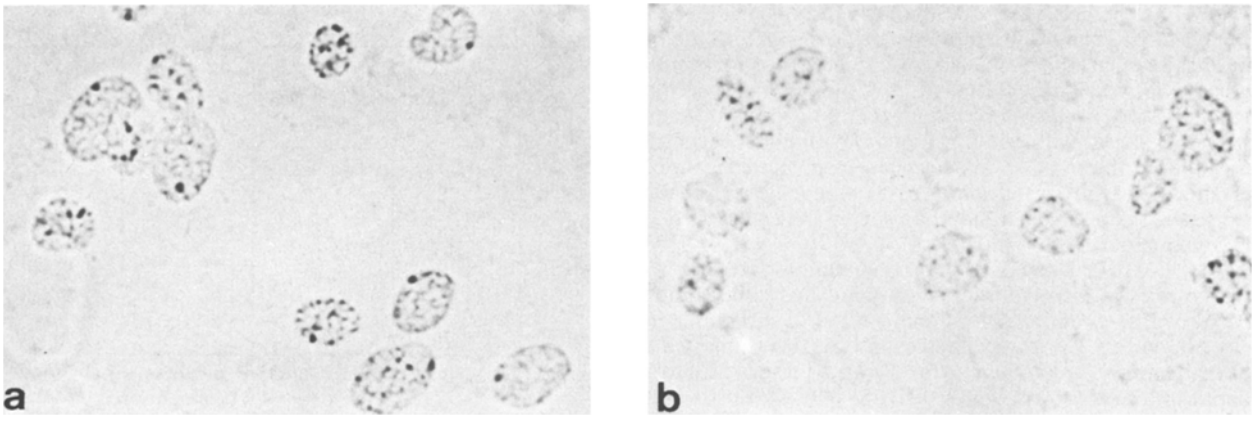

Interphase nuclei of unstained amniotic cells from female (a) and male (b) mouse embryos under brightfield microscopy. A dark mass adjacent to the inner nuclear membrane is identifiable in the cells from female mouse embryos, whereas no such mass can be seen in the male cells.

stained examination demonstrated consistently a clearly identifiable sex chromatin in the same position as that in which the dark mass had been seen.

As expected, by using a phase-contrast microscope with dark (positive) contrast (Olympus, PLL; absorbance rate 60%), we could obtain similar results. In retrospect, we found several early reports[3-7] that mentioned the possibility of visualizing sex chromatin of cultured cells under phase-contrast microscopy. Miles[4] has seen sex chromatin 'clumps' in cultured human amniotic cells. Unstained amniotic cells may be suitable specimens for nuclear sex diagnosis. Previously three groups of investigators[8-11] described the techniques for sex chromatin preparations of rodent amniotic cells and yolk sac. However, no mention was made of the possible use of unstained specimens.

Our preliminary attempts with rat embryos suggest that this method is also applicable. The method presented here may be useful for quick sex diagnosis of murine embryos for various purposes in experimental embryology.

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Differential expression of isozymes in relation to organogenesis in two closely related species[1]

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Summary. Two closely related species of Gentianaceae were established in tissue culture and transferred to a regeneration medium containing 2,4-D. Even though the age of cultures and all the environmental conditions were identical, only one of them expressed a clear morphogenic potential, and the differences are expressive in the isozyme banding patterns observed for creatine phosphokinase, lactate dehydrogenase and indoleacetaldehyde dehydrogenase. No differences in alkaline phosphatase isozymes were detected.
Key words. Gentianaceae; morphogenesis; caulogenesis; isozymes.

Exacum affine Balf. (German violet) and *Eustoma grandiflorum* (Raf.) Shinn (prairie gentian) are two closely related species belonging to the family Gentianaceae. The former is a herbaceous mesophyte and the latter is a succulent xerophyte. Evidence is given hereby showing that their morphogenic potential is different under identical cultural conditions, the difference being distinctly identifiable from their isozyme banding patterns for three of the four different enzymes studied.

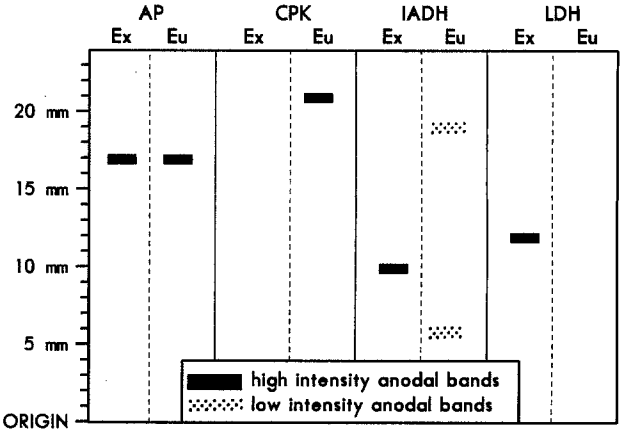
Exacum affine and *Eustoma grandiflorum* were established in tissue culture using MS medium supplemented with 1.0 mg/l of 2,4-D and 0.1 mg/l of kinetin. Transfers were made to a regeneration caulogenic MS medium supplemented with 0.03 mg/l of 2,4-D and 1.0 mg/l of kinetin. The explants were never exposed to exogenous IAA. The pH of the regulation medium was 5.8 upon establishment of the cultures and remained constant throughout the course of the experiment. Sequential manipulation has proved to be successful in the induction of various levels of morphogenesis[2, 3], but the isozyme differential in relation to morphogenesis in such closely related species has not been shown before. Within four weeks in the regeneration medium, the friable callus of

Exacum affine produced globular structures which are suspected to be primordial meristems developing from the superficial cells in the callus. There is precedence for similar observation[4, 5]. The callus of *Eustoma grandiflorum* did not reach this stage, which was not unexpected because different species respond differently to auxin/cytokinin concentrations. The object here was to grow them under identical chemical and physical conditions to study the differential expression of the genetic mechanisms as reflected by their isozyme patterns.

