the discharge of hypothalamic FSH releasing factor. Recent evidence on the control of FSH secretion shows that inhibin, a non-steroidal hormone secreted by the gonads, selectively controls FSH secretion[17]. It would be interesting to study whether pineal control of FSH secretion is mediated through the gonadal inhibin.

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PRO EXPERIMENTIS

Fluorometric estimation of dead cells in cell suspensions[1]

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Summary. An objective vitality test is proposed. It is based on the fluorescence increment of ethidium bromide in the presence of dead cells, which is proportional to cellular DNA under conditions previously defined.

Estimation of the proportion of dead cells in a given cell suspension is a very common procedure in experimental biology and medicine. Dye exclusion techniques[2] are most frequently used, but require subjective counting. Among various dyes excluded from living cells, ethidium bromide (EBr) offers the possibility of developing an objective method because it reacts quantitatively with nucleic acids[3]. Edidin[4] was the first to evaluate titers of cytotoxic antisera using the fluorometric measurement of EBr stained cell suspensions. His method, however, was not based on a true quantitative estimation of deoxyribonucleic acid (DNA) and was neither intended nor suited for the calculation of absolute or relative cell numbers. By making use of recent developments in the fluorometry of nucleic acids by EBr[5-7], especially dissociation of nucleoproteins by heparin and digestion of ribonucleic acid, we arrived at conditions where the DNA of dead cells (inside or outside the cell) is detected specifically and quantitatively. The proposed procedure may be used to carry out, in cell suspensions: a) relative counts of dead and living cells, and b) quantitative estimations of DNA or (by a factor) of the total number of cells present.

Materials. Ascites cells of a mouse sarcoma induced by UV light (UVT 14306)[8] were carried in the inbred strain XVII/Bln. Fluorometric measurements were performed with a Beckman SF 1078 spectrofluorometer.

Solutions (numbers refer to the table): (1) Cell suspension (about 10^6/ml) in PBS-GS, washed once. (2) Ribonuclease (RNase, EC 2.7.7.16, Ferak, Berlin), 50 µg/ml in PBS. (3) Heparin (Polfa, Warsaw), commercial solution (5000 IU/ml) diluted 1:300 with PBS. (4) Digitonin (VEB Ysat, Wernigerode), 1.5 mg/ml in ethanol. (5) PBS (Dulbecco's phosphate buffered saline)[9], pH 7.5. (6) PBS-GS (PBS plus 2 mg/ml glucose and 1% inactivated fetal calf serum from Flow Labs., Bonn). (7) Ethidium bromide (Serva, Heidelberg), 25 µg/ml in PBS. Solutions 2,4 and 7 can be stored at +4 °C for 1 month.

Proposed procedure. Assay mixtures according to the table, but without EBr, are prepared and incubated in a waterbath at 37 °C for 20 min. Thereafter EBr is added. As soon as constant temperature is reached, mixtures can be measured. Excitation is set at 365 nm, emission at 590 nm. In the case of filter fluorometers, the filter combination of Boer[10] can be used. The fluorometer reading of mixture A (F_A) is adjusted to the full scale of the instrument.

Assay mixtures

Mixture	1 Cell suspension	2 RNase	3 Heparin	4 Digitonin	5 PBS	6 PBS-GS	7 EBr
A	0.5	0.5	0.5	0.01	0.5	–	0.5
B	0.5	0.5	0.5	–	0.5	–	0.5
C	–	0.5	0.5	–	0.5	0.5	0.5

Volumes in ml, total volume 2.5 ml (in 4 ml cuvettes). Numbers of solutions refer to 'materials' section, where concentrations are given

Mixtures should be shaken before reading.

