Results and discussion. A typical morula stained with Hoechst dye and observed under fluorescent optics is shown in the figure, A. Although all the nuclei are readily counted in this focal plane they are not in sharp focus as the three-dimensional perspective is still maintained. Fragmented ova that may give the appearance of a morula can easily be distinguished by this approach (fig., B and C).

The squash technique is necessary to determine the number of cells in the mouse blastocyst since all the nuclei cannot be observed in a single focal plane. If the blastocyst is squashed with the zona pellucida intact, the nuclei may be partially expressed from the broken zona pellucida (fig., D), but the presence of the zona pellucida interferes with the intensity of fluorescence of nuclei left inside the zona. The figure, E, shows a blastocyst squashed after the zona pellucida was removed. The wavy appearance of the DNA is the result of the distortion of the nuclei under pressure. Exposure of the blastocysts to a 1% Triton X-100 solution for 20–30 s prior to squashing decreases the nuclear distortion as shown in the figure, F.

The results in table 1 show that fertilized one-cell ova remain viable up to the blastocyst stage in culture when exposed to Hoechst 33258 at a concentration of 1.0 µg/ml. Additionally, transfer of the treated ova to pseudopregnant females shows that the embryos are viable and normal at least to day 13 of gestation. Exposure to only the UV light from either the 50 or 100 W lamp at the times used does not adversely effect the survivability of the fertilized ova. However, the intensity of the UV light in combination with either 0.5 or 1.0 min of UV exposure has a dramatic effect on the number of blastocysts that develop from the one-cell stage, following treatment with 1.0 µg/ml of the DNA-binding dye. 81 and 57% of the ova exposed to the 50 W lamp for 0.5 and 1.0 min respectively, developed to the blastocyst stage in vitro compared to 0% when the ova were exposed to the 100 W lamp for similar periods of time. Lower concentrations of dye result in improved embryonic viability, but do not give intense images of fluorescence with the present optical equipment. It may be possible, however, to visualize nuclei with lower dye concentration if image intensifying equipment is employed[7]. Blastocysts exposed to the dye and UV light showed increased fluorescent intensity as the dye concentration increased.

The dye alone at various concentration does not appear to effect the viability of the blastocyst. Embryos exposed to the dye at concentrations as high as 200 µg × ml for 30 min showed no decrease in viability up to day 14 of pregnancy (table 2). In both experiments testing viability, fetuses were found to be normal on dissection, thus providing no evidence that dominant mutations effecting gross fetal morphology were caused by the dye or UV, either alone or in combination. However, it would be necessary to use a multiple recessive stock of mice and to allow their development to term to obtain a more complete test of the possible mutagenic effect of such treatment.

This technique for detecting nuclei can be useful as a quick and easy method to:

1) Count the number of cells at the compact morula or blastocyst stage of embryo development.

2) Distinguish between early cleavage stages and abnormal fragmented ova.

3) Detect the number and location of pronuclei particularly in the ova of species such as the cow and pig where it is difficult to visualize pronuclei under phase or Nomarski optics.

4) Determine that sperm penetration has occurred during in vitro fertilization of oocytes.

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A simplified method for isocratic HPLC analysis of polyamines

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Summary. A simple technique is described for the separation and analysis of polyamines in tissues and body fluids, utilizing precolumn clean-up on disposable CM-cellulose columns, followed by an automatable HPLC procedure. Complete separation and analysis takes 12–15 min per sample with sensitivity in the pmole range.

Key words. Polyamines; putrescine; spermidine; spermine; high pressure liquid chromatography.

Interest in the polyamines has expanded by major proportions due to the recognition that these substances serve as primary modulators of both normal and pathological cell growth, such as that seen during ontogeny, pathological or compensatory tissue hypertrophy, malignancy and infection[1–4]. Sensitive methods for the separation and analysis of polyamines by HPLC have been developed which typically require either multi-buffer elution with either postcolumn or precolumn derivatization[5–9], or an isocratic system requiring a prolonged period for column regeneration between samples[10]. We now describe a procedure which utilizes isocratic HPLC separation and thus does not require multiple pumps, column re-equilibration or other specialized equipment or techniques. The result is a complete separation of the polyamines in 12–15 min per sample with sensitivity comparable to that of more elaborate procedures.

Materials. Ortho-phthalaldehyde (OPA), CM-cellulose (0.72 meq/g, coarse grade), putrescine dihydrochloride, histamine dihydrochloride, spermidine phosphate, spermine diphosphate, agmatine sulfate, cadaverine dihydrochloride and Brij 35 were purchased from Sigma Chemical Corp. (St Louis, MO). 2-Mer-

