grown in the medium of BREWBAKER and KWACK[1]. The procedure recommended by ERDTMAN[2] was followed in acetolysis. The chemical constitution of the pollinial wall was tested by the histochemical methods described by JENSEN[3] and SOUTHWORTH[4].

The pollinia of *Calotropis* are flat structures with wide rounded bases and narrow apexes. Of the two longer edges, one is almost straight and the other is slightly convex. Under the microscope, the pollinium appears to have a cellular flat surface bordered with thick homogenous edges. At the time of germination, pollen tubes emerge from the distal half of the flat edges of the pollinia. As suggested by ZERONI and GAIL[5], the pollinial wall was cut open at various places to test the polarity of the growing pollen tubes. The direction of tube growth was not markedly affected even with the new outlets so opened.

Asymmetrical germination of pollinia has already been reported in *Asclepias*[5]. In 3 other members of Asclepiadaceae also we found that the pollen tubes emanate only through particular regions of the pollinia which appear to be genus-specific. Thus, the position of emergence is on the straight edge of the pollinium near the base in *Calotropis*, near its apex in *Dregea* and near the base on the convex edge in *Daemia*. Since this region has not been marked by any visible characteristics, histochemical methods were employed to study the nature of the pollinial wall with particular reference to the area of emergence of the tubes. These tests were confined to the pollinia of *Calotropis*.

Previous reports ascribe a waxy or cuticular[5,6] nature to the pollinium. However, no disintegrating effects on the wall were seen when the pollinia were placed in closed vials containing lipid solvents such as acetone, benzene or chloroform for as long as 3 days. Of the 3 solvents, benzene alone appeared to have dissolved some of the deposits on the corpusculum and connectives of the pollinia although the pollinial wall remained intact at the end of the treatment. It is therefore reasonably certain that 'waxy substances' are not the chief constituents of the pollinial wall. The suspected cuticular nature of the wall was also tested. Cutin is saponified by an alcoholic solution of KOH, but the pollinial wall is found to be insoluble in this reagent. In contrast, the wall is saponified in the sporopollenin solvent of fused KOH. It may be noted here that the reaction of the sporopollenin of the pollen exines to the above tests is also identical to that of pollinial walls. Lignin shares the solubility properties of sporopollenin[4], but the usual staining techniques, specific for this constituent, have not revealed its presence in the pollinia.

The sporopollenin composition of pollinial wall is further emphasized by acetolysis. Although it had no effect on the bag-like nature of the pollinia, acetolysis leaves an open area on the pollinial wall corresponding to the germinating region. The slit so revealed has well defined edges followed by clear cellular outlines all around. In the pollinia of *Calotropis* which we examined, this slit measured about 350–400 μm in length. Since the length of the opening is much greater than its breadth, it may be defined as a furrow, following the terminology suggested by FAGERI and IVERSEN[7].

Our study reveals some of the similarities between pollen and pollinial wall. The furrow on the pollinial wall and the germ pore on the pollen exine are really differentiated wall regions for the emanation of pollen tubes. Again, as in the case of the germ pore in pollen wall, the position and morphology of the pollinial furrow appear to have some diagnostic significance. Besides, the chemical composition of the two walls shows resemblances. Both are resistant to lipid solvents, alcoholic KOH and acetolysis. At the same time, acetolysis clears the regions of the pore and the furrow. The various tests mentioned above indicate that the pollinial wall, like that of the pollen exine, is composed of sporopollenin[8].

Zusammenfassung. Nachweis, dass das Pollinarium von *Calotropis gigantea* reichlich Sporopollenin enthält und dass die Pollenschläuche das Pollinarium durch eine präformierte Furche verlassen.

S. JALAJA and A. N. NAMBOODIRI

Department of Botany, University of Kerala, Kariyavattom 695 581 (Kerala, India),
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A Combination of Sister Chromatid Differential Staining and Giemsa Banding

When a cell is grown in a medium containing 5-bromodeoxyuridine (BrdUrd or the older abbreviation BUdR which is used throughout this paper) for two cell cycles, the two sister chromatids of each chromosome are different: one with bromouracil (BU) substituting thymine in both of the DNA strands and the other with only one BU-substituted DNA strand. LATT[1] demonstrated that when such cells are stained with the fluorochrome 33258 Hoechst and observed with UV optics, the chromatid with bifiliary substitution would show dull fluorescence or no fluorescence whereas the chromatid with unifiliary substitution would show bright fluorescence. In the preparations showing sister chromatid differential staining (SCD), sister chromatid exchanges (SCE) can be detected with excellent resolution.