The 3-month-old callus in the regeneration medium was frozen prior to masceration in a ground glass tissue homogenizer with a proportional amount (w:v) of the buffer for electrophoresis. The mascerated callus was then subjected to centrifugation at 15,000 × g for 5 min to remove cellular debris and the extracts were stored at − 40 °C prior to electrophoresis. Cellulose acetate electrophoresis was conducted using equipment and supplies obtained from Helena Laboratories, Beaumont, TX, USA. Procedures for observation of alkaline phosphatase (AP), creatine phosphokinase (CPK), and lactate dehydrogenase (LDH) isozymes were as described in the appropriate Helena Laboratories Technical Manual[6–8], using fluorescent-qualitative methods. The bridge buffer was tris-barbital-sodium barbital, pH 8.8. For detection of alkaline phosphatase (AP), cellulose acetate plates were electrophoresed for 20 min at 180 V. The substrate used for staining the isozymes consisted of 2.4 mM naphthol ASMX phosphoric acid dissolved in the bridge buffer. For creatine phosphokinase (CPK), the plates were electrophoresed for 10 min at 300 V and the substrate consisted of 14.8 mM creatine phosphate, 11.5 mM glucose, 1.03 mM ADP, 3.8 mM AMP, 1.7 mM NAD, 5.2 mM magnesium acetate, 0.4 U/ml yeast hexokinase and 0.8 U/ml glucose-6-phosphate dehydrogenase in 20% glucose. For lactate dehydrogenase (LDH), the plates were electrophoresed for 10 min at 300 V, and the substrate consisted of 60 mM lithium lactate, 5 mM NAD, and 100.2 mM tris buffer (pH 8.8) in 20% sucrose. Following the staining procedure, the fluorescent isozyme bands were observed in a Helena CPK Photo Vubox equipped with two long-wave ultraviolet lamps. The procedure used for detection of indoleacetaldehyde dehydrogenase (IADH) isozymes is being reported here for the first time. It was identical to that described above for LDH, except that the substrate contained indole-3-acetaldehyde instead of lithium lactate. One possible precursor to IAA in meristems is indole-3-acetonitrile mediated by the enzyme nitrilase. The results reported here prove that the precursor to IAA is indole-3-acetaldehyde which is converted to IAA in meristematic cells destined to undergo caulogenesis.

For AP, a single isozyme was observed for both species. LDH activity was observed for only *Exacum affine*. For CPK, a single isozyme was observed only for *Eustoma grandiflorum*. For IADH, a total of three anodal enzymes were detected with migration rates of 6 mm, 10 mm and 19 mm from origin (fig.). Caulogenesis is successful only in the presence of strong IADH activity (band in intermediate position in *Exacum affine*). It is of significance to note that a non-migratory, non-enzymatic fluorescent pigment was clearly observed in all sample cells containing *Exacum affine* extract. This was observed regardless of the isozyme staining procedure used, and its significance in organogenesis is unexplained at this time.

The fact that the differences between samples appear to be primarily caused by differences in level of isozyme activity rather than presence or absence of bands, suggests that a regulatory gene may be involved, which is triggered by the level of development of the tissues. This regulatory gene(s) could exert transcriptional, translational or post-translational control over the activity of the genes that specify the individual enzymes. The high intensity intermediate IADH band in *Exacum affine* found in all the samples, reflects the caulogenicity of this species. The cultures of *Eustoma* did not show any primordial globular structures which is reflected in the two low intensity bands. There is plenty of evidence in the literature as to the pathway of biosynthesis of IAA. It was proved as early as 1940 that production of auxin is an enzyme-mediated process[9]. Gordon and Nieva[10] and Gordon[11] proved later that IAA was formed in the leaves when incubated with tryptophan by two different pathways. In one of these pathways, the deamination of tryptophan yields indolepyruvic acid, and in the other, decarboxylation of tryptophan yields tryptamine. Both of these pathways lead to the production of indole-3-acetaldehyde, which is penultimate intermediary in the synthesis of IAA. The final conversion step is mediated by the enzyme indoleacetaldehyde dehydrogenase, in the absence of which IAA cannot be produced[12–16]. The regeneration medium described in these experiments did not contain exogenous IAA; it is imperative that when the explanted callus underwent organogenesis, this auxin was endogenously produced because the isozymes of the mediating enzyme indoleacetaldehyde dehydrogenase were detected in the tissues.



Isozymes observed in callus tissue samples. Calli are of the same age, and grown under identical conditions. AP, alkaline phosphatase; CPK, creatine phosphokinase; IADH, indoleacetaldehyde dehydrogenase; LDH, lactate dehydrogenase; Ex, *Exacum affine* (caulogenous); Eu, *Eustoma grandiflorum* (non-caulogenous).

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