captoethanol and sodium octyl sulfate were from Eastman-Kodak (Rochester, NY). Solutions required for sample preparation were 0.4 M $HClO_4$+2 mM disodium EDTA, 2 M KOH, 0.01 M phosphate buffer (pH 6.2), 0.02 M borate buffer (pH 9.0), 0.02 M borate buffer+0.15 M NaCl (pH 9.0) and 3 N HCl. The HPLC buffer consisted of 12.2 g $NaH_2PO_4 \cdot H_2O$+0.7 ml 85% H_3PO_4+25 mg sodium octyl sulfate per l of deionized and distilled water; retention time was adjustable by addition of varying amounts of methanol to the HPLC buffer. All HPLC solutions were filtered with a Millipore filter (type HA, 0.45 µm) prior to use. For fluorescence derivatization, a solution was prepared consisting of 250 ml of 0.2 M borate-bicarbonate buffer[11]+0.65 ml of 2-mercaptoethanol+1.1 ml of Brij 35+260 mg OPA dissolved in 5 ml methanol+750 ml H_2O, final pH 10.3. The analysis requires the following equipment: a single-piston HPLC pump (Beckman model 110B or equivalent), a standard 25 cm × 6.4 mm C_{18} reverse-phase column (10 µm, such as that made by Alltech) and a detection system with a flow cell and a filter-type or grating-type fluorometer connected to a chart recorder; we utilized a filter fluorometer with excitation at 390 nm and emission at 475 nm. The OPA reagent can be pumped with any standard peristaltic pump. Although most of our results were obtained with a Farrand ratio fluorometer and a 300 µl flow cell, considerable improvement can be obtained where needed by the use of more sophisticated and sensitive detection equipment and smaller volume flow cells. For the purpose of this communication, we wished to present the results as they would be obtained with routinely available, reasonably-priced facilities available to most laboratories.

To remove interfering substances such as aromatic and basic amino acids, monoamines, histamine, etc., samples were first passed through a disposable CM-cellulose column by a modification of the method of Endo[11]. Tissues of up to 1 g weight were homogenized with a Polytron in up to 5 ml of ice-cold 0.4 M $HClO_4$ with 2 mM disodium EDTA. After centrifugation at 26,000 × g for 10 min, the supernatant solution was frozen at 0 °C until assayed. An aliquot corresponding to < 300 mg of original wet wt of tissue was neutralized with 2 M KOH to a final pH of 5–6 and then placed on a 3 cm × 6 mm CM-cellulose column. The column was then washed with 7 ml of 0.01 M phosphate buffer (pH 6.2) and 7 ml of 0.02 M borate buffer (pH 9.0) after which polyamines were eluted in 7 ml of 0.02 M borate buffer with 0.15 M NaCl (pH 9.0). The eluate was acidified with 30 µl of 3 N HCl. Recovery of standard amounts of polyamines from CM-cellulose columns was complete (97–101% in five separate experiments).

HPLC separation, fluorescence derivatization and analysis. The CM-cellulose column eluate was injected (20–200 µl) into the HPLC at a buffer flow rate of 1 ml/min. Retention time was controlled by changing the methanol concentration; 12% methanol gave a typical run time of 15 min per sample. The C_{18} column effluent and OPA reagent (2.5 ml/min) were combined by a T-joint with a small dead-volume, allowed to react in a delay-coil (350 cm 1/32 in I.D. Tygon tube) and then passed through the flow cell; the use of OPA for detection of primary amines is well-established[12].

Sample chromatograms for mixtures of polyamine standards with typical interfering substances present and tissue samples from 5-day-old Sprague-Dawley rats are shown in figure 1. Even with the large-volume flow cell (300 µl), separation of the polyamines was complete both in standard samples and in those from tissues after the precolumn clean-up procedure; separation was adequate from equivalent amounts of histamine, cadaverine and agmatine, the latter two which elute with the polyamines[11]. Since histamine is removed in the CM-cellulose step and since cadaverine and agmatine are not usually present in significant

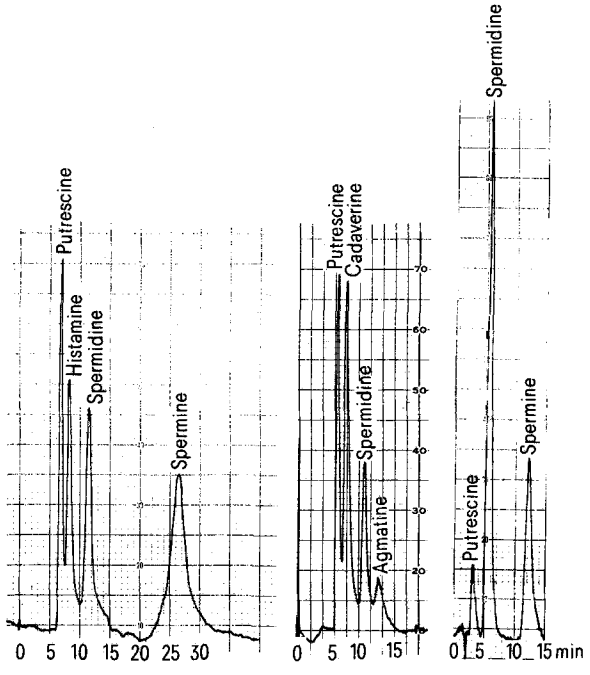

Figure 1. Separation of polyamines and interfering substances from standard mixtures (left, middle) and from kidney of 5-day-old rat (right). In left panel, standards contained 100 pmoles of putrescine, histamine and spermidine and 400 pmoles of spermine; in middle panel, all substances were injected at 250 pmoles; kidney sample represents injection of material from approximately 1.8 mg of tissue and contained 130 pmoles putrescine, 980 pmoles spermidine and 1090 pmoles spermine. The HPLC buffer contained 8% methanol (left, middle) or 12% methanol (right). Flow cell utilized for these tracings had a volume of 300 µl.