The fluorescence technique was later modified for Giemsa staining[2–4]. In these preparations, the bifiliary substituted chromatid stains lightly and the unifiliary substituted chromatid stains deeply. Again SCE are extremely clear.

Since these procedures already have important applications[5,6] and more uses are expected in the future, it

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would be advantageous to develop additional techniques in this direction. In the present report we describe procedures which combine sister chromatid differential staining and G banding.

Materials and methods. We used the following monolayer cell cultures as material for the present study: Chinese hamster cell line Don, the cactus mouse, *Peromyscus eremicus*, cell line 2352, primary cultures of Swiss mouse embryos, and human lymphocytes. For monolayer cultures each was given BUdR (5 µg/ml) for 2 cell cycles (24 h for Don and 30 h for others). For human lymphocyte cultures BUdR was added 24 h after initiation and reincubated for an additional 48 h. All cultures were grown in complete darkness when BUdR was in the medium. In all cases, a 2 h Colcemid treatment (0.05 µg/ml) was applied prior to harvest. Conventional air-dried preparations were made.

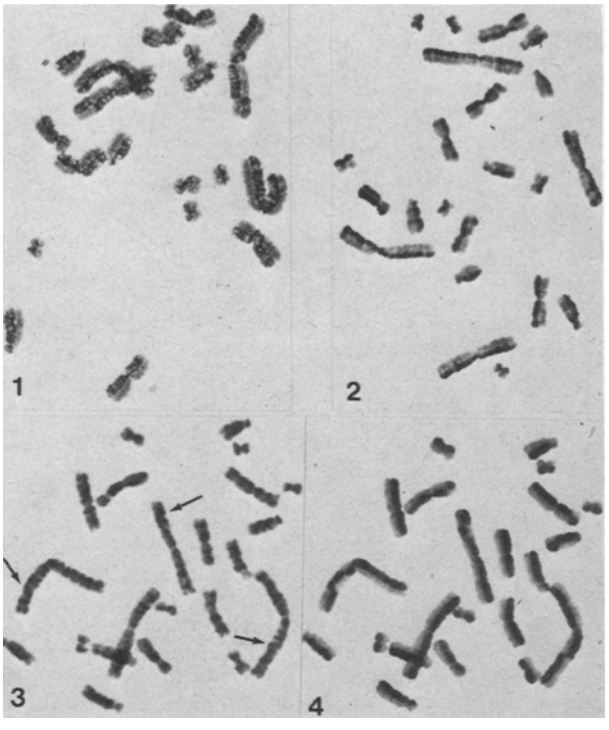

Fig. 1–4. Chinese hamster cells after grown in a medium containing 5-bromodeoxyuridine for two cell cycles. ×1350. 1. Trypsin treatment, showing G banding on deeply stained chromatid. 2. Trypsin treatment, showing G banding on lightly stained chromatid. 3. Urea treatment, showing typical G bands. Arrows indicate interrupted positive bands. 4. The same cell shown in Fig. 3, showing sister chromatid differential staining and exchanges.

