XX in the female and XY in the male. In meiosis of the latter, a large and a small vesicle could also be seen in severalp achytene configurations. The larger one was interpreted as the X chromosome and the smaller one as the Y. By comparison, the vesicles in *A. lituratus lituratus* may be interpreted, the smaller one as the Y_1, corresponding to the original Y, and the larger one as containing the remainder of the sex complement. The second protrusion observed in some pachytene figures probably corresponds to the Y_2.

In diplotene of *A. lituratus lituratus*, the sex vesicle was no longer observed. In diakinesis the trivalent is longitudinally disposed, both the Y connected end-to-end with the extremities of the X; the original Y (Y_1) with the short arm, and the Y_2, which shows negative heteropycnosis, with the long arm of the X (Figure 3). In *N. leporinus* the submetacentric X is distended, showing negative heteropycnosis; the Y is connected end-to-end with the short arm of the X.

It is interesting to emphasize that, while in meiotic prophase of *C. perspicillata*[7] the Y and 1 of the arms of the X synapse, in *A. lituratus lituratus* no Y shows synapsis behaviour with the X chromosome.

In late metaphase I the trivalent is folded in an arch-like configuration with both the Y side-by-side, but still connected end-to-end with the X. Apparently this configuration warrants the distribution of both Y to the pole opposite to the X, assuring the production of balanced gametes.

Two kinds of metaphases I were found in *A. lituratus lituratus*, with $n = 15$ containing 1 sex chromosome, the X and with $n = 16$ containing 2 sex chromosomes, the Y_1 and the Y_2.

Resumen. En el murciélago *Artibeus lituratus lituratus* el número de cromosomas es de $2n = 30$ en la hembra y de $2n = 31$ en el macho. El mecanismo de determinación del sexo es XX/XY_1Y_2. La translocación de un autosoma al X resultó en la diferenciación del neo-Y. Son descritos los aspectos del comportamiento del trivalente sexual durante la meiosis. Estos son comparados a los que presenta la especie *Noctilio leporinus* ($2n = 34$) cuyo mecanismo es XX/XY.

M. L. Beçak, R. F. Batistic, L. D. Vizotto and W. Beçak

Secção de Genética, Instituto Butantan, São Paulo (Brazil), 16 August 1968.

Chromosome Preparations of Bovine Leucocytes

The recent development of refined techniques has enabled the determination of somatic karyotypes of a variety of animals, either directly from bone marrow or other tissues established in culture. Unfortunately, the reliability of these various techniques is unsatisfactory since, the problems often encountered when utilizing such techniques have not been discussed nor mentioned in the literature. The purpose of this paper is to present a sufficiently reliable technique for bovine chromosome preparations from cultured peripheral-blood leucocytes and to comment on some of the technique's inherent problems. The technique is essentially similar to the ones suggested by Hungerford et al.[1] and Basrur and Gilman[2].

Materials and methods. Blood samples were collected in sterile, 15 ml graduated conical centrifuge tubes, containing 0.5 ml of an anticoagulant (heparin, 0.4 mg/10 ml blood) and 0.25 ml of an antibiotic mixture (penicillin-streptomycin), centrifuged at 800 rpm for 5–7 min (at room temperature) and then stored in the refrigerator at 4 °C for 40–48 h. This procedure was found to yield a high mitotic index.

The culturing procedure was carried out under ethylene glycol conditions as a precaution against contamination. All equipment used in the procedure was autoclaved for 20 min.

Approximately 1.5 ml plasma along with the buffy coat (layer of lymphocytes) was withdrawn with a sterile pipette, from the stored samples and diluted, in sterile, plastic T-flasks (Falcon), with a solution made up as follows: To 5 ml TC medium 199 were added 2 ml bovine serum (heat inactivated at 58 °C for 30 min), 0.25 ml penicillin-streptomycin (100 U and 100 µg respectively) and 0.25 ml bacto-phytohemagglutinin (M or P), Difco Laboratories, Detroit.

The cultures were swirled at room temperature to ensure thorough mixing and then horizontally incubated at 38 C° ($\pm$ 0.5 °C). Swirling of the cultures was continued periodically for about 2 h. Temperature constancy was found to be an absolute prerequisite for achieving desired results and therefore maintained throughout the incubation period.

Following 70½ h of incubation, colchicine (1 ml of 0.008 g + 100 ml water) was added to the cultures. Sterile precautions beyond this stage of the procedure were found unnecessary. The procedure of hypotonic pretreatment was begun immediately following the period of incubation in colchicine (4–5 h at 38 $\pm$ 0.5 °C). The contents of each flask were transferred to 15 ml graduated conical centrifuge tubes and centrifuged at 800 rpm for 15 min (room temperature approximately).

Hypotonic pretreatment in synergy with colchicine treatment is necessary for the swelling of cells and the dispersion of metaphase chromosomes. Therefore a fresh solution of 0.8% of sodium citrate was used as the hypotonic solution.

The supernatant was decanted from each centrifuge tube with a pipette. The 'button' of cells at the bottom of each tube was resuspended by tapping the outside wall of the tube vigorously and by a gradual addition of 4–5 ml of hypotonic solution. After 30 min in hypotonic solution the cells were centrifuged at 800 rpm for 15 min.

