be stored overnight or longer if desired).

18. Centrifuge 5 min at 500 rpm.

19. Remove supernatant and resuspend cells in several drops of freshly made fixative. The amount of fresh fixative may vary according to the cell concentration desired.

20. Make slides by using standard air-drying technique (Rothfels and Siminovitch, 1958) putting the cells on slides wet with water and chilled in refrigerator. Stain with any of the standard stains.

Preparations of avian chromosomes obtained by this technique are better than any obtained by the previously mentioned conventional techniques (Figure 1).

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