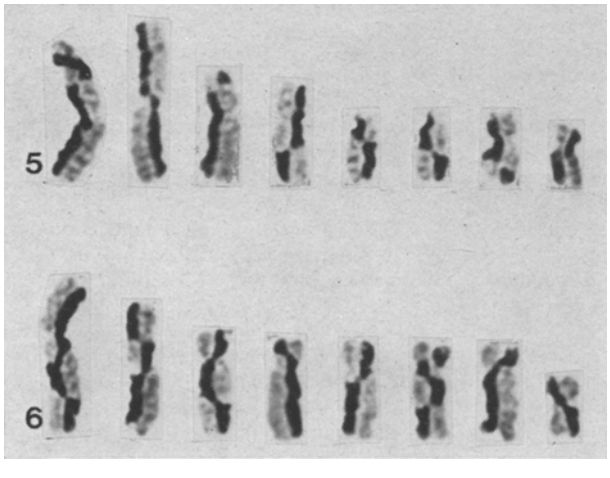

Fig. 5 and 6. Cut-out chromosomes from human lymphocytes after grown in a medium containing 5-bromodeoxyuridine for two cell cycles. Trypsin treatment, showing G band on the lightly stained chromatids. ×2000. 5. Showing SCE points relative to the position of G bands. 6. Showing deeply stained material connecting the deeply stained chromatid at SCE.

Two methods were used: 1. Trypsin treatment. We dilute the regular trypsin solution used in routine cell cultures with Hanks' balanced salt solution (without Ca and Mg) to 20% of its original strength. Slides, 2 days to 2 months old, are treated with this trypsin solution for 1–2 min, then quickly rinsed in Hanks' solution (without Ca and Mg), 70% ethanol, 95% ethanol, and air-dried again. They are then stained in 2% Giemsa prepared in 0.01 *M* phosphate buffer (pH 7) for approximately 2 min and then rinsed in deionized water. The procedure is in fact the same as the regular G banding technique, but it gives SCD as well as G banding. G bands may appear in either the lightly-stained or the deeply stained chromatid, depending upon the duration of the trypsin treatment. Brief treatment produces excellent SCD. A slightly longer treatment time yields G bands on the bifiliary substituted chromatid and longer treatment produces bands on both chromatids. Continued treatment obliterates the SCD pattern and only G bands are seen. Thus both patterns, SCD and G bands, can be produced on the same cells.

2. Urea treatment. The urea method of SHIRAISHI and YOSIDA[7] can be used effectively to achieve the same result but the SCD pattern is rapidly replaced by G bands. We prepare an 8M urea solution which is mixed with M/15 Sorensen's buffer (3:1) and the mixture is kept at 37 °C. Slides are dipped into this solution for a brief exposure (5–15 sec), rinsed, and stained in Giemsa the same way as before.

It is also feasible to induce G banding first by urea treatment[7] and later induce SCD of the same cells by the technique of KORENBERG and FREEDLENDER[4].

Results and discussion. Figure 1 presents a Chinese hamster metaphase showing SCD and G banding on the darkly stained chromatid, and Figure 2, on the lightly stained chromatid. Both were taken from trypsin-treated preparations. In general, when G banding is on the light chromatid, the bands are more distinct. Figure 3 presents the G banding pattern of a Chinese hamster metaphase treated with urea. The same cell is shown in Figure 4 revealing SCE patterns following the KORENBERG technique[4].

LATT[6] found that the points of SCE are usually at the Q^- bands. In G banding, it would mean G^- bands. Figure 5 presents several cut-out human chromosomes (trypsin treatment) which do not entirely confirm LATT's observation.

The actual point of exchange is difficult to determine since it is known that Giemsa positive bands are produced by differential contraction of portions of the chromatid at specific regions. As the chromosome condenses during late prophase into metaphase what may have been 3 or 4 minor positive bands may become one major positive band. An exchange in a Giemsa negative region would be easier to interpret but our data indicates that the exchange points are rarely within Giemsa negative areas but instead appear to be at the junction of positive and negative bands. The 'tilted' appearance of the G bands at points of exchange (Figure 3) causes us to suspect that the

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regions of the positive Giemsa bands are involved and that the exchange may be more complex than has been suggested. At present we have no better explanation for the interrupted positive G bands in the exchange regions.

Of particular interest with the trypsin procedure to induce SCD is that frequently one observes interchromatid connections at the point of SCE (Figure 6). Such connections have never been seen in 33258 fluorescence preparations or Giemsa preparations using procedures described previously by other investigators. It is possible that trypsin treatment distorts the distribution of nucleoproteins along the chromatids and the connecting stained material represents an artefact.

Since SCE may be a sensitive and accurate method for measuring genetic instability of mammalian and human cells, improvements in methodology (rapidity, simplicity as well as combination of techniques) should prove to be useful in future investigations. The procedures described in the present paper not only can reveal both SCD and G banding but are also rapid. The trypsin method requires a total of approximately 5 min.