Since optimal fixation was achieved when the amount of hypotonic surrounding the cells was minimal, most of

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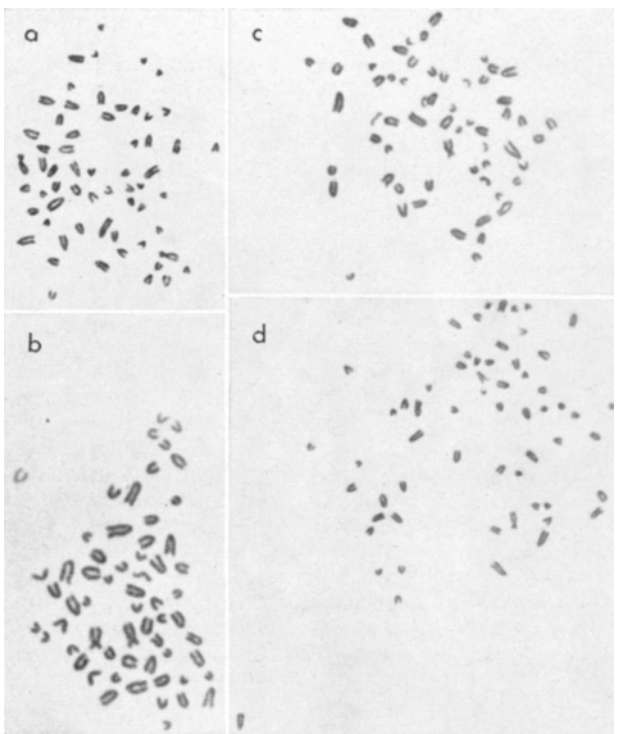
[2] P. K. Basrur and J. P. W. Gilman, Nature *204*, 1335 (1964).

the supernatant was decanted, without disturbing the cell 'button'. To avoid the clumping of cells, approximately 4 ml freshly prepared fixative, consisting of 3 parts absolute methanol (4 °C) and 1 part glacial acetic acid were then added with a continuous agitation of the cell 'button'. This procedure of centrifugation, decantation and addition of fixative was repeated 2 more times. Resuspension was best achieved by tapping the outside wall of the tube rather than by aspirating with a pipette. The total time in fixative could be varied from 90 min to 24 h or longer, without deterioration in the preparation.

Finally a suspension of cells was made in a small amount ($^1/_2$ ml) of fresh fixative. A drop or two of this 'hazy' suspension was then placed on a methanol-immersed, precleaned glass slide. The cells were allowed to spread on the slide and the methanol was ignited and allowed to burn off.

Preparations were stained with Feulgen since such preparations revealed a better morphology of the chromosomes. The procedure was as follows: Ignited slides were immersed in distilled H_2O for 5 min. The slides were drained and then placed in 5 *N* HCl (room temperature) for 40 min in a covered container[3]. The slides were quickly rinsed in distilled water and then placed in Feulgen stain in a dark, stoppered container for 30 min. Finally the slides were rinsed in tap water until the water remained clear of purple stain[4] and they were then mounted in Gurr's water-mounting medium.

Results and discussion. The common experience with peripheral blood cultures is that they are not always reliable and successful – unreliability and failure often being a result of an unawareness of the problems involved in the technique.



Bovine mitotic metaphase spreads obtained by the technique described. (a) Female cattle. × 1000. (b) Female bison. × 1000. (c) Male bison. × 1000. (d) Male cattle. × 700.

Our experience with peripheral-blood cultures indicates that the enigmatic factors of the technique are essentially the critical factors, often responsible for the failure of the growth of the cultures. The necessity for both phytohemagglutinin (PHA) (an extract of the red kidney bean *Phaseolus vulgaris*) and 'heat inactivated' serum in the culture medium remains an enigma. However, failure to include the forementioned constituents in the culture medium results in a failure of the growth of the cultures. The exact mechanism of action of PHA in peripheral blood cultures still remains undeciphered. The precise chemical nature of the 'active factor' in the bean extract, causing both a transformation in lymphocyte morphology and subsequent mitosis, has yet to be defined. Some state that the transformation in morphology ('blastogenesis') is the direct consequence of PHA attachment to a specific structure of the small lymphocyte[5,6]. The mitogenic effects are probably due to the ability of the extract to cause a rejuvenation of the lymphocytes. This in turn causes a general increase in metabolism and DNA synthesis, ultimately resulting in the division of some cells[7].

Evidence seems to suggest that the serum, when inactivated, contains a broad spectrum of growth factors (probably proteins), that can satisfy the requirements of cell growth and are actually utilized in the course of tissue cultures[8,9].

For increased reliability and success, modifications are constantly being adopted for utilization of the technique with different animals. However, seldom is a mention made of the various modifications. Therefore it was felt necessary to report our modifications in their entirety. The modified technique proves to be a dependable one, for the culture of leucocytes of bovines and other vertebrates as well. It produces ample well-spread metaphases (see Figure) for more thorough cytogenetic and karyotypic diagnoses[10].

Résumé. Les techniques perfectionnées de la culture des tissus ont rendu possible l'étude des chromosomes des vertébrés. Cependant, les résultats obtenus par l'emploi de ces techniques sont fréquemment sujets à caution parce que les difficultés inhérentes à ces problèmes ne sont pas suffisemment discutées. Certaines modifications qui ont donné de bons résultats sont ici suggérées pour la méthode de préparation des chromosomes bovins utilisant les leucocytes périfériques du sang. La technique en question peut être aussi bien appliquée aux autres vertébrés.

R. BHAMBHANI and J. KUSPIRA

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