$$\text{Calculation: \% dead cells} = \frac{F_X \times 100}{F_{Dig}} \quad (1)$$

where

$$F_X = F_B - F_C \quad (2)$$

$$F_{Dig} = F_A - F_C \quad (3)$$

Results and discussion. Basic details of the fluorometric assay of nucleic acids by EBr have been described elsewhere[3,5-7]. The present application of this method to suspensions of cells instead of solutions or homogenates has been extensively tested with UVT 14306 cells as a model, and confirmed with other cell types. The results are briefly summarized. No special problems of fluorometry emerged when using dilute cell suspensions instead of homogenates. There was a straight proportionality between the number or percentage of dead cells per assay mixture and fluorescence intensity, as long as the EBr concentration was not limiting (up to 2 µg DNA per ml). We found no measurable EBr uptake by living cells at 25 °C within at least 60 min. Treatment with RNase and heparin according to the proposed procedure caused no appreciable harm to living cells as judged by trypan blue exclusion. The fluorometer reading of a suspension of digitonin killed cells at 25 °C was stable for at least 3 h. Digitonin did not influence the DNA assay. An essential finding was that cell suspensions killed by digitonin revealed DNA values identical with those obtained after complete homogenization, which have been shown earlier to be quantitative[6]. This means that the method measures DNA outside and inside (dead) cells to the same extent. It follows that - by comparison with a standard (DNA[6], rhodamine B[7], or chicken erythrocytes) - quantitative estimations of DNA can be performed in cell suspensions simply after the addition of digitonin, thus avoiding the necessity of homogenization[6]. Very few substances interfere directly with the EBr assay[5], but some show unspecific fluorescence and, by enhancing the background level, decrease the sensitivity of the assay. For this reason, phenol red, a common pH indicator in tissue culture media, should be omitted, and higher amounts of serum should be avoided (or samples diluted with PBS-GS prior to the assay). Cells without EBr show only negligible fluorescence in most cases (UVT 14306 cells: 1% of F_{Dig}). If necessary, e.g. in cell suspensions heavily contaminated by erythrocytes, it can be taken into account by using 2 additional blanks:

D 0.5 ml cell suspension + 2.0 ml PBS
E 0.5 ml PBS-GS + 2.0 ml PBS

$F_D - F_E$ is the correction term which should then be subtracted from F_X (equation 2) as well as from F_{Dig} (equation 3).

In figures 1 and 2 results of the proposed fluorometric assay have been compared to those obtained by the conventional trypan blue exclusion test. Both methods agree with 2 remarkable exceptions. 1, in an early phase of heat damage (figure 1), the EBr assay seems to be more sensitive than the trypan blue test. This is in accordance with earlier microscopic observations[11]. 2, the fluorometric viability assay is clearly advantageous in cases where a number of cells are not only killed, but lysed or homogenized, and therefore no longer countable by microscope (figure 2). These cells are detected by the fluorometric assay as long as their DNA is not yet degraded.

The proposed method is especially useful in large series of estimations intended to examine dose-effect relations, kinetics, or the mode of action of toxic or membrane damaging agents. Automation would be possible.

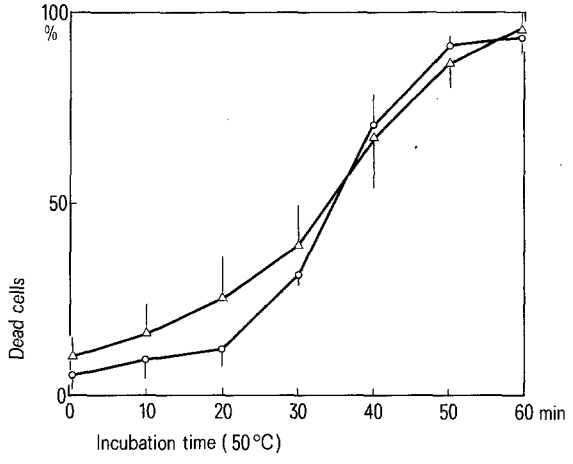

Fig. 1. Comparison of EBr assay and trypan blue exclusion test. Aliquots of a suspension of UVT 14306 cells were subjected to the damaging effect of incubation at 50 °C for various periods of time. Thereafter, viability was assessed by both methods. △, EBr assay; ○, trypan blue test (relative counts). Values are means of 3 experiments. Vertical bars denote SE.

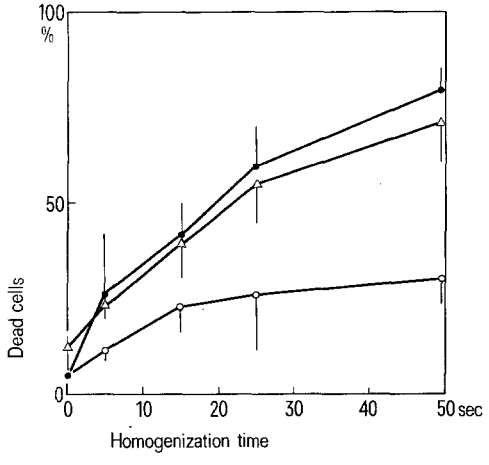

Fig. 2. Comparison of EBr assay and trypan blue exclusion test. Aliquots of a suspension of UVT 14306 cells were subjected to partial homogenization by Ultra Turrax TP 10N (Janke & Kunkel, Freiburg) in subsequent steps of 5 sec each at 0 °C in a volume of 5 ml. Thereafter viability was assessed by both methods. △, EBr assay; ○, relative trypan blue counts; ●, absolute trypan blue counts (dead cells calculated as the difference between original total count and the number of cells surviving). Values are means of 3 experiments. Vertical bars denote SE.

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