Summary. We report a procedure for combining sister chromatid differential staining and G banding in the same metaphase plate. Mammalian cells in culture are grown in medium containing 5-bromodeoxyuridine for two cell cycles, and conventional air-dried preparations are made. The slides are treated with a trypsin or a urea solution the same way as for regular G banding. This method is simple and fast and provides additional information for cytogeneticists[8].

S. PATHAK, A. D. STOCK and A. LUSBY[9]

Department of Biology, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston (Texas 77025, USA), and The University of Texas, Graduate School of Biomedical Sciences, Houston (Texas 77025, USA), 8 April 1975.

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[9] The University of Texas Graduate School of Biomedical Sciences, Houston, Texas 77025, USA.

Limulus Chromatophorotropin: Action on Isolated *Uca* Legs and in Various Crustaceans

The CNS of the horseshoe crab, *Limulus polyphemus*, contains a chromatophorotropin that disperses melanophore pigment in *Uca* species[1,2]. During our continuing study of this substance[3,4], we wondered if it acted directly or indirectly in *Uca* melanophores. We also wondered if this *Limulus-Uca*-chromatophorotropin, which for convenience we refer to as LUC, was capable of influencing pigment migration in chromatophores of other crustaceans. To obtain answers to these questions, we have tested the effects of LUC extracts on isolated legs of *Uca* species and on eyestalkless representatives of several crustacean families.

All animals (except *Gecarcinus lateralis*[5]) were obtained commercially and held in suitable artificial sea water (Instant Ocean) environments. Eyestalks were removed at least 1 day prior to experiments. Isolated legs, having all stage one[6] melanophores, were obtained from eyestalkless *Uca pugilator* and the recently described *U. panacea*[7]. Legs were removed proximal to the autotomy plane on the basiischium and placed in Syracuse glasses containing physiological saline[8]. The 2nd and 3rd walking legs were used; the right legs as controls and the left legs as experimentals. Extracts were prepared by thoroughly grinding lyophilized *Limulus* CNS in all-glass homogenizers in either saline (legs) or artificial sea water (crabs). The resultant mixture was then centrifuged, boiled for 10 min, recentrifuged, and the supernatant used for injection. Control legs were injected with 10 µl saline, and experimental legs with 10 µl of a 0.1 mg dry CNS/ml saline extract. Preliminary studies showed that melanophores of eyestalkless *Uca* exhibited pronounced responses to injections of 10 µl of one dry CNS/5 ml sea water LUC extract. Therefore, eyestalkless crabs were injected with a volume of that extract equal to the ratio eyestalkless crab weight/*Uca* weight. Control crabs were injected with an equal volume (10–150 µl) of sea water. All injections were made with a Hamilton 100 µl syringe equipped with a 30 gauge needle. Legs were injected through the autotomy plane into the merus, and crabs were injected at the base of the walking legs. All assays were performed in a darkened room. Isolated leg chromatophores were staged[6] before injection and at 15, 30, 60, 90, and 120 min after injections. Eyestalkless crab chromatophores were observed and staged[6] at similar intervals, or until the absence of response was evident. No attempt was made to quantify the response of eyestalkless crab chromatophores to LUC extracts. Instead, we observed the chromatophore response to extracts and to sea water for a period sufficient to demonstrate pigment dispersion or contraction, or no response, in those animals injected with extract. In positive experiments, LUC extracts produced obvious dispersion or concentration of pigment (different by two or more chromatophore stages from the control value) within 15–30 min after injection. Experiments on eyestalkless crabs were conducted three or more times for each species.

To test for a possible direct action of LUC, we first injected isolated legs with 10 µl of LUC extract or 10 µl of saline. The results of these experiments at 15, 30, 60, 90, and 120 min, are shown in Table I. Although the chromatophores of the control legs showed a response to saline injections, the response of legs from both species to LUC was significantly higher ($p < 0.005$, Student's t-test and Mann-Whitney U-test) at all intervals examined. Since these experiments demonstrated that LUC extracts could cause pigment dispersion in the melanophores of

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