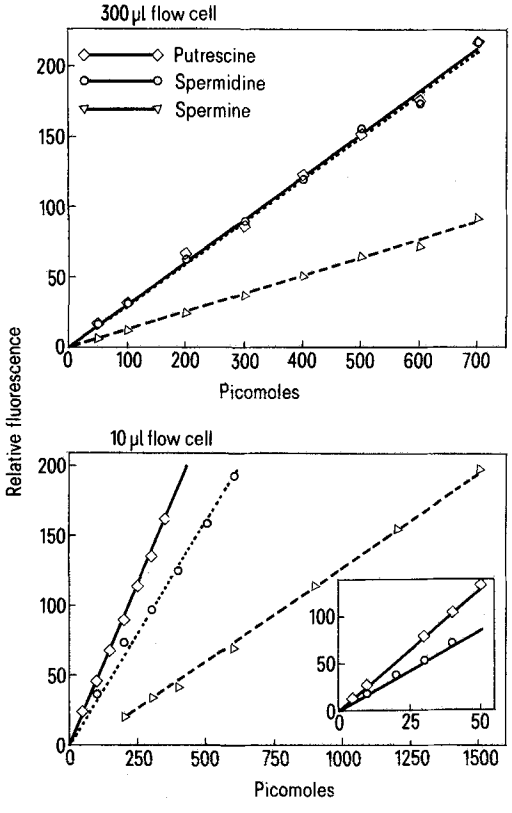

Figure 2. Standard curves for fluorescence emission of polyamine standards in 300 µl flow cell (top) and 10 µl flow cell (bottom).

amounts, tracings obtained from actual tissue samples usually show clean and complete separations (fig. 1). Total resolution of all interfering substances, when present, can be achieved by increasing the retention time (decreased methanol) and/or by utilization of a routinely-available (albeit more expensive) smaller volume flow cell (10 µl); as noted above, this is not usually necessary.

Under the conditions utilized here, the detector system was linear across a range representing 10–700 pmoles of putrescine or spermidine and 30–1500 pmoles of spermine (fig. 2); 10 pmoles of spermidine gave a 20 cm deflection of the recording pen with a signal-to-noise ratio of better than 10:1. Again, increased sensitivity and better separation can be achieved with the smaller volume flow cell, but the spermine standard curve may then become non-linear unless the OPA buffer flow rate is increased; the latter requires that high-pressure fittings be used in the postcolumn derivatization stage, a procedure not utilized here. Values for tissue polyamines were entirely comparable to those found with other methods (table)[13–16].

The method presented here offers a simple, rapid procedure for separation and analysis of polyamines in tissues and biological fluids. Although not critical for many situations, the sensitivity (in the pmole range) is comparable to that of other techniques which utilize precolumn derivatization or more elaborate separation equipment[5, 6, 8–10, 17]. Indeed, our procedure is suitable and sensitive enough for determinations in small tissue samples and fluids such as cerebrospinal fluid or serum. As is the case with all polyamine determinations, care must be taken to avoid contamination and surface adsorption when values are below 100 pmoles. Although the method was designed specifically for analysis with minimal laboratory equipment and cost, significant improvements can be obtained by using: a) less commonly-found resins in the precolumn clean-up, such as P-cellulose[18], which requires a smaller elution volume and thus increases the overall sensitivity; b) concentration of the sample after the pre-column phase by evaporation with N_2; c) more sophisticated HPLC equipment, smaller volume flow cells and more specialized and sensitive fluorescence detectors; and d) automating the HPLC phase of the analysis with an automatic sampler, such as the Waters WISP 710B. Routinely, 50 samples can be processed through the CM-cellulose column phase in a single hour and the columns can then be discarded or regenerated. Because derivatization occurs post-separation, there is no stability problem of the fluorescence product and samples can then be placed in an automated sampler; with such automation, the subsequent stages can all be performed 'hands-off' and around-the-clock. The use of an isocratic system eliminates the need for expensive, multi-pump HPLC equipment, buffer-switching devices or time-consuming column re-equilibration. Polyamine analyses can thus be performed on a routine basis in basic and clinical laboratories with a minimum of cost, equipment and personnel time.

Polyamine levels in tissues from 5-day-old rats

Determination	Tissue	HPLC method (nmoles per g tissue)	Literature values
Putrescine	Heart	74 ± 6	60–140
	Brain	123 ± 3	80–135
	Kidney	103 ± 9	60–115
Spermidine	Heart	496 ± 27	500–800
	Brain	430 ± 9	350–650
	Kidney	676 ± 47	520–700
Spermine	Heart	568 ± 51	450–600
	Brain	403 ± 16	300–420
	Kidney	692 ± 64	690–850

Data represent mean ± SE of eight animals. Literature values are from references 13–16.

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